

Neutrophil phenotypes in bronchial airways differentiate single from dual responding allergic asthmatics

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Abstract

Introduction: Allergic asthmatics with both an early (EAR) and a late allergic reaction (LAR) following allergen exposure are termed 'dual responders' (DR), while 'single responders' (SR) only have an EAR. Mechanisms that differentiate DR from SR are largely unknown, particularly regarding the role and phenotypes of neutrophils. Therefore, we aimed to study neutrophils in DR and SR asthmatics.

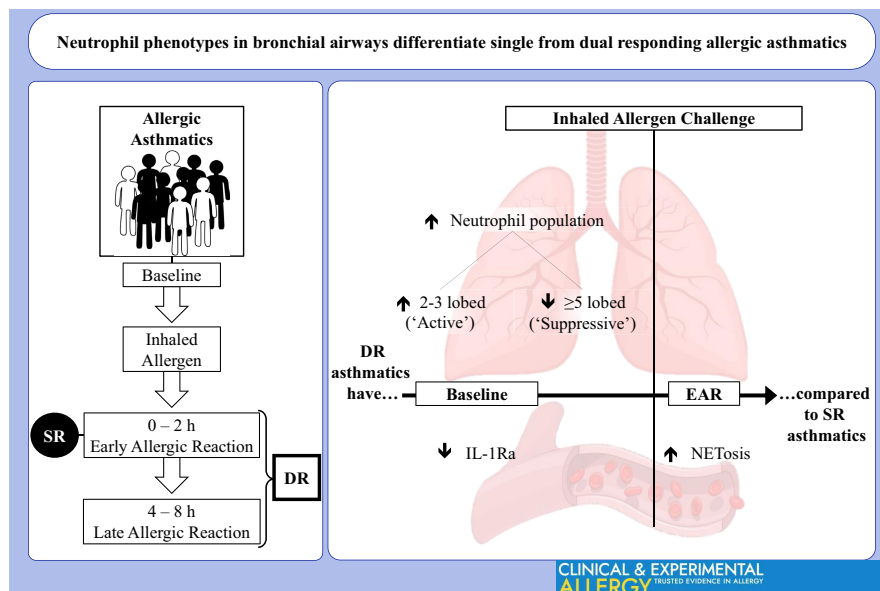
Methods: Thirty-four allergic asthmatics underwent an inhaled allergen challenge, samples were collected before and up to 24 h post-challenge. Cell differentials were counted from bronchial lavage, alveolar lavage and blood; and tissue neutrophils were quantified in immune-stained bronchial biopsies. Lavage neutrophil nuclei lobe segmentation was used to classify active (1–4 lobes) from suppressive neutrophils (≥ 5 lobes). Levels of transmigration markers: soluble (s)CD62L and interleukin-1Ra, and activity markers: neutrophil elastase (NE), DNA-histone complex and dsDNA were measured in lavage fluid and plasma.

Results: Compared with SR at baseline, DR had more neutrophils in their bronchial airways at baseline, both in the lavage ($p = .0031$) and biopsies ($p = .026$) and elevated bronchial neutrophils correlated with less antitransmigratory IL-1Ra levels ($r = -0.64$). DR airways had less suppressive neutrophils and more 3-lobed (active) neutrophils ($p = .029$) that correlated with more bronchial lavage histone ($p = .020$) and more plasma NE ($p = .0016$). Post-challenge, DR released neutrophil extracellular trap factors in the blood earlier and had less pro-transmigratory sCD62L during the late phase ($p = .0076$) than in SR.

Conclusion: DR have a more active airway neutrophil phenotype at baseline and a distinct neutrophil response to allergen challenge that may contribute to the development of an LAR. Therefore, neutrophil activity should be considered during targeted diagnosis and bio-therapeutic development for DR.

KEYWORDS

allergic asthma, CD62L, IL-1Ra, inhaled allergen challenge, late allergic reaction, NETosis, neutrophils



GRAPHICAL ABSTRACT

Allergic asthmatics underwent an inhaled allergen challenge with samples taken from the airway and blood at baseline and after the challenge. Dual responding allergic asthmatics (DR) had more 2- to 3-lobed (known as 'active') and less ≥ 5 -lobed ('suppressive') airway neutrophils correlating with less circulating IL-1Ra than single responders (SR) at baseline. Following an inhaled allergen challenge, DR had more circulating NETosis factors than SR during the early allergic reaction phase (EAR).

1 | INTRODUCTION

Approximately 50% of allergic asthma patients present with both an early (EAR) and a late allergic reaction (LAR) to inhaled allergen and are termed 'dual responders' (DR), while those who only have an EAR are termed 'single responders' (SR). The LAR, occurring between 4 and 8 hours (h) after allergen exposure, is associated with more extensive airway inflammation and can last up to several weeks.¹ Therefore, compared with SR, DR often reflects a more severe asthma clinical phenotype.² Despite having a reproducible type-2 immune response to inhaled allergen in both SR and DR subgroups,¹ no baseline biomarkers related to the type-2 immune response can presently predict the airway response in an individual (e.g.³). In contrast, some studies have shown differences in neutrophil-related biomarkers^{4,5} and less neutrophils in the blood between DR and SR.⁶ However, the neutrophil-related differences within the airway compartments and how they may contribute to the dual allergic response have not been investigated.

The roles of neutrophils in asthma were attributed to the release of damage markers that attract eosinophils,⁷ tissue repair^{7,8} and subsequent apoptosis.⁹ Yet, this may not be the case in a diseased state as an increased neutrophil presence in airways is often related to more severe asthma,¹⁰ uncontrolled asthma¹¹ or the use of oral corticosteroids.¹² Neutrophils are also one of the first innate immune cells to be recruited to the airways of allergic asthmatics and have been found to be needed for inducing airway inflammation, play role in exacerbating allergic inflammation, but also, be dampened by type-2 allergic inflammation¹³—though the mechanisms behind these roles are largely unknown.

Key Messages

- Dual responding allergic asthmatics have more baseline airway neutrophils with an active morphology than single responders.
- Increased airway neutrophils in dual responders were associated with less antimigration signalling rather than more chemoattraction.
- Post-challenge, dual responders release an active neutrophil response in the blood earlier than in single responders.

Chemoattractants of neutrophil migration into the tissue include interleukin-17 (IL-17), primarily released by T-helper 17 cells¹⁴ and IL-8, released by activated monocytes/macrophages and endothelial/epithelial cells.¹⁵ To regulate chemoattraction, IL-1 receptor agonist (IL-1Ra) is released by many cells, including monocytes and neutrophils, to reduce neutrophil recruitment to inflamed tissue—avoiding excessive self-damage.^{16,17} A major step in the migration of leukocytes, including neutrophils, from circulation to the airways is the shedding of CD62L (also called L-selectin).¹⁸ During inflammation, neutrophils can also undergo a process called NETosis, releasing neutrophil extracellular traps (NETs) that contain proinflammatory neutrophil elastase (NE), myeloperoxidase (MPO), self dsDNA and histone proteins.¹⁹ The NETosis process can cause damage to the surrounding tissue and has been reported to drive exacerbation

severity in a mouse asthma model.¹⁹ In general, neutrophils responding to tissue inflammation with NETosis are usually healthy, but a prolonged neutrophil response in the tissue can be concerning.

In contrast, a newly defined suppressive phenotype of CD62L(dim) neutrophils, that is speculated to be maturing directly from unsegmented to hypersegmented nuclei (i.e. five or more lobes),^{20–22} has been shown to appear in the blood as early as 3 h post-challenge.²³ Recently, we reported an increase in blood CD62L(dim) neutrophils 23 h post-challenge from the same blood samples as used in the present study.²⁴ However, it is more difficult to determine whether airway CD62L(dim) neutrophils originated as naturally occurring 'suppressive' CD62L(dim) in circulation or whether they were activated CD62L(high) neutrophils that have shed CD62L to transmigrate (i.e. move out of circulation between the endothelial cells) in response to an allergen challenge.²⁵ Presently, the best way to distinguish suppressive from active CD62L(dim) neutrophils in the airways is to assess the segmentation of neutrophil nuclei, where CD62L(dim) suppressive neutrophils are hypersegmented (i.e. ≥ 5 lobes),²¹ while active neutrophils commonly appear unsegmented ($=1$) to 4 lobes.²²

Due to the prolonged allergic response in DR, we hypothesised that there would be an altered neutrophil involvement in DR compared with SR. Therefore, we assessed the numbers and morphology of airway neutrophils and their related biomarkers in the airways and the plasma in DR versus SR before and after an inhaled bronchial allergen challenge.

2 | MATERIALS AND METHODS

2.1 | Subjects and study design

All subjects ($n = 34$) had mild to moderate allergic asthma and all were nonsmokers without or with a low dose of inhaled corticosteroids (ICS, max 400 μg of budesonide). All subjects signed a written informed consent and the Regional Ethics Review Board in Lund, Sweden, approved the study (2012/800). Each individual completed lung physiology measures and blood sampling before and at several time points following an inhaled bronchial allergen challenge. When FEV₁ dropped $\geq 20\%$ compared with baseline, the challenge was considered completed (starting at the 0 h time point). Subjects included 19 SR and 15 DR, and DR were defined by the second drop of $\geq 12\%$ in FEV₁ from baseline that was maintained between 4 and 8 h post-challenge (defined by Stenberg et al, 2017),²⁶ for subject characteristics see Table 1. The period of 0–2 h post-challenge was considered the 'early phase' and from 4 to 8 h was considered the 'late phase'. Study procedures including lung physiology (with lung reactance given as X5 and AX measured by impulse oscillometry referred to in this manuscript) and related findings have been previously reported.²⁶ The study design for relevant sample collections and timepoints is outlined in Figure S1. Briefly, a bronchoscopy was performed before and 24 h

TABLE 1 Patient characteristics (median $\pm$ IQR). Separated into single responders (SR) and dual responders (DR) for all patients. Allergen extract used was purified and standardized to eliminate the effects of LPS (ALK-Abelló, Hørsholm, Denmark)

	All patients		
	SR ($n = 19$)	DR ($n = 15$)	pValue
Sex (Male/Female)	11/8	6/9	.9
Age (years)	27 (26–41)	24 (22–30)	.036
BMI (kg/m^2)	23 (22–26)	24 (23–26)	.7
Regular use of ICS (n)	6	10	.5
Duration of asthma (years)	19.5 (10–24)	15.0 (13–21)	.9
ACT (score)	22 (20–24)	22 (20–24)	.5
Methacholine PD20 (μg)	208 (104–550)	228 (174–917)	.3
Number of sensitizations	5 (4–6)	4 (3–6)	.3
Allergen inhaled (n)			
Cat	10	8	N/A
Horse	4	2	
Birch	2	2	
D. pteronyssinus	2	1	
Grass	1	2	
FEV1% max drop 0–2 h after challenge	22.5 (21.2–24.9)	23.5 (21.1–26.3)	.5
FEV1% max drop 4–8 h after challenge	7.2 (4.7–8.7)	20.1 (14.8–24.0)	<.0001

Note: Statistical significance between SR and DR of all patients was determined by nonparametric Mann–Whitney test ($p < .05$ is bolded). Body mass index (BMI), inhaled corticosteroids (ICS), asthma control test (ACT) and forced expiratory volume in one second (FEV1).

post-challenge, and blood was collected before and 0, 0.5, 1, 2, 4, 6, 8 and 23 h post-challenge as previously described.²⁷

2.2 | Bronchial and alveolar lavage processing

A subgroup (SR, $n = 10$, DR $n = 8$) underwent a bronchoscopy (see Table S1 for subgroup characteristics) with bronchial biopsies, bronchial lavage and alveolar lavage before and 24 h post-challenge. Sterile phosphate-buffered saline (PBS) (3×50 ml) was instilled through a bronchoscope into the middle lobe and the first 50 ml was collected as the bronchial fraction and the remaining 100 ml was recovered as the alveolar fraction (herein referred to as bronchial lavage and alveolar lavage, respectively) similar to a previous study.²⁸ Cells were separated from the lavage fluid by centrifugation, cytopun and either stained with Kwick-Diff (Shandon) or fixed with formaldehyde. See Supplementary Methods for a detailed description of processing.

2.3 | Neutrophil nuclei segmentation differentiation

Scans (40 $\times$) of the Kwick-Diff stained lavage cytopins were assessed using 100 randomly selected neutrophils per slide identified in ImageScope (Lecia Biosystems) by a qualified technician. Each neutrophil was individually categorised according to the number of nucleus lobes, i.e. as unsegmented or two, three or four lobes or a hypersegmented nucleus of five or more lobes. The nucleus lobes had to be clearly separated to be counted as segmented (see examples in Figure S2). Percentage of each segmented neutrophil type was used to calculate the number of each segmented neutrophil subset per mL of lavage.

2.4 | Blood sampling

EDTA-treated whole blood was sampled before and at multiple time points post-challenge and used for plasma collection and a subgroup (Table S1) was sent for a differential count of WBC at the accredited Clinical Chemistry routine lab at Skåne's University Hospital in Lund, Sweden. See Supplementary methods for processing and analysis.

2.5 | Enzyme-linked immunosorbent assay

DuoSet ELISA kits from R&D systems were used as per the manufacturer's instructions to assess all the plasma time points for CD62L (R&D Systems) and all the plasma time points and lavages for NE (R&D Systems). The cell death detection ELISA (Roche) was used to detect cytoplasmic histone-associated DNA fragments (herein termed as 'histones') in lavage fluid and plasma as per the manufacturer's instructions. See Supplementary methods for processing.

2.6 | Protein concentrations using multiplex Luminex[®]

The Magnetic Luminex[®] Assay multiplex kit (bio-technique) was used to quantify IL-17, IL-8 and IL-1Ra in baseline, 6 h and 23 h plasma and lavage fluid and to quantify CD62L in lavage fluid. See Supplementary methods for reagent details and processing.

2.7 | Qubit dsDNA assay

The Qubit[®] dsDNA HS assay kit (Life technologies) was used to quantify dsDNA in lavage fluid and plasma samples as per the manufacturer's instructions.

2.8 | Immunohistochemical staining in biopsy specimens and cytopins

Biopsies were fixed in paraffin, and prestained using an antimacrophage cocktail of anti-CD68 (Dako) and anti-CD163 (LEICA) (to avoid nonspecific analysis of macrophage-based myeloperoxidase, MPO),²⁹ labelled with DAB precipitate, then stained for neutrophils using anti-MPO (Dako) (labelled with viny green). Slides were digitalized and all the viable airway tissue was captured using ImageScope. Images were processed to generate the percentage of neutrophil green area normalised to the total area of tissue.

A few alveolar cytopins, (5 paired samples of baseline and 24 h), were stained for CD62L (coloured with DAB) and counterstained with hematoxylin and eosin. Images of mixed lavage cells were processed using colour thresholds and cell count to produce an average CD62L stain area per cell. See Methods S1 for details on staining protocols and quantifications.

2.9 | Statistics

Statistics outlined in each figure legend were calculated from GraphPad Prism (version 9). Briefly, the Mann-Whitney U test was used to compare DR and SR; the Wilcoxon signed-rank test was used to compare paired timepoints (of 2–3 timepoints); the 2-way ANOVA corrected for multiple comparisons was used to compare more than 3 paired timepoints; and the Spearman's rank analysis was used for correlations. See Supplementary Methods for more details.

3 | RESULTS

3.1 | Dual responders have more airway neutrophils at baseline than single responders

At baseline, DR presented with significantly more neutrophils per mL in the bronchial lavage than in SR ($p = .0031$). In general, each

bronchial lavage had consistently more neutrophils than its paired alveolar lavage ($p = .0078$; Figure 1A). DR also had more macrophages ($p = .021$) in the baseline bronchial lavage than in SR. No other differences in the cell populations were observed between SR and DR per mL lavage (see Table S2 for all medians and p values). The total number of cells per total bronchial lavage volume also had more neutrophils in DR than in SR ($p = .0028$, Table S3)—but not macrophages. Both the increased numbers of baseline bronchial neutrophils and macrophages correlated with an increasing maximum decrease in FEV₁% during the late phase (Figure 1B). For the complete matrix of correlations between the decrease in FEV₁% drop during the early (1–2 h) and the late (4–8 h) phase and baseline bronchial cells, see Table S4.

The increased number of neutrophils in the DR airways at baseline was confirmed by staining for neutrophil-specific MPO in bronchial biopsies ($p = .026$; Figure 2A, B). The number of neutrophils in the tissue at baseline correlated with increased lung elasticity (measured as airway reactance and might reflect more peripheral obstruction³⁰) at both baseline and during the late phase (Figure 2C, D—see Table S5 for p values).

3.2 | Airway neutrophils from dual responders have less nuclei segmentation than single responders.

To differentiate the more active (1- to 4-lobed) neutrophils from the suppressive (hypersegmented) neutrophils in the lavages, we

assessed the proportion of each lobed neutrophil (see examples in Figure 3A). The majority of the bronchial lavage neutrophils in DR were unsegmented ($p = .0027$), 2-lobed ($p = .0047$) or 3-lobed ($p = .029$) as compared to SR at baseline (Figure 3B). DR also maintained significantly more 3-lobed neutrophils than SR at 24 h post-challenge ($p = .042$). There was no difference in the number of 4-lobed neutrophils between DR and SR at either time point. As numbers of hypersegmented neutrophils were very low, we merged the bronchial and alveolar counts per subject to compare total lavage occurrences (i.e. yes or no) of hypersegmented neutrophils. Overall, DR had less occurrences of hypersegmented neutrophils in the combined lavage (2/11 occurrences or 18%) than in SR (12/19 occurrences or 63%; Figure 3C).

The total number of alveolar neutrophils at baseline correlated with the number of sensitizations, of these specifically, the 2-lobed and 3-lobed neutrophils correlated with the number of sensitizations to different allergens (Figure 3D) when analysing all individuals (i.e. SR + DR), but this correlation did not occur with the hypersegmented neutrophils (see Table S6).

3.3 | Less CD62L in bronchial lavage from dual responders than single responders.

Since there is a known relationship between the leukocyte migration into the airways and CD62L, we assessed the CD62L levels in the

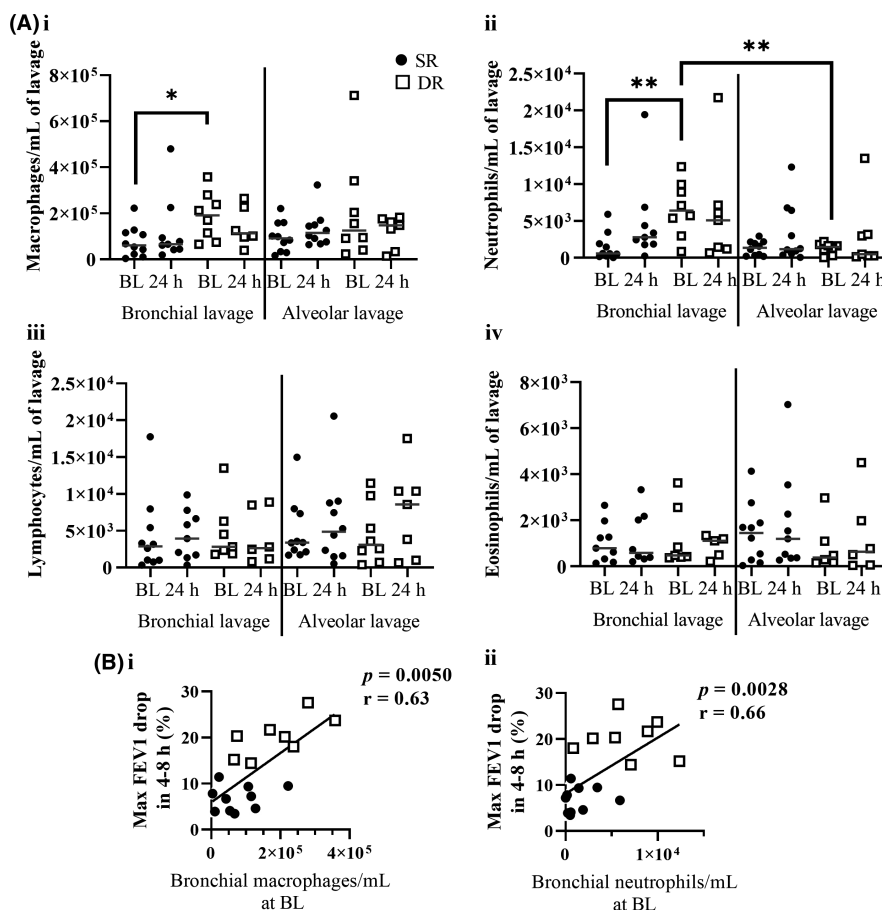


FIGURE 1 Lung lavage cell differentiation for dual responders (□DR) and single responders (●SR). Data compare SR and DR at baseline (BL) and 24 h post-challenge. (A) Number of the different bronchial and alveolar lavage cells per mL of lavage, differentiated to (i) macrophages, (ii) neutrophils, (iii) lymphocytes and (iv) eosinophils, $n = 6–10$. (B) Both bronchial (i) macrophages and (ii) neutrophils correlate with the late-phase per cent drop in FEV₁

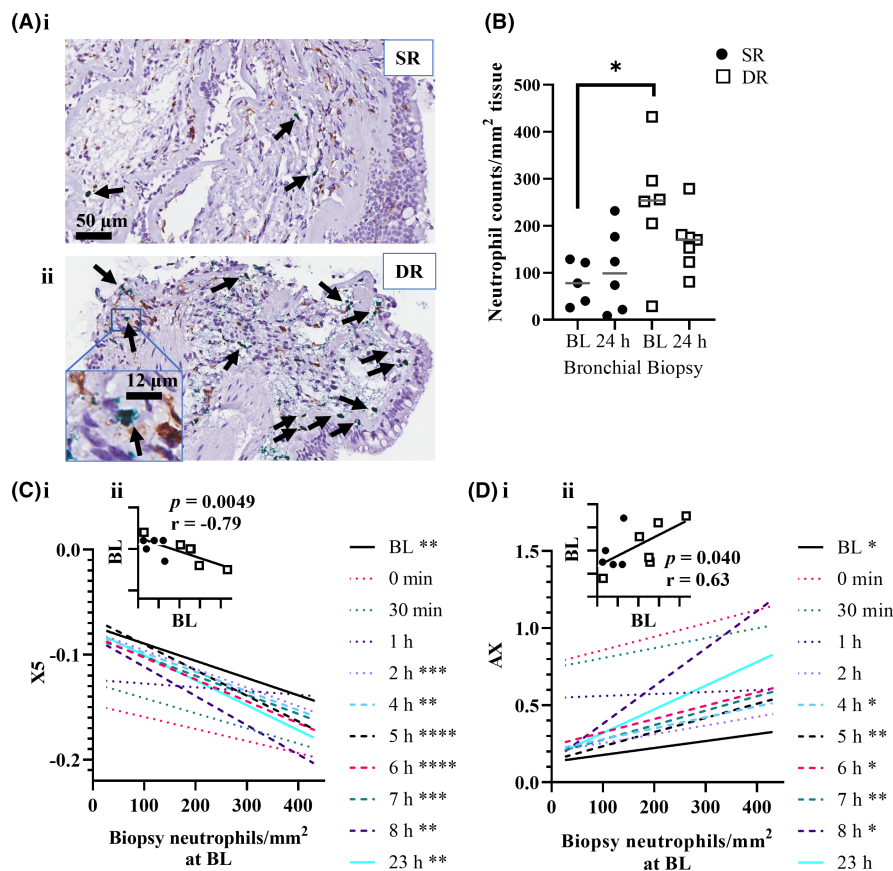


FIGURE 2 Neutrophils in bronchial biopsies. (A) Representative images of stained biopsies, in (i) single responders (SR) and (ii) dual responders (DR), at baseline (BL) stained for neutrophil MPO (green, indicated by black arrows). (B) Number of neutrophil cells/mm² of the tissue area, $n = 5-6$. Biopsy neutrophil numbers at baseline were correlated with peripheral reactance readings (Ci) X5 and (Di) AX from baseline and 23 h after challenge (solid lines) including the early-phase (dotted lines) and late-phase times (dashed lines). Both C and D contain inserts of (ii) baseline to baseline data with the same axis as the overall graph

airways (Figure 4). At baseline, soluble (s)CD62L levels in the bronchial lavage fluid were significantly lower in DR than in SR ($p = .021$, Figure 4A). To check whether this was due to less shedding in the lavage or whether the lavage leukocytes just had less CD62L on their surface overall, a few lavage slides were stained for CD62L on the cell surface (Figure 4B). When quantified for average stain area per cell, DR tended to have less CD62L in the cell surface than in SR at baseline, but samples were too few to statically confirm that DR leukocytes were CD62L(dim) (Figure 4C).

3.4 | Neutrophils comprise a large proportion of blood leukocytes that increase during the late phase

To assess the systemic immune response following an allergen challenge, WBC was differentiated from a subgroup of the subjects (SR: $n = 7$ and DR: $n = 10$) with similar characteristics as the whole study group (Table 1 and Table S1). There was a significant increase in the total blood leukocyte number from baseline to 4 h post-challenge ($p = .0005$), specifically in monocytes ($p = .0010$), lymphocytes ($p = .0010$) and neutrophils ($p = .0005$), but not in eosinophils nor basophils (Figure 5A). An increase at 4 h was expected as this is in line with previous reports.³¹ This was followed by a significant decrease in the total blood leukocyte number from 4 to 23 h post-challenge ($p = .0039$), specifically in monocytes ($p = .016$), lymphocytes ($p = .0039$) and neutrophils ($p = .016$), but again, not in eosinophils

nor basophils. There were no differences in total WBC counts between SR and DR at any time point (Figure S3).

The proportion of neutrophils among the blood leukocytes increased at 4 h in DR ($p = .0050$) but not in SR (Figure 5B). Concurrently, the proportion of blood lymphocytes and eosinophils dropped in DR but not SR (Figure S4). From 4 to 23 h post-allergen challenge, the proportion of neutrophils decreased significantly (DR: $p = .0018$; SR: $p = .023$) to significantly lower numbers than at baseline (DR: $p = .0078$; SR: $p = .063$). There was no change in the proportion of monocytes and basophils throughout the study (Figure S4); however, the blood monocyte and neutrophil numbers did correlate at baseline and 4 h post-allergen challenge, but no longer after 23 h (Figure S5).

3.5 | Dual responders present with less leukocyte-migratory sCD62L in plasma during the late phase than in single responders

Since there is a known relationship between the leukocyte migration into the airways and the shedding of CD62L, we assessed CD62L levels in the plasma (Figure 5C). Overall, DR had significantly less sCD62L than SR at all time points ($p = .0054$), markedly during the late phase at 6 h ($p = .0076$) and 8 h ($p = .033$) post-challenge. Higher concentrations of blood monocytes and neutrophils at baseline correlated with higher sCD62L just 30 minutes post-challenge

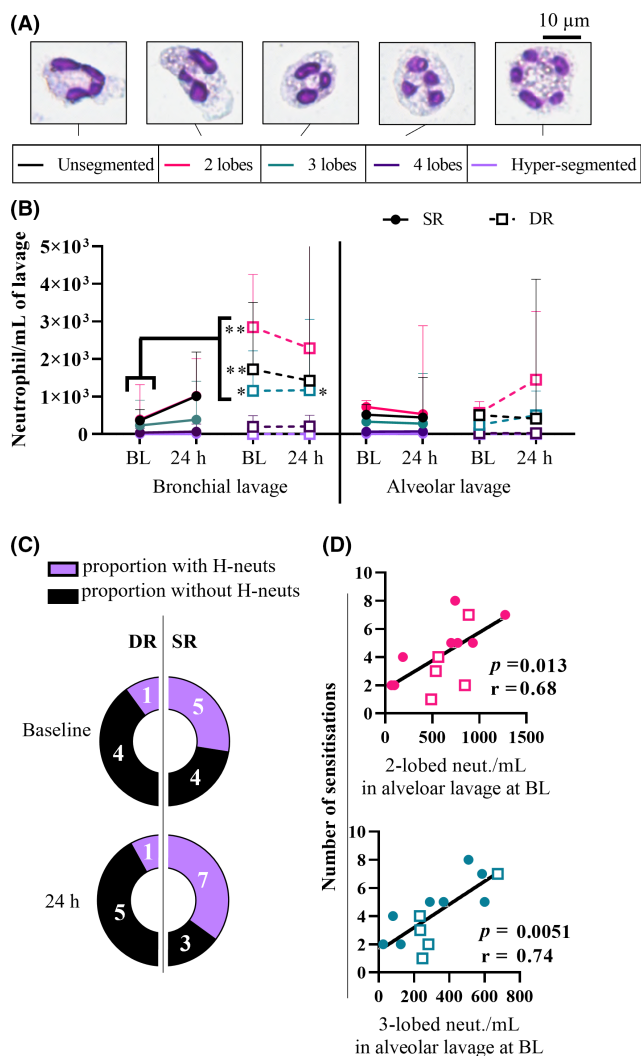


FIGURE 3 Morphological assessment of nuclei segmentation of lavage neutrophils. Data compare single responders (SR) and dual responders (DR) at baseline (BL) and 23 h post-challenge. (A) Representative images of the different segmented neutrophil nuclei. (B) Number of neutrophils with each type of segmentation/ml of lavage, $n(\text{SR}) = 8$, $n(\text{DR}) = 5$. (C) Hypersegmented neutrophils (H-neut) occurrence using combined alveolar + bronchial lavage cell numbers. (D) Correlations of 2- and 3-lobed neutrophils to the number of sensitisations

(Figure 5D, E) but only the neutrophils became inversely correlated with sCD62L levels—highlighting their strong relationship to shedding CD62L and migrating out of circulation as previously reported.³² For the complete matrix of correlations between sCD62L and blood leukocytes in this study, see Table S7.

3.6 | Lower levels of IL-1Ra (but not more IL-8 or IL-17) in the plasma correlate with more bronchial neutrophils at baseline

Since DR had significantly less sCD62L levels in the plasma, this may indicate differences in leukocyte migration patterns, we assessed the

neutrophil chemoattractants IL-8 and IL-17 and neutrophil antirecruitment IL-1Ra. In plasma, DR had less IL-8 at baseline ($p = .029$) but overall all IL-8 plasma readings showed low values^{33,34} (Figure 6Ai). Plasma IL-17 levels tended to increase in DR from baseline to 23 h ($p = 0.09$, paired Wilcoxon test) (Figure 6Bi). DR tended to have less IL-1Ra (median: 219 pg/mL) than SR (314 pg/mL) throughout with SR increasing IL-1Ra (BL-6h $p = .019$; Figure 6Ci). Meanwhile, the lavage levels of IL-8, IL-17 and IL-1Ra were similar in both DR and SR (Figure 6Aii, Bii, Cii). There was a significant correlation between IL-8 and IL-17 in alveolar lavage showing the chemoattractant condition in the lower airways was consistent at baseline (Figure S6A).

Of the three factors (Figure 6D), IL-1Ra plasma levels at baseline (but not bronchial lavage levels—see Figure S6B) correlated with the number of bronchial neutrophils at baseline (Figure 6Diii). Suggesting that DR has more bronchial neutrophils due to the lower antimigration signalling from IL-1Ra rather than an increased attraction signalling from IL-8 or IL-17.

3.7 | Dual responders release NETosis factors much earlier than single responders

Since DR had more neutrophils, in particular the more active 2- and 3-lobed neutrophils in the airways, we assessed whether DR had more tissue-damaging NETosis activity in the plasma and the lavage fluid. Following the allergen challenge, DR had increasing levels of histones in plasma already during the early phase (BL to 1h $p = .015$; Figure 7Ai). In contrast, SR had decreasing levels of histones during the early phase followed by a significant increase during the late phase (BL to 6h $p = .0045$). Although DR tended to have more dsDNA in the plasma throughout all time points, there was no significant difference between the two groups (Figure 7Bi). However, the NE levels followed a trend similar to the histone levels, with DR releasing more NE both during the early phase (BL to 0h $p = .037$, BL to 1h $p = .045$, BL to 2h $p = .0073$) and the late phase (BL to 8h $p = .0044$) compared with baseline, while NE in SR remained at baseline levels, only increased during the late phase (BL to 8h $p = .0009$; Figure 7Ci).

In the bronchial lavage (where more of the neutrophils resided) the histone levels tended to be higher 24 h post-challenge in DR (median: 2.3-fold) than in SR (0.5-fold, Figure 7Aii). The NETosis levels of dsDNA and NE tended to be higher in the DR (median: 12.4 ng/mL and 3.14 ng/mL, respectively) at baseline than in SR (median: 4.4 and 1.85 ng/mL, respectively; Figure 7Bii, Cii). NETosis factors in the alveolar lavage remained relatively the same.

Correlation analysis showed that higher numbers of 3-lobed active neutrophils in the bronchial lavage also had high levels of histone at baseline (Figure 7D). In addition, higher baseline numbers of 1- to 4-lobed active neutrophils, in contrast with hypersegmented neutrophils, correlated well with the level of histone in the lavage at 24 h post-challenge (see the matrix of correlations in Table S8A), whereby only 1- to 2-lobed neutrophils at 24 h maintained this correlation to histone levels at 23 h in the bronchial lavage (see the matrix of

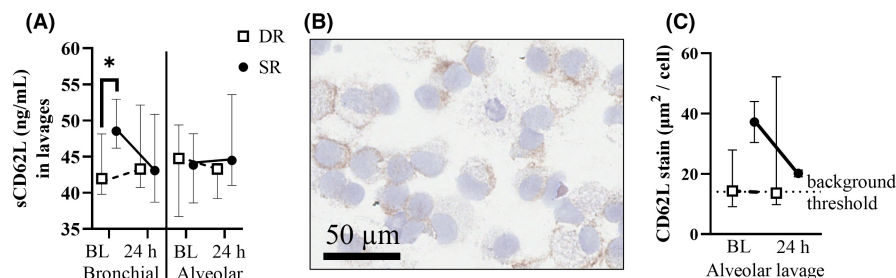


FIGURE 4 CD62L levels in lung lavage and on alveolar cells. Data compare single responders (●SR) and dual responders (□DR) at baseline (BL) and 24 h post-challenge. (A) ELISA of sCD62L in the lavage, $n(\text{SR}) = 10$, $n(\text{DR}) = 8$. (B) Representative image of CD62L stain (brown) on alveolar lavage cells. (C) CD62L stain is here as area of stain per cell, $n(\text{DR}) = 3$, $n(\text{SR}) = 2$

correlations in Table S8B). Of the more active 1- to 4-lobed neutrophils, the bronchial NE levels were correlated with unsegmented neutrophils numbers at baseline (Table S9A) and plasma NE levels were correlated with the bronchial 3-lobed (Figure 7E) and 4-lobed neutrophils 23/24 h post-challenge (see the matrix of correlations in Table S9B).

4 | DISCUSSION

Our data show that DR present with higher numbers of neutrophils within the airways at baseline compared with SR. These neutrophils are likely active neutrophils as they present with mostly 2- or 3-lobed segmented nuclei, that correlated with higher NETosis levels. The combination of less suppressive neutrophils and less IL-1Ra with no difference in chemoattractant levels suggests that DR may have had less antitransmigration signalling rather than more attractant signalling of neutrophils to the airways. After the allergen challenge, an early increase in NETosis factors and a later decrease of sCD62L indicate that DR had a differently timed neutrophil response to the allergen challenge than SR. These findings show, in agreement with our hypothesis, that neutrophils and their response take part in differentiating DR from SR responses in the airway environment and the overall response to an allergen challenge.

The higher number of airway neutrophils at baseline in DR was specially found in the bronchial lavage but not in the alveolar lavage. This location-specific increase is similar to the previously reported general increase proportion of neutrophils in the bronchial compared with the alveolar compartment in both asthmatics and healthy individuals.²⁸ Furthermore, the strong correlation of central biopsy neutrophils to the DR defining physiology during the late phase, provided a strong case for the more elevated airway neutrophils to become a distinguishing feature of DR asthma specifically before an allergen challenge. As allergen type was evenly distributed between SR and DR, the levels of natural allergen-associated LPS³⁵ are not likely to play a differentiating role in the levels of baseline neutrophils.³⁶

Morphological characterisation showed that the more abundant neutrophils in DR had lower nuclei segmentation. In general, 2- to 4-lobed neutrophils have previously been suspected to be mature

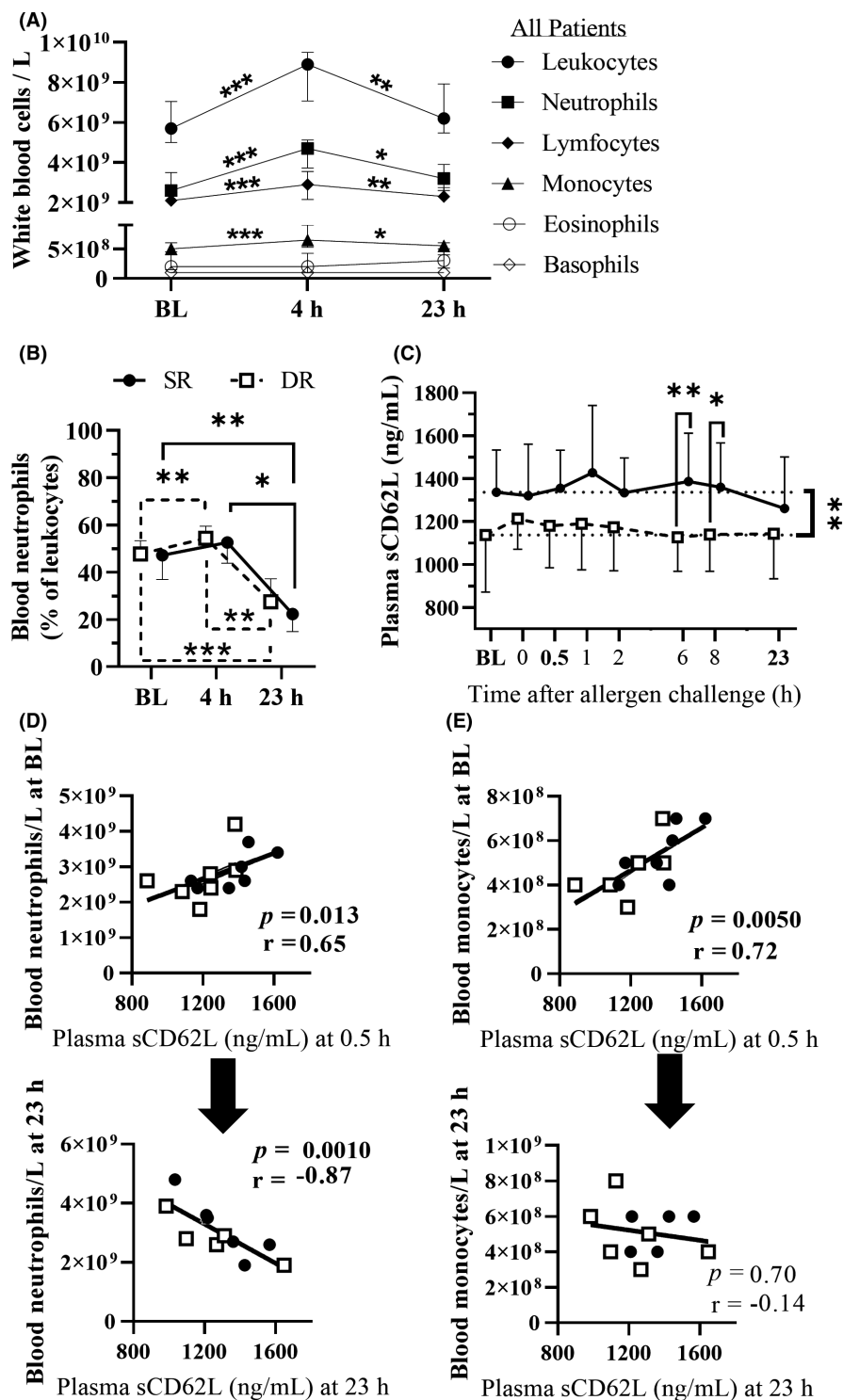
enough to be active.²² Here, the 3-lobed neutrophils were likely the best representation of active neutrophils, correlating with both NETosis factors and atopic sensitizations. NETosis factors, such as NE and histones, are commonly known to be released by active neutrophils and are often increased in severe asthma,³⁷ and more specifically neutrophilic asthma,³⁸ but are also increased during exacerbation.¹⁹

Interestingly, higher numbers of airway neutrophils and more sensitizations are key traits that distinguish more severe/uncontrolled asthma from milder asthma.^{7,11,39} The LAR response is representative of more severe disease,² suggesting that DR (mild/moderate) asthmatics are biologically closer to more severe asthmatics than their SR (mild/moderate) counterparts. This theory was further supported by the strong correlation, at both baseline and during the late phase, to clinical markers of peripheral obstruction (decreased X5 and increased AX) that has been reported in uncontrolled asthma with airway neutrophilia.³⁰ Our previous report on this same cohort also shows a high involvement of the peripheral airways in the DR.²⁶ Based on these similarities, one would think that the more promising treatments for severe or uncontrolled asthma based on targeting non-type-2 mechanisms may also be of benefit to DR.⁴⁰

In general, differences in neutrophil population numbers in the tissue can come from either increased migration into the tissue, decreased migration out of the tissue (e.g. less reverse transmigration¹⁷) or decreased cell death and clearance.⁴¹ Based on the low CD62L levels in the fluid and lavage cells in DR, the increased number of neutrophils seem to be newly migrated into the bronchial airways. Future flow cytometry of lavage and/or biopsy cells should be completed to confirm the migration status of the neutrophils. Nonetheless, increased migration could be due to increased chemoattraction either from specific signals or general tissue damage (such as the aforementioned obstruction), decreased antimigration signalling or increased permeabilization through the tissue.⁴¹

If the increased migration was due to increased chemoattraction, we would expect an increase in IL-8 and IL-17, especially at the tissue site to attract the neutrophils; however, this is not the case here. Instead, we find DR plasma has less IL-1Ra than SR similar to neutrophilic asthma.⁴² This compliments our findings as the IL-1R

FIGURE 5 Blood cell differentiation and plasma sCD62L levels. (A) Number of WBC/L of blood at baseline (BL), 4 h and 23 h post-challenge, $n(\text{BL}) = 17$, $n(4\text{h}) = 12$, $n(23\text{h}) = 13$. (B) Per cent of neutrophils relative to the total number of leukocytes at each time point, $n = 6$ –10. (C) ELISA of sCD62L in plasma, $n(\text{SR}) = 18$; $n(\text{DR}) = 12$. (D) Correlation of BL and 23 h blood neutrophils/L and (E) of BL and 23 h blood monocytes/L to sCD62L levels in plasma at 0.5 and 23 h post-challenge



(IL-1Ra target—not assessed here) has previously been reported to be a positive biomarker of airway neutrophilia and airway obstruction.⁴³ Glucocorticoids have been shown to decrease the amount of IL-1Ra released by neutrophils, which could be a contributing factor since more DR used ICS than SR in this study.⁴⁴ Based on these results, DR may benefit more from the nonchemoattractant targets tested as non-type-2 biotherapeutics than chemoattractant targets.⁴⁰

DR had less hypersegmented suppressive neutrophils to regulate immunoactivity—similar to how asthmatics have less T regulatory cells compared with healthy⁴⁵—adding to the theory that the immune response in DR is less regulated. To support our suppressive neutrophil findings, since a number of occurrences were low, we found a recent study that shows higher CC16 levels correlates with less suppressive neutrophils in a mouse model.⁴⁶ This coincides with our previously reported findings that these DR subjects had

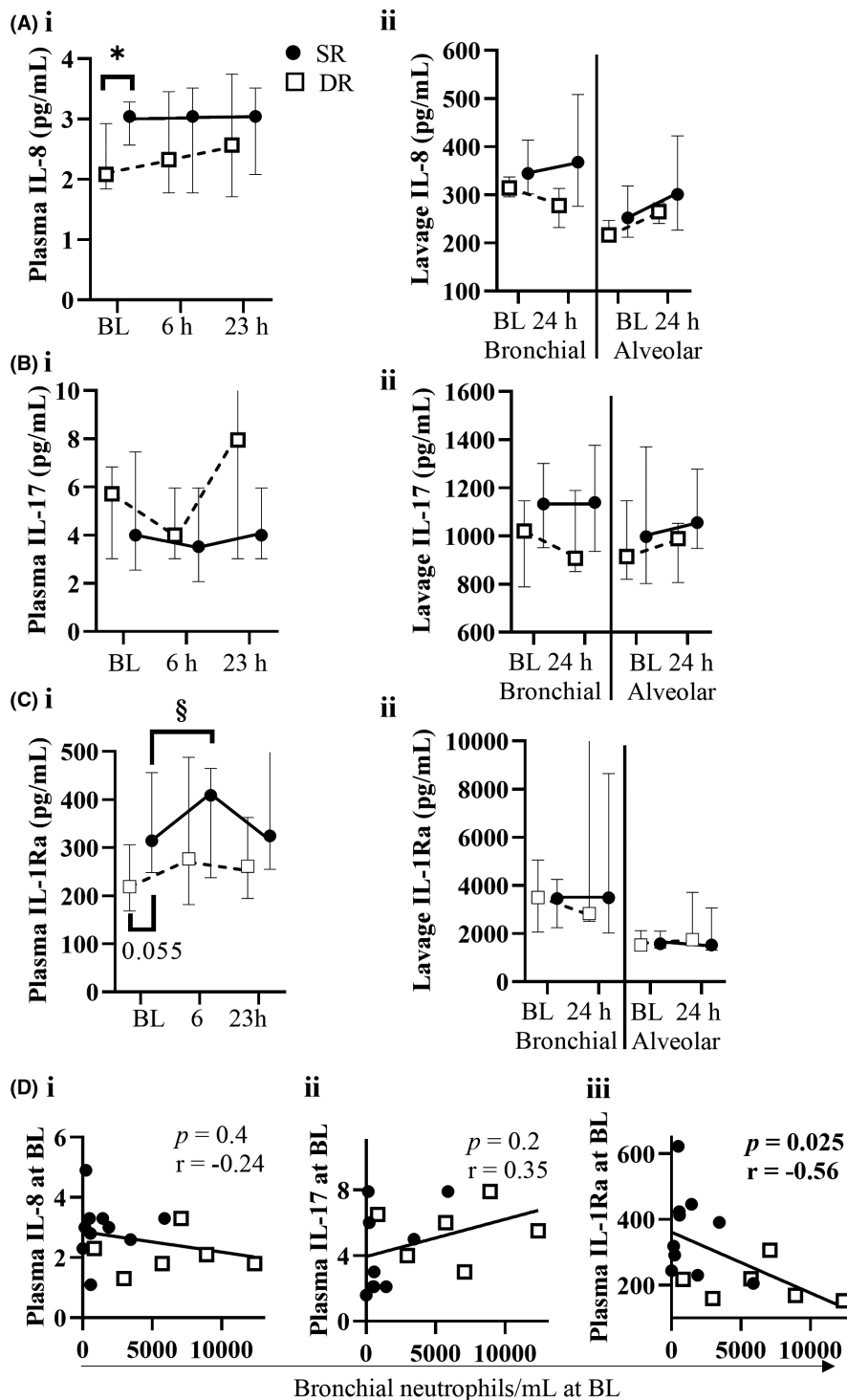


FIGURE 6 Levels of IL-8, IL-17 and IL-1Ra within the plasma and lung lavage. Luminex assay for single responders (●SR) and dual responders (□DR) at baseline (BL), 6 and 23 h post-challenge in the plasma for (Ai) IL-8, (Bi) IL-17 and (Ci) IL-1Ra, at BL and 23 h $n(\text{SR}) = 18$ and $n(\text{DR}) = 12$; at 6 h $n = 10$ each. In the lavages for (Aii) IL-8, (Bii) IL-17 and (Cii) IL-1Ra, $n(\text{SR}) = 10$ and $n(\text{DR}) = 8$. (D) Bronchial lavage neutrophils at baseline were correlated with each in the plasma for (i) IL-8, (ii) IL-17 and (iii) IL-1Ra

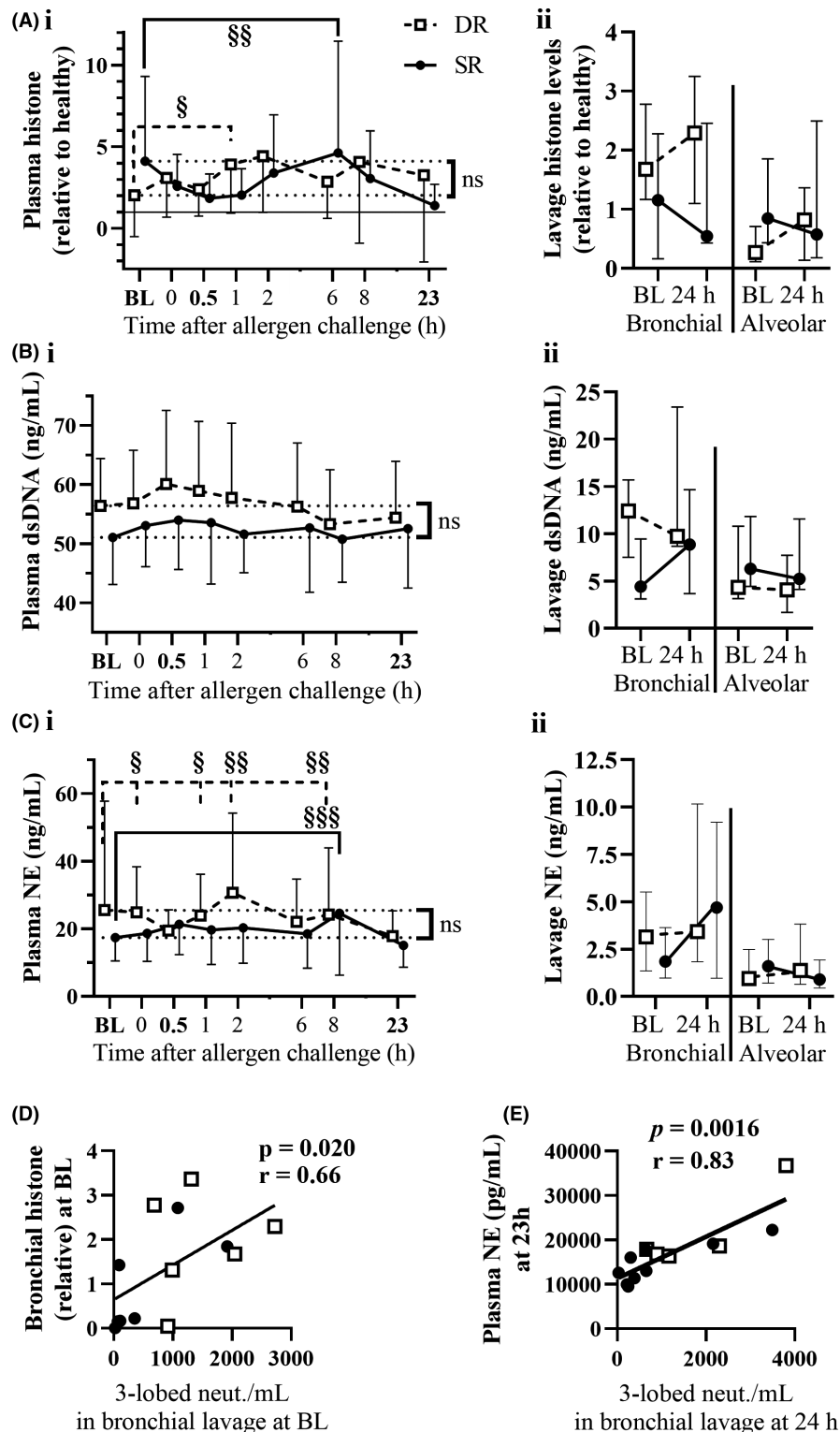
significantly higher CC16 levels than SR post-challenge.²⁷ With the current assessments, our study suggests that DR may lack sufficient cell types and signalling that could block neutrophil transmigration at baseline that could lead to a type of neutrophilic asthma.

After the allergen challenge, DR had less sCD62L in plasma than in SR—a state known to relate to lung injury inflammation.⁴⁷ Less sCD62L has been reported to occur when there is less leukocyte migration¹⁸ into the lungs⁴¹ while an acute rise (like in SR) can self-limit the neutrophil transmigration.⁴⁸ Neutrophils are also

often required for monocyte recruitment,⁴⁹ a cell in excess found to be associated with fatal asthma airway tissue⁵⁰ and were also increased here in DR airways, so they should be assessed further alongside neutrophils.

Using correlation analysis at baseline and 23 h post-challenge, we reason that sCD62L levels in the plasma might have given a good indication for neutrophil migration in these subjects. The pattern of sCD62L levels relative to baseline indicated that DR neutrophils may not have transmigrated during the early reaction and even less

FIGURE 7 Levels of NETosis factors in plasma and lung lavage. Data compare single responders (●SR) and dual responders (□DR) at baseline (BL) up to 23 h post-challenge. (Ai) ELISA of histones relative to healthy levels, (Bi) Qubit assay of dsDNA and (Ci) ELISA of NE in plasma, $n(\text{SR}) = 18$, $n(\text{DR}) = 12$. Lavage levels of (Aii) histones, (Bii) dsDNA and (Cii) NE. The number of 3-lobed bronchial neutrophils correlated with (D) bronchial histone levels at baseline and (E) plasma NE levels at 23/24 h post-challenge



during the late phase, meaning that DR neutrophils stayed in the blood long after the allergen challenge while SR neutrophils may have started migrating out before the late phase. This coincides with the early-phase release of NETosis factors in DR plasma but not SR. Suggesting that SR may have a more 'healthy' neutrophil response to the allergen challenge that may prevent the LAR from occurring or that the more active neutrophil response to the allergen challenge may give rise to the LAR.

5 | CONCLUSION

In conclusion, DR had more neutrophils within the airways that were mostly 2- or 3-lobed active phenotype at baseline than at SR. Correlation analysis indicates that an increase in neutrophils in DR airways comes from either more peripheral tissue obstruction and/or less antimigration signalling. Following an allergen challenge, DR released more NETosis factors during the early phase and shed less

transmigrating sCD62L during the late phase than SR. Overall, these findings indicate that the neutrophil response is differently regulated in DR than in SR, and this may be related to the LAR occurring in DR but not in SR. In the future, clinically, it is important to also target the excessive population of active neutrophils together with the type-2 inflammation in more difficult types of asthma such as the DR.

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CONFLICT OF INTEREST

All authors declare no conflict of interest.

AUTHOR CONTRIBUTION

NV, HS, ZD, LB and ET involved in conceptualization; NV, SE, DB, LB and ET involved in methodology; NV and SE involved in formal analysis; NV, HS, SE and DB involved in investigation; HS, JA, L-OC, LB, JE and ET involved in resources; NV involved in data curation and visualisation; NV and ET involved in writing-drafts and project administration; NV, HS, SE, ZD, SKG, L-OC, LB, JE and ET involved in writing-revisions; ZD, JA, SKG, L-OC, LB, JE and ET involved in supervision; NV, L-OC, LB, JE and ET involved in funding acquisition.

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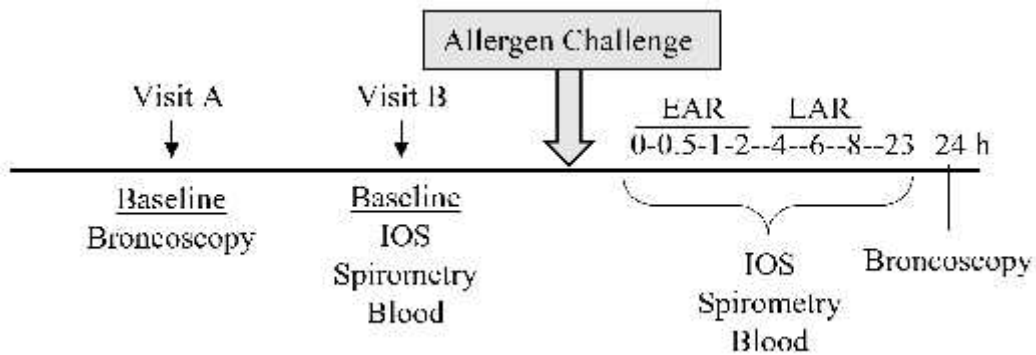
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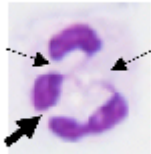
SUPPORTING INFORMATION

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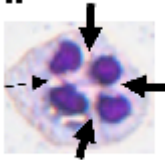
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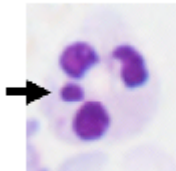
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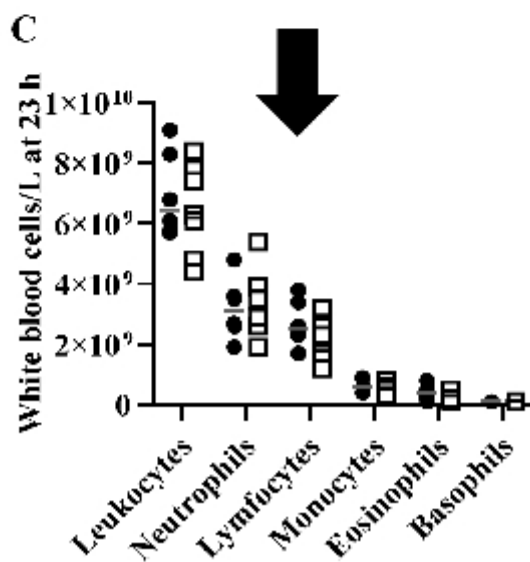
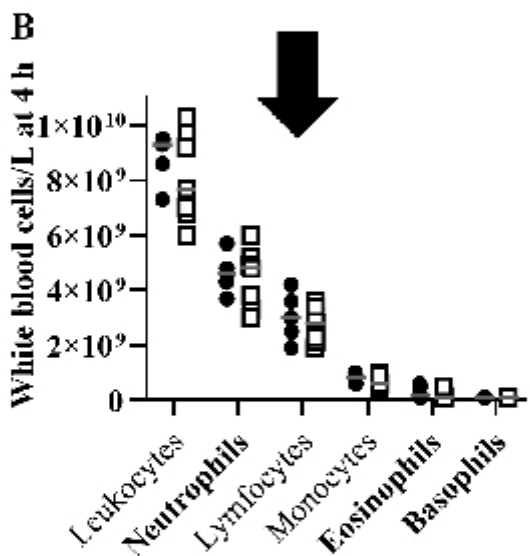
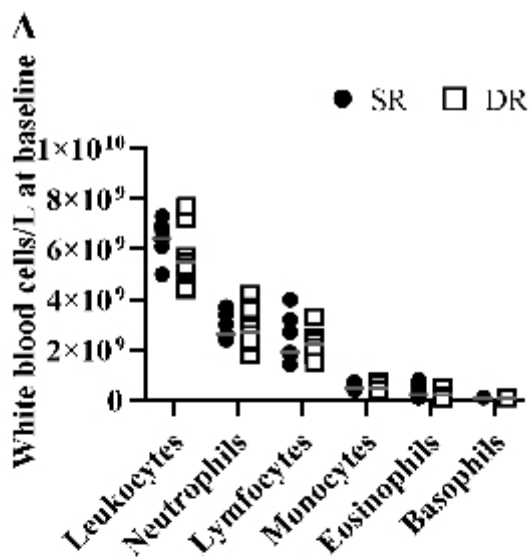


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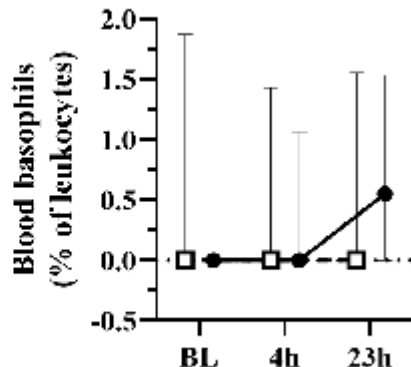
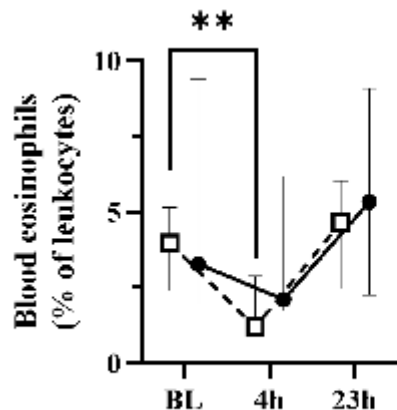
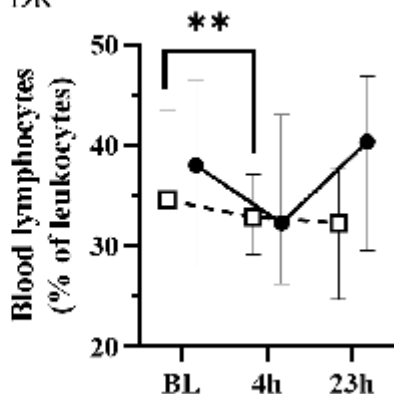
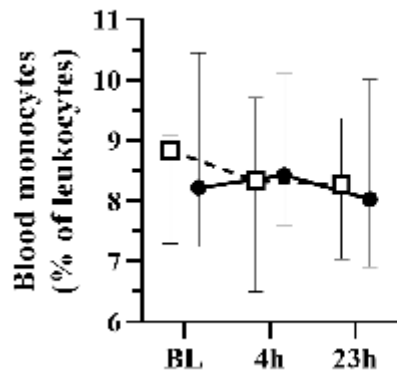


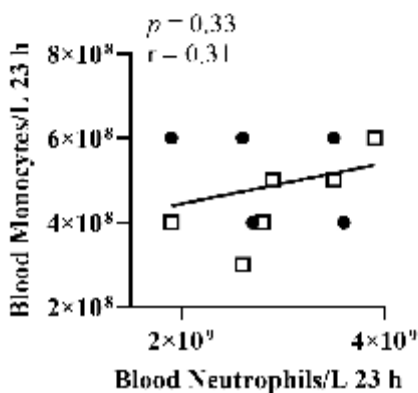
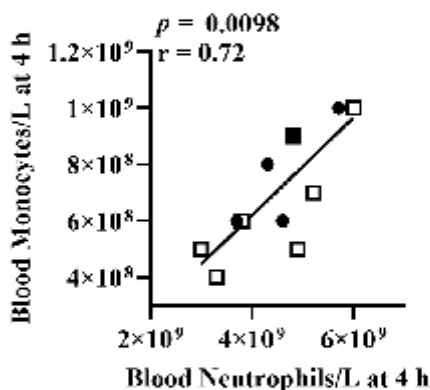
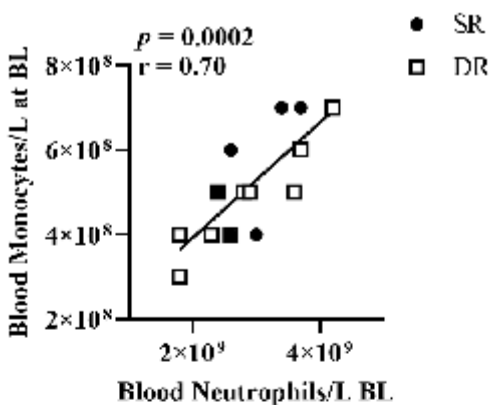
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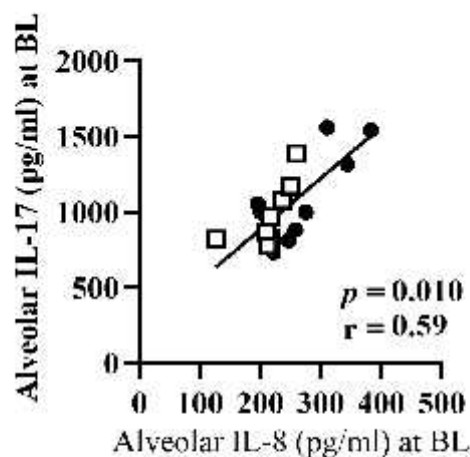
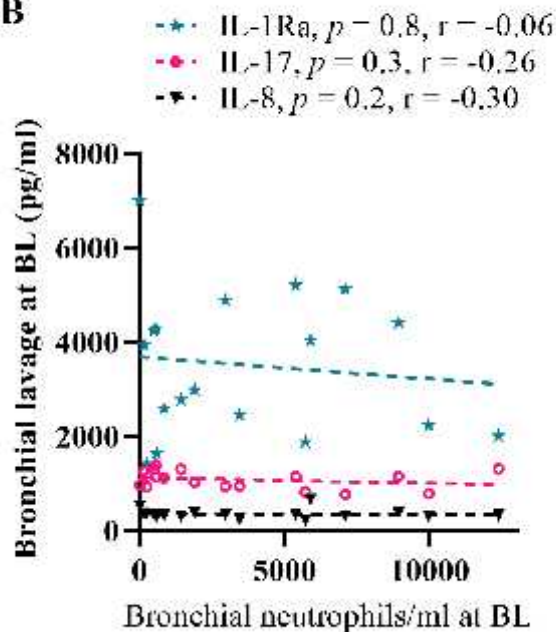




● SR □ DR





A**B**

SUPPLEMENTAL METHODS

Additional Abbreviations

RGB red, green, blue

Reagents source

Kwick-Diff (Kit cat: 9990700, Lot: VDF226, Shandon, Thermo Scientific, USA); CD62L DuoSet (Cat: DY728, lot: P231072, R&D Systems, Sweden) NE DuoSet (Cat: DY9167-05, lot: P206180 and P230408, R&D Systems, Sweden); Cell Death Detection ELISA kit (Cat: 11544675001, Roche, Merck, Germany); Magnetic Luminex® Assay multiplex kit (Cat: LXSAHM, Bio-technique); Qubit® dsDNA HS assay kit (Cat: Q32851, Life Technologies); anti-CD68 (clone: PG-M1, ref: M0876, lot: 20047721, Dako, Denmark); anti-CD163 (ref: NCL-CD163, lot: 6057985, LEICA, UK); anti-MPO (ref: A0398, lot: 20051734, Dako, Denmark); Dako Envision++ anti-rat/anti-mouse dual link system-HRP secondary (Ref: K4063, Lot: 11246748, Agilent Technologies, Denmark); Denaturing solution (ref: DN5001L, lot: 07021, Biocore medical); DAB (ref: K346711-2, Dako, Denmark), Vina Green™ Chormogen kit (ref: BRR807A, Biocare medical), formaldehyde 4% buffered (Ref: 02176, lot: 3614020, HistoLab, Sweden); rat anti-human CD62L (Ref: ab244495, Lot: GR3279841-5, abcam, Netherlands); Mayers hematoxylin (Ref: 01820, Lot: 2520307, HistoLab, Sweden)

Softwares

GraphPad Prism (v9.1.2, San Diego, CA 92108, USA); ImageScope (v12.3.3.5048, Leica Biosystems, USA) ImageJ (v1.52a, Wayne Rasband, National Institutes of Health, USA)

25

26 Bronchial and alveolar lavage cell differentiation

27 Bronchial lavage and alveolar lavage were filtered through a 100 µm cell strainer and
28 centrifuged for 10 min at 400 g within 30 minutes of collection. Lavage fluid was aliquoted
29 and put in -80°C until later analyses. The cells were thereafter re-suspended in PBS and
30 subjected to cytopsin (500 x g for 6 min at 30 000 cells/spot on SuperfrostPlus slides). Air
31 dried slides were fixed with methanol and stained with Diff Kwick (Shandon, cat: 9990700).
32 A minimum of 400 cells were differentially counted as macrophage, lymphocyte, neutrophil,
33 eosinophil or basophil. The percentage of each cell type was multiplied by the total number
34 of cells per lavage to give the total number of each cell type. The total number of each cell
35 type was also divided by the volume collected to produce the number of each cell type per
36 mL of lavage.

37

38 Blood sampling

39 White blood cell differentiation data was given as cells per litre (L) of blood. Plasma was
40 separated from EDTA-treated whole blood 30 minutes after collection (centrifugation at 2000
41 x g for 10 minutes) and stored at -80 until analysis.

42

43 Enzyme-linked immunosorbent assay

44 Based on a dilution series of a few samples, plasma was diluted to 1:800 for CD62L, 1:20 for
45 NE and used neat for histone-associated DNA fragments. Lavage fluid was used neat for
46 histone ELISA. Background absorbance and then average PBS sample readings were
47 removed from sample absorbance before extrapolating concentration of CD62L and NE

based on the hyperbola curve of standards as calculated in GraphPad Prism. The same background was removed from histone data and then all samples were normalised to the average healthy patient reading for plasma and lavage respectively and presented as such.

Protein concentrations using multiplex Luminex

Plasma samples at baseline, 6 h (to capture the LAR) and 23 h after the allergen challenge, and lavage fluids at baseline and 24 h after the allergen challenge were analysed using Luminex containing beads for IL-8, IL-17 and CD62L. Each plate was processed as per manufactures instructions where the samples were incubated at 4 °C overnight and the reader (MagpixTM, Luminex XMPA technology) was set to a minimum of 50 counts. All plasma samples (diluted 1:2) reached 50 counts, however, the lavage samples (diluted 1:2) averaged 22 counts – increasing the error slightly.

Immunohistochemical staining in biopsy specimens and cytopins

Neutrophils in biopsies

Biopsy tissue taken at baseline and 24 h after the allergen challenge were fixed in standard formaldehyde-based paraffin, sectioned at 3 µm and stored at 4°C until stained. Before staining, endogenous peroxidase was blocked with 0.3% H₂O₂. Firstly, macrophages were pre-stained (to exclude potentially confounding macrophages) with a anti-CD68 and anti-CD163 primary cocktail (1 h) and the secondary-HRP (30 m) and labelled with brown DAB precipitate (10 m). To prevent a cross reaction, tissue was then incubated in a denaturing solution (5 m). Then the neutrophils were stained with for anti-MPO (1 h) and the secondary-HRP (30 m) was labelled with vina green chromogen detection (10 m). Viable airway tissue images taken did not include the majority of the fat and smooth muscle cells present in the biopsy section. All slides were digitalized using Aperio and snapshots were taken at 40x

zoom using ImageScope software and imported into ImageJ for analysis using *colour threshold area* to generate the percentage of neutrophil green area normalized to the total area of tissue. Note that the excess light green (assumed to be released MPO into the tissue) was not included in the total green area by adjusting the *brightness* threshold.

CD62L on alveolar lavage cells

Alveolar cytopins, fixed in formaldehyde and stored in PBS (5 paired samples of baseline and 24 h), were stained using the abcam IHC protocol using 0.3% H₂O₂ (15 min) after the primary antibody (website: <https://www.abcam.com/protocols/immunostaining-paraffin-frozen-free-floating-protocol>). Slides were incubated with anti-human CD62L primary antibody (overnight at 4°C) then anti-rat/anti-mouse HRP-conjugated Dako EnVision secondary (1 h) coloured with DAB (30 min). Cells were counterstained with hematoxylin (60 s, developed in bi-carb water for 5 min) and eosin (5 s). Ten images of mixed lavage cells were processed in ImageJ using RGB *colour threshold area* and *cell count* to produce average CD62L stain area per cell.

ImageJ parameters

- Biopsy total tissue: Hue 0 - 255, Brightness reduced until background noise removed, average = 234
- Biopsy neutrophil MPO (green): Hue 30-150 adjusted to remove the background Brightness peak (within min 160, max 200)
- Lavage cytopsin CD62L (brown): Hue 0-20 + 225-255
- Lavage cytopsin cell count: Gaussian Blur, Sigma 2 and Maxima noise tolerance 4 (or 9 for dim staining)

97

98 **Statistics**

99 ELISA Standard curves and interpolation of data based on a hyperbola fit and data statistics
 100 were calculated using GraphPad Prism (version 9). Non-parametric Mann-Whitney U-test
 101 was used for data comparing SR and DR groups at each time point and Wilcoxon signed rank
 102 test was used to compare paired values between times when there was only two or three time
 103 points. Comparing DR to SR at each time point with more than three timepoints used mixed-
 104 effects analysis corrected using Šidák test. Comparing DR and SR separately for each time
 105 point to baseline used 2-way ANOVA with multiple comparisons corrected using the Dunnett
 106 test. Data that was normally distributed (determined by Kolmogorov-Smirnov test) used
 107 parametric ratio paired tests for changes over time and a Welch's t test for comparing SR and
 108 DR. Significance are shown in figures as $^{*/\$}p < 0.05$, $^{**/§§}p < 0.01$, $^{***/§§§}p < 0.001$ and
 109 $^{****/§§§§}p < 0.0001$, where '*' are significance between data presented and '\$' are
 110 significance between relative percentage of data to paired baseline value. Data points were
 111 only removed as outliers if they were four or more standard deviations above or below the
 112 mean of the entire assay (i.e. including all DR, SR and timepoint data for that assay).
 113 Statistics in correlation graphs in figures was only bolded if they showed a significant
 114 difference from zero (linear regression $p < 0.05$) and a strong correlation (Spearman's rank
 115 analysis $p < 0.05$; $-0.60 < R < 0.60$) when adjusted for multiple comparisons such as multiple
 116 timepoints of the same variable with and without any assay outliers included (calculated in
 117 excel as data above or below 2.5 standard deviations from the whole assay average).

118 **Specific Statistics for each Figure**

119 Table 1: Data is presented as median (IQR). Statistical significance between SR and DR of all
 120 patients was determined by non-parametric Mann-Whitney test ($p < 0.05$ is bolded). Body
 121 mass index (BMI), inhaled corticosteroids (ICS), kilounits per litre (kU/L), standardised

quality units (SQ-U), asthma control test (ACT), forced expiratory volume in one second (FEV1).

Figure 1: (A): Mann-Whitney U test was used for difference between SR and DR, Wilcoxon test was used for paired bronchial and alveolar lavage, line represents median, (B): Spearman's rank analysis of all patients (i.e. SR and DR), where bolded p and r value indicate strong correlation. $*p < 0.05$, $**p < 0.01$.

Figure 2: (B): Data was normally distributed (Kolmogorov-Smirnov test) and therefore difference between SR and DR used Welch's t test, (C, D): Spearman's rank analysis of all patients (i.e. SR and DR), where bolded p and r value indicate strong correlation. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

Figure 3: (B): Mann-Whitney U-test, data is presented as median+IQR, (D): Spearman's rank analysis of all patients (i.e. SR and DR), where bolded p and r value indicate strong correlation. $*p < 0.05$, $**p < 0.01$.

Figure 4: Statistics(A): Mann-Whitney U test, data is presented as median+IQR. $*p < 0.05$.

Figure 5: (A): Wilcoxon signed rank test, data is presented as median $\pm$ IQR (B): data was normally distributed (Kolmogorov-Smirnov test), therefore used ratio paired t test, data is presented as mean $\pm$ or - SD, (C) mixed-effects analysis, data is presented as mean $\pm$ or - SD, (D, E): Spearman's rank test of all patients (i.e. SR and DR), where bolded p and r value indicate strong correlation. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figure 6: (A, B, C): difference between SR and DR used Mann-Whitney U test, $*p < 0.05$, change over time used Wilcoxon, data presented as median $\pm$ IQR, with relative to baseline indicated by $§p < 0.001$. Outliers removed from plasma results include: 2 DR outliers in the IL-8, 2 SR outliers in the IL-17 and 1 DR outlier in the IL-17. (D): Spearman's rank analysis of all patients (i.e. SR and DR), where bolded p and r value indicate strong correlation.

146 Figure 7: (Ai, Bi, Ci): 2-way ANOVA was used for >3 timepoints, data presented as mean +
147 or – SD, with relative to baseline indicated by $^{\$}p < 0.05$, $^{ §§}p < 0.01$, $^{ §§§}p < 0.001$ (Aii, Bii,
148 Cii): data presented as median $\pm$ IQR for two timepoints (D, E): Spearman’s rank analysis of
149 SR+DR patients, where bolded p and r value indicate strong correlation.

150

SUPPLIMENTAL TABLES

Supplementary table 1: **Patient characteristics.** Separated into single responders (SR) and dual responders (DR) for the sub-group analysis.

	Bronchoscopy		WBC	
	SR (n = 10)	DR (n = 8)	SR (n = 7)	DR (n = 10)
Sex (Male/Female)	5/5	4/4	6/1	5/5
Age (years)	29	27	25	27
BMI (kg/m²)	23	24	23	24
Regular use of ICS (n)	4	6	1	8
Duration of asthma (years)	19	17	10	17
ACT (score)	22	22	19	21
Methacholine PD₂₀ (µg)	137	225	183	390
Number of sensitisations	5	3	4	3
Allergen inhaled (n)				
○ Cat	7	4	3	4
○ Horse	2	1	1	2
○ Birch	0	2	2	2
○ D. pteronyssinus	1	1	0	1
○ Grass	0	0	1	1
FEV1 % max drop 0-2 h after challenge	21.5	24.8	21.7	24.2
FEV1 % max drop 4-8 h after challenge	6.9	20.2	8.6	19.0

Data is presented as median in the sub-groups. Body mass index (BMI), inhaled corticosteroids (ICS), asthma control test (ACT), forced expiratory volume in one second (FEV1).

Supplementary table 2: **Number of each cell type per mL of bronchial and alveolar lavage.** Separated into baseline (BL) and 24 hours (h) after allergen challenge for single responders (SR) and dual responders (DR). Including total cells/ml in lavage and total ml of lavage collected.

Cells / ml of lavage (*10 ³)		Baseline (BL)			24 h after challenge			BL v 24 h	
		SR	DR	M-W <i>p</i> ^a	SR	DR	M-W <i>p</i> ^a	SR <i>p</i> ^b	DR <i>p</i> ^b
Macrophages	Alveolar	91 (32-160)	130 (53-310)	0.4	110 (74-150)	150 (34-180)	0.7	0.3	0.9
	Bronchial	61 (19-119)	191 (84-269)	0.021	66 (43-160)	110 (82-240)	0.2	0.6	0.6
Eosinophils	Alveolar	1.4 (0.2-2.1)	0.4 (0.1-1.6)	0.5	1.2 (0.4-2.9)	0.6 (0.5-2.6)	0.4	0.8	0.2
	Bronchial	0.8 (0.2-1.6)	0.5 (0.4-2.5)	0.8	0.6 (0.3-2.1)	1.1 (0.3-1.3)	1.0	>.9	0.6
Neutrophils	Alveolar	1.3 (0.3-2.1)	1.4 (0.5-1.8)	0.8	1.1 (0.5-6.5)	0.5 (0-3-3.2)	0.4	0.3	0.6
	Bronchial	0.6 (0.3-1.8)	6.4 (3.6-9.7)	0.0031	2.8 (1.8-5.6)	5.1 (1.2-7.1)	0.8	0.5	0.8
Lymphocytes	Alveolar	3.4 (2.0-7.5)	3.1 (1.1-8.6)	0.9	4.9 (1.6-8.8)	8.6 (1.0-10)	0.8	0.5	0.9
	Bronchial	2.9 (0.9-6)	2.8 (1.9-6.3)	0.6	3.9 (1.5-7.2)	2.6 (1.1-8.6)	0.9	0.8	>.9
All cells / ml	Alveolar	100 (35-165)	134 (56-312)	0.4	132 (76-164)	153 (35-197)	0.7	0.3	0.9
	Bronchial	64 (22-129)	202 (93-277)	0.012	72 (46-172)	106 (100-236)	0.2	0.5	0.7
Total ml lavage collected	Alveolar	60 (36-86)	79 (60-83)	0.4	79 (48-84)	85 (72-90)	0.3	0.5	0.7
	Bronchial	20 (14-31)	15 (14-21)	0.3	15 (13-21)	16 (10-27)	0.8	0.016	0.6

Data is presented as median (IQR). ^a Mann-Whitney test (M-W) was used for comparison between SR and DR at baseline and 24 h after allergen challenge. ^b Wilcoxon matched-pairs test was used for comparison between BL and 24 h within SR and DR. *p* < 0.05 is bolded.

Supplementary table 3: **Number of different cells per total volume of bronchial and alveolar lavage collected.** Separated by baseline (BL) and 24 hours (h) after allergen challenge for single responders (SR) and dual responders (DR). Including total number of cells per lavage.

Cells / lavage (*10 ⁵)		Baseline (BL)			24 h post challenge			BL v 24 h	
		SR	DR	M-W <i>p</i> ^a	SR	DR	M-W <i>p</i> ^a	SR <i>p</i> ^b	DR <i>p</i> ^b
Macrophages	Alveolar	57 (20-91)	90 (37-230)	0.2	69 (60-110)	100 (29-140)	0.9	0.2	0.7
	Bronchial	10.1 (5-25)	31.5 (16-53)	0.05	14 (5.8-22)	21 (6.4-79)	0.3	0.9	0.8
Eosinophils	Alveolar	0.5 (0.1-1.7)	0.3 (0.1-1)	0.7	0.7 (0.06-2.1)	0.3 (0.04-1.8)	0.5	0.3	0.3
	Bronchial	0.2 (0.05-0.4)	0.1 (0.07-0.3)	>.9	0.07 (0.04-0.3)	0.1 (0.02-0.2)	0.9	0.9	0.8
Neutrophils	Alveolar	0.8 (0.1-1.7)	1.1 (0.9-1.4)	0.5	0.8 (0.4-4)	0.7 (0.2-2.7)	0.6	0.3	0.4
	Bronchial	0.2 (0.05-0.4)	1.1 (0.8-1.4)	0.0019	0.6 (0.3-0.7)	0.5 (0.1-3.1)	>.9	0.3	>.9
Lymphocytes	Alveolar	2.3 (1.3-3.7)	2.5 (0.9-5.2)	0.8	3.4 (0.5-6.4)	6.2 (0.8-9.3)	0.5	0.4	0.2
	Bronchial	0.5 (0.1-1.5)	0.4 (0.3-1.7)	0.7	0.9 (0.2-1)	0.5 (0.2-1.2)	0.5	0.9	0.8
Total cells	Alveolar	66 (23-96)	95 (40-230)	0.2	80 (63-110)	110 (30-150)	0.9	0.2	0.6
	Bronchial	12 (5.9-26)	33 (18-55)	0.07	16 (5.6-23)	18 (8-80)	0.2	0.9	>.9

Data is presented as median (IQR). ^a Mann-Whitney test (M-W) was used for comparison between SR and DR at baseline and 24 h after allergen challenge. ^b Wilcoxon matched-pairs test was used for comparison between BL and 24 h within SR and DR. *p* < 0.05 is bolded.

Supplementary table 4: **Correlations between the max FEV% drop at 0-2h (early phase) and 4-8h (late phase) to all bronchial lavage baseline cells / mL using data from all patients (i.e. SR and DR).**

Baseline bronchial lavage cells	Max drop in FEV1%	
	Early phase	Late phase
Macrophages	p = 0.8	0.005
	r = -0.05	0.63
Neutrophils	p = 0.8	0.0028
	r = -0.08	0.66
Lymphocytes	p = 0.6	0.7
	r = -0.16	0.11
Eosinophils	p = 0.7	0.6
	r = 0.12	0.15

Supplementary table 5: **Correlations between all the impulse oscillometry (IOS) readings of X5 and AX to the bronchial biopsy baseline neutrophils / mm² using data from all patients (i.e. SR and DR).**

*Slope did not significantly differ from zero using simple linear regression.

IOS: Biopsy neutrophil at baseline			
		X5	AX
Baseline	<i>p</i> =	0.0049	0.040
	<i>r</i> =	-0.80	0.63
0h	<i>p</i> =	0.4	0.3
	<i>r</i> =	-0.29	0.35
0.5 h	<i>p</i> =	0.3	0.6
	<i>r</i> =	-0.39	0.16
1 h	<i>p</i> =	0.4	0.6
	<i>r</i> =	-0.28	0.18
2 h	<i>p</i> =	0.0005	0.013*
	<i>r</i> =	-0.89	0.73
4 h	<i>p</i> =	0.0048	0.013
	<i>r</i> =	-0.80	0.73
5 h	<i>p</i> =	<0.0001	0.0057
	<i>r</i> =	-0.94	0.79
6 h	<i>p</i> =	<0.0001	0.011
	<i>r</i> =	-0.94	0.75
7 h	<i>p</i> =	0.0006	0.0022
	<i>r</i> =	-0.89	0.84
8 h	<i>p</i> =	0.0025	0.011
	<i>r</i> =	-0.83	0.75
23 h	<i>p</i> =	0.0089	0.020
	<i>r</i> =	-0.76	0.70

Supplementary table 6: **Correlations between number of each identified nuclei-lobed sub-population of neutrophils/ml in bronchial or alveolar lavage at baseline versus number of allergen sensitisations using data from all patients (i.e. SR and DR).**

A) Baseline bronchial neutrophils		Unsegmented	2-lobed	3-lobed	4-lobed	Hyper-segmented	Total cells
Number of Sensitisations	<i>p</i> =	0.9	0.3	0.5	0.5	0.06	0.3
	<i>r</i> =	-0.042	-0.27	-0.20	-0.20	0.58	-0.33

B) Baseline alveolar neutrophils		Unsegmented	2-lobed	3-lobed	4-lobed	Hyper-segmented	Total cells
Number of Sensitisations	<i>p</i> =	0.09	0.013	0.0051	0.06	0.7	0.0098
	<i>r</i> =	0.50	0.68	0.74	0.54	-0.16	0.70

Statistics: Spearman's rank analysis of all patients, where bolded *p* and *r* values indicate a strong correlation.

Supplementary table 7 **Correlation matrix of data from all patients (i.e. SR and DR) for sCD62L in plasma versus the number of the different blood cells (per ml) before and for each time point after allergen challenge.**

A) Baseline blood cells	sCD62L in plasma								
		Baseline	0 h	0.5 h	1 h	2 h	6 h	8 h	23 h
Leukocytes	<i>p</i> =	0.9	0.9	0.2	0.7	0.7	0.3	0.8	0.3
	<i>r</i> =	0.050	0.015	0.37	0.13	0.13	0.32	0.081	0.33
Neutrophils	<i>p</i> =	0.2	0.5	0.013	0.2	0.2	0.2	0.3	0.9
	<i>r</i> =	0.38	0.22	0.65	0.40	0.34	0.34	-0.35	-0.050
Lymphocytes	<i>p</i> =	0.043	0.3	0.2	0.5	0.6	0.5	0.9	0.7
	<i>r</i> =	-0.60	-0.32	-0.40	-0.22	-0.17	-0.22	-0.028	0.13
Monocytes	<i>p</i> =	0.2	0.6	0.005	0.2	0.3	0.4	0.7	0.7
	<i>r</i> =	0.38	0.14	0.72	0.41	0.30	0.23	0.13	0.10
Eosinophils	<i>p</i> =	0.6	0.4	0.5	0.4	0.7	0.5	0.3	0.4
	<i>r</i> =	-0.19	-0.23	-0.20	-0.24	-0.14	0.19	-0.38	0.25

B) 23 h blood cells	sCD62L in plasma								
		Baseline	0 h	0.5 h	1 h	2 h	6 h	8 h	23 h
Leukocytes	<i>p</i> =	0.1	0.1	0.4	0.2	0.5	0.9	0.5	0.2
	<i>r</i> =	-0.52	-0.46	0.26	0.42	0.21	0.046	-0.24	-0.39
Neutrophils	<i>p</i> =	0.5	0.3	0.5	0.2	0.4	0.8	0.2	0.001
	<i>r</i> =	-0.24	-0.37	0.24	0.44	0.28	0.082	-0.43	-0.87
Lymphocytes	<i>p</i> =	0.2	0.4	0.2	0.2	0.5	0.096	0.3	0.4
	<i>r</i> =	-0.39	-0.25	0.41	0.45	0.22	0.51	0.35	0.25
Monocytes	<i>p</i> =	0.3	0.6	0.5	0.6	0.5	0.6	0.8	0.7
	<i>r</i> =	-0.37	-0.19	0.26	0.19	0.26	-0.17	-0.089	-0.14
Eosinophils	<i>p</i> =	0.048	0.4	0.7	0.9	0.4	0.6	0.2	0.3
	<i>r</i> =	-0.65	-0.27	-0.15	0.0063	-0.30	0.17	-0.46	0.29

Statistics: Spearman's rank analysis, where bolded *p* and *r* values indicate strong correlations.

Supplementary table 8: **Correlation matrix of data from all patients (i.e. SR and DR) for lobed-neutrophils in bronchial lavage at A) baseline and B) 24 h versus histone levels in bronchial lavage at each time point.**

A) Bronchial lobed neutrophils/ml at baseline		Unsegmented	2-lobed	3-lobed	4-lobed	Hyper-segmented	(All neutrophils)
Bronchial lavage histone at baseline	$p =$	0.08	0.09	0.020	0.1	0.8	0.020
	$r =$	0.51	0.49	0.66	0.45	0.06	0.61
Bronchial lavage histone at 24 h	$p =$	0.0061	0.0061	0.012	0.050	0.6	0.0048
	$r =$	0.73	0.73	0.69	0.56	0.19	0.68

B) Bronchial lobed neutrophils/ml at 24 h		Unsegmented	2-lobed	3-lobed	4-lobed	Hyper-segmented	(All neutrophils)
Bronchial lavage histone at baseline	$p =$	0.2	0.3	0.09	0.07	0.9	0.2
	$r =$	0.38	0.33	0.52	0.54	0.02	0.36
Bronchial lavage histone at 24 h	$p =$	0.038	0.022	0.2	0.3	0.6	0.06
	$r =$	0.59	0.64	0.39	0.30	0.18	0.51

Statistics: Spearman's rank analysis, where bolded p and r values indicate strong correlations.

Supplementary table 9: **Correlation matrix of data from all patients (i.e. SR and DR) for each lobed neutrophil in the bronchial lavage at baseline to A) neutrophil elastase (NE) in the bronchial lavage at baseline and B) in the plasma after the allergen challenge.** Note that there were no correlations to lavage NE at 23 h nor plasma NE at baseline.

A) Bronchial lobed neutrophils/ml at baseline		Unsegmented	2-lobed	3-lobed	4-lobed	Hyper-segmented	(All neutrophils)
Lavage NE at baseline	<i>p</i> =	0.038	0.05	0.3	0.4	0.1	0.05
	<i>r</i> =	0.59	0.55	0.31	0.27	-0.45	0.50

B) Bronchial lobed neutrophils/ml at 24 h		Unsegmented	2-lobed	3-lobed	4-lobed	Hyper-segmented	(All neutrophils)
Plasma NE at 23 h	<i>p</i> =	0.08	0.2	0.0016	0.0006	0.1	0.1
	<i>r</i> =	0.48	0.41	0.83	0.82	0.44	0.41

Statistics: Spearman's rank analysis of all patients, where bolded *p* and *r* values indicate strong correlations.