Airway Epithelial versus Immune Cell Stat1 Function for Innate Defense against Respiratory Viral Infection

Laurie P. Shornick, Audrey G. Wells, Yong Zhang, Anand C. Patel, Guangming Huang, Kazutaka Takami, Moises Sosa, Nikhil A. Shukla, Eugene Agapov and Michael J. Holtzman

*J Immunol* 2008; 180:3319-3328; doi: 10.4049/jimmunol.180.5.3319
http://www.jimmunol.org/content/180/5/3319

Supplementary Material  http://www.jimmunol.org/content/suppl/2008/02/20/180.5.3319.DC1

References  This article cites 47 articles, 22 of which you can access for free at: http://www.jimmunol.org/content/180/5/3319.full#ref-list-1

Subscription  Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions  Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts  Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Airway Epithelial versus Immune Cell Stat1 Function for Innate Defense against Respiratory Viral Infection

Laurie P. Shornick,* Audrey G. Wells,† Yong Zhang,* Anand C. Patel,‡ Guangming Huang,* Kazutaka Takami,* Moises Sosa,* Nikhil A. Shukla,* Eugene Agapov,* and Michael J. Holtzman2∗‡

The epithelial surface is often proposed to actively participate in host defense, but evidence that this is the case remains circumstantial. Similarly, respiratory paramyxoviral infections are a leading cause of serious respiratory disease, but the basis for host defense against severe illness is uncertain. Here we use a common mouse paramyxovirus (Sendai virus) to show that a prominent early event in respiratory paramyxoviral infection is activation of the IFN-signaling protein Stat1 in airway epithelial cells. Furthermore, Stat1−/− mice developed illness that resembled severe paramyxoviral respiratory infection in humans and was characterized by increased viral replication and neutrophilic inflammation in concert with overproduction of TNF-α and neutrophil chemokine CXCL2. Poor control of viral replication as well as TNF-α and CXCL2 overproduction were both mimicked by infection of Stat1−/− airway epithelial cells in culture. TNF-α drives the CXCL2 response, because it can be reversed by TNF-α blockade in vitro and in vivo. These findings pointed to an epithelial defect in Stat1−/− mice. Indeed, we next demonstrated that Stat1−/− mice that were reconstituted with wild-type bone marrow were still susceptible to infection with Sendai virus, whereas wild-type mice that received Stat1−/− bone marrow retained resistance to infection. The susceptible epithelial Stat1−/− chimeric mice also exhibited increased viral replication as well as excessive neutrophils, CXCL2, and TNF-α in the airspace. These findings provide some of the most definitive evidence to date for the critical role of barrier epithelial cells in innate immunity to common pathogens, particularly in controlling viral replication. The Journal of Immunology, 2008, 180: 3319–3328.

The epithelium is responsible for forming a barrier between the environment and the underlying host tissues and is often proposed to take an active role in the innate immune response to pathogens. Initial proposals included a sentinel role for epithelial cells in directing immune cell traffic and activation (1). However, this concept was difficult to prove because many of the systems for epithelial immune function were found to overlap with the ones found in immune cells themselves. For example, the expression of cell adhesion molecules such as ICAM-1 and chemokines such as CCL5 are part of the repertoire for both epithelial and immune cell compartments (2, 3). Thus, a loss of function approach was not selective for epithelial cells vs immune cells. An alternative concept is that epithelial cells may directly inhibit pathogen replication when these cells serve as the primary host cells. For example, epithelial cells of the respiratory tract are the primary targets for common respiratory viruses, and at least in vitro, these host epithelial cells may be capable of inhibiting viral replication based on production of antiviral IFNs (4). Nonetheless, it is generally assumed that innate and adaptive immune responses from immune cells are responsible for antiviral defense. Particular emphasis has been placed on an innate immune response from NK cells, plasmacytoid dendritic cells, and macrophages and an adaptive immune response leading to T cell or Ab-mediated clearance of viruses (5, 7).

In this work, we approached the issue of epithelial vs immune cell function in host defense using a mouse model for a common type of respiratory viral infection. The infectious agent used in this model is Sendai virus (SeV),† also known as mouse parainfluenza virus type I, which is the archetype member of the paramyxovirus family that includes respiratory syncytial virus (RSV), human metapneumovirus, and human parainfluenza viruses that more often infect humans (8–10). In a C57BL/6 genetic background, SeV replicates predominantly in airway epithelial cells (especially ciliated cells) and causes acute inflammation of the small airways (i.e., bronchiolitis). This pattern of illness provides a high fidelity model of lower respiratory infection with paramyxoviruses in humans (3, 11). Here we show that SeV replication in the airway epithelial cells causes production of IFNs and activation of the IFN-signaling molecule Stat1. Moreover, mice deficient in Stat1 (i.e., Stat1−/− mice) develop a more severe infection with higher viral titers and more extensive inflammation than their wild-type counterparts do. This pattern of disease is indistinguishable from severe RSV bronchiolitis in children (12). Unexpectedly, an analysis of chimeric mice showed that Stat1 function in the epithelial vs the immune cell compartment was critical for viral clearance and prevention of severe immunopathology. These findings were consistent with observed defects in the antiviral response in isolated airway epithelial cells. Together, the observations provide for a critical role of epithelial immune function in antiviral defense and suggest that a defect in epithelial IFN signaling may contribute to more severe forms of paramyxoviral infection in humans.

† Address correspondence and reprint requests to Dr. M. J. Holtzman, Washington University School of Medicine, Campus Box 8052, 660 South Euclid Avenue, St. Louis, MO 63110. E-mail address: holtzmann@wusl.edu

‡ Abbreviations used in this paper: SeV, Sendai virus; BAL, bronchoalveolar lavage; MOI, multiplicity of infection; mTEC, mouse tracheal epithelial cell; PI, postinoculation; RSV, respiratory syncytial virus; SeV-UV, UV-inactivated SeV.
Materials and Methods

Cell culture

Primary culture mouse tracheal epithelial cells (mTECs) were established on Transwell membranes using air-liquid interface conditions as described previously (13). SeV, strain 52, was obtained from American Type Culture Collection and stored at −70°C. Cultures were inoculated with SeV or an equivalent amount of UV-inactivated SeV (SeV-UV) in the apical compartment for 1 h at 37°C. Air-liquid interface conditions were re-established by washing the membrane with PBS.

Mice generation and inoculation

Wild-type C57BL/6 and B6.SJL mice were obtained from The Jackson Laboratory; same-strain Stat1−/− mice were obtained from David Levy (New York University, New York, NY) (14). All mice were housed in pathogen-free conditions in a biohazard barrier facility in microisolation cages, and the University Animal Studies Committee approved all procedures. After necropsy, mice were inoculated with SeV or SeV-UV diluted in 30 μl of PBS. After inoculation, mice were inspected and weighted daily. For TNFα neutralization, mice were treated with entanercept (Enbrel from Immunex) at 10 mg/kg given i.p. on postinoculation (PI) days 1, 3, 5, and 7, based on protocols described previously (15–17). Viral titer was monitored by plaque-forming assay as described previously (18).

RNA analysis

For oligonucleotide microarray analysis, total RNA was isolated from mTEC cultures using the Micro RNA Isolation kit (Stratagene). Linear amplification of transcript and hybridization to high-density oligonucleotide microarrays (Affymetrix Mouse Genome U74A) were performed as described previously (19). Microarray normalization and statistical analysis were performed using packages from the Bioconductor project executed in the R programming environment (20). Data were normalized using the empirical Bayes version of the GCRMA algorithm as implemented in the GCRMA package (21). Differential expression (SeV vs SeV-UV inoculation) was assessed using linear models and empirical Bayes-modulated F statistics as implemented in the LIMMA package (22). Differences in gene expression were considered significant if p values were <0.05 after adjustment for multiple testing as described previously (23), so that the false discovery rate was <5%. Visualization and plotting was performed using Spotfire DecisionSite for Functional Genomics. Microarray data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus under series accession number GSE10211.

Total lung RNA was isolated using the RNeasy mini kit (Qiagen). For real-time PCR, target mRNA levels were quantified with fluorogenic probe/primer combinations using the TaqMan One-Step system (PE Biosystems). The following were used for amplification: for SeV-specific RNA, forward primer nt 68–85, reverse primer nt 112–129, and probe nt 87–110 of accession number M30202; for mouse CXCL2, forward primer nt 704–726, reverse primer nt 775–797 and probe nt 731–757 of accession number NM_009140; for mouse Stat1 forward primer nt 755–771, reverse primer nt 803–820, and probe nt 776–791 of accession number NM_009283. Mouse TNFα primer and probe were from Applied Biosystems (Assay ID Mm0043259_g1). The TaqMan Rodent GAPDH control Kit was used to normalize expression between samples.

Western blotting

Lungs were homogenized in 1% Nonidet P-40, 0.05 M Tris (pH 8.0), 250 mM NaCl, 1 mM EDTA, containing 1 mM PMSF, 10 mM iodoacetatin, 10 mg/ml leupeptin, 1 mM orthovanadate, 2 mM sodium pyrophosphate, and 10 mM sodium fluoride. After SDS-PAGE, proteins were blotted onto polyvinylidene difluoride membrane (GE Healthcare) and incubated with mouse anti-human Stat1 Ab (BD Biosciences) or with rabbit anti-human phospho-Stat1 (Tyr701) Ab (Cell Signaling Technology). Secondary Abs were anti-mouse IgG1 (Roche Applied Science) and sheep anti-rabbit IgG (Millipore), respectively.

Immunostaining of tissues and cells

Lungs were fixed in 10% neutral buffered formalin (at 25 cm water pressure), dehydrated in ethanol, embedded in paraffin, and cut into 5-μm sections. Tissue sections were blocked with normal goat serum and then incubated with a 1/1500 dilution of rat anti-SeV Ab (BioReliance), rat anti-mouse neutrophil Ab (clone 7/4, Serotec), rat anti-human CD3 Ab (Serotec), or rat anti-mouse Mac-3 Ab (BD Biosciences) for 1 h at 25°C. Primary Ab binding was detected with biotinylated goat anti-rat Ab (1/500 dilution for 30 min at 25°C) and the VECTASTAIN ABC-AP kit. Sections were stained with an alkaline phosphatase red substrate and counterstained with hematoxylin. For immunostaining of mTECs, cells were fixed with 4% paraformaldehyde in PBS and stained with rat anti-SeV Ab (BioReliance) and mouse anti-β-tubulin IV (Sigma-Aldrich), followed by incubation with Cy-3-conjugated rabbit anti-rat IgG and FITC-conjugated donkey anti-mouse IgG.

Bromochondroelastic lavage (BAL) analysis

BAL was performed via tracheal cannulation with 1.0 ml of PBS with 2% FBS. Total cell counts were determined after hypotonic lysis. An aliquot of cells was taken for cytoospin centrifugation and Wright-Giemsa staining to determine differential cell counts. Protein levels for murine CXCL2 and TNFα were measured using Quantikine colorimetric sandwich ELISA kits (R & D Systems).

Bone marrow transfer

Bone marrow cells were harvested by flushing the femurs and tibias of donor mice with RPMI 1640 (Invitrogen Life Technologies) supplemented with 10% heat-inactivated FBS (HyClone), 2 mM t-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (R10). Cells were washed once and suspended in R10 containing the anti-Thy-1.2 mAb 30-H12 (1/10 dilution; American Type Culture Collection) and low-toxic rabbit complement (1/15 dilution; Accurate Chemical & Scientific Corp.). After incubation at 37°C for 45 min, the cells were washed, and 3 × 106 cells were used to reconstitute lethally irradiated (9.5–10 Gy) recipient mice. Chimeric mice were analyzed 8 wk after bone marrow transfer. Bone marrow engraftment was assessed by flow cytometry of lung immune cells. Lung cell suspensions were obtained as described previously (24). Briefly, we perfused lungs with DMEM containing 5% FBS, 1% nonessential amino acids, 2 mM glutamine, and 50 mM 2-ME. Lungs were cut into fragments and digested in DMEM containing these additives and 1 mg/ml collagenase (type IA; Invitrogen Life Technologies) for 20 min at 37°C. After digestion, the cell suspension was passed through a 40-μm pore size nylon cell strainer. Single-cell suspensions were stained with PE-conjugated mouse anti-CD45.1 and FITC-conjugated mouse anti-CD45.2 (BD Biosciences) for 30 min at 4°C. Data acquisition was performed using a FACSCalibur instrument (BD Biosciences) and CellQuest software. Data analysis was performed using FlowJo software (Treestar).
Lung sections were subjected to immunostaining for Stat1 and corresponding to Stat1, Stat1

imulation conditions from SeV-infected primary culture mTECs that were main-

alyzed using a paired real-time PCR, BAL cell counts, chemokine levels, and viral titer were ana-

Mouse survival was assessed by Kaplan-Meier analysis. Values for ELISA, Statistical analysis

ed using anti-Stat1 or anti-phospho-Stat1 (Tyr701) Ab. Arrows indicate bands corresponding to Stat1, Stat1β, phospho-Stat1, and phospho-Stat1β (p-Stat1 and p-Stat1β). c, Using inoculation conditions from a, corresponding lung sections were subjected to immunostaining for Stat1 and β-tubulin-IV. d, Lung sections were also subjected to immunostaining for phospho-Stat1 and 4′,6′-diamidino-2-phenylindole (DAPI). Arrows indicate a typical airway epithelial cell (Epi) and alveolar macrophage (Mac) with phospho-Stat1 localization to the cell nucleus. For c and d, bars are 20 μm.

performed using a BD Biosciences FACSCalibur flow cytometer, and data analysis was performed with FlowJo software (version 6.4.7; Tree Star).

**Statistical analysis**

Mouse survival was assessed by Kaplan-Meier analysis. Values for ELISA, real-time PCR, BAL cell counts, chemokine levels, and viral titer were analyzed using a paired t test. The significance level for all analyses was p < 0.05.

**Results**

**Viral infection drives Stat1 activation in airway epithelial cells**

Oligonucleotide microarrays were used to establish a profile for gene expression in wild-type airway epithelial cells after parainfluenza viral infection. Analysis was performed on mRNA isolated from SeV-infected primary culture mTECs that were main-

tained under physiological conditions. Relative to cells that were inoculated with UV-inactivated virus, we observed an epithelial response that was dominated by expression of IFN (in this case, IFN-β) and IFN-dependent genes. For example, genes with established IFN responsiveness and/or antiviral function encoded for at least 107 of the 136 virus-induced genes, including Stat1 (Fig. 1 and Supplementary Table I). Within this group of 107 genes, at least 12 genes are direct transcriptional targets of Stat1 (25), and at least 13 genes are capable of direct antiviral action. Taken together, genomic analysis suggested a primary role for Stat1 in the epithelial response to virus, especially SeV, infection.

Similar to the effect of viral infection on airway epithelial cells in vitro, SeV inoculation of wild-type mice also caused increases in epithelial Stat1 expression in vivo. Real-time PCR assays of whole-lung RNA showed increases in Stat1 mRNA levels that began at PI day 1 and lasted to day 5 (Fig. 2c). Western blot analysis of whole-lung lysates demonstrated corresponding increases in Stat1 and activated (phosphorylated) Stat1 protein levels on PI days 3–14 (Fig. 2b). Immunostaining with a Stat1-specific Ab indicated that increased Stat1 was expressed predominantly in airway epithelial cells and was localized to the cytoplasm of ciliated and nonciliated cells (Fig. 2c). Activation of lung Stat1, based on

*The on-line version of this article contains supplemental material.
tyrosine phosphorylation, developed in concert with Stat1 expression (Fig. 2b). Activation of epithelial Stat1, based on nuclear localization of phosphorylated Stat1, was detectable by PI day 1 and persisted to day 8 (Fig. 2d and data not shown). Detection of Stat1 expression and activation was most evident in airway epithelial cells but was also found in neighboring cells such as macrophages.

**Increased viral replication and inflammation in Stat1-null mice**

Based on the prominence of Stat1 activation and IFN-dependent gene expression after viral infection, we next assessed the effects of SeV infection in Stat1-deficient (Stat1−/−) mice. The critical function of Stat1 for protection against SeV infection was established when we found that Stat1−/− mice were highly sensitive to intranasal inoculation with SeV. For example, intranasal inoculation with 1 × 10⁵ PFU SeV caused no detectable mortality in wild-type mice, whereas inoculation with the same amount or even 10-fold less SeV caused 100% mortality in Stat1−/− mice (Fig. 3a). In conjunction with higher mortality rates, SeV titer was increased in the lungs of Stat1−/− mice at all measured time points after inoculation (Fig. 3b). Immunostaining with an anti-SeV Ab demonstrated that virus was predominantly localized to airway epithelial cells and occasionally to macrophages and was no longer detectable by PI day 8 in wild-type mice (Fig. 3c). This pattern was consistent with our previous reports in wild-type mice (3, 11). This pattern of SeV immunostaining was amplified in Stat1−/− mice at early time points (e.g., PI day 1). In addition, SeV was also present within an inflammatory exudate in the airway lumen of Stat1−/− mice at later time points (e.g., PI days 3 and 8).

We next characterized the inflammation found in the airways of Stat1−/− mice during SeV infection. Immunostaining with a neutrophil-specific Ab demonstrated that the prominent luminal exudate was composed mainly of neutrophils (Fig. 4a). Immunostaining for macrophages and T cells showed that these cells were also present in the exudate at lower numbers of cells (data not shown). The inflammation was quantified in BAL fluid cell counts. These results again indicated that Stat1−/− mice developed a marked increase in the number of neutrophils with lesser increases in the numbers of macrophages and lymphocytes (Fig. 4b). The neutrophilic inflammation in Stat1−/− mice was at its highest level at PI day 8, a time when the neutrophil infiltration into the lung is no longer detectable in wild-type mice. This excessive and prolonged inflammatory response is not found in wild-type mice even at a 100-fold higher SeV inoculum that causes comparable levels of mortality (data not shown).

Neutrophils are generally short-lived (3–4 days) after leaving the bone marrow (26), suggesting that the increased number of neutrophils in the airways of SeV-infected in Stat1−/− mice might reflect either a decrease in neutrophil apoptosis or an increase in...
neutrophil recruitment at the site of infection. We found that the majority of neutrophils in the airspace were undergoing apoptosis based on colocalization of TUNEL and anti-neutrophil Ab staining (data not shown). Infection with a higher level of SeV causes airway epithelial expression of ICAM-1 and ICAM-1-dependent inflammation (2, 11), but we found no increase in ICAM-1 expression in either Stat1−/− or wild-type mice at these low levels of viral inoculation (data not shown). We therefore reasoned that the increase in the numbers of neutrophils in Stat1−/− mice after infection may be due to excessive neutrophil recruitment driven by increased chemokine production. In support of this mechanism, we found selective increases in CXCL2 mRNA in lung tissue and corresponding protein in BAL fluid from Stat1−/− mice compared with Stat1+/+ mice after SeV infection (Fig. 4, c–e). Therefore, increased CXCL2 levels may be responsible at least in part for the increased neutrophil influx after viral infection in Stat1−/− mice.

Stat1 deficiency drives excessive chemokine production via TNF

Our in vivo studies of mice raised the possibility that at least some of the excess morbidity in Stat1−/− mice could be based on neutrophil-dependent inflammation driven by CXCL2 production. Indeed, CXCL2-dependent inflammation causes excessive mortality after Coxsackie virus infection in mice (27). We reasoned that airway epithelial cells could be the source of CXCL2 in this setting, so we directly assessed the capacity of Stat1−/− vs Stat1+/+ airway epithelial cells to produce CXCL2 in response to SeV infection. Initial experiments indicated that SeV inoculation of primary culture mTECs caused infection primarily in ciliated epithelial cells (Fig. 5a). This pattern of infection was therefore similar to that observed in vivo. Under these conditions, we found that Stat1−/− cells produced significantly higher levels of CXCL2 in response to SeV infection in comparison to Stat1+/+ cells (Fig. 5b). This finding suggested that Stat1 may provide an inhibitory influence on viral induction of CXCL2 gene expression, and this influence is lost in Stat1−/− cells.

We next assessed the epithelial mechanism for Stat1 control of CXCL2 production. We recognized that Stat1 may inhibit TNF-α production (28), and in turn, TNF-α-dependent signals may be necessary for CXCL2 production (29). Therefore, we questioned whether Stat1 deficiency may lead to increased TNF-α signaling and consequent CXCL2 production in Stat1−/− airway epithelial cells after SeV infection. Indeed, we next found significantly higher levels of TNF-α mRNA and protein production in Stat1−/− cells compared with Stat1+/+ cells after SeV infection (Fig. 5c and data not shown). Levels of TNF-α production were no different in Stat1−/− vs Stat1+/+ mouse macrophages after SeV infection (data not shown), suggesting that Stat1 control of TNF-α production is at least somewhat distinct for airway epithelial cells. We next determined whether increased TNF-α production was in fact causing increased CXCL2 production in Stat1−/− airway epithelial cells. For these experiments, we treated cells with the TNFRII fusion protein etanercept to achieve TNF-α blockade as described previously (15–17). We found that neutralization of TNF-α caused a significant decrease in CXCL2 production from Stat1−/− but not wild-type cells (Fig. 5b). Blocking TNF-α with a neutralizing Ab against the TNF type I receptor did not affect CXCL2 levels, suggesting that the increased CXCL2 production in Stat1−/− cells was the result of signaling through the TNF type II receptor (data not shown).

To determine whether epithelial Stat1 control of TNF-α and CXCL2 production might also be present in vivo, we returned to our studies of the mouse model of paramyxoviral infection. For these experiments, we decreased the SeV inoculum to 20 PFU. At this inoculum, Stat1−/− mice can recover from infection and can be monitored for evidence of airway inflammation. Under these conditions, we again found a significant increase in CXCL2 levels and neutrophilic inflammation in BAL fluid from Stat1−/− vs Stat1+/+ mice after SeV infection (Fig. 6, a and b). In addition, we found that neutralization of TNF-α with etanercept caused a significant decrease in CXCL2 levels and the numbers of neutrophils in the BAL fluid from Stat1−/− mice after SeV infection. Thus, the levels of CXCL2 and neutrophils were both significantly decreased in etanercept-treated vs untreated Stat1−/− mice at SeV postinoculation day 8 (Fig. 6, a and b). Together with the data obtained in vitro, the findings suggested that TNF-α production was responsible for increased CXCL2 levels and neutrophil influx in the Stat1−/− mice after SeV infection. Despite the significant decrease in the level of neutrophilic airway inflammation after etanercept treatment, we found no improvement in the survival rate or attendant weight loss in Stat1−/− mice treated after SeV infection (Fig. 6, c and...
Levels of SeV in lung tissue were also no different in etanercept-treated vs untreated mice at postinoculation day 8 (Fig. 6e). Thus, etanercept treatment appeared to have little effect on the morbidity associated with SeV infection of Stat1−/− mice, perhaps because the prolonged increase in viral replication was unchanged in these animals.

Epithelial Stat1 signaling controls viral replication

Our in vivo studies of mice also raised the possibility that Stat1 control of viral replication was the critical factor for survival after paramyxoviral infection. The decreased viral clearance in Stat1−/− mice did not appear to be due to defects in the adaptive immune response because we found equivalent numbers of SeV-tetramer positive CD8+ T lymphocytes in the lungs of Stat1+/+ and Stat1−/− mice (data not shown). Similarly, there was no difference in the levels of anti-SeV Abs in the serum of both Stat1+/+ and Stat1−/− mice (data not shown). Accordingly, we studied the possibility that epithelial control of viral replication was critical for survival after SeV infection. This possibility was investigated in further studies of isolated airway epithelial cells in vitro and then in chimeric mice in vivo.

FIGURE 6. Increased TNF-α driving CXCL2 production and neutrophil influx in Stat1−/− mice after SeV infection. a. Stat1−/− and Stat1+/+ mice were inoculated with SeV (20 PFU) with or without treatment with Enbrel (10 mg/kg given i.p.) on PI days 1, 3, 5, and 7, and CXCL2 levels in BAL fluid from PI day 8 were measured by ELISA. b. Stat1−/− and Stat1+/+ mice were inoculated and treated as in a. For each condition, mice were subjected to BAL on PI day 8, and BAL fluid cells were subjected to Wright staining and counting. c. Mice were inoculated as in b and monitored for survival by Kaplan-Meier analysis. d. Mice were inoculated as in b and monitored for weight loss. e. Mice were inoculated as in b and lung RNA from PI day 8 was analyzed for SeV mRNA by real-time PCR. All values represent mean ± SEM; *, significant difference between values for Stat1−/− mice without vs with etanercept treatment.

FIGURE 7. a. Deficient control of viral replication in Stat1−/− airway epithelial cells. a. mTECs from Stat1+/+ mice were inoculated with SeV (MOI 1.0) without or with preincubation with IFN-β (1000 U/ml for 12 h). On the indicated days after inoculation, cellular RNA was analyzed for SeV mRNA by real-time PCR. Values represent mean ± SEM; *, Significant decrease from corresponding no IFN-β treatment control. b. For conditions in a, corresponding analysis of mTECs from Stat1−/− mice. c. mTECs from Stat1+/+ and Stat1−/− mice were inoculated with SeV (MOI 0.001–1.0) without preincubation with IFN-β, and cellular RNA was analyzed on PI day 2. a and b: *, significant increase from corresponding Stat1+/+ cells.
For studies of isolated airway epithelial cells, we first compared viral replication rates between mTECs isolated from Stat1$^{-/-}$ or Stat1$^{+/+}$ mice. When well-differentiated mTEC cultures were infected with SeV at a relatively high viral multiplicity of infection (MOI) of 1, we observed productive infection in wild-type mTECs, but no difference in viral replication rates between Stat1$^{-/-} >>$ Stat1$^{+/+}$ and Stat1$^{-/-} >> $ Stat1$^{+/+}$ CD45.1 PE

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$

$10^4$ $10^3$ $10^2$ $10^1$ $10^0$
Stat1\(^{-/-}\) and Stat1\(^{+/+}\) cells (Fig. 7). However, pretreatment of cultures with IFN-\(\beta\) caused a marked decrease in viral titer in Stat1\(^{-/-}\) cells (Fig. 7a). Moreover, this beneficial effect on viral replication was not found in Stat1\(^{+/+}\) cells (Fig. 7b). These findings suggested that IFN-\(\beta\) activation of Stat1 protects cells that are not yet infected with virus. Therefore, the protective effects of Stat1 may be more evident at a lower MOI that allows for viral spread to neighboring cells. Indeed, we found a significant increase in viral replication rates in Stat1\(^{-/-}\) compared with Stat1\(^{+/+}\) airway epithelial cells at a viral MOI of 0.001 (Fig. 7c).

We next aimed to test whether epithelial Stat1 might also be protective against viral infection in vivo. Viral induction of Stat1 activation and expression is most prominent in airway epithelial cells, especially in the initial days after SeV infection. We generated bone marrow chimeras to better define whether epithelial vs immune cell Stat1 was responsible for the compromise in Stat1\(^{-/-}\) mice. The chimeras were generated by transferring bone marrow from wild-type B6.SJL mice (CD45.1) into irradiated Stat1\(^{-/-}\) mice (CD45.2) and from Stat1\(^{-/-}\) mice into irradiated wild-type B6.SJL mice. Engraftment was confirmed by flow cytometry analysis of CD45.1 vs CD45.2 alleles in peripheral blood and lung tissue immune cells (Fig. 8a and data not shown). This approach allowed us to dissect the role of Stat1 in the radiation-resistant compartment (especially the airway epithelium) compared with the radiation-sensitive hemopoietic cells (especially immune cells). Indeed, the usual activation of airway epithelial Stat1 was preserved in lethally irradiated Stat1\(^{-/-}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow but was lost in Stat1\(^{-/-}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow (Fig. 8b).

In this setting, we found that Stat1\(^{+/+}\) mice that received Stat1\(^{-/-}\) bone marrow retained resistance to SeV whereas Stat1\(^{-/-}\) mice reconstituted with wild-type bone marrow were still susceptible to infection with SeV even at 1 \(\times 10^4\) PFU (Fig. 8c). Stat1\(^{+/+}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow and Stat1\(^{-/-}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow were no different in their response to virus than Stat1\(^{+/+}\) and Stat1\(^{-/-}\) mice, respectively (data not shown). Accordingly, we compared the chimeras to native Stat1\(^{+/+}\) and Stat1\(^{-/-}\) mice. The relative susceptibility of the bone marrow chimeras to SeV infection correlated with the degree of immune cell infiltrate present in the airway. Thus, a prominent infiltrate was observed in the Stat1\(^{-/-}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow but not in the Stat1\(^{+/+}\) mice that received Stat1\(^{-/-}\) bone marrow. Immunostaining demonstrated that the infiltrate contained SeV and neutrophils (Fig. 8d). In concert with neutrophil influx, we detected increased levels of CXCL2 in BAL fluid from Stat1\(^{-/-}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow relative to Stat1\(^{+/+}\) mice that received Stat1\(^{-/-}\) bone marrow (Fig. 8e). In addition, viral titers were significantly increased in nonhemopoietic Stat1\(^{+/+}\) chimeras by PI day 8 (Fig. 8f). Thus, the pattern of illness for Stat1\(^{-/-}\) mice reconstituted with Stat1\(^{+/+}\) bone marrow was similar to that of Stat1\(^{-/-}\) mice as well as Stat1\(^{+/+}\) mice reconstituted with Stat1\(^{-/-}\) bone marrow. Moreover, the pattern of illness found in Stat1\(^{-/-}\) mice that received Stat1\(^{+/+}\) bone marrow was similar to Stat1\(^{+/+}\) mice as well as Stat1\(^{-/-}\) mice that received Stat1\(^{+/+}\) bone marrow. Taken together, these results suggest a critical role for the host airway epithelial cell in innate antiviral immunity by improving viral clearance.

**Discussion**

We report a series of experiments in support of the concept that the epithelial barrier takes an active role in host defense against viral infection. In particular, we studied airway epithelial cells in vitro and in vivo to show that: 1) Stat1 is up-regulated and activated in airway epithelial cells coinciding with the primary site of viral infection and replication; 2) Stat1 deficiency leads to marked increases in viral replication after infection; 3) Stat1 is required for IFN-dependent control of viral replication and consequent survival from infection; 4) Stat1 is also required for control of TNF-\(\alpha\) production and consequent increases in CXCL2 expression and neutrophilic inflammation; and 5) Stat1 expression in nonhemopoietic cells is critical for controlling viral replication and neutrophilic inflammation after infection whereas Stat1 reconstitution in immune cells fails to correct these immune defects in controlling viral replication or airway inflammation in Stat1\(^{-/-}\) mice. Together, these findings provide strong evidence for the role of Stat1 function in airway epithelial cells as a critical component of the innate immune response to respiratory viral infection.

The present results add significantly to previous reports of Stat1 function in antimicrobial defense. For example, the increased susceptibility of Stat1\(^{-/-}\) mice to SeV infection was also found for related human pathogens such as RSV and influenza virus infection (30, 31). Stat1\(^{-/-}\) mice have also been found to be extremely susceptible to Listeria monocytogenes, Leishmania major, vesicular stomatitis virus, mouse hepatitis virus, murine norovirus, and dengue virus (14, 32–34). However, these studies did not define the role of the host epithelial cell in controlling pathogen replication. In fact, Stat1 expression in immune cells (particularly dendritic cells) appeared to be required for immunity to L. major (35).

One study reported that bone marrow chimeric mice reconstituted with Stat1\(^{+/+}\) tissue cells but not Stat1\(^{-/-}\) immune cells showed a decrease in replication of Chlamydia pneumoniae in mice (36). In this study, however, neither the outcome from infection nor the inflammatory response was characterized. In addition, there was no determination of the cellular site for Stat1 expression or activation. Nonetheless, the previous data in combination with the present work may suggest a general requirement for Stat1 signaling in nonimmune host cells to control infection from a variety of microbial pathogens.

The full mechanism for Stat1 control of pathogen replication is uncertain, but our work provides insight into this issue as well. In particular, we found no difference in viral replication rates between Stat1\(^{+/+}\) and Stat1\(^{-/-}\) airway epithelial cells at relatively high viral inoculum (MOI 1). However, pretreatment of cultures with IFN-\(\beta\) caused a marked decrease in viral titer in Stat1\(^{+/+}\) cells, while this beneficial effect was not found in Stat1\(^{-/-}\) cells. We recently reported a similar benefit for other RNA viruses in comparing Stat1\(^{-/-}\) cells with Stat1\(^{+/+}\) cells reconstituted with Stat1 (37). In both cases, the findings suggest that Stat1 function may best protect uninfected host cells and thereby limit cell-to-cell spread of virus. Indeed, we were able to detect a significant increase in viral replication rates in Stat1\(^{-/-}\) airway epithelial cells at a lower viral inoculum (MOI 0.001). Thus, type I IFN released by infected tissue cells as well as immune cells (e.g., plasmacytoid dendritic cells) could drive Stat1 expression and activation in neighboring cells that would become prearmed against viral infection. It is also possible that Stat1-dependent IFN-independent effects on gene expression may also contribute to antiviral defense.

In addition to control of pathogen levels, we also found that Stat1 regulates the inflammatory response. In this case as well, Stat1 appears to exert its influence by its actions in host tissue cells rather than immune cells. Thus, Stat1\(^{-/-}\) epithelial cells but not macrophages produced higher levels of TNF-\(\alpha\) and CXCL2 in response to SeV infection in comparison with Stat1\(^{+/+}\) cells. Because there is no difference in viral replication between Stat1\(^{-/-}\) and Stat1\(^{+/+}\) cells under these conditions (i.e., in the absence of IFN pretreatment), it appears that Stat1 provides an inhibitory influence on viral induction of TNF-\(\alpha\) and CXCL2 gene expression.
Moreover, we found the same pattern in vivo in Stat1−/− mice. Here again, the exaggerated inflammatory response does not simply reflect increased viral load, because similar effects are not observed in wild-type mice even at a 10-fold higher viral inoculum that results in comparable levels of mortality.

The inflammatory response in SeV-infected Stat1−/− mice appeared relevant to human disease because an indistinguishable pattern of neutrophilic inflammation and neutrophil chemokine production is found in infants with severe RSV infection (38, 39). Indeed, airway obstruction with inflammatory cell debris is a prominent feature of fatal RSV infection in human infants and thus may contribute to a fatal outcome in this setting (12). We were unable to influence the outcome from SeV infection by inhibition of the TNF-α to CXCL2 to neutrophil cascade. We achieved only partial blockade of neutrophil tissue infiltration. This finding implied that other factors also contribute to neutrophil accumulation at the site of viral infection. Whether inhibition of these additional factors alone or in combination with antiviral treatment might then influence the outcome from infection still must be defined. However, the findings also implied that viral replication, which remained uncontrolled by anti-inflammatory treatment, was the basis for excess morbidity and mortality under these conditions. In that regard, other groups have found that TNF-α blockade was able to decrease the degree of body weight loss after RSV infection in wild-type as well as IFN-γ−/− mice (40). Perhaps this is because RSV (which is a human pathogen) does not replicate as efficiently in the mouse airways as SeV (which is a natural native mouse pathogen; Ref. 41). Thus, control of viral replication is not as important in the RSV as the SeV mouse model of severe respiratory illness.

For these reasons and others, our findings in the SeV model may hold special relevance to severe viral respiratory infection in humans. Thus, as noted in the preceding paragraph, Stat1−/− mice infected with SeV closely resemble humans with severe RSV infection. In that regard, we note that RSV has evolved the means to be relatively resistant to the IFN-induced antiviral state (42). This effect is based at least in part on the capacity of RSV proteins NS1 and NS2 to block IFN-αβ action by decreasing Stat2 levels (43). Moreover, this effect of RSV is specific for human Stat2 and is not found for mouse Stat2. This species-specific effect could be the reason that RSV infection of mice has a very different phenotype from human infection. Thus, viral titer and clearance rates are no different between wild-type and Stat1−/− mice after RSV infection, and the immune response is characterized by eosinophilic inflammation and Th2-type cytokine production in Stat1−/− mice after RSV infection (30).

In sum, we have developed a new model for severe respiratory viral infection that rests on SeV infection of Stat1−/− mice (as diagrammed in Fig. 9). Previous reports indicate that cellular and humoral responses of the adaptive immune system as well as immune cells of the innate immune system are required for host defense against SeV infection (3, 5–7). The findings in this new model provide strong evidence for a critical role for airway epithelial cell control of viral replication in defense against severe respiratory viral infection. The mechanism for defense likely depends on blockade of cell-to-cell spread of virus and consequent prevention of a prolonged increase in viral titer. The findings are reminiscent of severe paramyxoviral infection in humans. The observations may also be relevant to humans with Stat1 deficiency due to inherited defects in Stat1 signaling that are also highly susceptible to viral infection (44–46). Moreover, some patients with cystic fibrosis may exhibit acquired defects in Stat1 signaling, and these patients also exhibit increased neutrophilic inflammation in concert with neutrophil chemokine (IL-8) expression during viral infection (47). Others have suggested that patients with asthma may also exhibit a defect in IFN-β production and consequent susceptibility to respiratory viral infection (48). The present results therefore provide a rational basis for targeting the airway epithelial cell as the critical site to correct any defects in Stat1 function as a means for improving the outcome from respiratory viral infection.

**Acknowledgments**

We thank David Briner for expert technical assistance.

**Disclosures**

The authors have no financial conflict of interest.

**References**


