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Inflammatory impact of IFN-\(\gamma\) in CD8\(^+\) T cell-mediated lung injury is mediated by both Stat1-dependent and -independent pathways

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SIGNIFICANT LUNG INJURY frequently accompanies influenza viral infection, which is mediated by the direct effects of the virus and is a result of host immune responses. While CD8\(^+\) T cells are important for influenza viral clearance (11), it is believed that these cells can contribute to this injury, as dysregulated CD8\(^+\) T cell responses are associated with enhanced pulmonary pathology during influenza infection in mice and humans (5, 17). However, it is difficult to separate the direct effects of the viral infection on lung pathology from the effects of the CD8\(^+\) T cell effector functions, such as cytotoxicity and production of the proinflammatory cytokines IFN-\(\gamma\) and TNF-\(\alpha\). Using a transgenic mouse model of influenza infection in which influenza hemagglutinin (HA) is expressed by lung epithelial cells (6), we have identified critical mechanisms by which CD8\(^+\) T cells mediate influenza immunopathology in the absence of replicating virus. We previously demonstrated that, following alveolar antigen recognition, TNF-\(\alpha\) production by HA-specific CD8\(^+\) T cells induced lung epithelial cell chemokine expression, which resulted in extensive airway inflammation and lethal lung injury (4, 32).

While the role of TNF-\(\alpha\) production by CD8\(^+\) T cells in mediating influenza immunopathology is well characterized, the role of IFN-\(\gamma\)-production by CD8\(^+\) T cells in acute lung injury during influenza infection is equivocal. IFN-\(\gamma\) production by influenza-specific CD8\(^+\) T cells has been shown to mitigate the extent of lung injury during infection (31), and IFN-\(\gamma\) expression is important for a protective recall response against secondary challenge with influenza virus (2). The protective effects of IFN-\(\gamma\) during infection may be due in part to direct stimulatory effects of IFN-\(\gamma\) on CD8\(^+\) T cell responses (30). In contrast, antibody neutralization of IFN-\(\gamma\) during influenza infection has been shown to reduce the extent of influenza-specific CD8\(^+\) T cell-mediated lung injury and the magnitude of inflammatory cell infiltration into the lungs (1, 18). Moreover, IFN-\(\gamma\) production by virus-specific CD8\(^+\) T cells has been implicated in mediating immunopathology during respiratory syncytial viral infection (19), and IFN-\(\gamma\) is thought to have an important role in the development of lung injury during severe acute respiratory disease (12). Importantly, IFN-\(\gamma\) deficiency does not appear to impair influenza viral clearance (10), suggesting that IFN-\(\gamma\) may primarily function to dampen or exacerbate immunopathology during influenza infection.

The biological effects of IFN-\(\gamma\) are mainly mediated by rapid activation of the Jak/Stat1 pathway and the subsequent
changes in gene expression (21). The role of Stat1 in regulating IFN-γ-mediated immunopathology during influenza infection is unclear, as Stat1 deficiency during influenza infection results in the inability of the host to control viral replication and systemic spread of the virus (8). IFN-γ production by CD8+ T cells has been shown to activate Stat1 in neurons and mediate loss of dendrites and synapses (14). However, Stat1 deficiency has been shown to exacerbate pathogenicity in a model of central nervous system disease (29), suggesting that Stat1 may mediate anti-inflammatory effects, in addition to its obvious antiviral role. The increased pathology in the absence of Stat1 may be due in part to Stat1-independent IFN-γ signaling (9), as loss of Stat1 results in enhanced and sustained IFN-γ-induced Stat3 activation (23). Overexpression of Stat3 in lung epithelial cells results in enhanced inflammation during tumorigenesis (15), while loss of lung epithelial cell Stat3 expression attenuates allergic airway inflammation (26). The balance of Stat1 and Stat3 activation has been proposed to have profound effects on cytokine gene expression and inflammation (24), suggesting that Stat1-independent IFN-γ signaling may result in alternative activation of Stat3 and increased pulmonary inflammation.

In this study we investigated the roles of Stat1-dependent and -independent IFN-γ signaling in CD8+ T cell-mediated influenza immunopathology in the absence of replicating virus. Using an adoptive transfer model, in which wild-type (WT) or IFN-γ-deficient HA-specific CD8+ T cells were transferred into WT or Stat1-deficient HA-transgenic mice, we identified important roles for Stat1-dependent and -independent IFN-γ signaling in mediating the extent of CD8+ T cell influenza immunopathology.

METHODS

Mice. BALB/c mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice lacking IFN-γ [gene knockout (GKO)] were kindly provided by Dr. Thomas Braciale. Mice lacking Stat1 (Stat1−/−) were kindly provided by Dr. Joan Durbin. HA-transgenic mice expressing the influenza HA of A/Japan/57 virus in the lung are described elsewhere (4). To generate HA-transgenic mice lacking Stat1, HA-transgenic mice were bred with Stat1−/− mice. All animal studies were conducted in accordance with guidelines approved by the Institutional Animal Care and Use Committee at Geisel School of Medicine at Dartmouth University.

CD8+ T cell generation and adoptive transfer. WT and GKO CD8+ T cells specific for HA210–219 (a Kd-restricted epitope of A/Japan/57 HA; hereafter referred to as HA210) were generated as previously described (4) and restimulated in vitro with HA210–219 peptide-pulsed, irradiated syngeneic splenocytes to maintain bulk cultures of HA210-specific CD8+ T cells. HA210-specific CD8+ T cells were adaptively transferred into HA-transgenic mice by tail vein injection. In some experiments, CD8+ T cells were labeled with carboxyfluorescein succinimidyl ester prior to transfer to monitor T cell trafficking to the lung. Morbidity (as measured by weight loss) was monitored daily, and loss of ≥20% of the initial body weight was used as an end point in accordance with our Institutional Animal Care and Use Committee-approved protocol.

Bronchoalveolar lavage and tissue preparation. At the appropriate time points, lungs were lavaged in situ as previously described (4). The recovered fluid was centrifuged, and the supernatant was analyzed for cytokines and chemokines by Millipore mouse 32 Plex Luminox assay performed by DartLab (Lebanon, NH). Albumin concentrations in the bronchoalveolar lavage fluid (BALF) were determined by ELISA (Bethyl Laboratories, Montgomery, TX). Cells were counted on a hemocytometer with Trypan blue exclusion to determine the total number of viable cells, and cell populations were enumerated using the PROTOCOL Hema 3 stain set (Fisher, Houston, TX). For assessment of lung histology, lungs were inflated with 0.5% low-melting-point agarose in PBS and fixed in formalin, and sectioned slices were stained with hematoxylin-eosin. For primary lung type II cell gene and protein expression, cells were purified as described previously (16), and Western blot analysis was performed.

Mouse lung epithelial cell culture. Mouse lung epithelial (MLE) cells were cultured with HA210–219 peptide and cultured with HA210-specific CD8+ T cells. Alternatively, MLE cells were cultured with mouse recombinant TNF-α and IFN-γ (Biolegend, San Diego, CA). ELISA was used to analyze cytokine and chemokine production. Gene expression was analyzed as previously described (22).

Statistics. Statistical analysis was performed with GraphPad Prism (GraphPad Software, La Jolla, CA). Two-tailed unequal t-test with 95% confidence interval was used to analyze differences between groups, while two-way ANOVA with 95% confidence interval was used to analyze differences between groups over time.

RESULTS AND DISCUSSION

CD8+ T cell expression of IFN-γ enhances lung epithelial cell chemokine expression. First, using an in vitro system that reflects the interaction between CD8+ T cells and lung epithelial cells in vivo, we sought to characterize the effects of IFN-γ production by CD8+ T cells on lung epithelial cell function. MLE cells were pulsed with HA210–219 peptide and cultured with HA210-specific WT or GKO CD8+ T cells. As expected, there was no detectable production of IFN-γ by activated GKO CD8+ T cells, in contrast to WT cells (Fig. 1A). However, GKO CD8+ T cells were not impaired in other examined effector functions, including TNF-α production (Fig. 1A) and cytotoxicity (Fig. 1B). Loss of CD8+ T cell production of IFN-γ abrogated MLE production of IFN-inducible chemokines, IFN-γ-inducible protein-10 (IP-10) and monokine induced by IFN-γ (MIG), and resulted in reduced production of CCL2 and CXCL2 (Fig. 1C). IFN-γ and TNF-α appeared to act synergistically to induce MLE expression of CCL2 and CXCL2, as treatment of MLEC cells with TNF-α and IFN-γ increased production of CCL2 and CXCL2 compared with treatment with TNF-α alone (Fig. 1D). Furthermore, IFN-γ, but not TNF-α, was required for the induction of guanylate-binding protein and GTPase gene expression in MLE cells (Fig. 1, D and E). GTPases are localized to the endoplasmic reticulum and Golgi compartments and have been shown to have important roles in chemokine secretion (25, 27), suggesting that CD8+ T cell-produced IFN-γ functions synergistically with TNF-α to enhance lung epithelial cell chemokine release.

CD8+ T cell expression of IFN-γ enhances acute lung injury. Next, we examined the role of IFN-γ production by influenza-specific CD8+ T cells in the development of pulmonary pathology in a CD8+ T cell-mediated model of acute lung injury. We adoptively transferred HA210-specific WT or GKO CD8+ T cells into HA-transgenic mice. We observed a reduction in the total number of inflammatory cells infiltrating the airways and lung parenchyma, with attenuated alveolar damage on histology in GKO recipients, indicating that IFN-γ production by the transferred CD8+ T cells was critical for the full extent of pulmonary pathology (Fig. 2A). GKO recipients also exhibited reduced weight loss morbidity and increased oxygen saturation capability compared with WT recipients (data not shown). To confirm that reduced CD8+ T cell trafficking to the lung in GKO recipients was not responsible
for the attenuated injury that occurred in these mice, we labeled HA210-specific CD8\(^+\) T cells with carboxyfluorescein succinimidyl ester prior to transfer. We observed similar numbers of WT and GKO HA210-specific CD8\(^+\) T cells in the lung 24 h after transfer, suggesting that these cells had similar ability to traffic to the lung (Fig. 2B). Moreover, there were similar levels of TNF-\(\alpha\) in the airways of WT and GKO recipients 24 h after transfer, suggesting that these cells had similar capacity to recognize alveolar antigen and mediate effector activities (Fig. 2C). As expected, IFN-\(\gamma\) was not detected in GKO recipients, indicating that the transferred CD8\(^+\) T cells were the primary source of IFN-\(\gamma\) (Fig. 2D). We previously showed that expression of chemokines by alveolar epithelial cells is critical for CD8\(^+\) T cell-mediated acute lung injury (4, 33), and we observed significantly reduced levels of IP-10, MIG, CCL2, and CXCL2 in GKO recipients compared with WT recipients (Fig. 2E). Preliminary data also demonstrated greater induction of guanylate-binding protein and GTPases in lung epithelial cells in WT than GKO recipients (data not shown), indicating that CD8\(^+\) T cell production of IFN-\(\gamma\) increased chemokine release by lung epithelial cells, resulting in enhanced recruitment of inflammatory cells and the subsequent exacerbated pulmonary pathology.

Stat1 deficiency enhances CD8\(^+\) T cell-mediated acute lung injury independent of its antiviral activities. Since the biological effects of IFN-\(\gamma\) are primarily mediated by the Stat1 pathway (21) and we observed reduced Stat1 gene expression in GKO recipients compared with WT recipients (data not shown), we generated HA-transgenic mice that lacked Stat1 to examine whether Stat1-dependent IFN-\(\gamma\) signaling was required to mediate the full extent of CD8\(^+\) T cell-mediated lung injury. Because of the specific constraints of studying the specific impact of Stat1 deficiency in a viral infection (in which control of viral replication is severely impaired and it is impossible to control for antigen load), this model enabled us to demonstrate the specific impact of Stat1 deficiency on CD8\(^+\) T cell-mediated pulmonary immunopathology. To our surprise, we found that Stat1 deficiency resulted in enhanced morbidity and eventual death of all animals in the experiment at an otherwise nonlethal dose (in Stat1-sufficient animals) of transferred HA210-specific WT CD8\(^+\) T cells (Fig. 3A). Transfer of as few as 10\(^6\) HA210-specific CD8\(^+\) T cells was sufficient to induce death in Stat1\(^{-/-}\) HA-transgenic mice, whereas a CD8\(^+\) T cell transfer of \(\geq 10^7\) times more cells (10\(^7\)) was required for comparable inflammatory influx and lung injury in WT HA-transgenic mice (6). Stat1\(^{-/-}\) HA-transgenic mice exhibited...
enhanced infiltration of the airways and lung interstitium, with increased disruption of the alveolar air space, and this was dependent on IFN-γ production by the transferred CD8+ T cells, as lung injury was abrogated in Stat1−/− HA-transgenic recipients of GKO CD8+ T cells (Fig. 3B). Unlike viral infection, CD8+ T cell recognition of nonviral antigen expressed by alveolar epithelial cells does not result in type I IFN expression, further indicating that CD8+ T cell expression of IFN-γ is absolutely required for this process. Consistent with the enhanced lung injury observed on histology, levels of albumin, a marker of vascular leakage, in the airways were elevated (Fig. 3C) and peripheral oxygen saturation was reduced (data not shown) in Stat1−/− HA-transgenic mice compared with WT HA-transgenic mice. These data indicate that Stat1 has an important role in regulating the extent of immunopathology, in addition to its obvious role in antiviral immunity, and underscore a critical proinflammatory role for Stat1-independent IFN-γ signaling.

Stat1 deficiency enhances airway inflammatory responses. Next we sought to characterize the inflammatory responses that contributed to the enhanced morbidity and mortality in Stat1−/− HA-transgenic mice. The total number of cells recovered from the airways of Stat1−/− HA-transgenic mice increased 24 and 48 h after transfer of CD8+ T cells (Fig. 4A). While there were no changes in the frequency of macrophages, there were increases in the frequency of neutrophils and eosinophils in the airways of Stat1−/− HA-transgenic mice compared with WT HA-transgenic mice (Fig. 4B–D), and there were significant increases in the absolute number of macrophages, neutrophils, and eosinophils in Stat1−/− HA-transgenic mice compared with WT HA-transgenic mice (data not shown). We also observed an increase in matrix metallopro-
teinase 9 gene expression in the lungs of Stat1−/− HA-transgenic mice (data not shown), consistent with the enhanced neutrophil response in these mice, as expression of matrix metalloproteinase 9 by neutrophils has been shown to be important for neutrophil migration into the respiratory tract and the extent of lung injury during influenza infection (3). To elucidate the mechanisms contributing to the enhanced cellular responses in Stat1−/− HA-transgenic mice, we examined TNF-α and IFN-γ production by the transferred CD8+ T cells. There were no significant differences in the total levels of TNF-α and IFN-γ between Stat1−/− HA-transgenic and WT HA-transgenic mice (Fig. 4E), suggesting that HA210-specific CD8+ T cells were similarly capable of trafficking to the lungs, recognizing antigen, and being activated to express effector activities in WT and Stat1−/− HA-transgenic mice. Taken together, these data demonstrate that, following the induction of similar effector responses by the transferred CD8+ T cells, loss of Stat1 in the recipient mice results in an increase in airway inflammatory responses, which contributes to the enhanced pulmonary pathology.

Stat1 deficiency results in sustained Stat3 activation in lung epithelial cells and altered chemokine expression. As we previously described a critical role for lung epithelial cells in mediating lung injury following CD8+ T cell transfer (33), we next examined lung epithelial cell responses in Stat1−/− HA-transgenic mice. Stat1-dependent genes, such as suppressor of cytokine signaling 1, were ablated in the lung epithelial cells in Stat1−/− HA-transgenic mice following CD8+ T cell transfer (data not shown). Interestingly, in the absence of Stat1, we observed enhanced and prolonged Stat3 signaling in lung epithelial cells recovered from Stat1−/− HA-transgenic mice compared with WT HA-transgenic mice (Fig. 4F). Enhanced phosphorylation and sustained activation of Stat3 in lung epithelial cells of Stat1−/− HA-transgenic mice were evident 6 h after CD8+ T cell transfer, indicating that Stat3 activation in the Stat1−/− HA-transgenic mice was likely mediated by IFN-γ produced by the transferred CD8+ T cells (within 5–6 h after transfer). This is consistent with previous studies that have shown that IFN-γ activates Stat3 rapidly and in a sustained manner in Stat1−/− mouse embryonic fibroblasts (20, 23). As IFN-γ is absolutely required for CD8+ T cell-mediated lung injury in Stat1−/− HA-transgenic mice and Stat3 has been implicated in mediating airway inflammation (15, 26), it is likely that alternative activation of Stat3 by IFN-γ contributes to the dysregulated inflammatory responses in Stat1−/− HA-transgenic mice. Lung epithelial cell production of IP-10 and MIG was abrogated in Stat1−/− HA-transgenic mice, indicating that IFN-γ signaling through Stat1 was required for expression of these chemokines (Fig. 4G). CCL2 and CXCL2 expression was also reduced in Stat1−/− HA-transgenic mice (Fig. 4G). In contrast, levels of eotaxin were significantly increased in the airways of Stat1−/− HA-transgenic mice (Fig. 4I), consistent with the enhanced eosinophil response in these mice. Eotaxin release by airway smooth muscle cells has been shown to be dependent on Stat3 activation (7), and loss of lung epithelial cell Stat3 expression attenuates eosinophil airway infiltration during asthma (26), indicating that the enhanced eotaxin expression in Stat1−/− HA-transgenic mice may be due in part to the dysregulated Stat3 signaling in lung epithelial cells. We also observed increased levels of granulocyte-colony stimulating factor in the airways of Stat1−/− HA-transgenic mice (Fig. 4H), which may have contributed to the enhanced neutrophil mobilization observed in these mice. Thus it is likely that alternative activation of Stat3 regulates lung epithelial inflammatory gene expression in Stat1−/− HA-transgenic mice, resulting in severe lung injury.

Fig. 3. Stat1 deficiency exacerbates CD8+ T cell-mediated lung injury. A: survival of WT and Stat1−/− HA-transgenic mice after adoptive transfer of 2.5 × 106 HA210-specific WT CD8+ T cells. B: representative hematoxylin-eosin-stained whole lung histology 5 days after transfer of 2.5 × 106 HA210-specific WT CD8+ T cells into WT (top) or Stat1−/− (middle) HA-transgenic mice or transfer of 2.5 × 106 HA210-specific GKO CD8+ T cells into Stat1−/− HA-transgenic mice (bottom). C: BALF concentration of albumin 48 h after CD8+ T cell transfer. Values are means ± SD of 3 independent experiments with 2–4 mice per group.
Taken together, our data indicate that Stat1-dependent and -independent IFN-γ signaling mechanisms mediate CD8+ T cell-mediated immunopathology following influenza alveolar antigen recognition, in the absence of the complicating factor of replicating virus. While direct comparison of the impact of IFN-γ and Stat1 deficiencies on pulmonary pathology was complicated by the fact that different doses of CD8+ T cells were used in the two different models, our data show that in the presence of Stat1, IFN-γ and TNF-α produced by effector CD8+ T cells act synergistically to increase lung epithelial cell release of chemokines and enhance airway inflammatory cell infiltration and 2) in the absence of Stat1, IFN-γ signaling results in sustained Stat3 activation, increased recruitment of neutrophils and eosinophils to the airways, and lethal pulmonary pathology. While Stat3 binding to the IFN-γ receptor compensates for the deficiency of Stat1 binding, the net result is enhanced immunopathology. Interestingly, influenza virus is capable of inhibiting IFN-γ-induced Stat1 activation (28), but it is unknown whether the Stat3 pathway is also affected. It is tempting to speculate that viral interference with IFN-γ-induced Stat1 activation may skew the response toward enhanced and prolonged Stat3 activation and exacerbated pulmonary pathology. In contrast, it is also possible that type I IFN signaling in lung epithelial cells during influenza infection may trigger Stat1 activation, limiting alternative Stat3 activation and abrogating injury early after infection. However, the kinetics of type I IFN expression and the CD8+ T cell response to the virus may still permit the mechanisms described here to exacerbate pulmonary pathology, as type I IFN expression peaks shortly after infection (days 2–3) (13), while the peak CD8+ T cell response occurs later (days 8–10). Therefore, the presence of early Stat1 activation by type I IFN may not be sufficient to prevent the Stat1-dependent or -independent lung injury mediated by CD8+ T cell expression of IFN-γ following influenza infection. It has not been previously possible to isolate the specific impact of Stat1 deficiency on the immunopathology associated with immune-mediated viral clearance, since Stat1+/− mice are exquisitely permissive to viral replication. This study is the first to specifically demonstrate that Stat1 deficiency has an important impact on T cell-mediated immunopathology, independent of the impact on antiviral activities. Additionally, given our ob-
ervations that IFN-γ expression by influenza-specific CD8+ T cells largely serves a proinflammatory role, independent of any antiviral functions, and that IFN-γ expression by T cells is dispensable for influenza viral clearance, IFN-γ may be a novel target to limit the extent of immunopathology during acute influenza infection.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are disclosed by the authors.

AUTHOR CONTRIBUTIONS

C.V.R., M.P.D., J.E.D., and R.I.E. developed the concept and designed the research; C.V.R., M.P.D., and C.S.A. performed the experiments; C.V.R., M.P.D., A.K., C.S.A., and R.I.E. analyzed the data; C.V.R., M.P.D., C.S.A., J.E.D., and R.I.E. interpreted the results of the experiments; C.V.R., M.P.D., and C.S.A. prepared the figures; C.V.R. and M.P.D. drafted the manuscript; C.V.R., M.P.D., A.K., J.E.D., and R.I.E. edited and revised the manuscript; C.V.R., M.P.D., A.K., C.S.A., J.E.D., and R.I.E. approved the final version of the manuscript.

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