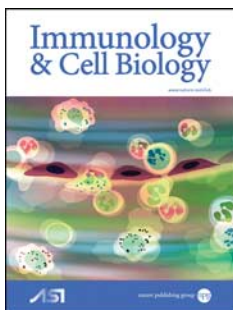


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A critical role of Gas6/Axl signal in allergic airway responses during RSV vaccine-enhanced disease.

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Running title: Gas6/Axl signaling in RSV-induced allergic inflammation

Abstract

Respiratory syncytial virus (RSV) is a common virus that causes lower respiratory infections across a wide range of ages. A licensed RSV vaccine is not available because vaccination with formalin-inactivated RSV (FI-RSV) and the subsequent RSV infection cause not only insufficient induction of neutralizing antibodies but also severe allergic airway responses, termed FI-RSV vaccine enhanced disease (FI-RSV VED). However, the underlying mechanism has not been identified, although a Th2-biased immune response is known to be a hallmark of this disease. Our previous studies have shown that growth arrest-specific 6 (Gas6)/Axl signaling leads to Th2-biased immune responses during fungus-induced allergic airway inflammation. Here, we show that Gas6/Axl signaling also leads to FI-RSV VED and partially identify the mechanism in mice. Inhibiting Gas6/Axl signaling using Gas6-deficient mice, neutralizing antibodies, and a specific inhibitor of Axl attenuated allergic airway hyperresponsiveness, including airway inflammation, goblet cell hyperplasia, and Th2 cytokine production, in addition to increasing interferon- γ levels and the production of RSV-neutralizing IgG2a in FI-RSV VED. Gas6 was produced in

lymph nodes during immunization with FI-RSV. Lymph node cells derived from immunized mice produced high levels of Gas6 and Th2 cytokines, but not IFN- γ , after restimulation with RSV. Finally, we found that dendritic cells stimulated with RSV-glycoprotein (G protein) produced Gas6 and that Axl signaling suppressed DC maturation and the induction of IL-12 production by the toll-like receptor 4 agonist RSV-fusion protein. Taken together, these results indicate that RSV-G protein-induced Gas6/Axl signaling causes allergic airway responses during FI-RSV VED.

Introduction

Respiratory syncytial virus (RSV) is a major cause of respiratory tract infection in individuals across a wide range of ages worldwide. It leads to 64 million cases of pneumonia and causes approximately 200,000 deaths annually(1, 2). Almost all infants are infected with RSV at least once by the time they are 2 years old, and the infection often recurs throughout life(3). Infants in the first six months, elderly individuals, and adults with underlying respiratory diseases are susceptible to lower respiratory tract infection with RSV(4-6). Despite these situations, currently no safe and effective RSV vaccines are available.

The major obstacles in the development of an RSV vaccine, formalin-inactivated RSV (FI-RSV), include not only the absence of neutralizing antibodies but also the occurrence of allergic airway inflammation during subsequent natural RSV infection. In the 1960s, the FI-RSV vaccine caused severe lung injuries in recipient children, and two infants died associated with an extended peribronchiolitis and alveolitis with a significant increase in the number of eosinophils present in the lung parenchyma that is currently called FI-RSV vaccine-enhanced disease (FI-RSV

VED)(7-9). FI-RSV VED was also observed in a mouse model: when FI-RSV-immunized mice were challenged with RSV, there were inappropriate Th2 immune responses, such as increased airway hyperresponsiveness (AHR), eosinophilia, and mucus goblet cell hyperplasia(10-13), although the underlying cause has not been determined.

Two major RSV surface proteins, fusion glycoprotein (F protein) and attachment glycoprotein (G protein), trigger the immune response. The RSV-F protein, the most effective viral protein in inducing protective immunity in animal model systems, binds to toll-like receptor 4 and triggers the activation of immune cells(14, 15). In contrast, the RSV-G protein binds to CX3C chemokine receptor 1 and is involved in Th2 immune responses, such as allergic inflammation, but the underlying mechanism is not clear(16, 17).

TAM (Tyro3, Axl, and Mertk) receptors, which belong to a distinct receptor tyrosine kinase protein subfamily, are expressed in various cells and tissues, and their ligand, growth arrest-specific 6 (Gas6), is involved in a variety of diseases(18-20). It has long been known that Gas6/TAM plays a role in the clearance of apoptotic cells

and as a pro-proliferative mediator in smooth muscle cells, fibroblasts, and cancer cells(21-24). In the field of immunology, it has been shown that Gas6/TAM signaling has immunosuppressive functions that inhibits toll-like receptor- and cytokine-driven immune responses *in vitro*(25, 26). Additionally, TAM receptor signaling in dendritic cells (DCs) controls the magnitude of the immune response by suppressing the activation of T cells *in vivo*(27). More recently, our study showed that signaling through Gas6 and its highest affinity receptor Axl, but not Mertk and Tyro3, is a novel inducer of Th2 immune responses in fungus-induced allergic airway inflammation(28-30).

Thus, the aim of the present study was to explore whether Gas6/Axl signaling facilitates allergic airway responses during FI-RSV VED. In this study, we addressed the role of Gas6/Axl in a well-described model of FI-RSV VED. Our data demonstrate that the induction of Gas6/Axl signaling by RSV-G protein leads to allergic airway inflammation via the regulation of DCs during FI-RSV VED.

Results

Gas6/Axl signaling facilitates allergic airway inflammation during FI-RSV VED.

To clarify the underlying mechanism, we first established a model of FI-RSV VED in WT mice; the schedule is shown in Figure 1A. Methacholine-induced AHR was significantly greater in mice with FI-RSV VED than control mice (**Figure 1B**). In line with the AHR results, severe airway inflammation was observed during FI-RSV VED (**Figure 1C**). Likewise, goblet cell hyperplasia was observed in the airway during FI-RSV VED (**Figure 1D**). In FI-RSV VED, eosinophilia was also observed in the tissue and bronchoalveolar lavage fluid (BALF) as previous reports on clinical and animal study (**Figure 1E, F**). These results suggest that Th2-biased immune reactions are predominant in this condition and cause allergic airway inflammation. Indeed, Th2 cytokines such as IL-4, IL-5, and IL-13, which are inducers of the allergic response observed in FI-RSV VED, were significantly higher in the lungs of mice experiencing FI-RSV VED than the control group (**Figure 1G**). The RSV titers in the lung from mice during FI-RSV VED on day 8 after virus infection were significantly higher than in that from non-immunized mice with FI-RSV (**Figure 1H**).

To study the role of Gas6/Axl signaling in FI-RSV VED, allergic responses were compared between WT and Gas6 KO mice. The allergic responses in Gas6 KO mice were significantly suppressed compared to those in the WT group (**Figure 2A**). Our model was non-lethal, but significant weight loss was transiently observed in WT mice on days 4 to 8 after RSV challenge. On the other hand, no weight loss in Gas6 KO mice was observed during this period (**Figure 2B**). The extent of allergic inflammation clearly correlated with AHR(31). In this model as well, peribronchial accumulation of inflammatory cells was clearly higher in the WT group than in the Gas6 KO group, as determined from histological analysis and the AHR results (**Figure 2C**). Additionally, goblet cell hyperplasia was observed in lung sections from the WT group, whereas a signal was hardly detected in lung sections from the Gas6 KO mice (**Figure 2D**). The number of eosinophils, which cause allergic inflammation, in the lungs of Gas6 KO mice was significantly less than that in WT mice (**Figure 2E**). The level of Th2 cytokines, such as IL-4, IL-5, and IL-13, in the homogenate from WT lungs was significantly greater than that in the homogenate from Gas6 KO mice (**Figure 2F**). Accordingly, the number of IL-4- and IL-13-producing CD4⁺ T cells in

lungs from Gas6 KO mice was also significantly lower than that in lungs from WT mice (**Figure 2G**).

Gas6 binds TAM receptors with different affinities: Axl $\geq$ Tyro3 $\gg$ Mertk(32, 33). Therefore, Axl could be the first choice of target to inhibit Gas6/TAM signaling in this model. Additionally, our previous findings revealed that anti-Axl mAb treatment ameliorates airway allergic inflammation during fungal asthma by suppressing Th2 cytokine production(28). In the current model as well, anti-Axl mAb treatment suppressed the allergic response during FI-RSV VED. The delivery of four anti-Axl mAb injections during immunization with FI-RSV significantly suppressed the increased AHR compared to the appropriate control group (**Figure 3A, B**). Other factors, such as body weight loss, airway inflammation accompanied by eosinophilia, and goblet cell hyperplasia, that contribute to the pathogenesis of allergic response in FI-RSV VED were also improved by anti-Axl mAb treatment (**Figure 3C–F**). Finally, as with Gas6 deficiency, anti-Axl mAb treatment suppressed Th2 cytokine production, which leads to pathogenesis (**Figure 3G**). However, since Gas6 will bind to Mertk when Axl is blocked by a monoclonal antibody, we injected mice with an Axl-specific

inhibitor, BGB324, which generally does not prevent binding between Gas6 and Axl, and examined the effects on allergic airway responses during FI-RSV VED. The results indicated that the injection of Axl inhibitors significantly attenuated the increase in AHR observed in the corresponding control group (**Supplemental Figure 1A, B**). Other factors, such as airway inflammation, goblet cell hyperplasia, and the production of Th2 cytokines, contributing to the pathogenesis of allergic response in FI-RSV VED were also improved by Axl inhibitor treatment (**Supplemental Figure 1C, D, E**). These results correspond reasonably well to the results that we observed for the anti-mAb treatment, suggesting that Gas6/Axl signaling facilitates allergic airway inflammation during FI-RSV VED.

Gas6 generated in lymph nodes during immunization with FI-RSV drives differentiation into Th2 cells.

Dendritic cells (DCs) engulf exogenous pathogens in the airways and then migrate to T cell-enriched lymph nodes via chemotactic signals. Exposure to allergens, in particular, then leads to interleukin (IL)-4-, IL-5-, and IL-13-induced Th2-dependent

airway inflammation and subsequent remodeling(34, 35). As Gas6/Axl signaling facilitates the allergic response during FI-RSV VED, we next investigated the source of Gas6 and its target cells during FI-RSV VED. Moreover, the development of FI-RSV VED is attributed to the regulation of acquired immunity; therefore, we investigated the cellular response in lymph nodes after immunization or infection with FI-RSV or RSV, respectively. The protein level of Gas6 in homogenates of the mediastinal lymph nodes (MLNs) from primary RSV-infected and FI-RSV VED mice was significantly greater than that in naïve mice (**Figure 4A**). Interestingly, the Gas6 level was also higher in homogenates of MLNs from FI-RSV-immunized mice without RSV infection, although Th2 cytokines were hardly detectable in this condition. When cells from FI-RSV-immunized mice were restimulated with RSV *in vitro*, they produced significantly more Gas6 than cells from non-immunized mice (**Figure 4B, C**). Furthermore, when lymph node cells from immunized Gas6 KO mice were restimulated with live RSV *in vitro*, they produced significantly more Th2 cytokines than cells from WT mice (**Figure 4C**). In contrast, the production of Th2 cytokines by lymph node cells isolated from FI-RSV-immunized Gas6 KO mice was significantly

reduced after restimulation with RSV, whereas IFN- γ production was significantly increased after restimulation. These results suggest that Gas6 regulates the differentiation of Th1 and Th2 cells via DCs during immunization with FI-RSV (**Figure 4C**). In line with this, we previously found that DCs and macrophages, but not T cells, produce high levels of Gas6, which facilitates the allergic response, and express TAM receptors—Axl in particular—in a fungal asthma model (30). Therefore, we again focused on DCs as the key cell subset driving subsequent immune responses. We then investigated whether these cells express Gas6 and Axl after FI-RSV and RSV stimulation *in vitro*. DCs constitutively produced Gas6 without any stimulation, and FI-RSV and RSV stimulation led to significant Gas6 production compared to control conditions (**Figure 4D**). Additionally, DCs constitutively express Axl without FI-RSV or RSV stimulation (**Figure 4E**). These results suggest that RSV- and FI-RSV-induced Gas6 autocrinely affects DCs via Axl in the lymph node and defines the subsequent Th1/Th2 immune response during immunization with FI-RSV.

Gas6/Axl signaling inhibits viral clearance by suppressing RSV-neutralizing IgG2a production during FI-RSV VED.

The inhibition of Gas6/Axl signaling via genetic deficiency, monoclonal antibody treatment, or inhibitor treatment ameliorated allergic responses during FI-RSV VED. Therefore, we investigated whether targeting Gas6/Axl signaling affects the induction of IgE, which induces the allergic response. As a result, the production of IgE was significantly less in Gas6 KO mice than in WT mice during FI-RSV VED (**Figure 5A**). Interestingly, we also found that RSV-F protein-specific IgG2a was hardly detected in serum from WT mice, whereas significant levels were seen in serum from Gas6 KO mice. The RSV titers in the lung of Gas6 KO mice were significantly lower than those in WT mice both at day 4 and at day 8 after virus infection during FI-RSV VED (**Figure 5B**). These results suggest that restoring IFN- γ production by lymph node cells from Gas6 KO mice could lead to increased production of IgG2a, which could be an effective neutralizing antibody against RSV. In fact, serum isolated from Gas6 KO mice during FI-RSV VED significantly reduced plaque formation by RSV *in vitro* relative to serum from WT mice (**Figure 5C**). These

results were observed in mice treated with the anti-Axl mAb during FI-RSV. When anti-Axl mAb was injected intraperitoneally every day from 2 days before FI-RSV injection to 1 day after the injection, the production of anti-RSV-F protein-specific IgE was significantly less than that in the IgG-treated group (**Figure 5D**). In contrast, the IFN- γ -induced IgG2a level was significantly higher in serum from anti-Axl mAb-treated mice than control IgG-treated mice. The RSV titer in the lung on days 4 and 8 was significantly lower in anti-Axl mAb-treated mice than in IgG-treated mice (**Figure 5E**). Furthermore, mice treated with the anti-Axl mAb showed significantly greater viral clearance than IgG-treated mice (**Figure 5F**). These results correlate with the results observed in Gas6 KO mice, suggesting that Gas6/Axl signaling increased the production of IgE, but not IgG2a, which efficiently neutralizes RSV. Therefore, targeting this signaling pathway might permit that appropriate regulation of humoral immunity in RSV infection.

RSV-G protein-induced Gas6/Axl signaling inhibits RSV-F protein-induced activation of DCs.

Gas6/Axl signaling facilitates allergic airway inflammation and reduces the production of effective antibodies after RSV infection. Therefore, we investigated which component of RSV is responsible for augmenting the Th2 immune response after FI-RSV vaccination and RSV infection. Since the RSV-G protein also induces a Th2-biased immune response during FI-RSV VED(17, 36), we hypothesized that there might be a relationship between RSV-G protein and Gas6 production. When exogenous RSV-G protein was added to DCs, Gas6 was produced in a dose-dependent manner, whereas Gas6 production was not induced by RSV-F protein (**Figure 6A, B**). We then tried to clarify the underlying mechanism by which Gas6/Axl signaling regulates the Th1/Th2 immune response during FI-RSV VED. IL-12 treatment during immunization elicits a Th1 immune response in FI-RSV VED and improves vaccine immunogenicity(13). We then examined IL-12 production specifically by DCs. Although RSV-G protein failed to induce IL-12 in DCs, incubating DCs with RSV-F protein resulted in a high level of IL-12 production. Importantly, addition of RSV-G protein with RSV-F protein diminished IL-12 induction in DCs, and neither FI-RSV nor RSV induced significant production of IL-12 by DCs (**Figure 6C**).

These results indicate that RSV-F protein is a strong stimulator of IL-12 production by DCs, whereas RSV-G protein has an opposing effect on the production of this cytokine. Moreover, the addition of both RSV-F and RSV-G significantly suppressed IL-12 production, in contrast to the addition of RSV-G protein alone. Similarly, the addition of recombinant Gas6 to DC cultures stimulated with RSV-F protein significantly suppressed IL-12 production regardless of the presence of RSV-G protein. In contrast, the suppression of IL-12 production from DCs treated with RSV-G protein, FI-RSV, and RSV was did not occur when the DCs were treated with the anti-Axl mAb. These results suggest that RSV-G protein-induced Gas6/Axl signaling suppresses RSV-F protein-induced IL-12 production in DCs. Additionally, an *in vivo* study also showed that Gas6 deficiency and anti-Axl mAb treatment, which show antiallergic effects, significantly increased the production of IL-12 in the lung during FI-RSV VED relative to that in the corresponding control group (**Figure 6D**). Finally, the expression of MHC II, CD40, CD80, and CD86, which are markers of DC activation, was also enhanced by RSV-F protein, but not RSV-G protein and RSV (**Figure 6E**). This RSV-F-induced expression was suppressed by the existence of

RSV-G protein as well as exogenous Gas6, whereas blockade of Gas6/Axl signaling ameliorated the suppression, suggesting that RSV-G protein-induced Gas6/Axl signaling alters DC characteristics such as antigen-presenting ability and activation. Taken together, RSV-G protein-induced Gas6/Axl signaling suppresses RSV-F protein-induced IL-12 production and the appropriate maturation of DCs, which may lead to proallergic responses and a reduction in suitable protection against RSV.

Discussion

Allergic airway responses are associated with Th2 immunity, and IL-25, IL-33, and TSLP have been identified as triggers in various allergic models(37-39). In contrast, our previous study showed that signaling from Gas6 and its receptor, Axl, is a novel inducer of allergic airway responses in a model of fungal asthma(28, 30). In this study, we also observed that Gas6/Axl signaling leads to allergic airway responses in RSV infection after immunization with FI-RSV (i.e., FI-RSV VED); this mechanism was previously unknown. Furthermore, we demonstrated that RSV-G protein, a Th2 inducer, leads to Gas6 production by DCs, which suppresses RSV-F protein-induced IL-12 and IFN- γ production via Axl during FI-RSV VED.

A mouse FI-RSV VED model has been established and widely used to clarify the mechanism underlying the pathogenesis of RSV-associated allergy(40-42). The structural changes in RSV-F protein that result from formalin fixation have been considered one of the causes of FI-RSV VED. Moreover, formalin effects are partially reversible by chemical reduction and boost Th2-biased responses in disease(43). However, UV irradiation of RSV also exacerbates the pathogenesis of RSV infection

after immunization with UV-inactivated RSV. The administration of UV irradiation-fixed RSV led to increased AHR (**Supplemental Figure 2A**), inflammation, including goblet cell hyperplasia and infiltration of eosinophils into the airways (**Supplemental Figure 2B, C**), and Th2 cytokine production (**Supplemental Figure 2D**) on days 4 and 8 after RSV infection. These results are consistent with reports by other groups and suggest that other mechanisms mediate the immune response that causes FI-RSV VED(44). In fact, RSV-G protein, but not F protein, induces the Th2-biased immune response that causes FI-RSV VED(17, 36, 45, 46). Although the molecular mechanism through which RSV-G protein induces Th2-biased immune responses in FI-RSV VED has not been determined, our findings show that Gas6/Axl signaling could be mechanism responsible for this induction.

We have identified two possible mechanisms through which Gas6/Axl signaling leads to a Th2-biased immune response in FI-RSV VED: 1) Gas6/Axl signaling directly induces Th2 immunity without modulating the Th1 immune response during allergic airway response, or 2) Gas6/Axl signaling skews the Th1/Th2 immune balance towards Th2 by suppressing the Th1 response, including IL-12 production

and subsequently IFN- γ . It is important that Gas6/Axl signaling dramatically enhances allergic immune responses during FI-RSV VED, even though it simultaneously attenuates RSV-F protein-induced activation of DCs with the concomitant suppression of MHC II, CD40, and CD80 expression. Furthermore, Gas6/Axl also strongly suppresses IFN- γ production in innate immune models(28, 47). These results seem to support the second hypothesis. However, further studies are needed to verify our hypothesis at the molecular and cellular levels while focusing on the mechanism by which Gas6/Axl suppresses IFN- γ in particular.

The roles of Gas6/Axl signaling in immune regulation have not yet been fully explained. Dr. Rothlin's group recently reported that the TAM family receptor tyrosine kinase Tyro3 is a negative regulator of type 2 immunity(48). These results seem to contradict our previous and current results, but we have already suggested distinct roles of TAM receptors in fungal asthma(28, 30). Another TAM family member, Mertk, shows the opposite effects of Axl and suppresses type 2 inflammation. As the affinity of Gas6 for Axl is significantly higher than its affinity for Mertk, the suppressive effects of Mertk appear with the effects of Axl when Gas6 reaches higher levels. The

use of different ligands (Gas6 and protein S) and receptors (TAM) generates diversity in efferocytosis and cancer progression; therefore, it is reasonable to consider that Th1/Th2 immune responses, such as allergic inflammation, are also regulated by these variations(21, 49). Although we showed that the combination of Gas6 and Axl leads to a Th2 immune response and reduces the induction of neutralizing antibodies against RSV, it might be necessary to carefully evaluate whether other combinations of ligands and receptors are involved in FI-RSV VED.

Targeting Axl might have applications not only in the development of a vaccine but also as a therapy for RSV infections that have unique characteristics. Recurrent RSV infection often causes asthma, particularly in childhood(50). Even healthy adults have recurrent RSV infection with cold-like symptoms throughout their life due to the failure to produce appropriate neutralizing antibodies(3). These effects might be due to Gas6/Axl signal-triggered immune suppression, which resembles the response during FI-RSV VED. Further studies investigating how Gas6/Axl signaling regulates the innate and adaptive immune responses associated with RSV infection will help resolve various issues associated with RSV infection.

In summary, we used gene targeting, monoclonal antibody, and inhibitor approaches to determine that the Gas6/Axl signal induced by RSV-G protein causes allergic airway responses and the lack of RSV neutralizing antibodies in FI-RSV VED. Thus, Gas6/Axl signaling might explain the earlier failures in the development of an RSV vaccine. Although further studies investigating the molecular mechanisms underlying the RSV-G protein-mediated induction of Gas6 and the Gas6/Axl signaling-mediated induction of Th2-biased immune reactions are required, the attenuation of Gas6/Axl signaling during vaccination and infection might represent a novel therapy for the prevention and treatment of RSV infection.

Materials and Methods

Ethics statement

All animal experiments were performed according to the guidelines of the Committees on Animal Handling and Ethical Regulations of the National Institute of Infectious Diseases (NIID), Japan, and the protocol was approved by the Institutional Animal Care and Use Committee of the institute.

Mice

Mice were maintained in a specific-pathogen-free facility in NIID. Wild-type C57BL/6J (WT) mice (7 weeks old) were obtained from Japan SLC, Inc. (Hamamatsu, Japan). Gas6-deficient (Gas6 KO) mice were generated as previously described and were subsequently bred and maintained in a specific pathogen-free colony at NIID(51). Gas6 KO mice were kindly provided by Dr. Russell Taichman (University of Michigan, Ann Arbor, MI, USA).

Model of FI-RSV VED in mice

RSV strain A2 was originally provided by Dr. Stokes Peeble at Vanderbilt University and propagated in cultured HEp-2 cells (American Type Culture Collection, VA). As shown in Supplemental Fig. S1, male C57BL/6J mice and Gas6 KO mice underwent primary sensitization with FI-RSV (10^5 PFU equivalent/mouse) and Imject Alum (Thermo Fisher Scientific, MA) via subcutaneous injection. Three weeks later, the mice were sensitized with FI-RSV (10^5 PFU equivalent/mouse) via intranasal (i.n.) administration three times every other day, followed by a final infection with live RSV (10^6 PFU/mouse) via i.n. instillation. Beginning on days 4 and 8 after RSV challenge, AHR was assessed in all groups of mice using flexiVent (SCIREQ, Montreal, Canada). Once the baseline airway resistance was established, increasing doses (i.e., 210 $\mu\text{g/kg}$ or 420 $\mu\text{g/kg}$) of methacholine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were administered via the tail vein, and AHR was monitored. In these studies, we observed that the 420 $\mu\text{g/kg}$ dose of methacholine evoked maximal changes in airway resistance, and these values are reported herein. Whole lung lobes from each mouse were dissected and snap frozen for cellular and proteomic analyses or fixed in 10% formalin for histological analysis. Sodium pentobarbital (0.04 $\mu\text{g/kg}$ of mouse

body weight; Kyoritsu Seiyaku Corporation, Tokyo, Japan) was used to anesthetize mice prior to intubation and ventilation.

Anti-Axl monoclonal antibody (mAb) and Axl inhibitor (BGB324) treatment of mice with FI-RSV VED

C57BL/6J mice received IgG (5 µg/dose; Genentech, CA) or anti-Axl mAb (5 µg/dose; Genentech) intraperitoneally every day from 2 days before to 1 day after the first FI-RSV injection with alum, for a total of 4 doses of anti-Axl mAb. Three weeks later, the mice were sensitized with FI-RSV (10^5 PFU equivalent/mouse) via i.n. administration three times every other day, followed by a final infection with live RSV (10^6 PFU/mouse) via i.n. instillation. Beginning on day 8 after RSV challenge, the allergic response was determined as described above. In another experiment, mice were treated with BGB324 (250 µg/dose; BerGenBio, Bergen, Norway) or hydroxypropyl methylcellulose (HPMC; Alfa Aesar, MA) in the same manner as the anti-Axl mAb treatment in FI-RSV VED as a control.

Cytokine ELISA

Murine Gas6, IL-4, IL-5, IL-13, IL-12p40, and interferon (IFN)- γ were measured in culture samples using a standardized sandwich ELISA (R&D systems, MN).

Recombinant murine proteins (R&D Systems) were used to generate standard curves.

The limit of ELISA detection for each cytokine was consistently >19.5, 15.6, 31.3, 62.5, 19.5, and 31.3 pg/ml for the Gas6, IL-4, IL-5, IL-13, IL-12p40 and IFN- γ sandwich ELISAs, respectively.

Bone marrow-derived DCs (BMDCs) and *in vitro* assay

BMDCs were prepared from control mice at various times before and after RSV challenge. To generate DCs, bone marrow cells were cultured with granulocyte-macrophage colony-stimulating factor (20 ng/ml; R&D Systems) for 6 days. DCs were sorted based on CD11c expression using magnetic-activated cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). DCs were then exposed to FI-RSV and RSV (multiplicity of infection = 1) and incubated for 48 h. The supernatant and DCs were collected for Gas6 ELISA and flow cytometric analysis of Axl,

respectively. In addition, DCs were stimulated with recombinant RSV-G protein (0.01–1 µg/ml; Sino Biological, Inc., Beijing, China) for 24 h, and the level of Gas6 in the supernatant was measured. Similarly, DCs were treated with recombinant Gas6 or anti-Axl mAb for 30 min and with RSV-F protein (1 µg/ml; Sino Biological Inc.) and/or RSV-G protein, FI-RSV, or RSV for 48 h. After this, the level of IL-12p40 in the supernatant was measured using a standard ELISA assay.

Flow cytometric analysis

Whole lung samples were obtained from all groups on day 8 after RSV challenge and incubated with collagenase type I (1 mg/ml; Thermo Fisher Scientific, Waltham, MA) and DNase I (1 µg/ml; Sigma-Aldrich, Saint Louis, MO) at 37 °C for 45 min. Whole lung cell suspensions were then incubated with anti-CD16/CD32 (2.4G2) and further labeled with fluorescent dye-conjugated mAbs (CD45 (BD Pharmingen, San Jose, CA Cat. No. 560694), CD11b (BD Pharmingen, 562127), Siglec-F (BD Pharmingen, 552126), CD4 (BD Pharmingen, 550954), and CD11c (BD Pharmingen, 553801), CD3 (eBioscience, CA, 17-5892-81) and CD8 (eBioscience, 11-0081-82)) for 15 min.

Similarly, tissue culture-generated cells were incubated with these antibodies for the same amount of time. For intracellular staining of cytokines, lung cells (10^6 cells per well) were stimulated with PMA (50 ng/ml) and ionomycin (1 μ M) in the presence of Golgistop (BD Cytofix/Cytoperm Kit; BD Biosciences Pharmingen) for 5 h. Cells were washed and stained with anti-CD45, anti-CD3, anti-CD4, and anti-CD8 antibodies. They were resuspended in a fixation/permeabilization solution (BD Cytofix/Cytoperm Kit; BD Pharmingen) and stained with anti-IL-4 (BioLegend, CA, 17-7041-81), anti-IL-5 (BD Pharmingen, 562048), and anti-IL-13 (BioLegend, 12-7133-81) antibodies for 30 min. For the *in vitro* study, BMDCs were stained with antibodies against MHC II (BD Pharmingen, 557000), CD40 (BD Pharmingen, 562846), CD80 (BD Pharmingen, 560528), and CD86 (BD Pharmingen, 561964) after blocking FcR with anti-CD16/CD32. Data were acquired on a FACSCanto II machine using FACS Diva software (BD Pharmingen), and all data were analyzed using the FlowJo software package (TreeStar, Ashland, OR).

Culture and stimulation of lymph node cells

Mediastinal lymph nodes (MLNs) were digested mechanically using an 18-gauge needle and enzymatically via incubation with collagenase I and DNase I in RPMI 1640 (Wako, Osaka, Japan) with 10% fetal calf serum (FCS). Following RBC lysis, cells were passed through a 40- μ m strainer and counted. Suspensions of total lymph node cells were cultured in complete medium and restimulated with RSV (multiplicity of infection = 1) for 48 h for measurement of Th cytokines in the culture supernatant.

Plaque assay and plaque reduction assay

For the immunoplaque assay, total lungs were homogenized, and serial dilutions of the supernatant were incubated in a 24-well plate with HEp-2 cell monolayers in Dulbecco's modified Eagle's medium (DMEM) and overlaid with methylcellulose, as described previously(52). After 4 days, viral plaques were visualized using a goat anti-RSV polyclonal antibody (Millipore, Temecula, CA). For the plaque reduction assay, serum from mice in each group was gradually diluted with DMEM containing 10% FCS, then added to 90% confluent HEp-2 cells for 7 h and replaced with

methylcellulose. On day 3, after adding the lung homogenates, the plaque assay was carried out with the anti-RSV antibody. The data are expressed as the percentage of plaque reduction compared to naïve serum, which was set to 1.

Detection of RSV-specific antibodies

RSV-specific antibodies were detected in sera collected on days 4 and 8 after RSV challenge during FI-RSV VED using direct ELISA; plates were coated with RSV-F protein. Serum samples for ELISA were diluted 1:10–1:1000; the data shown here are for the 1:100 dilution. Anti-RSV IgG (EMD Millipore, CA) was used as a secondary antibody before horseradish peroxidase was added (Bio-Rad, NC) and the OD at 450 nm was measured.

Preparation of UV-inactivated respiratory syncytial virus (RSV) and immunization.

RSV was irradiated with UV at $1350 \mu\text{W}/\text{cm}^2$ for 20 min to inactivate it. Instead of formalin-inactivated RSV, mice were immunized with UV-inactivated RSV prior to RSV infection to construct vaccine-enhanced disease.

Statistical analysis

All results are expressed as the mean $\pm$ standard error of mean. A two-tailed Student's *t*-test was used to determine statistical significance between groups. All statistical analyses were performed using GraphPad Prism software (ver. 6.0; San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

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Author contributions

T.S. designed and performed the experiments and analyzed the data. T.S. and M.A. wrote the manuscript.

Abbreviations:

Respiratory syncytial virus – RSV

Growth arrest-specific 6 – Gas6

Tyro3, Axl, and Mertk – TAM

Formalin-inactivated RSV vaccine-enhanced disease – FI-RSV VED

Dendritic cells – DCs

Immunoglobulin – Ig

Monoclonal antibody – mAb

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References

1. Garcia CG, Bhole R, Soriano-Fallas A, Trost M, Chason R, Ramilo O, et al. Risk factors in children hospitalized with RSV bronchiolitis versus non-RSV bronchiolitis. *Pediatrics*. 2010;126(6):e1453-60.
2. Nair H, Nokes DJ, Gessner BD, Dherani M, Madhi SA, Singleton RJ, et al. Global burden of acute lower respiratory infections due to respiratory syncytial virus in young children: a systematic review and meta-analysis. *Lancet*. 2010;375(9725):1545-55.
3. Fernald GW, Almond JR, Henderson FW. Cellular and Humoral Immunity in Recurrent Respiratory Syncytial Virus Infections. *Pediatric Research*. 1983;17:753-8.
4. Meissner HC. Selected populations at increased risk from respiratory syncytial virus infection. *Pediatr Infect Dis J*. 2003;22(2 Suppl):S40-4; discussion S4-5.
5. Suarez-Arrabal MC, Mella C, Lopez SM, Brown NV, Hall MW, Hammond S, et al. Nasopharyngeal bacterial burden and antibiotics: Influence on inflammatory markers and disease severity in infants with respiratory syncytial virus bronchiolitis. *J Infect*. 2015;71(4):458-69.
6. Matsumoto K, Inoue H. Viral infections in asthma and COPD. *Respir Investig*. 2014;52(2):92-100.
7. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. *Am J Epidemiol*. 1969;89:405-21.
8. Kim HW, Canchola JG, Brandt CD, Pyles G, Chanock RM, Jensen K, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. *Am J Epidemiol*. 1969;89:422-34.
9. Fulginiti VA, Eller JJ, Sieber OF, Joyner JW, Minamitani M, Meiklejohn G. Respiratory virus immunization. I. A field trial of two inactivated respiratory virus vaccines; an aqueous trivalent parainfluenza virus vaccine and an alum-precipitated respiratory syncytial virus vaccine. *Am J Epidemiol*. 1969;89:435-48.
10. Connors M, Giese NA, Kulkarni AB, Firestone CY, Morse HCr, Murphy BR. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV)

challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. *J Virol.* 1994;68:5321-5.

11. Cannon MJ, Openshaw PJ, Askonas BA. Cytotoxic T cells clear virus but augment lung pathology in mice infected with respiratory syncytial virus. *J Exp Med.* 1988;168(3):1163-8.

12. Graham BS, Bunton LA, Wright PF, Karzon DT. Role of T lymphocyte subsets in the pathogenesis of primary infection and rechallenge with respiratory syncytial virus in mice. *J Clin Invest.* 1991;88(3):1026-33.

13. Tang YW, Graham BS. Interleukin-12 treatment during immunization elicits a T helper cell type 1-like immune response in mice challenged with respiratory syncytial virus and improves vaccine immunogenicity. *J Infect Dis.* 1995;172(3):734-8.

14. Kurt-Jones EA, Popova L, Kwinn L, Haynes LM, Jones LP, Tripp RA, et al. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nat Immunol.* 2000;1(5):398-401.

15. Rallabhandi P, Phillips RL, Boukhvalova MS, Pletneva LM, Shirey KA, Gioannini TL, et al. Respiratory syncytial virus fusion protein-induced toll-like receptor 4 (TLR4) signaling is inhibited by the TLR4 antagonists *Rhodobacter sphaeroides* lipopolysaccharide and eritoran (E5564) and requires direct interaction with MD-2. *MBio.* 2012;3(4).

16. Varga SM, Wissinger EL, Braciale TJ. The attachment (G) glycoprotein of respiratory syncytial virus contains a single immunodominant epitope that elicits both Th1 and Th2 CD4+ T cell responses. *J Immunol.* 2000;165(11):6487-95.

17. Johnson TR, Graham BS. Secreted respiratory syncytial virus G glycoprotein induces interleukin-5 (IL-5), IL-13, and eosinophilia by an IL-4-independent mechanism. *J Virol.* 1999;73(10):8485-95.

18. Fourgeaud L, Traves PG, Tufail Y, Leal-Bailey H, Lew ED, Burrola PG, et al. TAM receptors regulate multiple features of microglial physiology. *Nature.* 2016;532(7598):240-4.

19. van der Meer JH, van der Poll T, van 't Veer C. TAM receptors, Gas6, and protein S: roles in inflammation and hemostasis. *Blood.* 2014;123(16):2460-9.

20. Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, et al. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med*. 2001;7(2):215-21.
21. Graham DK, DeRyckere D, Davies KD, Earp HS. The TAM family: phosphatidylserine sensing receptor tyrosine kinases gone awry in cancer. *Nat Rev Cancer*. 2014;14(12):769-85.
22. Lemke G, Burstyn-Cohen T. TAM receptors and the clearance of apoptotic cells. *Ann N Y Acad Sci*. 2010;1209:23-9.
23. Seitz HM, Camenisch TD, Lemke G, Earp HS, Matsushima GK. Macrophages and dendritic cells use different Axl/Mertk/Tyro3 receptors in clearance of apoptotic cells. *J Immunol*. 2007;178(9):5635-42.
24. Benzakour O, Kanthou C. The anticoagulant factor, protein S, is produced by cultured human vascular smooth muscle cells and its expression is up-regulated by thrombin. *Blood*. 2000;95(6):2008-14.
25. Rothlin CV, Ghosh S, Zuniga EI, Oldstone MB, Lemke G. TAM receptors are pleiotropic inhibitors of the innate immune response. *Cell*. 2007;131(6):1124-36.
26. Sharif MN, Sosic D, Rothlin CV, Kelly E, Lemke G, Olson EN, et al. Twist mediates suppression of inflammation by type I IFNs and Axl. *J Exp Med*. 2006;203(8):1891-901.
27. Carrera Silva EA, Chan PY, Joannas L, Errasti AE, Gagliani N, Bosurgi L, et al. T cell-derived protein S engages TAM receptor signaling in dendritic cells to control the magnitude of the immune response. *Immunity*. 2013;39(1):160-70.
28. Shibata T, Habel DM, Coelho AL, Kunkel SL, Lukacs NW, Hogaboam CM. Axl receptor blockade ameliorates pulmonary pathology resulting from primary viral infection and viral exacerbation of asthma. *J Immunol*. 2014;192(8):3569-81.
29. Shibata T, Hogaboam CM. Gas6'ing the innate immune response during experimental asthma. *Ann Am Thorac Soc*. 2014;11 Suppl 5:S303-5.
30. Shibata T, Ismailoglu UB, Kittan NA, Moreira AP, Coelho AL, Chupp GL, et al. Role of growth arrest-specific gene 6 in the development of fungal allergic airway disease in mice. *Am J Respir Cell Mol Biol*. 2014;51(5):615-25.
31. Porsbjerg C, Brannan JD, Anderson SD, Backer V. Relationship between airway responsiveness to mannitol and to methacholine and markers of airway inflammation,

peak flow variability and quality of life in asthma patients. *Clin Exp Allergy*. 2008;38(1):43-50.

32. Fisher PW, Brigham-Burke M, Wu SJ, Luo J, Carton J, Staquet K, et al. A novel site contributing to growth-arrest-specific gene 6 binding to its receptors as revealed by a human monoclonal antibody. *Biochem J*. 2005;387(Pt 3):727-35.

33. Nagata K, Ohashi K, Nakano T, Arita H, Zong C, Hanafusa H, et al. Identification of the product of growth arrest-specific gene 6 as a common ligand for Axl, Sky, and Mer receptor tyrosine kinases. *J Biol Chem*. 1996;271(47):30022-7.

34. Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol*. 2004;22:789-815.

35. Fahy JV, Corry DB, Boushey HA. Airway inflammation and remodeling in asthma. *Curr Opin Pulm Med*. 2000;6:15-20.

36. Johnson TR, Johnson JE, Roberts SR, Wertz GW, Parker RA, Graham BS. Priming with secreted glycoprotein G of respiratory syncytial virus (RSV) augments interleukin-5 production and tissue eosinophilia after RSV challenge. *J Virol*. 1998;72(4):2871-80.

37. Tamachi T, Maezawa Y, Ikeda K, Kagami S, Hatano M, Seto Y, et al. IL-25 enhances allergic airway inflammation by amplifying a TH2 cell-dependent pathway in mice. *J Allergy Clin Immunol*. 2006;118(3):606-14.

38. Stolarski B, Kurowska-Stolarska M, Kewin P, Xu D, Liew FY. IL-33 exacerbates eosinophil-mediated airway inflammation. *J Immunol*. 2010;185(6):3472-80.

39. Zhou B, Comeau MR, De Smedt T, Liggitt HD, Dahl ME, Lewis DB, et al. Thymic stromal lymphopoietin as a key initiator of allergic airway inflammation in mice. *Nat Immunol*. 2005;6(10):1047-53.

40. Connors M, Giese NA, Kulkarni AB, Firestone CY, Morse HC, 3rd, Murphy BR. Enhanced pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of interleukin-4 (IL-4) and IL-10. *J Virol*. 1994;68(8):5321-5.

41. Castilow EM, Olson MR, Varga SM. Understanding respiratory syncytial virus (RSV) vaccine-enhanced disease. *Immunol Res*. 2007;39(1-3):225-39.

42. Su YC, Townsend D, Herrero LJ, Zaid A, Rolph MS, Gahan ME, et al. Dual proinflammatory and antiviral properties of pulmonary eosinophils in respiratory syncytial virus vaccine-enhanced disease. *J Virol*. 2015;89(3):1564-78.
43. Moghaddam A, Olszewska W, Wang B, Tregoning JS, Helson R, Sattentau QJ, et al. A potential molecular mechanism for hypersensitivity caused by formalin-inactivated vaccines. *Nat Med*. 2006;12(8):905-7.
44. Delgado MF, Coviello S, Monsalvo AC, Melendi GA, Hernandez JZ, Batalle JP, et al. Lack of antibody affinity maturation due to poor Toll-like receptor stimulation leads to enhanced respiratory syncytial virus disease. *Nat Med*. 2009;15(1):34-41.
45. Haynes LM, Caidi H, Radu GU, Miao C, Harcourt JL, Tripp RA, et al. Therapeutic monoclonal antibody treatment targeting respiratory syncytial virus (RSV) G protein mediates viral clearance and reduces the pathogenesis of RSV infection in BALB/c mice. *J Infect Dis*. 2009;200(3):439-47.
46. Li C, Zhou X, Zhong Y, Li C, Dong A, He Z, et al. A Recombinant G Protein Plus Cyclosporine A-Based Respiratory Syncytial Virus Vaccine Elicits Humoral and Regulatory T Cell Responses against Infection without Vaccine-Enhanced Disease. *J Immunol*. 2016;196(4):1721-31.
47. Shibata T, Habel DM, Coelho AL, Hogaboam CM. Axl receptor blockade protects from invasive pulmonary aspergillosis in mice. *J Immunol*. 2014;193(7):3559-65.
48. Chan PY, Carrera Silva EA, De Kouchkovsky D, Joannas LD, Hao L, Hu D, et al. The TAM family receptor tyrosine kinase TYRO3 is a negative regulator of type 2 immunity. *Science*. 2016;352(6281):99-103.
49. Lew ED, Oh J, Burrola PG, Lax I, Zagorska A, Traves PG, et al. Differential TAM receptor-ligand-phospholipid interactions delimit differential TAM bioactivities. *Elife*. 2014;3.
50. Piedimonte G, Perez MK. Alternative mechanisms for respiratory syncytial virus (RSV) infection and persistence: could RSV be transmitted through the placenta and persist into developing fetal lungs? *Curr Opin Pharmacol*. 2014;16:82-8.
51. Angelillo-Scherrer A, de Frutos P, Aparicio C, Melis E, Savi P, Lupu F, et al. Deficiency or inhibition of Gas6 causes platelet dysfunction and protects mice against thrombosis. *Nat Med*. 2001;7(2):215-21.

52. Lukacs NW, Moore ML, Rudd BD, Berlin AA, Collins RD, Olson SJ, et al. Differential immune responses and pulmonary pathophysiology are induced by two different strains of respiratory syncytial virus. *Am J Pathol.* 2006;169(3):977-86.

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Figure Legends

Figure 1. A model of FI-RSV VED in mice. Timeline for establishing a model of formalin-inactivated-RSV enhanced disease (FI-RSV VED) (**A**). Airway hyperresponsiveness (AHR) to methacholine challenge (420 mg/kg) was determined in vehicle, FI-RSV-immunized (FI-RSV), RSV-infected (RSV), and FI-RSV VED mice on day 8 after RSV challenge (**B**). Representative hematoxylin and eosin-stained (**C**) and Periodic acid-Schiff (PAS)-stained (**D**) lung tissue sections obtained from mice on day 8 after RSV challenge. Original magnification: $\times 200$. Representative image of eosinophil (cell that has segmented nuclei and small brick-red granules within the cellular cytoplasm: yellow arrows) infiltration in lung tissue sections obtained from mice on day 8 after RSV challenge (**E**). Eosinophil counts in the bronchoalveolar lavage fluid (BALF) from each group of mice on day 8 after RSV challenge (**F**). The protein levels of IL-4, IL-5, and IL-13 in lung homogenates obtained from mice on day 8 after RSV challenge (**G**). Viral titers in lungs from FI-RSV-sensitized mice on day 8 after RSV challenge (**H**). These results are expressed as the mean $\pm$ standard error of the mean (SEM) for $n = 5$ (vehicle, FI-RSV, RSV) or 6 (FI-RSV+RSV). $*P < 0.05$, $**P <$

0.01, *** $P < 0.001$, **** $P < 0.0001$. Representative results from two independent experiments are shown.

Figure 2. Growth arrest-specific (Gas) 6 facilitates the allergic airway response during formalin-inactivated respiratory syncytial virus vaccine-enhanced disease (FI-RSV VED).

Airway hyperresponsiveness (AHR) to methacholine challenge (420 mg/kg) was determined in FI-RSV-sensitized wild-type (WT) mice or Gas6-deficient (Gas6 KO) mice on day 8 after RSV challenge (A). The change in body weight of FI-RSV-sensitized WT mice or Gas6 KO mice after RSV challenge (B). Representative hematoxylin and eosin-stained lung tissue sections from WT and Gas6 KO mice on day 8 after RSV challenge. Original magnification: $\times 40$ (top) and $\times 200$ (bottom) (C). Representative Periodic acid-Schiff (PAS)-stained lung tissue sections from WT and Gas6 KO mice on day 8 after RSV challenge. Original magnification: $\times 400$ (D). Eosinophil (Siglec-F⁺CD11b⁺CD11c⁻ in granulocyte on FCS/SSC) counts in lungs from both groups of mice (E). The protein levels of

interleukin (IL)-4, IL-5, and IL-13 in whole lung homogenates from both groups (**F**).

The number of IL-4-, IL-5-, and IL-13-producing CD45⁺CD3⁺CD4⁺ cells in lungs from WT and Gas6 KO mice on day 8 after RSV challenge (**G**). These results are expressed as the mean $\pm$ SEM for $n = 5$ (WT) or 6 (Gas6 KO). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Representative results from two independent experiments are shown.

Figure 3. Administration of an anti-Axl monoclonal antibody (mAb) during immunization prevents allergic airway response in FI-RSV VED. Timeline of anti-Axl mAb treatment in the FI-RSV VED model (**A**). Mice were injected with IgG (5 μ g/dose) or anti-Axl mAb (5 μ g/dose) intraperitoneally every day from 2 days before to 1 day after the first FI-RSV injection, for a total of 4 doses of antibodies (5 μ g/dose $\times$ 4 = 20 μ g total). Airway hyperresponsiveness (AHR) to methacholine challenge (420 mg/kg) was determined in control IgG- and anti-Axl mAb-treated mice with FI-RSV VED on day 8 after RSV challenge (**B**). The change in body weight of IgG- or anti-Axl mAb-treated mice over time after RSV challenge (**C**). Representative hematoxylin and eosin-stained lung tissue sections from IgG- or anti-Axl mAb-treated mice on day 8

after RSV challenge. Original magnification: $\times 40$ (top) and $\times 200$ (bottom) (**D**). Eosinophil counts in the bronchoalveolar lavage from both groups of mice (**E**). Representative Periodic acid-Schiff (PAS)-stained lung tissue sections from IgG- or anti-Axl mAb-treated mice on days 4 and 8 after RSV challenge (**F**). The protein levels of interleukin (IL)-4, IL-5, and IL-13 in bronchoalveolar lavage fluid from IgG- or anti-Axl mAb-treated mice on day 8 after RSV challenge (**G**). These results are expressed as the mean $\pm$ SEM for $n = 5$ (+IgG) or 6 (+Axl Ab). $*P < 0.05$, $**P < 0.01$. Representative results from two independent experiments are shown.

Figure 4. Gas6 is generated in lymph nodes during immunization with FI-RSV drives differentiation into Th2 cells. The protein levels of Gas6 in mediastinal lymph nodes (MLNs) after immunization with FI-RSV, RSV infection, and RSV infection post immunization with FI-RSV (FI-RSV + RSV) (**A**). The protein levels of Gas6 in the supernatant of MLN cells from naïve (indicated as –, white bar) and FI-RSV-immunized mice (FI-RSV, black bar) that were restimulated with RSV for 2 days (**B**). The protein levels of interleukin (IL)-4, IL-5, IL-13, and interferon (IFN)- γ in

the supernatant of MLN cells from FI-RSV-immunized wild-type (WT, white bar) and Gas6-deficient (Gas6 KO, black bar) mice that were restimulated with RSV for 2 days (+RSV) (**C**). The protein level of Gas6 in the supernatant of bone marrow-derived dendritic cells (BMDCs) stimulated with FI-RSV and RSV for 24 h (**D**). Flow cytometric analysis of Axl expression on BMDCs after stimulation with RSV and FI-RSV for 24 h (**E**). These results are expressed as the mean $\pm$ SEM for $n = 5-6$ (A-C) or 3 (D, E). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. Representative results from three (A-C) or two (D, E) independent experiments are shown.

Figure 5. Gas6/Axl signaling inhibits viral clearance by reducing RSV-neutralizing IgG2a production during FI-RSV VED. ~~Viral titer in lung from FI-RSV-sensitized wild type (WT) or Gas6-deficient (Gas6 KO) mice, at day 8 after RSV challenge (A).~~ Anti-RSV fusion glycoprotein (F protein) IgE (left) and IgG2a (right) levels in sera from FI-RSV-sensitized WT and Gas6 KO mice on days 4 and 8 after RSV challenge (**A**). Viral titers in lungs from FI-RSV-sensitized WT and Gas6 KO mice on day 8 after RSV challenge (**B**). Plaque reduction was determined by adding

serum from FI-RSV-sensitized WT or Gas6 KO mice to RSV and subsequently adding of HEp-2 cells (**C**). Anti-RSV-F protein IgG2a (left) and IgE (right) levels in serum from FI-RSV-sensitized IgG- or anti-Axl mAb-treated mice, at day 8 after RSV challenge (**D**). Viral titers in lungs from FI-RSV-sensitized control IgG- or anti-Axl mAb-treated mice, on day 8 after RSV challenge (**E**). Plaque reduction was determined by adding serum obtained from FI-RSV-sensitized IgG- or anti-Axl mAb-treated mice on day 8 to RSV and subsequently addition of HEp-2 cells (**F**). These results are expressed as the mean $\pm$ SEM for $n = 5-6$. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Representative results from two independent experiments are shown.

Figure 6. RSV-glycoprotein (G protein)-induced Gas6/Axl signaling inhibits RSV fusion glycoprotein (F protein)-induced activation of DCs. The production of Gas6 by bone marrow-derived DCs (BMDCs) stimulated with 0.01, 0.1, or 1 $\mu\text{g/ml}$ recombinant RSV-G protein (**A**) and 0.01, 0.1, or 1 $\mu\text{g/ml}$ RSV-F protein (**B**) for 24 h. Interleukin (IL)-12p40 production by BMDCs treated with recombinant Gas6 or anti-Axl mAb. These cells were treated with RSV-F protein and/or RSV-G protein or

RSV for 24 h (**C**). Representative data and MFI of MHC II, CD40, CD80, and CD86 expression on BMDCs treated with recombinant Gas6 or anti-Axl mAb. These cells were treated with RSV-F protein and RSV-G protein or RSV for 24 h (**D**). The protein level of IL-12p40 in lung homogenates from wild-type (WT) and Gas-deficient (Gas6 KO) mice (left panel) or lung homogenates from IgG- and anti-Axl mAb-treated mice (right panel) on day 8 after RSV challenge (**E**). These *in vitro* (D, n = 3) and *in vivo* (A, B, C, E, n = 5) results are expressed as the mean $\pm$ SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Representative results from three (D) or two (A, B, C, E) independent experiments are shown.

Figure 1

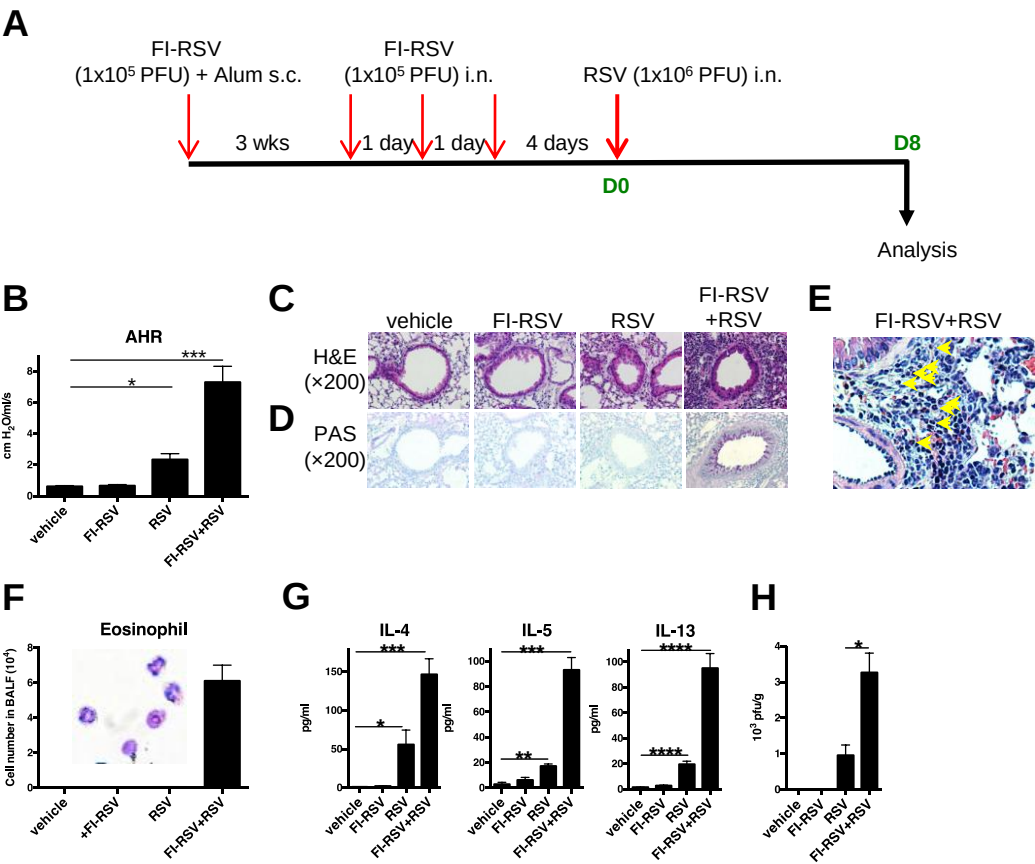


Figure 2

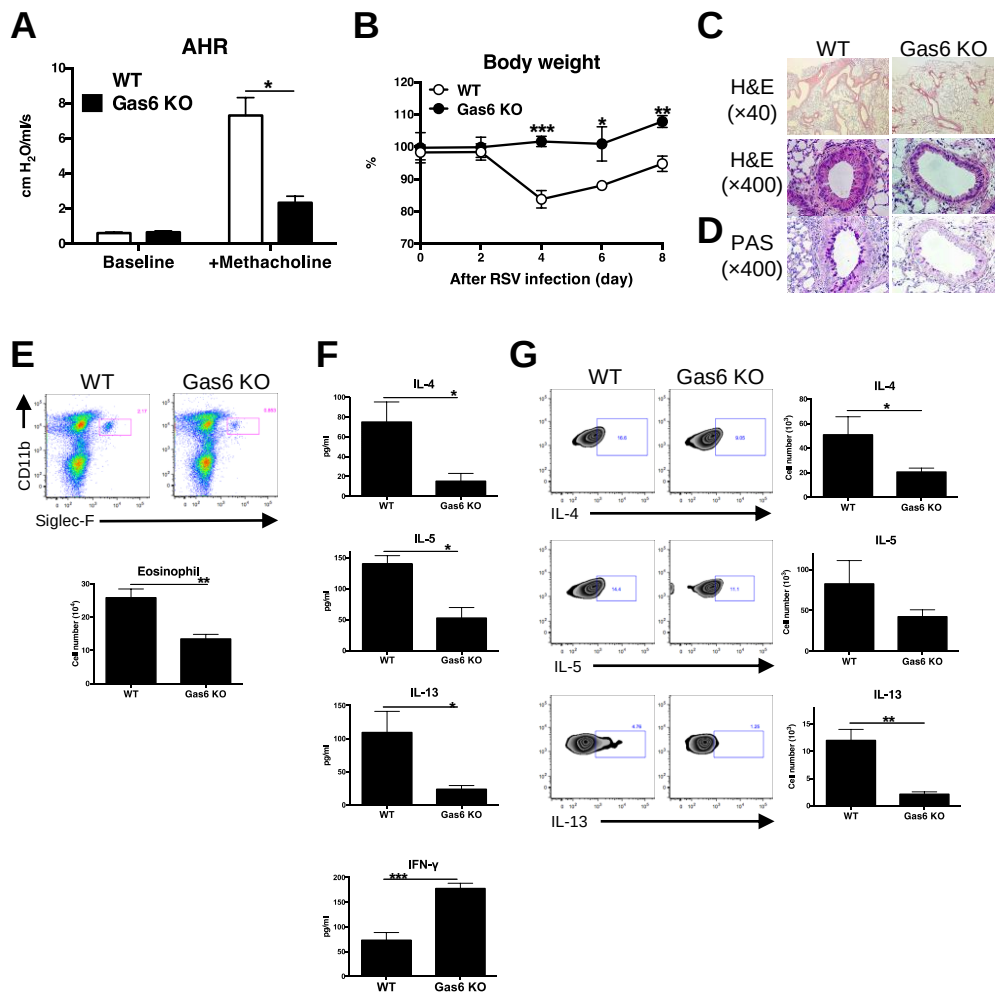


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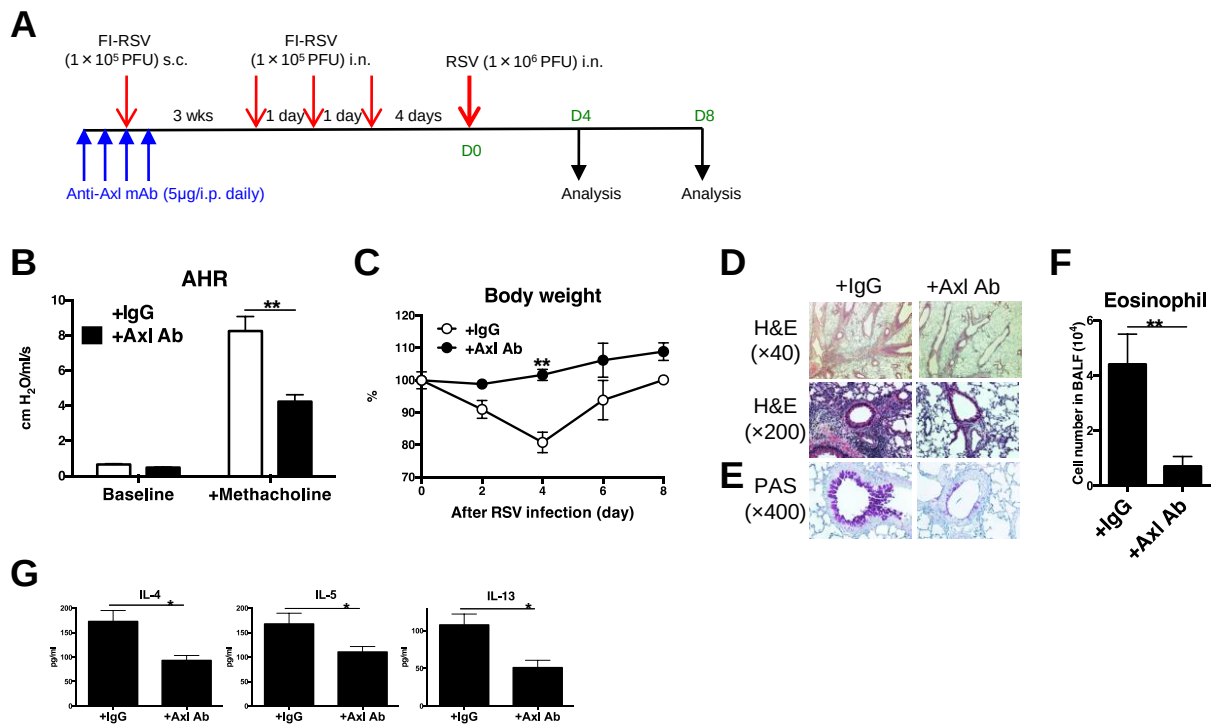


Figure 4

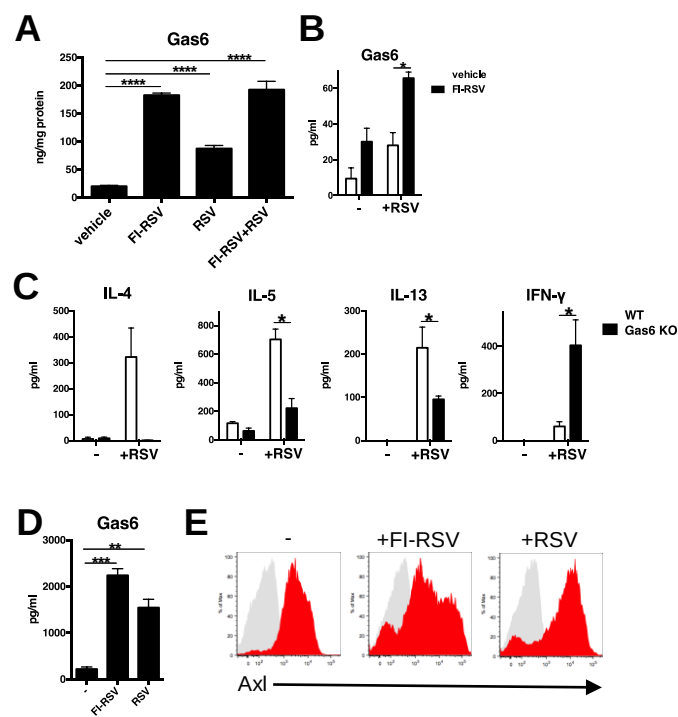


Figure 5

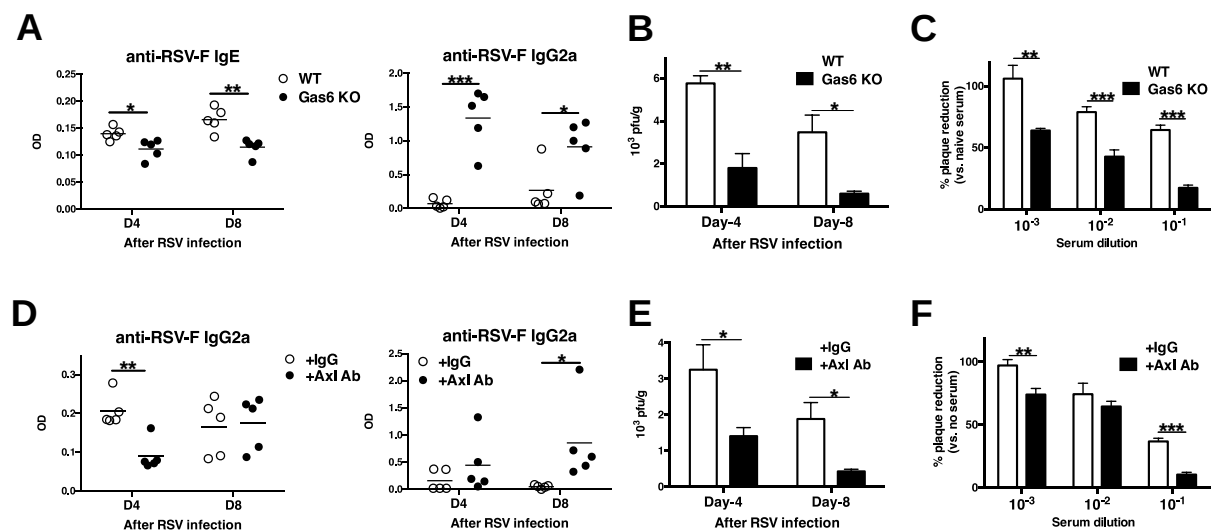


Figure 6

