

Tollip Inhibits IL-33 Release and Inflammation in Influenza A Virus-Infected Mouse Airways

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Abstract

Respiratory influenza A virus (IAV) infection continues to pose significant challenges in healthcare of human diseases including asthma. IAV infection in mice was shown to increase IL-33, a key cytokine in driving airway inflammation in asthma, but how IL-33 is regulated during viral infection remains unclear. We previously found that a genetic mutation in Toll-interacting protein (Tollip) was linked to less airway epithelial Tollip expression, increased neutrophil chemokines, and lower lung function in asthma patients. As Tollip is involved in maintaining mitochondrial function, and mitochondrial stress may contribute to extracellular ATP release and IL-33 secretion, we hypothesized that Tollip downregulates IL-33 secretion via inhibiting ATP release during IAV infection. Wild-type and Tollip knockout (KO) mice were infected with IAV and treated with either an ATP converter apyrase or an IL-33 decoy receptor soluble ST2 (sST2). KO mice significantly lost more body weight and had increased extracellular ATP, IL-33 release, and neutrophilic inflammation. Apyrase treatment reduced extracellular ATP levels, IL-

33 release, and neutrophilic inflammation in Tollip KO mice. Excessive lung neutrophilic inflammation in IAV-infected Tollip KO mice was reduced by sST2, which was coupled with less IL-33 release. Our data suggest that Tollip inhibits IAV infection, potentially by inhibiting extracellular ATP release and reducing IL-33 activation and lung inflammation. In addition, sST2 may serve as a potential therapeutic approach to mitigate respiratory viral infection in human subjects with Tollip deficiency.

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Introduction

Influenza virus, most commonly known as the flu, is an RNA virus that has three different subtypes: A, B, and C [1]. Influenza A virus (IAV), more specifically H1N1, is the virus responsible for the Spanish flu of 1918 and the pandemic of 2009, where approximately 60.8 million people were infected [2]. Of those hospitalized, asthma was one of the most common underlying medical conditions [3–5]. Like many other respiratory viruses, IAV is associated with asthma exacerbations [6–8]; however, the underlying mechanisms are not completely understood.

Toll-interacting protein (Tollip) is an adaptor protein that is constitutively expressed in epithelial cells, and has been implicated in regulating Toll-like receptor-mediated signaling, inhibiting the IL-1 signaling pathway, as well as maintaining autophagy and mitochondrial function [9–11]. Our group previously found that a genetic mutation in Tollip, rs5743899, led to reduced Tollip expression in human airway epithelial cells, increased neutrophil chemokine levels, and reduced lung function in asthma patients [12]. Additionally, we found that neutrophilic inflammation caused by a different RNA virus, rhinovirus, was attenuated by Tollip. More specifically, we found that Tollip inhibited neutrophilic inflammation through the inhibition of the IL-1 signaling pathway [13] in cultured human airway epithelial cells and mouse airways exposed to rhinovirus as well as type 2 cytokines IL-33 and IL-13. Tollip is one of the key immune negative regulators during virus infection, but the role of Tollip in regulating the production of type 2 cytokines such as IL-33 during viral infection has not been investigated.

Patients who presented with severe IAV infection had excessive neutrophilic inflammation and neutrophil extracellular trap formation [14]. During degranulation and release of neutrophil extracellular traps, neutrophil elastase (a serine protease) is also released and can further enhance inflammation by cleaving proteins such as IL-1 β and IL-33 into their mature forms, enhancing their activation [15, 16]. It is unclear if Tollip can inhibit excessive neutrophilic inflammation by reducing the release and activation of IL-33 during IAV infection.

IL-33, an alarmin, is an IL-1 family member cytokine, which can be released from lung epithelial cells upon injury and oxidative stress caused by viruses and allergens [8, 17]. The severity of IAV infection in mice has been positively correlated with IL-33 release into airways [18]. IL-33 can be released in its full length or pro-form that is approximately 30 kDa and functionally active, but it can also be cleaved into fragments ranging from 17 to 28 kDa with varying degrees of activity [19].

How IL-33 is released during viral infection remains unclear. It is known that IAV infection can cause mitochondrial stress (e.g., reactive oxygen species production) and damage the cells [20]. When there is injury to mitochondria, adenosine 5'-triphosphate (ATP) is released as a mechanism to initiate inflammatory signaling leading to increased inflammation [21, 22]. Patients with respiratory diseases such as chronic obstructive pulmonary disease and idiopathic pulmonary fibrosis have significantly more ATP released into bronchoalveolar lavage (BAL) fluid compared to healthy controls, which is associated

with an increase in IL-33 levels [21, 23]. It is unclear if IAV infection enhances extracellular ATP release in Tollip-deficient lungs. As Tollip is critical to facilitate the degradation of damaged mitochondria [11], we hypothesized that Tollip deficiency increases extracellular ATP release, subsequent IL-33 secretion, and inflammation during IAV infection. We further hypothesized that blocking excessive IL-33 signaling in Tollip-deficient mice with the IL-33 decoy receptor soluble ST2 (sST2) may reduce exaggerated lung inflammatory responses during viral infection.

Materials and Methods

IAV Preparation

The virus used in this study was pandemic influenza A/California/04/2009 (CA04) virus, which was kindly provided by Dr. Mari Numata from National Jewish Health (NJH). The virus was propagated in Madin-Darby canine kidney (MDCK; ATCC, Manassas, VA, USA) cells, as previously published [24–27]. Briefly, virus was propagated in MDCK cells in Dulbecco's Modified Eagles Medium (DMEM; Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal calf serum (MilliporeSigma, Burlington, MA, USA), L-glutamine, antibiotics, and 1.5 μ g/mL of N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Thermo Fisher Scientific, Waltham, MA, USA), harvested at 72 h post-infection, and tittered by quantitative plaque assay using MDCK cells [28].

Mice

Tollip knockout (KO) mice on a C57/BL6 background were obtained from Dr. Liwu Li at Virginia Polytechnic Institute and State University and bred at the NJH Biological Resource Center (BRC). Wild-type (WT) C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA) and housed with the KO mice at NJH BRC under pathogen-free housing conditions. All the experimental protocols were approved by the Institutional Animal Care and Use Committee at NJH.

Mouse Model of IAV Infection

Tollip KO and WT mice, both males and females from the age of 8–12 weeks with age and gender matched, were inoculated intranasally with 1×10^2 PFU/mouse of IAV or 50 μ L of PBS as control. Mice were observed and weighed daily and were sacrificed four days and seven days post-infection. We chose this dose of IAV based on our previous publication [28].

Mouse Model of IAV Infection with Apyrase Treatment

Tollip KO and WT mice were inoculated intranasally with 1×10^2 PFU/mouse of IAV. 24 h after IAV infection, mice were given 200 U/kg body weight of apyrase (MilliporeSigma) via oropharyngeal aspiration. Apyrase is an ATP converter that removes the phosphate group from ATP and ADP resulting in the culmination of AMP. On day five and day six post-infection, mice were given another round of 200 U/kg of apyrase. Mice were sacrificed seven days post-infection. We chose 200 U/kg based on previously published research

where mice on the same C57BL/6 background showed reduced airway inflammation after treatment with apyrase [29].

Mouse Model of IAV Infection with sST2 Treatment

Tollip KO and WT mice were infected with 1×10^2 PFU/mouse of IAV and simultaneously treated intranasally with either 5 μ g or 10 μ g of recombinant mouse ST2 (R&D Systems, Minneapolis, MN, USA), or IgG control, in a total volume of 50 μ L. 24 h after infection, mice were given another round of ST2 treatment. Mice were monitored and weighed daily. Four days post-infection, mice were sacrificed. The 5 μ g dose was selected from one of our previous publications [30], and the 10 μ g dose was selected after our pilot dose optimization study.

Mouse BAL Fluid and Lung Tissue Processing

Mice were euthanized by intraperitoneal injection of pentobarbital sodium (Fatal-Plus) in sodium chloride. Lungs were lavaged with 1 mL of sterile saline. Cell-free BAL fluid was used for Western blot analysis of IL-33 and ATP measurement using the ATP Assay Kit (MilliporeSigma). BAL fluid cell cytospin slides were stained with a Diff-Quick stain kit (IMEB, San Marcos, CA, USA) for cell differential counts. Leukocyte differentials were determined as a percentage of 500 counted leukocytes. Right lungs were submerged in 10% formalin and used for immunohistochemistry (IHC).

Mouse Trachea Epithelial Cell Isolation and Culture

In separate experiments, tracheal epithelial cells from naive WT and Tollip KO mice were isolated as previously reported [31]. Briefly, tracheas from WT and Tollip KO mice were incubated in a 0.1% protease (MilliporeSigma), 50 μ g/mL amphotericin B (MilliporeSigma), DMEM (Thermo Fisher Scientific, Waltham, MA) solution for up to 4 h at 4°C. Thereafter, the protease solution was quenched with DMEM plus 2% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA, USA). The cells were then expanded on irradiated 3T3 fibroblasts (ATCC, Manassas, VA) in the presence of recombinant mouse epidermal growth factor (mEGF; Corning Inc., Corning, NY, USA) and a Rho kinase inhibitor Y-27632 as described by us and others [31–33]. Once confluent, mouse trachea epithelial cells (mTECs) were put onto a collagen-coated 24-well plate in BronchiaLife medium (Lifeline Cell Technology LLC, Frederick, MD, USA) supplemented with mEGF.

IAV Infection in mTEC

Once cells were 100% confluent in the 24-well plate, 1x PBS (Thermo Fisher Scientific) was used to wash the cells one time. The cells were infected with either 1×10^3 PFU/well or 1×10^5 PFU/well of IAV in BronchiaLife medium with 1.5 μ g/mL of TPCK/trypsin or just BronchiaLife medium with 1.5 μ g/mL TPCK/trypsin as a control. Cells were incubated at 37°C for 2 h, after which, the cells were washed twice with PBS, and fresh BronchiaLife medium with mEGF was added. 48 h after infection, supernatants were harvested for ATP measurement. The 48-h time point was chosen based on our optimization study that showed peak ATP release was at 48 h post-IAV infection (data not shown).

Effect of ATP on IL-33 Release in mTEC for Measuring IL-33 Release

WT mTECs were treated with 10 μ M of ATP disodium salt (MilliporeSigma). 48 h after treatment, supernatants were harvest-

ed for measuring IL-33 release. 10 μ M of ATP was chosen based on previously published research that showed human airway epithelial cells treated with ATP-released IL-33 into the supernatant [23].

To demonstrate ATP-induced IL-33 in airway epithelial cells was functionally active, an aliquot (250 μ L) of the conditioned media (supernatant) from mTECs treated with or without ATP was centrifuged to remove cell debris and then treated for 30 min at 37°C with apyrase (2U/mL) and/or sST2 (1 μ g/mL), which was then added to naive WT mTECs. After 48 hrs, the supernatant was harvested for CXCL1 (a neutrophilic chemokine) measurement.

Immunohistochemistry

Immunostaining of IL-33 was performed on formalin-fixed mouse lung tissues. 5 μ m paraffin-embedded sections were deparaffinized in two washes of xylene and rehydrated in graded alcohol series. Tissue sections were then heated at 95°C in sodium citrate buffer (10 mM, pH6.0) for 20 min for antigen retrieval, and endogenous peroxidase was blocked by 0.3% H₂O₂ in distilled water. After blocking with 1.5% normal goat serum, the sections were incubated with either a polyclonal goat anti-mouse IL-33 antibody (1:50, AF3626; R&D Systems, Minneapolis, MN, USA) or an isotype control overnight at 4°C in a humidified chamber. Subsequently, the tissue sections were incubated with the biotinylated secondary antibody and then the avidin-biotin-peroxidase complex (VECTASTAIN Elite ABC horseradish peroxidase kit; Vector Laboratories, PK-6105, Burlingame, CA, USA). 3-Amino-9-ethylcarbazole (MilliporeSigma, Burlington, MA, USA) was used as chromogen to develop peroxidase-dependent color reaction.

Quantification of IL-33 Staining in Mouse Airway Epithelium

The positivity of IL-33 immunostaining in airway epithelium (no hematoxylin counterstaining) was quantified by using the NIH ImageJ software. By applying the density slice function of the software, IL-33 positive area and the total area of the airway epithelium were obtained. The positivity of IL-33 was calculated by dividing the positive area over the total area of the airway. The average values in three medium-sized airways per mouse were used for statistical analysis.

Western Blot Analysis

IL-33 released into the BAL fluid of Tollip KO and WT mice was measured via Western blot. Equal volume of BAL was separated on 20% SDS-polyacrylamide gels, transferred onto PVDF membranes, blocked with Western blocking buffer, and incubated with a goat anti-mouse IL-33 antibody (R&D Systems, Minneapolis, MN, USA) overnight at 4°C. Western blotting of soluble E-cadherin was performed to indicate the level of lung epithelial integrity or injury. Briefly, equal volume of mouse BAL fluid was separated on 8% SDS-polyacrylamide gels, transferred onto PVDF membranes, blocked with blocking buffer, and incubated with a goat anti-mouse E-cadherin antibody (R&D Systems) overnight at 4°C. After washing in PBS with 0.1% Tween-20, the membranes were incubated with the appropriate horseradish peroxidase-linked secondary antibodies and developed using a Fotodyne imaging system (Fotodyne, Inc., Hartland, WI, USA).

Densitometry was performed using the NIH ImageJ software. The densitometric values in equal volumes of BAL fluid sample were used to indicate protein levels. Protein levels in WT PBS controls were used to normalize those in other groups of mice.

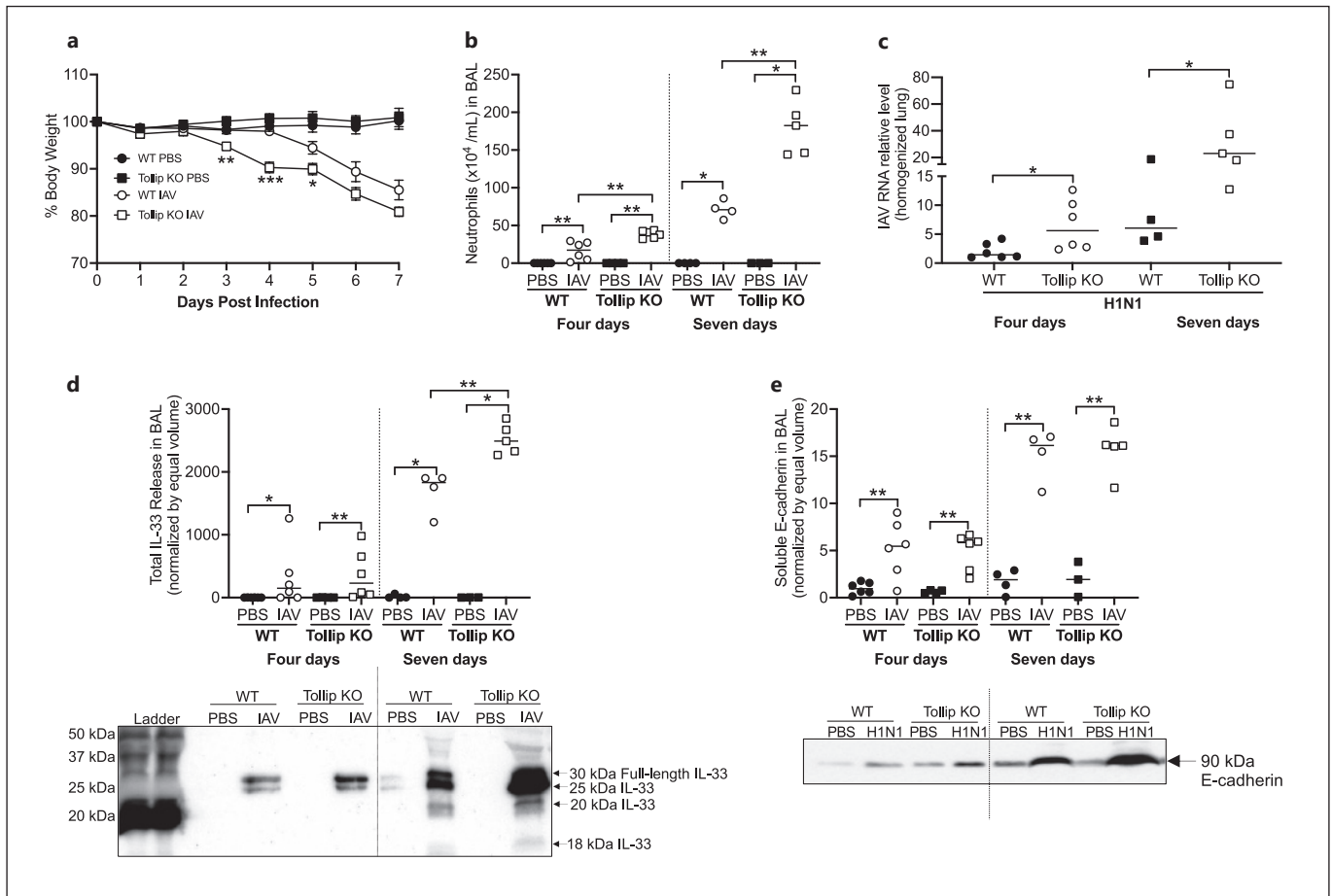


Fig. 1. Tollip-deficient (KO) mice infected with IAV exhibit greater weight loss, inflammation, viral load, and IL-33 release. WT and Tollip (KO) mice were intranasally infected with 1×10^2 PFU/mouse of IAV or treated with PBS. **a** IAV-infected Tollip KO mice lost significantly more weight 4 days post-infection. **b** Tollip KO mice had significantly more neutrophils in BAL fluid four days and seven days post-infection. **c** Tollip KO mice had significantly higher viral load in homogenized lung tissue compared to WT mice. **d** Tollip KO mice

had significantly more total IL-33 (full length and cleaved fragments) secreted into BAL fluid seven days post-infection. **e** IAV infection increased the soluble E-cadherin levels in BAL fluid of WT and Tollip KO mice. Western blots were normalized by loading equal volume (25 μ L) of BAL fluid. Black circles: WT PBS; black squares: Tollip KO PBS; white circles: WT IAV; white squares: Tollip KO IAV. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. p values represent a Mann-Whitney test. Replicates from one individual experiment.

Reverse Transcription and Quantitative Real-Time PCR

Intracellular IAV was measured by reverse transcription and quantitative real-time PCR. Lung tissue was homogenized, and RNA was isolated using the TRIzol reagent method. A total of 500 ng of RNA underwent reverse transcription to produce cDNA using a Bio-Rad T100 thermocycler. Custom-made primers and probe (Integrated DNA Technologies, Coralville, IA) for IAV were 5'-GACCRATCCT-GTCACCTCTGAC-3' (forward), 5'-AGGGCATTYTG-ACA-AAKC-3' (reverse), and 5'-TGCAGTCCTCGCTCACTGGC-ACG-3' (probe) [34].

Statistical Analysis

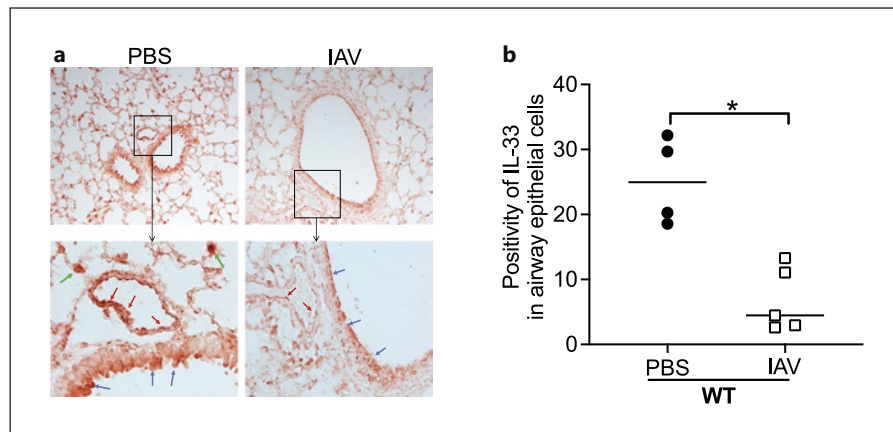
Nonparametric data were analyzed using a Mann-Whitney test for two group comparisons. Parametric data were analyzed using unpaired t tests for two group comparisons. A p value < 0.05 was considered statistically significant.

Results

Tollip-Deficient Mice with IAV Infection Show Enhanced Weight Loss, Lung Inflammation, Viral Load, and IL-33 Release

After three days of a low-dose IAV infection, Tollip-deficient (KO) mice began to lose significantly more weight than WT mice. This significant weight loss continued until five days post-infection, with day four being the most significant. While mice continued to lose weight during seven days of infection, the weight loss difference between WT and Tollip KO mice was no longer significant at days six and seven (Fig. 1a).

Fig. 2. IAV infection reduces intracellular IL-33 found in airway epithelial cells of WT mice. **a** IHC of WT mouse lung tissues showing IL-33 staining in epithelial cells (blue arrow), endothelial cells (red arrow), and alveolar type II cells (green arrow). **b** Morphometric quantitative analysis of IL-33 in airway epithelium of WT mice showing less IL-33 following IAV infection. * $p < 0.05$. p value represents a Mann-Whitney test. Replicates from one individual experiment.



IAV infection significantly increased neutrophils in BAL fluid in both strains of mice. However, Tollip KO mice had significantly more neutrophils than WT mice four days post-infection. At seven days post-infection, increase in neutrophils was observed in both strains of IAV-infected mice, but Tollip KO mice continued to have significantly more neutrophils than WT mice (Fig. 1b).

Viral load was measured by reverse transcription and quantitative real-time PCR in homogenized lung tissue of WT and Tollip KO mice (Fig. 1c). Four days and seven days past infection, Tollip KO mice had significantly higher viral load compared to WT mice, suggesting impaired viral clearance from Tollip KO mouse lungs.

IL-33 release into the BAL fluid was measured via Western blot (Fig. 1d). While mice infected with IAV for four days had significantly more release of total IL-33 (both the full length and cleaved fragments) compared to PBS controls, there was not a significant difference between WT and Tollip KO strains. However, infected Tollip KO mice after seven days of infection had significantly more IL-33 in the BAL fluid than WT mice.

It was previously shown that soluble E-cadherin was increased in BAL fluid of mice infected with respiratory syncytial virus to indicate the disruption of the airway epithelial barrier [35]. To determine if increased IL-33 release seen in Tollip KO mice was associated with excessive lung injury caused by IAV infection, soluble E-cadherin was measured in the BAL fluid (Fig. 1e). After four and seven days of IAV infection, both WT and Tollip KO mice had significantly more soluble E-cadherin than their uninfected counterparts. Interestingly, there was no significant difference of soluble E-cadherin levels between WT and Tollip KO mice, suggesting that the excessive IL-33 found in the BAL fluid of Tollip KO mice may not be

related to more severe loss of lung epithelial cell integrity during IAV infection.

Localization of IL-33 in IAV-Infected Mouse Lungs

IHC was done to visualize where IL-33 was located in mouse lung tissue. As can be seen in Figure 2a, IL-33 is localized to airway epithelial cells, endothelial cells, and alveolar type II cells. In WT mice infected with IAV, there was a significant reduction in intracellular IL-33, specifically in the airway epithelial cells (Fig. 2b). This suggests that IAV infection may promote IL-33 release from airway epithelium into the airway lumen.

Role of ATP in Tollip Deficiency-Mediated IL-33 Release in vivo

While we have shown IAV infection might promote IL-33 release into the airway lumen, it remains unclear as to how IL-33 is being released. There are reports suggesting that IL-33 can be actively released via ATP without the involvement of cell death. For example, extracellular ATP can be increased by epithelial cell stress [36, 37], which is associated with IL-33 release [21, 23, 38].

We found that ATP levels were increased in BAL fluid of IAV-infected (vs. PBS control) WT and Tollip KO mice (Fig. 3a). Interestingly, Tollip KO mice had significantly higher levels of ATP than WT mice. To determine if ATP contributes to the excess IL-33 release in Tollip KO mice, we treated mice with apyrase, an ATP converter. After apyrase treatment, extracellular ATP levels were significantly reduced in both WT and Tollip KO mice with IAV infection. Importantly, excess IL-33 release in BAL fluid of IAV-infected was significantly reduced by apyrase treatment (Fig. 3b). However, apyrase did not decrease IL-33 levels in IAV-infected WT mice. Apyrase

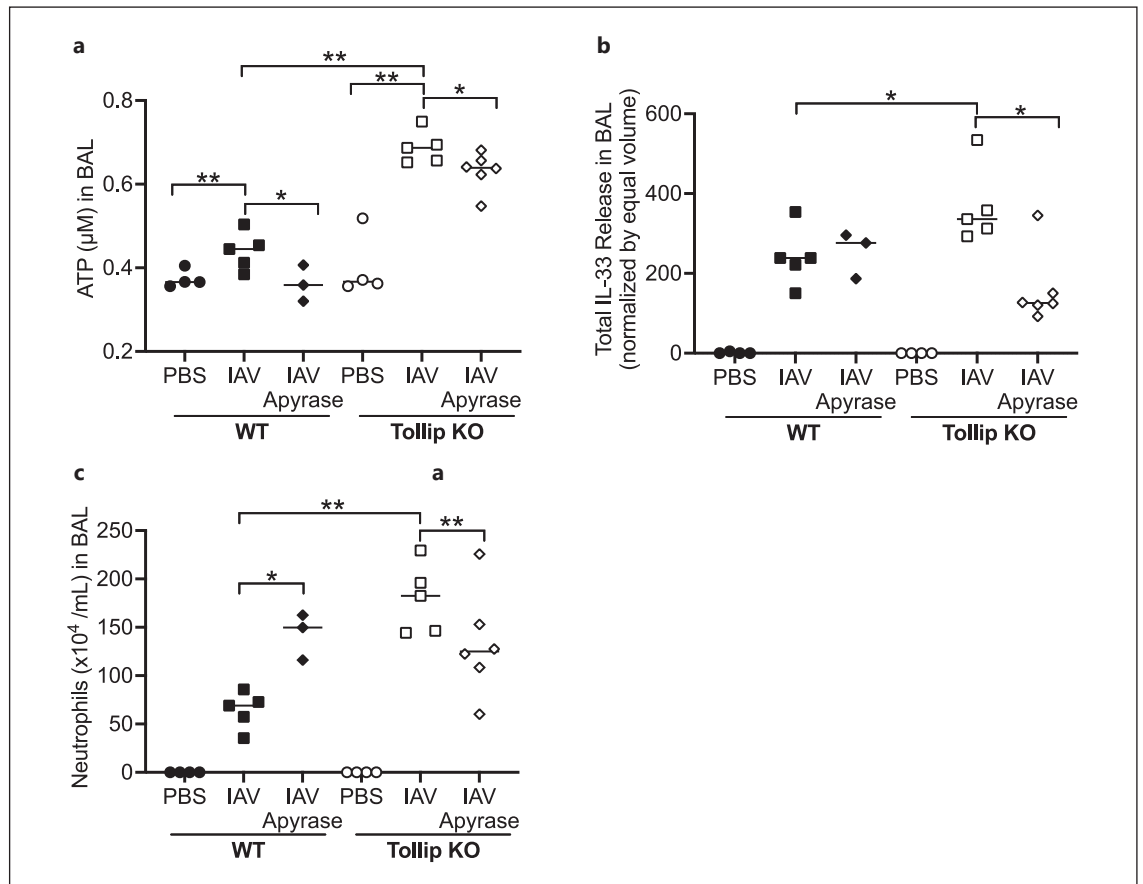


Fig. 3. A converter of extracellular ATP reduces excessive release of ATP and IL-33 release, and inflammation in IAV-infected (seven days) Tollip-deficient (KO) mice. **a** IAV-infected Tollip KO mice had significantly more extracellular ATP in BAL fluid than infected WT mice, which was reduced by apyrase treatment. Apyrase treatment significantly reduced total IL-33 (full length and

cleaved fragments) release (**b**) and neutrophil levels in IAV-infected Tollip KO mice (**c**). Western blots were normalized by loading equal volume (25 µL) of BAL fluid. * $p < 0.05$; ** $p < 0.01$. p values represent a Mann-Whitney test. Replicates from one individual experiment.

treatment also significantly reduced the neutrophils in the BAL fluid of Tollip KO mice, but not WT mice (Fig. 3c).

Role of ATP in IL-33 Release following IAV Infection *in vitro*

To determine if airway epithelial cells may contribute to the ATP release seen *in vivo* following IAV infection, WT and Tollip KO mTECs were infected with varying doses of IAV, and extracellular ATP was measured from the supernatants (Fig. 4a). WT and Tollip KO cells had similar ATP levels in their supernatant under baseline conditions. The lower dose of IAV did not show an increase in ATP levels in WT cells 48 h after infection compared to PBS controls; however, Tollip KO cells trended

to increase ATP compared to the PBS control cells. It is important to note that infected Tollip KO cells had significantly more ATP in the supernatants compared to infected WT cells. The high dose of IAV showed a significant increase in ATP levels for both strains of cells, with Tollip KO continuing to have significantly more extracellular ATP release. Interestingly, the lactate dehydrogenase (LDH) levels, which is a reliable indicator of cellular cytotoxicity, remained similar between low dose of IAV and controls; however, the high dose of IAV showed a significant increase in LDH levels that were similar among WT and Tollip KO cells (Fig. 4b). These data suggest that IAV infection in Tollip-deficient cells enhances extracellular ATP release in the absence or presence of cellular cytotoxicity.

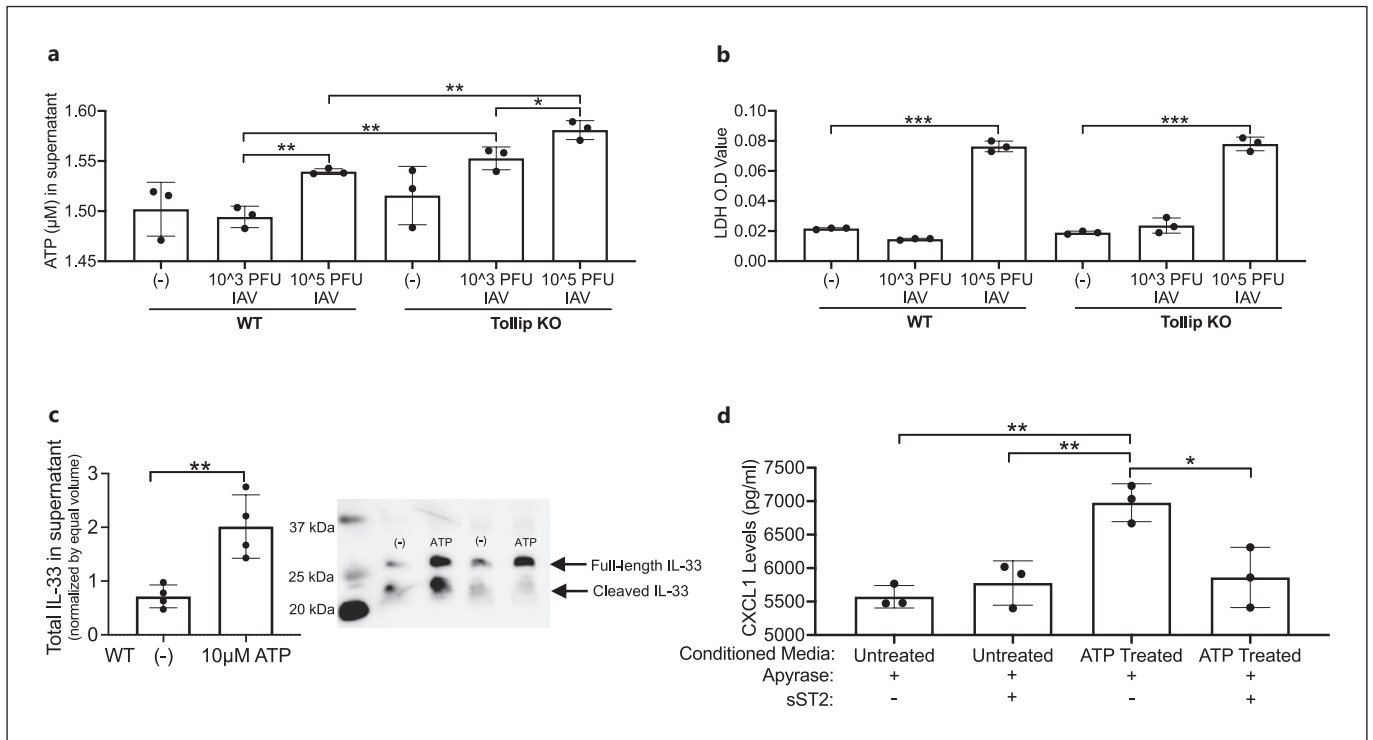


Fig. 4. IAV-induced extracellular ATP directly enhances IL-33 release in mTECs. WT C57BL/6 and Tollip KO mTECs were infected with two different doses of IAV for 48 h when (a) Tollip KO cells infected with a high dose of IAV had significantly higher levels of extracellular ATP than WT cells, while (b) LDH (a marker of cytotoxicity) levels were similar among WT and Tollip KO cells. c WT mTECs treated with 10 μM ATP for 48 h showed a significant

increase of total IL-33 (full length and cleaved fragments) release. Western blots were normalized by equal volume loading. d Conditioned media (supernatants) treated with 10 μM of ATP in the presence of apyrase increased neutrophilic chemokine CXCL1 in naïve WT cells, which was inhibited by sST2. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. p values represent unpaired parametric t tests. Replicates from two individual experiments.

To determine if extracellular ATP directly increases total IL-33 release, mTECs from WT mice were treated with ATP. Western blot analysis showed that ATP significantly increased the release of total IL-33 (both the full length and cleaved fragments) (Fig. 4c). To determine whether released IL-33 by extracellular ATP is functionally active, conditioned media (supernatants) from mTECs treated with or without ATP was added to naïve WT mTECs to measure CXCL1, a neutrophilic chemokine. Before conditioned media were added to mTECs, they were treated with apyrase and/or sST2 (an IL-33 decoy receptor [39]) to prevent any effect of ATP on additional IL-33 release or other IL-33-mediated effects, and to confirm the specificity of IL-33 activity/action in ATP-treated epithelial conditioned media. We found that ATP-treated conditioned media in the presence of apyrase significantly increased CXCL1 compared with untreated conditional media (Fig. 4d), which was significantly

inhibited by the addition of sST2, suggesting the functional activity of extracellular ATP-induced IL-33 release.

sST2 Treatment Inhibits IAV-Induced Lung Inflammation in Tollip KO Mice

To determine if amplified IL-33 signaling is partially responsible for the increased neutrophilic inflammation following IAV infection in Tollip KO mice, we treated the mice with sST2. A low dose of sST2 treatment was able to significantly reduce airway neutrophilic inflammation in WT mice; however, it did not reduce neutrophils in Tollip KO mice (Fig. 5a). The dose of sST2 was doubled in Tollip KO mice as greater neutrophilic inflammation was seen in IAV-infected KO mice. The higher dose of sST2 was able to significantly reduce IAV-induced neutrophilic inflammation. sST2 treatment was also able to significantly reduce IAV-mediated IL-33 release in Tollip KO

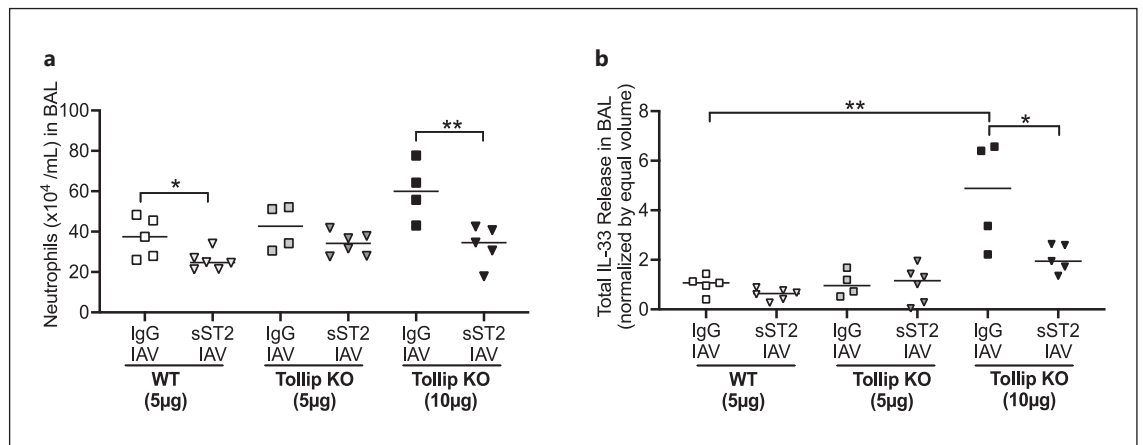


Fig. 5. Effect of IL-33 signaling pathway inhibition by sST2 on IAV-mediated lung neutrophilic inflammation. **a** sST2 treatment (10 µg/mouse) during IAV infection (four days) significantly reduced excessive neutrophilic inflammation in Tollip KO mice. In WT mice, sST2 at 5 µg/mouse was sufficient to reduce neutrophilic

inflammation. **b** Excessive IL-33 release was reduced with sST2 treatment (10 µg/mouse) during IAV infection (four days) in Tollip KO mice. Western blots were normalized by equal volume loading. **p* < 0.05; ***p* < 0.01. *p* values represent a Mann-Whitney test. Replicates from one individual experiment.

mice, but not in WT mice (Fig. 5b). This suggests that sST2 is effective in inhibiting the IL-33/ST2L signaling axis and neutrophilic inflammation.

Discussion

This study, for the first time, shows that during IAV infection Tollip deficiency promotes excessive neutrophilic inflammation in part through extracellular ATP release and the ensuing IL-33 release and activation. We have also shown that by treating Tollip-deficient mice with recombinant mouse sST2 protein to block IL-33 signaling, there is a reduction of IAV-mediated lung neutrophilic inflammation.

Our group, and others, have shown that Tollip can inhibit inflammation and promote an antiviral response to several different species of viruses including rhinovirus [12, 13], HIV [40], and iridovirus [41]. Whether and how Tollip regulates inflammation during IAV infection remains poorly understood. In this report, we have clearly demonstrated that Tollip deficiency promoted lung neutrophilic inflammation and increased viral load following IAV infection, which is associated with greater release of the activated isoform of IL-33. The role of IL-33 during viral infections remains highly complex. Initially, IL-33 release was thought to act exclusively as an alarmin that is released due to injury or cell death caused by allergens, infections, and/or mechanical stress [42]. Recently, it has

been found that IL-33 plays a crucial role in neutrophilic and non-neutrophilic inflammation [43]. Our current data show that IAV infection promotes the release of IL-33 into the BAL fluid, which is significantly enhanced by Tollip deficiency. To determine if this release was due to injury (e.g., loss of epithelial integrity) caused by the virus, soluble E-cadherin was measured. E-cadherin is one of the cell-adhesion molecules seen in the epithelial tight junctions [44]. E-cadherin can be cleaved and released into the lumen of the airway by proteases such as neutrophil elastase, a serine protease released by neutrophils [45]. These data suggest that while IAV infection is causing injury, seen by the increase in E-cadherin in the BAL fluid, Tollip deficiency does not further enhance the injury. Therefore, the IL-33 release induced by a nonlethal IAV infection in Tollip-deficient mice may not be explained by amplified epithelial injury. As previous studies suggest, infection of a murine-specific Pneumovirus species in an allergic asthma model increased IL-33 release and impaired the host interferon response [46]. In our future studies, we will determine if Tollip may protect the lung from viral infection and IL-33 release in allergen-challenged mice.

While our data show that IAV infection induces IL-33 release, the underlying mechanism is unknown. Different mechanisms (e.g., passive and active) have been proposed for IL-33 release. While cell injury including necrosis and apoptosis initiates the passive release of IL-33, new mechanisms of active IL-33 release or activation during viral

infection have been elucidated. Emi-Sugie et al. [47] reported that treatment of endothelial cells with a low dose of poly I:C, a synthetic dsRNA molecule that mimics viral infection, showed enhanced IL-33 mRNA and protein expression without cell injury. Igarashi et al. [48] concluded that microRNA-29, which can be induced by respiratory syncytial virus, was able to inhibit sST2 release in human bronchial epithelial cells, leading to greater activation of the IL-33/ST2L signaling pathway. Importantly, mitochondrial stress and the ensuing extracellular ATP release have been implicated in the active release of IL-33 [49]. Our data show that following IAV infection Tollip deficiency promotes extracellular ATP release without amplified epithelial injury. When extracellular ATP was converted into AMP by apyrase, there was a significant reduction in IL-33 levels and neutrophilic inflammation, especially in Tollip KO mice. While apyrase was able to reduce ATP levels in Tollip sufficient mice, there was an increase in neutrophils in WT mice. Li et al. [50] concluded that the removal of systemic ATP by apyrase in WT septic mice did not inhibit the purinergic signaling mechanisms required for neutrophil chemotaxis, but still improved overall survival. Therefore, the exact mechanism of neutrophil recruitment via extracellular ATP release in different settings or disease models needs further investigation.

This study suggests that the epithelial cells are one of the cell types responsible for ATP-mediated IL-33 release during IAV infection. Others have reported and our IHC staining verifies that IL-33 can be released from many types of cells including airway epithelial cells, alveolar type II cells, endothelial cells, and fibroblasts [51]. Our *in vitro* experiments with mTECs suggest that Tollip-deficient airway epithelial cells may contribute to the enhanced extracellular ATP and IL-33 release seen *in vivo* resulting in enhanced neutrophilic inflammation. The resulting consequence of enhanced IL-33 levels is unclear. IL-33 has been implicated in inflammation and tissue injury/repair. As discussed above, we have clearly demonstrated the role of IL-33 in lung inflammation in the context of Tollip deficiency. Interestingly, IL-33 release may have some beneficial role in tissue repair [52–54]. When we added exogenous recombinant mouse IL-33 to our mouse airway epithelial cell injury model (i.e., scratch test), IL-33 promoted repair in Tollip sufficient cells, where the wound healed completely. In Tollip-deficient cells, IL-33 inhibited the repair process (online suppl. Fig. 1a, b; for all online suppl. material, see www.karger.com/doi/10.1159/000525315). This finding suggests the dependency of IL-33-mediated tissue repair on Tollip.

In the current study, we also demonstrated the efficacy of sST2 in inhibiting excessive neutrophilic lung inflammation in IAV-infected Tollip-deficient mice. sST2, the decoy receptor that sequesters IL-33 before it can bind to the transmembrane ST2 receptor, has been proposed to suppress the IL-33/ST2 signaling pathway. Our group has previously found that sST2 treatment after rhinovirus infection and type 2 cytokine exposure inhibits the release of neutrophil chemoattractant IL-8 release from human epithelial cells [13]. Here, we report that by inhibiting the IL-33/ST2 signaling pathway in the early stages of IAV infection, sST2 inhibited excessive neutrophilic inflammation, especially in Tollip-deficient mice. Our data suggest the potential therapeutic implication of using sST2 to prevent or treat excessive airway inflammation in patients with Tollip deficiency associated with genetic variants of Tollip or other conditions.

There are some limitations to the current study. The first is that the current study focuses on IAV infection without other disease components such as allergen challenge or cigarette smoke exposure to address the role of Tollip deficiency-mediated excessive IL-33 release in exacerbations of eosinophilic asthma or chronic obstructive pulmonary disease. While our data show neutrophilic inflammation following the infection, there were no eosinophils present. There are several possibilities to explain the absence of eosinophils in our model. First, IAV infection is known to induce Th1 cytokines such as IFN- γ , which is considered to inhibit eosinophilic inflammation [55–58]. Second, as already mentioned, we did not add an allergic component such as allergen challenge in our mouse model. Lastly, it is possible that when the Th1 response diminishes over time, Tollip deficiency-related excessive IL-33 may initiate eosinophilic inflammation, which needs future experimental confirmation. The second limitation is that while this report shows that extracellular ATP increases IL-33 release, more studies should be done to see if Tollip deficiency promotes mitochondrial dysfunction such as excessive ROS release, further explaining the increase in extracellular ATP levels.

The results of our study, for the first time, demonstrate that Tollip inhibits inflammation induced by IAV infection, potentially through the regulation of extracellular ATP-mediated IL-33 release. Furthermore, by reducing extracellular ATP after infection, or by blocking the IL-33/ST2 pathway during infection, excessive neutrophilic inflammation in Tollip-deficient lungs can be reduced.

Statement of Ethics

The Institutional Animal Care and Use Committee (IACUC) at NJH approved all studies involving animals under protocol AS2792-03-23.

Conflict of Interest Statement

Monica Kraft has received grants from Sanofi, ALA, Chiesi, and AstraZeneca; royalties from Elsevier; and consulting fees from AstraZeneca and Sanofi outside the submitted work. The rest of the authors have no conflicts to declare.

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Author Contributions

Nicolette Schaunaman and Hong Wei Chu conceived the study. Nicolette Schaunaman, Kris Genelyn Dimasuay, and Diana Cervantes performed all experiments, and Nicolette Schaunaman performed the data analysis. Nicolette Schaunaman, Kris Genelyn Dimasuay, Diana Cervantes, Liwu Li, Mari Numata, Monica Kraft, and Hong Wei Chu discussed and interpreted all results. Nicolette Schaunaman wrote the manuscript. All authors read and approved the final manuscript.

Data Availability Statement

All data generated or analyzed during this study are included in this article or its supplementary material files. Further inquiries can be directed to the corresponding author.

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