Interference with Intraepithelial TNF- α Signaling Inhibits CD8⁺ T-Cell-Mediated Lung Injury in Influenza Infection

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Abstract

CD8⁺ T-cell-mediated pulmonary immunopathology in respiratory virus infection is mediated in large part by antigen-specific TNF- α expression by antiviral effector T cells, which results in epithelial chemokine expression and inflammatory infiltration of the lung. To further define the signaling events leading to lung epithelial chemokine production in response to CD8⁺ T-cell antigen recognition, we expressed the adenoviral 14.7K protein, a putative inhibitor of TNF- α signaling, in the distal lung epithelium, and analyzed the functional consequences. Distal airway epithelial expression of 14.7K resulted in a significant reduction in lung injury resulting from severe influenza pneumonia. *In vitro* analysis demonstrated a significant reduction in the expression of an important mediator of injury, CCL2, in response to CD8⁺ T-cell recognition, or to TNF- α . The inhibitory effect of 14.7K on CCL2 expression resulted from attenuation of NF- κ B activity, which was independent of I κ -B α degradation or nuclear translocation of the p65 subunit. Furthermore, epithelial 14.7K expression inhibited serine phosphorylation of Akt, GSK-3 β , and the p65 subunit of NF- κ B, as well as recruitment of NF- κ B for DNA binding *in vivo*. These results provide insight into the mechanism of 14.7K inhibition of NF- κ B activity, as well as further elucidate the mechanisms involved in the induction of T-cell-mediated immunopathology in respiratory virus infection.

Introduction

INFECTION WITH RESPIRATORY VIRUSES RESULTS IN CONSIDERABLE PULMONARY IMMUNOPATHOLOGY, a major component of which appears to result from the host-specific immune responses (5,21,30). CD8⁺ T cells infiltrate the lung in response to respiratory virus infection such as influenza (18,20). The function of CD8⁺ T cells in viral defense is mediated by several effector activities, including perforin and Fas Ligand (FasL)-mediated cytotoxicity, as well as secretion of cytokines such as TNF- α and IFN- γ that are involved in antiviral responses (35,36). CD8⁺ T-cell functions are critical for the resolution of respiratory virus infection, though they play an important role in tissue injury as well (26). Despite intensive investigation, the degree to which the antiviral and immunopathological effector mechanisms are separable remains

unclear. We have previously shown that CD8⁺ T-cell recognition of alveolar epithelial antigen results in severe lung inflammation, which is dependent on antigen-specific expression of TNF- α by effector T cells, triggering epithelial cell production of a variety of inflammatory mediators (11,42,45). Excessive chemokine production is known to result in inflammatory infiltration that can lead to tissue injury and organ failure (6,7,41), which we observed in our model. Among these chemokines, CCL2 (MCP-1) plays a particularly important role in the recruitment of inflammatory monocytes and lymphocytes, a key event in many inflammatory processes (8,34), and we have shown that its inhibition *in vivo* significantly abrogates CD8⁺ T-cell-mediated lung injury (45). Influenza virus infection induces CCL2 expression in lung epithelial cells that directs trans-epithelial migration of monocytes (3,17). The enhanced inflammatory response and

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leukocyte recruitment occurring after infection with highly pathogenic strains of influenza has been suggested to be dependent on TNF- and i-NOS-producing dendritic cells, as well as the CCR2 receptor (1,23). Epithelial CCL2 is rapidly induced in response to CD8⁺ T-cell recognition *in vitro* and *in vivo* (32,43,45), and appears to require both TNF receptors, TNF-R1 and TNF-R2 (24).

The adenovirus genome E3 region contains several genes that encode proteins which inhibit TNF- α - or Fas L-mediated apoptosis of target cells (4,22), and the 14.7K protein has been shown to protect against *in vitro* cytolysis by CD8⁺ T cells or by TNF- α (13). Compared with the wild-type adenovirus, mutants lacking 14.7K resulted in enhanced inflammatory influx and lung injury in murine adenovirus pneumonia (38). Transgenic mice expressing 14.7K in the alveolar epithelium are resistant to LPS-mediated lung inflammation, and displayed a decrease in mononuclear influx (16). However, signal transduction events involved in adenoviral 14.7K regulation of gene epithelial expression are poorly understood, notwithstanding the fact that analysis of these pathways is likely to inform the mechanisms of immunopathology associated with a variety of respiratory epithelial infections. In this report, we investigated the effects of epithelial adenoviral 14.7K expression on CD8⁺ T-cell- and TNF- α -mediated NF- κ B activation, CCL2 production, and the induction of pulmonary immunopathology in influenza infection.

Materials and Methods

BALB/c, SP-C-14.7K, and SP-C-HA-14.7 mice were used at 12–16 wk of age. The SP-C-14.7K and SP-C-HA mice have been backcrossed onto the BALB/c background for 10 generations. The double transgenic (SP-C-HA-14.7) strain was generated by interbreeding mice expressing either HA or 14.7 under the transcriptional control of the surfactant protein C (SP-C) promoter, which directs expression to the distal airway (alveolar and bronchiolar) epithelium (10,16). All experiments were conducted in strict accordance with the guidelines of the National Institute of Health (NIH) and the Dartmouth Medical School Institutional Animal Care and Use Committee (IACUC), including the requirement that any animal that lost 20% of its initial weight after infection would be euthanized. CD8⁺ T-cell bulk lines and clones were activated *in vitro* prior to adoptive transfer (43), and injected via the tail vein. Weight loss was monitored daily, and lungs were harvested at appropriate times for histology or chemokine analysis. Lung tissue extracts were assayed for CCL2 using a sandwich ELISA (BD Pharmingen, San Diego, CA), according to the manufacturer's instructions. In some experiments, mice were infected intranasally with 5×10^3 TCID₅₀ of influenza A/PR/8/34. Five days after virus infection, the animals were anesthetized, a tracheostomy tube was inserted, and whole-lung diffusing capacity was determined by CO uptake (a surrogate for oxygen transfer), as previously described (11). In some experiments the partial arterial pressure of oxygen was measured in samples from the ventral tail artery, as previously described (42). Viral titers in whole-lung homogenates were determined as TCID₅₀ in MDCK cells (43). Mouse TNF- α and IFN- γ were purchased from Genzyme (Boston, MA) and PBL (New Brunswick, NJ), respectively. For *in vitro* studies, murine alveolar epithelial

cells stably expressing H-2Kd (MLE-Kd; 46) were treated with synthetic peptide representing the Kd-restricted 210–219 epitope of the A/Japan/57 HA and co-cultured with HA210-specific CD8⁺ T cells, or treated with soluble TNF- α (20 ng/mL) or IFN- γ (1000 U/mL). In co-culture experiments, MLE-Kd and CD8⁺ T cells were separated after incubation using mouse anti-CD8-bound magnetic beads. Total RNA was prepared from cells using the RNeasy kit (Qiagen, Valencia, CA). RT-PCR was performed using primer sequences as previously described (32), and RETROscript (Ambion, Austin, TX), according to the manufacturer's protocol. Cell extracts were prepared and proteins were separated by electrophoresis using 8–10% SDS-PAGE gels (25). Proteins in the gel were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA), subjected to immunoblotting with the indicated antibodies, and visualized by enhanced chemiluminescence (Pierce Protein Research Products, Rockford, IL). Antibodies to I κ B- α and NF- κ B (p50 and p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-Akt (serine 473), Akt, phospho-GSK-3 β , GSK-3 β , phospho-p65 (S468), and horseradish peroxidase (HRP)-linked secondary antibodies were purchased from Cell Signaling Technology (Boston, MA). Chromatin immunoprecipitation (ChIP) assays were performed using the EZ-ChIP kit (Upstate/Millipore, Billerica, MA), according to the manufacturer's directions, using PCR primers spanning the NF- κ B site in the CCL2 promoter (39).

Results

To study events in intraepithelial TNF- α signaling that lead to inflammatory chemokine induction upon CD8⁺ T-cell recognition, we stably expressed the adenoviral 14.7K protein in MLE-Kd alveolar epithelial cells, and studied CCL2 induction triggered either by T-cell antigen recognition, or by treatment with soluble TNF- α . As shown in Fig. 1A, both CD8⁺ T-cell recognition and treatment with recombinant TNF- α induced CCL2 expression in control MLE-Kd cells, but this was significantly abrogated in 14.7K-expressing MLE-Kd. The effect of 14.7 appeared to be specific for CCL2, because induction of another important chemokine, CXCL2 (MIP-2), was not significantly altered under similar conditions. In addition, 14.7K expression had no impact on the expression of guanylate binding protein (GBP-2) and IFI-47, genes known to be induced by interferon- γ , another antigen-dependent product of CD8⁺ T cells.

The NF- κ B pathway plays an important role in TNF- α induction of CCL2 expression (29). The predominant NF- κ B heterodimer consists of p65 and p50 subunits in the cytoplasm, and are bound to an inhibitor of NF- κ B (I κ B- α) protein that prevents its activation and translocation into the nucleus (12,28). We therefore tested whether the adenoviral 14.7K protein would inhibit I κ B- α degradation or p65 nuclear translocation. Western blot analysis revealed that adenoviral 14.7K did not inhibit TNF- α -induced I κ B- α degradation or p65 nuclear translocation in MLE-Kd cells (Fig. 1B). Recruitment of NF- κ B subunit p65 to the NF- κ B-responsive element has been shown to be a major regulatory event in transcriptional activation by TNF- α (29,39). ChIP revealed that the p65 and p50 subunits were recruited to the CCL2 promoter in response to TNF- α in control MLE-Kd but

FIG. 1. Stable expression of adenovirus 14.7K abrogates NF- κ B activation and CCL2 induction by CD8⁺ T-cell recognition or TNF- α treatment in MLE-Kd cells. **(A)** MLE-Kd cells stably transfected with either empty pcDNA 3.1 vector or one expressing adenoviral 14.7K were treated with synthetic peptide along with CD8⁺ T cells, or soluble TNF- α , for the indicated time periods. The levels of CCL2, CXCL2, GBP-2, IFI-47, 14.7K, and β -actin were determined by RT-PCR. These data are representative of three independent experiments. **(B)** Cells were treated with TNF- α (20 ng/mL) for 20 min. Equal amounts of lysates were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies to Ik-B α , β -actin, p65, and SP1. **(C)** Cells were treated with TNF- α (20 ng/mL) for 15 or 30 min or were left untreated. After *in vivo* cross-linking, chromatin was isolated from the cells and immunoprecipitated with antibodies against the p65 or p50 subunits of NF- κ B. Genomic PCR was performed using primers that span the NF- κ B binding region from the mouse CCL2 promoter. Control PCR was performed using purified genomic DNA from the input samples. Results are representative of two independent experiments. **(D)** Cells were treated with TNF- α (20 ng/mL) for 30 min and the lysates were analyzed by immunoblotting using antibodies to p-Akt (serine473), total Akt, pGSK-3 β (serine9), total GSK-3 β , p-p65 (serine468), and total p65. The blots shown are representative of three independent experiments.

not in adenoviral 14.7K-expressing cells (Fig. 1C), suggesting that adenoviral 14.7K inhibits transcriptional activation of CCL2 by interfering with NF- κ B recruitment to its promoter, rather than by inhibition of nuclear translocation. In order to understand the mechanisms involved in this inhibition, we examined several other candidate signal transduction pathways triggered by TNF- α signaling. In addition to the IKK pathway, TNF-receptor activation regulates multiple pathways in parallel, including mitogen-activated protein kinases (MAPK), such as extracellular-regulated kinases (ERK), Jun N-terminal kinases (JNK), and p38 kinase. In addition, TNF- α signaling may regulate phosphoinositide 3'-kinase (PI3K), Akt, and glycogen synthase kinase-3 β (GSK-3 β), which are involved in multiple phosphorylation events and the recruitment of various transcription factors and co-regulators (12,28,39,40,44). Among these kinases, GSK-3 β is unique in

that it is constitutively active under normal conditions and inactivated in response to PI3K/Akt-mediated inhibitory phosphorylation (9). Interestingly, GSK-3 β has been reported to be required for NF- κ B recruitment to κ B-responsive elements, and induction of a subset of NF- κ B regulated genes, including CCL2, but not CXCL2 (39). TNF- α has been reported to stimulate phosphorylation on serine9 of GSK-3 β in a variety of cells (14,15,19), so we performed Western blot analysis on 14.7K-expressing MLE-Kd cells for GSK-3 β after treatment with TNF- α . As shown in Fig. 1D, TNF induction of serine9 phosphorylation of GSK-3 β was attenuated in MLE-Kd cells expressing 14.7K compared with control MLE-Kd cells, suggesting that this may be an important mechanism regulating at least some of the transcriptional activities induced in epithelial cells by TNF-receptor signaling. In an effort to further understand the

mechanisms of this inhibition, we performed a similar analysis of serine473 phosphorylation of Akt, which is upstream of GSK-3 β , and found that this was also attenuated by 14.7K (Fig. 1D). In contrast, TNF- α -induced ERK and p38 phosphorylation was unaffected in both cells (data not shown). TNF- α -induced serine9 phosphorylation of GSK-3 β has been shown in other studies to be important for the serine468 phosphorylation of the p65 subunit of NF- κ B (15), and therefore we asked whether 14.7K expression would impact TNF- α induction of serine468 phosphorylation of p65 in MLE-Kd cells. Western blot analysis revealed that serine468 phosphorylation of p65 was indeed attenuated in 14.7K-expressing MLE-Kd cells compared with control cells (Fig. 1D). Abrogation of serine phosphorylation of GSK-3 β and p65 by adenoviral 14.7K may be a key mechanism by which TNF- α -receptor induction of NF- κ B activation and CCL2 expression are suppressed in MLE-Kd cells, and sug-

gests that this may represent an important regulatory event in epithelial antiviral immunity.

To evaluate the physiological and pathological consequences of inhibiting this aspect of epithelial TNF-receptor signaling in the setting of an effector CD8 $^{+}$ T cell response to an acute virus infection, we used a well-characterized model involving intranasal influenza infection, followed by adoptive transfer of HA-specific CD8 $^{+}$ effector cells (45). Transgenic animals expressing the 14.7K protein on the distal airway (alveolar and bronchiolar) epithelium under control of the surfactant protein C promoter (16) were infected and then used as recipients of activated CD8 $^{+}$ T cells transferred intravenously. As shown in Fig. 2A, infection of both transgenic and WT animals with A/PR8/34 resulted in a considerable decrement in CO uptake, a surrogate measure for oxygen transfer capability (or diffusing capacity; 11), and this was significantly worse in WT mice after transfer of CD8 $^{+}$

FIG. 2. Adenoviral 14.7K expressed in alveolar epithelium is protective against CD8 $^{+}$ T-cell-mediated lung injury *in vivo*. **(A)** Groups of WT (BALB/c) mice or SPC-14.7K transgenic mice were infected intranasally with A/PR8/34 influenza, and then used as recipients of adoptively transferred activated CD8 $^{+}$ T cells specific for the Kd-restricted epitope in A/PR8/34 HA. Five days later CO uptake (whole-lung diffusing capacity) was measured. Data represent mean $\pm$ SD (4–7 animals per group). **(B)** Virus titers on day 4 after infection with A/PR8/34 alone (white bars), or with infection followed by adoptive transfer of specific CD8 $^{+}$ T cells (black bars). **(C)** HA-specific CD8 $^{+}$ T cells (5×10^6) were transferred into wild-type HA $^{+}$ mice (blue line), or SPC-14.7K/HA $^{+}$ double transgenic mice (red line), and body weight was monitored every day for 4 d. The data represent the mean $\pm$ SD of two experiments with 3 mice per group ($*p < 0.0001$). **(D)** Lung histopathology in WT HA $^{+}$ (i) and 14.7K/HA $^{+}$ (ii) mice 4 d after adoptive transfer of HA-specific CD8 $^{+}$ T cells. Representative H&E-stained lung sections are shown at 40 $\times$ and 100 $\times$ (inset) magnification. **(E)** ELISA for CCL2 in whole-lung extracts 24 h after transfer of CD8 $^{+}$ T cells into WT-HA $^{+}$ or 14.7K/HA $^{+}$ mice. Data represent mean $\pm$ SD from two experiments, with 3 mice per group. Color images available online at www.liebertonline.com/vim.

effectors, as we have previously shown (46). Interestingly, there was no additional decrement in the 14.7K transgenic mice after T-cell transfer compared to infection alone, suggesting that the injury attributable to CD8⁺ T-cell effector activities was ablated by inhibition of this epithelial TNF-receptor-dependent signaling pathway by 14.7K. CD8⁺ T-cell transfer alone (without infection) had no impact on CO uptake, and these measurements were similar to those observed in unmanipulated mice (data not shown). Clearance of virus was delayed in 14.7K transgenic mice as well, which had detectable lung viral titers 4d after infection and transfer, while the WT animals did not (Fig. 2B).

In order to specifically focus on the immunopathology triggered by antigen-specific T-cell activities, without the variable of viral clearance, we performed a set of experiments in a model in which lung injury is mediated exclusively by CD8⁺ T-cell recognition of transgenically-expressed target antigen in the distal airways. Mice expressing both 14.7K and influenza HA on the alveolar epithelium were generated by breeding 14.7K transgenic mice with mice expressing HA under the control of the same promoter (10), resulting in alveolar epithelial expression of both transgenes. Adoptive transfer of activated HA-specific CD8⁺ T cells IV resulted in significant morbidity (as measured by weight loss) in WT-HA⁺ compared with 14.7K-HA⁺ mice (Fig. 2C). All of the WT HA⁺ animals were euthanized after day 4 because of their weight loss. Pulmonary infiltrates in WT-HA⁺ lungs consisted largely of a macrophage and neutrophil influx, whereas the macrophage influx was attenuated in 14.7K-HA⁺ mice (Fig. 2D). Consistent with the histopathology, CCL2 expression in lung was significantly attenuated in 14.7K-HA⁺ compared with WT-HA⁺ mice (Fig. 2E). Measurement of arterial Po₂ revealed a significant reduction in the alveolar-arterial oxygen gradient in 14.7⁺HA⁺ T-cell recipients compared with 14.7-HA⁺ T-cell recipients (29.1 ± 1.4 versus 38.6 ± 2.3 ; $p < 0.05$; data not shown), indicating a relative preservation of functional gas exchange capacity afforded by 14.7K expression.

Discussion

The TNF- α and NF- κ B pathways are central regulators of immune responses, cell survival, and apoptosis, and are modulated by a large number of pathogens for their survival (31,33). There are numerous examples of evasive strategies that unrelated viruses have evolved to counter these systems (2,27), in addition to adenovirus, indicating their potential significance in antiviral immune responses. In this study, we focused primarily on the immunopathological impact of inhibition of this pathway by 14.7K, using influenza infection as a model in which immune-mediated lung injury is likely an important contributor to the clinical outcome. We have previously shown that CD8⁺ T-cell expression of TNF- α and alveolar epithelial expression of TNF-R1 and TNF-R2 on lung epithelial cells are required for significant T-cell-mediated lung injury (24,43), and that induction of epithelial chemokine expression, particularly CCL2, is a primary contributor to the immunopathology (37,45). In this study, we found that distal airway epithelial CCL2 induction was abrogated by adenoviral 14.7K through the inhibition of TNF- α -receptor-induced GSK-3 β phosphorylation and recruitment of NF- κ B to the CCL2 promoter. GSK-3 β is required for NF- κ B

recruitment and induction of a subset of NF- κ B-regulated genes including CCL2, but not CXCL2 (39). Overexpression of GSK-3 β has been shown to enhance, while its inhibition has been shown to abrogate, CCL2 induction and the inflammatory responses associated with chronic renal allograft disease (14). Targeted deletion of GSK-3 β in mice has been shown to abrogate NF- κ B activation mediated by TNF- α (19). Whether the 14.7K protein inhibits Akt and GSK-3 β serine phosphorylation directly or by modulation of upstream signaling pathways remains to be determined. In any case it provides an interesting tool for analyzing the critical biochemical underpinnings of the immunopathological responses to virus infection, and of those mediated primarily by the host immune response.

Our *in vivo* experiments support previous findings indicating the importance of TNF- α in CD8⁺ T-cell-mediated lung injury (43,46). Although the system we use arguably reflects a model of secondary rather than primary T-cell responses to influenza infection, it provides a unique opportunity to focus our studies on the epithelial responses to activated T-cell recognition in viral infection, which occur much more slowly in the primary response. During influenza infection, viral antigens are presented on both epithelial and non-epithelial cells in the lungs, yet interference with TNF- α signaling exclusively in distal airway epithelial cells resulted in abrogation of CD8⁺ T-cell-mediated lung injury (as well as viral clearance). This underscores the importance in influenza infection (and undoubtedly others) of the interaction between CD8⁺ T cells and airway epithelial cells as the key event in immune-mediated lung inflammation and viral clearance. Understanding the signal transduction pathways involved in host epithelial inflammatory responses and viral evasion/inhibition of these pathways may lead to novel strategies to mitigate pulmonary immunopathology in respiratory virus infection.

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Author Disclosure Statement

No conflicting financial interests exist.

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