

Prolonged inflammatory resolution in allergic asthma relates to dysfunctional interactions between neutrophils and airway epithelium



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ABSTRACT

Background: Allergic asthma is a heterogeneous disorder involving chronic airway inflammation, reversible airflow limitation, and tissue remodeling, causing chronic airflow limitation. Most of the asthma research has been focused on elucidating the proinflammatory pathways underlying disease pathogenesis. Paradoxically, the necessity of appropriate termination and resolution of inflammation has not been recognized until recently. The latter has led to the concept of chronic inflammation developing as a result of lack of specific “stop” signals for the inflammatory process.

Objective: To investigate the interaction between neutrophils and airway epithelium during inflammatory resolution in patients with allergic asthma.

Methods: An in vitro scratch assay with cultured epithelial cells, based on live-imaging microscopy, was used to evaluate regeneration and the influence of neutrophils on resolution. Epithelial cells and autologous neutrophils were derived from healthy donors and patients with allergic asthma. Supernatants and cells were collected for enzyme-linked immunosorbent assay and transcriptional analyses at the end of the experiment.

Results: Healthy epithelial cells regenerated faster than epithelial cells from patients with allergic asthma. Autologous neutrophils improved the regeneration of healthy epithelial cells but not asthmatic epithelial cells. Interleukin (IL)-8 and β -catenin were down-regulated in healthy epithelial cells after resolution, but not in allergic asthmatic epithelial cells.

Conclusion: The prolonged duration of inflammation in the respiratory tract in patients with allergic asthma could be due to the impaired healing pattern of epithelial cells and their compromised interactions with the neutrophils.

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Introduction

Allergic asthma is a heterogeneous disease characterized by chronic inflammation and a disrupted epithelium.¹ Most of the research efforts in allergic asthma have been focused on proinflammatory pathways. Consequently, many of the current therapeutic targets focus on blocking the initiating or amplifying inflammatory components. The necessity of appropriate termination and resolution of inflammation has not until recently been recognized. This has led to the idea of allergic asthma as a persisting airway inflammatory process, caused by a lack of specific “stop” signals for inflammatory responses.²

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The resolution of inflammation in healthy airways is an active process with specialized mediators and cellular mechanisms that are enlisted to restore tissue homeostasis. Acute inflammation is initiated within minutes of recognition of a danger signal. The inflammatory process is then generally self-limited and resolves within hours or days.^{3,4} When this process is dysfunctional or disturbed, the inflammation becomes chronic, causing more or less permanent changes in airway structure and function.²

The epithelium in the airway is a protective barrier against inhaled particles, such as allergens and viruses, and is tightly regulated during normal conditions.⁵ The epithelium modulates inflammatory responses by attracting immune cells to the site of inflammation and by regulating tissue regeneration. In asthma, eosinophils and neutrophils are attracted to the site of inflammation, and it is well established that eosinophils play a dominant role in the pathophysiology, whereas the role of neutrophils has generally been

overlooked. Neutrophils have been considered short-lived and fairly simple immune cells acting primarily in the acute phase of inflammation. They are among the first cells to be recruited to the site of inflammation and infection, contributing to airway hypersensitivity and bronchoconstriction.^{6–8} The neutrophil also has a role in the resolution of inflammation; however, the resolution mechanisms in asthma remain to be firmly established.⁹ We have previously revealed that expression of “don’t eat me” markers is increased and “eat me” markers is decreased on neutrophils in patients with allergic asthma.¹⁰ This could prolong the neutrophils’ presence in the tissue, preventing the resolution of inflammation and increasing their interaction with epithelial cells.

Tissue regeneration is regulated by many active signaling pathways in the epithelium, in particular those responsible for tissue growth and wound healing. One of these pathways is the canonical WNT signaling pathway which includes β -catenin, a protein involved in gene transcription and cell adhesion. The β -catenin signaling in epithelial cells can be activated by binding to neutrophils through intercellular adhesion molecule 1 (ICAM-1). β -catenin plays a crucial role in the regeneration of cells and tissues in the lung and controls tissue growth, healing, and resolution.^{11–13}

The epithelium affected by allergic asthma is mostly located in the bronchi. These cells can be retrieved using bronchial brushing or bronchoalveolar lavage (BAL), which is a rather invasive procedure. Nasal epithelial cells, which can be retrieved with a less invasive procedure than BAL, have similar inflammatory responses as bronchial airway epithelial cells. This makes nasal epithelial cell cultures suitable for functional studies of the lower airways.^{14,15}

To investigate the resolution of inflammation in allergic asthma, the present study is designed to characterize the interaction between neutrophils and airway epithelium during wound healing.

Methods

Ethics Statement

The study was approved by the local ethical committee in Stockholm, Karolinska Institutet, and all donors of epithelium and neutrophils gave their written informed consent. All methods were performed in accordance with the relevant guidelines and regulations.

Patient

Patients with allergic asthma and allergic rhinitis (AR) had a history of acute asthmatic response to birch, grass, or both birch and grass pollen. They exhibited a positive allergy screening result to skin prick test with sensitization to timothy and/or birch. All samples were collected outside pollen season, and none of the patients experienced any symptoms of their allergies. The patients underwent fractional concentration of exhaled nitric oxide measurement and spirometry with inhaled Terbutalin 0.5 mg/dose (Bricanyl). Blood immunoglobulin E and blood eosinophils and neutrophils were measured (eTable 1). There were 3 males and 5 females included with an age range from 24 to 56 (median: 35) years. None of the subjects used systemic or nasal corticosteroids 3 weeks before the visit. Those taking antihistamines were asked to refrain from them within 3 days before the investigation.

Healthy controls exhibited a negative ImmunoCap test and had no history of allergic or asthmatic ailments. There were 7 males and 7 females included with an age range from 19 to 50 (median: 33.5) years. Both the patients with asthma and the healthy controls were sampled on 2 separate occasions. The first visit included an introduction to the study, blood sampling in heparin tubes, and nasal brush epithelial cell (human nasal epithelial cells [HNECs], human nasal epithelial cells from allergic asthmatic donors [aHNECs], human nasal epithelial cells from healthy donors [hHNECs]) collection for culture (Fig 1A–C). The second visit included blood collection in heparin tubes (Fig. 1D–E).

Isolation and Culture of Human Nasal Epithelial Cells

Approximately 2 weeks before the scratch assay, HNECs were isolated by nasal brushing as described previously.¹⁶ Cells were maintained in collagen-coated tissue culture flasks in keratinocyte serum-free medium (Gibco, Waltham, MA), supplemented with 0.005 μ g/mL epidermal growth factor (Gibco, Waltham, MA), 0.05 mg/mL bovine pituitary extract (Gibco, Waltham, MA), 40 U/mL penicillin, 40 μ g/mL streptomycin, and 0.025 μ g/mL Fungizone (complete keratinocyte serum-free medium). Furthermore, 10 ng/mL of ciprofloxacin was added to the media for the first passage and removed in the later passages. All cells were cultured in a

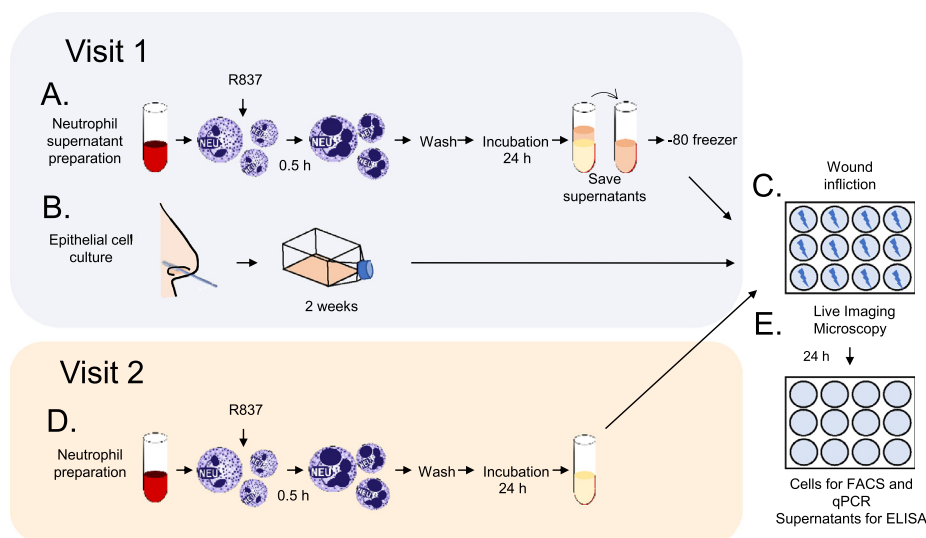


Figure 1. Schematic overview of the method. Visit 1: A. Neutrophil supernatant preparation. Neutrophils were isolated from blood from patients with allergic asthma and healthy controls. The neutrophils were subsequently stimulated by R837 (Imiquimod) for 30 min, washed, followed by a 24 hour incubation. The supernatants from the activated neutrophils were then stored in -80 degrees until the scratch assay was performed. B. Epithelial cell culture. Nasal epithelial cells were isolated from patients with allergic asthma and healthy controls. The cells were cultured for about 2 weeks before harvest and wound infliction (C). Visit 2: D. Neutrophil preparation. The same neutrophil procedure as on visit 1 is performed, but instead of saving the supernatants, the activated neutrophils were added to the epithelial cells with wound inflictions (C). E. The cells were incubated for 24 hours and the epithelial regeneration was monitored by live imaging microscopy. The cells were collected for FACS, qPCR and ELISA analysis after 24 hours.

humidified chamber at 37°C with 5% carbon dioxide. Cells from passages 2 to 5 were used.

Neutrophil Preparation and Scratch Assay

On the day of the scratch assay, fresh peripheral blood was collected in heparin tubes. The neutrophils were isolated using the MACSExpress Neutrophil Isolation Kit and the Erythrocyte Depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Isolated neutrophils were resuspended in a complete medium (CM) consisting of RPMI 1640 (Invivogen, San Diego, California) with 20% sterile filtered autologous plasma (0.2 μ m filter, Sarstedt, Nümbrecht, Germany), 100 U/mL penicillin and 100 μ g/mL streptomycin (Invivogen, San Diego, California), to a concentration of 2×10^6 cells/mL. Furthermore, 5×10^5 neutrophils were taken to a fluorescence activated cell sorting (FACS) tube for staining as control. The remaining neutrophils were activated with 30 μ g/mL R837 (Imiquimod), an imidazoquinoline amine analog to guanosine (Invivogen) for 30 minutes. The cells were washed twice in CM, and 5×10^5 neutrophils were taken aside for antibody staining for FACS analysis (eTable 2A and eFig 1). The remaining neutrophils were resuspended in keratinocyte serum-free medium. The neutrophils were added to the plate with epithelial cells in different concentrations. For supernatant (SN) preparation, neutrophils were isolated on the same occasion as the HNEC isolation. The same concentration of neutrophils as in the scratch assay was added to a plate without epithelial cells and incubated for 24 hours (Fig 1). The medium was then removed, centrifuged free from cells, and frozen at -80°C for approximately 2 weeks. The SN samples were thawed on the day of the scratch assay and added to wells with epithelial cells. The epithelial cells were cultured in 24-well plates for 24 hours before the scratch assay. On the day of the assay, a scratch was inflicted using a 1000- μ L tip. The wells were washed with phosphate-buffered saline (PBS), and autologous neutrophils and autologous SN were added. Neutrophils from healthy control participants were added in the same manner as the autologous neutrophils in the experiments where epithelial cells from patients with allergic asthma were used. The wells were then monitored with a live-imaging microscope (Leica DMI6000) taking an image every hour for

18 hours. For example, pictures of the wounds from healthy donors and donors with allergic asthma (Fig. 2D). The images were then analyzed with ImageJ where the wound was measured. Supernatants from the plate were collected at the end of the experiment and stored at -80°C . HNECs and neutrophils were collected for FACS analysis (eTable 2B and eFig 2) and stored in RNAProtect for further ribonucleic acid (RNA) analysis.

Fluorescence Activated Cell Sorting Analysis of Blood, Human Nasal Epithelial Cells, and Neutrophils

Blood was used for FACS analysis of the neutrophils and eosinophils. The blood was treated with ammonium chloride erythrocyte lysis solution (0.8 mM NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA). The cells were stained with antibodies for 20 minutes (eTable 2C). After the scratch assay, neutrophils and HNECs were divided between FACS tubes. The neutrophils were washed from the HNECs using PBS, and the HNECs were removed from the plate using 0.1 % trypsin. The neutrophils and HNECs were stained for 20 minutes with antibodies (eTable 2A and B). Cells were washed with PBS and resuspended in PBS with 1% PFA. Cells were analyzed on an LSRFortessa analyzer (BD) and identified based on forward- and side-scatter properties and cell-specific antibodies. The collected data were processed using FlowJo software (BD).

Ribonucleic Acid Preparation and Quantitative Polymerase Chain Reaction

RNA from neutrophils was isolated using the RNeasy Micro Plus Kit (Qiagen, Hilden Germany) according to the manufacturer's instructions. The quantity and quality of RNA were determined by a spectrophotometer using the wavelength absorption ratios 260/280 and 260/230. Furthermore, 400 ng RNA was reverse transcribed into complementary deoxyribonucleic acid using the Omniscript Reverse Transcription Kit (Qiagen, Hilden Germany) with Oligo(dT)18 primers (Thermo Fisher Scientific, Waltham, Massachusetts) on an xPeltier Thermal Cycler 200 (MJ Research, Watertown, Massachusetts). All quantitative polymerase chain reaction (qPCR) reactions used the QuantiFast Multiplex PCR Kit (Qiagen, Hilden Germany) and were

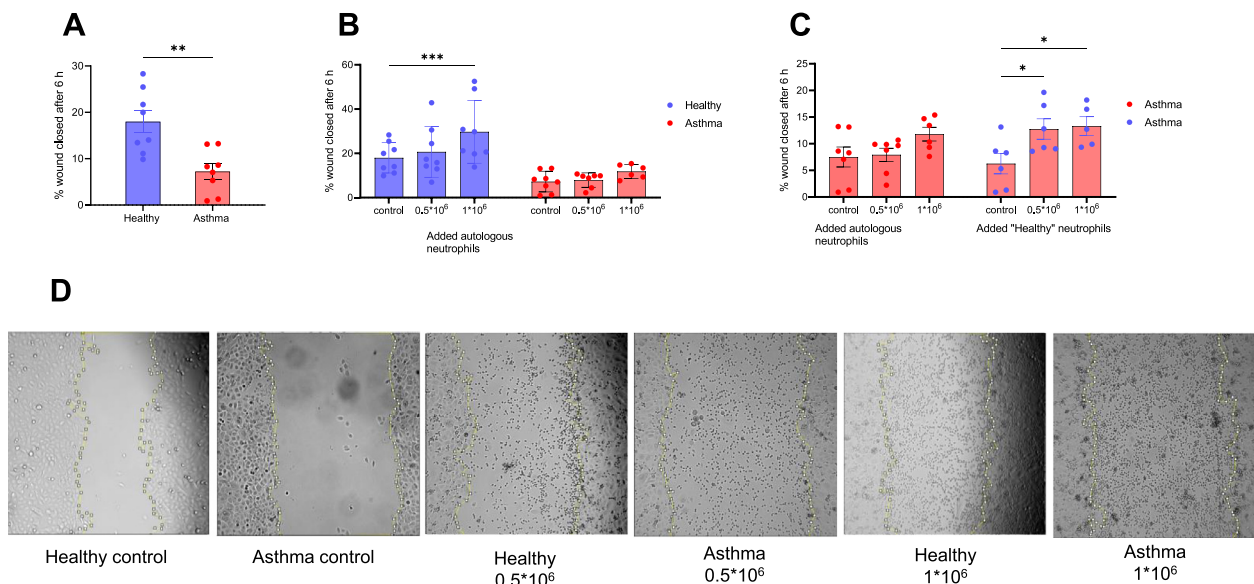


Figure 2. Epithelial regeneration after 6 hours, with/without coculture with activated neutrophils. (A) Epithelial regeneration without neutrophils (n = 8). (B) Healthy (n = 8) and allergic asthmatic epithelial regeneration with/without neutrophils (control: n = 8, 0.5×10^6 : n = 7 and 1×10^6 : n = 6). (C) Allergic asthmatic epithelial cells with autologous (control: n = 7, 0.5×10^6 : n = 7 and 1×10^6 : n = 6) or healthy neutrophils (control: n = 6, 0.5×10^6 : n = 6 and 1×10^6 : n = 5). (D) Example pictures of wounds after 6 hours. The area of each wound has been highlighted manually to increase the readability, see yellow marking.

performed on a Stratagene mx3000p machine (Agilent Technologies, Santa Clara, California). All reactions were run with the following program: activation at 95°C for 5 minutes followed by 40 cycles of 2-step cycling with denaturation at 95°C for 45 seconds and annealing or extension at 60°C for 45 seconds. Primers and probes for reference genes were obtained from Sigma Aldrich (KiCqStart Probe Assays, GAPDH fwd: ACAACGAATTTGGCTACAGC, rev: AGTGA-GGGTCTCTCTCTCC, probe: [Cy5]ACCACCAGCCCCAGCAAGAGCACAA [QQD]), ACTB fwd: CAAGATGAGATTGGCATGGC, rev: CACATTGTGA-ACITTTGGGGG, probe: [HEX]TGACAGCAGTCGGTTGGAGCGAGCA[OQA]). The following target primers were obtained at Thermo Fisher Scientific: β -catenin (CTNNB1) Hs00355045_m1, CD16 (FCGR3A), Hs02388314_m1, and CD62L (SELL) Hs00174151_m1. The $2^{-\Delta\Delta Ct}$ values were calculated, and the average of the control group was set to 1.

Measurement of Interleukin-8 and Tumor Necrosis Factor Alfa

Cell-free supernatants from the wound healing assay were analyzed for levels of interleukin (IL)-8 (antigen sensitivity: 31.20–2000 pg/mL) and tumor necrosis factor alfa (TNF- α) (15.6–1000 pg/mL) using enzyme-linked immunosorbent assay (R&D Systems Minneapolis, Minnesota) according to the manufacturer's instructions.

Statistic

Statistical analyses were performed with GraphPad Prism version 9.3.1 (GraphPad Software, La Jolla, California). All data are illustrated as mean plus or minus SEM. A *P* value of less than .05 was considered statistically significant (**P* < .05, ***P* < .01, ****P* < .001). A D'Agostino and Pearson omnibus normality test was used to determine whether the data were normally distributed. For 2 sets of data, a *t* test was performed as the data were normally distributed data. For more than 2 sets of data, a one-way analysis of variance or two-way analysis of variance with Dunnett's posthoc test was performed.

Results

Patients With Allergic Asthma and Healthy Controls Are Comparable

The proportion of neutrophils and eosinophils is somewhat higher in the blood from the group with allergic asthma than that from the healthy control group. Antigen expression and the activation state of the neutrophils and eosinophils in the blood were not significantly different between those with allergic asthma and healthy controls before the experiment started (eTable 3A and B and eFig 1). The isolated neutrophils had a purity of $88.42\% \pm 13.92\%$ which was considered acceptable.

Reduced Regeneration in Human Nasal Epithelial Cells From Donors With Allergic Asthma

The status of the epithelial regeneration was evaluated 6 hours after the scratch. The hHNEC had $17.9\% \pm 2.43\%$ of the wound closed (*n* = 8) compared with $7.2\% \pm 1.65\%$ in the group with asthma (*n* = 8). Thus, the healing process was significantly protracted in cells from asthma donors (Fig 2A). The experiment was monitored for 18 hours, and all the downstream analyses were performed after 18 hours. There was no significant difference between the wound for patients with allergic asthma and that of the healthy controls after 18 hours (Fig 2A).

Neutrophil-Dependent Wound Healing

To understand the neutrophil's role in the healing and regeneration of HNEC, different numbers of autologous activated neutrophils

were added to the epithelial wound as cocultures. The status of the epithelial regeneration was evaluated 6 hours after the scratch. hHNEC reached $20.6\% \pm 4.07\%$ and $29.7\% \pm 5.02\%$ wound closure when cocultured with 500,000 and 1 million autologous neutrophils, respectively. The epithelial regeneration in the coculture with 1 million neutrophils was significantly increased compared with the control ($17.9\% \pm 2.43\%$). When autologous neutrophils from donors with allergic asthma were cultured with the aHNEC, no significant improvement in epithelial regeneration was found. The aHNEC reached $7.9\% \pm 1.23\%$ and $11.8\% \pm 1.29\%$ closure when cocultured with 500,000 and 1 million autologous neutrophils, respectively, compared with aHNEC without the addition of activated neutrophils ($7.2\% \pm 1.65\%$) (Fig 2B).

Neutrophils From Healthy Donors Improve the Regeneration of Human Nasal Epithelial Cells From Donors With Allergic Asthma

The same type of coculture of aHNEC, as stated previously, was then repeated, but this time, the autologous neutrophils were exchanged with neutrophils from the healthy donors. The regeneration after 6 hours was $12.8\% \pm 1.92\%$ and $13.3\% \pm 1.75\%$ closure for coculture with 500,000 and 1 million healthy neutrophils, respectively. The corresponding control, without neutrophils, reached $6.3\% \pm 1.93\%$ closure. In contrast with autologous neutrophils, neutrophils from healthy donors improved the regeneration process of the aHNEC (Fig 2C).

Interleukin-8 Decreases During the Regeneration of Human Nasal Epithelial Cells From Healthy Donors, but Not Human Nasal Epithelial Cells From Donors With Allergic Asthma

The production of IL-8 was evaluated in the supernatant after 18 hours. hHNECs exhibited $11,263 \pm 2318$ pg/mL of IL-8 compared with 5858 ± 1433 and 3777 ± 604 pg/mL in supernatant when cocultured with 500,000 and 1 million autologous neutrophils, respectively. In supernatants from aHNEC, the corresponding IL-8 levels were $10,620 \pm 3166$, 7268 ± 2248 , and 7327 ± 3196 pg/mL in control, coculture with 500,000 and 1 million autologous neutrophils, respectively. The IL-8 production was significantly down-regulated in hHNECs cocultured with 1 million autologous neutrophils compared with the control. No down-regulation of the IL-8 production was found in the aHNEC (Fig 3A).

Tumor Necrosis Factor Alfa Decreases During the Regeneration of Both Human Nasal Epithelial Cells From Healthy Donors and Human Nasal Epithelial Cells From Donors With Allergic Asthma

The supernatant level of TNF- α seemed to decrease after coculture with neutrophils. However, this reduction did not reach statistical significance and occurred in supernatants from both patients with asthma and controls. Hence, the involvement of TNF- α in the regeneration of the airway epithelium might be limited in both groups (Fig 3B).

β -Catenin Changes During Regeneration of Human Nasal Epithelial Cells From Healthy Donors, but Not Human Nasal Epithelial Cells From Donors With Allergic Asthma

The levels of β -catenin RNA were also measured and the $2^{-\Delta\Delta Ct}$ values were calculated. The levels of β -catenin were down-regulated in hHNECs after neutrophil exposure compared with the aHNECs. There was no significant down-regulation of β -catenin in aHNECs (Fig 4).

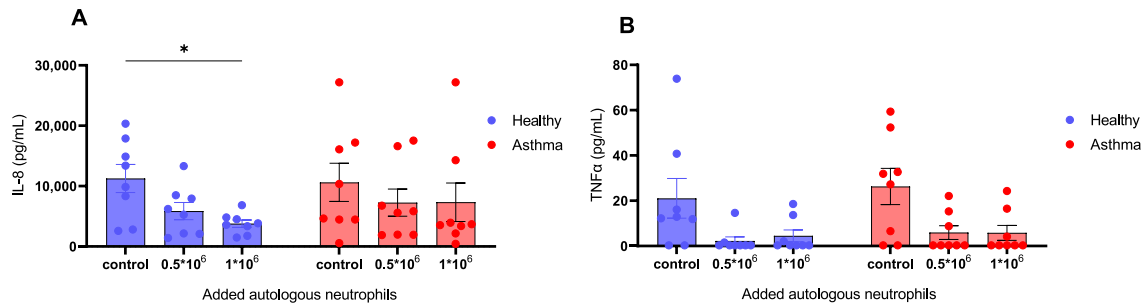


Figure 3. (A) IL-8 and (B) TNF- α secretion during coculture between neutrophils and epithelial cells after 18 hours ($n = 8$). The control is epithelial cells without neutrophils. IL-8, interleukin-8; TNF- α , tumor necrosis factor alpha.

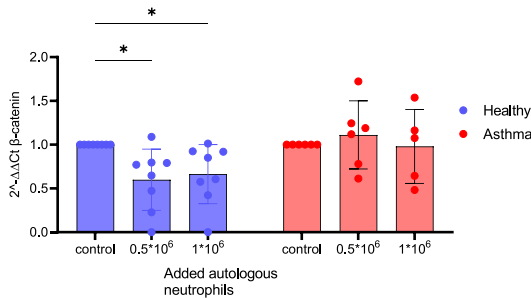


Figure 4. β -catenin mRNA expression during neutrophils and epithelial cells coculture after 18 hours. With healthy ($n = 8$) and allergic asthmatic cells (control: $n = 6$, 0.5×10^6 : $n = 6$ and 1×10^6 : $n = 5$). The control is epithelial cells without neutrophils. mRNA, messenger ribonucleic acid.

Neutrophilic Modulation on Epithelial Cells Is Dependent on Direct Cell-to-Cell Interaction

To find out whether the neutrophilic enhancement of epithelial cell closure was because of direct or indirect contact between the neutrophil

and the epithelial cell, supernatants from activated neutrophils were cocultured with autologous epithelial cells. There was no difference in the healing process when the effect of supernatants from neutrophils from both healthy donors and donors with asthma was evaluated (Fig 5A).

The surface expression of ICAM-1 was measured on the HNEC after 18 hours. There was an increase in the expression of ICAM-1 on HNEC when cultured with supernatant from autologous activated neutrophils compared with the control. In contrast, no significant difference in ICAM-1 expression was found in the aHNEC during the same circumstances. hHNEC had an MFI for ICAM-1 of $10,057 \pm 3159$, $15,645 \pm 5522$, and $15,502 \pm 5403$ for control, culture with supernatant from 500,000 neutrophils and 1 million neutrophils, respectively. Whereas the culture from the aHNEC had MFI levels at 9866 ± 1724 , 9619 ± 1107 , and 9487 ± 729 for control, culture with supernatant from 500,000 neutrophils and 1 million neutrophils, respectively (Fig 5B).

Furthermore, neither IL-8 (Fig. 6A) nor TNF- α (Fig. 6B) was reduced at 18 hours when HNECs were cultured with supernatants from activated neutrophils. This stands in contrast to the reported reduction of IL-8 after cocultures of hHNECs with neutrophils (discussed previously) and of TNF- α for both aHNECs and hHNECs (discussed previously).

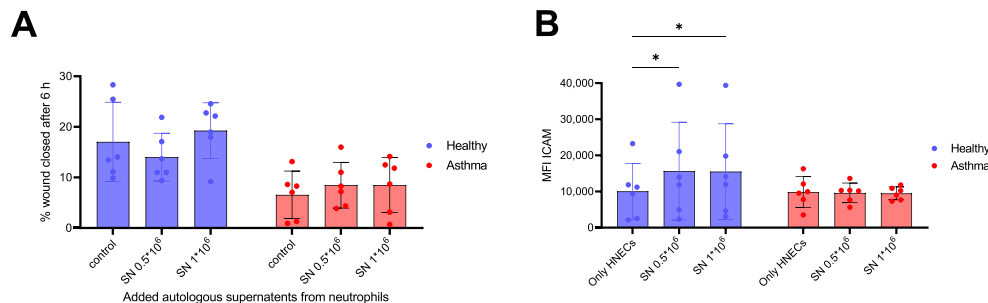


Figure 5. (A) Regeneration of epithelial cells with SN from activated neutrophils after 6 hours ($n = 6$) and (B) ICAM-1 expression on epithelial cells cultured with the same conditions after 18 hours ($n = 6$). The control is epithelial cells without neutrophils. HNECs, human nasal epithelial cells from donors with allergic asthma; ICAM-1, intercellular adhesion molecule 1; SN, supernatant.

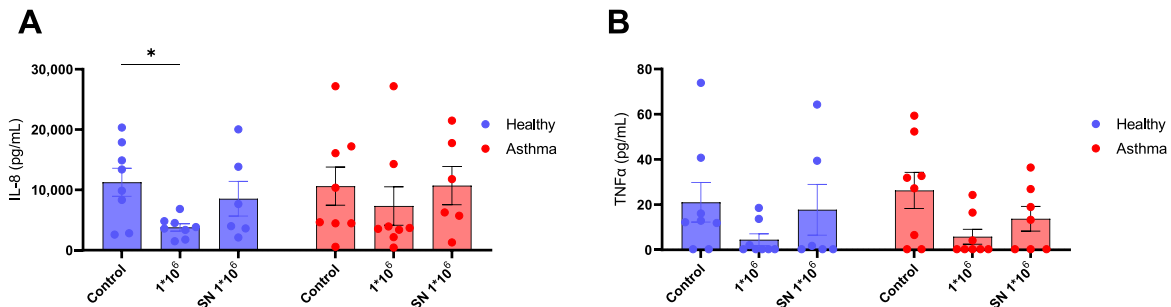


Figure 6. IL-8 and TNF- α production from epithelial cells with/without neutrophils (1×10^6) or SNs (SN 1×10^6) from activated neutrophils after 18 hours. (A) IL-8 production (control: $n = 8$, 1×10^6 : $n = 8$ and SN 1×10^6 : $n = 6$ for both healthy and asthma). (B) TNF- α production with healthy (control: $n = 8$, 1×10^6 : $n = 8$ and SN 1×10^6 : $n = 6$) and allergic asthma (control: $n = 8$, 1×10^6 : $n = 8$ and SN 1×10^6 : $n = 7$). The control is epithelial cells without neutrophils. IL-8, interleukin-8; SN, supernatant; TNF- α , tumor necrosis factor alpha.

No Difference in Neutrophil Activation After In Vitro Activation Between Healthy and Disease

The stimulation of purified neutrophils with R837 was evaluated using the activation markers CD11b, CD66b, and CD62L. The activation levels of neutrophils before and after the stimulation with R837 are the same between neutrophils from healthy donors and donors with allergic asthma (eFig 3).

Discussion

This study reveals that the regeneration pattern of “asthmatic” aHNEC is impaired when compared with “healthy” hHNEC. Neutrophils from healthy donors increase the regeneration rate of both the hHNEC and aHNEC. In contrast, neutrophils from donors with asthma do not alter the regeneration rate. The neutrophil-induced improved regeneration seems to possibly be mediated through interaction with ICAM-1. Furthermore, there seems to be a dysregulation of the β -catenin expression in aHNEC which is not found in hHNEC.

Isolated cells from patients with allergic asthma and healthy controls were identical in their state of activation indicating that the effects found in the scratch assay are actual effects of the intervention and not an effect of allergic asthma per se. The purity of the isolated neutrophils was acceptable, and their state of activation was similar, with identical levels of CD11b, CD66b, and CD62L being the same (eFig 3).

The fact that the aHNEC demonstrates impaired healing is in line with our expectations, and it supports the idea of a model representing the allergic airway.¹⁷ What is remarkable is that neutrophils, which in the past have been more associated with airway damage, now increase the regeneration rate in a healthy setup. This is not the case in the diseased setup. Still, neutrophils from a healthy donor could increase the regeneration rate of aHNEC, but not to the same extent as in the healthy setup, suggesting that an ideal healing mechanism relays not only on the neutrophil but also on the epithelium.

IL-8 is known to decrease in the epithelium when enough neutrophils have reached the site of inflammation.¹⁶ This is a safety mechanism to prevent an adverse number of neutrophils from gathering in the tissue. This mechanism is impaired in the cocultures of patients with allergic asthma. This is in line with previous results revealing a reduced epithelial IL-8 production when neutrophils come in contact with the healthy epithelium but not when they come close to epithelium from donors with asthma.¹⁸ In contrary to what was expected, IL-8 was not decreased in wells containing 1,000,000 healthy donor neutrophils. This is probably caused by neutrophils themselves, releasing IL-8 into the supernatant.

To further investigate whether the neutrophilic enhancement of epithelial cell closure was because of direct or indirect contact between the neutrophil and the epithelial cell, supernatants from activated neutrophils were cocultured with autologous epithelial cells. The ICAM-1 expression was measured on the HNEC in both coculture and supernatant culture after 18 hours. The increased ICAM-1 expression on the hHNEC in the supernatant culture compared with the control is a second indication that wound healing is mediated by cell-cell interactions. The HNECs have increased their ICAM expression because nothing has bound the epithelial cells.

The role of β -catenin in inflammation seems to be complex and has traditionally been associated with airway remodeling in chronic asthma.¹⁹ A complete inhibition of β -catenin with a specific shRNA is known to significantly aggravate inflammation, leading to an accumulation of neutrophils and lymphocytes and the production of proinflammatory cytokines such as IL-1 β , TNF- α , and IL-6 in the BAL fluid.²⁰ It has also been found that the β -catenin pathway often is dysregulated in impaired tissue restoration.^{11,21} β -catenin has to be transiently activated to promote the resolution of inflammation and reach homeostasis.²² The role of neutrophils in lung tissue repair has likewise been associated with β -catenin.^{23,24} Presently, β -catenin

was still expressed in aHNEC after the resolution, which was not found in hHNEC, indicating a dysregulation of the β -catenin in aHNEC. Hence, if this pathway should be targeted in future therapeutic attempts, a fine-tuned approach will probably be necessary to induce relief of symptoms rather than aggravate the disease.

The qPCR analysis was conducted on the HNEC from the coculture. The cell layer was washed once before the analysis to minimize the neutrophil contamination in the qPCR. To determine the level of neutrophil contamination, qPCR on neutrophil-associated genes was performed. CD16 and CD62L generated no Ct values when the same concentration of complementary deoxyribonucleic acid was analysed as in the other qPCR analyses. It is therefore likely that the levels of β -catenin measured were solely generated by epithelial cells.

A limitation of the study is the rather small sample size and the use of nasal epithelial cells instead of bronchial epithelial cells. Previous studies have revealed that nasal epithelial cells could be suitable for functional studies of the lower airways.^{14,15} Bronchial epithelial cells can be retrieved using bronchial brushing or BAL, but the derivation of nasal epithelium is much less invasive. The difference in inflammatory response between nasal epithelial cells and bronchial airway epithelial cells has been studied in vitro¹⁵ and learned to be negligible; the bronchial and the nasal airways have also been found to be close to identical on a transcriptional level.¹⁴ The bronchial epithelium from patients with allergic asthma is usually considered to be more affected by the disease and therefore more fragile than the nasal epithelium.⁵ It is, therefore, likely that the effects found in the present setup might be even more pronounced if the study was conducted using bronchial epithelial cells. Moreover, the use of nasal epithelial cells makes the findings relevant for AR. Last, ideally, the β -catenin levels should have been measured in the middle of the wound healing procedure to better understand the kinetics of β -catenin expression. This would have improved our ability to speculate on a specific role of β -catenin in the airway inflammatory resolution process.

Finally, it is tempting to conclude that the resolution of airway inflammation of the epithelial barrier is impaired in patients with allergic asthma. The speed of recovery can be increased during normal circumstances through direct contact between activated neutrophils and epithelial cells, something that is not happening in asthma. Targeting the failed interaction between neutrophils and epithelium might prove a novel therapeutic goal for the treatment of allergic asthma and AR. β -catenin, found to be up-regulated in the asthmatic samples, might conjure a specific target.

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Disclosures

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Supplementary Data

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Supplementary Data

eTable 1
Subject Characteristics and Baseline Physiology

Subject characteristics	Allergic asthmatics (n = 8)
Specific IgE (kE/L)	170 (65–300)
Exhaled NO ₅₀ (ppb)	31 (12.0–74.3)
FEV1 (% of predicted)	95 (93.3–102.3)
FVC (% of predicted)	82.5 (74–107.8)
MFEF (% of predicted)	77.7 (60.3–89.5)

Abbreviations: IgE, immunoglobulin E; FEV1, forced expiratory volume in 1 second; FVC, forced vital capacity; MFEF, maximal forced expiratory flow.
NOTE. Data are expressed as a median (interquartile range).

eTable 2
Antibody Panels Used for Flow Cytometry Analysis. A, Panel Targeting Neutrophil Specific Antigens Used on Purified Unstimulated and Stimulated Neutrophils. B, Panel Targeting Antigens on Neutrophils and Eosinophils in the Blood. C, Panel Targeting Antigens on HNECs

Table 2A.			
Antigen	Fluorochrome	Clone	Antibody supplier
CD15	BV711	W6D3 and 7C3.rMAb	BD
CD62L	BV510	DREG-56	BD
CD11b	AF700	ICRF44	BD
CD16	V450	3G8	BD
CD66b	PERCP5.5	G10F5	BD
Table 2B.			
Antigen	Fluorochrome	Clone	Antibody supplier
ICAM-1/CD54	FITC	BBA20	R&D
CD326/EpCAM	APC	EBA-1	BD
Table 2C.			
Antigen	Fluorochrome	Clone	Antibody supplier
CD15	BV711	W6D3 and 7C3.rMAb	BD
CD16	FITC	3G8	BD
CD11b	AF700	ICRF44	BD
Siglec8	APC	7C9	BioLegend
CD69	PE	G44-26	BD
CD44	BV421	G44-26	BD
CD125	BV786	A14	BD

Abbreviations: HNEC, human nasal epithelial cell from donors with allergic asthma; ICAM-1, intercellular adhesion molecule 1.

eTable 3
Neutrophil and Eosinophil Status in the Blood

Table 3A					
		% Eosinophils		% Neutrophils	
Healthy		0.8750 ± 0.1838		10.17 ± 2.776	
Asthma		2.69 ± 1.025		10.68 ± 2.669	
Table 3B					
Cell type	Antigen	Healthy	Allergic asthma	Clone	Antibody supplier
Eosinophils	CD11b	3.505 ± 2.012	13.91 ± 12.06	ICRF44	BD
	CD44	2.890 ± 0.3497	2.748 ± 0.3527	G44-26	BD
	CD69	1.777 ± 0.4362	2.263 ± 0.9302	FN50	BD
	CD125	5.057 ± 0.3966	5.462 ± 1.244	A14	BD
Neutrophils	CD11b	1.813 ± 0.6357	3.23 ± 2.594	ICRF44	BD
	CD44	2.612 ± 0.4203	2.642 ± 0.4117	G44-26	BD
	CD69	1.282 ± 0.1878	1.095 ± 0.2453	FN50	BD
	CD125	5.608 ± 0.8704	4.533 ± 1.156	A14	BD

NOTE. Neutrophils were gated using CD15, eosinophils using Siglec8, and specific antigens were analyzed. Antigen expression on neutrophils and eosinophils was similar for patients with allergic asthma and the healthy controls, that is, they had the same proportions of activated neutrophils and eosinophils before the resolution assay. Data are expressed as mean percent ± SEM, n = 12.

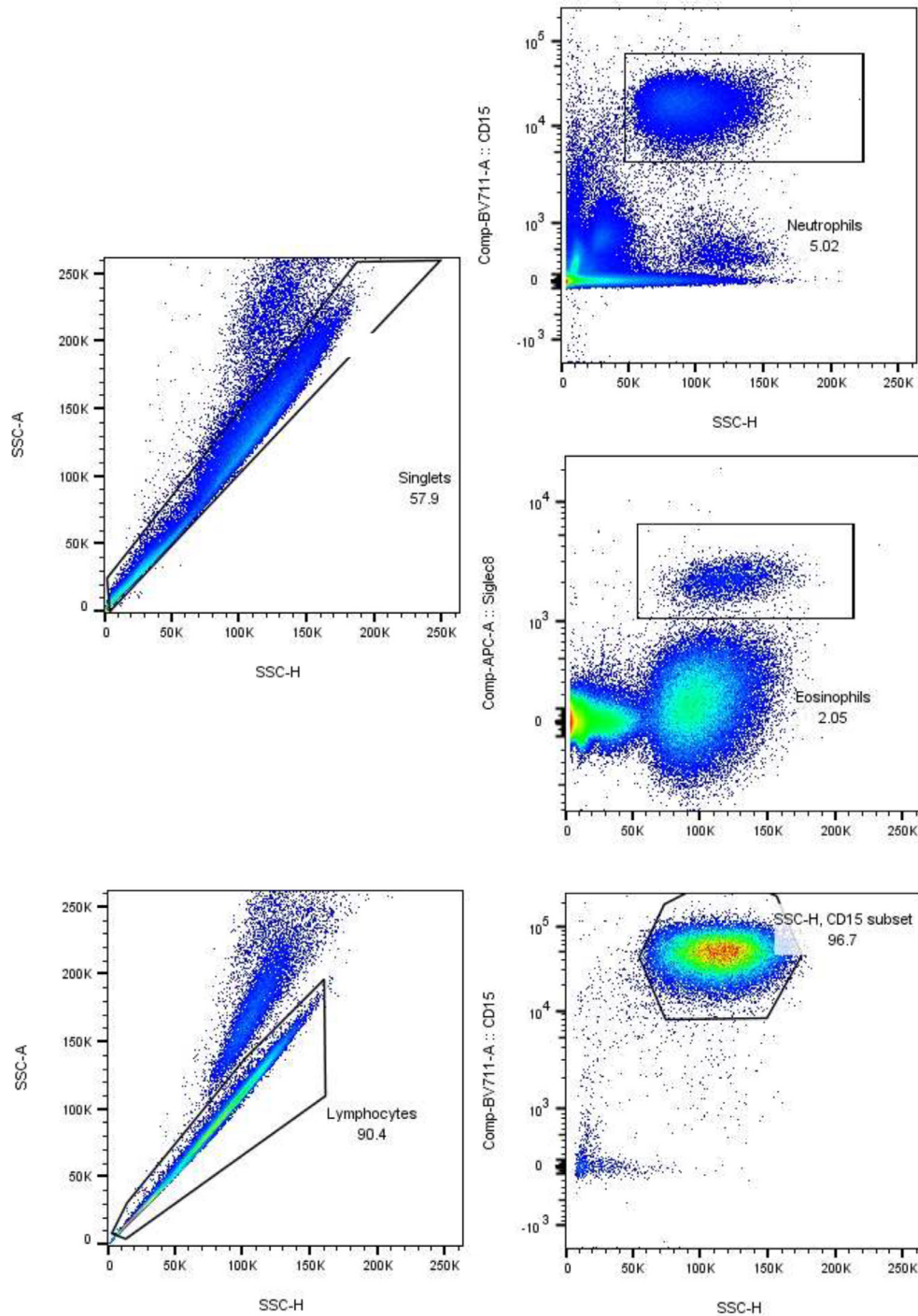
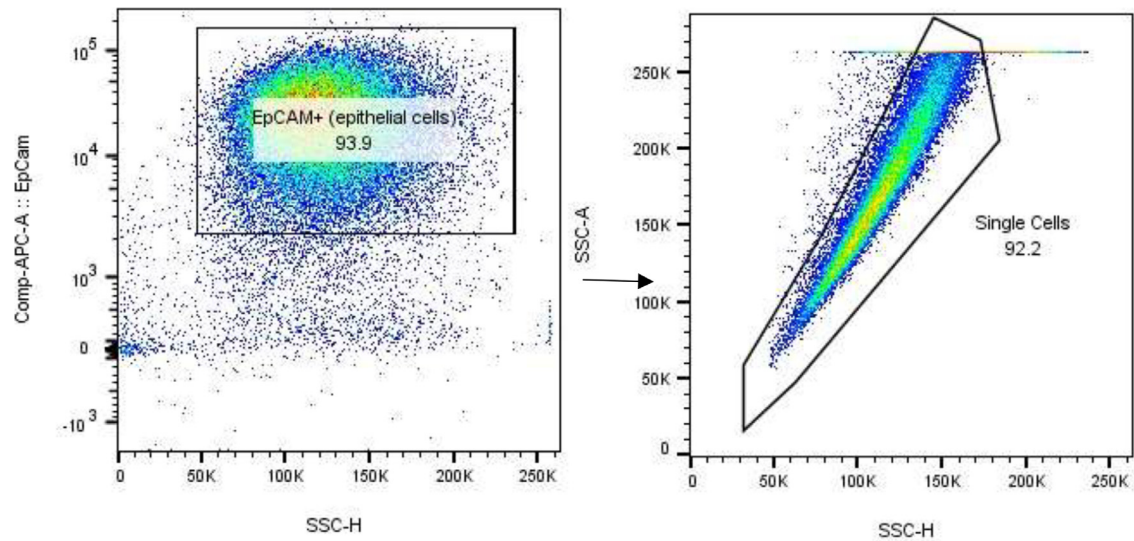
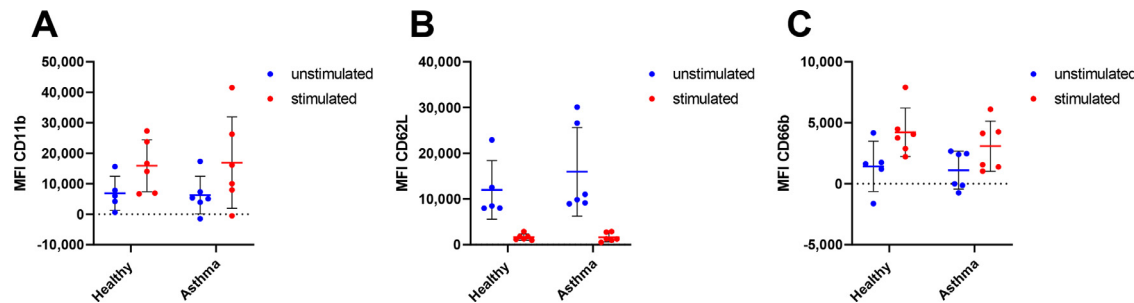


Figure 1. (A) Gating strategy for neutrophils and eosinophils from the whole blood. Single cells were selected, CD15 was used to gate neutrophils, and Siglec8 was used to gate eosinophils. (B) Gating strategy for neutrophils purified from whole blood. Single cells were selected, and CD15 was used to gate neutrophils.



eFigure 2. Gating strategy for HNECs. HNECs were gated using Epcam and single cells were gated. HNEC, human nasal epithelial cell.



eFigure 3. CD11b, CD62L, and CD66b on neutrophils from healthy and disease. Neutrophils have been purified from whole blood and activated with R837. The unstimulated and stimulated neutrophils from healthy donors and donors with allergic asthma have the same activation pattern for the markers A: CD11b; B: CD62L; and C: CD66b.