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Characteristics of CD56-positive cells in guinea pig lung in the dynamics of experimental allergic inflammation

S. S. Popko*[A,B,C,D,F], V. M. Yevtushenko[A,E,F], H. A. Zidrashko[E,F]

Zaporizhzhia State Medical University, Ukraine

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

The aim of this work is to study morphometric characteristics and distribution of CD56-positive cells in guinea pig lung in the dynamics of experimental allergic inflammation.

Materials and methods. We studied the distribution and quantitative changes of CD56-positive cells in guinea pig lung in the dynamics of experimental allergic inflammation using histological, histochemical, immunohistochemical, morphometric and statistical methods.

Results. The number of CD56-positive cells increased in the dynamics of experimental ovalbumin-induced allergic inflammation. The increase in the mean number of CD56-positive cells was found in the early period of allergic inflammation (on the 30th day, experimental group II) by 64.5 % (P*/** < 0.001) compared to the control group and by 56.4 % (P* < 0.01) compared to the 23rd day of examinations (experimental group I). The following increase in the mean number of CD56-positive cells by 60.2 % (P*/** < 0.001) was detected in group III compared to the 23rd day of the experiment (group I). However, the mean number of CD56-positive cells was shown to be decreased by 51.5 % (P*/** < 0.001) in group IV compared to the 36th experimental day (group III).

Conclusions. CD56-positive cells are located in the pulmonary interstitium. The number of CD56-positive cells is statistically significantly increased in group III in the late stages of the allergic inflammation indicating an active involvement of these cells in maintaining allergen-induced airway inflammation.

Key words: CD56-positive cell, lung, guinea pigs, allergy, immunohistochemical staining, neuroendocrine cells.

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***E-mail:** kluchkosv@gmail.com

Характеристика CD56-позитивних клітин у легенях морської свинки в динаміці експериментального алергічного запалення

С. С. Попко, В. М. Євтушенко, Г. А. Зідрашко

Мета роботи – вивчення морфометричної характеристики та розподілу CD56-позитивних клітин у легенях морських свинок у динаміці експериментального овальбумін-індукованого алергічного запалення.

Матеріали та методи. Вивчили розподіл і кількісні зміни CD56-позитивних клітин у легенях морських свинок у динаміці експериментального алергічного запалення з використанням гістологічного, гістохімічного, імуногістохімічного, морфометричного та статистичного методів.

Результати. У динаміці експериментального овальбумін-індукованого алергічного запалення спостерігали збільшення кількості CD56-позитивних клітин. Середня кількість CD56-позитивних клітин збільшилась у ранньому періоді алергічного запалення (на 30 добу, II експериментальна група) на 64,5 % (p*/** < 0,001) порівняно з контрольною групою, на 56,4 % (p* < 0,01) порівняно з 23 добою спостереження (I експериментальна група). Надалі збільшення середньої кількості CD56-позитивних клітин встановили в пізньому періоді алергічного запалення (на 36 добу) на 60,2 % (p*/** < 0,001) порівняно з 23 добою експерименту (I експериментальна група). Спостерігали зниження середньої кількості CD56-позитивних клітин на 44 добу експерименту на 51,5 % (p*/** < 0,001) порівняно з 36 добою спостереження в III експериментальній групі.

Висновки. CD56-позитивні клітини локалізуються в легеневому інтерстиції. Найбільш статистично значуще збільшення кількості CD56-позитивних клітин виявили у тварин III експериментальної групи в пізньому періоді алергічного запалення, що свідчить про активну участь CD56-позитивних клітин у підтримці процесу алергічного запалення в легенях морських свинок.

Ключові слова: CD56-позитивна клітина, легеня, морська свинка, алергічне запалення, імуногістохімічне забарвлення, нейроендокринна клітина.

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Характеристика CD56-положительных клеток в лёгких морской свинки в динамике экспериментального аллергического воспаления

С. С. Попко, В. М. Евтушенко, Г. А. Зидрашко

Цель работы – изучение морфометрической характеристики и распределения CD56-положительных клеток в лёгких морских свинок в динамике овальбумин-индуцированного экспериментального аллергического воспаления.

Материалы и методы. Изучили распределение и количественные изменения CD56-положительных клеток в лёгких морских свинок в динамике экспериментального аллергического воспаления с использованием гистологического, гистохимического, иммуногистохимического, морфометрического и статистического методов.

Результаты. В динамике экспериментального овальбумин-индуцированного аллергического воспаления наблюдали увеличение количества CD56-положительных клеток. Среднее количество CD56-положительных клеток увеличилось в раннем периоде аллергического воспаления (на 30 сутки, II экспериментальная группа) на 64,5 % (p*/** < 0,001) по срав-

Ключевые слова: CD56-положительная клетка, лёгкое, морская свинка, аллергическое воспаление,иммуногистохимическое окрашивание, нейроэндокринная клетка.

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нению с контрольной группой, на 56,4 % (p* < 0,01) по сравнению с 23 сутками наблюдения (I экспериментальная группа). Последующее увеличение среднего количества CD56-положительных клеток отмечено в позднем периоде аллергического воспаления (на 36 сутки) на 60,2 % (p*/** < 0,001) по сравнению с 23 сутками эксперимента (I экспериментальная группа). Однако показано снижение среднего количества CD56-положительных клеток на 44 сутки эксперимента на 5,15 % (p*/** < 0,001) по сравнению с 36 сутками наблюдения в III экспериментальной группе.

Выводы. CD56-положительные клетки локализуются в лёгочном интерстиции. Наиболее статистически значимое увеличение количества CD56-положительных клеток установлено у животных III экспериментальной группы в позднем периоде аллергического воспаления, что свидетельствует об активном участии CD56-положительных клеток в поддержании процесса аллергического воспаления в лёгких морских свинок.

Neural cell adhesion molecules (NCAMs), or CD56, are specifically expressed on neural, peripheral neuroectodermal, neuroendocrine cells and tumors. These molecules constitute the immunoglobulin super-family of cell-surface adhesion proteins involved in direct cell-cell adhesion [1]. CD56 is also found on natural killer cells, natural killer-like T cells, dendritic cells and seromucous glands [1,2]. There are three basic isoforms of CD56 (NCAM-120, NCAM-140 and NCAM-180) generated by alternative splicing from a single gene, differing in the intracellular domain length [2,3]. CD56 is often considered as a marker of neural lineage commitment due to its discovery location [1,4]. One of the most important key cells of local immunity in lung are pulmonary neuroendocrine cells (PNECs) [5–8]. Given the expression of neuroendocrine differentiation markers by PNECs, there is a high probability that PNECs positively interact with CD56 [1,9]. PNECs transmit signals directly to innate lymphoid cells 2 (ILC2) for reception and response to environmental stimuli that enter the airways [10]. PNECs are involved in both innate and adaptive immune responses to inhaled allergens [11]. Nowadays, neuronal mechanisms of activation of innate and adaptive imunity are actively studied by scientists worldwide [12,13]. Still, the CD56-positive cell distribution in guinea pig lung in the dynamics of experimental allergic inflammation remains an opened question.

Aim

The aim of our work is to study morphometric characteristic and distribution of CD56-positive cells in guinea pig lung in the dynamics of experimental allergic inflammation.

Materials and methods

Forty-eight sexually mature male guinea pigs (450–600 gram) were weighed and kept in a vivarium of Zaporizhzhia State Medical University with free access to ovalbumin (OVA)-free food and water. The experimental protocol was followed the published guidelines (Strasbourg, 1986; Kyiv, 2006). Animals were assigned equally into six experimental groups of 8 guinea pigs each. Groups I–IV were OVA-sensitized (Sigma Aldrich, USA) guinea pigs with the use of aluminum hydroxide (alum) adjuvant (AlumVax Hydroxide vaccine adjuvant, OZ Biosciences, France), followed by OVA aerosol challenge. Group V were sensitized and exposed to saline guinea pigs and served as a control. Group VI was presented by intact animals (norm). The experiment was ended in each experimental endpoint (23, 30, 36 and 44 days).

Allergic airway inflammation was induced by subcutaneous injection of OVA solution and aerosol challenge with OVA through nasal inhalation (0.5 mg/mL per animal) mixed with alum (10 mg/mL in saline per animal) on days 0, 7 and 14. From day 21 to day 28, the animals were exposed to aerosolized OVA (10 mg/mL in saline) for 15 min using a nebulizer device (Little Doctor International, Singapore, LD-211C) coupled to a plastic chamber [14].

Lungs were removed and fixed immediately in 10 % neutral buffered formalin. Formalin-fixed, preserved by progressive alcohol dehydration, paraffin wax-embedded lung specimens were selected for histological preparation of 5-μm-thick sections and stained with hematoxylin and eosin. Laidlaw silver impregnation was used to identify pulmonary neuroendocrine cells and neuroepithelial bodies [15]. Argyrophilic granules of neuroendocrine cells have the ability to accumulate silver ions, while metallic silver appears only on light or after adding an external reducing agent. The argyrophilic reaction product was deposited in the cytoplasm in the form of small dark brown granules.

Paraffin-embedded sections were immunohistochemically stained using monoclonal antibodies Mo a-Hu CD56 Antigen, Clone T199 (Thermo Scientific, USA). Dewaxing and rehydration with simultaneous high-temperature antigen retrieval was performed by heating with an autostainer using a PT-module (Thermo Fisher Scientific, USA) in Dewax & HIER buffer H (Thermo Fisher Scientific, USA) (pH = 9.0). The sections were incubated with 3 % H_2O_2 to block endogenous peroxidase activity and proteins were removed. Incubation with primary antibodies was performed according to the manufacturer's instructions, visualization of the IHC reaction was performed using an UltraVision Quanto HRP + DAB System (Thermo Scientific, USA). Sections were stained with Mayer's hematoxylin and embedded in the Cytoseal. Complex morphometric examinations were carried out under a Carl Zeiss Primo Star microscope equipped with a digital Axiocam for photomicrographs using the ZEISS ZEN 2011 software. Following the immunohistochemical staining on the serial cross sections, the total number of CD56-positive cells per unit area of 5000 μm^2 was counted, on ten areas of three sections of each specimen captured from the middle lobe of the right lung using a microscope with an oil immersion objective (×1000).

Data were represented as mean ± standard deviation [SD] for all parameters. The data were analyzed using the standard software package Microsoft Office Excel 2010 and Statistica for Windows 13 (StatSoft Inc., No. JPZ804I382130ARCN10-J), the libraries SciPy (BSD License), NumPy (BSD License), pandas-profiling (MIT License), pandas (BSD License). We used the library Matplotlib (BSD License) for the Python programming language to visualize the processed data. The hypothesis of the normal data distribution was verified using the Kolmogorov–Smirnov test and the Shapiro–Wilk test. Statistical significance

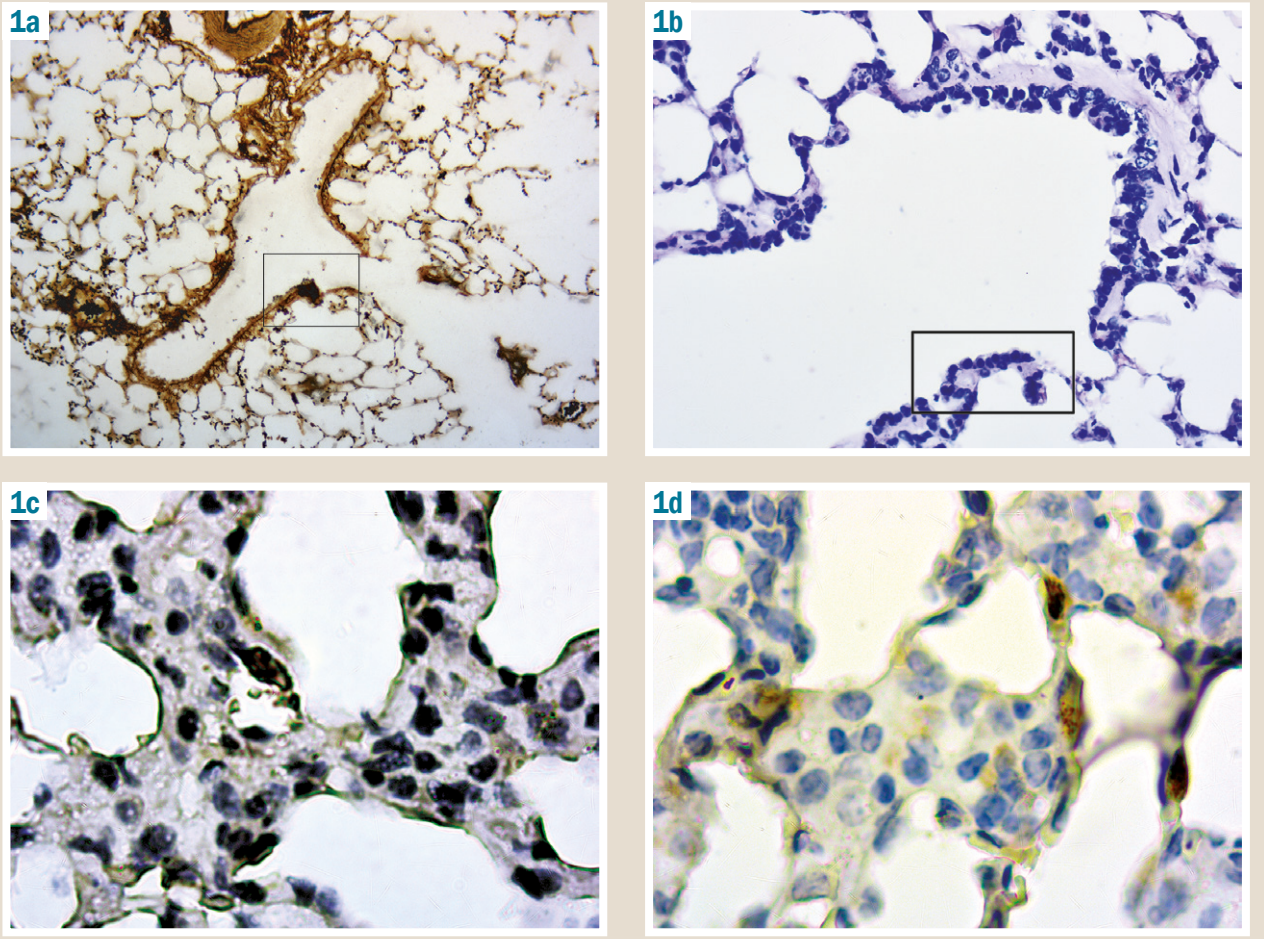

Fig. 1. Distribution of CD56-positive cells and cells stained positively with silver impregnation in guinea pig lung. Group II (**1a, 1c**). Group III (**1b, 1d**). **1a:** cells stained positively with silver impregnation located in bifurcation of terminal bronchiole to respiratory bronchiole; **1b:** neuroepithelial body surrounded by bronchiolar exocrinocytes located in bifurcation of small airways to terminal bronchioles; **1c, 1d:** CD56-positive cells located in pulmonary interstitium. **Staining: 1a**: Laidlaw silver impregnation; **1b:** haematoxylin and eosin; **1c, 1d:** immunohistochemical identification of CD56-positive cells. **1a, 1b:** ×400. **1c, 1d:** ×1000.

between different groups were calculated using the Student t-test (P*) for continuous variables and Whitney–Mann U-test (P**) for variables with abnormal distribution. The data were expressed as the median (Me) with interquartile range (Q1; Q3). The results were considered significant at a level of $P < 0.05$.

Results

A low number of CD56-positive cells are normally present in guinea pig lung. It is of note, that CD56-positive cells showed a diffuse cytoplasmic staining in pulmonary interstitium in the specimens. However, it is worth remarking that CD56 was not found in the epithelial layer of bronchial mucosa, therefore type I PNECs ("opened" type) did not express CD56. Nevertheless, we revealed positively stained by silver impregnation cells in the epithelium of the terminal airways (*Fig. 1a*). Apparently, it was possible to identify these cells as PNECs type I, localized, as far as is known, in bifurcation of terminal airways, at the junction of small airways and terminal bronchioles or the latter and respiratory bronchioles. PNECs were often collected into neuroepithelial bodies – clusters of 6–8 cells, surrounded by bronchiolar exocrinocytes or club cells (*Fig. 1b*). The latter, according to our observations, were confined to the terminal and respiratory bronchioles, had a cuboidal or pyramidal shape, the narrowed apical part protruding into the airway lumen.

The second type of PNECs ("closed" type) was revealed in the pulmonary interstitium, frequently in the wall of respiratory bronchioles and in alveolar ducts. Oval-shaped PNECs II type with long cytoplasmic processes were frequently localized between capillary endotheliocytes and alveolocytes. Similar cells with such morphological features showed a positive reaction with anti-CD56 mAb in our study (*Fig. 1c, 1d*).

A difference in the number of CD56-positive cells in guinea pig lung was not statistically verified ($P^*/^{**} > 0.05$) between animals of the intact and control groups, indicating no influence of the experimental protocol on the morphometric data changes (*Fig. 2*). Therefore, we compared the results of the experimental and control groups.

The mean number of CD56-positive cells was 2.88 ± 0.09 per 5000 μm^2 in the intact group animals, but it was found to be increased in the dynamics of experimental OVA-induced allergic inflammatory process. The mean number of CD56-positive cells increased in the early stages of allergic inflammation (on the 30th day, group II) by 64.5 % ($P^*/^{**} < 0.001$) in comparison with the control, and by

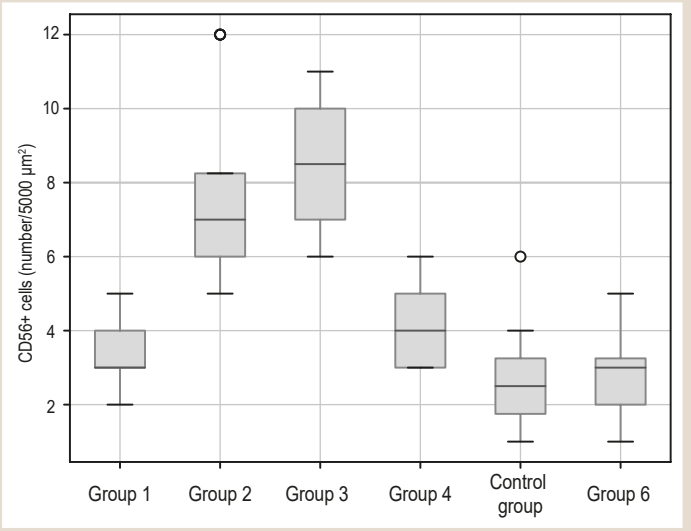

Fig. 2. Immunohistochemical quantification of CD56-positive cells in guinea pig lung after OVA administration. ***:** P < 0.05 (t-test); ****:** P < 0.05 (U-test) compared to the control animals. Me (Q1; Q3). M ± SD. N = 8. The median (Me) is shown by the green line. Outlier is shown by the circle.

56.4 % (P* < 0.01) compared to the 23rd day of examination (group I) (*Fig. 2*).

The following increase in the mean number of CD56-positive cells by 60.2 % (P*/** < 0.001) was detected in group III compared to the 23rd day of the experiment (group I) and by 67.6 % (P*/**< 0.001) compared to the control group. However, the mean number of CD56-positive cells was shown to be decreased by 46.8 % (P*/** < 0.01) in group IV compared to the 30th day (group II) and by 51.5 % (P*/** < 0.001) compared to the 36th experimental day (group III).

Discussion

In the present study, we have proved quantitative changes of CD56-positive cells in OVA-sensitized and challenged guinea pig lung in the dynamics of experimental OVA-induced allergic inflammation. We used guinea pigs (Cavea porcellus – mammals in the Caviidae family) for the model of allergic inflammation. These animals, due to many similarities in immunological reactions, the respiratory system sensitivity, susceptibility to allergic diseases to humans, are a useful model for studying the allergic airway inflammation [16].

We tested the use of CD56 NCAM as the marker for immunohistochemical identification of PNECs in our study. The results presented also have demonstrated for the first time the increased mean number of CD56-positive cells in OVA-sensitized and challenged guinea pig lung at the early stages of allergen-induced airway inflammatory process using the model of allergic asthma. We have observed the statistically significant increase (P*/** < 0.001) in the mean number of CD56-positive cells, beginning in group II, which was about 3 times the number of the control animals. The increased number of CD56-positive cells has been detected at the late stages of allergen-induced airway inflammatory process. We have shown a downward trend in the mean number of CD56-positive cells in group IV (4.12 ± 0.40 per 5000 μm², P** < 0.001) compared to that in the previous period of examinations, since the CD56-positive cell mean number was much the same as in the group V.

In addition, given the similar results with the use of CD56 in studies of other scientists [17,18] showing a strong CD56 staining pattern in small cell lung carcinoma (small cell carcinomas with positive CD56 staining indicate a neuroendocrine phenotype), CD56 may also be a useful marker for PNECs. To assess peripheral nerve sheath tumor, scientists used CD56 staining and confirmed neuronal origin of tumor cells to differentiate from other phenotypes [19]. Our findings are in agreement with the above reports. Hence, it would be possible to use CD56 NCAM as a marker for immunohistochemical identification of pulmonary neuroendocrine cells II ("closed") type, if it is confirmed by other markers of neuroendocrine differentiation [20–23].

On the other hand, CD56 is a marker of natural killer cells, but, in fact, can be expressed by other immune cells, such as gamma/delta and alpha/beta T cells, dendritic cells, monocytes [24,25]. Natural killer cells are prototypes of innate lymphoid cells and expressed in humans by CD56 in the absence of CD3 [1,26,27]. The reason why NK cells and other immune cells express CD56 remains to be determined. Potentially, the presence of CD56 displays the degree of differentiation and activation, similar to HLA-DR or CD69 [27].

Conclusions

1. The most statistically significant increase in the mean number of CD56-positive cells is observed in group III by 67.6 % (P*/** < 0.001) in comparison with the control animals and by 60.2 % (P*/** < 0.001) as compared to the 23rd day of the experiment (group I), indicating an active involvement of CD56-positive cells in maintaining allergen-induced airway inflammatory process.

2. The study results revealed the applicability of anti-Human monoclonal antibody CD56 cross-reaction with the cells of guinea pig lung. Hence, it would be possible to use CD56 neural cell adhesion molecule as the marker for immunohistochemical identification of pulmonary neuroendocrine cells II ("closed") type, if it is confirmed by other markers of neuroendocrine differentiation.

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Information about authors:
Popko S. S., MD, PhD, Associate Professor of the Department of Histology, Cytology and Embryology, Zaporizhzhia State Medical University, Ukraine.
ORCID ID: 0000-0002-5533-4556

Yevtushenko V. M., MD, PhD, DSc, Head of the Department of Histology, Cytology and Embryology, Zaporizhzhia State Medical University, Ukraine.
ORCID ID: 0000-0002-6858-6488
Zidrashko H. A., MD, PhD, Associate Professor of the Department of Histology, Cytology and Embryology, Zaporizhzhia State Medical University, Ukraine.

Відомості про авторів:

Попко С. С., канд. мед. наук, доцент каф. гістології, цитології та ембріології, Запорізький державний медичний університет, Україна.
Євтушенко В. М., д-р мед. наук, професор, зав. каф. гістології, цитології та ембріології, Запорізький державний медичний університет, Україна.
Зідрашко Г. А., канд. мед. наук, доцент каф. гістології, цитології та ембріології, Запорізький державний медичний університет, Україна.

Сведения об авторах:

Попко С. С., канд. мед. наук, доцент каф. гистологии, цитологии и эмбриологии, Запорожский государственный медицинский университет, Украина.
Евтушенко В. М., д-р мед. наук, профессор, зав. каф. гистологии, цитологии и эмбриологии, Запорожский государственный медицинский университет, Украина.
Зидрашко Г. А., канд. мед. наук, доцент каф. гистологии, цитологии и эмбриологии, Запорожский государственный медицинский университет, Украина.

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