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Enhanced Tim3 Activity Improves Survival after Influenza Infection

Josalyln L. Cho,* Marly L. Roche,* Barry Sandall,+ Abraham L. Brass,§,* and Benjamin D. Medoff,*†

Influenza is a major cause of morbidity and mortality in the United States. Studies have shown that excessive T cell activity can mediate pneumonitis in the setting of influenza infection, and data from the 2009 H1N1 pandemic indicate that critical illness and respiratory failure postinfection were associated with greater infiltration of the lungs with CD8⁺ T cells. T cell Ig and mucin domain 3 (Tim3) is a negative regulator of Th1/Tc1-type immune responses. Activation of Tim3 on effector T cells has been shown to downregulate proliferation, cell-mediated cytotoxicity, and IFN-γ production, as well as induce apoptosis. In this article, we demonstrate that deletion of the terminal cytoplasmic domain of the Tim3 gene potentiates its ability to downregulate Tc1 inflammation, and that this enhanced Tim3 activity is associated with decreased phosphorylation of the TCR-CD3ζ-chain. We then show that mice with this Tim3 mutation infected with influenza are protected from morbidity and mortality without impairment in viral clearance or functional heterotypic immunity. This protection is associated with decreased CD8⁺ T cell infiltration of the lungs with CD8⁺ T cells (8–11). In addition, cytokine levels correlated positively with severity of illness scores (8). In this situation, inhibition of T cell activity leads to mitigation of the lung inflammation (12–14). However, it also can lead to profound immunosuppression, which in the case of viral pneumonitis can reduce viral clearance (12–15). It follows that a therapeutic strategy that could control excessive T cell-mediated injury without significant immunosuppression or impairment in the ability to clear infections would be an ideal treatment for severe influenza pneumonitis.

T cell Ig and mucin domain 3 (Tim3) is a type-1 transmembrane receptor with immunoregulatory properties on effector T cells and APCs (16). Tim3 is expressed on Th1 and Tc1 cells, and has been shown to be a negative regulator of the Th1/Tc1 response (17–19). On these cells, activation of Tim3 via ligand binding results in decreased T cell proliferation (20), decreased T cell-mediated cytotoxicity (21, 22), decreased IFN-γ production (23, 24), and induction of apoptosis (25). However, Tim3 is also expressed on NK cells and APCs, and on APCs, activation of Tim3 has a proinflammatory role (25). One of the Tim3 ligands has been identified as galectin-9 (gal-9) (23), a β-galactoside–binding lectin that is induced by IFN-γ and has a number of immunoregulatory functions (26–28). Binding of Tim3 by gal-9 has been shown to result in phosphorylation of a highly conserved tyrosine residue (human Y265; murine Y256) (29), but the overall mechanisms by which Tim3 signals are relatively undefined. Furthermore, there are multiple other tyrosine residues in the Tim3 cytoplasmic domain with relatively unknown signaling functions (30). Overall, these data suggest that the signaling pathways of Tim3 are highly complex and likely depend on the cell type on which it is expressed.

Animal models of immunoinflammatory disease including experimental autoimmune encephalitis (17, 19, 23, 31), experimental autoimmune arthritis (32), and transplantation (33, 34) have suggested that the predominant effect of Tim3 activation in vivo is anti-inflammatory. Studies suggest that Tim3 plays a similar role during acute viral infection of the eye, by limiting the Ag-specific CD8⁺ effector T cell response and thereby immune-mediated bystander injury (21, 22). We therefore hypothesized that Tim3 would play an important role in regulating the immune response to influenza infection. In an effort to explore both the functional role of Tim3 in influenza pathogenesis and mechanisms of Tim3 signaling, we generated a Tim3 mutant mouse (Tim3mut) with pres-
of a highly conserved tyrosine kinase motif (Y256) and deletion of a portion of the remaining distal cytoplasmic domain. In this article, we demonstrate that this deletion enhances the negative regulatory activity of Tim3 on CD8+ T cells and is associated with decreased phosphorylation of CD3ζ, a potentially novel mechanism for the inhibitory activity of Tim3. Furthermore, the enhanced ability of Tim3 to downregulate the Tc1 response in Tim3mut mice is associated with reduced morbidity and mortality during influenza infection. Importantly, mutation of Tim3 had no adverse effect on viral clearance or the development of cellular immunity.

Materials and Methods

Generation of Tim3 mutant mice

Mice with a deletion of the terminal cytoplasmic domain of the Tim3 gene were generated using modified bacterial artificial chromosome (BAC) technology as previously described (35). In brief, BAC clones spanning the Tim3 locus were obtained from Research Genetics (Invitrogen Life Technologies, Carlsbad, CA). Two sequences flanking exon 7 were cloned into the 5' and 3' insertion sites of the pSKY replacement vector. BAC host cells were transformed with the pBADredex plasmid, which helped produce electroporation-competent cells. The linear fragment released from the pSKY backbone was then electroporated into the BAC host, and the transformants were selected for simultaneous resistance to chloramphenicol (from the BAC backbone) and zeocin (from the insert). After selection, homologous recombinants were identified by PCR. Tim3.5/prco primers identify a predicted 440-bp PCR product in the mutant BAC, confirming 5' targeting. To confirm 3' targeting, an additional primer Tim3.2 was included in the PCR. As predicted, the PSV/Tim3.2 primers identify a 490-bp PCR product in the mutant BAC and 326-bp product in the wild-type (WT) BAC. The modified BAC was electroporated into embryonic stem cells, and fluorescence in situ hybridization (FISH) was performed as described previously (35), confirming successful targeting in clones B9, C6, and D8. Mice from clones B9 and C6 were born in the expected Mendelian frequency and were healthy. Founder mice were crossed with C57BL/6 mice for 10 generations.

Mice

WT C57BL/6 mice were purchased from National Cancer Institute (Rockville, MD). The OT-I and OT-II TCR transgenic mice in the C57BL/6 background are bred and maintained in our facility. In addition, OT-I and OT-II mice in the C57BL/6 background were crossed with Tim3mut mice. CC10-OVA mice in the C57BL/6 background were generated as previously described (36), bred, and maintained in our facility at 6–8 wk of age and were sex matched for all experiments. All protocols were approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

Flow cytometry

Single-cell suspensions of spleen, blood, lymph nodes, bronchoalveolar lavage (BAL), fluid, and lung were prepared and RBCs were lysed. Lung lobes were removed, minced with scissors, and then digested for 45 min in RPMI 1640 with 0.28 Wunsch U/ml Liberase (Roche Applied Science, Indianapolis, IN) and DNase 30 U/ml (Sigma-Aldrich, St. Louis, MO) at 37°C to extract leukocytes from lung tissue. The digested tissues were then strained through a 70-μm filter before RBC lysis. Samples were blocked with purified CD16/CD32 mAb (BD Biosciences, San Diego, CA) and then stained with fluorescently labeled Abs to CD4, CD8, CD25, CD69, and Tim3 (BD Biosciences except for Ab to Tim3, which was obtained from R&D Systems, Minneapolis, MN). Some samples were also stained with fluorescently labeled tetramer to influenza nucleoprotein (366–374) (Tet; National Institutes of Health Tetramer Facility, Atlanta, GA). For the apoptosis experiments, some cells were treated with 10-μM full-length recombinant gal-9 (Novus Biologicals, Littleton, CO) for 6 h. Cells were then stained with Annexin V and propidium iodine (BD Biosciences). Flow cytometry was performed on an Accuri C6 analytical flow cytometer (Accuri Cytometers, Ann Arbor, MI) and analyzed using FCS Express software (DeNovo Software, Los Angeles, CA).

OT-I and OT-II T cell preparation and adoptive transfer

Isolation and preparation of OT-I and OT-II T cells were performed as described previously (36, 37). In brief, spleens and lymph nodes were harvested, single-cell suspensions were prepared, and CD8+ or CD4+ T cells were isolated using an Ab-mediated magnetic negative selection kit (EasySep Mouse T Cell Enrichment Kit; StemCell Technologies, Van-}

cover, BC, Canada). Effector OT-I T cells were prepared by culturing purified CD8+ OT-I T cells with irradiated APCs from spleens of C57BL/6 or Tim3mut mice with 700 ng/ml SIINFEKL peptide, 2 μg/ml anti-CD28, 10 ng/ml IL-2, and 10 ng/ml IL-12. After 5 d of culture, CD8+ T cells were purified using gradient centrifugation. For the CC10-OVA adoptive transfer experiments, 2 × 103 effector CD8+ T cells were resuspended in sterile PBS and injected i.p. OT-II T cells were polarized to Th1 cells by coculturing with irradiated APCs with 100 ng/ml OVA peptide, 1 μg/ml anti-CD28, 1 ng/ml IL-12, and 10 μg/ml anti-IL-4. To achieve maximal Tim3 expression, we restimulated CD4+ T cells with APCs and Ag on day 7 and purified on day 10.

Proliferation assays

In vitro proliferation studies, purified CD8+ and CD4+ T cells were labeled with CFSE (Invitrogen) and incubated for 48–72 h. Proliferation was assessed by CFSE dilution on flow cytometry. In vivo proliferation studies were performed by injecting 2 mg BrdU (BD Pharmingen) i.p. into mice at day 6 after influenza infection. Tissues were collected 24 h later, and single-cell suspensions were prepared. BrdU labeling was performed according to the manufacturer’s protocol, and samples were analyzed by flow cytometry.

Cytotoxicity assay

Purified effector OT-I T cells were coincubated with Ag-loaded EL4 target cells. OT-I–mediated cytotoxicity was measured using a commercially available bioluminescence kit (aCella Tox; Cell Technology, Mountain View, CA) according to the manufacturer’s protocol.

Western blot and immunoprecipitation

Some cells were treated with 10 μM gal-9 for 2 or 5 min. Cells were lysed using ice-cold Nonidet P-40 lysis buffer (Invitrogen) containing a protease inhibitor mixture (Complete Mini, Roche Applied Science) and a phosphatase inhibitor mixture (Calbiochem, La Jolla, CA). Protein was quantified using a commercially available system (Pierce BCA Protein Assay Kit; Thermo Scientific, Rockford, IL) according to the manufacturer’s protocol. Equal amounts of protein were subjected to SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was incubated with an anti-phosphotyrosine Ab (clone 4G10; Millipore, Billerica, MA) and visualized by the ECL detection system (Amersham Pharmacia, GE Healthcare, Pittsburgh, PA). For immunoprecipitation (IP), cells were lysed, protein was quantified as described earlier, and equal amounts of protein were subjected to IP using an anti-CD3Δζ Ab (Abcam, Cambridge, MA) and a commercially available kit (Pierce Co-Immunoprecipitation Kit; Thermo Scientific) according to the manufacturer’s protocol. Eluted protein was then subjected to Western blotting as described earlier using an anti-phosphotyrosine Ab.

Virus and infections

Influenza A/Puerto Rico/8/34 (PR8) was obtained from the American Type Culture Collection (VR-1469; Manassas, VA) and grown in Madin–Darby canine kidney cells (MDCK) in our laboratory. Influenza A/Hong Kong/8/68-x31 (x31) was provided by Dr. Troy Randall (Trudeau Institute) and grown in embryonated chicken eggs in our laboratory. The recombinant virus influenza A/WSN/33 OVAx (WSN-OVAx) was generated and kindly provided by Dr. David Topham (University of Rochester) (38). Viral pool titers were measured by MDCK plaque assay, and appropriate infecting doses were determined by in vivo titration. Mice were infected with a dose of influenza PR8 that leads to ~80% mortality (lethal dose 80 [LD80]; 105 PFU) at 6–8 wk of age. For heterotypic memory experiments, mice were infected with a nonlethal dose of x31 virus (5 × 107 PFU) and allowed to recover for 4 wk. They were then infected with 3-fold the LD80 of PR8 (3 × 107 PFU) and followed for 28 d. For assessment of Ag-specific IFN-γ production, mice were infected with a nonlethal dose of WSN-OVAx virus (200 PFU). Viruses were diluted to the appropriate dose using sterile PBS. Mice were anesthetized with ketamine (80 mg/kg)-xylazine (12 mg/kg), and 30 μl virus was administered intranasally.

Histopathology

Mice were sacrificed at day 3 or 7 postinfection. Lung lobes were inflated with 10% buffered formalin and then placed in formalin. Paraffin-embedded, 4-μm sections were prepared and stained with H&E. Histology was evaluated for severity by an investigator blinded to the genotype of the mice.
BAL fluid analysis

Mice were sacrificed at day 3 or 7 postinfection. BAL fluid was obtained by infusing six 0.5-ml washes of cold PBS with 0.12% 2 mm EDTA intratracheally. Differential cell counts were obtained after spinning 1.5 × 10⁷ cells onto slides and staining with Hema-3 (Fisher Scientific, Pittsburgh, PA). Differential counts were performed on at least 200 cells per slide. Cells were also analyzed by flow cytometry as described earlier. Cytokine protein levels were analyzed using a commercially available bead-array (Procarta Cytokine Assay; Affymetrix, Santa Clara, CA) according to the manufacturer’s protocol.

Quantitative real-time PCR

RNA was purified using either chloroform extraction or a purification column (RNeasy; Qiagen, Valencia, CA). After a DNase step, 1 µg RNA was converted to cDNA (Applied Biosystems, Carlsbad, CA). Specific primers for sequence detection of message for the Tim3 extracellular domain were forward primer 5'-CACCATCGAGGAGAAC-3' and reverse primer 5'-AGGCTCATCAGTACCAGC-3'; and for the Tim3 cytoplasmic domain were forward primer 5'-GAGGAAATATCTCA- CACCATTGGAAGAC-3' and reverse primer 5'-CAGAAATGAGGCC- GAGCCTTTTTAAGTTG-3'. The primers for specific cytokines were selected from the Massachusetts General Hospital Primer Bank (http://pga.mgh.harvard.edu/primerbank/). Samples underwent amplification in the presence of SYBR Green (Applied Biosystems). The reaction was analyzed in real time during amplification by the PCR machine (Masterecyler EP Realplex; Eppendorf, Hauppauge, NY).

Intracellular staining

Ag-specific staining. EL4 cells were pulsed with 700 ng/ml SINFEKL peptide and then coincubated for 24 h with CD8+ T cells isolated from the lungs of mice 7 d postinfection with WSN-OVA virus. The cells were then exposed to GolgiStop (BD Biosciences) to block cytokine secretion, fixed, and permeabilized using a commercially available kit (BD Biosciences) and stained for IFN-γ.

PMA/ionomycin. CD8+ T cells were isolated from the lungs of mice 7 d postinfection with PR8. They were stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml), exposed to GolgiStop, fixed, permeabilized, and stained as described earlier. Results were analyzed by flow cytometry.

Viral titers

Infectious viral titers were determined using a modified MDCK cell assay as previously described (39, 40). In brief, lungs were harvested on day 3 or 7 after influenza infection, homogenized, serially diluted, and added to duplicate wells containing confluent monolayers of MDCK cells for 12 h. The cells were then immunostained for surface expression of hemagglutinin and quantitatively imaged. In addition, the number of viral RNA copies per lung was determined by quantitative RT-PCR. RNA was prepared from whole-lung homogenates at days 3, 7, 10, and 14 using TRIzol (Sigma-Aldrich), and 1 µg RNA was converted to cDNA (Applied Biosystems). Quantitative PCR (QPCR) was performed as described earlier to amplify the polymerase gene of the PR8 influenza virus using the following primers: forward primer 5'-CGTCCAAATCTCCTGCTGA-3' and reverse primer 5'-CATGTTGCTCTTTCCATCCA-3'.

Results

Generation of a Tim3 mutant mouse

To investigate mechanisms of Tim3 signaling, we generated a BAC construct with disruption in the terminal portion of the cytoplasmic domain (exon 7) of the Tim3 gene by the zeomycin-resistance gene. The resulting mutant BAC is shown in Fig. 1A. Recombinants were identified by PCR (Fig. 1B), and the construct was injected into embryonic stem cell clones. Targeting was confirmed by FISH analysis of several embryonic stem cell clones (Fig. 1C), which were used to generate Tim3mut mice. We performed sequence analysis of the cytoplasmic domain on genomic DNA. As shown in Fig. 1D, the mutation resulted in deletion of aa 263–280, while preserving two proximal tyrosine amino acids in the cytoplasmic tail. One of the tyrosines has previously been shown to be phosphorylated by the tyrosine kinase Itk after gal-9 binding (29), and both of the proximal tyrosines can be phosphorylated by the Src family tyrosine kinases Lck and Fyn (30). However, the signaling mechanisms of the deleted tyrosine residues are unknown. We further confirmed the deletion by RT-PCR analysis of splenocytes from Tim3mut mice using primers directed at either the extracellular domain or the deleted portion of the cytoplasmic domain. This demonstrated normal expression of RNA encoding the extracellular domain, but we were unable to detect RNA encoding the targeted cytoplasmic domain (Fig. 1E).

Tim3mut mice were viable, fertile, and grossly normal in size and appearance. Analysis of lymph nodes, spleen, and lung from 6–8 wk old mice demonstrated normal percentages of CD4+, CD4+ Fopx3+, and CD8+ T cells compared with WT mice. In addition, there were no differences in Tim3 expression on naive CD4+ or CD8+ T cells isolated from these mice (Fig. 1F–H).

Deletion of the terminal cytoplasmic domain potentiates the ability of Tim3 to downregulate Tc1 cells

To study the functional effects of the Tim3 mutation in vitro, we crossed Tim3mut mice with OT-I and OT-II mice, which are transgenic for a TCR specific for an OVA peptide bound to class I and class II MHC, respectively. First, we assessed whether surface expression of the extracellular domain of Tim3 was normally upregulated on CD8+ and CD4+ T cells from Tim3mut mice after activation. CD8+ and CD4+ T cells were isolated from WT and Tim3mut OT-I and OT-II mice, and stimulated in vitro into Tc1 and Th1 cells using APCs and specific Ag. As expected, few naive T cells from WT and Tim3mut mice expressed Tim3. OT-II T cells required two rounds of in vitro polarization for maximal upregulation of Tim3 expression. We found no significant differences in the surface staining of the extracellular domain of the mutant form of Tim3 in either CD8+ or CD4+ T cells compared with WT (Fig. 2A, 2B).

Activation of Tim3 on T cells has been shown to suppress effector T cell functions such as proliferation and cytotoxicity. To assess the functional effects of this Tim3 mutation, we first compared proliferation of Tim3mut OT-I with WT OT-I T cells by CFSE dilution. Because Tim3 is also expressed on APCs, we stimulated the T cells with either WT or Tim3mut APCs. WT and Tim3mut naive CD8+ and CD4+ T cells, which express little to no Tim3, had normal proliferation and no differences were observed (data not shown). However, when we examined proliferation of activated effector CD8+ T cells at the time of peak Tim3 expression (day 5), there was a marked reduction in CD8+ T cell proliferation that was independent of Tim3 expression. CD8+ T cells from WT and Tim3mut mice were isolated from mice at day 5, coincubated with OVA-loaded EL4 target cells or unloaded cells, and cytotoxicity was assessed using a bioluminescent assay. This demonstrated a significant reduction in CD8+ T cell-mediated cytotoxicity by Tim3mut T cells when compared with WT T cells that was again independent of Tim3 expression on APCs (Fig. 2C). No differences were observed in CD4+ T cell proliferation in similar experiments (data not shown). We next assessed CD8+ T cell-mediated cytotoxicity by stimulating WT and Tim3mut OT-I T cells in vitro into effector T cells. CD8+ T cells were purified at day 5, coincubated with OVA-loaded EL4 target cells or unloaded cells, and cytotoxicity was assessed using a bioluminescent assay. This demonstrated a significant reduction in CD8+ T cell-mediated cytotoxicity by Tim3mut T cells when compared with WT T cells that was again independent of Tim3 expression on APCs (Fig. 2D). Taken together, these data suggest that deletion of the terminal cytoplasmic domain potentiates the ability of Tim3 to downregulate proliferation and cytotoxicity of Tc1 cells. This activity is independent of Tim3 expression on APCs and is not replicated in Th1 cells.

Deletion of the terminal cytoplasmic domain eliminates gal-9–mediated CD8+ T cell apoptosis

The activation and clonal expansion of T cells is followed by a death phase, during which the majority of effector T cells are eliminated (41). In addition to its inhibitory effects on proliferation and cytotoxicity, Tim3 has been demonstrated to mediate
T cell apoptosis (21). We therefore assessed whether there were differences in gal-9–mediated apoptosis by activating CD8+ T cells in vitro, incubating them alone or with 10 μM gal-9 for 6 h, then staining with Annexin V and propidium iodide. There were no differences in apoptosis at baseline between WT and Tim3mut CD8+ T cells. However, although gal-9 was able to induce apoptosis on WT T cells, it had no effect on Tim3mut T cells (Fig. 2E). These studies indicate that deletion of the terminal cytoplasmic domain results in loss of gal-9–mediated apoptosis, and that the negative regulatory and apoptotic functions of Tim3 may be differentially regulated.

Enhanced negative regulation of the Tc1 response is associated with decreased phosphorylation of the TCR–CD3ζ-chain

To confirm that the Tim3 mutation had altered signaling in CD8+ T cells, we assessed tyrosine phosphorylation by Western blot. WT and Tim3mut OT-I T cells were isolated, stimulated in vitro with WT APCs, and purified on day 5. The CD8+ T cells were then incubated alone or with 10 μM gal-9 for 2 or 5 min. Protein lysates were blotted and probed with an anti-phosphotyrosine Ab (4G10). As expected, there were no differences between naive WT and Tim3mut CD8+ T cells at baseline, when Tim3 is expressed on very few T cells. However, at day 5 after activation, there was appreciably decreased phosphorylation of a 23-kDa protein in Tim3mut T cells (Fig. 3A). Phosphorylation of this protein further decreased after 2 and 5 min of gal-9 stimulation. Similar experiments in WT and Tim3mut CD4+ T cells failed to demonstrate any appreciable differences in phosphorylation (data not shown).

Based on the 23-kDa size of the differentially phosphorylated protein, we hypothesized that this might represent the TCR–CD3ζ-chain. Inhibition of CD3ζ phosphorylation has been shown to reduce cytokine production and proliferation in T cells (42). To test whether the identified protein was CD3ζ, we repeated the experiment and then used an Ab to CD3ζ to immunoprecipitate the protein from cellular extracts. Precipitated protein was blotted and probed with 4G10. Phosphorylated CD3ζ typically results in
the presence of two bands between 18 and 23 kDa. The IP increased the sensitivity of the detection, allowing both phosphorylated forms of CD3ζ to be visualized. Loading was assessed by reprobing the blot with anti-CD3ζ Ab. These results clearly demonstrate that there is less CD3ζ phosphorylation in the activated Tim3mut OT-I T cells compared with activated WT OT-I T cells both before and after gal-9 stimulation (Fig. 3B). These findings confirm that signaling differences are induced by the Tim3 mutation. In addition, given the functional role of CD3ζ phosphorylation for T cell effector functions, these data suggest a novel mechanism for the negative regulatory activity of Tim3 on CD8+ effector T cells.

Tim3 expression is upregulated on T cells recruited to the lung after influenza infection

To explore whether Tim3 plays an important role in regulating the adaptive immune response during influenza infection, we infected WT mice with a dose of PR8 that is lethal in ∼80% (LD80) and

FIGURE 2. Deletion of the terminal cytoplasmic domain potentiates the ability of Tim3 to downregulate Tc1 lymphocytes. WT and Tim3mut OT-I and OT-II cells were stimulated in vitro and purified at day 5 or 10, respectively. Representative flow cytometry data of Tim3 surface expression on WT and Tim3mut (A) OT-I CD8+ T cells at days 0 (upper plots) and 5 (lower plots), and (B) OT-II CD4+ T cells at days 0 (upper plots) and 10 (lower plots). (C) Activated effector CD8+ T cell proliferation 72 h after purification assessed by CFSE dilution (two independent experiments; p < 0.05, two-way ANOVA). (D) CD8+ T cell-mediated cytotoxicity against Ag-loaded target cells or unloaded cells as a control assessed using a bioluminescent assay (two independent experiments; p < 0.05 and p < 0.01, two-way ANOVA). (E) Assessment of apoptosis by Annexin V+ staining before and after stimulation with 10 μM gal-9 for 6 h (one experiment; p < 0.05, Student t test).

FIGURE 3. Mutation of Tim3 results in decreased phosphorylation of the TCR–CD3ζ-chain. WT and Tim3mut OT-I cells were stimulated in vitro into effector cells and purified at day 5. Some samples were treated with 10 μM gal-9 for 2 or 5 min, and protein lysates were prepared. (A) Anti-phosphotyrosine (4G10) Western blot. Phosphorylation differences can be seen in a 23-kDa protein. β-actin is shown as a loading control. (Representative blots from two independent experiments). (B) Lysates were immunoprecipitated using an anti-CD3ζ Ab, blotted, and probed with 4G10. Phosphorylation differences can be seen in the 18- and 23-kDa bands. Total CD3ζ is shown as a loading control. Representative blots from two independent experiments.
determined Tim3 expression on CD8+ and CD4+ T cells in the lung, draining thoracic lymph node (tLN), and spleen at various time points. During influenza infection, priming of naive virus-specific T cells occurs within 72 h. Priming is followed by sustained proliferation and accumulation of large numbers of virus-specific effector T cells that traffic to the airways and lung parenchyma at days 6–7 postinfection (43–45). These cells continue to accumulate from days 7–10 postinfection, resulting in rapid clearance of virus (46, 47). In naïve animals, few T cells express Tim3. Upregulation of Tim3 expression is seen on T cells in both the tLN and lung by day 3 postinfection and peaks at day 7. Levels of Tim3 expression are greatest on CD8+ T cells in the lung, with nearly 40% of these cells expressing Tim3 at day 7 (Fig. 4, Supplemental Fig. 1). These results demonstrate significant upregulation of Tim3 on CD8+ T cells at the site of inflammation, suggesting that Tim3 may play an important role in modulating the effector functions of these cells during influenza infection.

**Tim3mut mice are protected from morbidity and mortality during influenza infection**

We next investigated the role of the Tim3 mutation in vivo by infecting WT and Tim3mut mice with LD80 influenza PR8. In this model, morbidity and mortality is dependent on immune-mediated injury to the lung manifest as a severe pneumonitis, and prior work has suggested that CD8+ effector T cells are one of the critical mediators of this immunopathology (6, 48, 49). Mice were followed for weight loss and survival for 28 d. Mortality in Tim3mut mice was significantly reduced compared with WT mice (Fig. 5A). In addition, Tim3mut mice lost significantly less weight than their WT counterparts (Fig. 5B). However, we found no significant differences in immunohistopathology on lung sections taken at days 3 or 7 after PR8 infection (Supplemental Fig. 2).

We therefore assessed the cellular infiltrate to the airways and lungs following PR8 infection. Analysis of BAL fluid and lung tissue at days 3 and 7 postinfection demonstrated no differences in mononuclear, neutrophilic, or T cell infiltration of the respiratory system, including the number of Ag-specific CD8+ T cells as assessed by staining with class I Tet (Fig. 5C–H, Supplemental Figs. 3, 4). We also did not detect differences in the number of NK cells in BAL fluid or lung tissue at day 3 postinfection (data not shown).

**FIGURE 4.** Tim3 expression is upregulated on cells during influenza infection. WT mice were infected with influenza PR8 and tissues collected at various time points. Percentages of Tim3-expressing (A) CD8+ T cells and (B) CD4+ T cells in the lungs, tLN, and spleen at days 0, 3, 7, and 10 following influenza infection (n = 3 mice/group per time point).

Both human and mouse studies have demonstrated that the magnitude of cytokines produced postinfluenza infection may determine the extent of immunopathology, so we next assessed the levels and expression of inflammatory cytokines. Although we found no significant differences between WT and Tim3mut mice at day 3 postinfection (Supplemental Fig. 3), at day 7, there was a trend toward lower expression of inflammatory cytokines in whole-lung homogenates from Tim3mut mice, with significantly less IFN-γ expression in Tim3mut mice after correction for multiple comparisons (p < 0.01; Fig. 6A). However, despite differences in cytokine RNA levels, we were unable to detect differences in cytokine protein levels in BAL fluid from WT and Tim3mut mice at day 3 or 7 postinfection (data not shown). Discrepancies between RNA levels of the cytokines versus protein levels may relate to compartmentalization of protein secretion to different lung structures (i.e., airway versus interstitium), accumulation of protein in the lung, or the sensitivity of the protein assay. These data demonstrate that deletion of the terminal cytoplasmic domain of Tim3 is protective against morbidity and mortality during infection with influenza A, and that this protection is associated with decreased IFN-γ RNA expression.

**Tim3mut CD8+ T cells have impaired effector functions**

Because Tim3 is expressed on multiple cell types that play an important role in the immune response to influenza, we wanted to further characterize the CD8+ T cell-specific effects. Because CD8+ and CD4+ T cells are major sources of many key inflammatory cytokines, we isolated these cells from lung tissue at day 7 postinfection. CD8+ T cells from Tim3mut mice expressed lower RNA levels of TNF, IFN-γ, and IL-6 (Fig. 6B). However, no differences in cytokine expression were observed in CD4+ T cells (data not shown). We further investigated these findings by isolating CD8+ T cells from lung tissue at day 7 postinfection and performing intracellular staining for IFN-γ. For these experiments, some mice were infected with a recombinant influenza A virus expressing OVA protein (WSN-OVA), whereas others were infected with PR8. Using Ag-specific stimulation of CD8+ T cells isolated from the lungs of mice infected with WSN-OVA or PMA and ionomycin stimulation of CD8+ T cells isolated from the lungs of mice infected with PR8, we were able to confirm lower levels of IFN-γ production in Tim3mut mice (Fig. 6C, 6D, Supplemental Fig. 4). Next, we examined in vivo CD8+ T cell proliferation in WT and Tim3mut mice. Mice were infected with BrdU at day 6 following influenza infection. Twenty-four hours later, lungs were collected, BrdU was labeled, and proliferation was assessed by flow cytometry. Consistent with our in vitro data, we found fewer BrdU+ CD8+ T cells in the lungs of Tim3mut mice compared with WT (Fig. 6E, Supplemental Fig. 4). Notably, because mice were injected with BrdU at day 6 postinfection, these experiments provide us with only a snapshot of effector T cell proliferation at the time of maximal Tim3 expression. Therefore, despite differences in the number of proliferating cells, we may not see differences in total cell numbers. Finally, to confirm that Tim3mut CD8+ T cells have impaired ability to mediate pneumonitis, we used a CD8+ T cell-specific model of severe lung injury. In this model, activated OT-I cells are adoptively transferred into a transgenic mouse that expresses membrane-bound OVA in airway lining cells (CC10-OVA) (36, 50). After transfer, OT-I cells are recruited into the lung where they proliferate and mediate lung injury, leading to severe pneumonitis and death. OT-I cells were isolated from WT and Tim3mut mice, activated into effector cells in vitro, and adoptively transferred into CC10-OVA mice by i.p. injection. The adoptively transferred OT-I cells are the only cell type affected...
by the Tim3 mutation, allowing us to specifically assess the role of the Tim3 mutation in regulating CD8+ T cell effector functions. Effector activity of CD8+ T cells is correlated with mortality in this model (36, 50). As shown in Fig. 6F, CC10-OVA mice that received Tim3mut OT-I cells had improved survival compared with mice that received WT OT-I cells, which is consistent with enhancement of the negative regulatory activity of Tim3 on these cells. Taken together, these data demonstrate that Tim3mut CD8+ T cells have decreased production of inflammatory cytokines, decreased proliferation, and decreased effector function compared with WT cells.

**Tim3mut mice have normal viral clearance**

We postulated that the reduced cytokine production and cytotoxicity of Tim3mut CD8+ T cells might lead to impaired viral clearance. To test this, we collected lungs from WT and Tim3mut mice following PR8 infection. Using QPCR to measure viral copies and a previously described MDCK infectivity assay to assess viral titer (39, 40), we found no differences in viral clearance between WT and Tim3mut mice (Fig. 7, Supplemental Fig. 3). These data demonstrate that Tim3mut CD8+ T cells have decreased production of inflammatory cytokines, decreased proliferation, and decreased effector function compared with WT cells.

**Tim3mut mice have intact functional heterotypic immunity**

Recently published data suggest that Tim3–gal-9 interactions may limit the magnitude and efficiency of CD8+ T cell memory responses (21, 51). We therefore assessed whether enhanced Tim3 activity would affect heterotypic memory. For these experiments, mice were infected with a nonlethal dose of x31 virus, an H3N2 subtype. Mice recovered for 4 wk and then were challenged with an otherwise lethal dose of PR8 (3 x LD80), an H1N1 subtype. Effective immunity depends on CD8+ T cell memory in this model. Tim3mut mice lost less weight than WT controls during the x31 infection, consistent with our findings in acute PR8 infection (Fig. 8A). Despite reduced CD8+ T cell effector function, Tim3mut mice had no weight loss and 100% survival similar to WT mice after secondary infection with PR8 (Fig. 8B). These data demonstrate that despite decreased effector functions of CD8+ T cells, functional heterotypic memory responses are intact in Tim3mut mice.

**Discussion**

These data clearly demonstrate that enhancement of the negative regulatory activity of Tim3 is protective against morbidity and mortality in a murine model of influenza infection. This protection is associated with decreased CD8+ T cell effector function including proliferation, cytotoxicity, and inflammatory cytokine expression. Experiments using an adoptive transfer model of pneumonitis confirm that the effects of the Tim3 mutation specifically on CD8+ T cells can limit lung injury and mortality. Despite the decrease in CD8+ T cell effector function, viral clearance and functional heterotypic immunity remain intact. These findings suggest that enhancing the inhibitory activity of
Tim3 may be beneficial in reducing immune-mediated lung injury postinfluenza infection. Despite extensive characterization of its regulatory function, the signaling mechanisms of Tim3 remain largely undefined. Tim3 has a well-established role in inhibiting effector T cell function (52, 53). However, unlike other molecules that have been shown to inhibit effector T cells, there are no obvious inhibitory signaling motifs in the cytoplasmic tail. The cytoplasmic tail of Tim3 is considerably more complex than that of other Tim family members, containing five tyrosine residues that are good candidates for phosphorylation and several predicted serine/threonine kinase binding sites (54, 55). One of these tyrosine residues (human Y265; murine Y256) is phosphorylated after gal-9 binding by the tyrosine kinase Itk (29). This residue is intact and presumably available for signaling in our mutant construct. By deleting the terminal cytoplasmic domain of Tim3, we have demonstrated several novel aspects of Tim3 signaling. First, we have demonstrated that signaling through Tim3 involves not only the highly conserved Y256 but also occurs in the terminal cytoplasmic domain. Further, we have shown that deletion of the terminal cytoplasmic domain potentiates the ability of Tim3 to downregulate Tc1 effector functions, suggesting that these residues are negative regulators of Tim3 function.

In a recent publication, a Tim3 truncation mutation similar to ours was generated to examine the role of cytoplasmic tyrosine residues in coupling T cell activation with downstream signaling events (30). Consistent with our data, this mutant construct demonstrated enhanced Tim3 signaling and activity relative to WT Tim3, confirming that the distal tyrosine residues of the cytoplasmic tail have negative regulatory activity on Tim3 signaling. Interestingly, their data also suggested that Tim3 might play a role in T cell activation. However, it should be noted that these experiments used an ectopic expression system whereby Tim3 was expressed at high levels on naive T cells before activation. This is not the case with primary naive T cells or naive T cells in vivo, which require several days of activation before there is significant upregulation of Tim3. It is well documented that TCR signaling is different in naive T cells compared with T cells after activation.

FIGURE 6. *Tim3mut* CD8+ T cells express lower levels of inflammatory cytokines during influenza infection. Cytokine expression by QPCR in (A) whole-lung homogenates (*n* = 12 mice/group from 3 independent experiments; *p* < 0.01, two-way ANOVA after correction for multiple comparisons) and (B) CD8+ T cells isolated from lungs (*n* = 6 mice/group from one experiment; *p* < 0.05, *p* = 0.09, and *p* < 0.05, Student *t* test) of WT and *Tim3mut* mice at day 7 following influenza infection. Intracellular staining for IFN-γ production by CD8+ T cells isolated from lungs at day 7 (C) postinfection with WSN-OVA, and stimulation with OVA peptide (*n* = 3 mice/group; *p* < 0.05, two-way ANOVA), and (D) postinfection with PR8 and stimulation with PMA and ionomycin (*n* = 3 mice/group; *p* < 0.01, two-way ANOVA after correction for multiple comparisons; experiment was repeated with similar results). (E) In vivo proliferation assessed at day 7. Numbers of CD8+ BrdU+ T cells from whole-lung homogenates are shown (two independent experiments, *n* = 4 mice/group; *p* < 0.05, Student *t* test). (F) Survival of CC10-OVA mice after adoptive transfer of activated effector WT or *Tim3mut* OT-I cells (*n* = 7 mice/group from two independent experiments; *p* < 0.005, log-rank test).

FIGURE 7. *Tim3mut* mice have normal viral clearance. Viral titers in WT and *Tim3mut* mice at day 7 following influenza infection as assessed by (A) QPCR (*n* = 12 mice/group from 3 independent experiments) and (B) MDCK infectivity assay (*n* = 10 mice/group from 2 independent experiments).
Thus, we suspect that both mutants have enhanced Tim3 activity, but the effects of Tim3 are different in naïve T cells during activation versus effector T cells at the peak of the inflammatory response. Given the expression pattern of Tim3 in vivo, it seems likely that the predominant effect of enhanced Tim3 signaling will be on effector T cells, resulting in an anti-inflammatory role during viral infection.

The signaling differences we observed in effector T cells are associated with decreased phosphorylation of TCR–CD3ζ. Work by others has recently demonstrated that much like Tim1, Tim3 activity is coupled to TCR signaling pathways, and that Tim3 interacts with multiple proteins involved in TCR signal transduction (30, 59). However, to our knowledge, our studies are the first to suggest a potential interaction between Tim3 and signaling through CD3ζ. Interestingly, recent data suggest that Tim3 may be phosphorylated by the tyrosine kinase Lck, which also phosphorylates CD3ζ (42). Phosphorylation of CD3ζ provides a critical link between Ag recognition by the TCR and diverse intracellular signal-transduction pathways. In addition, it has been suggested that CD3ζ plays a role in controlling T cell survival (60), and inhibition of CD3ζ phosphorylation has been shown to reduce cytokine production and proliferation in T cells (42).

Furthermore, chronic immune dysfunction in diseases such as HIV, chronic HCV, and certain malignancies has been associated with loss of expression of CD3ζ and with increased expression of Tim3 on T cells (20, 60–62). Thus, downregulation of CD3ζ phosphorylation by Tim3 is one potential mechanism by which Tim3 may mediate its counterregulatory effects on T cells.

Although this mutation enhanced the suppressive function of Tim3, it eliminated gal-9–mediated apoptosis, suggesting that the distal cytoplasmic portion of Tim3 plays an important role in this process. Apoptosis of T cells is a highly regulated and complicated process separate from activation (41). Although some studies have demonstrated that gal-9 can induce CD8+ T cell apoptosis (21), in disease processes such chronic viral infection Ag-specific CD8+ T cells that express high levels of Tim3 persist despite the presence of gal-9, suggesting that they are not susceptible to gal-9–mediated cell death (20, 63). These cells have been described as exhausted, a process by which highly activated effector cells are rendered dysfunctional through hierarchical loss of effector functions. Tim3 has been functionally linked to this process in studies that demonstrate that blockade of Tim3 results in restoration of effector functions (20, 61). Thus, it would appear that Tim3 is able to mediate its inhibitory effects separately from its effects on apoptosis. Our Tim3 mutation may provide important clues as to why exhausted T cells can persist.

Influenza primarily infects respiratory epithelial cells, leading to their necrosis (64). The resulting inflammation promotes matura-

**FIGURE 8.** Tim3mut mice have preserved functional heterotypic immunity. WT and Tim3mut mice were infected with influenza x31, allowed to recover for 4 wk, then reinfected with an otherwise lethal dose of influenza PR8. (A) Weight loss as a percentage of original weight following infection with influenza x31 (n = 4 mice/group; p < 0.001, two-way ANOVA; experiment was repeated with similar results). (B) Weight loss as a percentage of baseline after rechallenge with an otherwise lethal dose of influenza A/PR8 (n = 4 mice/group; experiment was repeated with similar results).
mutation did not result in any appreciable phenotypic or signaling differences in CD4+ T cells. Furthermore, our in vitro studies did not show an effect of our Tim3 mutation on APCs, and we did not detect any differences in cellular infiltrate or IFN-γ expression in our in vivo influenza model at day 1 (data not shown) or day 3, during which time inflammation is primarily driven by the innate immune system.

In summary, we have shown that Tim3 is an important regulator of the adaptive immune response to influenza and that enhanced Tim3 activity can protect against morbidity and mortality postinfection. Experiments performed both in vitro and in vivo demonstrate a significant defect in effector functions of Tim3+/+ CD8+ T cells, suggesting that enhancing Tim3 activity may reduce immune-mediated lung injury postinfluenza infection, in part, because of inhibition of CD8+ T cell effector function. Finally, our experiments suggest a potentially novel mechanism for Tim3-mediated downregulation of T cell effector function via alteration of CD3ζ phosphorylation.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental Material

Enhanced Tim3 Activity Improves Survival After Influenza Infection$^{1,2,3}$

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SUPPLEMENTAL FIGURE LEGEND

Figure S1: Representative flow cytometry for Tim3 expression during influenza infection. Tim3 expression is shown on CD8\(^+\) and CD4\(^+\) T cells at day 7 following infection in the lung, draining thoracic lymph node (tLN) and spleen.

Figure S2: Histopathology following influenza infection. Representative lung histology from WT and \(Tim3^{mut}\) mice at day 3 (A and B, respectively) and 7 (C and D) after influenza infection. Lung sections are stained with H&E and shown at 20X power. Bars denote 50 \(\mu m\).

Figure S3: Response to influenza infection at day 3 in WT and \(Tim3^{mut}\) mice
WT and \(Tim3^{mut}\) mice were infected with influenza PR8 and tissues were collected at day 3 post infection. Percentages and absolute numbers of cells in the BAL (A and D, respectively), lungs (B and E) and cytospin preparations (C and F) from WT and \(Tim3^{mut}\) mice at day 3 after influenza infection (n=9 mice per group from 2 independent experiments). G - Cytokine expression by QPCR in whole lung homogenates at day 3 after influenza infection (n=9 mice per group from 2 independent experiments). H - Viral titers at day 3 after influenza infection as assessed by QPCR on whole lung homogenates (n=9 mice per group from 2 separate experiments). I – Time course of viral clearance in WT and \(Tim3^{mut}\) mice. QPCR was performed on whole lung homogenates from days 3, 7, 10 and 14 (n=3-12 mice per group per time point).
**Figure S4: Representative flow cytometry data from influenza experiments.**

WT and *Tim3<sup>mut</sup>* mice were infected with influenza PR8. Lungs were collected and analyzed at day 7 post-infection for A – influenza nucleoprotein 366-374-specific CD8<sup>+</sup> T cells (Tet), IFN<sub>γ</sub> expression by intracytoplasmic staining B – using antigen-specific stimulation after WSN-OVA<sub>i</sub> infection and C – using PMA and ionomycin stimulation after PR8 infection, and D – proliferation by BrdU.
Figure S1

Lung

ILN

Spleen

CD8$^+$ lymphocytes 39.3%

CD4$^+$ lymphocytes 11.4%

8.2%

2.1%

7.2%

4.6%

Tim3

Tim3

Tim3

Tim3

Tim3

Tim3
Figure S2

A  WT

B  Tim3<sup>mut</sup>

C

D  Day 7

Day 3
Figure S3
Figure S4