

**Enhanced allergic responsiveness after early childhood infection with respiratory viruses:
Are long-lived alternatively-activated macrophages the missing link?**

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Abstract

Early childhood infection with respiratory viruses, including human rhinovirus (HRV), Respiratory Syncytial Virus (RSV), and influenza, is associated with an increased risk of allergic asthma and severe exacerbation of ongoing disease. Despite the long recognition of this relationship, the mechanism linking viral infection and later susceptibility to allergic lung inflammation is still poorly understood. We discuss the literature and provide new evidence demonstrating that these viruses induce the alternative activation of macrophages. Alternatively-activated macrophages (AAM) induced by RSV or influenza infection persisted in the lungs of mice up to 90 days after initial viral infection. **Several** studies suggest that AAM contribute to allergic inflammatory responses, **although their mechanism of action is unclear. In this commentary**, we propose that virus-induced AAM provide a link between viral infection and enhanced responses to inhaled allergens.

Introduction:

Genetics and the environment contribute approximately equally to the development of asthma¹⁻⁴. However, how sensing of the environment is linked to asthma inception and exacerbation is not well understood⁵. Early childhood infection with respiratory viruses, including human rhinovirus (HRV), and Respiratory Syncytial Virus (RSV) is associated with an increased risk of subsequent development of asthma⁶ and HRV and influenza are associated with exacerbation of ongoing disease⁷. Despite the long recognition of this relationship, the mechanism linking viral infection and later susceptibility to allergic lung inflammation remains to be elucidated.

Respiratory viruses first encounter lung epithelial cells and alveolar macrophages (AM), initiating a cascade of innate immune responses⁸⁻¹¹. These viruses contain pathogen-associated molecular patterns (PAMPS) that directly stimulate pattern recognition receptors (PRRs) and can result in release of host-derived damage-associated molecular patterns (DAMPS). Many groups have focused on the effects of viral infection on epithelial cells and the communication between activated epithelial cells, innate lymphoid cells, and dendritic cells (DC)^{8-10,12-14}. Virus-infected epithelial cells can produce cytokines including TSLP, IL-33, and IL-25 that establish a “Th2-promoting” environment by programming lung DC¹⁰ and activating innate lymphoid cells^{10,13}. Such DCs are thought to promote the differentiation of helper T cells to the Th2 type that produce high levels of IL-4, IL-5, and IL-13¹⁰.

While these pathways clearly contribute to responses to respiratory insults, the role of the macrophage has been largely ignored. Macrophages are found in abundance in the lungs and airways, and are one of the first cells exposed to both viruses and aeroallergens. Macrophages

exhibit great plasticity and can adopt different phenotypes that promote different immune environments¹⁵⁻¹⁸. Macrophages exposed to strong inflammatory stimuli, *e.g.*, IFN- γ and LPS, differentiate into highly microbicidal, classically activated macrophages (CAM, also termed M1). CAMs produce a strong pro-inflammatory cytokine profile that can result in tissue damage. Conversely, exposure of macrophages to IL-4 or IL-13 leads to development of alternatively activated macrophages (AAM, also called M2) that counter the strong pro-inflammatory CAMs and are associated with wound healing¹⁶. **Several studies provide evidence suggesting that AAM actively participate** in the severity of allergic lung inflammation¹⁹⁻²¹, **although, as we discuss below, their contribution remains controversial in the field. In addition, the mechanism by which increased numbers of AAM contribute to the severity of allergic asthma is unclear. AAM produce a number of enzymes, cytokines, and chemokines that may play important roles in inflammation, but a single critical factor has not yet been identified in allergic lung inflammation.** While enhanced allergic lung inflammation has been linked to AAM by others using mouse models and in studies of asthma patients²²⁻²⁷, it is not yet known if AAM contribute to the initiation of allergic lung responses after viral infection or to exacerbation of allergic lung responses after viral infection.

Burden and Risk for Asthma

Asthma is a chronic disease of the small airways resulting in wheezing, coughing, and shortness of breath. Asthma ranks as the most common cause of non-communicable disease in children and poses a significant disease burden^{28,29}. Asthma is the most common cause of pediatric hospitalizations and is the most common cause for missed school days in children ages 5-17 years²⁸. For children aged 0-17 years of age, 2% of all ambulatory visits and 2.3% of emergency room visits were attributed to asthma³⁰. Despite advances in drug delivery and

standardization of management, about 170 children die annually in the United States from asthma exacerbations/complications^{28,29}. Weiss et al³¹ estimated that the annual healthcare cost of childhood asthma-related illnesses is 3.2 billion US dollars. Inner city children and low-income families are disproportionally affected and have higher morbidity and mortality due to asthma. In contrast, children living on farms have a reduced risk of asthma and allergies due to exposures to a greater diversity of microbes³². Similarly, children with more exposure to other children (siblings) at home or at a day care setting are less likely to develop asthma³³.

Positive family history, prenatal exposure to cigarette smoke, prematurity, and sensitization to allergens such as house dust mite and molds have been associated with increased risk for childhood-onset asthma³⁴. The role of interferons is considered vital in the development of wheezing and asthma in children, particularly after viral infections. Immature peripheral blood interferon- γ (IFN- γ) responses to viruses and mitogen have been linked with recurrent wheezing^{35,36}. Similarly, deficiency in the interferon response from airway epithelial cells has been implicated in asthma. Impairment of airway barrier, an important component of innate immunity, provides a potential mechanism by which environmental exposures can lead to asthma. Exposure to tobacco smoke or viruses in children with impaired airway barrier could lead to severe lower respiratory tract infection, enhanced viral replication, and subsequent increased risk for asthma^{37,38}.

Viral infection and asthma

Infection with respiratory viruses has been associated with both the inception and exacerbation of asthma. It is well understood that wheezing in early life is a risk factor for asthma in later life, and the most common cause of wheezing in early life is viral infection. HRV

and RSV infections in early childhood have been well-studied and their association with asthma has been widely reported^{39,40}. Recently, a link between human metapneumovirus (hMPV) infection and subsequent asthma has been demonstrated⁴⁰. Furthermore, viral infections are the most common cause of asthma exacerbation in children. About 80% of exacerbations are attributed to viral infections and HRV accounts for nearly two thirds of the cases. Children with asthma exacerbation due to influenza infection tend to have worse outcomes. The causative link between viral infection and subsequent development of asthma-like systems has been investigated in animal models.

A number of groups have established mouse models for virus-enhanced responses to later allergen exposure using adult or neonatal mice^{23,41-45}. Infection of neonatal mice (7-8 days old) with RSV⁴², HRV⁴¹, or influenza⁴⁴ induced rapid production of IL-13, IL-4, and TSLP in the lungs that preceded enhanced responses to allergens administered 7-10 days after infection.

This enhancement can also occur in older mice. To demonstrate that RSV infection enhances the subsequent response to allergen, 4 wk old BALB/c mice were infected with RSV or PBS (as a control). Ten days later, mice were treated with house dust mite (HDM) or PBS 4 times, and 48 hrs after the last exposure, bronchoalveolar lavage (BAL) cells and lungs were analyzed, representing 27 days between initial RSV infection and analysis (Fig. 1). RSV infection alone (RSV/PBS) induced a significant increase in total cell numbers found in the airways; these cells were predominantly macrophages (M) (Fig. 1A,B). As expected, HDM alone (PBS/HDM) induced an increase in the numbers of airway eosinophils (E). Strikingly, mice infected with RSV prior to HDM treatment (RSV/HDM) exhibited a *6-fold increase in the numbers of eosinophils in the airways compared to HDM exposure alone*. We observed only few airway

neutrophils (P) or lymphocytes (L) in the airways under these conditions (Fig. 1B). Prior exposure to RSV also significantly increased the percentage of eosinophils in the BAL (Fig. 1C). Enhanced responsiveness to allergen was also reflected in the analysis of lung sections (Fig. 1D). The HDM-induced inflammatory response was increased in mice previously infected with RSV. Prior infection with RSV increased the HDM-induced eosinophilia in the lung tissue and the development of PAS+, mucus-producing epithelial cells. RSV alone induced some inflammation predominantly surrounding blood vessels; however, this infiltrate was monocytic as observed under high power (100x). These results are consistent with those published by the Gelfand laboratory⁴⁶. Importantly, we detected Ym1/2+ macrophages in the lungs of mice treated with either RSV or HDM indicating that RSV-induced AAM remain in the lungs for at least 27 days post-infection. Furthermore, our data show that a 10-day interval between infection and initial allergen exposure is sufficient to reveal enhanced responsiveness.

Viral infection and AAM

Respiratory Syncytial Virus. RSV infection of primary murine macrophages and the RAW 264.7 macrophage cell line *in vitro* leads to early (hours) induction of pro-inflammatory genes (*e.g.*, IL-12, cyclooxygenase (COX)-2, etc.), followed by macrophage-derived IL-4 and IL-13 (measured by qRT-PCR, ELISA, and intracellular IL-4 staining of F4/80+ BAL cells), and the anti-inflammatory cytokine, IL-10⁴⁷. AAM marker expression (*e.g.*, Arg1, Fizz1, Ym1, MR, and others) peaked at 24-48 h *in vitro* and *in vivo*⁴⁷ as well as TSLP and IL-33 mRNA (unpublished observations), cytokines associated with allergic inflammation, a common sequella of RSV infection^{48,49}. The IL-4R α chain mediates IL-4- and IL-13-induced, STAT6-dependent signaling⁵⁰. RSV-infected IL-4R α ^{-/-} and STAT6^{-/-} macrophages or mice did not differentiate into AAM and exhibited enhanced and prolonged pro-inflammatory responses (*e.g.*, IL-12, COX-

2)⁴⁷. RSV-infected TLR4^{-/-} macrophages failed to differentiate into AAM, and produced low levels of PPAR γ ⁴⁷ a transcription factor required for induction of AAM markers⁵¹⁻⁵⁴. RSV-infected IFN- β ^{-/-} macrophages produced decreased levels of IL-4, IL-13, and IL-4R α □□□□□also failed to differentiate into AAM⁴⁷. Furthermore, treatment of RSV-infected WT macrophages with anti-IL-4 and/or anti-IL-13 antibodies reduced Arg1 mRNA while enhancing COX-2 mRNA (Fig. 2). Thus, RSV-induced AAM differentiation requires macrophage-derived IL-4/IL-13 and IL-4R α -, TLR4-, and IFN- β -mediated signaling. RSV-induced lung pathology (e.g., primarily alveolitis, peribronchiolitis) was significantly worse in IL-4R α ^{-/-} than WT mice, due to the failure to produce AAM. Adoptive transfer of WT macrophages to IL-4R α ^{-/-} mice reconstituted RSV-induced lung AAM, and mitigated IL-12 levels and pathology⁴⁷.

We previously reported that treatment of RSV-infected cotton rats with indomethacin, a pan-COX inhibitor, reduced RSV-induced pathology⁵⁵ and that Parecoxib, a COX-2-specific inhibitor, increased levels of RSV-induced AAM *in vivo*⁴⁷. Arachidonic acid is a substrate for both COX-2 and 5/15-lipoxygenase (5/15-LO). We observed a strong inverse relationship between COX-2 and 5/15-lipoxygenase (LO) expression that is reflected in CAM or AAM responses, respectively^{47,56} (Fig. 3). RSV-infected 5-LO^{-/-} mice and 5-LO^{-/-} or 15-LO^{-/-} macrophages, failed to develop AAM markers, but produced increased COX-2 mRNA⁵⁶. 5/15-LO-specific inhibitors blocked RSV-induced AAM differentiation *in vitro*; conversely, 5/15-LO-derived lipoxins or resolvins partially restored to RSV-infected 5-LO^{-/-} macrophages the ability to differentiate into AAM⁵⁶□□Indomethacin therapy significantly reduced lung pathology of RSV-infected 5-LO^{-/-} mice⁵⁶. Collectively, our data support the model proposed in Fig. 3: the extent of lung injury induced by RSV depends on the relative balance of COX-2 and 5/15-LO that controls

CAM and AAM differentiation. More recently, we published that administration of IL-4/anti-IL-4 complexes or azithromycin, agents shown to induce AAM in vivo⁵⁷⁻⁵⁹, and rosiglitazone, a PPAR γ agonist⁶⁰, each increased RSV-induced AAM and mitigated pathology in mice⁶¹. This balance between viral-induced tissue damage and AAM differentiation may become dysregulated in some individuals leading to inflammatory diseases such as allergic inflammation and asthma.

Influenza. Imai et al⁶². reported that chemical or microbial lung insults produce an oxidized phospholipid DAMP, OxPAPC, that elicits TLR4-, TRIF-, and IL-6-dependent signaling in lung macrophages, leading to acute lung injury (ALI). This led to our finding that TLR4^{-/-} mice are highly resistant to lethal infection by mouse-adapted influenza A/PR/8/34 (PR8)^{63,64} and to our hypothesis that therapeutic blockade of TLR4 signaling should mitigate influenza-induced ALI and lethality. Eritoran (E5564; Eisai Inc.), the most potent, synthetic TLR4 antagonist known^{65,66}, strongly binds the TLR4 co-receptor, MD2⁶⁷, thereby blocking MD2-LPS interactions. Eritoran administered *as late as 6 days p.i.*, protected mice significantly from PR8-induced ALI and lethality, and blocked cytokine production in lungs⁶⁴. Eritoran also protected cotton rats infected with non-adapted influenza from ALI. We recently confirmed the importance of TLR4 in PR8-induced lethality: mice treated on days 2 and 4 p.i. with anti-TLR4, but not control IgG, were significantly protected from lethality. In addition to blocking influenza-induced lethality when given therapeutically, Eritoran blocked not only the TLR4-mediated cytokine production induced by DAMPs, including OxPAPC and HMGB1, but also blunted accumulation of OxPAPC in the lungs and the release of HMGB1 into the serum^{64,68}.

Influenza infection may also predispose individuals to secondary bacterial pneumonia and is associated with increased mortality. *Streptococcus pneumoniae* and *Staphylococcus aureus* are

frequently isolated^{69,70}. Influenza-induced ALI was much more severe compared to that induced by RSV infection and we failed to detect AAM markers in mice that survived an LD₉₀ of influenza (data not shown); however, a sublethal dose of influenza that resulted in reduced ALI led to an early wave of pro-inflammatory cytokine induction (*e.g.*, TNF- α , IL-6) that overlapped with the induction of AAM markers⁷¹. Under these conditions, the mice exhibited increased susceptibility to an otherwise non-lethal dose of *Streptococcus pneumoniae*⁷¹. This suggests that the hyper-susceptibility to *Streptococcus pneumoniae* after influenza infection may be mediated by long-lived AAM that have a reduced capacity to produce proinflammatory cytokines and chemokines that would normally contribute to clearance of the bacteria (Fig. 4). This hypothesis is consistent with the observations of Didierlaurent et al. who observed that alveolar macrophages exhibited a diminished sensitivity to TLR agonists as late as 60 days after infection with influenza or RSV⁷². However, this study did not determine if the alveolar macrophages were, in fact, AAM. Thus, it is tempting to speculate that the development of AAM after influenza infection is intended to mitigate the host inflammatory response to virus infection, but in doing so, enhances susceptibility to secondary bacterial infection by diminishing cytokine production. Thus, we propose that such a population of long-lived AAM could also contribute to enhanced allergic responses to inhaled substances long after the initial respiratory viral infection is resolved.

To evaluate the longevity of AAM in the lung after RSV and influenza infection, BALB/ByJ mice were infected with RSV or a sublethal dose of influenza and cytokine and AAM marker mRNA measured over 120 days (Fig. 5). IL-1 β , TNF- α , and COX-2 mRNA were up-regulated at 6 days for both RSV and PR8; however, the PR8-infected mice exhibited sustained pro-inflammatory mRNA through 14 days post-infection (Fig. 5A). For AAM-

associated genes, Mannose Receptor, Ym1, and Arginase-1 mRNA were up-regulated by RSV as early as 1 week post-infection and remained above background, even at 120 days. For influenza infection, induction of AAM gene expression was slightly delayed, with maximum expression at 30 days that was also sustained above background through 120 days. The abundance of AAM in the lungs of mice after viral infection was analyzed by immunohistochemistry for Ym1, a member of the chitinase family and marker for mouse AAM. We observed a significant increase in numbers of Ym1+ macrophages over PBS treated mice within 6 days of RSV infection; this increase was maintained for 90 days post-infection. We also observed a significant increase in numbers of Ym1+ macrophages 14 days after infection with PR8 that lasted for 90 days post-infection.

Human rhinovirus (HRV). Rhinovirus infections are associated with 60-70% of the episodes of asthma exacerbations in school age children⁷³⁻⁷⁵, and in exacerbations of symptoms in chronic obstructive pulmonary disease (COPD) patients⁷⁶, and are a major cause of hospitalizations due to acute respiratory illness in young children in the USA⁷⁷. Until recently, animal models for studying HRV infection were very limited. Recent data suggested the BALB/c mouse as a model for rhinovirus-induced disease and exacerbation of allergic airway inflammation⁷⁸. Using HRV1B, Bartlett *et al.* demonstrated that infection is localized in the lungs and induces airway and pulmonary inflammation and mucin production, albeit accompanied by low viral replication. In OVA-sensitized/challenged BALB/c mice, subsequent infection with RV1B increased lung infiltration with neutrophils, eosinophils, and macrophages. This was associated with increased airway cholinergic hyperresponsiveness. Administration of anti-eotaxin-1, an eosinophil chemokine, attenuated the RV infection-induced eosinophilia. Eotaxin was present in lung macrophages and such cells stained for AAM markers including Arginase 1, Ym1, Mgl2, and IL-

10²⁷. The role of AAM in airway responsiveness was demonstrated by experiments in which macrophages were depleted from OVA-sensitized/challenged mice following RV infection²⁷. BALB/c mice were infected with RV. On day 35 post-infection, mice exhibited airway hyperresponsiveness and mucus metaplasia that was blocked by administration of anti-IL-13 antibody. In a similar model of allergic asthma, Hong et al⁷⁹ reported that OVA-treated IL-4R α knockout mice had neutrophilic airway inflammation in contrast to wild-type mice that exhibited exacerbated airway hyperreactivity associated with AAM. Blanco et al. recently reported that HRV-16 infection of cotton rats (*Sigmodon hispidus*) resulted in tracheal epithelial cell defoliation associated with peribronchiolar infiltration, bronchiolitis, perivascular infiltration alveolar septal infiltration and alveolar exudates, as well as mucus cell hypertrophy/hyperplasia⁸⁰.

Role of AAM in asthma inception and exacerbation

The potential for AAM to actively contribute to allergic lung inflammation has been long ignored, but recent reports have begun to shed light on their contribution to allergic disease. Asthmatics have greater numbers of macrophages in their lungs as compared to healthy controls^{25,81}. Asthmatics also demonstrate increased levels of chitinase family members in their blood and lung lavage fluid⁸². Single-nucleotide polymorphisms found in *CHI3L1* (YKL40), *CHIA* (AMCase), and *MRC1* (mannose receptor), all AAM markers, have been associated with asthma⁸³⁻⁸⁵. Peripheral blood monocytes isolated from children undergoing viral-induced asthma exacerbations had elevated expression of AAM signature genes including *MRC1*²⁶. Kim *et al.* found increased numbers of IL-13+ macrophages in bronchoalveolar lavage samples from asthma patients as compared to healthy controls²³. Furthermore, immunohistochemistry of biopsy specimens taken from the lungs of asthma patients and healthy controls demonstrated an

increase in AAM (defined by expression of mannose receptor, CD206, and stabilin-1) in the lungs of asthmatic patients²⁵. The percentage of AAM in the lung tissue correlated with peak expiratory flow variation, suggesting that AAM may regulate asthma severity.

Several reports using mouse models also support an important role for AAM in allergic lung inflammation. AAM were shown to contribute to the enhanced asthma response observed in female mice as compared to male mice²⁴. Female mice had greater numbers of AAM in lung tissue than male mice and showed enhanced allergic lung inflammation. Adoptive transfer of *in vitro* differentiated AAM by intranasal instillation enhanced allergic lung inflammation observed in male mice. Using a bone marrow chimeric model, the Keegan laboratory showed a strong correlation between the severity of allergic lung inflammation and the presence of IL-4R α ⁺ macrophages¹⁹ that were also positive for Ym1/2, an AAM marker²⁰. Furthermore, adoptive transfer of IL-4R α ^{+/+} but not IL-4R α ^{-/-} bone marrow-derived macrophages (BMM) in the presence of Th2 effector cells was sufficient to enhance allergic lung inflammation in the OVA model²⁰. Adoptive transfer of IL-4R α ⁺ BMM significantly enhanced the percentage of eosinophils, and levels of CCL11/**eotaxin**, CCL5/RANTES, and CCL2/**MCP-1** in the bronchoalveolar lavage (BAL) fluid²⁰. Ym1/2⁺ AAM were detected in the lungs and airways of mice receiving IL-4R α ⁺ BMM, but not IL-4R α ⁻ BMM. Nieuwenhuizen *et al.* analyzed responses in a neutrophil- and macrophage-specific knock-out of the IL-4 α and found that the ability to induce AAM during an allergic response was not absolutely necessary to support allergic lung inflammation⁸⁶. However, the LysM-cre-mediated deletion was later shown to be incomplete; IL-4R α was not deleted on immature monocytes and macrophages (LysM low) during ongoing inflammation induced by *Shistosoma mansoni* infection while it was deleted on mature, resident lung macrophages (LysM-high)⁸⁷. Using an alternate macrophage deleting approach, diphtheria

toxin (DT) treatment of Cd11b-DT receptor mice, Borthwick *et al.* recently showed that macrophage depletion with DT after priming of mice with the allergen house dust mite (HDM) resulted in decreased AAM responses in the lung and decreased eosinophilic inflammation in response to HDM challenge⁸⁸.

These findings from the studies in human asthma patients and mouse models of allergic lung inflammation **strongly suggest** that AAM are important, active contributors to Th2-driven inflammation and are not just bystander cells responding to the Th2 cytokines. It is reasonable to propose that AAM contribute to asthma severity and, therefore, may play a role in asthma exacerbation by viral infection. The placement of AAM early in asthma inception is less established but should be carefully considered.

Summary. As discussed above, viral infection induces AAM that are quite long-lived in the lung. We continued to detect AAM in the lungs of mice 90 days after infection (Fig. 5), long after virus infection is cleared. It is tempting to suggest that these long-lived macrophages may maintain “innate memory” or training that leads them to respond vigorously to a subsequent stimulus⁸⁹. If that stimulus happens to be an allergen, an overly robust Th2 type response could develop (Fig. 6). Thus, it is possible that the enhanced responsiveness to allergen exposure that occurs after viral infection is mediated by long-lived AAM.

In this scenario, viral infection would induce the formation of lung AAM. First, PAMPS found in RSV, influenza, or RV would stimulate PRRs on AMs and lung epithelial cells that stimulates rapid production of proinflammatory cytokines, followed by production of Th2-promoting cytokines including IL-4, IL-13, IL-25, IL-33, and TSLP. These cytokines act on AMs as well as granulocytes (mast cells, basophils, eosinophils) and innate lymphoid cells such as invariant NK T-cells (iNKT) or the lineage negative innate helper cell (Ih2) to produce more

IL-4 or IL-13. Second, IL-4 or IL-13, in combination with IL-33, would drive AAM differentiation mediated by autocrine and paracrine mechanisms. Third, these long-lived AAM remain in the lung with the potential to rapidly respond to innate activation.

A subsequent exposure to allergen could provide this innate activation. We propose that allergen-associated molecular patterns (AAMPS)²¹ stimulate PRR expressed on long-lived AAM leading to the rapid production of IL-4, IL-13, IL-25, IL-33, and TSLP and chemokines. **Lung epithelial cells could also contribute to the production of a subset of these cytokines including IL-25, IL-33, and TSLP.** These cytokines can activate dendritic cells (DCs), and other innate immune cells (step 4). Activated DCs migrate to the draining lymph node, and with the help of IL-4- producing cell such as basophils, stimulate naive CD4⁺ T-cells to become allergen-specific Th2 effectors or memory cells (step 5). Subsequently, Th2 effectors migrate to the site of inflammation producing high levels of IL-4, IL-5, and IL-13. These cytokines mediate mucus secretion, airway hyperreactivity (AHR), chemokine production, eosinophilic infiltration, and further AAM differentiation (step 6). Lung epithelial cells and AAM produce chemokines and other factors that could recruit more inflammatory cells from the blood including eosinophils and monocytes. Further exposure to allergen or infection with virus would reactivate this cycle stimulating memory Th2 cells to produce high levels of IL-4, IL-5, and IL-13⁹⁰, thereby establishing a positive amplification loop. If this model turns out to be accurate, then new avenues for therapeutic intervention can be identified and explored.

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Supplemental Materials

Materials and Methods used for the experimental data shown in this commentary are described in Supplemental Materials

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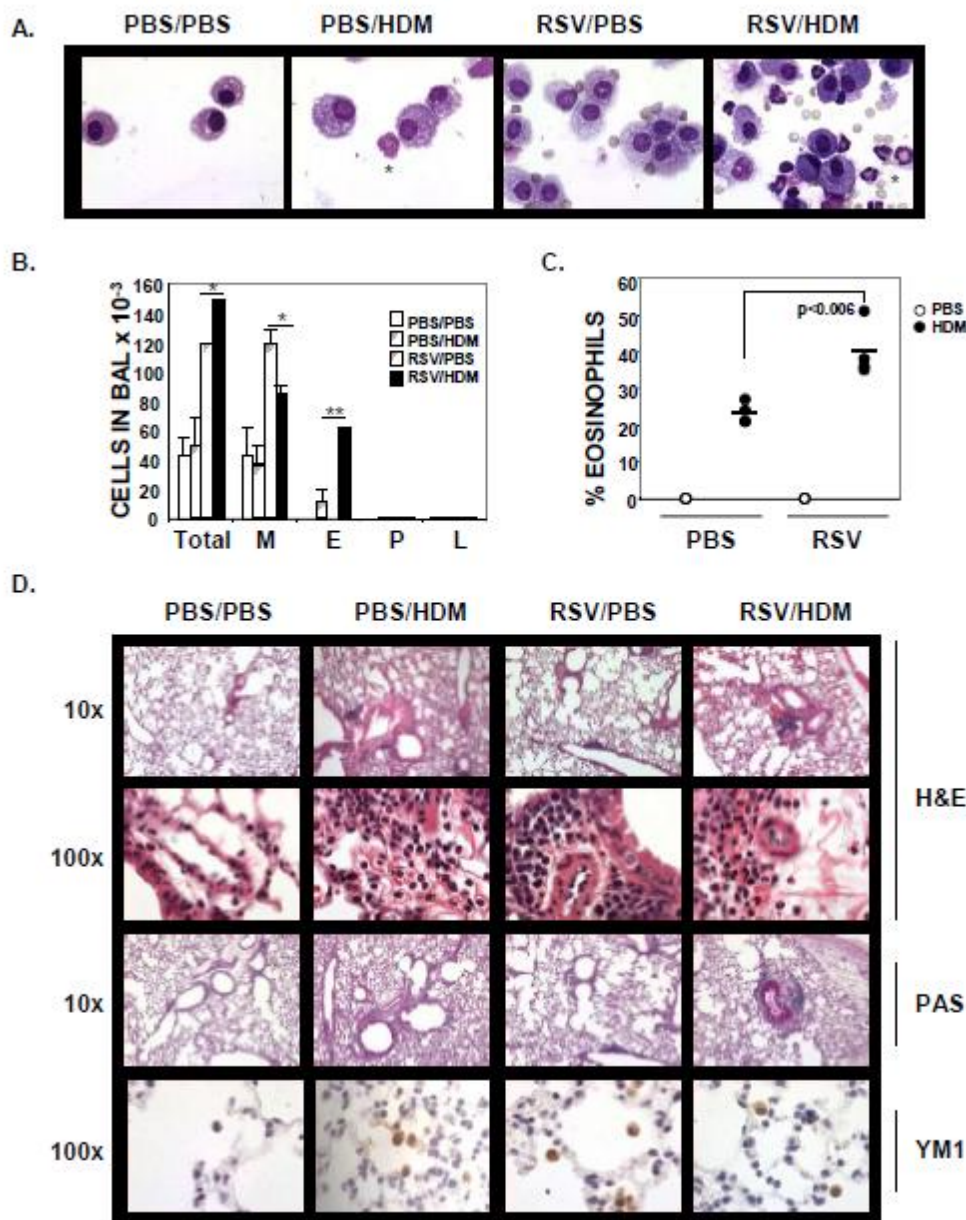


Figure 1: Prior infection with RSV enhances responses to allergen. 4-week old Balb/c mice were mock- or RSV-infected on day 0. Mice were treated 10 days later with PBS or HDM (50 μ g) i.n. on days 10,15, 20, and 25. Forty-eight hour later (day 27), BAL fluid and lungs were harvested. A. Representative images of cytopins stained with Wright-Giemsa (100x). Examples of eosinophils are marked by asterisks. B. Differential counts of total cells macrophages (M), eosinophils (E), polymorphonuclear leukocytes (P), and lymphocytes (L) in the BAL (n=3, mean +/- SEM). C. Percent

eosinophils in the BAL of each mouse (n=3, bar denotes the mean value). D. Representative images of lung sections stained with H&E (10x, 100x), PAS (10x), and anti-YM1 (100x).

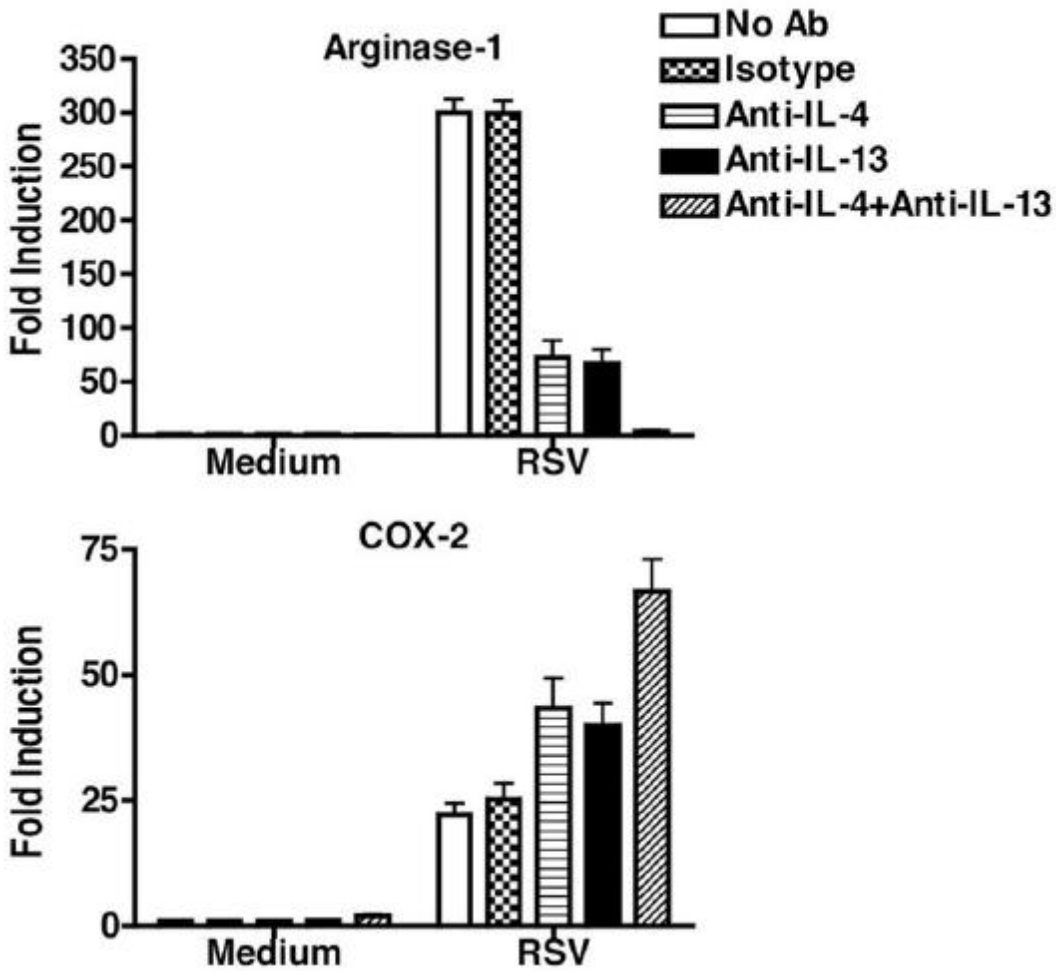


Figure 2. RSV infection of macrophages induces arginase-1 and suppresses COX-2 mRNA in an IL-4 and IL-13-dependent manner. Macrophages were prepared from highly purified thioglycollate-elicited C57BL/6J mice. Macrophages were infected with RSV (MOI = 2) for 48 h in the presence or absence of isotype control, anti-IL-4, and/or anti-IL-13 antibodies. Arginase-1 and COX-2 mRNA were measured by qRT-PCR 4 h post-infection.

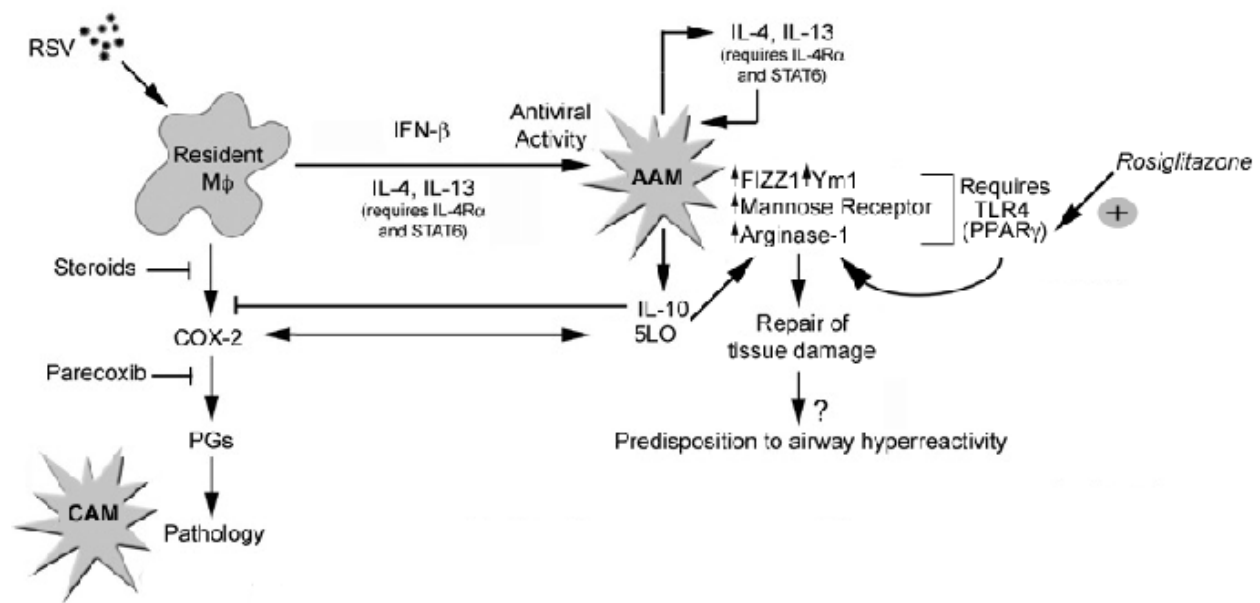


Figure 3: RSV infection induces a response balanced by pro-inflammatory and tissue repair

pathways. RSV infects multiple cell types in the lung, including macrophages, which result in the initial production of pro-inflammatory cytokines and chemokines that also recruit more inflammatory cells such as monocytes. The production of inflammatory cytokines and COX-2 lead to the differentiation of CAM as well as the resulting observed pathology. Later, through a complex process that is IL-4Rα-, TLR4-, IFN-β-, and lipoxygenase-dependent, AAM differentiation occurs, counteracting the earlier inflammatory insults through (i) production of anti-inflammatory cytokines such as IL-10, (ii) induction and activation of PPARγ through TLR4, (iii) induction and activation of the 5- and 15-lipoxygenase pathway preventing further production of COX-2 and the damaging prostaglandins, and (iv) repair of damaged tissue through production of enzymes such as FIZZ1 and Ym1. Although the induction of AAM is beneficial to the host initially, if the AAM phenotype persists it may lead to hypersensitivity to allergic triggers such as HDM.

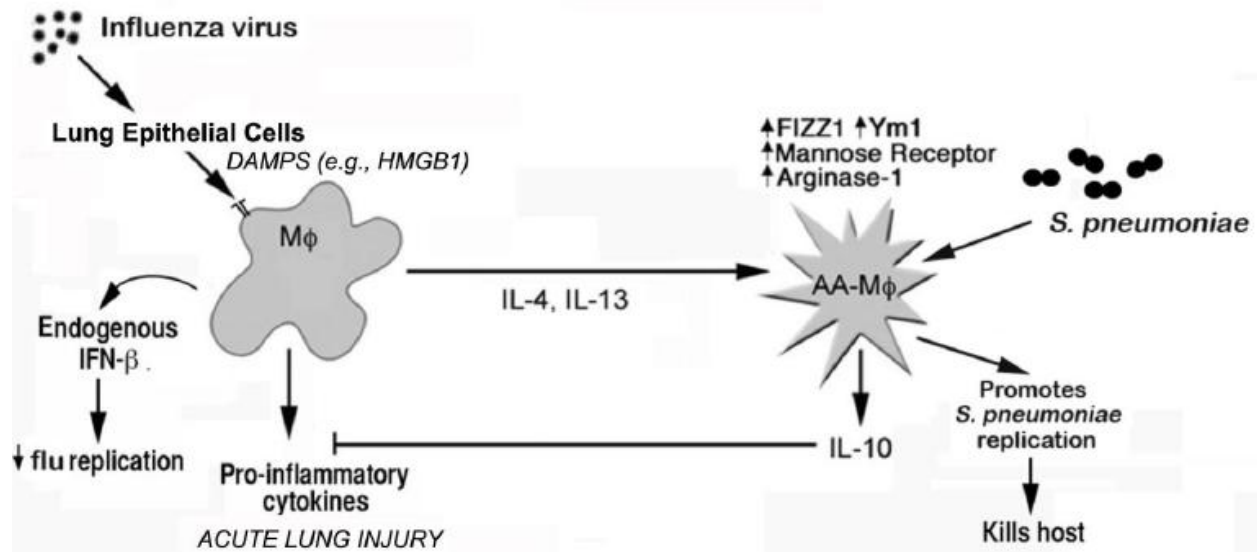


Figure 4: Model of influenza-induced AAM consequences. Infection with a sub-lethal dose of influenza virus induces an initial CAM response along with the production of various DAMPs. Over time, after viral clearance and during a recovery phase, the lung macrophages differentiate into AAM as shown by measurement of IL-4, IL-13, and markers associated with AAM (arginase-1, FIZZ1, and Ym1) that appear to be long-lived. Influenza-recovered mice that are challenged with a sub-lethal dose of *Streptococcus pneumoniae* serotype 3 within two weeks of the initial viral infection quickly develop a fatal pulmonary infection with rapid replication of *S. pneumoniae* observed along with a blunted inflammatory response.

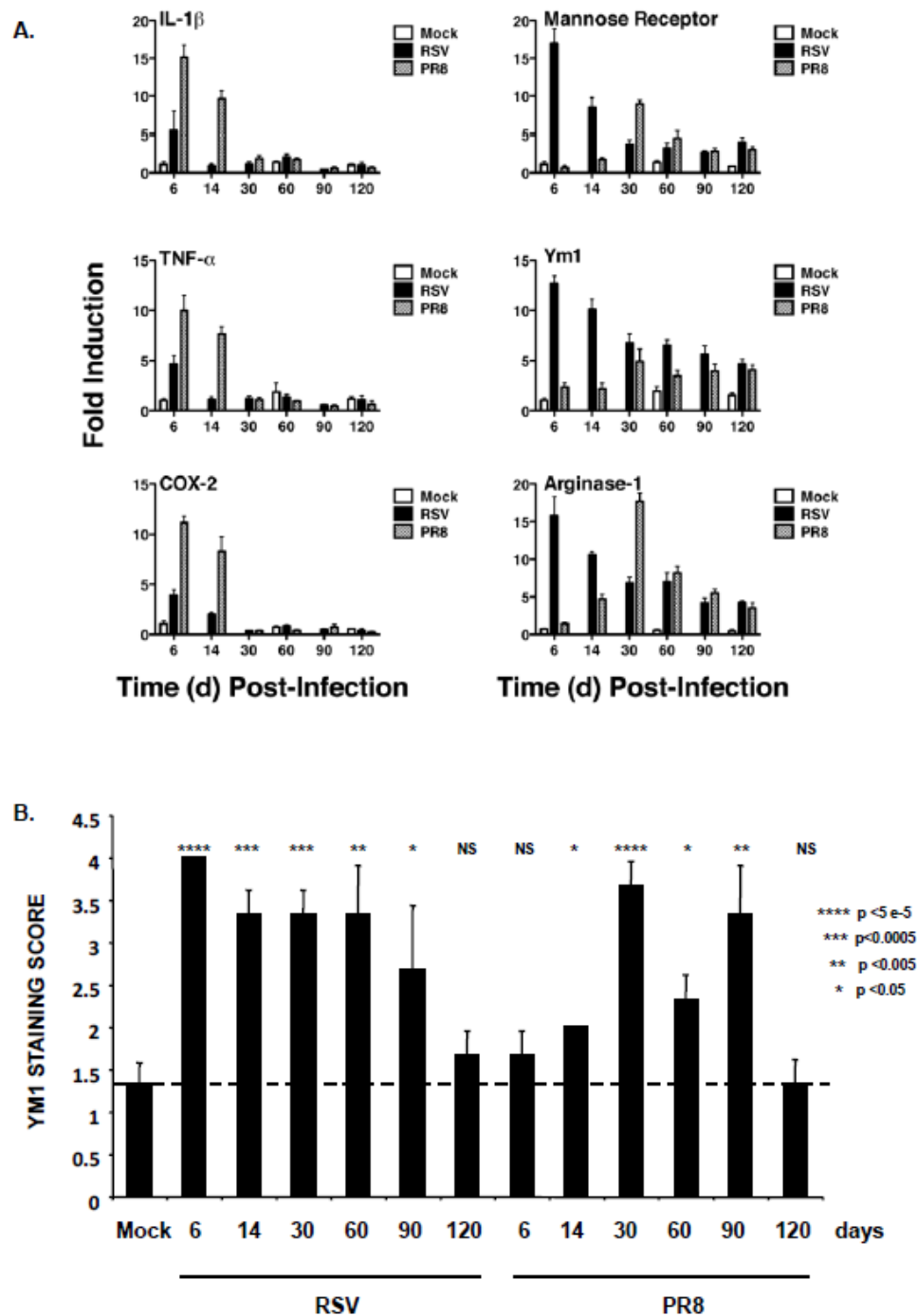


Figure 5. AAM remain in the lung long after virus infection. BALB/cJ mice were mock infected with PBS or infected with RSV (2×10^6 PFU/mouse i.n.) or influenza A/PR/8/34 (1000 TCID₅₀/mouse i.n.). Mice were euthanized on various days post-infection up until day 120 ($n=3$ mice/group). One lobe of each lung was harvested and processed for RNA. (A) Total RNA was isolated and the relative abundance of

mRNA for specific genes was evaluated as relative gene expression normalized to mock-infected. (B) The second lobe was prepared for histology and scored for staining for Ym1+ macrophages in a blinded fashion on a scale of 0-4 as described in the Supplemental Materials. Mean score +/- SEM is shown.

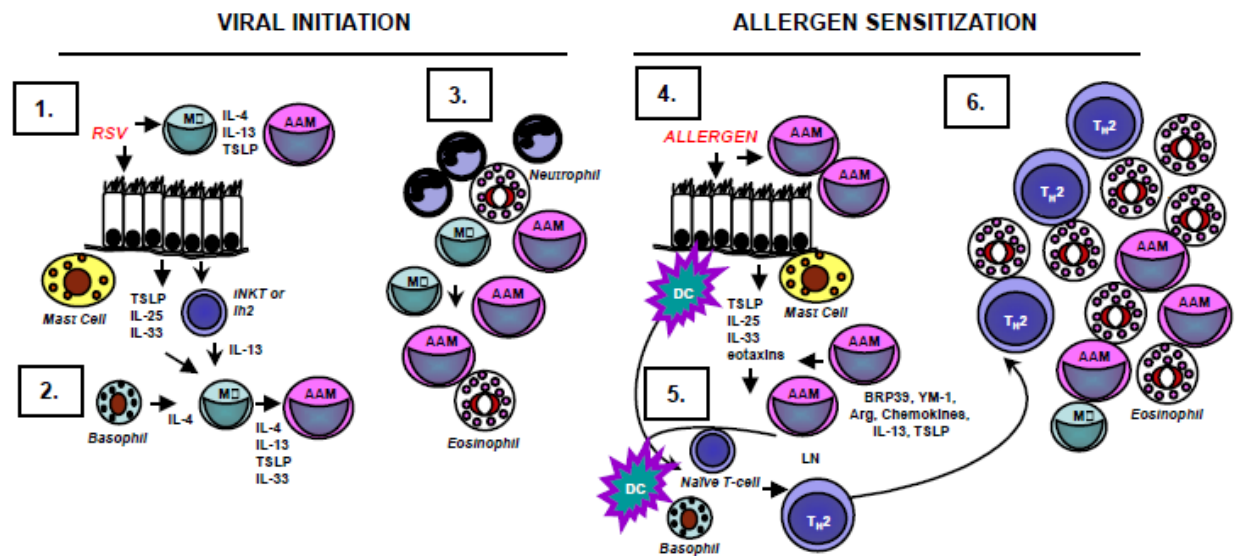


Figure 6. Role for AAM in virus-induced enhancement to allergen exposure. Initiation by virus infection. (1) PAMPS found in RSV, flu, or RV stimulate PRRs on AMs and lung epithelial cells that stimulates rapid production of proinflammatory cytokines, followed by production of Th2-promoting cytokines including IL-4, IL-13, IL-25, IL-33, and TSLP. These cytokines act on AMs as well as granulocytes (mast cells, basophils, eosinophils) and innate lymphoid cells such as invariant NK T-cells (iNKT) or ILC2 to produce more IL-4 or IL-13. (2) IL-4 or IL-13, in combination with IL-33, drive AAM differentiation mediated by autocrine and paracrine mechanisms. (3) Long-lived AAM remain in the lung.

Allergen exposure. (4) Molecular patterns found in allergens (AAMPS) stimulate PRR expressed on long-lived AAM leading to the rapid production of IL-4, IL-13, IL-25, IL-33, and TSLP and chemokines. **Lung epithelial cells could also contribute to the production of a subset of these cytokines including IL-25, IL-33, and TSLP.** These cytokines activate dendritic cells (DCs), and other innate immune cells. (5) Activated DCs migrate to the draining lymph node, and with the help of IL-4- producing cells such as basophils, stimulate naive CD4⁺ T-cells to become allergen-specific Th2 effectors or memory cells. (6) Th2 effectors migrate to the site of inflammation producing high levels of IL-4, IL-5, and IL-13 mediating the Type 2 inflammatory response.