

GM-CSF treatment prevents respiratory syncytial virus–induced pulmonary exacerbation responses in postallergic mice by stimulating alveolar macrophage maturation

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Background: Human respiratory syncytial virus (RSV) is a frequent cause of asthma exacerbations, yet the susceptibility of asthmatic patients to RSV is poorly understood.

Objective: We sought to address the contribution of resident alveolar macrophages (rAMs) to susceptibility to RSV infection in mice that recovered from allergic airway eosinophilia.

Methods: Mice were infected with RSV virus after clearance of allergic airway inflammation (AAI). The contribution of post-AAI rAMs was studied *in vivo* by means of clodronate liposome-mediated depletion, adoptive transfer, and treatment with recombinant cytokines before RSV infection.

Results: After clearing the allergic bronchial inflammation, post-AAI mice had bronchial hyperreactivity and increased inflammatory cell influx when infected with RSV compared with nonallergic mice, whereas viral clearance was comparable in both mouse groups. Post-AAI rAMs were necessary and sufficient for mediating these proinflammatory effects. In post-AAI mice the residing CD11c^{hi} autofluorescent rAM population did not upregulate the terminal differentiation marker sialic acid–binding immunoglobulin-like lectin F and overproduced TNF and IL-6 through increased nuclear factor κ B nuclear translocation. In line with these results, post-AAI lungs had reduced levels of the rAM maturation cytokine GM-CSF. Intratracheal administration of GM-CSF induced final rAM maturation in post-AAI mice and prevented the increased susceptibility to RSV-induced hyperreactivity and inflammation.

Conclusion: Defective production of GM-CSF leads to insufficient post-AAI rAM maturation in mice that recovered from an AAI, causing increased susceptibility to RSV-induced immunopathology. Promoting the differentiation of post-AAI rAMs might be a therapeutic option for preventing RSV-induced exacerbations in human asthmatic patients. (J Allergy Clin Immunol 2015;■■■:■■■-■■■.)

Key words: Allergic asthma, respiratory syncytial virus–induced exacerbation, alveolar macrophage, GM-CSF

Allergic asthma is a chronic inflammatory disease of the airways affecting more than 300 million persons worldwide. In asthmatic patients pulmonary accumulation of inflammatory leukocytes and cytokines together with lung structural remodeling and airway hyperreactivity (AHR) eventually results in recurrent episodes of airway obstruction, wheezing, and shortness of breath, which are clinically referred to as exacerbation reactions.¹ Although the mechanisms of asthmatic exacerbations are still poorly understood, it is increasingly clear that the majority of exacerbations are associated with acute viral respiratory tract infections, including rhinoviruses and influenza. However, the most severe asthmatic exacerbation reactions are often induced by respiratory syncytial virus (RSV) infections.²

RSV is the leading cause of infant hospital admission worldwide and causes 70% of bronchiolitis-associated hospitalizations in the developed world.³ Although CD8⁺ T cells^{4,5} and neutralizing antibodies⁵⁻⁷ are pivotal for viral clearance, a number of studies emphasize the importance of alveolar macrophages as key players in the early immune response to RSV infection.^{8,9} It has even been suggested that alveolar macrophages, rather than adaptive immune cells, are critical determinants of the severity of RSV-induced bronchiolitis.⁹

Resident alveolar macrophages (rAMs) constitute the predominant cell population in the alveolar spaces of healthy subjects and serve as important innate sentinels in the recognition of invading pathogens and other airborne particles. However, in rAMs these innate responses are tightly regulated, thus avoiding excessive inflammatory responses that can hamper pulmonary gas exchange.¹⁰ However, inflammatory insults as such can also negatively affect the anti-inflammatory function of rAMs. In the case of allergic inflammation, we recently reported the presence of a hyperinflammatory rAM phenotype exhibiting increased proinflammatory reactivity on exposure to Toll-like receptor (TLR) 3, TLR4 and TLR7 ligands.¹¹

We have now verified to what extent the increased inflammatory reactivity of rAMs instigated by a prior allergic bronchial inflammation might affect the outcome of a subsequent infection

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Abbreviations used

AAI:	Allergic airway inflammation
AHR:	Airway hyperreactivity
BAL:	Bronchoalveolar lavage
DC:	Dendritic cell
DMEM:	Dulbecco modified eagle medium
HBSS:	Hank balanced salt solution
IRF:	Interferon regulatory factor
NF- κ B:	Nuclear factor κ B
OVA:	Ovalbumin
PE:	Phycoerythrin
PFA:	Paraformaldehyde
PFU:	Plaque-forming units
rAM:	Resident alveolar macrophage
RSV:	Respiratory syncytial virus
RT:	Room temperature
Siglec-F:	Sialic acid-binding immunoglobulin-like lectin F
TLR:	Toll-like receptor

with RSV. Our results show that post-allergic airway inflammation (post-AAI) lungs are prone to increased pulmonary inflammation and AHR in response to RSV infection and that the hyperinflammatory phenotype of post-AAI rAMs is at the origin of the observed inflammation exacerbation in post-AAI mice. Furthermore, we found that the majority of the post-AAI rAM population displayed an immature phenotype. Full maturation of this immature post-AAI rAM population by GM-CSF treatment prevented the exacerbated nature of the lung response to RSV infection, resulting in decreased bronchoalveolar inflammation and AHR after RSV infection.

METHODS**Mice**

Six- to 8-week old female BALB/c mice were purchased from Charles River Laboratories Italy (Calco, Italy) and housed under specific pathogen-free conditions in individually ventilated cages in a controlled day/night cycle and given food and water *ad libitum*. All experiments were approved by the local animal ethics committee of Ghent University in accordance with European guidelines (directive 2010/63/EU; Belgian Royal Decree of April 6, 2010).

Mouse model of RSV-induced inflammation exacerbation in allergen-sensitized and challenged mice

A mouse model of RSV-induced inflammation exacerbation in allergen-sensitized and challenged mice was established, as described in the [Methods](#) section in this article's Online Repository at www.jacionline.org. In some experiments mice were treated with recombinant GM-CSF (provided by the VIB Protein Service Facility, Ghent, Belgium; see the [Methods](#) section in this article's Online Repository).¹²

Alveolar cell isolation, cell culture, and differential cell counts

Bronchoalveolar lavage (BAL) and *ex vivo* cultures of rAMs incubated for the indicated time with RSV A2 were performed, as described in the [Methods](#) section in this article's Online Repository. Total and differential numbers of BAL cells were determined by means of flow cytometry, as described in the [Methods](#) section in this article's Online Repository.

Determination of AHR

Lung function was assessed by using flexiVent (SCIREQ, Montreal, Quebec, Canada) invasive measurement of dynamic lung compliance, as described in the [Methods](#) section in this article's Online Repository.

rAM depletion and reconstitution

Depletion of the residing alveolar phagocyte population was achieved by means of intratracheal instillation of a 30% solution of clodronate-filled liposomes (N. Van Rooijen, VUC Amsterdam, Amsterdam, The Netherlands), as described in the [Methods](#) section in this article's Online Repository.

Cytokine measurement

Protein levels of mouse IL-6 and TNF in BAL fluid and culture supernatants were quantified with the Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, Calif).

Total RNA preparation and real-time quantitative PCR

RNA isolation was performed with the RNeasy Plus Micro Kit (Qiagen, Hilden, Germany). cDNA was synthesized with an iScript cDNA synthesis kit (Bio-Rad Laboratories). Real-time quantitative PCR was performed on a LightCycler 480 (Roche Molecular Systems, Basel, Switzerland) by using the sensiFAST SYBR No-ROX kit (Bioline, London, United Kingdom). Primers for ubiquitin-specific peptidase 18 (*Usp18*), interferon-induced protein with tetratricopeptide repeats 2 (*Ift2*), and 60S ribosomal protein L13 a (*Rpl13a*; reference housekeeping gene for normalization) were used.

Nuclear translocation assays

BAL cells were infected *in vitro* with RSV. At the indicated time points, cells were stained intracellularly for nuclear factor κ B (NF- κ B) p65, interferon regulatory factor (IRF) 3, or IRF-7, as described in the [Methods](#) section in this article's Online Repository. Stained cells were imaged with a Leica TCS SP5 AOBS confocal microscope (Leica, Wetzlar, Germany). Images were acquired with LAS AF software (Leica) and subsequently analyzed with Volocity software (PerkinElmer, Beaconsfield, United Kingdom).

Statistics

Statistical analysis was performed with GraphPad Prism 5 software (GraphPad Software, La Jolla, Calif). Following outlier statistics, the Kolmogorov-Smirnov test was used to check Gaussian distribution of parameters to choose between 1-way ANOVA and the Kruskal-Wallis nonparametric test. Differences in means between each 2 independent experimental groups were analyzed by using an unpaired *t* test or the nonparametric Mann-Whitney *U* test at a 95% CI. No statistical analysis was done for data of pooled samples.

RESULTS**Post-AAI mice have increased pulmonary inflammation in response to RSV infection**

A mouse model of AAI was set up to examine the influence of a preceding allergic bronchial inflammation on a subsequent pulmonary RSV infection. BALB/c mice were sensitized against the model allergen ovalbumin (OVA) by means of repeated intraperitoneal immunization with aluminum hydroxide as an adjuvant. Subsequently, the sensitized mice were repeatedly exposed to nebulized OVA, generating an eosinophilic airway inflammation reminiscent of the immunopathology of mild-to-moderate asthma (see [Fig E1, A](#), in this article's Online Repository at www.jacionline.org).

Within 15 days after the last of 7 OVA exposures, eosinophilic bronchial inflammation was cleared. Absolute cell numbers returned to basal levels (see Fig E1, B), and the alveolar cell population again consisted of more than 85% macrophages, which is comparable with levels seen in naive control mice (see Fig E1, C). Also, T_H2-associated cytokines were no longer detectable in BAL fluid (data not shown). At this time point, mice were infected by intranasal administration of RSV A2. Readout of bronchoalveolar inflammation was performed at days 1, 3, and 6 after infection (see Fig E1, D). RSV infection of naive mice resulted in moderate bronchoalveolar inflammation (Fig 1, A). A modest infiltration of mainly neutrophils was present at the early innate phase (days 1 and 3) of infection, which was subsequently supplemented with an influx of CD8⁺ T cells at day 6 after infection (Fig 1, B). In contrast, post-AAI mice mounted a severe 3- to 4-fold increased pulmonary inflammatory response from day 1 throughout days 3 and 6 (Fig 1, A and B). In accordance with the increased inflammatory response compared with that seen in RSV-infected naive mice, RSV-infected post-AAI mice had increased AHR to methacholine at day 3 after infection (Fig 1, C). Strikingly, in spite of the increased inflammatory cell recruitment in post-AAI lungs, RSV titers in post-AAI alveoli did not differ significantly from RSV titers in naive lungs at day 3 after infection (Fig 1, D).

Post-AAI rAMs contribute to increased RSV-induced inflammation

rAMs were depleted 3 days before RSV infection by means of intratracheal instillation of clodronate liposomes to determine to what extent post-AAI rAMs contributed to the exacerbated inflammatory response to RSV infection in post-AAI lungs (see Fig E1, D). Depletion of naive rAMs increased RSV-elicited lung inflammation by almost 3-fold (Fig 1, A), confirming the anti-inflammatory role of the cells early in infection.¹³ In contrast, depletion of post-AAI rAMs significantly reduced bronchoalveolar inflammation to levels almost identical to those seen in naive mice infected with RSV (Fig 1, A). At days 1 and 3, this decrease in inflammatory cell recruitment was mainly due to a reduction in the recruitment of myeloid cells, neutrophils, and CD4⁺ T cells (Fig 1, B). At the later adaptive stage of infection (day 6), diminished CD8⁺ T-cell recruitment was observed (Fig 1, B). In addition, although depletion of naive rAMs increased AHR to methacholine, depletion of post-AAI rAMs consistently tended to ameliorate this disease parameter, as indicated by the increased average airway compliance from 0.01 to 0.02 mL/cm H₂O, although statistical significance was not reached (Fig 1, C). Finally, depletion of naive rAMs resulted in increased alveolar viral titers at day 3 after infection (Fig 1, D), thus confirming a role for naive rAMs in the clearance of RSV infection. In contrast, depletion of post-AAI rAMs did not influence viral clearance. Indeed, nearly equal numbers of viral particles (± 400 plaque-forming units [PFU]/500 μ L of BAL fluid) were retrieved from the alveoli of post-AAI rAM⁺ and rAM⁻ mice at day 3 after infection (Fig 1, D), thus indicating that post-AAI rAMs do not contribute to viral clearance.

Adoptive transfer of post-AAI rAMs reinstates RSV-induced inflammation exacerbation

We next investigated whether the differential outcome of depletion of naive versus post-AAI rAMs on RSV-elicited

bronchoalveolar inflammation was due to intrinsic differences between both rAM populations or rather reflected secondary differences instigated by lung structural cells. To address this issue, the lungs of naive mice were depleted from rAMs by means of clodronate liposome administration and subsequently reconstituted with rAMs derived from naive or post-AAI mice ($\pm 90\%$ purity, see Fig E2 in this article's Online Repository at www.jacionline.org). The reconstituted lungs were subsequently exposed to RSV, and the BAL cell infiltrate was quantified at day 1 after infection (see Fig E3 in this article's Online Repository at www.jacionline.org). The response profile observed was similar to the response observed with the rAM depletion approach used previously (Fig 1). Indeed, reconstitution with rAMs isolated from post-AAI lungs exerted a worsening of RSV-elicited bronchoalveolar inflammation compared with reconstitution with naive rAMs (Fig 2). Thus adoptive transfer of post-AAI rAMs to naive lungs is sufficient to transfer the increased susceptibility for RSV-associated inflammatory exacerbation, as observed in post-AAI mice.

Post-AAI rAMs exhibit increased NF- κ B activity on RSV challenge

Both cell populations were isolated and cultured *in vitro* for 24 and 48 hours in the presence or absence of RSV to further confirm the differential inflammatory response of post-AAI rAMs. Compared with naive rAMs, post-AAI rAMs showed increased secretion of the proinflammatory cytokines IL-6 and TNF at both time points (Fig 3, A, upper panels). Interestingly, RSV-induced type I interferon responses, quantified through expression of the type I interferon-inducible genes *Usp18* and *Ifit2*,¹¹ were similar in both rAM phenotypes (Fig 3, A, lower panels). IL-6 and TNF are mainly induced by NF- κ B, whereas type I interferons are elicited by members of the IRF transcription factor family.¹⁴ Importantly, equal amounts of viral genome-derived RNA transcripts were retrieved from both rAM cultures at each time point (see Fig E4 in this article's Online Repository at www.jacionline.org). Therefore it is unlikely that the observed differences in cytokine secretion between naive and post-AAI rAMs were due to differences in susceptibility to RSV infection.

To further confirm the apparent discrepancy between NF- κ B and IRF activity, we monitored the nuclear translocation of both transcription factors using confocal imaging. In agreement with the cytokine and gene expression data depicted in Fig 3, A, *in vitro* RSV challenge of post-AAI rAMs resulted in about 2-fold more nuclear NF- κ B p65 compared with that seen in naive rAMs after 16 hours of culture with RSV (Fig 3, B, left panel). In contrast, levels of nuclear IRF-3 and IRF-7 (Fig 3, B, middle and right panels), the main type I interferon-inducing transcription factors in RSV-stimulated macrophages,¹⁴ did not differ significantly between both rAM populations. The increased NF- κ B proinflammatory reactivity of post-AAI rAMs to RSV challenge was also apparent *in vivo* when comparing IL-6 and TNF levels in the BAL fluid of naive and post-AAI mice 6 hours after RSV infection (see Fig E5 in this article's Online Repository at www.jacionline.org).

Post-AAI rAMs display an immature phenotype that can be rescued by exogenous GM-CSF administration

To elucidate the mechanism underlying the hyperinflammatory phenotype of post-AAI rAMs, we analyzed the expression of

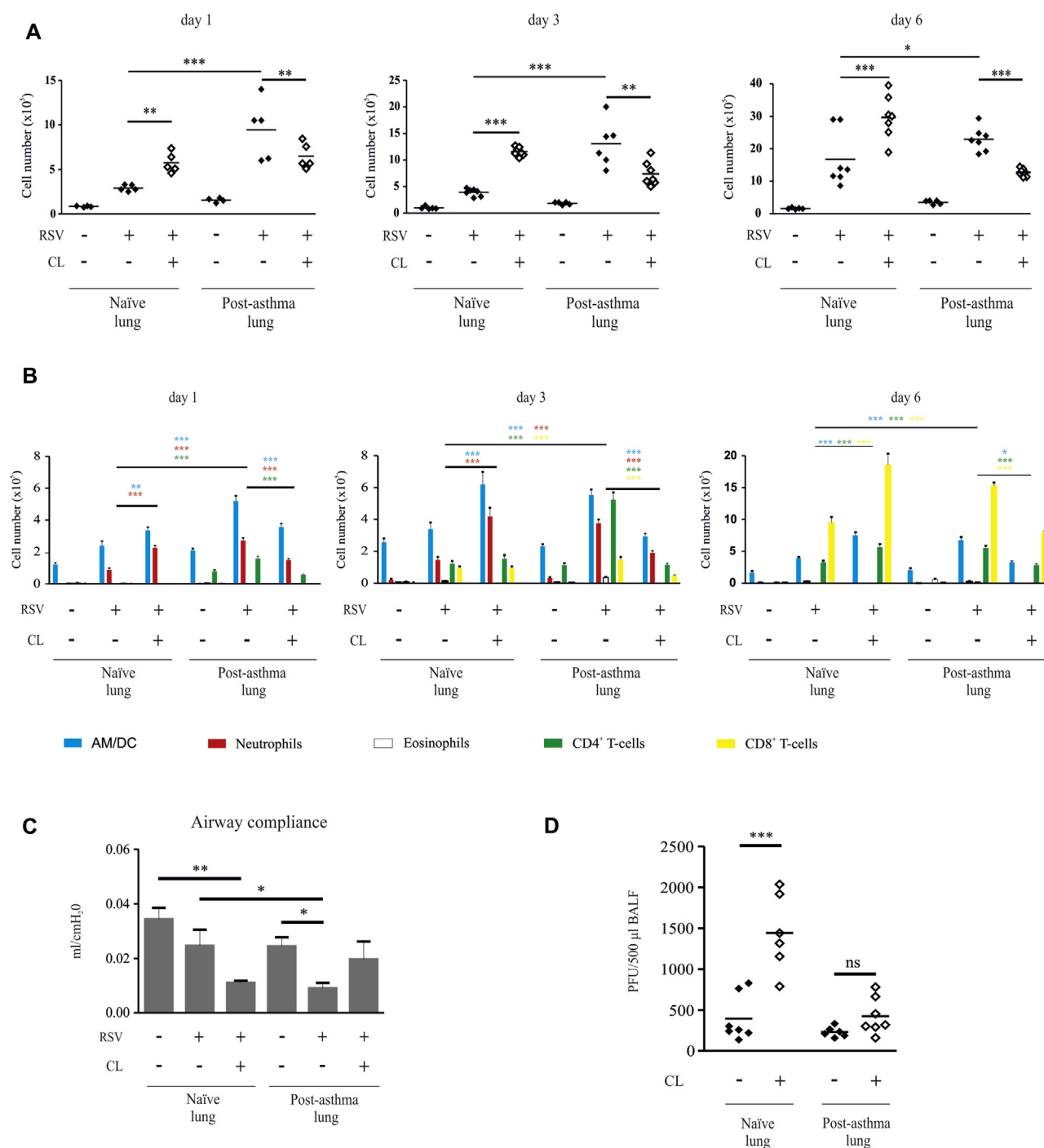


FIG 1. RSV infection induces an exacerbation reaction in post-AAI mice. **A** and **B**, Average total BAL cell counts (Fig 1, **A**) and average BAL cellular composition (Fig 1, **B**) of mock and RSV-infected naïve and post-AAI mice ($n = 5-6$) were determined on days 1, 3, and 6 after infection by means of flow cytometry. **C**, Naïve and post-AAI mice ($n = 5$) were exposed to aerosolized methacholine (800 mg/kg) at day 3 after infection, and airway compliance was measured. **D**, BAL fluid from naïve and post-AAI mice ($n = 6-7$) was collected at day 3 after infection. Viral recovery was assessed by using a plaque assay. All experiments were performed at least twice, and data from representative experiments are shown. Error bars represent means $\pm$ SEMs. CL, Clodronate liposomes. * $P < .05$, ** $P < .01$, and *** $P < .001$.

rAM maturation markers. Mature naïve rAMs are typically CD11c^{high}Autofluo^{high}CD64^{high} and sialic acid-binding immunoglobulin-like lectin F (Siglec-F)^{high} cells (Fig 4, **A**, upper panels).¹⁵ Although homogeneously CD11c^{high}Autofluo^{high}

CD64^{high}, post-AAI rAMs showed heterogeneous Siglec-F expression (Fig 4, **A**, lower panels), suggesting that the majority of post-AAI rAMs exhibited an immature phenotype. In agreement with this, we found significantly lower levels of

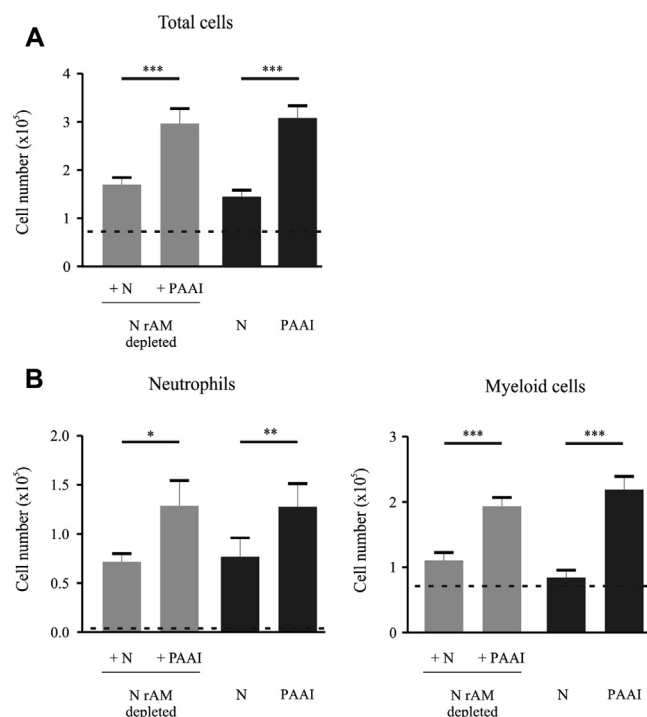


FIG 2. Adoptive transfer of post-AAI rAMs to naive recipient mice exacerbates inflammation during subsequent RSV infection. Average BAL total cell numbers (**A**) and average BAL neutrophils (*left panel*; **B**) of RSV-infected naive mice ($n = 5$) that received naive (*N*) or post-AAI (*PAAI*) rAMs or PBS intratracheally were determined on day 1 after infection by means of flow cytometry, as discussed previously. Data represent results from 1 of 2 independent experiments. The dotted line represents cell numbers of mock-infected naive mice. Error bars represent means $\pm$ SEMs. * $P < .05$, ** $P < .01$, and *** $P < .001$.

GM-CSF, the pivotal growth factor for rAM development,¹⁵⁻¹⁷ in the BAL fluid of post-AAI mice (**Fig 4, B**). Given the immature phenotype of post-AAI rAMs and the reduced BAL fluid levels of GM-CSF, we reasoned that administration of GM-CSF could rescue post-AAI rAM development and thus complete their maturation. Treatment of post-AAI mice by means of intratracheal delivery of GM-CSF for 2 consecutive days promoted the development of mature Siglec-F^{high} cells in a dose-dependent manner, shifting the Siglec-F^{low}/Siglec-F^{hi} ratio from 70/30 to 33/67 at the highest GM-CSF dose (**Fig 4, C**). Strikingly, the increased maturation of post-AAI rAMs directly affected RSV-induced exacerbation responses. Indeed, treatment of post-AAI mice with GM-CSF at days -2 and -1 before RSV infection (see **Fig E6** in this article's Online Repository at www.jacionline.org) nearly abolished the exacerbated inflammatory response of the post-AAI lungs, reducing the number of inflammatory cells to a level similar to that observed in naive lungs (**Fig 5, A**). In line with this reduced inflammation, treatment of post-AAI mice with GM-CSF before infection also significantly reduced the level of AHR to methacholine in these mice (**Fig 5, B**). This reduction of RSV-induced inflammation and AHR was not accompanied by an impaired antiviral activity in GM-CSF-treated post-AAI lungs because BAL fluid from both placebo- and GM-CSF-treated post-AAI mice contained equal RSV titers at day 3 after infection (**Fig 5, C**).

DISCUSSION

Several types of viruses, such as rhinoviruses, influenza virus, and RSV, have been detected in respiratory tract secretions from patients with asthma exacerbations. Although RSV infection does not result in dramatic clinical outcomes in a healthy adult, it is the instigator of the most severe exacerbation reactions in patients with asthma.¹⁸ In our present study we examined the influence of a preceding allergic bronchial inflammation on a subsequent pulmonary RSV infection. We found that, compared with naive mice, acute RSV infection of post-AAI mice induced an increased pulmonary inflammatory response, which was accompanied by increased AHR to methacholine. Furthermore, we show that in this mouse model of RSV-induced inflammation exacerbation, post-AAI rAMs act as crucial instigators of the exacerbated inflammatory response. Depletion of this rAM population before RSV infection abrogated the exacerbated inflammatory response, whereas reconstitution of naive lungs with post-AAI rAMs was sufficient to reinstate the exacerbated inflammation otherwise observed in post-AAI lungs. Strikingly, in all these conditions, increased pulmonary inflammation was not accompanied by an increased clearance of RSV, thus showing the futile nature of the exacerbated inflammation.

Naive rAMs are generally considered key immune regulators in the lung, dampening immune responses to preserve the lung gas exchange function. Murine naive rAMs have been shown to actively suppress local T-cell proliferation¹⁹ to downregulate the antigen presentation capacity of pulmonary dendritic cells (DCs)²⁰ and to protect against the development of AHR.²¹ In line with this, a substantial body of literature demonstrates that depletion of naive rAMs before lung challenge with allergen increases the overall allergic airway response in mice.²²⁻²⁶ Next to allergic airway disease, murine naive rAMs also fulfill an anti-inflammatory role during RSV infection, as evidenced by our present study and studies described in the literature.^{9,13} This anti-inflammatory role of naive rAMs stands in strong contrast to the pronounced proinflammatory reactivity of post-AAI rAMs to RSV challenge. The hyperinflammatory character of post-AAI rAMs was apparent during *in vitro* and *in vivo* RSV infection and represented a direct effect resulting from an intrinsic increase in NF- κ B p65 signaling and leading to increased proinflammatory cytokine secretion, including IL-6 and TNF. Yet post-AAI rAMs exhibited an increase only in NF- κ B p65 reactivity, whereas type I interferon signaling remained unaltered. Also, Bartlett et al²⁷ reported a similar uncoupling of proinflammatory NF- κ B p65 and antiviral type I interferon signaling pathways in a model of rhinovirus-induced asthma exacerbation. In this study NF- κ B p65-driven inflammation could be inhibited without impairing the antiviral activity mediated by the type I interferon pathway.²⁷ It will be worthwhile to investigate to what extent targeting NF- κ B transcription activity in post-AAI rAMs can suppress inflammation while preserving type I interferon-mediated antiviral defenses.

Interestingly, alternative activation of macrophages has been reported to elicit a similar proinflammatory yet futile antibacterial response.²⁸ However, when we alternatively activated naive rAMs with IL-4, the cells did not mimic the increased inflammatory responsiveness to RSV of post-AAI rAMs (see **Fig E7** and accompanying text in this article's Online Repository at www.jacionline.org). Thus although post-AAI rAM exhibited

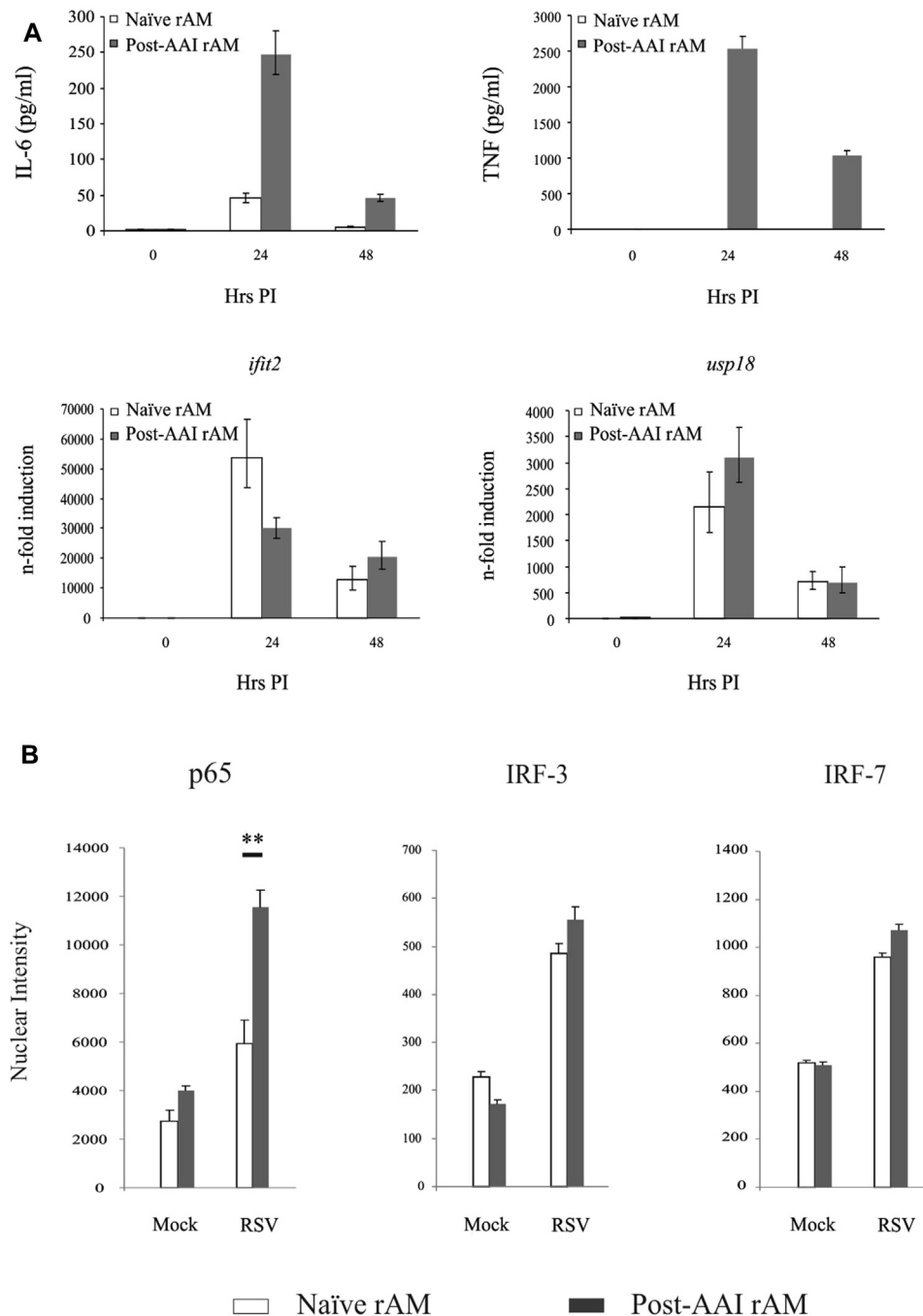
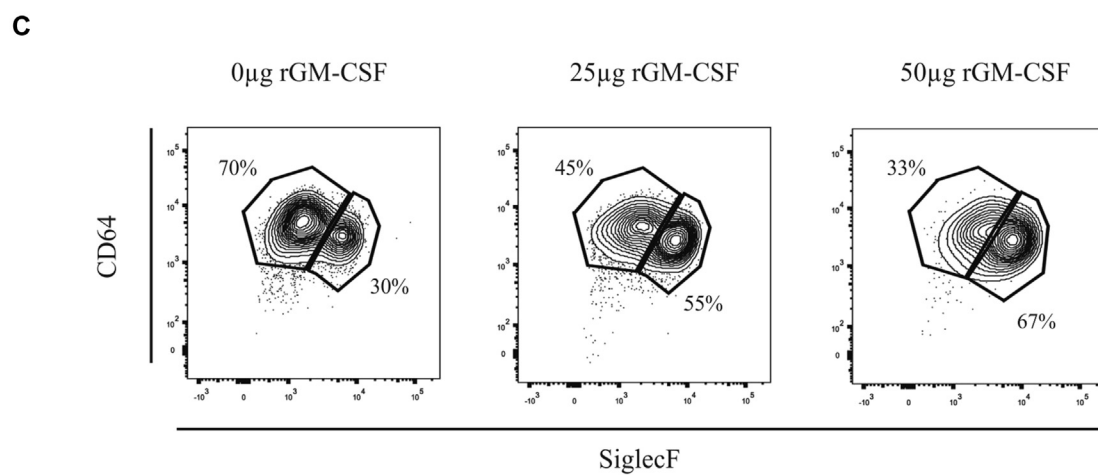
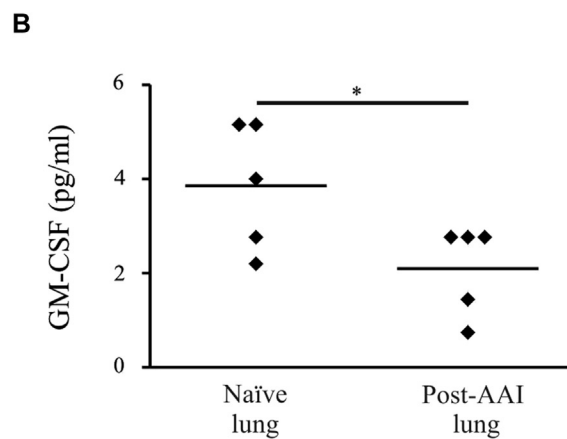
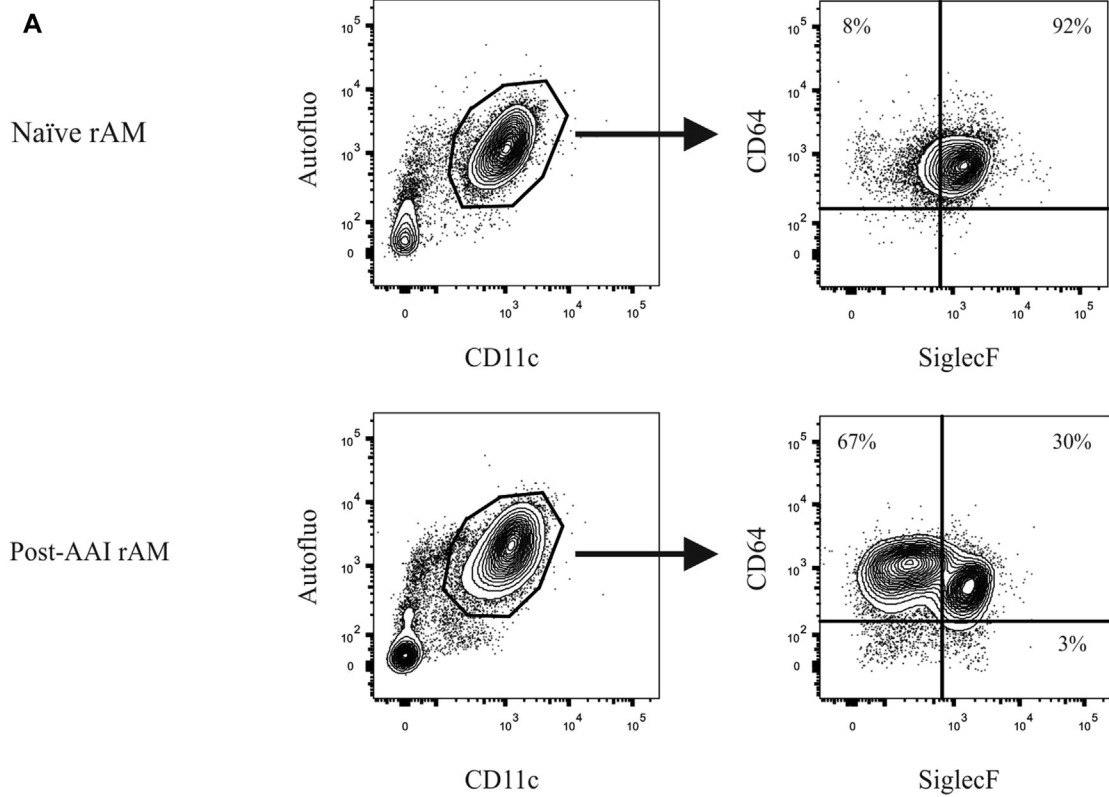


FIG 3. Inflammatory cytokine response of naive and post-AAI rAMs to *in vitro* and *in vivo* RSV infection. Pooled naive and post-AAI rAMs were isolated from BALB/c mice ($n = 8$) and infected *ex vivo* with RSV (multiplicity of infection = 5) for 24 or 48 hours or mock infected as a control. **A**, IL-6 (left panel) and TNF (right panel) levels in culture supernatants were determined by using the Bio-Plex suspension array system. Values represent average cytokine concentrations $\pm$ SEMs of triplicate culture conditions. Interferon-induced protein with tetratricopeptide repeats 2 (*Ifit2*) and ubiquitin-specific peptidase 18 (*Usp18*) gene expression in both rAM populations was determined by using real-time quantitative PCR. Values represent average n-fold inductions compared with mock-infected naive rAMs $\pm$ SDs of triplicate real-time quantitative PCR reactions. **B**, Nuclear translocation of p65 (left graph), IRF-3 (middle graph), and IRF-7 (right graph) was determined in naive and post-AAI rAMs 16 hours after *in vitro* RSV infection by using confocal imaging. Values represent the average nuclear fluorescent intensity of p65, IRF-3, or IRF-7 staining in cells of 10 randomly selected microscopic fields $\pm$ SEMs. Data represent results from 1 of 2 independent experiments. Error bars represent means $\pm$ SEMs. ** $P < .01$.



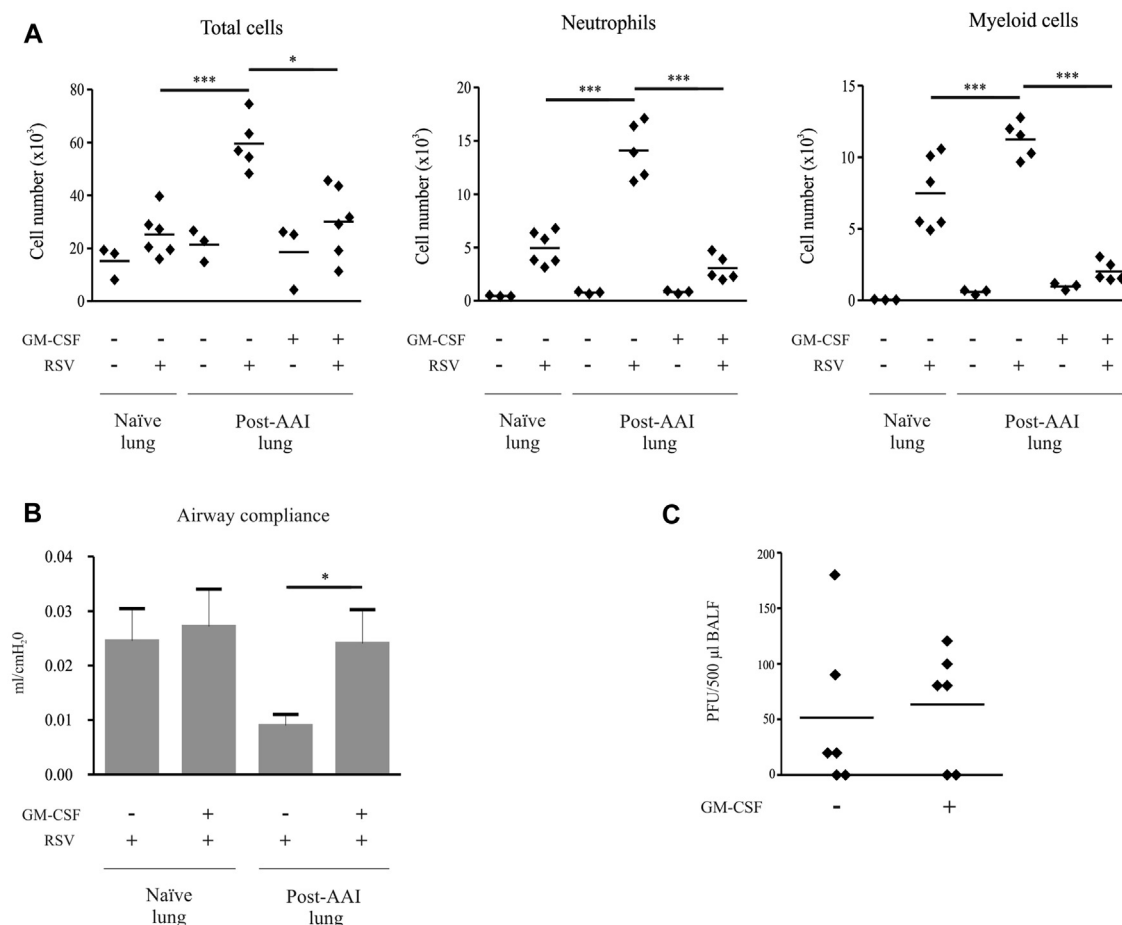


FIG 5. Prophylactic treatment with GM-CSF of post-AAI mice decreases RSV-induced inflammation exacerbation and AHR to methacholine. **A**, Average BAL absolute cell numbers and average BAL neutrophils and myeloid cells of GM-CSF- or placebo-treated post-AAI and naïve mice ($n = 3-6$) were determined on day 3 after infection by using flow cytometry. **B**, Naïve and post-AAI mice ($n = 5$) were exposed to aerosolized methacholine at day 3 after infection, and airway compliance was measured. **C**, BAL fluid from naïve and post-AAI mice ($n = 6$) was collected at day 3 after infection. Viral recovery was assessed by using a plaque assay. Data represent results from 1 of 2 independent experiments. * $P < .05$ and *** $P < .001$.

increased mRNA levels of *Arg1* and *Ym1*, 2 hallmarks of alternative macrophage activation, exposure to IL-4 alone does not seem to be sufficient for eliciting the post-AAI rAM phenotype. Instead, a more direct mechanism is suggested by our observation that a majority of the post-AAI rAM population were immature Siglec-F^{low} cells. An immature phenotype of post-AAI rAMs was further indicated by the decreased levels of GM-CSF, the pivotal rAM growth factor,¹⁵⁻¹⁷ we found in the alveolar airspaces of post-AAI mice. Accordingly, we achieved full maturation of the immature post-AAI rAM subset by means of intratracheal administration of exogenous GM-CSF. Strikingly, this conversion

to a mature phenotype abolished the exacerbated inflammatory response to RSV infection of the post-AAI lungs. These results clearly identify the predominantly immature status of post-AAI rAMs as being at the basis of the hyperinflammatory reactivity of the cells and their causal role in RSV-induced exacerbation. These results also emphasize the important role reduced GM-CSF levels can have in maintaining the immature hyperinflammatory status of post-AAI rAMs. However, the exact mechanism underlying reduced levels of GM-CSF in post-AAI lungs remains to be elucidated. Research of the past decades identified GM-CSF as an important factor in the onset and

FIG 4. Exogenous GM-CSF rescues the immature phenotype of post-AAI rAMs. **A**, Naïve and post-AAI rAMs were isolated by means of BAL ($n = 5$) and analyzed by using flow cytometry for intrinsic autofluorescence intensity and surface expression of CD11c, CD64, and Siglec-F. Shown are representative cytograms of a single mouse per group. **B**, BAL fluid was harvested from naïve and post-AAI BALB/c mice ($n = 5$), and protein levels of GM-CSF were analyzed by using the Bio-Plex suspension array system. Values represent average cytokine concentrations in picograms per milliliter. **C**, Post-AAI BALB/c mice ($n = 5$) received PBS, 25 µg of GM-CSF, or 50 µg of GM-CSF intratracheally on days 0 and 1. BAL was performed at day 2, and surface expression of Siglec-F by rAMs was analyzed by means of flow cytometry. rAMs were identified as Autofluo⁺CD11c⁺CD64⁺ cells within the live single-cell gate. Shown are representative cytograms of a single mouse per group. Data represent results from 1 of 3 independent experiments. * $P < .05$.

pathogenesis of allergic airway disease.²⁹⁻³⁴ Indeed, airway epithelial cell-derived GM-CSF was found to act as an adjuvant facilitating allergen sensitization in mice.³²⁻³⁴ In addition, during the effector phase of the allergic airway response, GM-CSF has been shown to exert a proinflammatory role.²⁹⁻³¹ Indeed, administration of exogenous GM-CSF to the lungs of OVA-sensitized and OVA-challenged mice increased airway eosinophilia,^{29,30} whereas prophylactic administration of neutralizing anti-GM-CSF antibodies decreased AAI on OVA inhalation.^{31,33} Finally, after clearance of inflammation, the present study identifies GM-CSF as a homeostatic regulator controlling the maturation and inflammatory status of rAMs. This notion is supported by our results in which administration of exogenous GM-CSF to post-AAI lungs promoted the maturation of post-AAI rAMs and exerted a clear anti-inflammatory effect during subsequent RSV infection by reducing neutrophil infiltration and AHR. The failure of endogenous GM-CSF to fully mature post-AAI rAMs might in fact result from the significantly reduced basal GM-CSF levels we consistently found in post-AAI lungs. Anti-inflammatory feedback mechanisms triggered in the course of the allergic response to reduce the pathogenesis-promoting activities of GM-CSF might very well be responsible for the reduced GM-CSF levels we observed in post-AAI lungs. TGF- β is abundantly produced during AAI and is dominantly present in post-AAI lungs.³⁵ TGF- β could suppress GM-CSF secretion by lung cells and hence inhibit post-AAI rAM maturation. Furthermore, a study by Sawada et al³⁶ reported the inhibitory effect of IL-4 on GM-CSF production. Although we did not detect any IL-4 in the BAL fluid of post-AAI mice, the ubiquity and prolonged presence of this cytokine during the allergic airway response could have imprinted lung cells to down-regulate GM-CSF production over an extended period of time. Thus although suppression of GM-CSF production by IL-4, TGF- β , or both might help to control the acute allergic response during allergic disease, suppressed GM-CSF production after allergic disease might increase the inflammatory sensitivity of the post-AAI lung by compromising the (functional) maturation of post-AAI rAMs.

In conclusion, our results uncover a cascade of events in which a prior allergic bronchial inflammation causes a maturation defect in post-AAI rAMs, resulting in hyperinflammatory reactivity of the cells to RSV infection, which in turn contributes to onset of an RSV-induced exacerbation reaction. We propose immature post-AAI rAMs are therapeutic targets for treating RSV-induced exacerbations in human asthmatic patient.

We thank Evelien Van Hamme for technical assistance with the confocal imaging experiments.

Key messages

- Post-AAI rAMs contribute to the development of increased RSV-induced inflammatory airway responses and AHR in post-AAI mice through increased proinflammatory NF- κ B signaling.
- Post-AAI rAMs display an immature phenotype that can be rescued by exogenous GM-CSF.
- Inducing full maturation of post-AAI rAMs by GM-CSF prevents RSV-induced exacerbations in post-AAI mice.

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METHODS

Mouse model of RSV-induced inflammation exacerbation in allergen-sensitized and challenged mice

BALB/c mice were immunized 2 times with a weekly single intraperitoneal injection of 20 μ g of grade V chicken egg OVA (Sigma-Aldrich, St Louis, Mo) adsorbed on 1 mg of AlOH₃ (aluminum hydroxide; Sigma-Aldrich) in endotoxin-free PBS (Lonza, Walkersville, Md). Seven days after the second immunization, OVA/aluminum hydroxide-sensitized mice were subsequently exposed to 7 OVA aerosols (30 min/d for 7 consecutive days) consisting of 1% grade III OVA (Sigma-Aldrich) in endotoxin-free PBS.

After clearance of the eosinophilic inflammation (day 15 after the last OVA aerosol), mice were exposed to RSV infection. To this end, mice were anesthetized with isoflurane by using the drop-jar method for approximately 30 seconds. Mouse lungs were subsequently infected with RSV by means of intranasal instillation of 1×10^7 PFU of RSV A2 at the indicated time points. RSV titers in stock solutions and BAL fluid were determined by using a plaque assay.

Human RSV A2

Human RSV A2 was obtained from ATCC (VR-1540) and propagated on Hep-2 cells (ATCC, CCL-23). Infections of Hep-2 cells were performed at a multiplicity of infection of 0.05 in serum free Opti-MEM medium (Life Technologies, Grand Island, NY). Three hours after infection, the inoculum was removed and replaced with Dulbecco modified eagle medium (DMEM; Life Technologies) supplemented with 2 mmol/L L-glutamine, 1% penicillin/streptomycin, and 1% FBS (all from Life Technologies). When syncytia were abundant, which is typically at day 3 after infection, the culture medium was collected. Polyethylene glycol 6000 (Sigma-Aldrich) was added to the clarified culture medium to a final concentration of 10% (vol/vol). The viral particles were precipitated by means of gentle stirring for 4 hours at 4°C and subsequently pelleted by means of centrifugation. The pelleted virus was resuspended in cold Ca²⁺- and Mg²⁺-free Hank balanced salt solution (HBSS; Life Technologies) containing 20% sucrose, placed in aliquots, snap-frozen, and finally stored at -80°C. Three vials were used for plaque assay on Vero cells (ATCC, CCL-81) to quantify the stock viral titer. Sterility of the viral stock was confirmed by inoculating 5×10^7 PFU in Hep-2 growth medium without antibiotics, LB Broth medium (Life Technologies), and YPD Broth medium (Life Technologies).

RSV plaque assay

BAL samples were diluted in serum-free DMEM medium and added to Vero cell cultures. After 3 hours, the cultures were washed and incubated for 72 hours in DMEM supplemented with 2 mmol/L L-glutamine, nonessential amino acids (Life Technologies), and 2% FBS containing 0.6% Avicel RC-851 (FMC Biopolymer, Philadelphia, Pa). After infection, the cells were washed 4 times with PBS and subsequently fixed in 2% paraformaldehyde (PFA; Sigma-Aldrich). After overnight fixation at 4°C, the PFA solution was removed, and the cells were washed twice with PBS. Subsequently, the cells were permeabilized with PBS containing 0.2% Triton X-100 (Millipore, Nottingham, United Kingdom) for 5 minutes and blocked with PBS containing 1% BSA (Sigma-Aldrich) for 1 hour. The viral plaques were stained with a polyclonal goat anti-human RSV serum (AB1128, Chemicon International, Temecula, Calif; 1:4000). After washing 3 times with 1% BSA in PBS, cells were incubated with horseradish peroxidase-conjugated anti-goat IgG antibodies (SC2020; Santa Cruz Biotechnology, Dallas, Tex) for 30 minutes. Nonbinding antibodies were removed by washing 4 times with PBS containing 1% BSA and 0.01% Triton X-100 and once with PBS. Finally, the plaques were visualized with TrueBlue peroxidase substrate (KPL, Gaithersburg, Md).

rAM depletion and reconstitution

Mice were sedated by means of intraperitoneal injection of a ketamine/xylazine mixture (80 mg/kg ketamine and 5 mg/kg xylazine). Subsequently,

80 μ L of a 30% solution of clodronate-containing liposomes in endotoxin-free PBS was administered by means of intratracheal instillation to anesthetized mice. Instillation of 80 μ L of endotoxin-free PBS was used as a control. Seventy-two hours after clodronate liposome instillation, rAM-depleted recipient lungs were reconstituted by means of intratracheal administration of 3×10^5 post-AAI rAMs or naive rAMs as a control. CD4⁺ T cells present in the BAL fluid were first depleted with CD4 (L3T4) Microbead-mediated MACS (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer's instructions. Reconstituted recipient mice were challenged with RSV A2 16 hours after reconstitution.

BAL and alveolar cell isolation and culture

Mice were anesthetized by means of intraperitoneal injection of Nembutal (pentobarbital; 125 mg/kg in PBS; Lundbeck, Valby, Denmark), and a small incision was made in the trachea to allow passage of a lavage cannula. Lungs were lavaged 4 times with 1 mL of HBSS supplemented with 0.05 mmol/L EDTA (Sigma-Aldrich). Optionally, a first lavage with 0.5 mL of HBSS-EDTA was performed, and the BAL fluid was isolated by means of centrifugation and harvesting of the supernatant. The BAL cell pellets were washed and resuspended in PBS for further analysis.

Naive and post-AAI rAMs isolated by means of BAL were cultured in culture medium RPMI 1640 (Life Technologies) supplemented with 10% heat-inactivated FBS, 2 mmol/L L-glutamine, 1 mmol/L pyruvate, 100 U/mL penicillin/streptomycin, and 55 μ mol/L 2-mercaptoethanol (all from Life Technologies). All cultures were enriched for macrophages by means of plastic adhesion for 1 hour at 37°C and 5% CO₂. In some experiments cells were stimulated with 10 ng/mL recombinant murine IL-4 (BD PharMingen, San Jose, Calif) for the indicated time.

Differential BAL cell counts

Differential numbers of BAL cells were determined by using flow cytometry. Fc-blocked (1 μ g/mL; clone 2.4G2; BD Biosciences, San Jose, Calif) BAL cells were stained with anti-mouse major histocompatibility complex (MHC) class II-eFluor450 (0.4 μ g/mL; clone M5/114.15.2; eBioscience, San Diego, Calif), CD3e-Alexa Fluor 488 (1 μ g/mL; clone 245-2C11; BD Biosciences), Siglec-F-phycoerythrin (PE; 1 μ g/mL; clone E50-2440; BD Biosciences), CD4-peridinin-chlorophyll protein complex (1 μ g/mL; clone RM4-5; BD Biosciences), CD8-PE-Cy7 (1 μ g/mL; clone 53-6.7; BD Biosciences), CD11b-allophycocyanin-Cy7 (1 μ g/mL; clone M1/70; BD Biosciences), CD11c-PE-TR (2 μ g/mL; clone N418; Life Technologies), and CD64-Alexa Fluor 647 (1 μ g/mL; clone X54-5/7.1; BD Biosciences). Sytox Red dead cell staining (Life Technologies) was added to exclude dead cells from the analysis. All samples were measured on a FACS LSRII flow cytometer and analyzed using FACSDiva software (both from BD Biosciences). Within the live (Sytox Red⁻) cell gate, CD4⁺ and CD8⁺ T lymphocytes were identified as CD3e⁺CD4⁺ and CD3e⁺CD8⁺ cells, respectively. Alveolar macrophages and DCs were further identified within the CD3e⁺CD11c⁺ cells based on their expression of Siglec-F, CD11b, and MHC class II. Finally, neutrophils and eosinophils were identified as CD3e⁺CD11c⁻Siglec-F⁻CD11b⁺ and CD3e⁺CD11c⁻Siglec-F⁺CD11b⁺ cells, respectively, in the remaining cell population.

Determination of airway hyperresponsiveness

AHR to methacholine (Sigma-Aldrich) was measured with the flexiVent (SCIREQ) invasive measurement of dynamic compliance. Methacholine is a nonspecific spasmogenic stimulus that triggers responsiveness and constriction of the airways. Mice were anesthetized intraperitoneally with 15 mg/kg xylazine, followed by an intraperitoneal injection of 54 mg/kg Nembutal (pentobarbital) 15 minutes later, just before they were started on the ventilator. Next, an 18-gauge needle was inserted into the trachea. Mice were connected to a computer-controlled small-animal ventilator and quas sinusoidally ventilated with a tidal volume of 10 mL/kg at a frequency of 150 breaths/min and a positive end-expiratory pressure of

2 cm H₂O to achieve a mean lung volume close to spontaneous breathing. After baseline measurement, mice were challenged for 10 seconds with saline aerosol and methacholine aerosol. The peak response was calculated as the mean of the 3 maximal values and used for calculation of airway dynamic compliance, which was expressed as milliliters per centimeter of H₂O.

Nuclear translocation assays

Naive and post-AAI rAMs were isolated and cultivated in a 15-well microplate (Ibidi, Planegg/Martinsreid, Germany). Both rAM populations were infected with RSV for the indicated time points. Afterward, the cells were fixed with a 2% solution of PFA for 10 minutes at room temperature (RT). Subsequently, the cells were permeabilized with a 0.2% Triton X-100 solution in PBS for 3 minutes at RT. After a blocking step of 1 hour with a 1% BSA solution in PBS, cells were intracellularly stained with a rabbit anti-NF- κ B p65, anti-IRF-3, or anti-IRF-7 antibody (all from Life Technologies) for 2 hours at RT. Detection of the different rabbit antibodies was subsequently achieved through incubation of a goat anti-rabbit antibody labeled with an Alexa Fluor 568 fluorescent group (Life Technologies) for 1 hour at RT. Vectashield supplemented with 4,6 diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories, Peterborough, United Kingdom) was finally added to mount the cell cultures. Stained cells were imaged with a Leica TCS SP5 AOBS confocal microscope by using the 488-nm argon laser line. Images were acquired with LAS AF software (Leica) and subsequently analyzed with Volocity software (PerkinElmer).

Mouse recombinant GM-CSF

Mouse recombinant GM-CSF was produced and purified, as outlined by Jacobs et al.^{E1} Briefly, the protein was produced by using the glycoengineered GlycoSwitch-Man5 *Pichia pastoris* yeast strain, a production strain for homogenously N-glycosylated proteins. The mouse GM-CSF gene sequence was cloned into the *P pastoris* pPIC9 expression vector (Life Technologies), which was subsequently transfected into the yeast cells. Culture supernatants were collected 60 hours after transfection, and GM-CSF protein was isolated by means of size exclusion chromatography (GE Healthcare, Fairfield, Conn), yielding a solution of GM-CSF with a purity of greater than 95%, as determined by using SDS-PAGE. The GM-CSF concentration was determined by using the Pierce BCA assay (Life Technologies), and endotoxin levels were determined by using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway Township, NJ). The LAL assay revealed endotoxin levels of 0.15 EU/mg GM-CSF.

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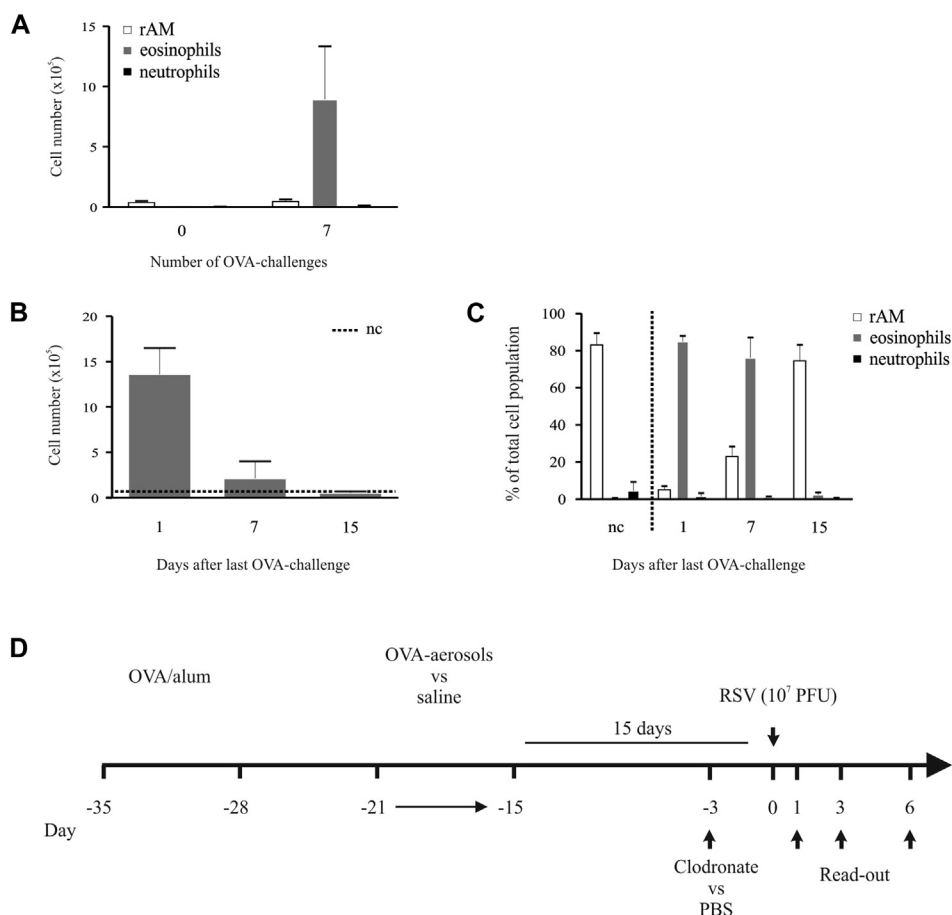


FIG E1. Mouse models of AAI and RSV-induced exacerbation. BALB/c mice were sensitized twice with a weekly single intraperitoneal injection of OVA/aluminum hydroxide. Seven days after the last immunization, sensitized mice ($n = 5$) were exposed to a 1% OVA aerosol for 30 min/d for 7 consecutive days or exposed to PBS as a naive control. **A**, Cellular composition in BAL fluid from mice exposed to 7 OVA challenges or PBS. **B** and **C**, Total cell numbers (Fig E1, **B**) and differential BAL cell composition (Fig E1, **C**) at the indicated time points after the last OVA challenge. *nc*, Naive control. **D**, Experimental setup of the mouse model for RSV-induced exacerbation. On day 0 (day 15 after the last OVA exposure), naive and post-AAI mice were inoculated intranasally with 1×10^7 PFU RSV or a mock solution as a control. Readout was performed at days 1, 3, and 6 after infection. Naive and post-AAI mice, respectively, received clodronate liposomes intratracheally 3 days before intranasal inoculation with RSV or received PBS as a control to deplete naive and post-AAI rAMs before infection.

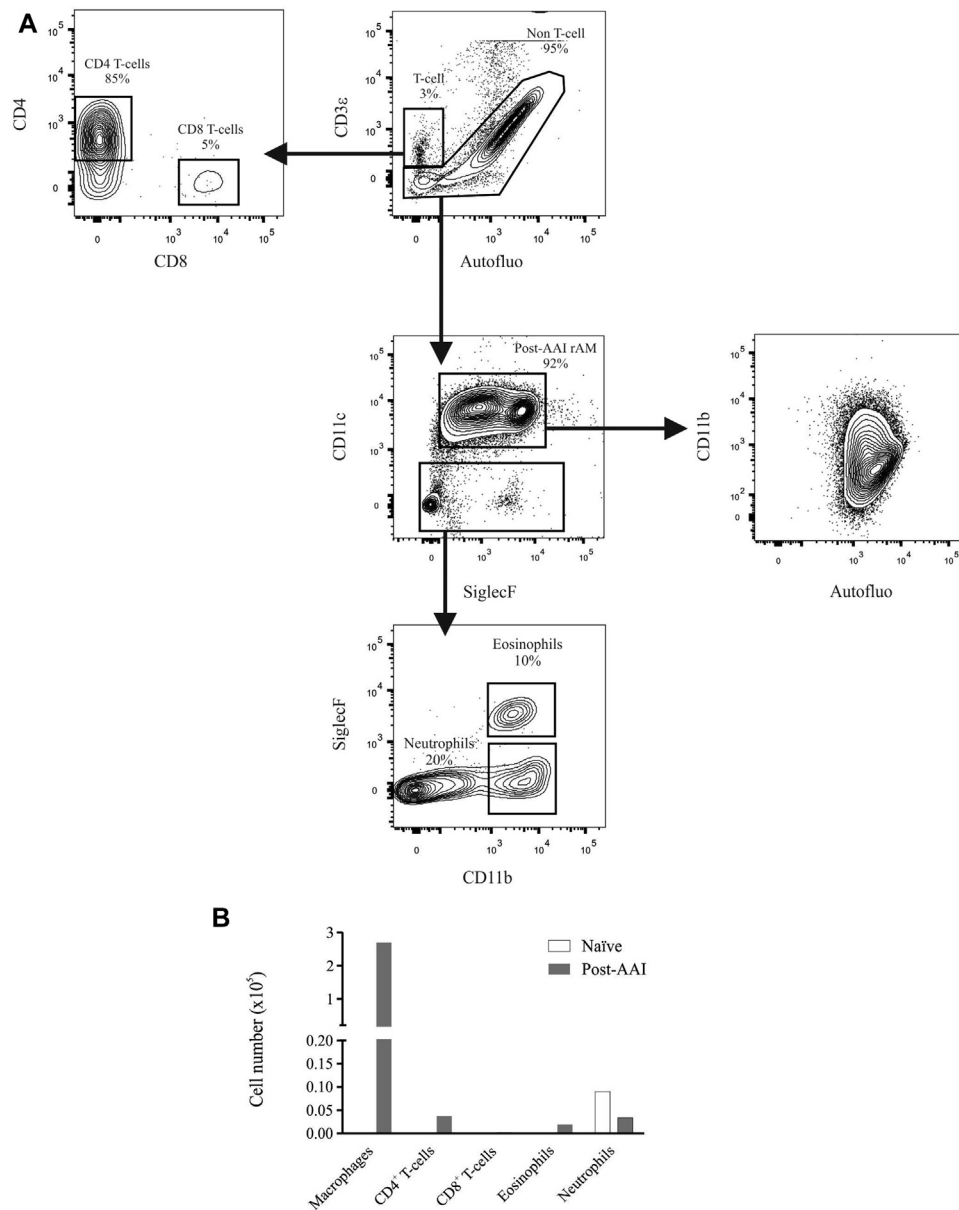


FIG E2. Differential and absolute composition of the adoptively transferred naive and post-AAI BAL cells. BAL cells were isolated from post-AAI mice, and CD4⁺ cells were removed by means of MACS. Cellular composition was determined by using flow cytometry. **A**, Within the cell gate, CD4⁺ and CD8⁺ T lymphocytes were identified as CD3⁺CD4⁺ and CD3⁺CD8⁺ cells, respectively. Post-AAI rAMs were further identified as CD3⁺CD11c⁺Siglec-F⁺Autofluo^{high}CD11b^{low} cells. Finally, neutrophils and eosinophils were identified as CD3⁺CD11c⁺Siglec-F⁺CD11b⁺ and CD3⁺CD11c⁺Siglec-F⁺CD11b⁺ cells, respectively. Percentages are relative to the parental cell population. **B**, Absolute cell numbers of the different cell populations present in the adoptively transferred naive and post-AAI BAL cells.

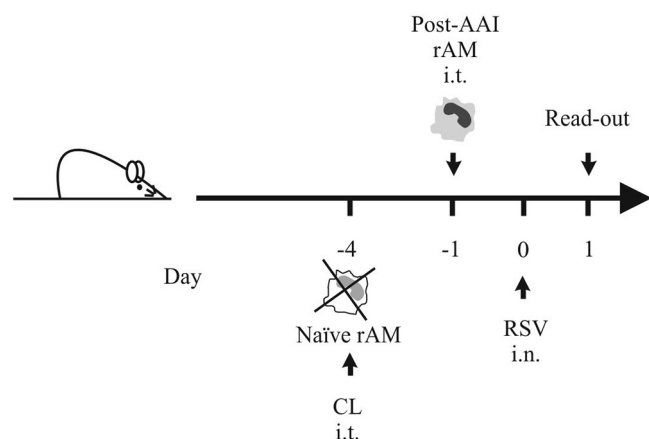


FIG E3. Schematic representation of the adoptive transfer experiment protocol used to investigate the intrinsic contribution of post-AAI rAMs to RSV-elicited bronchoalveolar inflammation. Naïve rAMs were depleted by means of intratracheal (*i.t.*) instillation of clodronate liposomes (*CL*) at day -4 ($n = 5$). Subsequently, naïve and post-AAI rAMs (3×10^5 cells) were transferred intratracheally to recipient mice at day -1. Reconstituted lungs were exposed to RSV at day 0, and BAL fluid was isolated at day 1 after infection. *i.n.*, Intranasal.

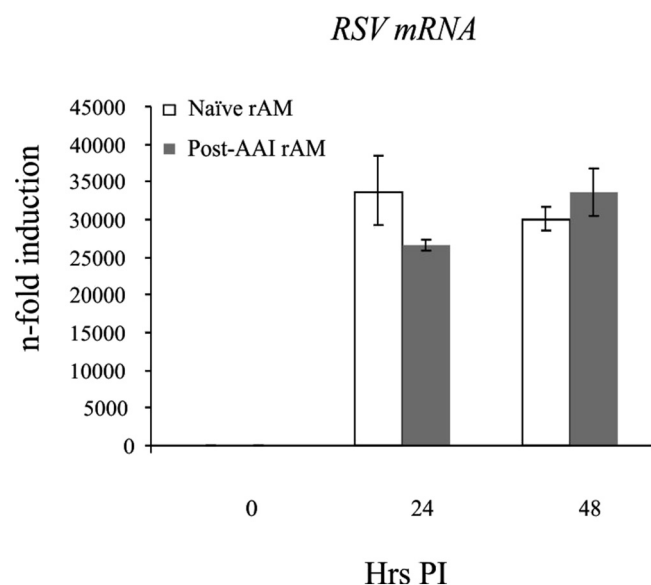


FIG E4. Naïve and post-AAI rAMs exhibit equal susceptibility to *in vitro* RSV infection. Pooled naïve and post-AAI rAMs were isolated from BALB/c mice ($n = 8$) and infected *ex vivo* with RSV (multiplicity of infection = 5) for 24 or 48 hours or mock infected as a control. Expression of RSV mRNA as a marker for viral replication was determined by using real-time quantitative PCR. Values represent average n-fold inductions compared with mock-infected naïve rAMs $\pm$ SD of triplicate real-time quantitative PCR reactions. Data are representative for 2 independent experiments. *Hrs PI*, Hours post infection.

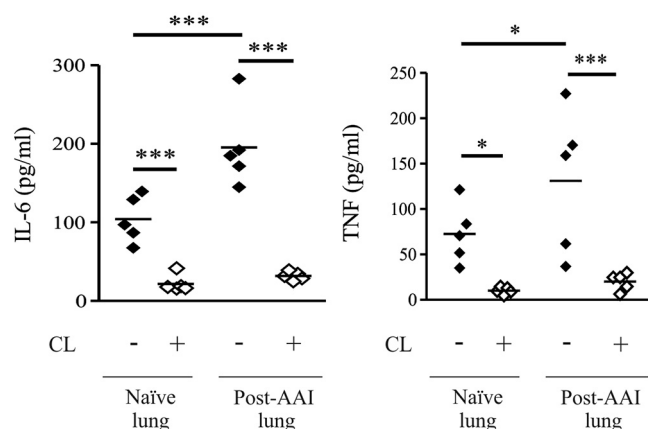


FIG E5. Inflammatory cytokine response of naive and post-AAI rAMs to *in vivo* RSV infection. Naive and post-AAI rAMs were depleted by means of intratracheal instillation of clodronate liposomes ($n = 5$). Three days later, 1×10^7 PFU RSV was inoculated through the intranasal route. PBS was administered as a control. BAL fluid was collected 6 hours after RSV infection. BAL fluid levels of IL-6 (*left panel*) and TNF (*right panel*) were measured by using the Bio-Plex suspension array system. Data represent results from 1 of 2 independent experiments. Error bars represent means $\pm$ SEMs. * $P < .05$ and *** $P < .001$.

To confirm increased NF- κ B proinflammatory reactivity of post-AAI rAMs to RSV challenge *in vivo*, we compared IL-6 and TNF levels in the BAL fluid of naive and post-AAI mice 6 hours after RSV infection. At this time point, no infiltration of inflammatory leukocytes was apparent, identifying rAMs and airway epithelial cells as the major potential cytokine sources. As shown, BAL fluid from post-AAI mice 6 hours after infection contained 2-fold increased IL-6 and TNF levels compared with those in RSV-infected naive mice. Depletion of rAMs in naive and post-AAI lungs abolished the alveolar IL-6 and TNF production in response to RSV by 5- to 7-fold, suggesting that both rAM populations were a major source of both cytokines.

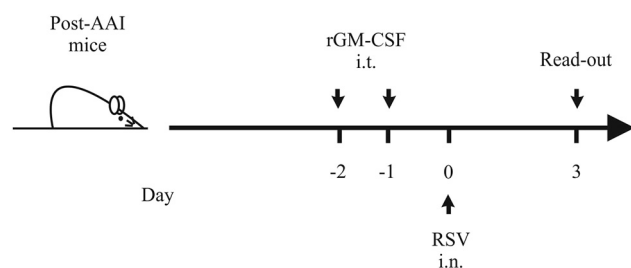


FIG E6. Schematic representation of the experimental protocol used to investigate the effect of prophylactic GM-CSF treatment of post-AAI mice on RSV-elicited bronchoalveolar responses. Naive and post-AAI BALB/c mice ($n = 3-6$) were treated with intratracheal (*i.t.*) instillation of $50 \mu\text{g}$ of GM-CSF at days -2 and -1 before RSV infection. Readout of the pulmonary inflammatory responses, AHR to methacholine, and RSV titers in BAL fluid was performed at day 3 after infection. *i.n.*, Intranasal.

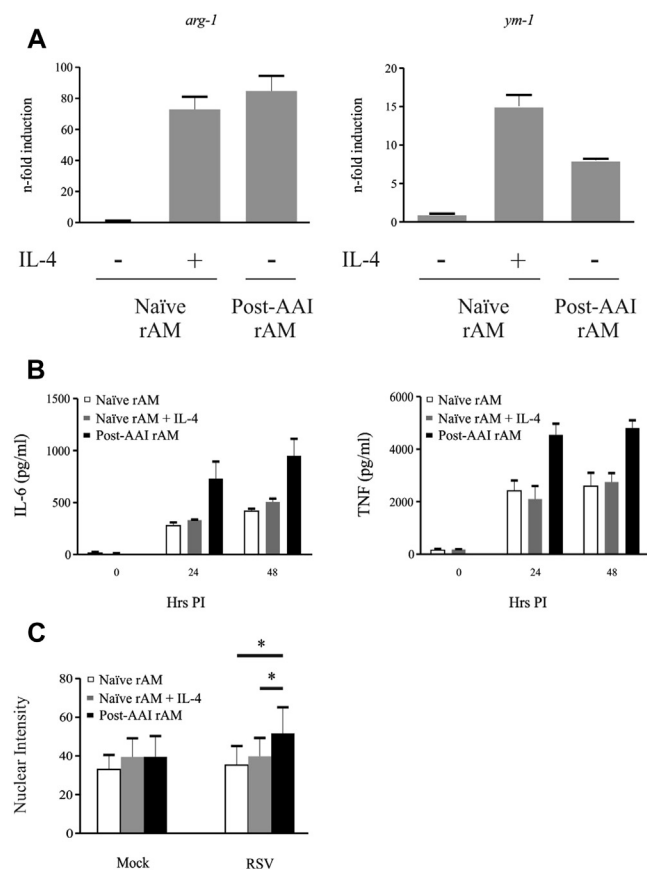


FIG E7. Alternately activated naive rAMs do not mimic the proinflammatory phenotype of post-AAI rAMs in response to RSV. Pooled rAMs from naive and post-AAI mice were isolated by means of BAL and cultured *in vitro*. Naive rAMs were stimulated with IL-4 (10 ng/mL) or left untreated for 24 hours. Post-AAI rAMs were left untreated for 24 hours. **A**, Transcription levels of *Arg1* and *Ym1* were subsequently determined by using real-time quantitative PCR. Values represent average n-fold inductions compared with untreated naive rAMs $\pm$ SDs of triplicate real-time quantitative PCR reactions. **B**, After the pretreatment period, naive and post-AAI rAM cultures were infected with RSV (multiplicity of infection = 5) or mock infected as a control for another 24 or 48 hours. IL-6 and TNF levels in culture supernatants were subsequently determined by using the Bio-Plex suspension array system. Values represent average cytokine concentrations $\pm$ SEMs of triplicate culture conditions. **C**, Nuclear translocation of p65 was determined in naive and post-AAI rAMs 16 hours after *in vitro* RSV infection by means of confocal imaging. Values represent the average nuclear fluorescent intensity of p65 in cells of 10 randomly selected microscopic fields $\pm$ SEM. * $P < .05$.

We previously demonstrated that, compared with naive rAMs, post-AAI rAMs exhibit an increased basal alternative activation, as indicated by increased expression levels of *Arg1* mRNA.^{E2} Because proper antiviral responses require classical activation of the macrophage, the observed alternative activation of post-AAI rAMs could be responsible for the aberrant responsiveness of the cells to RSV challenge. To address this question, we exposed naive rAMs to IL-4, a potent inducer of alternative activation, and determined whether this would alter the natural inflammatory response to RSV in a similar manner as post-AAI rAMs. Naive rAMs were pretreated *in vitro* with IL-4 for 24 hours to induce an alternative activation phenotype. As shown in Fig E7, A, mRNA levels of *Arg1* (left panel) and *Ym1* (right panel), 2 marker genes for alternatively activated macrophages,^{E3} were markedly increased in IL-4-treated naive rAMs compared with levels in untreated rAMs (Fig E7, A). Moreover, these expression levels were comparable with expression levels observed in post-AAI rAMs (Fig E7, A), thus confirming the previously reported alternative activation of post-AAI rAMs.^{E2} We next verified to what extent both rAM populations exhibited a similar inflammatory response to RSV infection. To this end, naive rAMs pretreated with IL-4 and post-AAI rAMs

were cultured for another 24 to 48 hours in the presence or absence of RSV. Strikingly, alternatively activated naive rAMs showed IL-6 and TNF secretion levels well below the levels observed with post-AAI rAMs and comparable with the levels of untreated naive rAMs (Fig E7, B). The differential responsiveness of alternatively activated naive and post-AAI rAMs to RSV infection was also observed when quantifying p65 nuclear translocation. Compared with alternatively activated or untreated naive rAMs, post-AAI rAMs exhibited significantly increased nuclear p65 levels (Fig E7, C). Together, these data indicate that alternative activation of rAMs by IL-4 does not mimic the increased inflammatory response to RSV of post-AAI rAMs.