



ORIGINAL ARTICLE

Asthma and Lower Airway Disease

Eosinophils capture viruses, a capacity that is defective in asthma

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Abstract

Background: Activated eosinophils cause major pathology in stable and exacerbating asthma; however, they can also display protective properties like an extracellular antiviral activity. Initial murine studies led us to further explore a potential intracellular antiviral activity by eosinophils.

Methods: To follow eosinophil-virus interaction, respiratory syncytial virus (RSV) and influenza virus were labeled with a fluorescent lipophilic dye (DiD). Interactions with eosinophils were visualized by confocal microscopy, electron microscopy, and flow cytometry. Eosinophil activation was assessed by both flow cytometry and ELISA. In a separate study, eosinophils were depleted in asthma patients using anti-IL-5 (mepolizumab), followed by a challenge with rhinovirus-16 (RV16).

Results: DiD-RSV and DiD-influenza rapidly adhered to human eosinophils and were internalized and inactivated (95% in ≤ 2 hours) as reflected by a reduced replication in epithelial cells. The capacity of eosinophils to capture virus was reduced up to 75% with increasing severity of asthma. Eosinophils were activated by virus in vitro and in vivo. In vivo this correlated with virus-induced loss of asthma control.

Conclusions: This previously unrecognized and in asthma attenuated antiviral property provides a new perspective to eosinophils in asthma. This is indicative of an imbalance between protective and cytotoxic properties by eosinophils that may underlie asthma exacerbations.

KEYWORDS

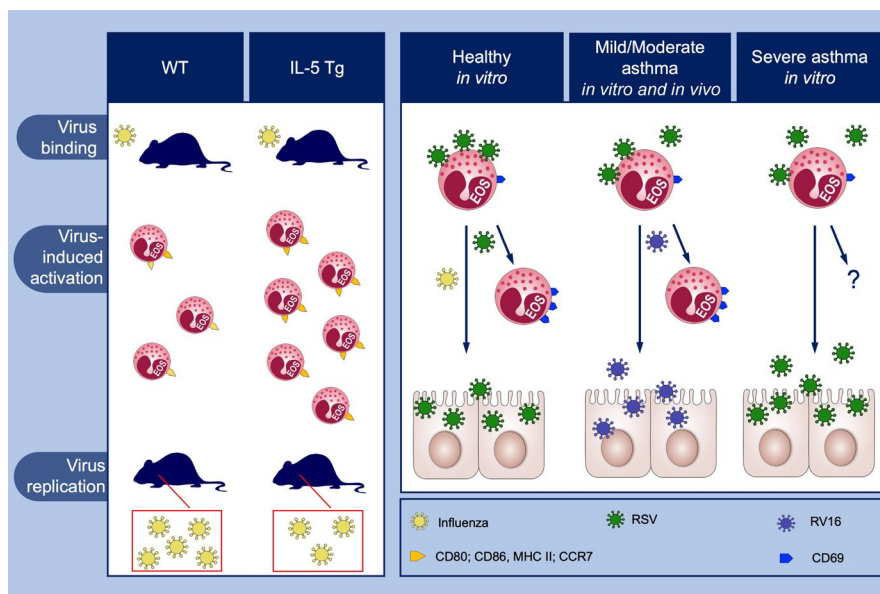
CD69, exacerbation, influenza, rhinovirus_16, RSV

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GRAPHICAL ABSTRACT

1,19 – dioctadecyl - 3, 3, 39, 39 – tetramethylindocarbocyanine (DiD)-respiratory syncytial virus and DiD-influenza adhered to human eosinophils. Both viruses were internalized and inactivated by eosinophils. The capacity of eosinophils to capture virus was reduced with increasing severity of asthma. IL-5 Tg: IL-5 transgenic; RSV: Respiratory syncytial virus; WT: Wild-type

1 | INTRODUCTION

Asthma is a chronic but heterogeneous inflammatory airway disease characterized by episodes of variable symptoms like wheezing, coughing, chest tightness, and shortness of breath.¹ Patients can experience periods of acute worsening of their asthma symptoms (exacerbations), which are predominantly triggered by respiratory viral infections, and to a lesser extent by allergens and air pollution.^{2,3} Exacerbations are paralleled by increased airway inflammation. These symptoms severely impact the patient's quality of life and even are life-threatening in severe asthma. Corticosteroids, the cornerstone in anti-inflammatory treatment of asthma, are not always effective during exacerbations and in severe asthma, despite enhanced doses. Therefore, there is a need for specific interventions tailored to the underlying inflammatory process. This was illustrated by the recent finding that eosinophil depletion by monoclonal anti-IL-5 antibody (eg, mepolizumab) treatment in moderate to severe eosinophilic asthma attenuated corticosteroid use and reduced exacerbations,^{4,5} the latter of which was also shown for treatment with anti-IL-5 receptor antibody.⁶

Airway eosinophilia is a hallmark of allergic asthma and of severe nonatopic late-onset asthma. It has been associated with both allergen- and respiratory virus-induced asthma exacerbations, airway hyperresponsiveness, and remodeling.⁷ In asthma, eosinophils are considered predominantly pro-inflammatory and contributing to tissue damage by the release of specific cytotoxic granular constituents and reactive oxygen species.^{8,9} However, earlier and recent studies have provided further support to earlier claims that eosinophils can also display protective regulatory functions in asthma.^{13,14} Eosinophils have been found to accumulate in airways of asthma patients during virus-induced exacerbations suggesting that

eosinophils may also be actively involved in the antiviral response. Extracellular eosinophil-derived neurotoxin (EDN) displays RNase activity and can enter viral capsids to degrade RNA from respiratory syncytial virus (RSV).^{13,16,17} In vivo relevance of this activity was shown in eotaxin2/IL-5 double transgenic (tg) mice that were protected against a lethal pneumonia virus infection.¹⁶ Apparently in line with these findings, we recently found that anti-IL-5-mediated depletion of eosinophils resulted in enhanced influenza X31 viral loads in house dust mite-sensitized mice, whereas the enhanced morbidity was reduced.²⁰

These initial findings urged us to further study the antiviral activity of eosinophils. Here, we show that mouse and human eosinophils efficiently capture and rapidly reduce infectivity of respiratory viruses. In line herewith, we²¹ showed that depletion of eosinophils by anti-IL-5 treatment significantly enhanced viral loads in airways from asthma patients challenged with rhinovirus 16 (RV16). Intriguingly, we here found that DiD-labeled virus interacted less with eosinophils from asthma patients compared to those from healthy controls, which may lead to a less effective virus inactivation. These findings support a newly recognized antiviral activity of eosinophils and place the role of eosinophils during virus-induced asthma exacerbations in a new perspective.

2 | RESULTS

2.1 | Murine lung eosinophils capture influenza virus and are activated *in vivo*

In a mouse model of eosinophilia (IL-5 transgenic (tg) mice), recovery after infection with a nonlethal dose of influenza (PR8) was faster

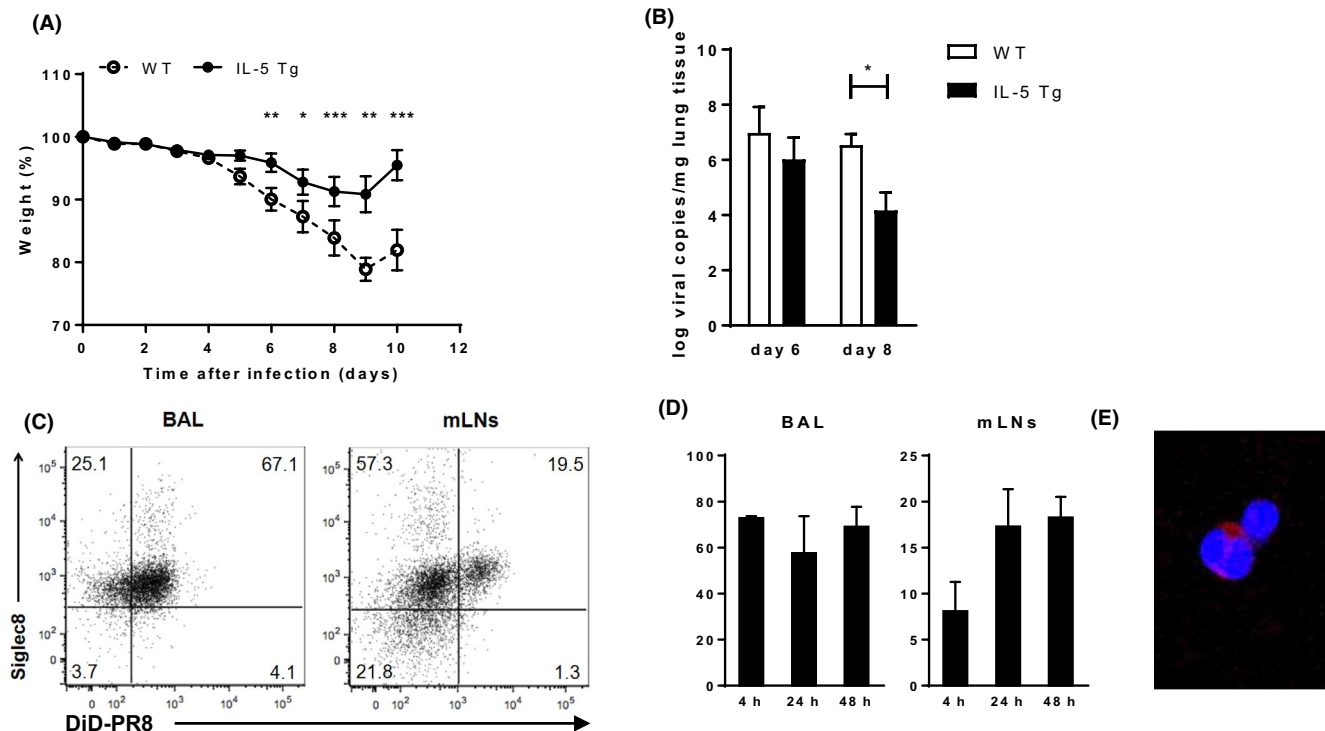


FIGURE 1 Reduced virus-induced pathology in mice with eosinophilia. A, Weight loss of IL-5 tg mice and controls after a PR8 infection. B, Viral load in the lungs at day 8 after PR8 infection. C, Representative flow cytometry plots of DiD-labeled PR8 uptake by BALF and mLN eosinophils from IL-5 tg mice. D, Quantification of DiD-labeled PR8 in BALF (left panel) and mLN (right panel). E, Representative confocal microscopy image of DiD-labeled PR8 virus with BALF eosinophils. Data are representative of 2 experiments with 3 (C-E) mice each or combined from 2 experiments with 2-12 mice (A,B) and expressed as mean $\pm$ SEM.: 2-way ANOVA (for A) or normal t test (B); * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

as compared to controls (WT), as was apparent from the reduced weight loss, a surrogate marker of inflammation (Figure 1A). In concordance, the pulmonary viral load at day 8, but not yet at the viral peak at day 6, was significantly lower than in WT mice (Figure 1B). IL-5 tg mice have elevated blood and bronchoalveolar lavage (BAL) eosinophils at homeostasis, and upon infection, the eosinophils were recruited to the airways (Figure S1A). The BAL inflammatory profile upon influenza infection did not differ with respect to the recruitment of other immune cells (Figure S1B). By using DiD-labeled PR8,²² purified by density gradient centrifugation, we traced virus not only to alveolar macrophages ($CD11c^{high} CD11b^{low} SigIF^{+}$) as was expected, but also to eosinophils ($F4/80^{+} CD11c^{-} CD11b^{+} SigIF^{+}$ cells) in BAL and draining mediastinal lymph nodes (mLN) of IL-5 tg mice (Figure 1C). The differentiation between alveolar macrophages and eosinophils is shown in Figure S1D. Within 4 hours after infection, more than 60% of the BAL eosinophils were associated with DiD-labeled PR8 (Figure 1D), as was confirmed by confocal microscopy (Figure 1E). Part of these PR8⁺ eosinophils migrated to the draining mLN (Figure 1D). Both BALF and mLN PR8⁺ eosinophils showed elevated expression of CD86, CD80 (only mLN), MHCII, and CCR7 compared to their PR8⁻ counterpart (Figure S1C). So besides their potential extracellular antiviral activity, these data indicate that murine pulmonary eosinophils can also capture virus in vivo and become activated.

2.2 | Rapid capture and inactivation of respiratory viruses by human eosinophils

Next, we extended these findings to human cells by exposing blood eosinophils from healthy donors to DiD-labeled respiratory syncytial virus (RSV) and influenza (PR8) in vitro. After 2 hours coculture, for both viruses, three distinct staining patterns were observed by confocal microscopy: (a) those showing separate viral particles spread across the eosinophil membrane (diffuse staining), (b) those with predominantly clustered virus (clustered staining), and (c) few cells without virus (Figure 2A,B). Similar staining patterns were found for BAL eosinophils (Figure 2C). DiD labeling of virus itself did not lead to aggregation of the virus as shown by confocal microscopy, indicating that the viral clusters on the eosinophils result upon interaction with virus.

After 25 minutes, about 70% of blood eosinophils were DiD-positive, predominantly diffuse staining (Figure 2D). After 90 minutes, nearly all eosinophils were DiD-positive, either diffusely or clustered, to an equal extent. At 120 minutes, most eosinophils were DiD-positive with the majority showing diffuse staining. The number of viral particles (DiD label) associated per eosinophil over time increased as shown by increased mean fluorescence intensity, although this varies per donor (Figure 2E).

To exclude that association of DiD-labeled virus by eosinophils is part of productive infection, eosinophils were incubated with

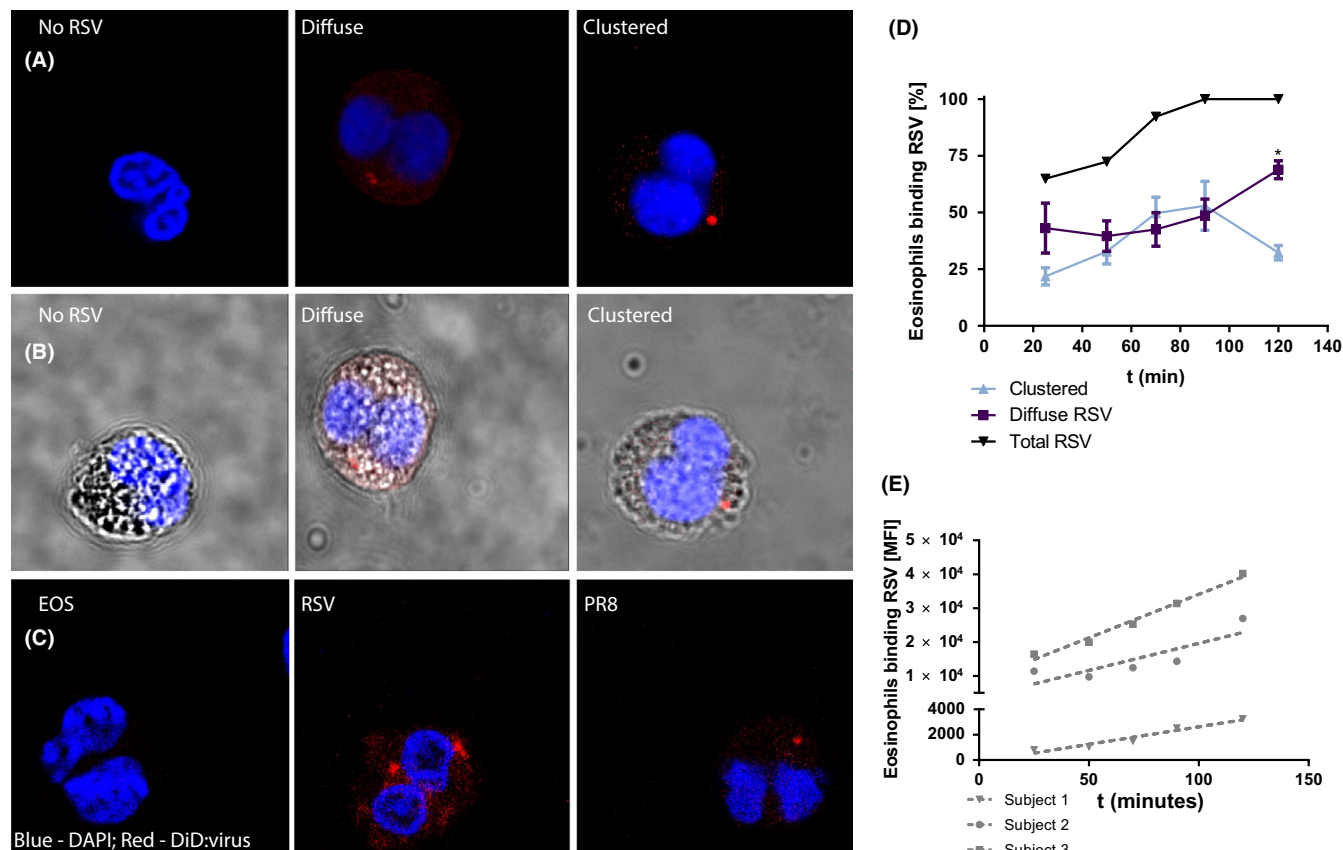


FIGURE 2 Rapid capture and inactivation of virus by human eosinophils. A, Representative confocal microscopy images of peripheral blood eosinophils incubated with DiD-labeled RSV (red) for 2 h. B, Bright-field images corresponding to (A). C, BALF eosinophils co-incubated with either DiD-labeled RSV or PR8 for 2 h. D, Patterns of virus associated with eosinophils over time, assessed by confocal microscopy ($n = 4$). E, RSV+ eosinophils over time, assessed by flow cytometry (G,H: $n = 3$). RSV (red), influenza (pink), heat-inactivated RSV (black) binding at 37°C and at 4°C (blue) to both peripheral blood (F,H: $n = 6$) and BALF eosinophils (g and i: $n = 2$). J, RSV load in epithelial cells 24 h after exposure to lysates from eosinophils exposed for 2 h to RSV (Healthy). RSV only is RSV added directly to the epithelial cells ($n = 9$). Ct value of 27.89 (5000 c/PCR) refers to a virus concentration of 6250 c/mL. Data are expressed as mean $\pm$ SEM. Paired t test: * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$

heat-inactivated DiD-labeled RSV (HI:RSV) or GFP-tagged RSV, which expresses GFP upon viral replication.²³ Eosinophils and HI:RSV adhered (Figure 2F) to a similar extent as viable RSV, indicative of uptake instead of infection. Also, RSV-GFP, which infects and replicates in epithelial cells, did not replicate in eosinophils (Figure S1). Furthermore, the capacity of RSV to infect epithelial cells was reduced by 95% when RSV was incubated with eosinophils for 2 hours (Figure 2J). Together this indicates that eosinophils rapidly capture viral particles and effectively reduce their infectivity.

Imaging of RSV-exposed eosinophils by electron microscopy revealed viral particles with the typical RSV structure and the approximate size (120–300 nm) within eosinophils, but only in the cytoplasm and not enclosed by a membrane (Figure 3). Combined these findings confirm uptake of viral particles by eosinophils.

2.3 | Eosinophils from asthma patients capture less DiD-labeled virus

As respiratory viral infections in asthma patients trigger acute worsening of asthma symptoms, we set out to determine how the

antiviral properties of eosinophils from asthma patients compared to those from healthy controls. Strikingly, blood eosinophils from mild to severe asthma patients (Table S1) displayed reduced levels of DiD-labeled RSV. Whereas more than 85% of eosinophils from healthy donors bound RSV, eosinophils from mild to moderate asthmatics could be divided into two subgroups: either binding identical to those from controls or up to a 40% reduced binding (Figure 4A). Interestingly, eosinophils showing binding similar to those from controls were all derived from patients using inhaled corticosteroids. In severe asthmatics, all on either high doses of inhaled corticosteroids or oral corticosteroids, a further reduced capacity by eosinophils to bind virus was observed. In some donors, only 20% of eosinophils bound RSV while others showed a moderate reduced binding capacity compared to that of healthy controls. To clarify whether eosinophils from asthma patients also display an aberrant capacity to reduce infectivity of virus, epithelial cells were exposed to a cell lysate of eosinophils from severe asthmatics and healthy controls exposed for 2 hours to RSV. Eosinophils from healthy controls reduced RSV infectivity (Figure 4B; $P < 0.0001$), and possibly slightly less by eosinophils

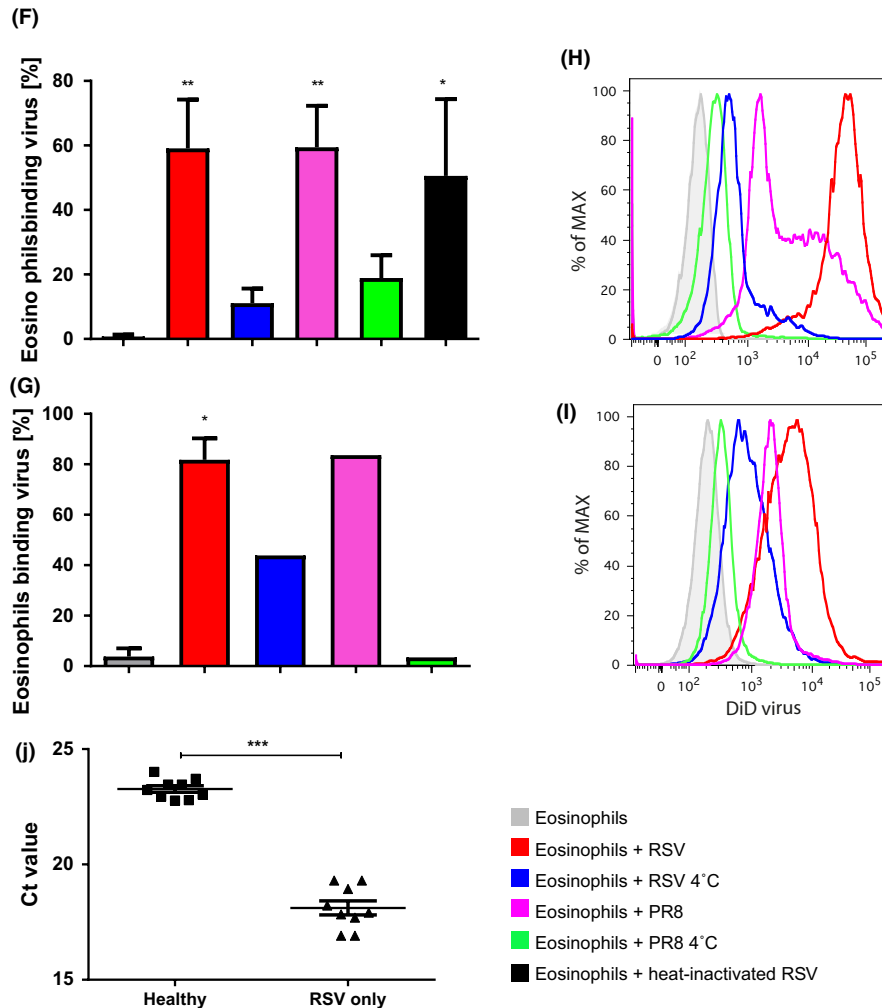


FIGURE 2 Continued.

from patients, as compared to epithelial cells directly infected with RSV (RSV only). In our analysis, we also considered that some severe asthma patients used oral corticosteroids ($n = 4$) ($n = 3$ for virus infectivity) or only inhaled corticosteroids ($n = 5$) ($n = 4$ for virus infectivity). We stratified the severe group by OCS use, and we examined a significant difference between severe asthmatics without OCS (–OCS) and healthy individuals (Figure S5A), but we do not see this in severe asthmatics on OCS (+OCS) and healthy subjects. There is also no difference between severe asthmatics (total group) and healthy subjects. Virus binding, however, is independent of OCS use (Figure S5B).

Together this indicates that eosinophils from asthma patients bind less RSV and may have a reduced capacity to inactivate viruses as compared to eosinophils from healthy individuals.

2.4 | Eosinophils are activated by virus interaction

Exposure of human blood eosinophils for 2 hours to RSV as well as to PR8 significantly enhanced expression of CD69 and trend wise upregulated CD11b expression. CD66b and CD62L expression were unaffected by viral exposure (Figure 5A). Eosinophils can synthesize

mediators de novo as well as release preformed mediators selectively.²⁴ Within 2 hours, RSV and PR8 increased IL-8 secretion by eosinophils (Figure 5B), whereas IL-6 secretion was increased by PR8, but not by RSV. Neither virus induced the release of eosinophil cationic protein (ECP) (Figure 5B). Together this suggests that eosinophils exposed to virus can drive subsequent inflammatory responses in a virus-specific manner.

2.5 | CD69 correlates to FEV₁ in RV-16-induced loss of asthma control

To extend these in vitro findings, in a separate study, mild to moderate asthmatics (patient characteristics in Table S2) receiving eosinophil-depleting mepolizumab (anti-IL-5) or placebo were challenged with low-dose (10TCID₅₀) RV16.²¹ In this experimental exacerbation model, loss of asthma control as monitored by forced expiratory volume in 1 second (FEV₁) started at 4–5 days after RV16 exposure, peaked around day 7, and was paralleled by increased airway inflammation.²⁵ CD69 expression by eosinophils recovered from BALF of patients receiving placebo or mepolizumab at day 7 after RV16 exposure strongly correlated inversely with FEV₁ %pred (relative

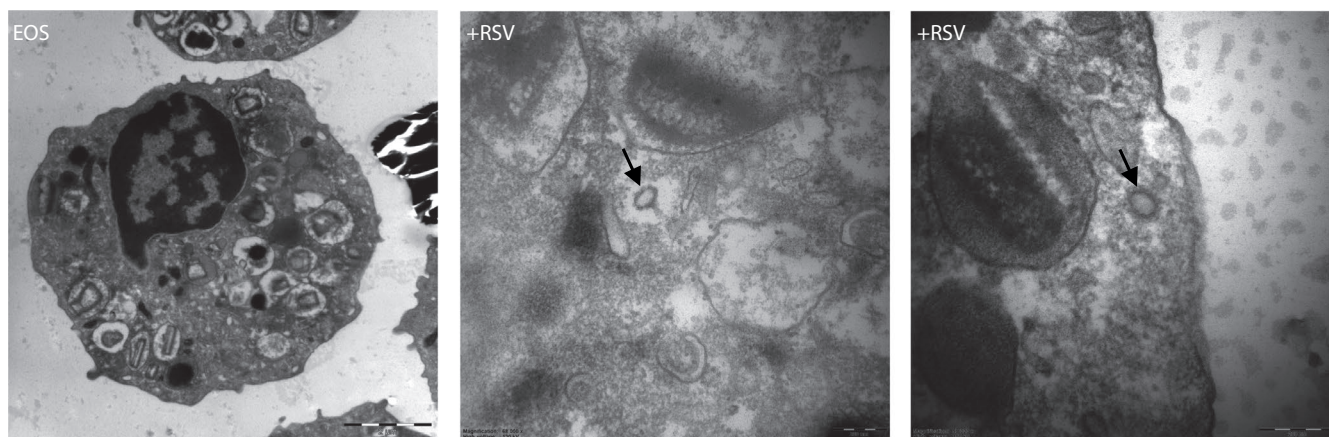


FIGURE 3 Representative EM images of eosinophils incubated with RSV (MOI 10). Intact RSV particles were found free in the cytoplasm but never surrounded by a plasma membrane (left panel)

to predicted FEV_1 ; Figure 6A right panel). This relationship was not found at day 0 (before RV16 was given, Figure 6A left panel), indicating that CD69 expression by eosinophils in RV16-challenged asthma patients relates to the activation of eosinophils by RV16. Interestingly, eosinophils from patients treated with mepolizumab showed no significant increase in CD69 expression after RV16 exposure (Figure 6B).

3 | DISCUSSION

Eosinophils exert prominent cytotoxic properties that can harm the respiratory mucosa and attenuate lung function in stable and exacerbating asthma.^{13,26} In the present study, however, we provide *ex vivo* and *in vivo* evidence that murine and human eosinophils can rapidly capture various respiratory viruses and significantly reduce infectivity of these viruses. Eosinophils that bound virus became activated, as reflected by altered expression of cell surface molecules like CD69 and the release of pro-inflammatory cytokines such as IL-6

and IL-8. In line herewith, RV16-induced loss of asthma control in patients revealed a strong correlation with CD69 expression by eosinophils. Intriguingly, eosinophils from asthma patients displayed a reduced capacity to bind virus.

Together this indicates that human eosinophils may be important scavengers of virus at the respiratory mucosa *in vivo*, preventing further viral propagation. With the murine allergic house dust mite model for severe asthma exacerbations, we showed that depletion of eosinophils using the anti-IL-5 antibody TRFK5 resulted in enhanced viral titers.²⁰ Similarly, in our human study, depletion of eosinophils followed by a RV16 challenge resulted in enhanced viral titers.²¹ These studies underline the *in vivo* antiviral activity of eosinophils and are in accordance with earlier murine studies showing that the number of eosinophils influenced killing of pneumonia virus of mice (PVM), although the mechanisms proposed were distinct from that shown here.^{16,18,19,27} Electron microscopy of eosinophils incubated with virus showed intact viral particles in the cytoplasm, but we failed to detect viral particles enclosed by a membrane, which would have supported the concept of intracellular inactivation of viral particles. During the infectious cycle,

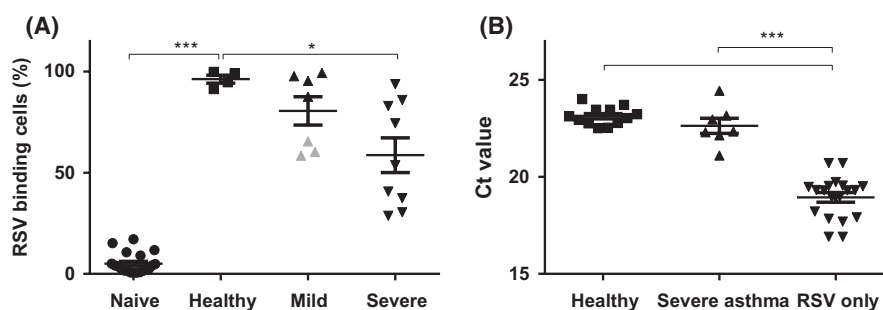


FIGURE 4 Eosinophils from asthma patients have a reduced capacity to bind and inactivate virus, but still inactivate virus *in vivo* (A). DiD-labeled RSV determined by flow cytometry. Eosinophils without exposure to DiD-labeled RSV (Naive), and after exposure to DiD-labeled virus for eosinophils from 4 healthy (Healthy), 8 mild to moderate asthmatics (Mild asthma) and 9 severe asthmatics (Severe asthma) for 16 h. Eosinophils from mild asthma patients not on ICS are depicted in gray. B, Infectious RSV (depicted by Ct-values) derived from lysates from RSV co-incubated with eosinophils, reflected by 24-h replication on epithelial cells. RSV only is RSV added directly to the epithelial cells ($n = 16$). Healthy refers to lysates from eosinophils from healthy donors ($n = 4$) and Severe asthma to those from severe asthma patients ($n = 7$). Ct value of 27.89 (5000 c/PCR) refers to a virus concentration of 6250 c/mL. Data are expressed as mean $\pm$ SEM. Paired or unpaired *t* test * $P < 0.02$, *** $P \leq 0.0001$

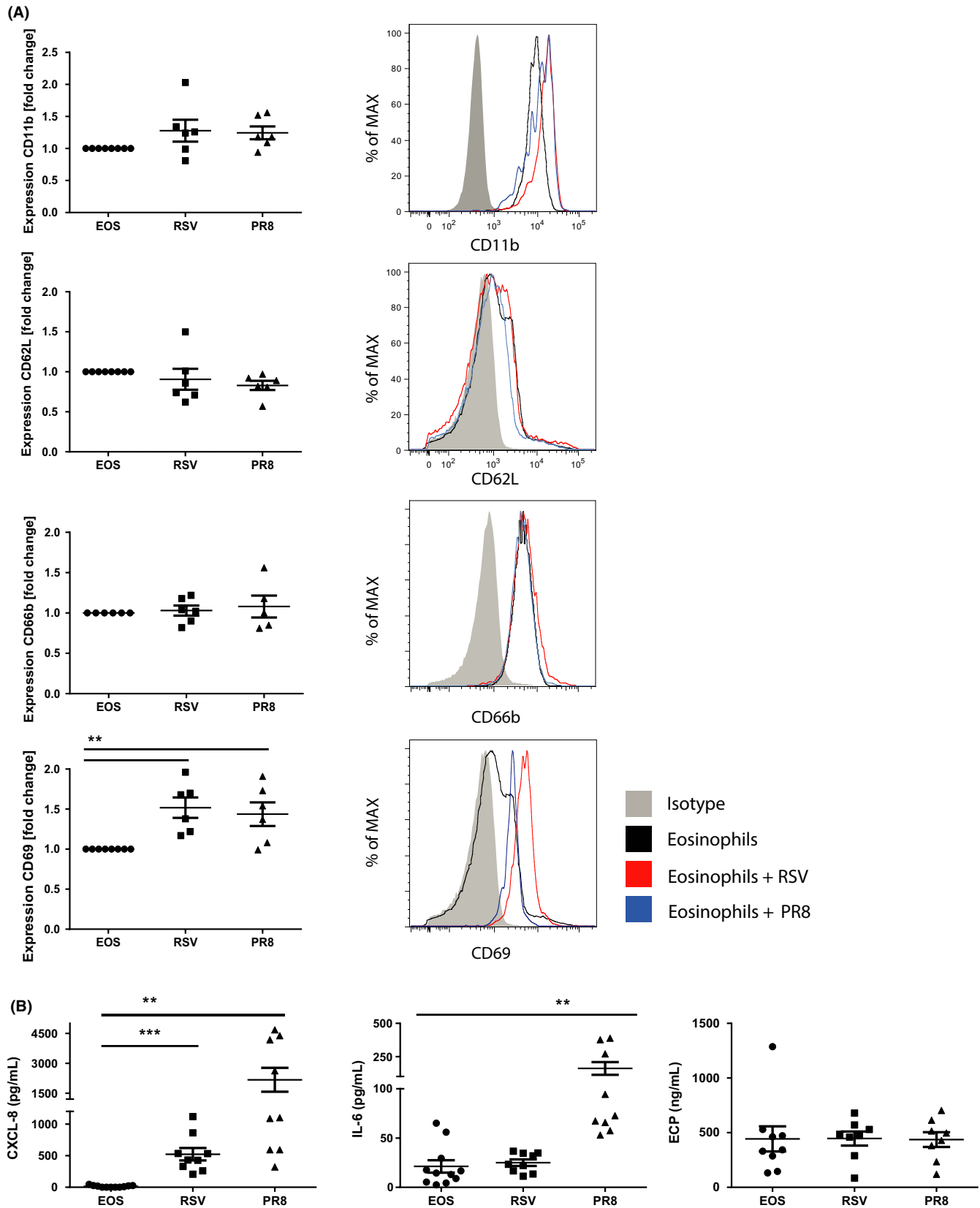


FIGURE 5 Eosinophils are activated upon exposure to virus in vitro (A). CD11b, CD62L, CD66b, and CD69 expression on blood eosinophils from healthy subjects after 2-h incubation with DiD-labeled RSV or influenza; gray, isotype-matched antibodies ($n = 6$). B, IL-8, IL-6, and ECP secretion of blood eosinophils from healthy subjects after 2-h incubation with RSV (MOI 10) or PR8 (MOI 2) ($n = 9$). Data are expressed as mean $\pm$ SEM. Paired t test: ** $P < 0.01$; *** $P < 0.001$

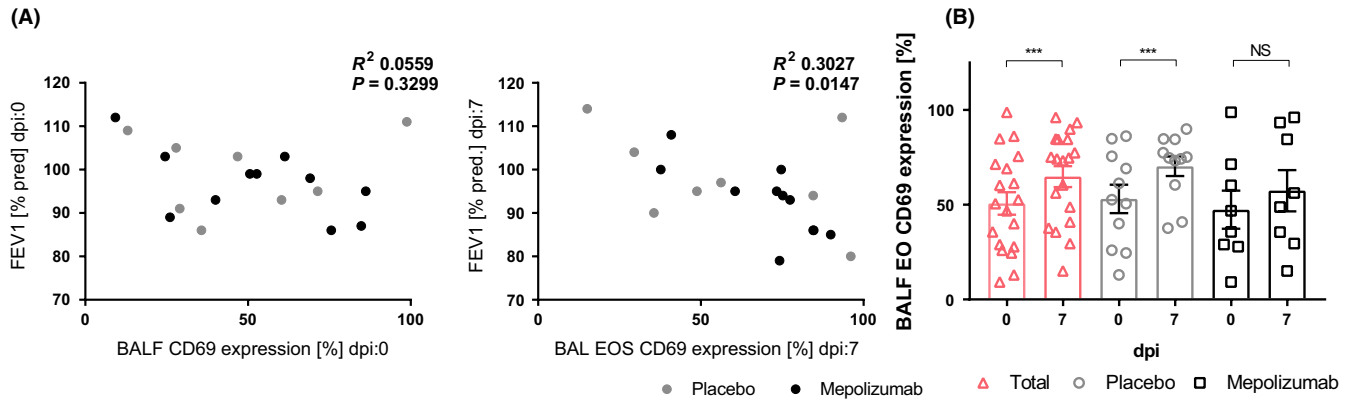


FIGURE 6 Eosinophils are activated upon exposure to virus in vivo (A). FEV₁ of asthma patients before RV16 challenge does not correlate (left) with CD69 expression on BALF eosinophils, whereas they correlate after RV16 challenge (right) (placebo- and mepolizumab-treated groups (Pearson r : -0.55 ; $P = 0.01$) and for placebo-treated group alone (Pearson r : -0.74 ; $P < 0.01$)). (b) After RV16 exposure in vivo, eosinophils become more activated (all mild asthmatics (pink; $n = 19$), placebo (gray; $n = 11$) and mepolizumab (black; $n = 8$)). Data are expressed as mean $\pm$ SEM. Paired t test or Pearson correlation: *** $P < 0.001$

RSV fuses with the plasma membrane,^{28,29} and thus, the presence of intact viral particles is indicative of viral uptake. Elucidation of the precise mechanisms by which viruses are inactivated by eosinophils, however, awaits further studies. Like eosinophils, macrophages also serve as a sink for virus; however, contrary to our findings for eosinophils, this involves an infectious cycle.^{30,31}

Recently, in a murine study, CD101-low eosinophils residing in lung tissue with a regulatory signature were distinguished from inflammatory CD101-high eosinophils, which typically are recruited into the lung.¹⁵ There are some indications that both eosinophil types exist in humans. In the present study, we did not distinguish between either types, although our findings with human eosinophils relate to circulating and recruited eosinophils and thus likely inflammatory eosinophils. In another study, we reported that CD101+ eosinophil influx in HDM-sensitized mice was boosted in response to house dust mite (HDM) and further increased by influenza infection.³² Interestingly, blocking IL-33 signaling, which drives influenza-induced exacerbation in HDM-sensitized mice, resulted in a significant reduction of CD86+ MHC-II+ CD101- eosinophils, but did not affect the inflammatory (CD101+) eosinophil population.³² As blocking of IL-33 signaling was paralleled by an increased clearance of virus, we consider it unlikely that resident eosinophils contributed to this. Although we did not distinguish between resident and inflammatory eosinophils in our anti-IL-5 studies,^{20,31} the increase in viral load upon anti-IL-5 treatment is again suggestive of an antiviral role for inflammatory eosinophils and not resident cells, as the presence of the latter appears independent of IL-5.¹⁵ Further studies, particularly in humans, however, are needed to clarify the role of resident eosinophils in the antiviral response.

As a consequence of viral binding in vitro, eosinophils become activated as reflected by increased expression of various activation markers and a fast release of mediators like IL-6 and IL-8, but not that of ECP. It is important to realize here that the DiD-labeled virus was purified on a density gradient, and thus, the activation of eosinophils is unlikely to be due to pro-inflammatory mediators released by cells

to produce the virus. Thus, eosinophils activated by virus can exert virus-specific immunomodulatory roles locally.³³ The IL-8 release can in part explain the influx of neutrophils during a viral infection. During a RSV infection in infants, neutrophilic inflammation is considered the most important cause of acute bronchiolitis.³⁴ At early time points of a RSV infection, particularly eosinophilic degranulation products are found,³⁵ which may emphasize the important role of eosinophils in RSV infections.

Also, the strong inverse correlation between CD69 expression by eosinophils and lung function (FEV₁) in asthma patients 7 days after a challenge with RV16 is in line with virus-induced activation of eosinophils and the cytotoxic properties of eosinophils in asthma (6-8). Although in our in vitro studies eosinophils failed to release ECP upon activation by viruses, it is possible that in an in vivo setting ECP is released. In our RV16 challenge study in asthma patients,²¹ we found an increase in ECP although it did not reach significance. Along these lines, it is possible that in more severe patients and with a more virulent virus, the release of ECP is more prominent. This is important as ECP has ribonuclease activity, which may inactivate viruses, but on the other hand, ECP also exerts cytotoxic, neurotoxic, fibrosis-promoting, airway-inflammatory, and immune-regulatory functions.^{36,37} Besides the activation of eosinophils, the murine studies indicate that after viral exposure, eosinophils migrated to the draining lymph nodes and increased expression of MHCII and co-stimulatory molecules. This points to an additional role for eosinophils in antigen presentation, as has been proposed earlier.⁴⁰

It is known that eosinophils in asthma patients are primed, for example, by IL-5.⁴¹ This could lead to a more pronounced virus-induced activation of eosinophils in asthma patients and to the release of ECP and other granular proteins. In line herewith, CD69 expression on eosinophils did not increase in patients treated with mepolizumab. Remarkably, eosinophils from asthma patients displayed a reduced capacity to bind and inactivate virus, which was particularly evident in patients with severe asthma. So, on the one hand, eosinophils in asthma patients may be less capable of clearing virus, whereas on

the other hand, virus may more profoundly activate eosinophils, because of their primed state. Therefore, both the number of eosinophils and their activation state control their antiviral response. As eosinophils from asthma patients are already activated in the circulation, this could explain their reduced capacity to bind viruses. Interestingly, mild to moderate asthma patients on corticosteroids, which attenuates eosinophil activation, had a higher antiviral activity, in support of the previous.⁴² The effect of corticosteroids may relate to inhibition of mediators that activate eosinophils but also to direct effects on eosinophils, such as by stabilizing the eosinophil's cell membrane.⁴³ In the reported studies, we used predominantly PR8 and RSV to show the direct interaction between virus and eosinophils, whereas we used RV16 in the human challenge model. Although the apparent uniform interaction of RSV and PR8 with eosinophils may indicate a general mechanism applicable also to RV16, we have not been able to test that. RV16 is known to rapidly inactivate after thawing, which is incompatible with DiD labeling of RV16. Alternatively, we could not use another virus than RV16 in our human challenge model and so there is uncertainty whether RV16 is actually captured by eosinophils. However, exposure to RV16 resulted in eosinophil activation and, upon depletion of eosinophils, in enhanced viral titers,²¹ all in line with findings for RSV and PR8.

In summary, our data show that eosinophils are capable of rapid capture and inactivation of virus, which has been so far an unrecognized property of eosinophils. In addition, we found that eosinophils particularly from severe asthma patients are defective in these properties, which may lead to enhanced viral loads. These reduced antiviral and enhanced cytotoxic properties of eosinophils from asthma patients likely underlie, at least in part, the pathogenesis of virus-induced asthma exacerbations. These findings also lead to a new perspective of therapeutic benefits in reducing eosinophils in the airway. Novel therapeutics like anti-IL-5 and IL-5R are currently aimed at reducing eosinophils and eosinophil cytotoxic compounds, however, consequently also reduce the potential protective properties of eosinophils. Whether the current dosing (100 mg s.c.), which differs from what we used here (750 mg i.v.), and whether long-term treatment with anti-IL5 or anti-IL-5R has additional effects are currently unknown. Based on the apparent maintenance of the antiviral properties of eosinophils from mild to moderate asthma patients by corticosteroids, we speculate that tapering anti-IL-5 and IL-5R treatments to limit activation of eosinophils, rather than eradicate eosinophils, may lead to even better clinical results.

4 | METHODS

4.1 | General methods

4.1.1 | Virus and cells

Influenza strain A/PR/8/34, RSV-A2 (a kind gift from AIMM therapeutics), and GFP-RSV (a kind gift from Dr Mark Peepels (Nationwide Children's Hospital, Columbus, Ohio, USA) and Dr Peter Collins (NIH, USA) were used. RSV was propagated in HE-P2 cells in IMDM (Lonza)

culture medium supplemented with 1% FCS and influenza in NCI-H292 cells in RPMI-1640 with 1% FCS. At day 1-3 postinfection, when cytopathic effects were observed, the supernatant was harvested. Cell debris was removed by centrifugation at 3000 g for 10 minutes, and the supernatant was snap frozen and stored at -80°C (Figure S4).

4.1.2 | DiD labeling of virus

1,19 - dioctadecyl - 3, 3, 39, 39 - tetramethylindocarbocyanine (DiD) (Molecular Probes, Invitrogen) was dissolved in DMSO at a concentration of 20 mg/mL and used to label influenza A/PR/8/34 or RSV-A2. Influenza and RSV were incubated at room temperature for 30 minutes with 10 µL or 2 µL DiD, respectively, followed by density gradient centrifugation to obtain purified labeled virus, essentially as described elsewhere.²² DiD labeling varied between preparations, and so for comparisons, a single batch of DiD-labeled virus was used. Direct DiD staining of eosinophils yielded homogeneous staining of the cell membrane, distinct from the staining seen upon exposure to DiD-labeled virus.

4.1.3 | Confocal microscopy

Coverslips were precoated with 50 µg/mL poly-L-lysine (Life technologies) for 2 hours at 37°C. Eosinophils (2.5×10^4) were seeded onto the precoated coverslips and fixated for 5 minutes in 3.7% paraformaldehyde. These were either immediately mounted with ProLong Gold (life technologies) anti-fade reagent with DAPI (nuclear staining). Immunofluorescence images were made using the Confocal Microscope SP-8 X SMD (Leica Microsystems) with a 63x/1.30 Oil CS2 objective.

4.1.4 | Electron microscopy

Eosinophils were fixed in 1% glutaraldehyde and 4% PFA in 0.1 mol/L sodium cacodylate buffer (McDowell fixative) and postfixed with 1% osmium tetroxide (OsO₄, Electron microscopy sciences) in cacodylate buffer. Samples were dehydrated in serial dilutions of alcohol and embedded into Epon (LX-112 resin Ladd research). Ultrathin Epon (50-85 nm) sections were collected on formvar-coated grids, counterstained with uranyl acetate and lead citrate, and visualized with transmission electron microscope (FEI Tecnai 12). For negative staining of RSV (Figure S3), the particles were allowed to adhere to carbon-coated grids, washed with PBS, and postfixed with 1% glutaraldehyde in PBS. Then, grids were washed with several drops of dH₂O and counterstained with uranyl acetate.

4.2 | Murine studies

4.2.1 | Animals

IL-5 transgenic mice (NJ.1638) were obtained from Drs. James and Nancy Lee (Mayo Clinic).⁴⁴ All animals (male; C57BL/6J; NJ.1638) were housed in standardized specific pathogen-free conditions.

4.2.2 | Influenza infection

IL-5 transgenic mice or negative littermates were infected in with 10 TCID₅₀ influenza A/PR/8/34 and killed after 2, 4, 6, 8, or 10 days. For visualization of virus, in separate experiments (Figure 1C-G), animals were infected with DiD-labeled influenza as previously described⁴⁵ and killed after 4, 24, or 48 hours.

All animal data are derived from at least two independent experiments with group sizes of 6-8 animals, except the infection with DiD-labeled influenza, which was performed twice with 3 animals per group.

4.3 | Human studies

4.3.1 | Rhinovirus 16 challenge in asthma patients under mepolizumab or placebo

This study was intended to clarify whether the attenuation of eosinophil numbers in asthma patients impacted the inflammatory response to a viral infection. To facilitate this, we aimed to not affect asthma pathophysiology otherwise than attenuating eosinophil numbers, as has been reported in detail in Ref.³¹ In a double-blind placebo-controlled two-armed trial, mild to moderate, steroid-naïve asthma patients, characteristics shown in Table S2, received one infusion containing 750 mg mepolizumab or placebo, which was known to attenuate eosinophil numbers for up to 4 weeks. Two weeks after infusion, patients were intranasally inoculated with 100 TCID₅₀ of rhinovirus type 16 to induce loss of asthma control (NCT01520051). Bronchoalveolar lavages (8 × 20 mL) were performed 0 days before and 7 days after RV16 infection with comparable recoveries. As the initial procedure for immunophenotyping of BALF cells was not satisfactory and we did not always obtain paired samples (before vs after rhinovirus challenge), we lost samples for analyses. For the specific analyses reported here, we only had complete data sets from eight patients on mepolizumab and 11 on placebo. This, however, did not result in a selection bias as there were no statistical significant differences between the placebo(P)- and mepolizumab(M)-treated groups as is shown in the Table S2, like there were no significant differences in the P- vs M-treated groups in table 1 in Ref.²¹

4.3.2 | DiD-labeled virus capture assay

Eosinophils used for this assay were isolated from peripheral blood from healthy, mild (different from those participating in the RV16 challenge study) to moderate and severe asthma patients, characteristics shown in Table S1 (described in more detail in Supplementary methods). Eosinophils were incubated with either A/PR/8/34 or RSV-A2 at a MOI of 2 and 10, respectively. Different conditions were used; the cells maintained in PBS supplemented with 1% BSA and were either incubated at 4°C or 37°C for 2 hours at 95% humidity and 5% CO₂.

4.3.3 | Virus degradation assay

Eosinophils were incubated with RSV-A2 at a MOI of 0.5, and the cells were incubated at 37°C for 2 hours at 95% humidity and 5% CO₂. Eosinophils were subsequently lysed by a three times freeze-thaw cycle and spun down for 5 minutes at 400 g. H292 cells were incubated with eosinophil lysates for 1 hour, and RNA is collected after 24 hours (see also Figure S4 for explanation of the study set-up). As a control, H292 cells were exposed directly to RSV-A2 at a MOI of 0.5. Virus titer was determined as described elsewhere.⁴⁶

4.4 | Statistics

Data from the RV16 challenge study for cell populations, cell activation markers, and also the asthma-related parameters were normally distributed and were expressed as mean ± SEM and analyzed using GraphPad Prism 6.0 software. The relevant statistical analyses for other analyses are described in legends to the figures. $P < 0.05$ was considered significant.

4.5 | Study approval

The Animal Care and Use Committee of the University of Amsterdam approved all experiments. The human study protocol was approved by the local ethics committee, and the participants provided written informed consent.

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CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

YSSP and SMB designed the study, did experiments, analyzed the data, and wrote the manuscript. AD, BSD, TD, EPH, LR, and DP did experiments and analyzed the data. CJM provided blood from patients and together with PIB performed the bronchoscopies. NNW analyzed data. LK and PJS contributed to the design of the study and wrote the manuscript. RL designed the study, analyzed the data, and wrote the manuscript.

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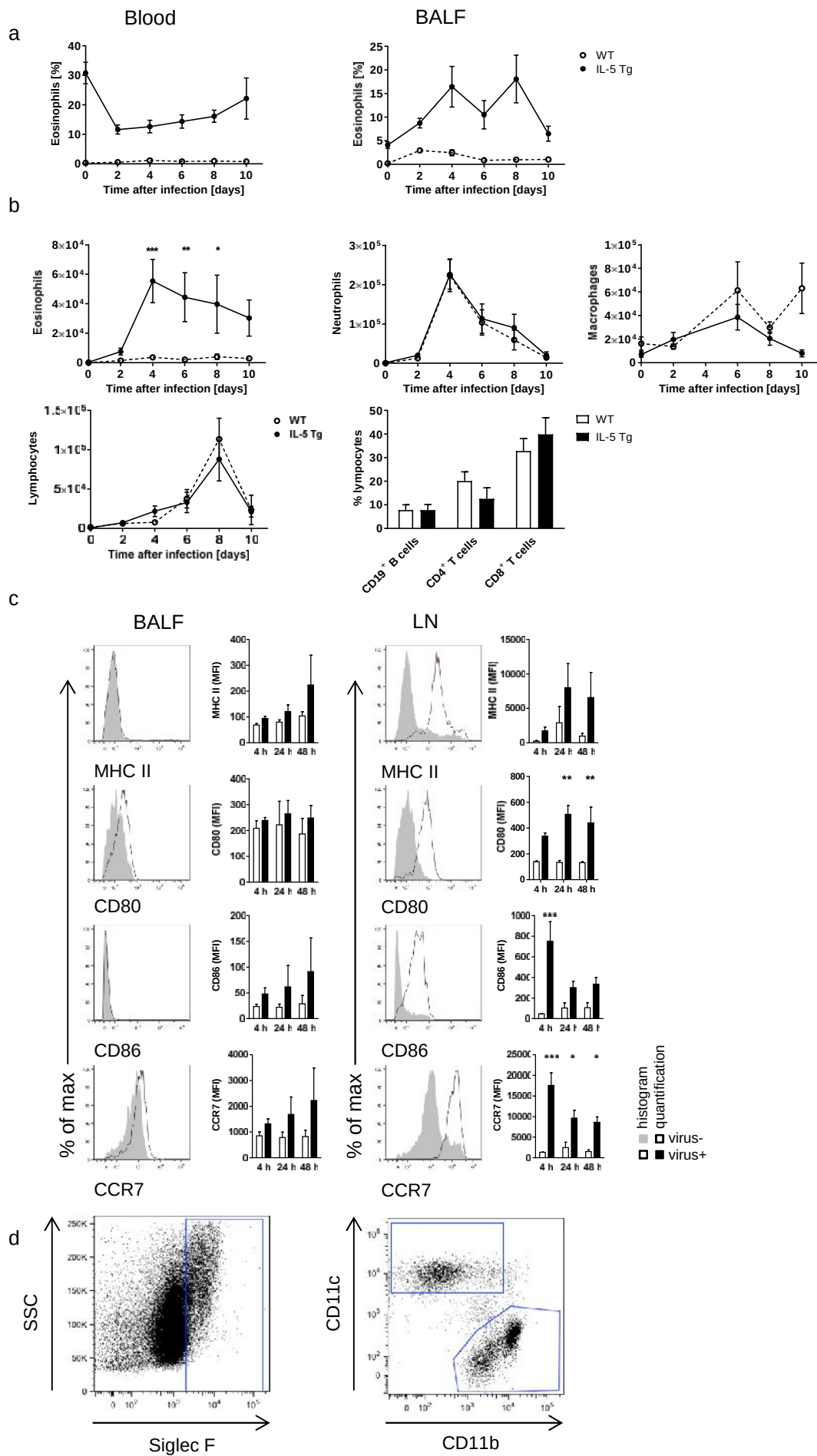
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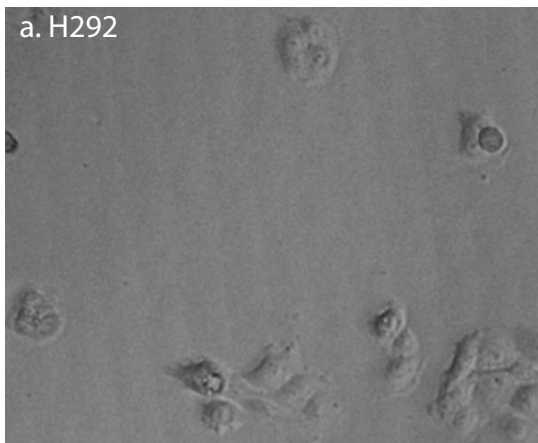
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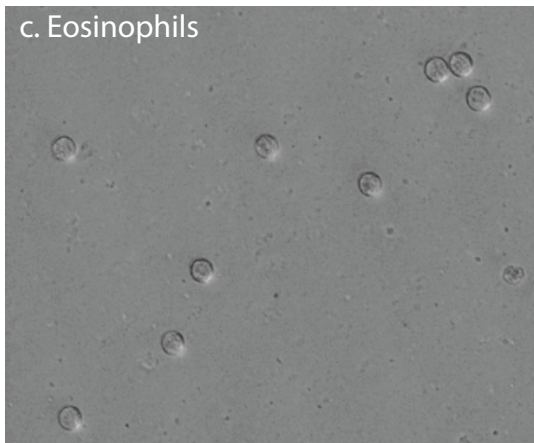
How to cite this article: Sabogal Piñeros YS, Bal SM, Dijkhuis A, et al. Eosinophils capture viruses, a capacity that is defective in asthma. *Allergy*. 2019;74:1898-1909. <https://doi.org/10.1111/all.13802>



a. H292



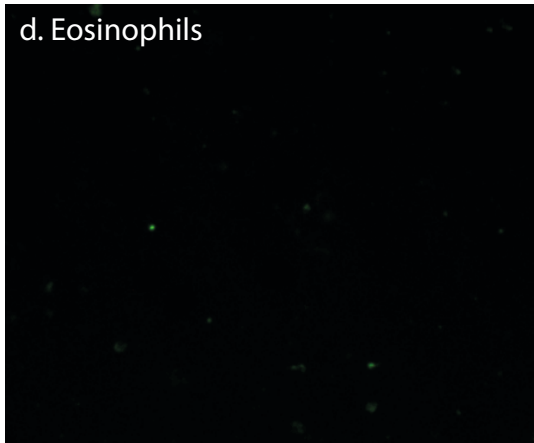
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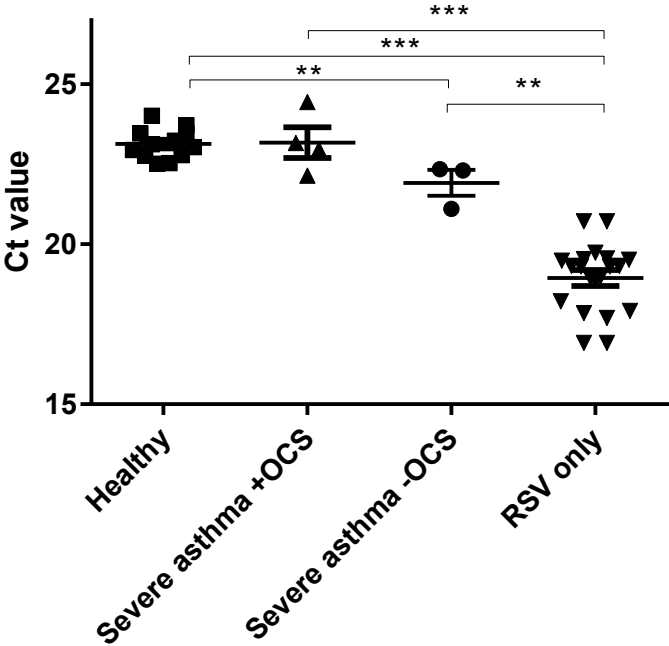
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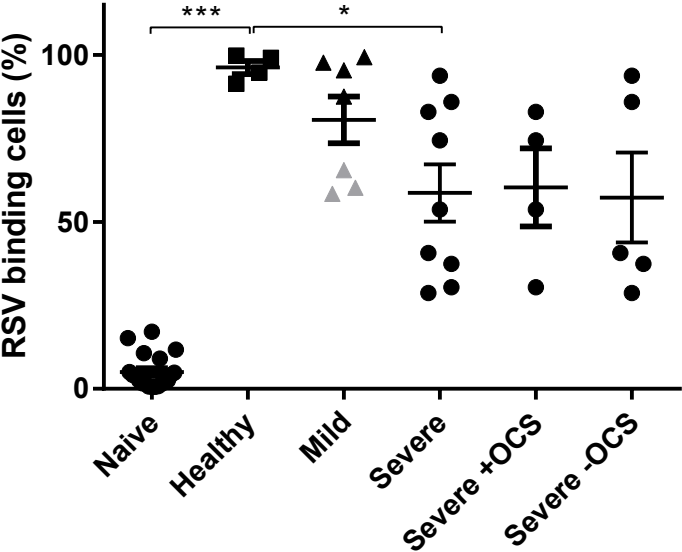
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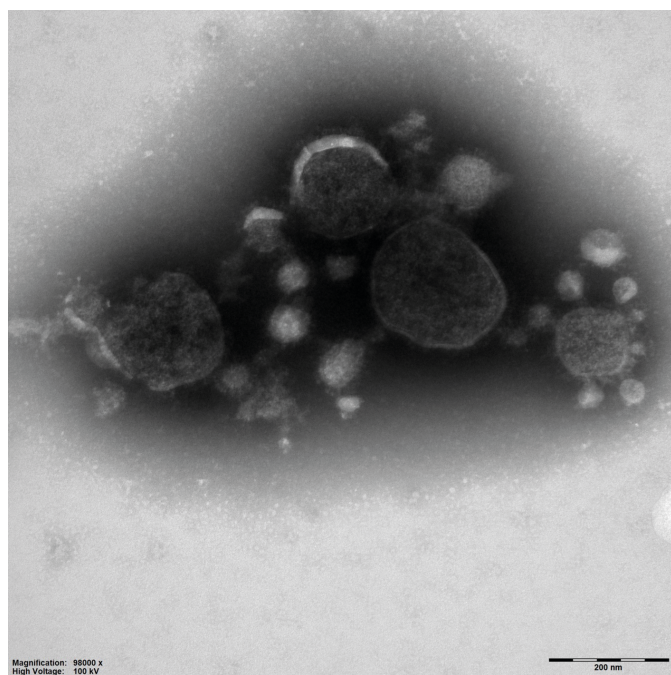
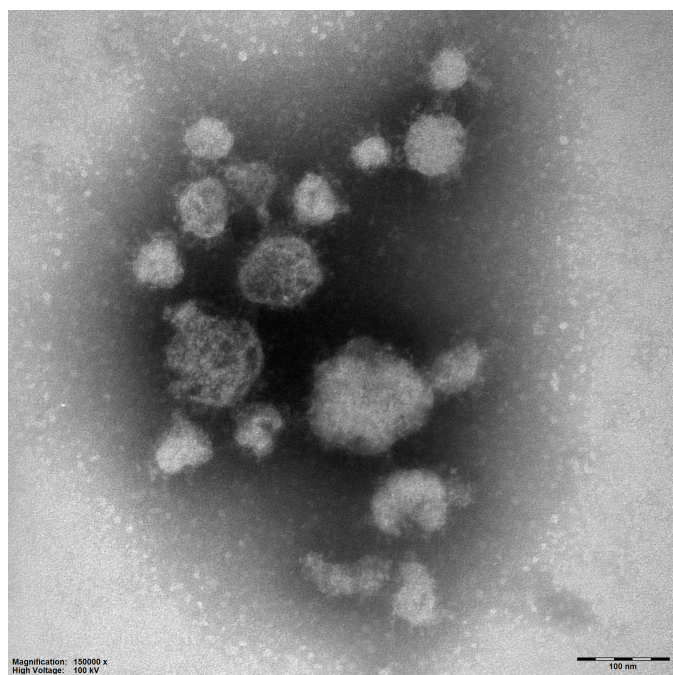


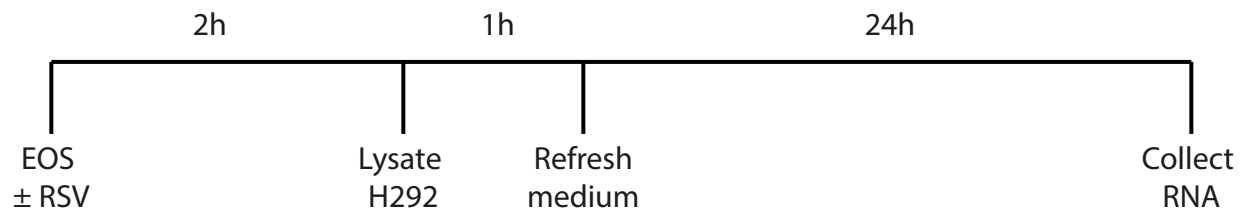
a



b







Patient	Asthma	Gender	FEV1 [L]	M/P	ICS/OCS	Allergy
18	Mild	M	4.58	M	N	Y
19	Mild	F	3.28	M	N	Y
20	Mild	F	2.93	P	N	Y
21	Mild	F	2.31	M	N	Y
22	Mild	F	4.37	M	N	Y
23	Mild	M	4.11	P	N	Y
24	Mild	F	2.97	P	N	Y
25	Mild	F	3.34	M	N	Y
26	Mild	F	4.00	P	N	Y
27	Mild	M	5.09	P	N	Y
28	Mild	F	2.52	M	N	Y
29	Mild	F	3.92	P	N	Y
30	Mild	F	3.71	M	N	Y
31	Mild	M	4.86	P	N	Y
32	Mild	F	3.04	P	N	Y
33	Mild	F	3.86	M	N	Y
34	Mild	F	3.21	P	N	Y
35	Mild	M	5.27	P	N	Y
36	Mild	F	2.90	P	N	Y

	Placebo ^a		Mepolizumab ^a	
FEV1%pred	102.09	6.89	103.08	9.95
FVC	4.38	0.95	3.91	0.78
Male/Female	4/11		1/7	
Age (yr)	22.00	4.15	23.77	5.46
IgE (kU/L)	436.28	705.22	220.04	269.21

Patient	Asthma	Gender	FEV ₁ [L]	ICS	OCS
1	Moderate	F	4.46	Y	N
2	Mild	M	5.28	N	N
3	Severe	F	2.34	Y	N
4	Mild	M	4.74	N	N
5	Moderate	F	2.23	Y	N
6	Moderate	M	3.37	Y	N
7	Moderate	F	3.57	Y	N
8	Moderate	F	3.98	Y	N
9	Severe	F	4.05	Y	N
10	Severe	M	3.80	Y	N
11	Severe	M	4.55	Y	Y
12	Severe	M	3.27	Y	N
13	Severe	M	3.62	Y	N
14	Severe	M	2.97	Y	Y
15	Severe	F	1.83	Y	Y
16	Severe	M	2.43	Y	Y
17	Mild	M	3,62	N	N

Supplementary Figures

S1. Cell recruitment and activation after influenza infection in mice with eosinophilia. (a) Presence of eosinophils in the blood and BAL of WT and IL-5 tg mice upon influenza infection. (b) Total numbers of eosinophils, neutrophils, macrophages and lymphocytes in the lung parenchymal tissue of WT and IL-5 tg mice upon influenza infection and the frequency of CD19⁺ B cells, CD4⁺ and CD8⁺ T cells within the lymphocyte population. (c) Representative flow cytometry plots (grey: virus- and white: virus+) and quantification of MHCII, CD80, CD86 and CCR7 expression on virus-positive (black bars) and virus-negative eosinophils (white bars) in BALF and mLNs of IL-5 tg mice. (d) Representative flow cytometry plots representing alveolar macrophages (CD11c^{high} CD11b^{low} SigIF⁺ cells) and eosinophil (F4/80⁺ CD11c⁻ CD11b⁺ SigIF⁺ cells) populations in IL-5 tg mice. Data are representative of 2 experiments with 3 (c) mice each or combined from 2 experiments with 2-12 mice (a,b) and expressed as mean $\pm$ SEM.: 2-way ANOVA (for a,b) or normal t-test (c); * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

S2. RSV replicates in epithelial NCI-H292 cells, but not in eosinophils. Cells were exposed to RSV-GFP at MOI:10 for 48 h. Bright-field images of H292 cells (a) and eosinophils (c) show the morphology of cells, while fluorescence images show viral replication in H292 cells in green (b) and no replication in eosinophils (d).

S3. Visualization of RSV by a negative stain using EM. A high magnification (x98,000) shows the difference sizes of RSV ranging from 120-300nm. The scale bar is indicated on the image.

S4. Time line of lysate experiment. Eosinophils were primarily incubated with or without RSV for two hours and subsequently lysed. Epithelial NCI-H292 cell line were incubated with these lysates for one hour and then the medium was refreshed. After 24 hours RNA was collected from the H292 cell line and quantified by PCR.

S5. Eosinophils from severe asthma patients not using oral corticosteroids have a reduced capacity to inactivate virus *in vivo*, but bind virus similarly compared to eosinophils from severe asthma patients on oral corticosteroids. (a) Infectious RSV (depicted by Ct-values) derived from lysates from eosinophils exposed for 2 h to RSV, subsequently administered to epithelial cells and left for 24 h to enable replication of RSV. RSV only is RSV added directly to the epithelial cells (n=16). Healthy refers to lysates from eosinophils from healthy donors (n=4) and similarly for lysates from eosinophils from severe asthma patients (n=4) using oral corticosteroids (+OCS) and those (n=3) not using oral corticosteroids (-OCS). Ct value of 27.89 (5000 copies/PCR) refers to a virus concentration of 6250 copies/mL. (b) DiD-labelled RSV determined by flow cytometry. Eosinophils without exposure to DiD-labelled RSV (Naive), and after exposure to DiD-labelled virus for eosinophils from 4 healthy (Healthy), 8 mild to moderate asthmatics (Mild) and 9 severe asthmatics (Severe) from which 4 are using oral corticosteroids (+OCS) and 5 are not using oral corticosteroids (-OCS) for 16h. Data is expressed as mean $\pm$ SEM. Paired or unpaired t-test * $p < 0.02$, ** $p < 0.01$, *** $p \leq 0.0001$.

Table S1. Baseline values for mild, moderate and severe asthma patients (GINA). Mild asthma not using inhaled (ICS) or oral corticosteroids (OCS), moderate asthma patients using ≤ 500 mg ICS and severe asthma patients using ≥ 1000 mg ICS.

Table S2. Baseline values for mild asthma patients (GINA) not using inhaled (ICS) or oral corticosteroids (OCS). There were no statistical significant differences between the placebo(P)- and Mepolizumab(M)-treated groups.^a Unpaired t-test or Fisher exact test is used; Mean $\pm$ sd, or ratio

Supplementary methods

Murine studies:

Bronchoalveolar lavage

The trachea was exposed through a midline incision and cannulated with a sterile 22-G Abbocath-T catheter (Abbot B.V., Hoofddorp, The Netherlands). Bronchoalveolar lavage (BAL) was performed by instilling and retrieving 1 mL of sterile saline into the right lung. The retrieved BAL fluid was centrifuged at 260 *g* for 10 min at 4°C, and the pellet was resuspended in 0.2 mL of sterile PBS. Cell counts were determined for each BALF sample by Trypan Blue staining and differential cell counts were performed on cytospin preparations stained with modified Giemsa stain.

Cell suspensions from lymph nodes

For analysis of cellular composition mediastinal lymph nodes were obtained. lymph node cell suspensions were obtained by grinding the tissue through 70 µm nylon sieves. Red blood cells were lysed using ammonium chloride buffer. Cell counts were performed before staining for flow cytometry analysis.

Viral load

Viral load was determined by real-time quantitative PCR in lung cell suspension as described previously (van der Sluijs, van Elden et al. 2003). The viral load present in a sample was calculated using a standard curve of particle-coated influenza virus included in every assay run. The following primers were used: 5'-GGACTGCAGCGTAGACGCTT-3' (forward); 5'-CATCCTGTTGTATATGAGGCCCAT-3' (reverse), 5'-CTCAGTTATTCTGCTGGTGCACCTTGCC-3'(50-FAM labeled probe).

Flow cytometry

For mice, single cell suspensions were stained for 30 min on ice with CD3 (145-2C11), CD4 (GK1.5), CD19 (1D3), MHC II (M5), CD11c (N418), CCR7 (4B12), CD80 (16-10A1) (all from eBiosciences), CD8 (63-6.7) SiglecF (E50-2440, BD Biosciences), F4/80 (BM8, Biolegend), CCR3 (83101, R&D systems), CD86 (GL-1, Biolegend), in the presence of Fc blocker (CD16/CD32, eBiosciences). Eosinophils were identified as CD11c⁻ CD11b⁺ CCR3⁺ SiglecF⁺.

Human studies:

Purification of eosinophils

Eosinophils for in vitro studies (Figures 2 up to 5b) were obtained from peripheral blood from 9 healthy donors and 8 mild to moderate and 9 severe asthma patients (Supplementary Table S1). Ethical approval was obtained from the local ethics committee (METC 2014_339) and informed consent was obtained from the participants. Granulocyte pellet was obtained using a Ficoll gradient (1.077 g/ml) and subsequently lysed twice in erythrocyte lysis buffer on ice. Eosinophils were obtained by negative selection (CD16, CD14, CD3) via MACS cell separation (Miltenyi). Purity was checked by a Diff-Quick stain and flow cytometry and was >95%.

Additional information on RV16 challenge (MATERIAL) study

The study has been described in detail (Sabogal Piñeros, Bal et al. 2018), but relevant information is provided here for convenience. All asthma patients (baseline values in supplementary table S2) were steroid-naïve, used only short-acting β 2-agonists, had a positive skin prick test against at least 1 out of 12 aero-allergens, had a methacholine bromide (MeBr) PC20 $\leq$ 9.8mg/ml and baseline FEV1 $\geq$ 80%, were aged between 18 and 50 years, non-smoking and had no exacerbation for at least 6 weeks prior to screening. Lung function tests were performed according to the European Respiratory Society (ERS) recommendations. Finally, patients with a RV16 antibody titer above 1:4 were excluded. All participants provided written

informed consent. The study protocol was reviewed and approved by the internal ethical review committee and in accordance with the declaration of Helsinki.

Flow cytometry

To analyze virus binding by human eosinophils, eosinophils were identified by Siglec8 (7C9; Biolegend) and CCR3 (61828; R&D) positive and CD16 negative (3G8; Bio Legend). Viral particles stained with DiD were visualized in the APC channel. Activation of eosinophils was determined using: CD66b (G10F5; Bio Legend), CD69 (FN50; BD Pharmingen), CD11b (ICRF44; BD Pharmingen) and CD62L (DREG-56; Bio legend). Viability was assessed using viability dye (FVD eFluor® 780; eBioscience) and Trypan blue exclusion. Data acquisition was done using FACSCanto II (BD Biosciences) and data were analyzed using Flowjo (Treestar).

Assays

Human IL-8 ELISA. Micro-titer plates were pre-treated with goat anti-mouse Ig antibody (0.36 µg/ml; DAKO) in PBS at 4°C overnight. MAB208 (0.1µg/ml; R&D) was used as primary antibody and BAF208 (40ng/ml; R&D) as secondary antibody.

Human IL-6 ELISA. Micro-titer plates were pre-treated with mouse anti-human IL-6 antibody (1 µg/ml; Sanquin) in PBS at 4°C overnight. Sheep-anti-human IL-6 biotin (Sanquin) was used as a secondary antibody.

Human ECP ELISA. Micro-titer plates were pre-treated with mouse anti-human ECP antibody (1 µg/ml; Diagnostics Development) in PBS at RT overnight at 4°C. Biotin-labeled rabbit anti-ECP (Diagnostics Development) was used. Absorbance values were read at 450-540nm.