ПРОСТРАНСТВЕННОЕ РАСПОЛОЖЕНИЕ НЕЙТРОФИЛОВ И ЭОЗИНОФИЛОВ В СЛИЗИСТОЙ ОБОЛОЧКЕ ГЛАВНОГО БРОНХА ПРИ ИНДУЦИРОВАННОМ АЛЛЕРГИЧЕСКОМ ВОСПАЛЕНИИ ДЫХАТЕЛЬНЫХ ПУТЕЙ У МЫШЕЙ

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Резюме. В ответ на антигены, ежедневно попадающие с током воздуха в респираторный тракт человека, в слизистой оболочке дыхательных путей формируется клеточных иммунный ответ. Аллергическое воспаление дыхательных путей характеризуется эозинофил-опосредованным иммунным ответом, однако, в случаях тяжелой астмы, в респираторном тракте также присутствуют нейтрофилы. Для исследования механизмов, регулирующих развитие воспаления по тому или иному пути, широко используют модели с использованием мышей. Данные о пространственном расположении нейтрофилов и эозинофилов в респираторном тракте необходимы как для понимания механизмов развития аллергической реакции в дыхательных путях, так и для оценки потенциала лекарственных препаратов, однако недостаточно изучены. В данной работе была разработана и охарактеризована модель, позволяющая наблюдать активацию аллергического воспаления в дыхательных путях в ответ на введение экстракта гриба *Aspergillus fumigatus* на ранней стадии. Адекватность модели была подтверждена путем оценки доли эозинофилов в крови и в бронхоальвеолярных смывах. С использованием иммуногистохимического окрашивания тотального препарата главного бронха мыши и лазерной сканирующей конфокальной микроскопии было охарактеризовано пространственное расположение нейтрофилов и эозинофилов: на обращенной в просвет дыхательного пути стороне эпителиального барьера, в стенке главного бронха или в подслизистом слое в непосредственной близости от гладкой мускулатуры. Активацию аллергического иммунного ответа определяли по достоверному увеличению эозинофилов в периферической крови мышей по сравнению с интактными мышами. В этот период времени количество эозинофилов в бронхоальвеолярном смыве было также достоверно выше, чем у интактных мышей. Как в бронхоальвеолярных смывах, так и в слизистой главного бронха в исследуемый интервал времени эозинофилы были преобладающей клеточной популяцией по сравнению с нейтрофилами. Анализ пространственного расположения клеток в слизистой главного бронха выявили преимущественное расположение эозинофилов в подслизистом слое, меньше в стенке глав-

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ного бронха и еще меньше – на обращенной в просвет стороне эпителия. В то же время нейтрофилы были в основном обнаружены в просвете дыхательного пути и в подслизистом слое, но не в стенке дыхательного пути. Полученные результаты свидетельствуют о том, что в ответ на последующие ингаляции аллергена мигрировать через стенку дыхательного пути в просвет будут с большей вероятностью эозинофилы, чем нейтрофилы. Таким образом, эозинофилы могут быть ответственны за повреждение эпителия в процессе развития аллергического воспаления дыхательных путей. При этом расположенные в непосредственной близости от клеток гладкой мускулатуры нейтрофилы наряду с эозинофилами могут быть вовлечены в индукцию бронхоспазма.

Ключевые слова: нейтрофилы, эозинофилы, аллергическое воспаление дыхательных путей, слизистая оболочка дыхательных путей, Aspergillus fumigatus, модель на мышах

SPATIAL CHARACTERISTICS OF NEUTROPHILS AND EOSINOPHILS IN CONDUCTING AIRWAY MUCOSA OF MICE WITH INDUCED ALLERGIC AIRWAY INFLAMMATION

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Abstract. Daily inhaled antigens induce cellular immune response in the airways. In case of allergens, allergic airway inflammation is usually represented by eosinophils, however, neutrophil infiltration is also observed during severe asthma. Animal models contribute to investigation of the mechanisms that involve the switching to eosinophil- or neutrophil-mediated inflammation. Data about the spatial location of eosinophils and neutrophils in the airways are necessary for both the understanding of allergic airway inflammation mechanisms and the drag potential estimation, however, not completely investigated. In the present study, we characterized the model of *Aspergillus fumigatus* extract-induced allergic airway inflammation that allowed investigating the early stage of inflammation development. The model adequacy was confirmed according to the blood and bronchoalveolar lavage eosinophilia. Using immunohistochemical staining of conducting airway as a whole-mount and confocal laser scanning microscopy, we estimated neutrophil and eosinophil spatial location: in the luminal side of the epithelium, in the airway wall or in the submucosal compartment close to the smooth muscle layer. An allergic airway response activation was detected upon significant elevation of blood eosinophil percentage compared to intact mice. Simultaneously, the number of eosinophils in the bronchoalveolar lavage was also significantly increased compared to the intact mice. At this time point, eosinophils predominated both in bronchoalveolar lavages and in conducting airway mucosa compared to neutrophils. Spatial location of conducting airway mucosal cell analysis demonstrated that eosinophils mostly located in the submucosal compartment, in a lesser extent in the airway wall, and a few eosinophils were detected in the luminal side of the epithelium. Neutrophils mainly infiltrated the luminal side of the epithelium, and a few neutrophils were detected in the submucosal compartment, while no neutrophils were detected in the airway wall. The data suggests that in response to the further allergen challenge, evidently eosinophils but not neutrophils will migrate through the airway wall to the airway lumen. Thus, eosinophils can be expected to damage airway epithelium in allergic airway inflammation development. Simultaneously, neutrophils located in close proximity to the smooth muscle layer together with eosinophils can contribute to bronchoconstriction induction.

Keywords: neutrophils, eosinophils, allergic airway inflammation, airway mucosa, Aspergillus fumigatus, mouse model, confocal microscopy

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Introduction

Asthma is a complex disease that comprises of different mechanisms that result in the common symptoms such as dyspnea, cough, and respiratory stress [8]. Traditionally, two forms of asthma have been assumed: “T helper 2 (Th2)-high” and “Th2-low” [4]. The former is mainly, but not always, associated with airway eosinophilia and responsiveness to steroid-treatment [4]. Th2-low or severe asthma is mostly characterized with neutrophil infiltration and resistance for steroid therapy; however, some research indicates neutrophilic asthma as a subtype of Th2-low asthma endotype [1, 2]. Interestingly, in eosinophilic asthma, patients have about 2-3 % of eosinophils in the sputum, while no less than 40-60 % of sputum neutrophils contribute for neutrophilic asthma diagnosis [1].

To investigate the mechanisms of asthma, animal models, particularly, mouse models are required. Mouse models almost simulate the Th2-high asthma characteristics; however, eosinophil and neutrophil recruitment is dependent on the mouse strain [5, 10]. Recent studies demonstrated that in response to single provocation with *Alternaria alternata* extract, C57BL/6 mice exhibited eosinophil-mediated immune response, while in BALB/c mice the response was characterized by neutrophil infiltration [13]. Besides genetic factors, the environmental factors play important role in the type of immune response formation, and multiple allergen supplementation contributes to eosinophil-mediated response [5]. The effect of eosinophils and neutrophils on the further disease development is not identical, thus neutrophils can induce acute airway inflammation, while eosinophils are associated with chronic long-lasting disease [5, 7, 10]. In this view, the spatial distribution of neutrophils and eosinophils in asthma is important, but not completely investigated aspect.

In the present study, using immunohistochemistry of whole-mount conducting airways of BALB/c mice with *Aspergillus fumigatus* extract-induced allergic airway inflammation and confocal laser scanning microscopy we detected neutrophils and eosinophils in conducting airway mucosa. We characterized the precise position of the cells: in the luminal side of the airway epithelium, in the airway wall, and in the submucosal compartment. The observations helped to expect potential role of eosinophils and neutrophils in asthma manifestation.

Materials and methods

In this study, we used BALB/c mice (Pushchino, Russian Federation) 18-20 g, 10-12 weeks. Mice were housed in the animal facility of IBCH RAS. Animal experiments were performed under the protocol 308/2020 approved by the Institutional Animal Care and Use Committee IBCH RAS. The animals were given standard food and tap water *ad libitum* and housed under regular 12 h dark:light cycles at 22 °C.

Mice were treated with 4 μg / mouse / application of *A. fumigatus* extract (Greer, USA) oropharyngeally with the 72 h intervals between the applications. Before each application, the peripheral blood was collected from the tail vein. For the cell analysis, blood was collected to the test-tubes with heparin, washed twice with hemolysis buffer and transferred to DPBS via centrifugation for 5 min at 380 g. 72 h after the 5th extract application, mice were euthanized and bronchoalveolar lavages (BALs) were obtained using DPBS and a cannula (Abbocath, EU). The samples were centrifuged for 5 min at 500 g. Blood and BAL cells were sediment to the glass slides using centrifuge Cytospin 2 (Shandon, UK), and stained using DiffQuick (Abris, Russia). Differential cell count was performed using stereomicroscope Zeiss Primo Star (Carl Zeiss, Germany) and objective 40×.

After BAL obtaining, lungs were inflated-fixed with 2% paraformaldehyde and stored at +4 °C overnight. The main axial airways of the left and right lower lobes were cut out using a stereomicroscope (Carl Zeiss) and micro-scissors (BBraun, Germany).

For immunohistochemistry, specimens were washed with DPBS at 150 rpm using orbital shaker (ApexLab, Russia), permeabilized with 0.3% Triton X-100. Non-specific antibody binding was blocked with 4% normal donkey serum in 0.5% BSA-DPBS. Specimens were stained with rat anti-mouse Ly6G-AlexaFluor488, and rat anti-mouse SiglecF-APC antibodies (all BioLegend, USA, in dilution 1:50) at +4 °C overnight. Specimens were washed with 0.3% Triton X-100 for 5 × 1 h, and stained with Phalloidin-Atto425 (Sigma Aldrich, USA, in dilution 1:50). Specimens were placed on slide glasses, and covered with Prolong Gold mounting medium (ThermoFisher, USA), and stored at -20 °C until use.

Three-dimensional images were obtained using an inverted confocal laser scanning microscope Zeiss LSM980 (Carl Zeiss). A 63× oil objective was used to image the region of interest for the further quantification. At least 4 z-stacks were acquired from each specimen. 405, 488 and 633 nm lasers and respective beam splitters were used. Fluorescence was detected using Lambda mode with the following spectral unmixing using the ZEN software (Carl Zeiss).

The image stacks were analyzed using Imaris 9.8 (Oxford Instruments, UK). The surfaces of epithelium and smooth muscles were created based on Phalloidin-Atto425 signal. Neutrophil and eosinophil surfaces were created based on Ly6G-Alexa488 and SiglecF-APC signals, respectively. The surfaces were created via three-dimensional surface rendering using volume and intensity mean thresholds. Cell surfaces were quantified automatically using intensity max in phalloidin channel and Z-position filters. Images were shown as maximal intensity projections. The final image processing was performed using Adobe Photoshop CS version 5 (Adobe Systems, USA).

Statistical analysis was carried out using GraphPad Prism software. The data were presented as medians and interquartile ranges. The data were analyzed using the Mann–Whitney U test. The difference in values at $p \leq 0.05$ was considered significant.

Results and discussion

Neutrophils are known to be a dominant population in the peripheral blood in steady state, however, upon allergen application, eosinophil infiltration was detected (Figure 1A, B). Thus, five *A. fumigatus* extract applications resulted in significant elevation of eosinophil percentage compared to that in intact mice – before the 1st application (Figure 1A, B). The percentage of periphery blood neutrophils subsequently decreased (Figure 1A, B). Thus, the time point at 72 h after the 5th application was selected as the time of switching to eosinophil-mediated response (Figure 1A, B, dotted line).

Mice were sacrificed at 72 h after the 5th extract application (Figure 1A, B, dotted line), and the BAL cell number was detected. At this time point,

eosinophil number increased significantly compared to intact mice, while just a small number of neutrophils was detected in BALs (Figure 1C).

To detect the presence of neutrophils and eosinophils in conducting airway mucosa, analysis of the high-resolution images of the airway whole-mounts was made. The analysis demonstrated that at this time point, both neutrophils ($Ly6G^+$ cells with typical to segmented granulocyte morphology) and eosinophils ($SiglecF^+$ cells with typical to segmented granulocyte morphology) were detected in conducting airway mucosa and submucosal compartment of mice with induced allergic airway inflammation (Figure 2A). Phalloidin staining allowed to visualized actin-rich fibers of the epithelial barrier and smooth muscle layer, and to detect airway wall – the compartment bordered by the epithelium and smooth muscles (Figure 2A).

Epithelium, smooth muscles, and eosinophils were built via surface rendering (Figure 2B, C). Using thresholds on Z-position and intensity maximum in Actin channel, the number of eosinophils in the luminal side of the epithelium (Figure 2D, red), in the airway wall (Figure 2D, yellow), and in the submucosal compartment (Figure 2D, orange) were detected. Due to irregular structure of the submucosal compartment, only attached to the smooth muscle layer submucosal cells were considered for quantitative analysis and the cells that laid deep in the tissues were exclude from the estimation (Figure 2D, magenta). The same principle was used for the neutrophil position characterization and estimation (data not shown).

Quantitative analysis demonstrated that both eosinophil and neutrophil numbers were significantly higher in the airways of mice with induced allergic airway inflammation compared to intact mice (Figure 2E). Eosinophils dominated in conducting airway mucosa of mice with induced allergic airway inflammation compared to neutrophils (Figure 2E). Simultaneously, eosinophils mostly located in the submucosal compartment, less in the airway wall and only a few eosinophils were observed in the luminal side of the epithelium (Figure 2F). Opposite to eosinophils, neutrophils were mostly located in the luminal side of the epithelium, little to no neutrophils were detected in the airway wall, and a small number was detected in the submucosa close to the smooth muscle (Figure 2G). Some neutrophils were detected deep in the submucosa, however, it was not possible to conclude, whether these neutrophils were in the lung vessels or in the tissue (data not shown).

Thus, five applications of *A. fumigatus* extract led to the blood eosinophil percentage elevation and eosinophil domination above neutrophils both in BAL and in the conducting airway, including conducting airway mucosa, and in the submucosal compartment. In conducting airways, eosinophils mostly located in the submucosal area close to the smooth muscles, while neutrophils migrate to the airway lumen.

Spatial aspects of cellular immune response in sites of inflammation is important and intensively investigated area of immunology [6]. Eosinophil-intraepithelial dendritic cell interactions were characterized in the tracheal mucosa of mice with ovalbumin-induce allergic airway inflammation [14]. Together with eosinophils, neutrophils also play important role in the local immune response to allergens. Thus, ovalbumin-sensitized and challenged mice reacted with enhanced neutrophil infiltration in response to *A. fumigatus* conidia application [12]. Moreover, neutrophils mig-

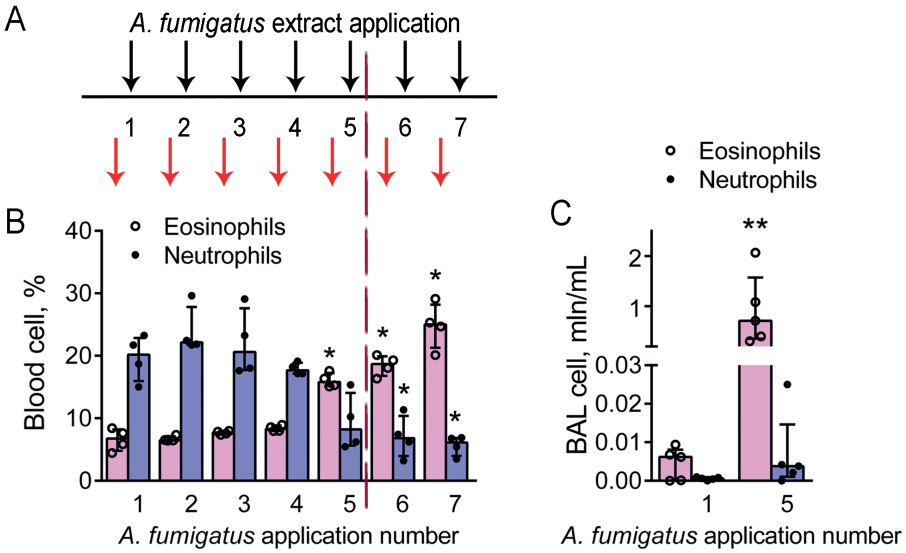

Figure 1. Blood and BAL cell redistribution in allergic airway inflammation

Note. A. The scheme of allergic airway inflammation induction via o.ph. applications of *A. fumigatus* extract (black arrows). Blood samples were collected before (T0), 72 h after the first (T2), and 72 h after the fifth (T6) extract applications (red arrows). B. Percentage of blood eosinophils (rose bars) and neutrophils (blue bars) at 72 h after the respective *A. fumigatus* extract application. C. Quantitative analysis of BAL eosinophils (rose bar) and BAL neutrophil (blue bar) before the 1st and before the 6th *A. fumigatus* extract application. Data are shown as median and i.q.r., significant difference between indicated groups: * $p \leq 0.05$, ** $p \leq 0.01$.

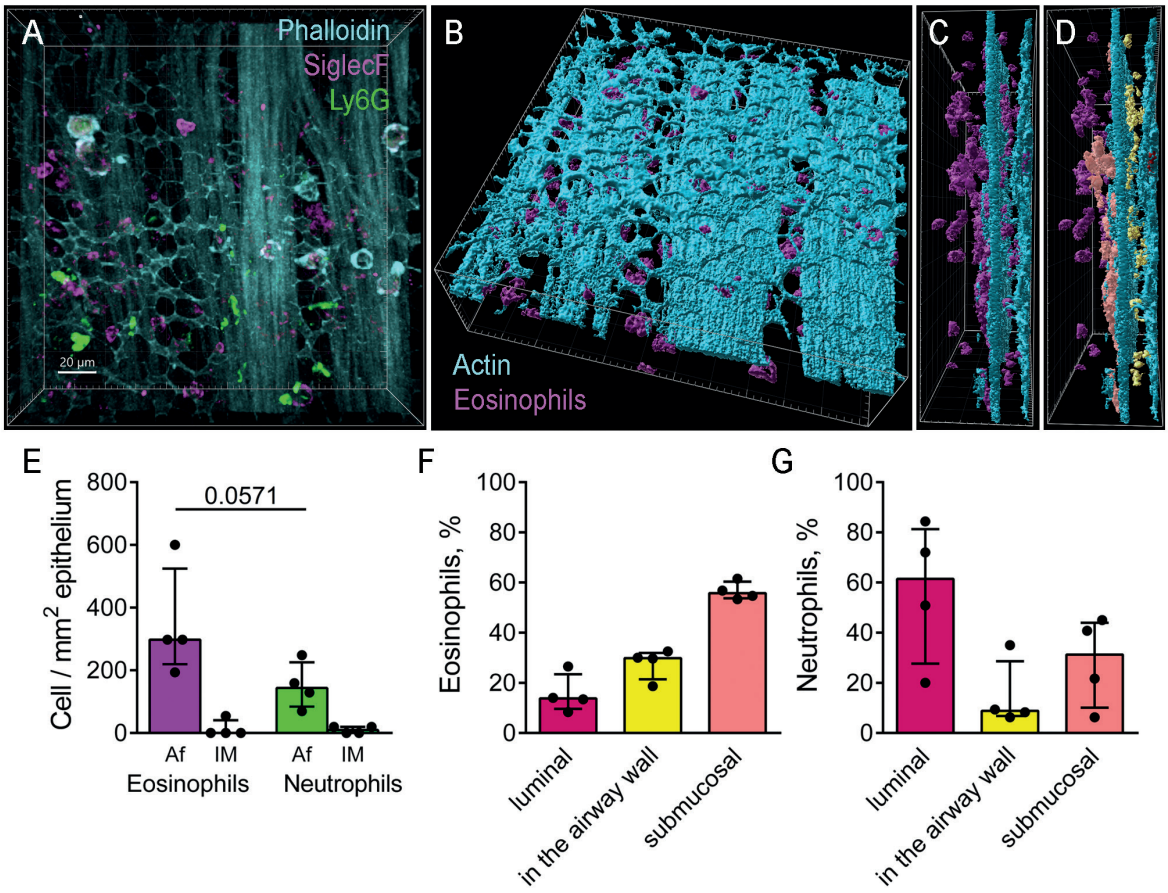

Figure 2. Eosinophil and neutrophil distribution in conducting airway mucosa

Note. A. Representative image of the region of conducting airway mucosa of mouse with induced allergic airway inflammation. Actin–rich epithelial and smooth muscle fibers (cyan), neutrophils (green) and eosinophils (magenta) are represented as maximum intensity projections. Scale bar 20 μm. B. Epithelium and smooth muscles, and eosinophils presented in A are shown via surface rendering. C. Side view of the image presented in B demonstrating eosinophils in relation to the airway wall (epithelium and smooth muscles). D. The sample of analysis of the eosinophil distribution: in the submucosal area that attached to smooth muscles (orange), in the airway wall (yellow), and in the luminal side of the epithelium (red). E. Quantitative analysis of eosinophils and neutrophils in conducting airway mucosa of mice with *A. fumigatus*-induced allergic airway inflammation (Af) and intact mice (IM). F, E. Percentage of eosinophils (F) and neutrophils (E) in the luminal side of epithelium (red bar), in the airway wall (yellow bar), and in the submucosal compartment (orange bar).

rated to the luminal side of the airway epithelium and internalized fungal conidia [12].

While *A. fumigatus* conidia applied to mice induce strong neutrophil-mediated response, the regular applications of small doses of *A. fumigatus* conidia or extract result in the eosinophil recruitment to the airways [11, 13, 14]. The data obtained in the present study demonstrate that despite the adherence of BALB/c mice to neutrophil-mediated response, multiple airway challenge with relatively low doses of the allergen – *A. fumigatus* extract, induced eosinophil recruitment.

Opposite to neutrophils that were mostly represented in the airway lumen, eosinophils infiltrated airways and accumulated near by the smooth muscles, from the side of submucosal compartment. From the one side, in response to the further allergen challenges eosinophils are more likely than neutrophils migrate through the airway wall to the airway lumen. In this case, upon migration, eosinophils can contact the smooth muscles that can trigger bronchoconstriction induction [5]. Migrating through the airway wall, eosinophils can also induce the damage of the airway epithelium that can activate the cascade of inflammatory response. From the other side, accumulation of eosinophils in the submucosal compartment may indicate the inability of eosinophils to migrate to the airway lumen due to the absence of the necessary signals or triggers and such inability can explain the protective effect of eosinophils in acute lung injury [5, 7].

Conclusion

Thus, in the present study we traced the redistribution of neutrophils and eosinophils in blood and airways of mice upon allergic airway inflammation development. We identified microanatomical location of the myeloid effector cells (neutrophils and eosinophils) in conducting airway mucosa and submucosal compartment. The data can explain implication of neutrophils and eosinophils to distinct asthma symptoms.

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