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Dividing neutrophils in subsets, reveals a significant role for activated neutrophils in the development of airway hyperreactivity

Running head: CD16^{high} CD62L^{dim} neutrophils have a significant role in the development of airway hyperreactivity

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Dividing neutrophils in subsets, reveals a significant role for activated neutrophils in the development of airway hyperreactivity

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Abstract

Background: Previous research has emphasized the importance of eosinophils in allergic asthma, while paying less attention to neutrophils. The known functionality of neutrophils in the inflammatory process has recently changed and knowledge about subsets of neutrophils, as characterized by their expression of CD16 and CD62L, has surfaced. Their specific roles in asthma are still unknown.

Objective: To study the functional differences between subsets of neutrophils by characterising the impact of individual subsets on airway smooth muscle reactivity.

Methods: The direct effect of neutrophils on airway hyper-responsiveness was assessed by co-culturing different subsets of neutrophils (produced by LPS *in vitro* stimulation) with human isolated small airways or murine tracheae with subsequent evaluation of smooth muscle reactivity to bradykinin in myographs. Supernatants and tissue were saved for ELISA and immunohistochemistry.

Results: The CD16^{high}CD62L^{dim} neutrophils were found to enhance the response to bradykinin in both human isolated small airways and murine tracheae. No such effects were obtained for the other subsets. The response is due to an upregulation of bradykinin receptor 2 through release of TNF α from the neutrophil.

Conclusions & Clinical Relevance: The present study introduces a new concept regarding the role of neutrophils and defines a novel direct link between a specific activated neutrophil subset and airway smooth muscle, establishing neutrophils as important players in the development of asthmatic airway hyperactivity.

ABBREVIATIONS:

BK - Bradykinin

LPS - lipopolysaccharide

PBS - Phosphate Buffered Saline

IL-1 β - Interleukin 1 beta

IL-1Ra - Interleukin 1 receptor

TNF α - Tumor necrosis factor alpha

RPMI 1640 - Roswell Park Memorial Institute 1640

DMEM - Dulbecco Modified Eagle Medium

NGS - Normal goat serum

Introduction

Inflammatory cells, such as neutrophils and eosinophils, are found in the airways and lungs of asthmatic patients. They contribute to chronic inflammation, airway obstruction and airway hyperresponsiveness¹. Previous research has emphasized the importance of eosinophils in allergic asthma, with less attention being devoted to neutrophils. The latter have been easy to overlook, as they are present in the lung at all times, even when the asthmatic patients seem to have no symptoms². Nevertheless, neutrophils have been shown to increase in number in the lung upon viral infections³, colonisation of bacteria⁴, and in uncontrolled asthma⁵.

A recent study by Pillay *et al.* identified that neutrophils can be subdivided into three subsets, based on their expression of CD16 and CD62L, each subset representing a different stage of maturity and activity. The CD16^{dim} CD62L^{high} subset is considered the immature neutrophil, recently

released from the bone marrow. These neutrophils are not found in the circulation in healthy donors, but appear 180 minutes after an LPS injection to the blood and are even more pronounced in severely injured patients. The CD16^{high} CD62L^{high}-expressing neutrophils are mature, but non-activated, whereas the CD16^{high} CD62L^{dim}-expressing neutrophils are considered to be mature and activated, playing an important role in inflammatory diseases such as systemic inflammation ⁶. Subsets of neutrophils with similar antigen presentation characteristics have been produced *in vitro* using LPS and we have

shown that these subsets have specific roles in allergic rhinitis ⁷ and head and neck squamous cells cancer ⁸.

Bradykinin has been proposed as a sensitive indicator for AHR ^{9,10}, found to be increased in broncho-alveolar lavage (BAL) fluid from asthmatic patients as a result of allergen challenge¹¹. In the presented paper we have investigated the role of activated neutrophils, produced by LPS *in vitro* stimulation, on Bradykinin induced AHR.

Methods

Ethics Statement

Human lung tissue (2010/181-31/2) and blood (2014/299-13) were obtained at the Karolinska University Hospital, Stockholm, and were approved by the local ethics committee at Karolinska Institute. All participants provided written informed consent. All animal procedures were sanctioned by the regional animal experimental ethical review board at Karolinska Institute, Stockholm (Stockholm norra djurförsöksetiska nämnd; ethical permit no. N153-11 and N258-13).

Neutrophil purification for later co-culture with isolated tissue

Fifteen healthy, non-allergic, non-asthmatic volunteers were recruited to provide blood samples to be used in neutrophil purification. Fresh peripheral blood was collected from in lithium heparin tubes.

Neutrophils were purified using ficoll-paque (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's instructions. The granulocyte rich pellet was treated with ammonium chloride erythrocyte lysis solution (0.8 % NH₄Cl, 10 mM KHCO₃ 0.1 mM EDTA). Neutrophils were then resuspended in complete medium (CM) consisting of RPMI 1640 (Invivogen, San Diego, CA, USA) with 20% autologous plasma, 100 U/mL penicillin and 100 µg/mL streptomycin (Invivogen, San Diego, CA, USA), to a concentration of 2 x 10⁶ cells /mL. To mimic different subsets of neutrophils in clearly defined groups, CD16^{high} CD62L^{high} neutrophils, that constituted half of all the neutrophils purified from the blood, were treated with 1 µg/mL lipopolysaccharide *In vitro* (LPS, L2654, Sigma, St. Louis, MO, U.S.A) for 2 h at 37°C in humidified 5% CO₂ in atmospheric air. All the cells stimulated with LPS were subsequently found to be in the CD16^{high} CD62L^{dim} subset. The remaining LPS were then washed twice with PBS to ensure minimal LPS contamination and resuspended to a concentration of 2 x 10⁶ cells /mL in CM and used for the co-culture. The risk of remnant LPS in the cell suspension that become added to the culture plate, is infinitesimal, it falls short of 2,6 pg/ml. Steroid resistance of non-activated neutrophils was assessed by pre-treating the cells with 7.6 µM fluticasone for 30 min before adding the LPS. To assess the role of IL-1β, IL-1βRA was added to the activation and co-culture set-up in one experiment. The supernatants from these experiments were saved in -80 prior to ELISA.

To confirm the establishment of the correct population and the purity of neutrophils (94-97 %), the cells were analysed with flow cytometry.

In order to further compare our *in vitro* subsets with the *in vivo* subsets of Pillay et al⁶ an expanded panels of antigens were investigated focusing on CD11b, CD11c, CD66b, CD49d, CD64 and CD95. As presented in Supplement table 1, the present neutrophil subgroups appeared in this aspect to be similar with the subsets of Pillay et al⁶.

In vitro reactivity measurements of the airway smooth muscle

Two patients, 1 female (age 71) and 1 male (age 50) undergoing lobectomy were recruited for the purpose of acquiring macroscopically healthy human lung tissue in an investigation approved by the regional ethical review board in Stockholm. After providing consent, lung tissue samples were collected. Within 3 h of resection, human isolated small airways, with a diameter of 0.5–2 mm, were identified based on their visual appearance (thin and almost transparent wall, bone white colour, presence of air bubbles and mucus, as well as lack of blood) and dissected free under ice-cold Krebs-Henseleit buffer solution (KH, Sigma, St. Louis, MO, U.S.A) and cut into intact rings. The rings were then divided into three groups: Control, CD16^{high} CD62L^{high} and CD16^{high} CD62L^{dim} treated (n=7).

Male Balb/C mice (weight 19–25 g, 8-14 weeks old) were housed in plastic cages with adsorbent bedding in temperature and light/dark cycle (12-hour/12-hour) controlled rooms. Food and water were available *ad libitum*. Animals were handled in accordance with the Federation for European Laboratory Animal Science Associations guidelines. The heart-lung package was rapidly removed and transferred to ice-cold Dulbecco's modified Eagle medium (DMEM, Sigma, St. Louis, MO, U.S.A) supplemented with 100 U/mL penicillin and 100 µg/mL streptomycin. The trachea was dissected free of surrounding connective tissue and cut along the cartilage into eight equally sized rings.

During the co-culture with neutrophils, murine and human airway segments were individually placed in 48-well plates (Ultra-low attachment; Sigma, St. Louis, MO, U.S.A.) with 300 µL DMEM and incubated at 37°C in humidified 5% CO₂ in atmospheric air for 24 h. The segments were then moved to individual wells containing 300 µL CM and cultured, in presence or absence of, newly purified LPS activated neutrophils (CD16^{high} CD62L^{high} and CD16^{high} CD62L^{dim}) for 2 h. In one set of experiments, 100 µg/mL etanercept was added to the co-culture. In another set of experiments, 5 µg/mL actinomycin D or 10 µg/mL IL-1Ra was added to the tissue 1 hour before the neutrophils were added and remained present during the co-culture period.

The changes in smooth muscle contraction were evaluated in myographs. Murine and human intact airway ring segments were placed in a tissue myograph (Organ Bath Model 700MO; DMT A/S, Aarhus, Denmark) containing 5 mL baths with KH buffer at 37°C, continuously bubbled with carbogen (5% CO₂ in O₂) to maintain a stable pH of 7.4 and free from neutrophils. Changes in the smooth muscle contraction were monitored and isometric force was measured (ADInstruments, Hastings, United Kingdom). During an equilibration phase of 1 h for mice and 1.5 h for human, the tension of the tissue was set to 0.8 mN for murine tissue and 1.5 mN for human tissue. To confirm tissue viability, contractile responses were induced by KCl (60 mM) followed by a wash and an additional 30-minute equilibration periods. In presence of indomethacin (3µM), atropine (1 µM) and captopril (10 µM), bradykinin was cumulatively added and the force change registered. Following functional assessment in the myograph, the tracheal segments were immersed in a fixative solution consisting of 4% paraformaldehyde, embedded in paraffin and saved for immunohistochemistry.

Measurement of IL-1β and TNFα with ELISA from the murine co-culture

Cell-free culture supernatants were analysed for levels of IL-1β (antigen sensitivity: 0.125-8 pg/mL) and TNFα (0.5-32 pg/mL) using ELISA (R&D Systems Minneapolis, MN, USA) according to the manufacturer's instructions.

Immunohistochemistry on isolated murine tissue

Tracheal rings were cut into 5 µm thick sections using a microtome. Sections was deparaffinised in ultraclear (5 min x 2) and rehydrated in 99.5%, 95% and 70% EtOH followed by dH₂O. Antigen retrieval was performed with warm citrate buffer for 25 min. Sections were then incubated with 1% Triton-X100 in PBS to permeabilise the tissue. Inhibition of the endogenous peroxidase was accomplished by treating the tissue with 1% H₂O₂ for 10 min followed by incubation with 10% normal goat serum (NGS, Abcam, Cambridge, UK) for 30 min to block nonspecific binding. The sections were subsequently incubated with primary antibodies towards bradykinin B2

receptors (1:50, SC-25671, Santa Cruz Biotechnology) or vehicle as a control, diluted in 10 % NGS, overnight in 4°C. The secondary antibody was then added (biotinylated goat anti-rabbit IgG, 1:200, BA-1000, Vectorlabs, Peterborough, United Kingdom) for 1 hour at room temperature. Staining was amplified with an Avidin-Biotin-Complex kit (Vectastain, PK6100, Vectorlabs) and subsequently visualized using 3,3'-Diaminobenzidine (DAB, SK-4100, Vectorlabs) according to the manufacturer's instructions. The sections were counterstained with Mayer's Haematoxylin. Images were captured with a Nikon Digital Sight DS-U1 camera, coupled to a Nikon Eclipse TE2000-U microscope, using NIS Elements F. 2.20 software. For analysis, images were initially deconvoluted to separate the DAB and hematoxylin channels. The DAB channel was thresholded to a constant value for all pictures, and the total area of staining measured.

Antibodies and flow cytometry

Antibodies targeted against CD45 (BD-562312 labelled PE-CF594), CD16 (BD-555406 labelled FITC), CD62L (BD-555544 labelled PE) CD11b (Beckman, IM0530 labelled FITC), CD11c (BD56130 labelled BV711), CD66b (BD562254 labelled Percp 5.5), CD49d (BD564593 labelled BB515), CD64 (BD561194 labelled Percp 5.5) and CD95 (Beckam IM2446U labelled PE) were obtained from BD (BD Biosciences, San Jose, Calif) or Beckman Coulter (Immunotech, Beckman Coulter, Marseille, France) and titrated to the optimal concentration. Cells were identified based on forward and side scatter properties and analysed on LRSFortessa analyzer (BD). The collected data was processed using FlowJo software (©Tree Star, Inc, Ashland, USA).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism software (version 6.0, Graph Pad Software, La Jolla, CA). All data are shown as mean $\pm$ SEM. A p-value of <0.05 was considered statistically significant (*P <0.05 , **P < 0.01 , ***P < 0.001). A D'Agostino & Pearson omnibus normality test was used to determine if the data was normally distributed. For more than two

sets of data, a one-way ANOVA with Bonferroni post-hoc test was performed. For two sets of data, Mann-Whitney test was performed.

Results

In vitro activation of neutrophils from healthy volunteers

After the neutrophils were purified, they were activated with LPS and stained with antibodies to ensure the right subset prior to further experimentation (**figure 1**). Further flow cytometric analysis of these subgroups revealed CD16^{high} CD62L^{dim} neutrophils increased the expression of CD11b, CD11c and CD66b compared with CD16^{high} CD62L^{high} neutrophils and no difference could be demonstrated on the CD49d, CD95 or CD64 expressions (**supplement table 1**). Pre-treatment with fluticasone did not inhibit the activation of neutrophils. After 2 h of LPS, the percentage of activated neutrophils reached 97% with fluticasone treatment, and 99% without fluticasone treatment.

Myograph measurements of neutrophil-treated airways

Different subsets of neutrophils were co-cultured with isolated airways from man and mouse, and subsequent effects on the airway smooth muscle were evaluated in myographs. The CD16^{high} CD62L^{dim} neutrophils enhanced the response to bradykinin (BK) in both human bronchi (p=0.02) and murine tracheae (p=0.001, **figure 2A, B respectively**). The Emax in segments co-cultured with activated CD16^{high} CD62L^{dim} was significantly stronger than the maximal contraction from segments co-cultured with CD16^{high} CD62L^{high} (**figure 2A, B**)

Bradykinin B2 receptor expression in airway smooth muscle

Stimulation with the CD16^{high}CD62L^{dim} neutrophils enhanced the contractile response to bradykinin. Addition of actinomycin D, which inhibits transcription, 1 h prior to addition of neutrophils, decreased the subsequent bradykinin-induced smooth muscle response in mouse trachea (p=0.01, **figure 3A**). Further, presence of CD16^{high} CD62L^{dim} neutrophils during the culture period increased the immunohistostaining of the bradykinin B2 receptor; no such amplification was seen neither in the controls (p=0.004) nor when CD16^{high} CD62L^{high} neutrophils were added (p=0.03, **figure 3B**).

IL-1 β and TNF α secretion from CD16^{high} CD62L^{dim} neutrophils

The supernatants from the murine co-culture were analysed with ELISA to measure IL-1 β and TNF α . The CD16^{high} CD62L^{dim} neutrophils produced significantly more IL-1 β (p=0.002) and TNF- α (p=0.008) than the CD16^{high} CD62L^{high} neutrophils (**figure. 4A, B**). When the IL-1 receptor antagonist (IL-1RA) was added co-culture in two concentrations, the higher concentration (1 mg/ml) inhibited the TNF- α production (p=0.047, **figure 4B**), whereas the lower concentration (0.1 mg/ml), despite being numerically reduced, showed no significant decrease. Blocking TNF- α with Etanercept resulted in a 53% inhibition of the bradykinin induced airway contraction in mice (p=0.04, **figure 4C**). IL-1Ra was also used in an attempt to block IL-1 β response on the smooth muscle. The use of IL-1Ra on the smooth muscle neither affected the response induced by the CD16^{high} CD62L^{dim} neutrophils nor the smooth muscle by itself (**Figure 5**).

Discussion

This study details that CD16^{high} CD62L^{dim} neutrophils have the ability to directly enhance the smooth muscle response to bradykinin in both human isolated small airways and murine tracheae (**figure 6**).

Bradykinin is a potent pro-inflammatory peptide that is increased in broncho alveolar lavage fluid from allergen challenged asthmatic patients¹¹. It limits the local airway flow in asthmatic patients through bradykinin B2 receptor mediated smooth muscle contraction. No such contraction can be induced in healthy controls¹². Thus, bradykinin has been suggested as a more sensitive marker for airway hyperreactivity than methacholine^{9,10}. To assess the potential relationship between various neutrophil subsets and asthmatic hyperactivity, neutrophil subsets were co-cultured with murine trachea, and bradykinin-induced smooth muscle contractions were evaluated in myographs. CD16^{high} CD62L^{dim} neutrophils were found to enhance the airway response to bradykinin. No corresponding effect was found using the CD16^{high} CD62L^{high} neutrophils. Using a limited number of human isolated small airways this effect could be confirmed in man, suggesting that CD16^{high} CD62L^{dim} neutrophils have the ability to alter the reactivity of the airways, potentially restricting the expiratory airflow in asthmatic lungs.

It is important to notice that the present approach to induce neutrophil subgroups is not exactly the same as the one used by Pillay and co-workers⁶. We used an *in vitro* set up where LPS was added to neutrophils after sorting them out from the blood, whereas Pillay et al.⁶ added LPS *in vivo* directly in the blood stream. Pillay et al provide an antigen based characterization of their neutrophil subgroups and it is of significance that we ended up with an identical classification of our *in vitro* activated neutrophils. So even if our *in vitro* activated neutrophil subgroups not necessarily are completely identical with the in previously described *in vivo* active cells we have made what appears to be a good attempt to mimic them. So albeit there is a slight difference in the methodology, the end product in terms of active CD16^{high} CD62L^{dim} neutrophils seems to be very similar.

To investigate the mechanisms behind the described neutrophil- induced local airway smooth muscle hyperreactivity, isolated tracheal tissues were co-cultured with different neutrophil subsets and the supernatants were analysed. The CD16^{high} CD62L^{dim} neutrophils were found to produce more IL-1 β and TNF- α than CD16^{high} CD62L^{high} neutrophils. The addition of an IL-1 receptor antagonist (IL-1Ra) during this procedure resulted in the inhibition of the TNF- α production, thus confirming that IL-1 β enhances the secretion of TNF α from neutrophils. However, IL-1 β and IL-1Ra had no effect on the smooth muscle. Further, an anti-TNF α antibody, Etanercept, reduced the bradykinin response in the myograph set up. The tentative roles of IL-1 β and TNF α suggested by these experiments are well in line with previous reports about the role of these cytokines in the development of smooth muscle hypersensitivity towards bradykinin¹³⁻¹⁵. There might be other sources of TNF α ¹⁶ but the present experiments focus on the potential role of neutrophils. The presented Actinomycin D experiments supported the idea of a link between airway smooth muscle hyperreactivity and up-regulation of the bradykinin B2 receptor. The increased bradykinin B2 receptor immunostaining of the in neutrophil-exposed tracheal tissue caused by the presence of CD16^{high} CD62L^{dim} neutrophils further validates this assumption. Together, this suggests that CD16^{high} CD62L^{dim} neutrophils induce an upregulation of the bradykinin B2 receptor via an IL-1 β -induced stimulation of TNF- α secretion, thus amplifying the effect of bradykinin in tracheal smooth muscle.

To achieve activated neutrophils (CD16^{high} CD62L^{dim}) for the *in vitro* experiments, the CD16^{high} CD62L^{high} neutrophils were treated with LPS, which is the major component of the membrane in gram-negative bacteria. Since bacterial infections in the airway are known to cause asthma exacerbation¹⁷, it is tempting to speculate that there is a role for CD16^{high} CD62L^{dim} neutrophils in infection-induced exacerbation in asthma patients. We could also demonstrate that the activation was not affected by corticosteroids which is in line with previous observations by others¹⁸. With the advent of phenotyping and cluster analysis, the “one size fits all” approach to asthma treatment has been questioned¹⁹ and a large subgroup of patients with severe steroid-insensitive asthma has been identified to be in need of novel treatment opportunities. As corticosteroids do not affect neutrophils and oral corticosteroids have even been shown to induce neutrophil recruitment into

the airways of patients with mild asthma, treatments focusing on the neutrophils may offer new possibilities¹⁸.

To summarize, as the perception of the neutrophil changes, from an expendable homogeneous population of cells that kill bacteria towards a more heterogeneous group of cells with specified roles in inflammation, a new predictive role for activated neutrophils in airway hyperreactivity becomes more apparent. The present findings of a specific neutrophil subset, CD16^{high} CD62L^{dim}, causing a marked increase of bradykinin reactivity, must encourage us to re-evaluate the traditional concepts of allergic asthma. By doing so we open up for new treatment strategies, particularly in patients where corticosteroids are not an option or are not sufficient.

Acknowledgments

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Figure 1. Differences between CD16^{high} CD62L^{high} and CD16^{high} CD62L^{dim} neutrophils. A dot-plot of neutrophils that shows the change in CD62L caused by LPS stimulation, with CD16^{high} CD62L^{high} in gray (A) and CD16^{high} CD62L^{dim} in black (B).

Figure 2. Neutrophils stimulated airways and airway contraction. (A) Isolated human airways segments ($n=7$) and (B) isolated murine airway segments ($n=8-10$) were cultured for 24 h and then incubated for 2 h with newly-purified, inactivated neutrophils (■), activated neutrophils (●), or control vehicle (▲). The segments were then rinsed and the contractile response to KCl and bradykinin tested. Exposure to activated neutrophils caused a long-standing hyperactive response to bradykinin. All results are expressed in mean $\pm$ SEM, one-way ANOVA with Bonferroni post-hoc test, * $P < .05$, ** $P < .01$, *** $P < .001$.

Figure 3. CD16^{high} CD62L^{dim} Neutrophils induces an upregulation of the bradykinin receptor 2. (A) Control tissues (▲), and tissues exposed to CD16^{high} CD62L^{dim} (●) as well as CD16^{high} CD62L^{dim} exposed tissue treated with Actinomycin D (○). The presence of CD16^{high} CD62L^{dim} neutrophils caused an upregulation of the bradykinin receptor ($n=8$).

Actinomycin D treatment returned the bradykinin-enhanced airway contractility back to normal. (B) Immunostaining of the tracheal tissue ($n=13-15$) revealed a higher presence of bradykinin B2 receptor in the CD16^{high} CD62L^{dim}-cultured tracheal segments than in the CD16^{high} CD62L^{high}-cultured segments. The difference between CD16^{high} CD62L^{dim} treated and control tissues were even more pronounced. All results are expressed in mean $\pm$ SEM and one-way ANOVA with Bonferroni post-hoc test used or for data with only two data points, Mann-Whitney test * $P < .05$, ** $P < .01$, *** $P < .001$.

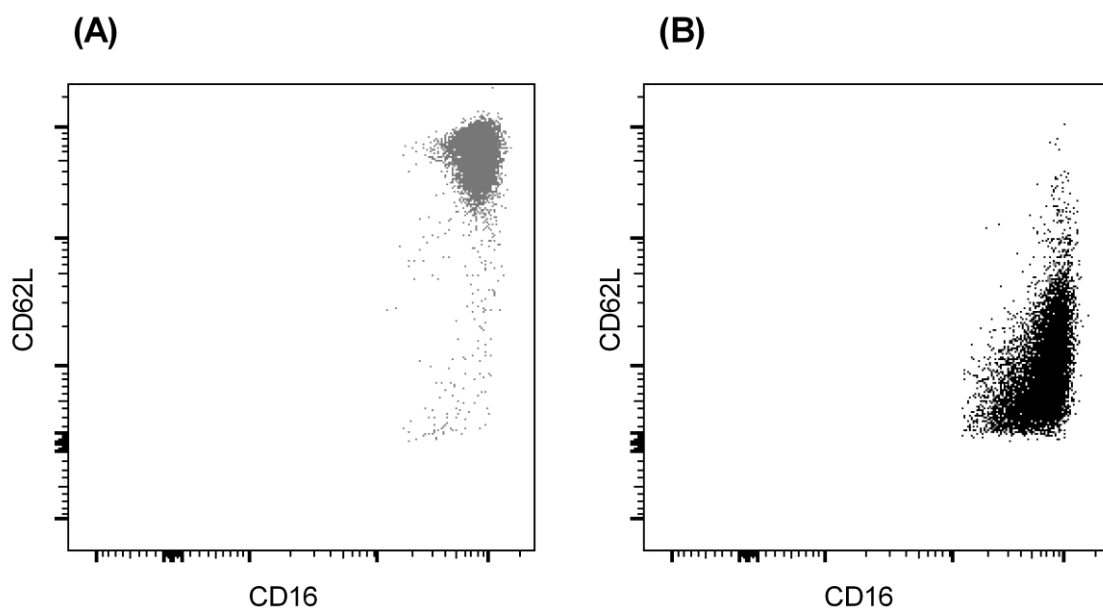
Figure 4. Differences between CD16^{high} CD62L^{high} and CD16^{high} CD62L^{dim} neutrophils. (A, B) The secretion of IL-1 β and TNF- α derived from CD16^{high} CD62L^{dim} subsets ($n=4$). The TNF- α secretion upon IL-1Ra (1 mg/ml) treated cells. (C) Control tissues (▲) and tissues exposed to CD16^{high} CD62L^{dim} (●) as well as CD16^{high} CD62L^{dim} exposed tissue treated with Etanercept (○). The Etanercept treatment reduced the bradykinin-enhanced contraction in the organ bath by 53% ($n=8$). All results are expressed in mean $\pm$ SEM and one-way ANOVA with Bonferroni post-hoc test used or for data with only two data points, Mann-Whitney test * $P < .05$, ** $P < .01$, *** $P < .001$.

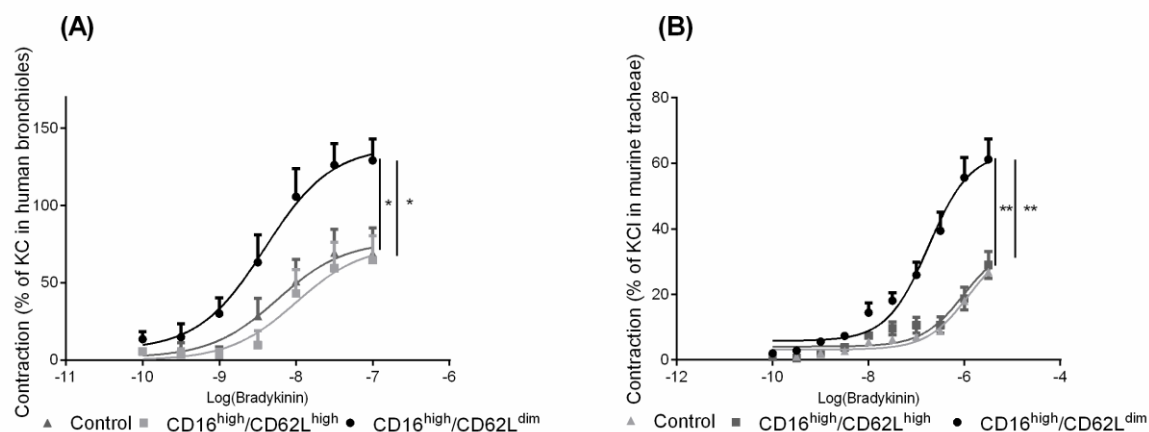
Figure 5. IL-1 β had no effect on the upregulated bradykinin response. Control tissues, $n=4$ (□) and tissues exposed to CD16^{high} CD62L^{dim}, $n=3$ (□) as well as CD16^{high} CD62L^{dim} exposed tissue treated with IL-1RA, $n=3$ (○).

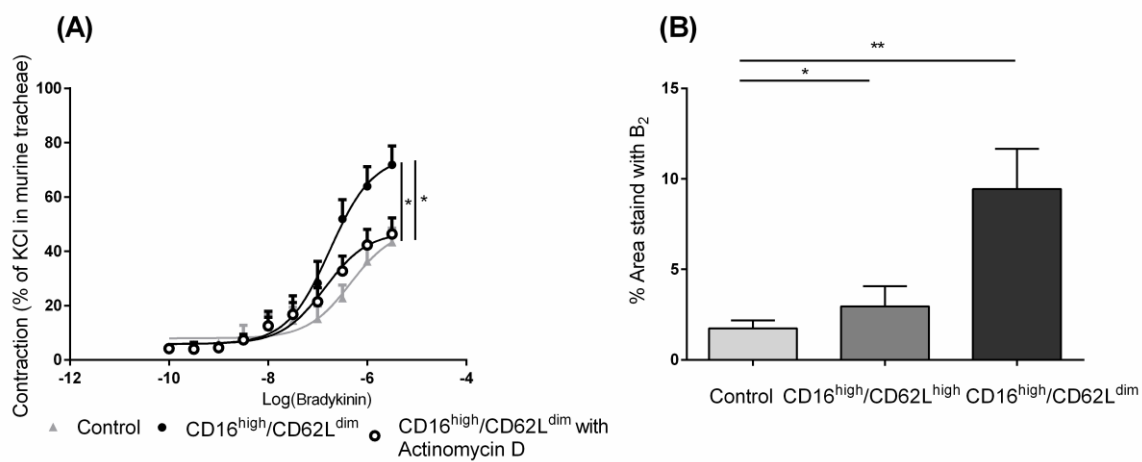
Figure 6. Graphical image of the findings in the article. The CD16^{high}CD62L^{dim} neutrophils may migrate to the lung where they induce upregulation of the bradykinin receptor, via TNF- α . The activated CD16^{high}CD62L^{dim} neutrophils enhance airway hyperactivity and may have a role in acute exacerbation in asthmatic patients.

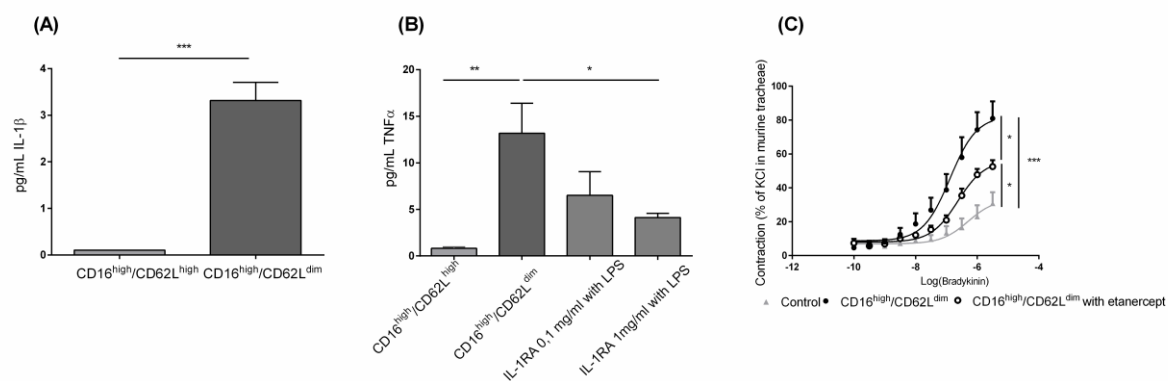
Supplement Tabell 1. Neutrophil subsets characterized by antigen identified by Pillay et al before and after LPS stimulation *in vitro*. Neutrophil subsets were gated using CD16 and CD62L and specific antigens were analyzed.

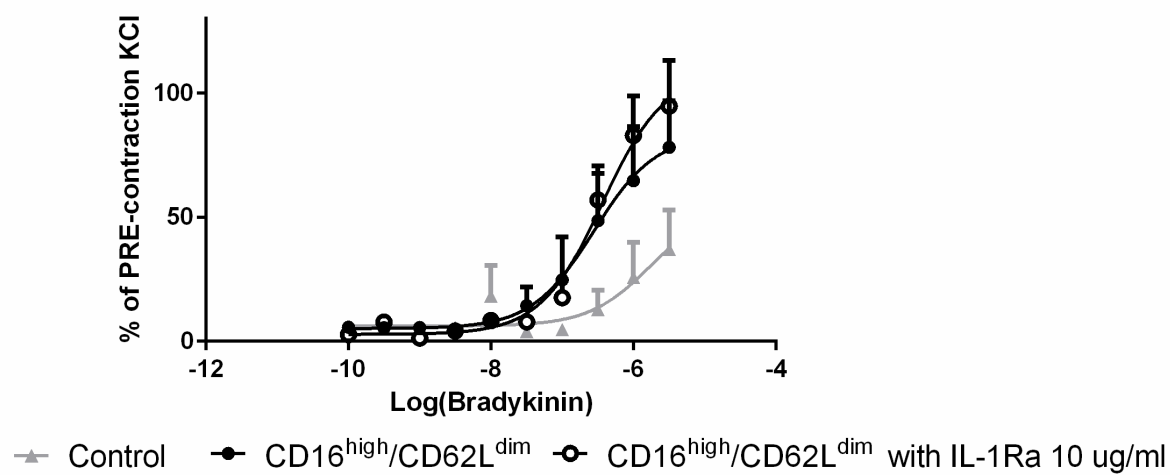
Antigen expressions on the different neutrophil subsets were equal to the results reported by Pillay et al. Data are expressed as mean $\pm$ SEM n=4

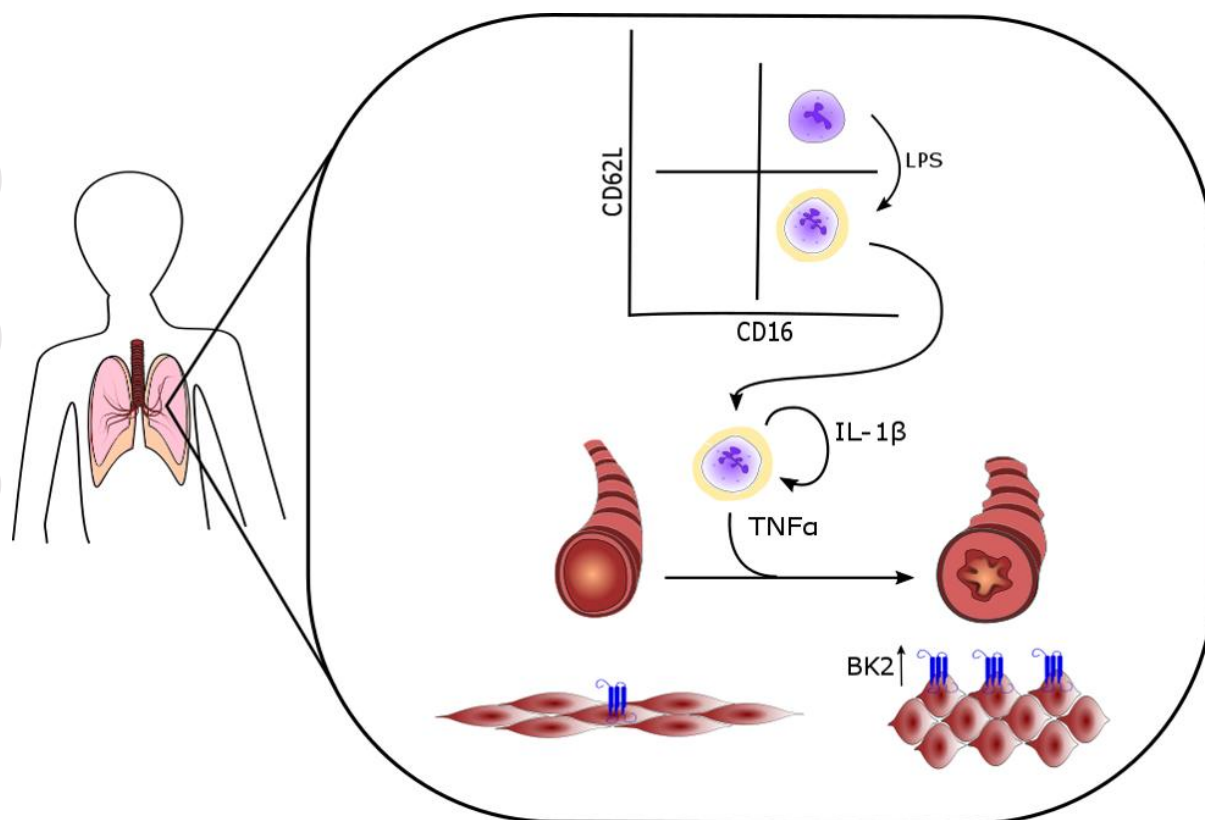












Supplementary information

Supplement Tabell 1. Neutrophil subsets characterized by antigen identified by Pillay et al before and after LPS

stimulation *in vitro*. Neutrophil subsets were gated using CD16 and CD62L and specific antigens were analyzed.

Antigen expressions on the different neutrophil subsets were equal to the results reported by Pillay et al. Data are expressed as mean $\pm$ SEM n=4

Antigen	CD16 ^{high} / CD62L ^{high}	CD16 ^{high} / CD62L ^{dim} 1 μ g/ml	Clone	Antibody supplier
CD11b	9827,75 $\pm$ 457,41	27732,75 $\pm$ 398,79	Bear1	Beckman Coulter
CD11c	1609 $\pm$ 97,65	4489,25 $\pm$ 264,74	B-ly6	BD
CD66b	5068,75 $\pm$ 207,91	18267,5 $\pm$ 1220,57	G10F5	BD
CD49d	478,25 $\pm$ 24,37	582,75 $\pm$ 14,98	9F10	BD
CD95	2606 $\pm$ 583,68	2846,25 $\pm$ 660,08	7C11	Beckman Coulter
CD64	638,75 $\pm$ 67,75	776,5 $\pm$ 84,12	10.1	BD