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ORIGINAL ARTICLE

Phosphodiesterase 4 inhibitors attenuate virus-induced activation of eosinophils from asthmatics without affecting virus binding

Yanaika Shari Sabogal Piñeros[1,2] | Tamara Dekker[1,2] | Barbara Smids[1,2] | Christof J. Majoor[2] | Lara Ravanetti[1,2] | Gino Villetti[3] | Maurizio Civelli[3] | Fabrizio Facchinetti[3] | René Lutter[1,2]

[1]Department of Experimental Immunology, Amsterdam Infection & Immunity Institute, Amsterdam, The Netherlands

[2]Department of Respiratory Medicine, Amsterdam University Medical Centres, University of Amsterdam, Amsterdam, The Netherlands

[3]Corporate Pre-Clinical R&D, Chiesi Farmaceutici S.p.A., Parma, Italy

Correspondence
Yanaika Shari Sabogal Piñeros, Amsterdam University Medical Centers, University of Amsterdam, room K0-154, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands.
Email: y.s.sabogalpineros@amc.uva.nl

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Abstract

Acute respiratory virus infections, such as influenza and RSV, are predominant causes of asthma exacerbations. Eosinophils act as a double-edged sword in exacerbations in that they are activated by viral infections but also can capture and inactivate respiratory viruses. Phosphodiesterase type 4 (PDE4) is abundantly expressed by eosinophils and has been implicated in their activation. This exploratory study aims to determine whether these opposing roles of eosinophils activation of eosinophils upon interaction with virus can be modulated by selective PDE4 inhibitors and whether eosinophils from healthy, moderate and severe asthmatic subjects respond differently. Eosinophils were purified by negative selection from blood and subsequently exposed to RSV or influenza. Prior to exposure to virus, eosinophils were treated with vehicle or selective PDE4 inhibitors CHF6001 and GSK256066. After 18 hours of exposure, influenza, but not RSV, increased CD69 and CD63 expression by eosinophils from each group, which were inhibited by PDE4 inhibitors. ECP release, although not stimulated by virus, was also attenuated by PDE4 inhibitors. Eosinophils showed an increased Nox2 activity upon virus exposure, which was less pronounced in eosinophils derived from mild and severe asthmatics and was counteracted by PDE4 inhibitors. PDE4 inhibitors had no effect on binding of virus by eosinophils from each group. Our data indicate that PDE4 inhibitors can attenuate eosinophil activation, without affecting virus binding. By attenuating virus-induced responses, PDE4 inhibitors may mitigate virus-induced asthma exacerbations.

KEYWORDS

degranulation, eosinophil_cationic_protein, NADPH_oxidase, CD69, CD63, neutrophil

Abbreviations: ECP, eosinophil cationic protein; EDN, eosinophil-derived neurotoxin; FEV1, forced expiratory volume in 1 second; ICS, inhaled corticosteroids; MBP, Major Basic Protein; Nox2, NADPH oxidase 2; PDE4, phopsphodiesterase-4; ROS, reactive oxygen species; RSV, respiratory syncytial virus.

1 | INTRODUCTION

Asthma is a chronic inflammatory disease characterized by airway hyperresponsiveness and periodic reversible airway obstruction.[1-4] In asthma, eosinophils are considered predominantly damaging by the release of cytotoxic granular proteins like eosinophil-derived neurotoxin (EDN), eosinophil cationic protein (ECP) and major basic protein (MBP).[5-7] In addition, activated eosinophils can generate large amounts of cytotoxic reactive oxygen species (ROS). Its key role in asthma pathophysiology[2,8] is illustrated by the fact that therapy tailored to sputum eosinophil counts is superior over those based upon clinical criteria.[9]

Acute worsening of asthma symptoms, referred to as an exacerbation, is often triggered by respiratory virus infections, such as respiratory syncytial virus (RSV), influenza[10] and rhinoviruses.[11,12] Although eosinophils are considered damaging during exacerbations, there are several indications that they also display protective properties such as antiviral activities. It is known that eosinophils are activated by RSV to release mediators that promote viral clearance in a TLR7-dependent pathway[13] and that EPO and EDN with antiviral activities are released by BAL eosinophils in response to a viral infection.[14] In a parallel study, we recently showed that eosinophils display intracellular antiviral activities.[15] Eosinophils rapidly capture, internalize and inactivate 95% of respiratory viruses within 2 hours. This antiviral activity was paralleled by a mild activation of eosinophils, 2 hours after exposure to virus.[16] Respiratory viruses induced a marked increase in CD69 cell surface expression, smaller increase in CD11b expression and reduced CD62L expression.[17] There was no enhanced CD63 expression, a tetraspanin and marker of degranulation, which is in line with no ECP release, one of the granular constituents.

From a therapeutic perspective, it is of interest to control the activation status of eosinophils and differentially manipulate protective and damaging properties of eosinophils. The majority of current therapies are focused on the reduction in eosinophil numbers, since especially high sputum eosinophil counts correlate with the severity of asthma symptoms.[18] Corticosteroids play an important role in the maintenance therapy of asthma, particularly by attenuating eosinophil numbers. It is widely recognized, however, that long-term use of corticosteroids causes major side effects and not all aspects of asthma are responsive to corticosteroids.[19,20] Recent alternative approaches involve antibodies that target the IL-5/IL-5 receptor axis such as Mepolizumab, Benralizumab, and Reslizumab, potentially affecting eosinophil recruitment, survival, and activation.[21] Various studies with these antibodies have shown a reduction in peripheral and airway eosinophils and blood ECP levels,[22] a reduction in asthma exacerbations,[23] but also contradictory outcomes like improvement or no effect on FEV1.[21]

A potential alternative approach that may differentially modulate protective and damaging properties of eosinophils is the use of pharmacological inhibitors of phosphodiesterase-4 (PDE4), which is prominently expressed in eosinophils.[24] PDE4 belongs to a family of 11 iso-enzymes (PDE1-11), which differ in substrate specificity, cellular distribution, and regulatory function. PDE4 control cellular cAMP levels by hydrolysis to 5′AMP and thereby attenuate the availability of cAMP, thus amplifying immune and inflammatory responses.[25-27] Selective inhibitors specifically designed for inhaled treatment, such as CHF6001[24] and GSK256066[28] may combine potent anti-inflammatory activity with limited systemic exposure and, consequently, improved tolerability. CHF6001, in particular, was shown to be more potent than Roflumilast in eliciting anti-inflammatory effects on virus-inducible cytokines in human bronchial epithelial cell lines.[29] Both CHF6001 and GSK256066 are effective in reducing allergen challenge responses in asthma patients.[27,30] While GSK256066 clinical development appears discontinued (ClinicalTrials.gov identifier: NCT00549679), CHF6001 is currently undergoing phase 2 clinical trials (ClinicalTrials.gov identifier: NCT02986321) in COPD patients as inhaled agent. For the two PDE4 inhibitors, a different tolerability profile has been reported, with GSK256066 showing nausea-like behaviors and emesis in ferrets at much lower doses (~10-fold) than CHF6001.[31] Indeed, while CHF6001 showed an excellent safety profile,[32] dose-limiting side effects of GSK256066 could have determined its discontinuation. Nevertheless, GSK256066 remains a highly potent and selective PDE4 inhibitor useful as a tool compound. Given the potential therapeutic role of PDE4 inhibitors in viral-induced asthma exacerbations, we explored the differential modulation of protective and damaging properties of eosinophils upon exposure to virus by these two nearly equipotent and highly selective PDE4 inhibitors, CHF6001 and GSK256066.[24] In addition, we assessed whether eosinophils from asthma patients behaved differently from those from healthy individuals. We assessed their effects on phenotypic activity markers (CD69, CD63, and ECP) and NADPH oxidase (Nox2) activity by eosinophils and, in comparison, by neutrophils from both healthy controls and asthma patients.

2 | MATERIALS AND METHODS

2.1 | Drugs

CHF6001 and GSK256066 were synthesized at Chiesi Farmaceutici S.p.A., Parma, Italy.[24,33]

2.2 | Patients and healthy individuals

Eosinophils and neutrophils were obtained from peripheral blood of healthy volunteers, mild to moderate (≤500 mg fluticasone) and severe asthmatics (≥1000 mg fluticasone). Ethical approval was obtained from the local Medical Ethics committee and informed consent from participants.

2.3 | Human eosinophil and neutrophil purification

HBSS (Lonza, Basel, Switzerland) was added to the collected peripheral blood and subsequently placed onto a Ficoll gradient (1.077 g/

mL) and centrifuged for 25 minutes at 1350 *g* at RT. The granulocyte pellet was lysed twice in erythrocyte lysis buffer on ice to get rid of erythrocytes. Eosinophils were obtained by negative selection (CD16) using MACS cell separation (Miltenyi). Neutrophils were obtained from the CD16-positive fraction. Purity was checked by Diff-Quick staining and flow cytometry and was >90 and >98% for eosinophils and neutrophils, respectively.

2.4 | Virus

Influenza, strain A PR/8/34, and respiratory syncytial virus (RSV)-A2 were used. RSV was propagated in HEp-2 cells in IMDM (Lonza) culture medium supplemented with 1% FCS and influenza on NCI-H292 cells (ATCC CRL 1848) in RPMI-1640 with 1% FCS. At day 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.

2.5 | Viral exposure

Eosinophils and neutrophils were maintained in RPMI-1640 supplemented with 10% FCS. All cells were incubated at 37°C, 95% humidity and 5% CO_2. Eosinophils and neutrophils were incubated with either influenza A PR/8/34 or RSV-A2 at a MOI: 2 and 5, respectively. Different conditions were used depending on the analyses; the eosinophils were incubated 18 hours for flow cytometry and the release of ECP. Prior to analysis by FACS, cells were re-suspended and washed with cold PBS containing 0.5% BSA with 2 mmol/L EDTA. For Nox2 activity, eosinophils and neutrophils were measured during 30 minutes after adding virus or fMLP. To determine binding of DiD-labeled RSV, eosinophils were maintained 18 hours with DiD-labeled RSV at MOI: 5.

2.6 | Compounds

All PDE4 inhibitors were dissolved in DMSO at a concentration of 10 mmol/L and final dilutions were made in the assay buffer (0.1% final DMSO concentration in the assays). In exploratory studies we utilized a range of concentrations (0.01-10 nmol/L) of GSK256066 and CHF6001 and selected as fixed test concentrations 0.1 nmol/L in eosinophils and 1.0 nmol/L in neutrophils in line with their subnanomolar inhibitory potency against PDE4 isoforms [28] Cells were preincubated with PDE4 inhibitors 30 minutes before exposure to virus or stimulus.

2.7 | Assays

2.7.1 | Amplex Red hydrogen peroxide assay

Hydrogen peroxide release from cells was measured using Amplex Red (Invitrogen) following manufacturer's instructions. Eosinophils and neutrophils were pretreated with PDE4 inhibitors for 30 minutes in Krebs-Ringer phosphate buffer. Subsequently, cells were treated with 1 μmol/L fMLP (Bio-connect), RSV-A2 or influenza A P/R/8 and directly measured for 30 minutes at 30s intervals at 37°C. The production of resorufin (fluorescence) was measured using a BIOTEK plate reader (synergy HT), with excitation at 530nm and emission at 590nm.

2.7.2 | Flow cytometry

To analyze the activation of human granulocytes, eosinophils were identified as Siglec8-positive (7C9; Bio Legend) and CD16-negative (3G8; Bio Legend) and Annexin V-negative (120F; IQP). Neutrophils were identified as CD16-positive (3G8; Bio Legend) and Annexin V-negative. A total of 50 000 granulocytes were incubated with mAbs for 30 minutes at 4°C, and 10 minutes with Annexin V at 4°C. An assessment of the activation of cell-surface markers was made by the use of mAbs against the following molecules: CD63 (H5C6; Bio Legend), CD69 (FN50; BD Pharmingen). Cells were washed in PBS containing 0.5% BSA. Data acquisition was done using FACSCanto II (BD Biosciences).

2.7.3 | Human ECP ELISA

ECP was measured using ECP monoclonal capture antibody (clone 614, Diagnostics Development), ECP standard (ImmunoCAP ECP Calibrator) and biotinylated polyclonal detection antibody (Diagnostics Development) as described elsewhere.[34]

2.7.4 | DiD labeling of virus

1,19-dioctadecyl-3,3,39,39-tetramethylindodicarbocyanine (DiD) (Molecular Probes, Invitrogen, Carlsbad, CA) was dissolved in DMSO at a concentration of 20 mg/mL and used to label RSV-A2. RSV was incubated at room temperature for 30 minutes with 2 μL DiD solution, followed by density gradient centrifugation to obtain purified labeled virus, essentially as described elsewhere.[35] All the comparative experiments were performed with the same batch of DiD-labeled virus.

2.8 | Statistics

Flow cytometry data were expressed as mean ± SEM and analyzed using FlowJo (Treestar), whereas that for quantification with GraphPad Prism 5.0 software and that for ELISA's using GEN5 data analysis software (BioTek); paired and unpaired t-tests were used, as indicated in the legends to the figures, and $P < .05$ was considered significant. The number of analyses is provided in the legends to the figures. To correct for multiple testing, FDR (Benjamini-Hochberg) corrected *P*-values (Q = 5%) were used.

2.9 | Ethics approval and consent

Medisch Ethische Toetsingscommissie—AMC; the ethics approval number: NL48912.018.14; All participants provided written informed consent.

3 | RESULTS

3.1 | Activation and degranulation in eosinophils, before and after exposure to virus, and the effect of PDE4 inhibitors

It is known that eosinophils exposed to virus for 2 hours can be mildly activated as reflected by an enhanced CD69 expression, whereas CD63 expression and ECP release are unaffected.[15] Here we addressed whether these markers were affected 18 hours after exposure to virus. Baseline parameters for eosinophils from controls and patients are provided in Figure 1. Notably, baseline values show small, though significant changes for CD63 and CD69 expression between eosinophils from healthy controls and asthma patients, whereas baseline ECP release by eosinophils from healthy donors was strikingly enhanced compared to those from asthma patients (Figure 1A). Interestingly, baseline differences were found for eosinophils from controls and patients, but not for neutrophils (Figure 1B). Differences cannot be explained by differences in apoptosis (AnnV).

To compare the effect of PDE4 inhibitors, data were normalized to baseline values. CD69 expression (Figure2A) and release of ECP (Figure 2C) by eosinophils from healthy subjects (white), but not that of CD63 (Figure 2B), were reduced by PDE4 inhibitors CHF6001 and GSK256066. In contrast, eosinophils from mild to moderate asthma patients (gray) and severe asthma patients (black) were less responsive to PDE4 inhibition, apart from inhibition of ECP release by eosinophils from mild to moderate asthma patients (Figure 2C). Exposure to influenza enhanced CD69 and CD63 expression, but not ECP release, by eosinophils from healthy subjects and patients. The PDE4 inhibitors apparently prevented the influenza-induced expression of CD69 and CD63, and reduced ECP release, although for the latter CHF6001 was only effective in eosinophils form severe asthma patients (Figure 2C). Exposure to RSV did not enhance CD69 and CD63 expression and ECP release, but exposure to RSV potentiated the inhibitory effect of PDE4 inhibitors, but not of GSK256066 on patient's eosinophils.

PDE4 inhibitors also attenuated CD69 and CD63 expression and MPO release by neutrophils (Figure S1) although the effects were less outspoken than that for eosinophils, particularly when the cells were exposed to virus.

3.2 | Effect of PDE4 inhibitors on Nox2 activity in virus-exposed eosinophils and neutrophils

Nox2 activity is comparable at baseline for eosinophils from healthy controls and patients (Figure 1A). Overall, eosinophils from

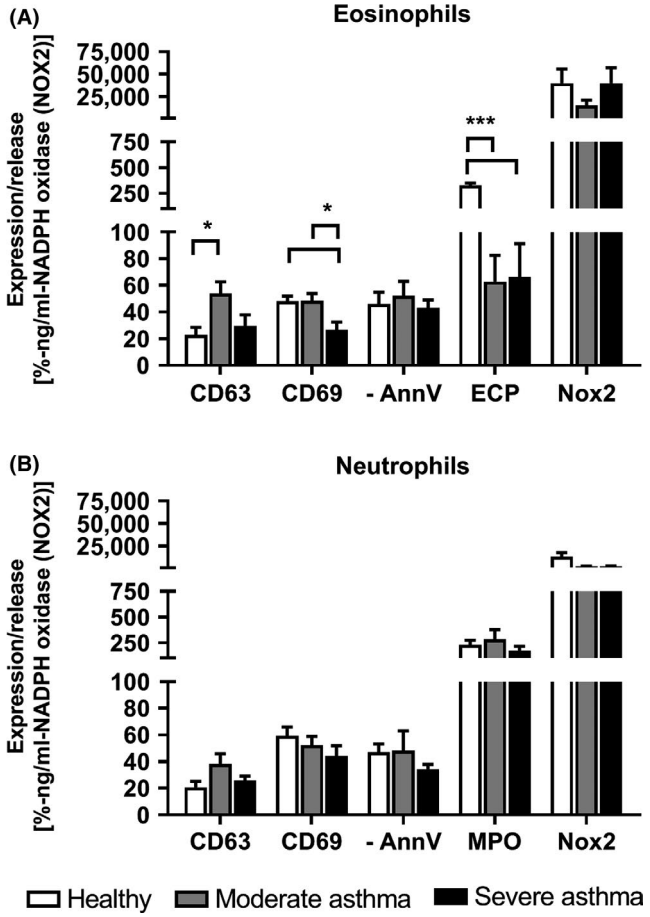


FIGURE 1 Activation and degranulation of eosinophils and neutrophils at baseline. Baseline parameters for eosinophils (A) and neutrophils (B) from healthy subjects (white bars), mild to moderate asthmatics (gray bars) and severe asthmatics (black bars). CD63, CD69 and Annexin V-negative cells (–AnnV) are presented as percentage of total cells (scale 0-100), ECP and MPO as ng/ml (scale 250-750) and NADPH oxidase (Nox2) as Vmax [530/25;590/35] (scale 25000-75000). *P < .05; ***P < .001, by unpaired t-test, n = 8 per group and included an FDR (Benjamini-Hochberg) corrected p-values (Q = 5%)

healthy individuals responded upon exposure to virus with an increased Nox2 activity, although this did not reach statistical significance, whereas eosinophils from patients responded less to virus (Figure 3A). Eosinophils from severe asthma patients displayed a significantly lower Nox2 activity to influenza compared to those from healthy individuals (P = .044). As fMLP induced Nox2 activity in eosinophils from asthma patients similar to that by eosinophils from healthy subjects, this indicates that eosinophils from severe asthma patients respond aberrantly to influenza (Figure 3A) and possibly also to RSV. In contrast to eosinophils, baseline Nox2 activity is higher for neutrophils from healthy controls as opposed to those from patients, but virus did not induce neutrophil Nox2 activity, whereas exposure to fMLP did (Figure 3B).

GSK 256066 inhibited Nox2 activity in eosinophils and also upon exposure to virus, but for the latter not for eosinophils from healthy controls (Figure 3A). CHF6001 significantly inhibited baseline and RSV-induced Nox2 activity by eosinophils from severe asthma

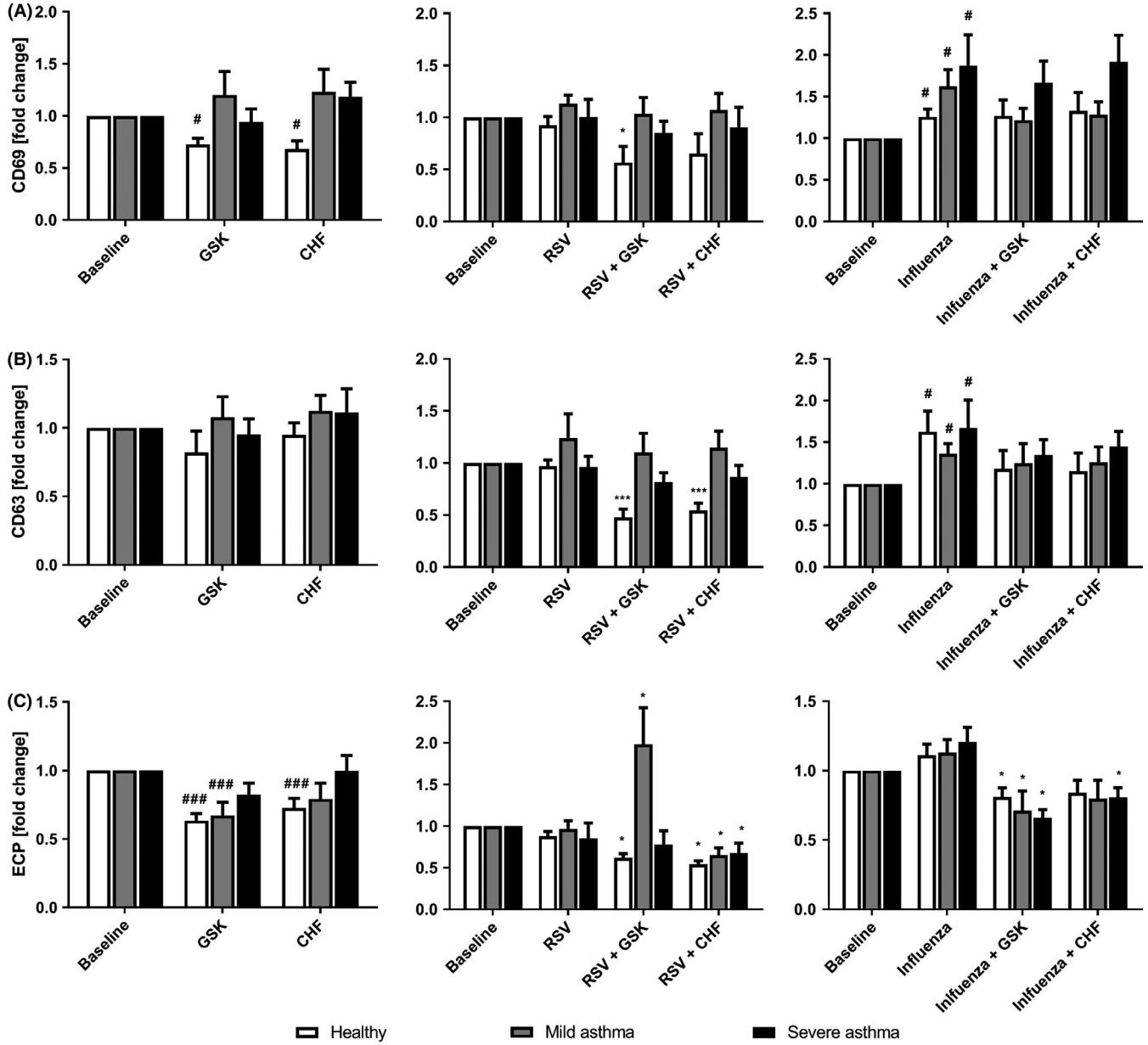


FIGURE 2 Activation and degranulation of eosinophils at baseline, after exposure to virus with/without pretreatment with PDE4 inhibitors. A, CD69 expression in eosinophils from healthy subjects (white; n = 7), from mild to moderate asthmatics (gray; n = 7) and from severe asthmatics (black; n = 9). B, As (A) but for CD63 expression. C, as (A) but for ECP release. $^{\#}$Significant vs baseline: $^{\#}P < .05$; $^{\#\#\#}P < .001$. *Significant vs virus only: $^{*}P < .05$; $^{***}P < .001$ by paired *t* test and included FDR (Benjamini-Hochberg) corrected *P*-values (Q = 5%)

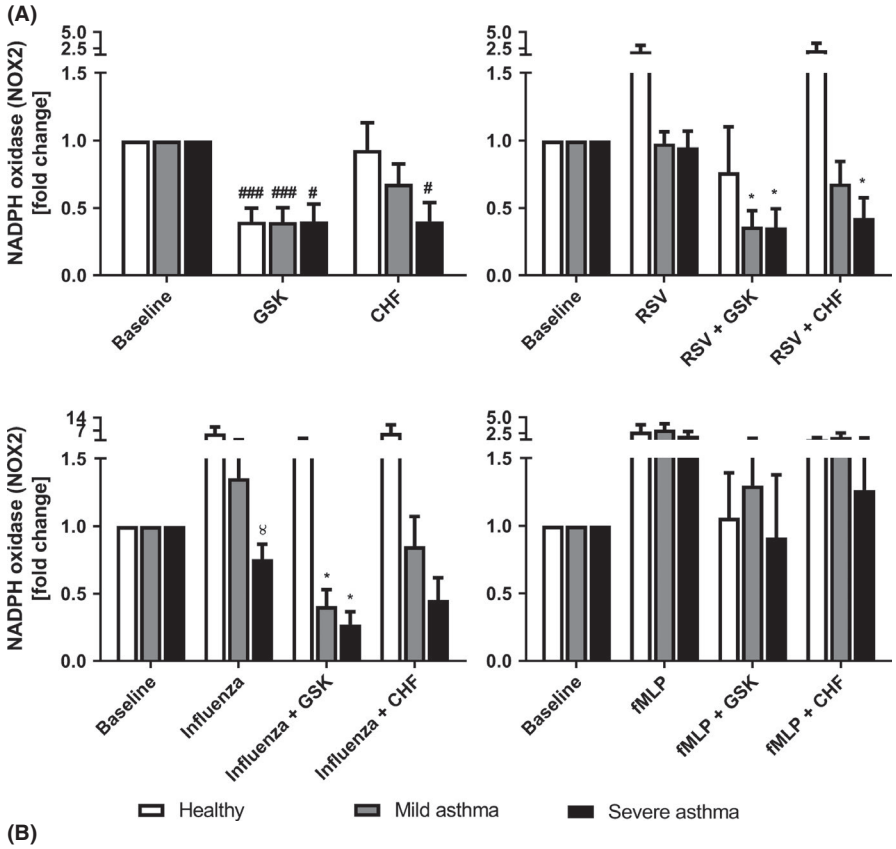
patients only. The Nox2 activity by eosinophils from mild to moderate asthma patients was not different between those using corticosteroids or not (data not shown). Both GSK256066 and CHF6001 profoundly reduced Nox2 activity in neutrophils, in the absence or presence of influenza ($P < .001$) or fMLP, but RSV exposure made neutrophils unresponsive to both PDE4 inhibitors (Figure 3B).

3.3 | Binding and inactivation of RSV by eosinophils

We investigated whether eosinophils incubated for 18 hours with RSV were able to directly bind RSV. We showed that PDE4 inhibitors had no effect on binding of virus by eosinophils from healthy controls or from asthma patients (Figure 4). There was a tendency for eosinophils from patients to bind less RSV than that by eosinophils form healthy controls as reported recently.[15]

4 | DISCUSSION

In this study we report that PDE4 inhibitors attenuate baseline ECP release, Nox2 activity and CD69 expression, although the extent of the effects differed between eosinophils derived from healthy subjects or patients. In line with our previous study[15] we found that



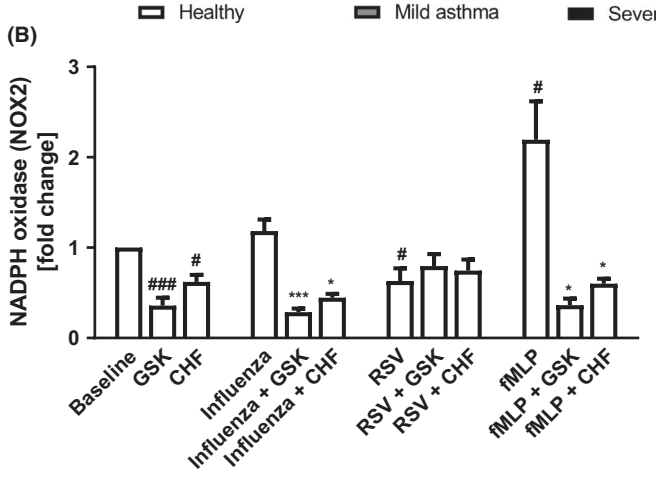


FIGURE 3 Eosinophil and neutrophil Nox2 activity is attenuated by PDE4 inhibitors GSK256066 and CHF6001. A, Baseline Nox2 activity is significantly reduced by GSK256066 in eosinophils from healthy subjects (white; n = 8), mild to moderate (gray; n = 8) and severe (black; n = 9) asthma patients. CHF6001 was only effective in severe asthmatics. After preincubation with inhibitors, eosinophils were stimulated with RSV, influenza or with fMLP. B, Neutrophils from healthy individuals showed a reduced Nox2 activity and were not stimulated after virus exposure. Neutrophils were responsive to both PDE4 inhibitors, but RSV exposure eradicated inhibition by PDE4 inhibitors. $^{\#}$Significant vs normalized baseline value: $^{\#}P < .05$; $^{\#\#\#}P < .001$, by paired *t* test. *Significant vs virus only: $^{*}P < .05$; $^{***}P < .001$ by paired *t* test; ^{8}significant vs healthy controls; $^{8}P < .05$ by unpaired t-test and included FDR (Benjamini-Hochberg) corrected *P*-values (Q = 5%)

eosinophils were activated up to 18 hours after exposure to virus, where influenza was more potent than RSV. We showed that eosinophils, but not neutrophils, display an enhanced Nox2 activity in response to influenza. In line with the defective binding of virus by eosinophils from asthma patients, virus-induced Nox2 activity was also reduced particularly in eosinophils from severe asthma patients. PDE4 inhibited virus-induced activation of eosinophils, particularly ECP release and Nox2 activity, the latter more prominent in eosinophils from asthma patients.

In our previous study,[15] we assessed the activation of eosinophils by virus over a 2 hours period. In this study we extended the investigation for the activation markers to 18 hours of incubation with viruses, showing that eosinophils maintain their activated state (CD69) and even may be slightly more activated as CD63 expression and apparently also ECP release increased. Secretory granules membranes are highly enriched in CD63,[36] while ECP is within the granules.[37] Besides secretory granules, CD63 is also present in lysosome-related organelles, although its origin is still uncertain.[36] This different intracellular compartmentalization may explain why both PDE4 inhibitors can suppress ECP release without substantially affecting CD63 cell surface expression.

The limited Nox2 response to viral exposure by eosinophils from patients is unlikely to be due to a defective Nox2, as the fMLP-induced Nox2 activity seems robust and not different from those from healthy individuals. The generation of ROS by Nox2 is important in the microbial defense,[38] but it remains to be determined whether ROS generated by virus-exposed eosinophils contribute to the reduced infectivity of virus. If so, the reduced activation of Nox2 by patient's eosinophils to virus limits their antiviral response. On the other hand, the generation of ROS by Nox2 is important in the

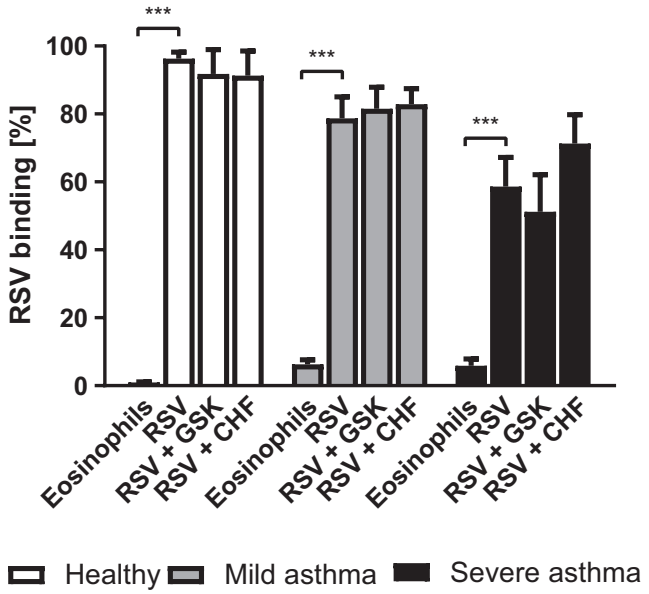


FIGURE 4 PDE4 inhibitors GSK256066 and CHF6001 do not modulate binding of RSV to eosinophils. Eosinophils from healthy donors (white; n = 4), mild to moderate (gray; n = 8) and severe asthmatics (black; n = 9) were exposed to DiD-labeled RSV for 18h. Eosinophils from severe asthmatics (black) showed trendwise reduction in RSV binding compared to eosinophils from healthy subjects (white), which was not affected by PDE4 inhibitors. ***$P < .001$ by paired *t* test; and included FDR (Benjamini-Hochberg) corrected *P*-values (Q = 5%)

microbial defense[38] but also causes tissue damage, including acute lung injury during influenza infection, and drives resistance against antibiotics of *Staphylococcus aureus* induced-pneumonia,[39-41] which may be limited by PDE4 inhibitors.

The attenuated virus-induced Nox2 activity by eosinophils from patients apparently correlated with the attenuated binding of virus by their eosinophils suggesting that this binding triggers Nox2 activation. A similar correlation, however, was not found for other markers of activation/degranulation. Interestingly, virus-induced Nox2 activity seems a specific response of eosinophils, since neutrophils did not upregulate Nox2 activity in response to either RSV or influenza. Neutrophils, which also express PDE4[42] and are relevant in severe asthma,[18,43-45] were also examined. Interestingly, virus exposure did not significantly affect phenotypic activation markers or Nox2 in neutrophils, whereas PDE4 inhibition profoundly reduced neutrophilic Nox2 activity in the presence or absence of influenza, but not upon RSV infection. This suggests that neutrophils do become activated by influenza, even though this did not lead to an enhanced Nox2 activity.

The current findings were obtained in vitro after a relatively short incubation with PDE4 inhibitors and therefore it would be of interest in future to study longer treatment periods and in an in vivo setting. This was an exploratory study and therefore additional experiments are necessary to gain further insights in the effects of PDE4 inhibitors on the activation and degranulation of these granulocytes.

CHF6001 failed to inhibit Nox2 activity in eosinophils from healthy controls and from mild to moderate asthma patients, but did profoundly inhibit Nox2 activity in eosinophils from severe asthma patients. This could indicate that eosinophils from severe asthma patients are more sensitive to inhibition of this specific pathway. Overall, the PDE4 inhibitors GSK256066 and CHF6001 showed very similar effects, with GSK256066 being apparently slightly more potent in accordance with potency differences between the two compounds against PDE4.[24]

In summary, this exploratory study indicates that eosinophils respond specifically to virus and that capacity is partially reduced in eosinophils from asthma patients. By demonstrating that PDE4 inhibitors modulate virus-induced eosinophil activation, and particularly Nox2 activity in eosinophils from asthmatics, we revealed a potential role for PDE4 inhibitors in controlling virus-induced responses that may be relevant in asthma exacerbations.

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CONFLICT OF INTEREST

G. Villetti, M. Civelli, F. Facchinetti are employees of Chiesi Farmaceutici S.p.A. Y. S. Sabogal Piñeros, T. Dekker, B. Smids, CJ Majoor and L. Ravanetti have no conflict of interest. R. Lutter has received grants from Medimmune, Nutrileads, Chiesi, Foresee, Lung Foundation and Stichting Astmabestrijding, none of which has influenced this manuscript.

AUTHORS' CONTRIBUTIONS

Y. S. S. P., T. D., and B. S. did the experiments and analyzed the data. Y. S. S. P also wrote the manuscript. CJM, L. R., G. V., M. C., F. F., and R. L. contributed to the design of the study and amended the manuscript.

ORCID

Yanaika Shari Sabogal Piñeros https://orcid.org/0000-0001-7824-9058
Fabrizio Facchinetti https://orcid.org/0000-0003-3505-4159

REFERENCES

1. Anandan C, Nurmatov U, Van Schayck OCP, Sheikh A. Is the prevalence of asthma declining? Systematic review of epidemiological studies. *Allergy*. 2010;65(2):152-167.
2. Sont JK, Willems LNA, Bel EH, van Krieken JHJM, Vandenbroucke JP, Sterk PJ. Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide to long-term treatment. *Am J Respir Crit Care Med*. 1999;159(4):1043-1051.
3. To T, Stanojevic S, Moores G, et al. Global asthma prevalence in adults: findings from the cross-sectional world health survey. *BMC Public Health*. 2012;12(1):204.
4. Asher MI, Montefort S, Björkstén B, et al. Worldwide time trends in the prevalence of symptoms of asthma, allergic rhinoconjunctivitis, and eczema in childhood: ISAAC Phases One and Three repeat multicountry cross-sectional surveys. *The Lancet*. 2006;368(9537):733-743.
5. Garofalo R, Kimpen JLL, Welliver RC, Ogra PL. Eosinophil degranulation in the respiratory-tract during naturally acquired respiratory syncytial virus-infection. *J Pediatr*. 1992;120(1):28-32.

6. Grunberg K, Smits HH, Timmers MC, et al. Experimental rhinovirus 16 infection - Effects on cell differentials and soluble markers in sputum in asthmatic subjects. *Am J Respir Crit Care Med*. 1997;156(2):609-616.
7. Harrison AM, Bonville CA, Rosenberg HF, Domachowske JB. Respiratory syncytial virus-induced chemokine expression in the lower airways - Eosinophil recruitment and degranulation. *Am J Respir Crit Care Med*. 1999;159(6):1918-1924.
8. Liu J-N, Suh D-H, Yang E-M, Lee S-I, Park H-S, Shin YS. Attenuation of airway inflammation by simvastatin and the implications for asthma treatment: is the jury still out[quest]. *Exp Mol Med*. 2014;46:e113.
9. Green RH, Brightling CE, McKenna S, et al. Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet (London, England)*. 2002;360(9347):1715-1721.
10. Jacoby DB. Virus-induced asthma attacks. *JAMA*. 2002;287(6):755-761.
11. Schaller M, Hogaboam CM, Lukacs N, Kunkel SL. Respiratory viral infections drive chemokine expression and exacerbate the asthmatic response. *Journal of Allergy and Clinical Immunology*. 2006;118(2):295-302.
12. Folkerts G, Busse WW, Nijkamp FP, Sorkness R, Gern JE. Virus-induced airway hyperresponsiveness and asthma. *Am J Respir Crit Care Med*. 1998;157(6):1708-1720.
13. Phipps S, Lam CE, Mahalingam S, et al. Eosinophils contribute to innate antiviral immunity and promote clearance of respiratory syncytial virus. *Blood*. 2007;110(5):1578-1586.
14. Percopo CM, Dyer KD, Ochkur SI, et al. Activated mouse eosinophils protect against lethal respiratory virus infection. *Blood*. 2014;123(5):743-752.
15. Sabogal Pineros YS, Bal SM, Dijkhuis A, et al. Eosinophils capture viruses, a capacity that is defective in asthma. *Allergy*. 2019;1-12.
16. Na HJ, Hamilton RG, Klion AD, Bochner BS. Biomarkers of eosinophil involvement in allergic and eosinophilic diseases: review of phenotypic and serum markers including a novel assay to quantify levels of soluble Siglec-8. *J Immunol Methods*. 2012;383(1–2):39-46.
17. Johansson MW. Activation states of blood eosinophils in asthma. *Clin Exp Allergy*. 2014;44(4):482-498.
18. Fahy JV. Eosinophilic and neutrophilic inflammation in asthma. *Proc Am Thorac Soc*. 2009;6(3):256-259.
19. Creticos PS. Treatment options for initial maintenance therapy of persistent asthma: a review of inhaled corticosteroids and leukotriene receptor antagonists. *Drugs*. 2003;63(2):1-20.
20. Calverley PMA. Effect of corticosteroids on exacerbations of asthma and chronic obstructive pulmonary disease. *Proc Am Thorac Soc*. 2004;1(3):161-166.
21. Garcia G, Taillé C, Laveneziana P, Bourdin A, Chanez P, Humbert M. Anti-interleukin-5 therapy in severe asthma. *Eur Respir Rev*. 2013;22(129):251-257.
22. Büttner C, Lun A, Splettstoesser T, Kunkel G, Renz H. Monoclonal anti-interleukin-5 treatment suppresses eosinophil but not T-cell functions. *Eur Respir J*. 2003;21(5):799-803.
23. Castro M, Wenzel SE, Bleecker ER, et al. Benralizumab, an anti-interleukin 5 receptor α monoclonal antibody, versus placebo for uncontrolled eosinophilic asthma: a phase 2b randomised dose-ranging study. *Lancet Respir Med*. 2014;2(11):879-890.
24. Moretto N, Caruso P, Bosco R, et al. CHF6001 I: A novel highly potent and selective phosphodiesterase 4 inhibitor with robust anti-inflammatory activity and suitable for topical pulmonary administration. *J Pharmacol Exp Ther*. 2015;352(3):559-567.
25. Manning CD, Burman M, Christensen SB, et al. Suppression of human inflammatory cell function by subtype-selective PDE4 inhibitors correlates with inhibition of PDE4A and PDE4B. *Br J Pharmacol*. 1999;128(7):1393-1398.
26. Hertz AL, Bender AT, Smith KC, et al. Elevated cyclic AMP and PDE4 inhibition induce chemokine expression in human monocyte-derived macrophages. *Proc Natl Acad Sci*. 2009;106(51):21978-21983.
27. Singh D, Petavy F, Macdonald A, Lazaar A, O'Connor B. The inhaled phosphodiesterase 4 inhibitor GSK256066 reduces allergen challenge responses in asthma. *Respir Res*. 2010;11(1):26.
28. Tralau-Stewart CJ, Williamson RA, Nials AT, et al. GSK256066, an exceptionally high-affinity and selective inhibitor of phosphodiesterase 4 suitable for administration by inhalation: in vitro, kinetic, and in vivo characterization. *J Pharmacol Exp Ther*. 2011;337(1):145-154.
29. Edwards MR, Facchinetti F, Civelli M, Villetti G, Johnston SL. Anti-inflammatory effects of the novel inhaled phosphodiesterase type 4 inhibitor CHF6001 on virus-inducible cytokines. *Pharmacol Res Perspect*. 2016;4(1):e00202.
30. Singh D, Leaker B, Boyce M, et al. A novel inhaled phosphodiesterase 4 inhibitor (CHF6001) reduces the allergen challenge response in asthmatic patients. *Pulm Pharmacol Ther*. 2016;40:1-6.
31. Villetti G, Carnini C, Battipaglia L, et al. CHF6001 II: a novel phosphodiesterase 4 inhibitor, suitable for topical pulmonary administration–in vivo preclinical pharmacology profile defines a potent anti-inflammatory compound with a wide therapeutic window. *J Pharmacol Exp Ther*. 2015;352(3):568-578.
32. Mariotti F, Govoni M, Lucci G, Santoro D, Nandeuil MA. Safety, tolerability, and pharmacokinetics of single and repeat ascending doses of CHF6001, a novel inhaled phosphodiesterase-4 inhibitor: two randomized trials in healthy volunteers. *Int J Chron Obstruct Pulmon Dis*. 2018;13:3399-3410.
33. Armani E, Amari G, Rizzi A, et al. Novel class of benzoic acid ester derivatives as potent PDE4 inhibitors for inhaled administration in the treatment of respiratory diseases. *J Med Chem*. 2014;57(3):793-816.
34. Majoor CJ, van de Pol MA, Kamphuisen PW, et al. Evaluation of coagulation activation after rhinovirus infection in patients with asthma and healthy control subjects: an observational study. *Respir Res*. 2014;15:14.
35. Ho AWS, Prabhu N, Betts RJ, et al. Lung CD103+ dendritic cells efficiently transport influenza virus to the lymph node and load viral antigen onto MHC class I for presentation to CD8 T cells. *Journal of Immunology*. 2011;187(11):10.
36. Lee JJ. Chapter 3 - Eosinophil structure and cell surface receptors A2 In: Rosenberg HF editor. *Eosinophils in health and disease*. Boston: Academic Press; 2013:19-38.
37. Davoine F, Lacy P. Eosinophil cytokines, chemokines, and growth factors: emerging roles in immunity. *Front Immunol*. 2014;5:570.
38. Pizzolla A, Hultqvist M, Nilson B, et al. Reactive oxygen species produced by the NADPH oxidase 2 complex in monocytes protect mice from bacterial infections. *J Immunol*. 2012;188(10):5003-5011.
39. von Dessauer B, Bongain J, Molina V, Quilodrán J, Castillo R, Rodrigo R. Oxidative stress as a novel target in pediatric sepsis management. *J Crit Care*. 2011;26(1):103.e1–103.e7.
40. Sun K, Yajjala VK, Bauer C, et al. Nox2-derived oxidative stress results in inefficacy of antibiotics against post-influenza *S. aureus* pneumonia. *J Exp Med*. 2016;213(9):1851-1864.
41. Vlahos R, Stambas J, Bozinovski S, Broughton BRS, Drummond GR, Selemidis S. Inhibition of Nox2 oxidase activity ameliorates influenza a virus-induced lung inflammation. *PLoS Pathog*. 2011;7(2):e1001271.
42. Schett G, Sloan VS, Stevens RM, Schafer P. Apremilast: A Novel PDE4 inhibitor in the treatment of autoimmune and inflammatory diseases. *Ther Adv Musculoskelet Dis*. 2010;2(5):271-278.
43. Kamath AV, Pavord ID, Ruparelia PR, Chilvers ER. Is the neutrophil the key effector cell in severe asthma? *Thorax*. 2005;60(7):529-530.
44. Nakagome K, Matsushita S, Nagata M. Neutrophilic inflammation in severe asthma. *Int Arch Allergy Immunol*. 2012;158(s1):96-102.

45. Bruijnzeel PLB, Uddin M, Koenderman L. Targeting neutrophilic inflammation in severe neutrophilic asthma: can we target the disease-relevant neutrophil phenotype? *J Leukoc Biol*. 2015;98(4):549–556.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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