



Anti-inflammatory role of CD11b⁺Ly6G⁺ neutrophilic cells in allergic airway inflammation in mice

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ABSTRACT

Asthma is a chronic inflammatory disease driven by overactivation of T helper cell type 2 (Th2) responses. In the present study, we investigated the functional relevance of CD11b⁺Ly6G⁺ neutrophilic cells in allergic airway inflammation *in vivo*. Allergic airway inflammation in mice was induced by house dust mite (HDM) or ovalbumin (OVA) sensitization and challenge. CD11b⁺Ly6G⁺ neutrophilic cells and T cell phenotypes were quantified by flow cytometry. To assess the functional *in vivo* relevance, CD11b⁺Ly6G⁺ neutrophilic cells were adoptively transferred intravenously or intratracheally and consequences on airway inflammation were studied. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells attenuated Th2 and Th17 responses and airway inflammation *in vivo*. Collectively, our results demonstrate that CD11b⁺Ly6G⁺ neutrophilic cells suppress airway inflammation in allergic mice *in vivo*. Adoptive cellular transfer of suppressive neutrophilic cells may represent an attractive therapeutic strategy for allergic airway inflammation.

1. Introduction

Asthma is the most common chronic lung disease characterized by lung tissue inflammation, intermittent airway obstruction, airway remodeling and airway hyperresponsiveness [1]. T cell dysregulation, particularly T helper (Th)2 immune responses characterized by interleukin (IL)-4, IL-5 and IL-13 release, eosinophil and mast cell recruitment and immunoglobulin (Ig)E production, is the hallmark of allergic asthma [2–5]. Beyond Th2, Th17 cells are increasingly recognized as a potentially pathogenic T cell subset in asthma [6–8]. Based on the T cell predominance, current therapeutic approaches aim to control overshooting T cell responses in allergic asthma.

While neutrophils have been traditionally regarded as merely pro-inflammatory innate immune cells, recent evidence indicates that neutrophilic cells can act immunomodulatory by dampening adaptive immune responses, particularly T cell responses [9]. This immunomodulatory potential of neutrophilic cells has been attributed to plasticity of conventional neutrophils and/or to a new suppressive myeloid cell phenotype, termed myeloid-derived suppressive cells (MDSCs) [10]. MDSCs were initially identified in cancer patients, represent a group of phenotypically heterogeneous myeloid cells that

potently suppress T cell responses [11]. In mice, these cells can be subdivided into neutrophilic/granulocytic [PMN-MDSCs, (Ly-6G^{high}CD11b⁺Ly-6C^{intermed})] and monocytic suppressive cells [M-MDSCs, (Ly-6G^{low}CD11b⁺Ly-6C^{high}SSC^{low})] [12]. It has been shown that exposure of mice to LPS triggers the recruitment of a MDSC-like phenotype (CD11b⁺Ly6G^{int}Ly6C^{low/-}F4/80⁺CD80⁺) into the lung associated with resolution of inflammation [13]. Furthermore, CD11b⁺Gr1^{int}F4/80⁺MDSC-like cells accumulate in allergic airway inflammation and suppress reactivation of primed Th2 cells in a TLR4- and MyD88-dependent pathway through IL-10 and arginase (Arg)1 [14,15]. Given the high plasticity of neutrophils, including T cell suppressive anti-inflammatory functions, the distinction between suppressive neutrophils and PMN-MDSCs remains challenging. The current study was designed to quantify MDSC-like suppressive neutrophilic cells in allergic airway inflammation *in vivo* and to therapeutically assess whether intravenously (IV) or intratracheally (IT) adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells influences the outcome of allergic airway inflammation.

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2. Material and methods

2.1. Murine studies

Six to eight weeks-old female BALB/c mice were purchased from Charles River Laboratories. The mouse model of allergic airway inflammation was performed as reported previously using HDM or OVA [16]. Intratracheal procedures were carried out under antagonizable anesthesia. Briefly, HDM (Greer Laboratories, Lenoir, North Carolina, USA) extracts (100 µg) were delivered intratracheally on days 0, 7, 14 (Supplementary Fig. 1A). In OVA model, mice were sensitized subcutaneously (SC) with 50 µg OVA (Grade V, Sigma-Aldrich) on days 0, 7, 14 and challenged with 1% aerosolized OVA for 30 min using an ultrasonic nebulizer (PARI BOY SX; PARI GmbH) once a day on days 26, 27, 28 (Supplementary Fig. 1B). Control mice received the same volume of PBS in place of HDM or OVA in sensitization and challenge stages of the protocol. All animal studies were approved by state authorities (Regierungspräsidium Tübingen, Germany) and were carried out according to the guidelines of the German law of protection of animal life. Suppressive neutrophilic or monocytic MDSC-like cells were phenotypically characterized as described previously [17] by using the markers CD11b, Ly6G (suppressive neutrophilic cells) and Ly6C (suppressive monocytic cells). Flow cytometry was performed on a FACS Calibur (BD). For adoptive transfer experiments, CD11b⁺ Ly6G⁺ neutrophilic cells were isolated from the bone marrows of six to eight weeks-old female BALB/c mice by MACS (MDSC isolation kit, Miltenyi Biotec, Germany). Adoptive transfer was performed by transferring 10×10^6 CD11b⁺ Ly6G⁺ neutrophilic cells per animal into female allergic BALB/c mice *via* lateral tail vein (IV) or IT injection on the last day of HDM challenge or in OVA model two days before and on the second day of OVA antigen challenge (10×10^6 in total, 5×10^6 each day). Control mice received 50 µl of PBS from IT route or 200 µl of PBS from IV route as a carrier of CD11b⁺ Ly6G⁺ neutrophilic cells in adoptive transfer stages of the protocol. As no significant effect was observed for control PBS IV or IT administrations, these data were not included in the results figures. To control for baseline variation in cell percentages among control and allergic airway inflammation groups and to allow a comparison of relative suppressive cell changes across treatment groups and different tissues, we have in line with previous publications [17], normalized our data to x-fold increases of CD11b⁺ Ly6G⁺ neutrophilic cells.

2.2. Flow cytometry

Single cell suspensions from mouse BALF and lungs were prepared. Murine suppressive cells were characterized using surface markers CD11b (clone M1/70), Ly6G (clone 1A8) and Ly6C (clone AL-21) (all from BD Biosciences). Bone marrow was used for control gating. Activation of T cell was measured using T cell surface markers: CD4 (GK1.5) and activation markers CD69 (clone H1.2F3) and CD25 (clone 3C7) (all from Biolegend). For T cell subtyping, cell surface and intracellular staining was performed using following markers: CD4 (GK1.5), IL-17A (clone TC11-18H10.1), IL-4 (clone 11B11), IFN-γ (clone XMG1.2) and IgG1 κ isotype control (clone RTK2071) with respective fluorescent tags (all from Biolegend). Intracellular staining was followed by permeabilization of cells with Cytofix/Cytoperm (BD Biosciences). Flow cytometry was performed using BD FACS Calibur (BD Bioscience) and data were analyzed with FlowJo software.

2.3. Measurement of cytokines in BALF

Cytokines in BAL supernatants were assayed using ELISAs (R&D System) for IL-4, IL-5, IL-13, IL-17A/F, IL-23, IL-10, IL33, (BioLegend) for TNFα, IL25, and TSLP according to the manufacturer's protocol.

2.4. Measurement of immunoglobulin in serum

Blood was collected into test tubes from retro-orbital sinus, allowed to clot and centrifuged (1000g) to obtain the serum. Total IgE and OVA specific IgE (BioLegend) levels were assayed by ELISA according to the manufacturer's protocol.

2.5. Measurement of inflammatory cell infiltration in BALF

Lungs were lavaged with 2 ml of phosphate-buffered saline (PBS). The cells were counted on a haemocytometer. The eosinophils count was performed on cytopsin preparations stained with Giemsa stain (Merck, Darmstadt, Germany) using light microscopy.

2.6. Quantitative real-time RT-PCR

Total RNA was obtained from whole lung homogenates using Precellys Tissue RNA Kit (peQlab) and reverse-transcribed into cDNA using a 20-µl reaction mixture (abm or BIO-RAD). qPCR analyses for *Tbet*, *Gata3*, *Foxp3* and *Rorγt* were performed on a ViiA 7 cyclor (Life Technologies) using QuantiTect Primers (QIAGEN), normalizing control with *GAPDH* as a reference gene. PCR was performed at 95 °C with initial denaturation for 2 min, followed by 40 cycles of amplification at 95 (15 s), 55 (30 s), and 72 °C (1 min).

2.7. Statistics

All statistical analyses were performed in GraphPad Prism v6 (San Diego, CA, USA). In all data the columns represent the mean. Mann-Whitney *U* test was used to determine the significant differences in all the data set. A *p* value < 0.05 was considered significant.

3. Results

3.1. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells dampen allergic airway inflammation *in vivo*

Using flow cytometric myeloid cell phenotyping (Fig. 1A), we assessed CD11b⁺Ly6G⁺ neutrophilic and CD11b⁺Ly6C⁺ monocytic cell populations, previously linked with suppressive MDSC-like capacities, in the bronchoalveolar (BALF) and pulmonary tissue compartments in a model of HDM-induced allergic airway inflammation. These studies demonstrated that HDM-driven airway inflammation itself did not recruit CD11b⁺Ly6G⁺ neutrophilic or CD11b⁺Ly6C⁺ monocytic cell populations *per se* into the lung (Fig. 1B, C), while total cell numbers and eosinophils increased indicating ongoing allergic inflammation in the airway compartment (Fig. 2A, B). Adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells from IV and IT routes significantly increased the respective neutrophilic / monocytic subset in BAL fluid (Fig. 1B, C) and significantly decreased the BALF total cell numbers, and eosinophils in HDM murine model of allergic airway inflammation (Fig. 2A, B). Adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells further decreased total IgE, IL-4, -5, -13, -17A/F, -23, -33, -25, TSLP and TNFα production in the HDM model (Fig. 2C, D). Furthermore, adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells decreased the number and activity of CD4⁺ and the number of Th17 cells in the lung compartments of HDM-induced allergic airway inflammation (Fig. 3A–D), suggesting that these CD11b⁺Ly6G⁺ neutrophilic cells confer suppressive capacities.

In contrast to the HDM model, CD11b⁺Ly6G⁺ neutrophilic or CD11b⁺Ly6C⁺ monocytic cell populations significantly decreased in the BAL fluids and lung tissues in OVA-induced allergic airway inflammation (Fig. 4A, B). In line with the HDM model, adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells from IV and IT routes significantly increased the respective neutrophilic / monocytic subset in the lung compartments (Fig. 4A, B) and significantly decreased the BALF total

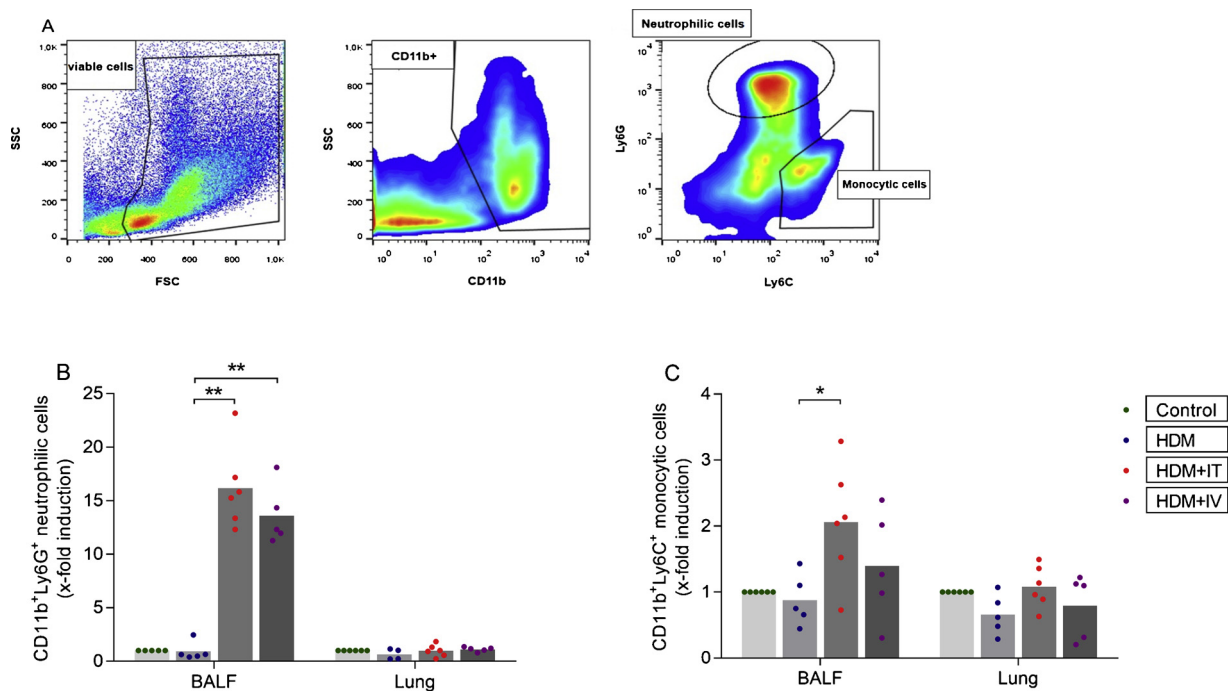


Fig. 1. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells are recruited to the lung compartment in HDM model of allergic airway inflammation. Representative gating strategies of suppressive neutrophilic and monocyte cells (A). Suppressive neutrophilic and monocyte cells were quantified by flow cytometry in BAL fluid and lung tissues of HDM model (B, C). All bar graphs show mean $\pm$ SEM and represent single experiment with 5–6 mice per experiment. * $P < 0.05$, ** $P < 0.01$.

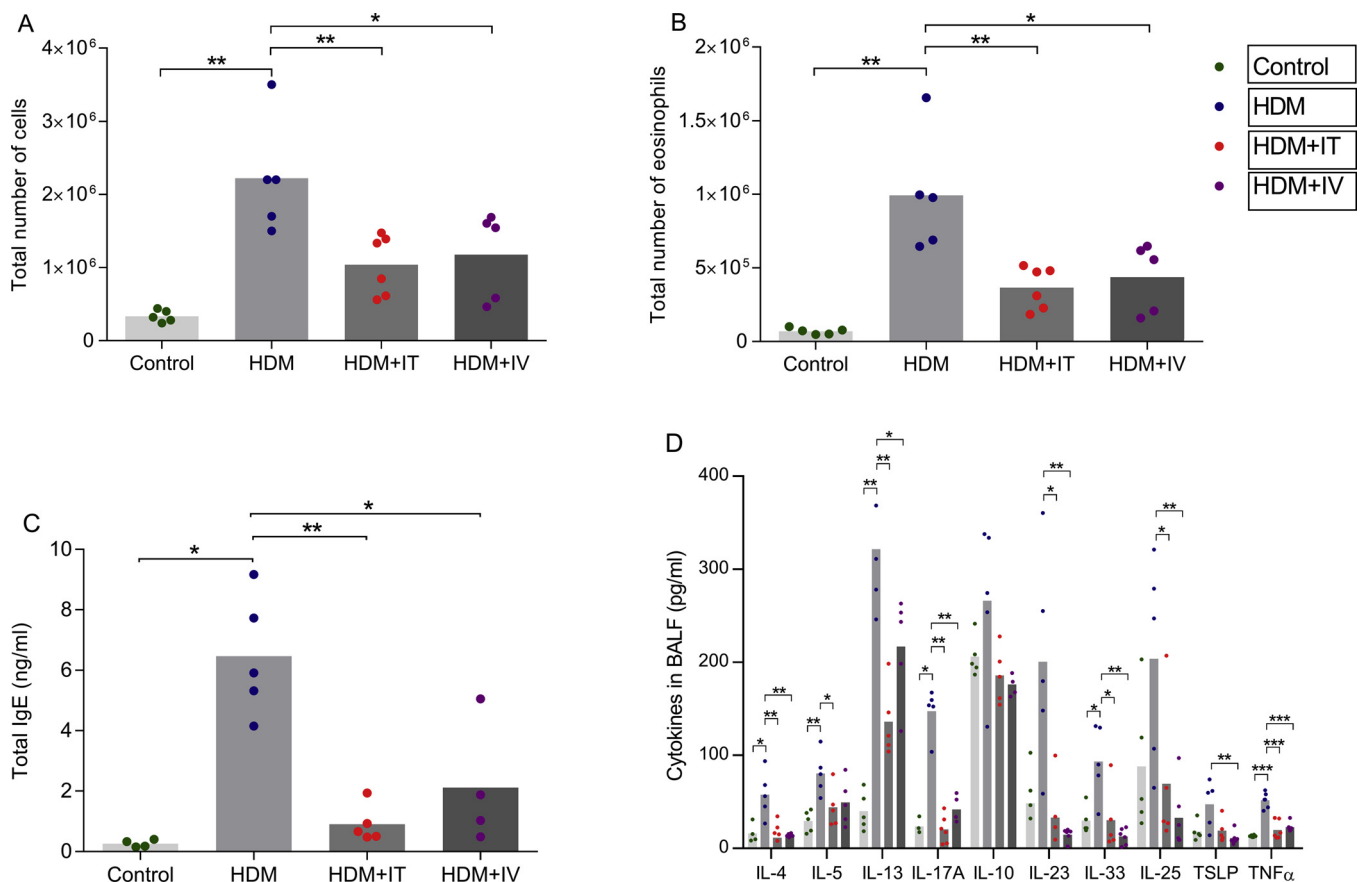


Fig. 2. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells dampen allergic airway inflammation HDM model of allergic airway inflammation. Total inflammatory cells (A) and eosinophil numbers (B) were quantified by cyto-spin in BAL fluid of HDM model. Total IgE in serum (C) and inflammation-related mediators (IL-4, -5, -13, -17A/F, -23, -33, -25, TSLP and TNF α) in BAL fluid were measured by ELISA in HDM model. (D). All bar graphs show mean $\pm$ SEM and represent single experiment with 5–6 mice per experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

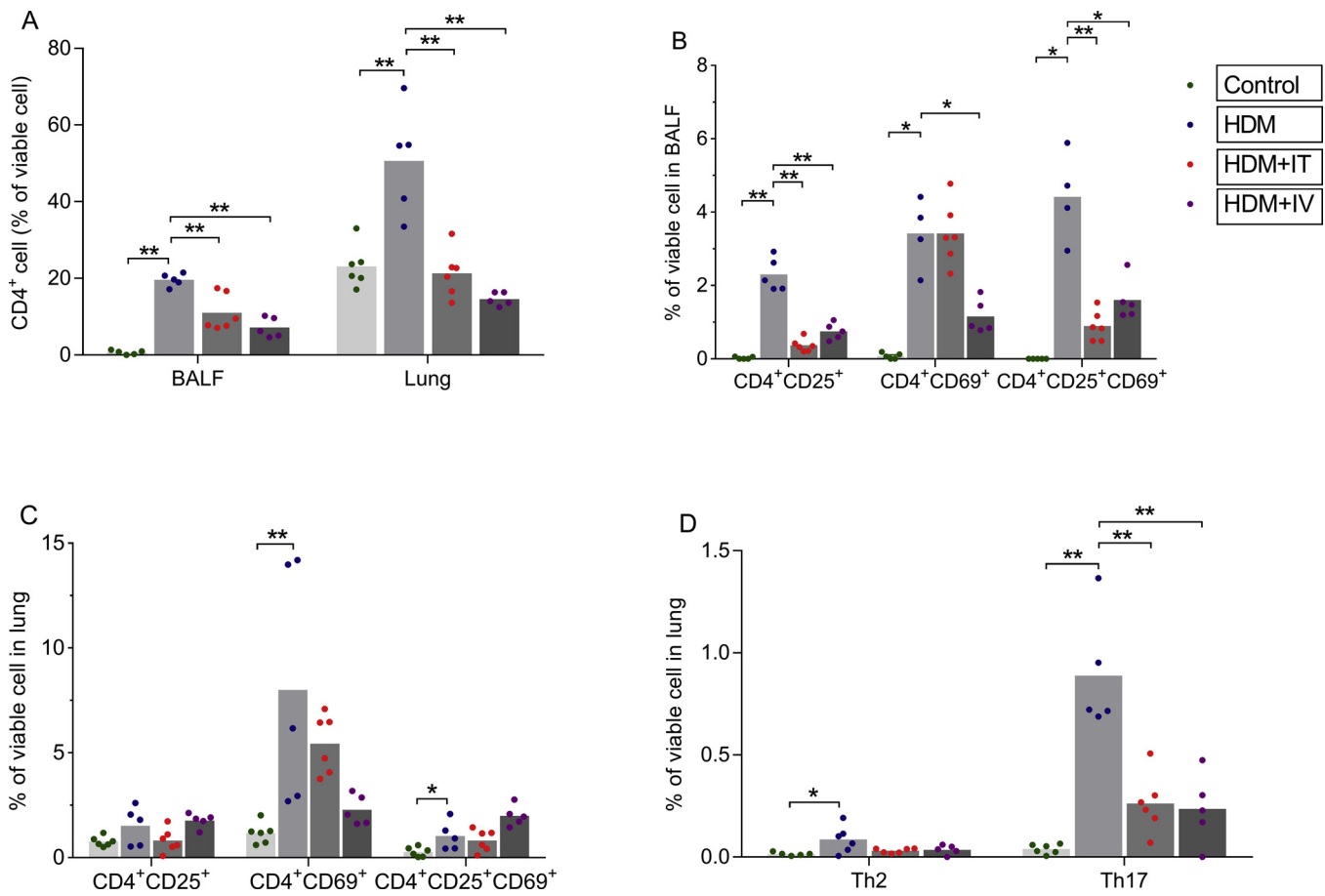


Fig. 3. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells reduce CD4⁺ cell numbers, CD4⁺ cell activity and Th17 cell numbers in HDM model of allergic airway inflammation.

CD4⁺ cell numbers (percentage of viable cells) were quantified by flow cytometry in BAL fluid and lung tissues of HDM model (A). Activation of CD4⁺ cells (expression of CD25 and CD69 marker) was quantified by flow cytometry in BAL fluid and lung tissues of HDM model (B, C). CD4⁺CD25⁺ cells are CD69⁻ and CD4⁺CD69⁺ cells are CD25⁻. Th2 and Th17 cell numbers (percentage of viable cells) were quantified by flow cytometry in lung tissues of HDM model (D). All bar graphs show mean $\pm$ SEM and represent single experiment with 5–6 mice per experiment. * $P < 0.05$, ** $P < 0.01$.

cell numbers, and eosinophils in OVA-induced allergic airway inflammation (Fig. 5A, B). Adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells did not change the production of OVA specific IgE and total IgE (Fig. 5C, D), but reduced IL-4, IL-17A/F, IL-33 and TNF α in the BAL fluid in the OVA model of allergic airway inflammation (Fig. 6). Adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells further decreased CD4⁺ cell numbers and activity in BALF as well as Th2 and Th17 cell numbers in the lungs tissues of OVA-induced allergic airway

inflammation (Fig. 7A–D). Adoptive transfer of CD11b⁺Ly6G⁺ suppressive neutrophilic cells further resulted in a significant decrease of *Gata-3* and *Rory-t* expression and increased the expression of *Foxp3* (IT route) in both HDM and OVA models of allergic airway inflammation (Fig. 8A, B).

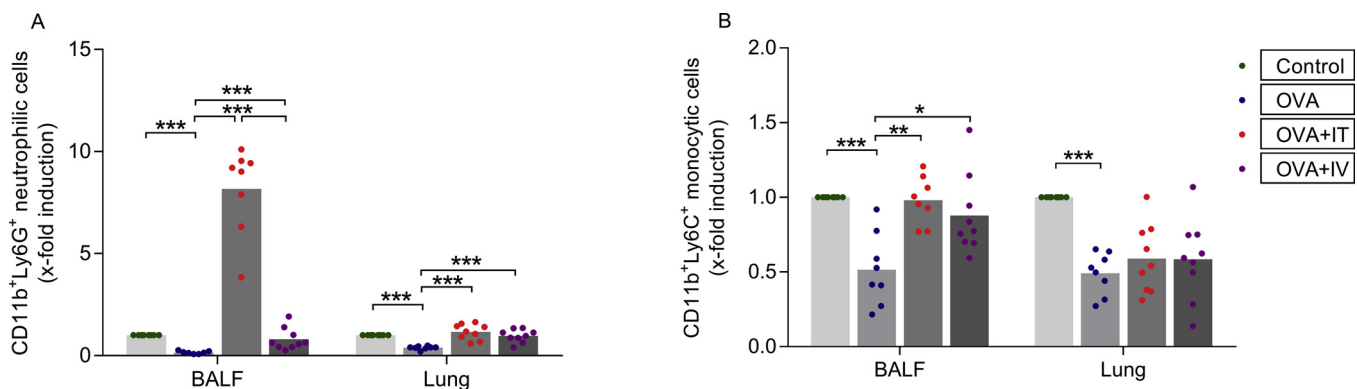


Fig. 4. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells are recruited to the lung compartment in OVA model of allergic airway inflammation.

Suppressive neutrophilic and monocyte cells were quantified by flow cytometry in BAL fluid and lung tissues of OVA model (A, B). All bar graphs show mean $\pm$ SEM and represent single experiment with 8–9 mice per experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

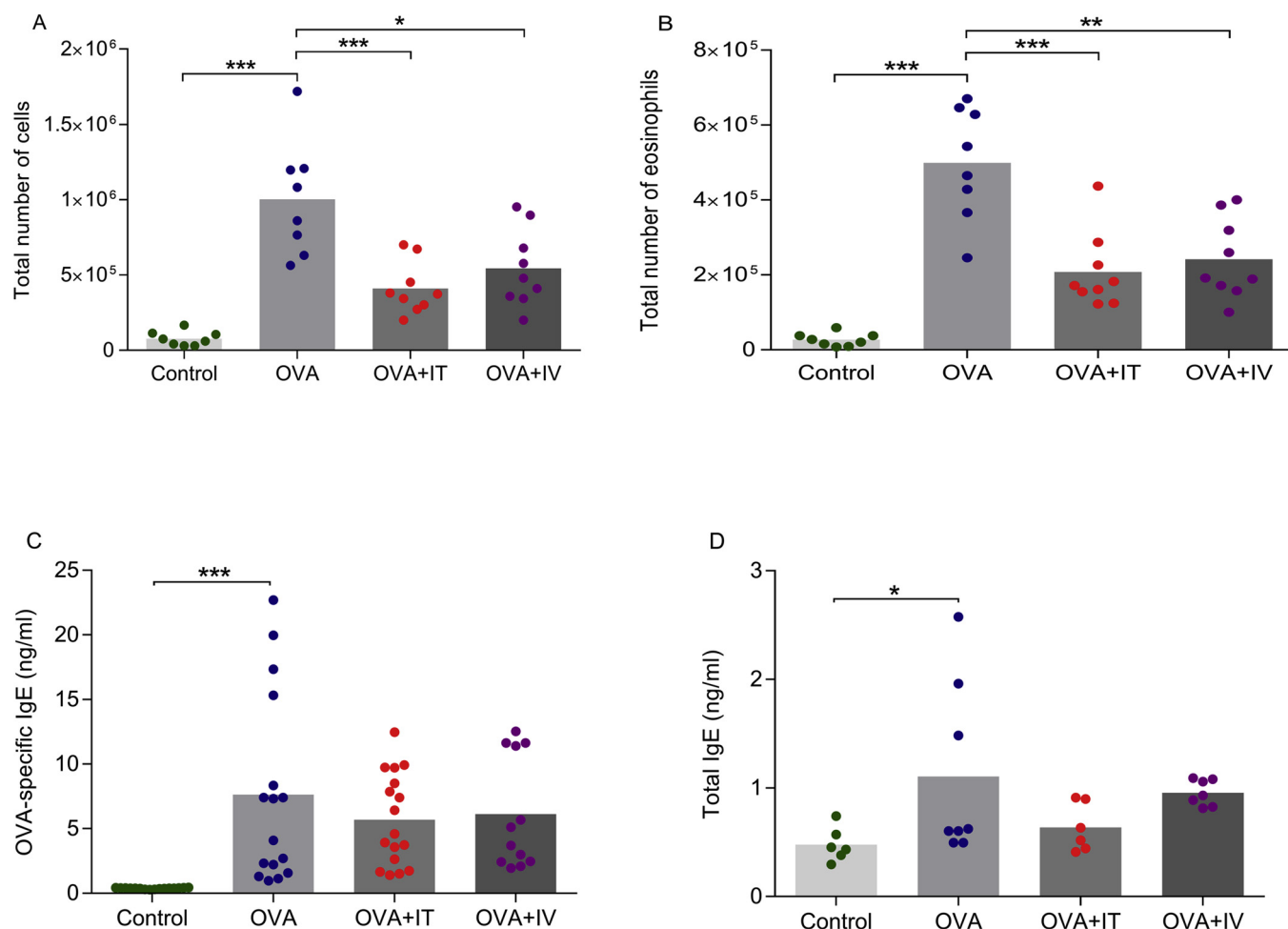


Fig. 5. Adoptively transferred CD11b⁺Ly6G⁺ neutrophils reduce the number of inflammatory cells but do not change OVA specific IgE and total IgE in OVA model of allergic airway inflammation.

Total inflammatory cells (A) and eosinophil numbers (B) were quantified by cytospin in BAL fluid of OVA model. OVA-specific (C) and total IgE in serum (D) were measured by ELISA in OVA model. All bar graphs show mean $\pm$ SEM and represent single experiment with 8–9 mice per experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

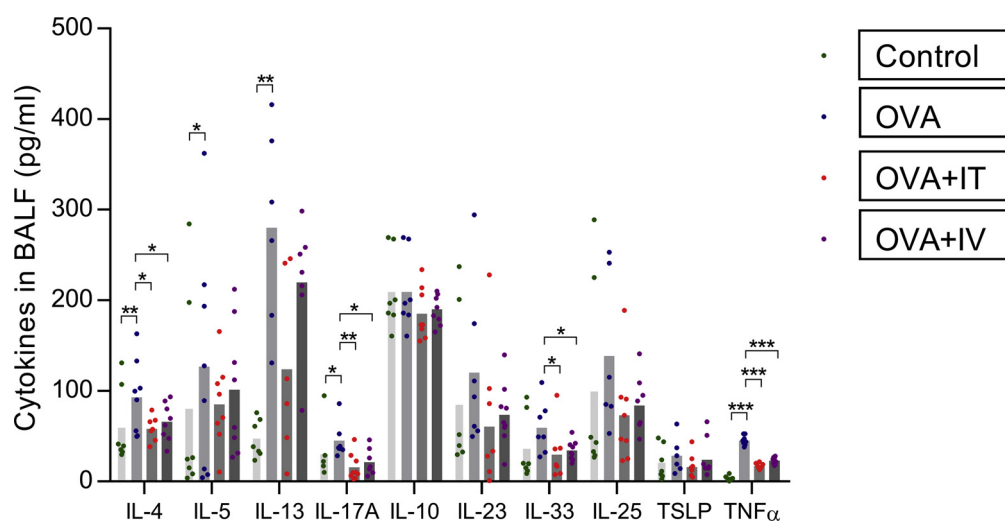


Fig. 6. Adoptively transferred CD11b⁺Ly6G⁺ neutrophils dampen pro-inflammatory cytokines production in OVA model of allergic airway inflammation.

Inflammation-related mediators (IL-4, -5, -13, -17A/F, -23, -33, -25, TSLP and TNF α) were measured by ELISA in BAL fluid of OVA model. All bar graphs show mean $\pm$ SEM and represent single experiment with 8–9 mice per experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

4. Discussion

Asthma, a chronic pulmonary condition, comprises a range of heterogeneous immune-related mechanisms, including both the innate and the adaptive arm of the immune system [1–4]. Beyond β_2 -adrenergic

agonists and long acting anticholinergics as bronchodilators, inhaled anti-inflammatory corticosteroids, mepolizumab and omalizumab have been used as medications of asthma treatment [18]. However, given our expanding understanding of the immune mechanisms involved in the pathogenesis of asthma, new and more specific/tailored anti-

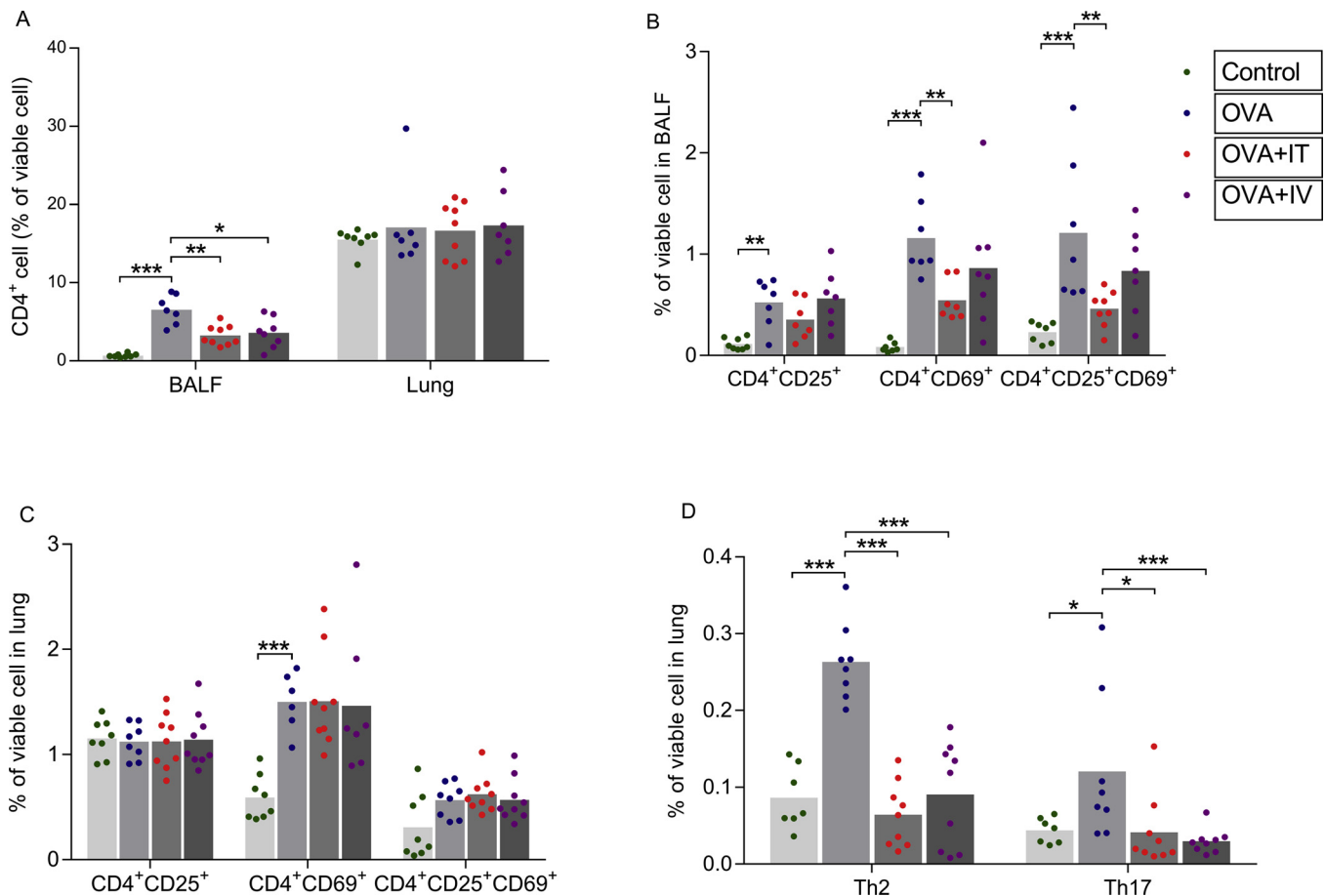


Fig. 7. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells reduce CD4⁺ cell numbers, CD4⁺ cell activity and Th2, Th17 cell numbers in OVA model of allergic airway inflammation.

CD4⁺ cell numbers (percentage of viable cells) were quantified by flow cytometry in BAL fluid and lung tissues of OVA model (A). Activation of CD4⁺ cells (expression of CD25 and CD69 marker) was quantified by flow cytometry in BAL fluid and lung tissues of OVA model (B, C). CD4⁺CD25⁺ cells are CD69⁺ and CD4⁺CD69⁺ cells are CD25⁺. Th2 and Th17 cell numbers (percentage of viable cells) were quantified by flow cytometry in lung tissues of OVA model (D). All bar graphs show mean $\pm$ SEM and represent single experiment with 8–9 mice per experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

inflammatory approaches are required. Asthma is characterized by dysbalanced T cell responses. Besides Tregs, innate immune cells that suppress overshooting T cell activities, termed myeloid-derived suppressor cells (MDSCs) have been proposed to play a role in autoimmune and allergic disease conditions, yet their functional impact on disease outcome *in vivo* remained poorly understood. Therefore, in order to assess a novel therapeutic approach in asthma, we aimed to investigate the functionality and efficacy of MDSC-like suppressive neutrophilic cells therapy in two murine models of allergic airway inflammation.

Previous studies provided evidence that a non-migratory MDSC-like cell population (CD11b⁺Gr1^{int}F4/80⁺ cells) accumulated in a model of LPS-induced lung inflammation and dampened lung dendritic cell-mediated reactivation of primed Th2 cells, suggesting that CD11b⁺Gr1^{int}F4/80⁺ cells MDSC-like cells contribute to the protective effects of LPS in allergic airway inflammation, yet the precise phenotype and function of these cells remained only partially understood [14]. Another study demonstrated that MDSCs significantly increase in human peripheral blood during the onset of asthma and positively correlated with serum levels of IL-10 and negatively IL-12, suggesting that circulating MDSCs modulate the immune signature in asthma in humans [19]. Cyclooxygenase and its product PGE2 and production of nitric oxide have been involved in the anti-inflammatory activity of MDSCs in allergic airway inflammation [20,21]. Moreover, glucocorticosteroids were found to induce MDSCs-like cells, defined by the surface expression of CD11b and Ly-6G [22]. When viewed in combination, these previous studies suggested that MDSC-like cells

accumulate in allergic airway inflammation and may affect disease outcome. Inspired by these previous investigations, we comprehensively studied the effect of adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells in two independent allergic airway inflammation models *in vivo*. We showed that CD11b⁺Ly6G⁺ neutrophilic cells adoptive transfer reduced airway inflammation, the production of IgE and the number of eosinophils in allergic airway inflammation. Furthermore, adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells regulated pulmonary T cell activities, Th17/Th2/Treg gene signatures in allergic airway inflammation *in vivo*.

In a previous study from our group, it has been shown that *Foxp3*-modified mRNA ameliorated Th2 and Th17 inflammation by reducing inflammatory cell infiltration to the lung through an IL-10-dependent pathway [16]. Adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells was able to suppress the upregulation of *Gata3* and *Rory-t* as well as the downregulation of *Foxp3* in murine OVA and HDM allergic airway inflammation models. Biochemical findings confirmed the anti-inflammatory role of adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells by showing a reduction of pro-inflammatory cytokines release in allergic airway inflammation. Taken together, adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells was capable of dampening allergic lung inflammation based on biochemical and immunological read-outs.

Our study has several limitations. First, the clinical translatability of our studies to the human situation is challenging, particularly as we showed efficacy for adoptive transfer *via* the intratracheal route, an approach not feasible for clinical routine. Nevertheless, a cellular anti-

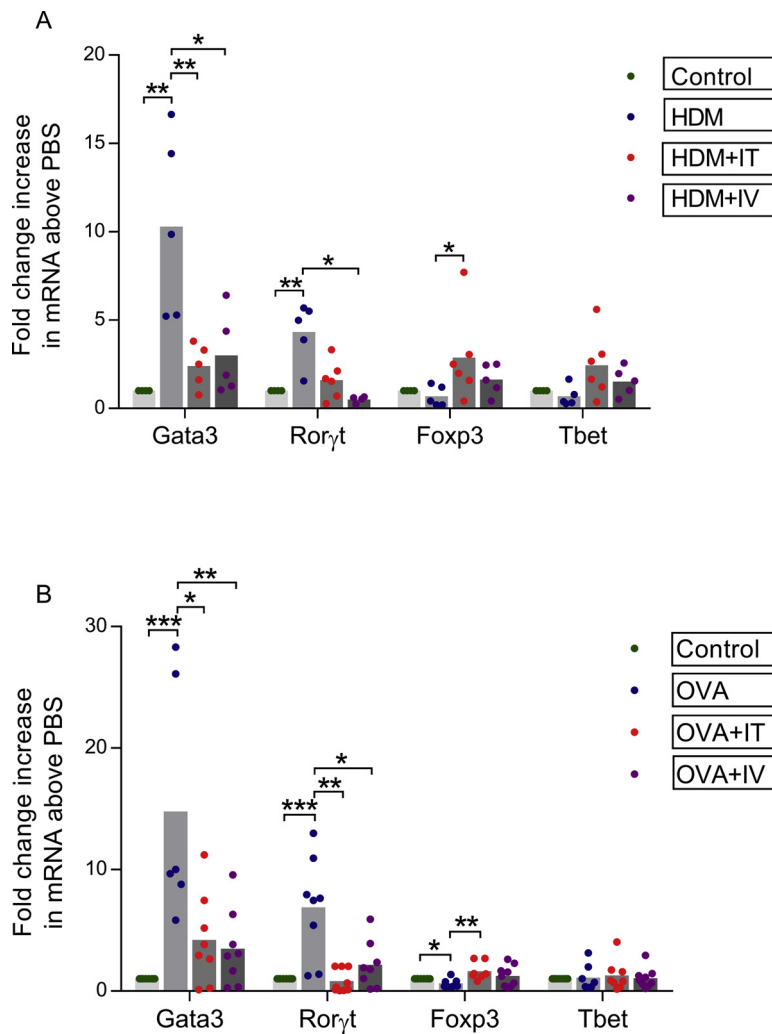


Fig. 8. Adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells dampen pro-inflammatory gene expression in HDM and OVA models of allergic airway inflammation.

Gene expression was measured for *Gata-3*, *Ror γ -t*, *Foxp3*, *T-bet* in whole lung lysates of HDM (A) and OVA (B) models of allergic airway inflammation. Gene expression data were normalized to *GAPDH* expression. All bar graphs show mean $\pm$ SEM and represent single experiment with 8–9 mice per experiment. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

inflammatory cell therapy approach utilizing suppressive neutrophilic cells might be conceivable in an autologous setting, comparable to lessons learned from studies utilizing mesenchymal stem cells (MSCs) [23]. Second, the phenotypic and functional distinction between MDSCs and neutrophils is still challenging, particularly given the interspecies differences on putative MDSC markers and the emerging plasticity of neutrophils. Therefore, we decided to phenotypically term our cell population of interest CD11b⁺Ly6G⁺ neutrophilic cells with anti-inflammatory properties. Third, we were unable to track the fate of adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells in our system, which should be dissected in future studies. Fourth, we were not also able to differentiate endogenous *versus* adoptively transferred CD11b⁺Ly6G⁺ neutrophilic cells in this project. Fifth, the mechanistic anti-inflammatory effect of CD11b⁺Ly6G⁺ neutrophilic cells in the airway microenvironment remains elusive and may include T cell-related suppressive (MDSC-like) and unrelated pro-resolving downstream effects, such as uptake by macrophages and release of anti-inflammatory mediators, a topic worth further investigation.

5. Conclusion

Collectively, our studies demonstrate that adoptively transferred

CD11b⁺Ly6G⁺ neutrophilic cells inhibit Th2/Th17-dominant inflammation in allergic airway inflammation in mice *in vivo*. Overall, the development of novel therapeutic agents that enhance suppressive neutrophilic cell activities in allergic airway inflammation may represent an attractive therapeutic strategy for the future.

Conflict of interest

D. H. works for a pharmaceutical company. No other conflicts of interest, financial or otherwise, are declared by the authors.

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Author contributions

NN, HÖ, CS and SK performed experiments. AH and BN provided scientific and technical help. DH and SK designed the study, supervised experiments, analyzed the data and wrote the manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.imlet.2018.10.007>.

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Online Data Supplement

Anti-inflammatory role of CD11b⁺Ly6G⁺ neutrophilic cells in allergic airway inflammation in mice

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Figure S1: House dust mite (HDM) (A) and ovalbumin (OVA) (B) murine models of allergic airway inflammation and adoptive transfer of CD11b⁺Ly6G⁺ neutrophilic cells.