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Word
C-Kit+ cells can modulate asthmatic condition via differentiation into pneumocyte-like cells and alteration of inflammatory responses via ERK/NF-κB pathway

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OBJECTIVE(S): The exact role of the progenitor cell types in the dynamic healing of asthmatic lungs is lacking. This investigation was proposed to evaluate the effect of intratracheally administered rat bone marrow-derived c-kit+ cells on ovalbumin-induced sensitized male rats.

MATERIALS AND METHODS: Forty rats were randomly divided into 4 groups; healthy rats received phosphate-buffered saline (PBS) (C); sensitized rats received PBS (S); PBS containing C-kit− cells (S+C-kit−); and PBS containing C-kit+ cells (S+C-kit+). After two weeks, circulating CD4+/CD8+ T-cell counts and pulmonary ERK/NF-κB signaling pathway as well as the probability of cellular differentiation were assessed.

RESULTS: The results showed that transplanted C-Kit+ cells were engrafted into pulmonary tissue and differentiated into epithelial cells. C-Kit+ cells increased the number of CD4+ cells in comparison with the C group (P<0.001); however, they diminished the level of CD8+ cells (P<0.01). Moreover, data demonstrated increased p-ERK/ERK ratio (P<0.001) and NF-κB level (P<0.05) in sensitized rats compared with the C group. The administration of C-kit+, but not C-Kit−, decreased p-ERK/ERK ratio and NF-κB level compared with those of the S group (P<0.05).

CONCLUSION: The study revealed that C-Kit+ cells engrafted into pulmonary tissue reduced the NF-κB protein level and diminished p-ERK/ERK ratio, leading to suppression of inflammatory response in asthmatic lungs.

INTRODUCTION

Asthma is a complex pathological condition with an allergic response in the broncho-pulmonary segment that threatens human health (1, 2). It was suggested that asthma coincides with the chronic inflammatory response which is caused by the activity of CD4+ T lymphocytes, namely, type 2 T helper (Th2)-immune response (3, 4). Upon stimulation of Th2 cells, arrays of cytokines are released into the asthmatic niche (5, 6). As a correlate, the dynamic balance of Th1/Th2 cells and significant production of interleukins (ILs) such as IL-4, -5 and -13 are thought to be involved in the recruitment of different subsets of inflammatory cells and subsequent airway hyper-responsiveness (7, 8). Moreover, the activity of antigen-primed CD8+ T cells is indispensable for the development of allergen-induced airway inflammation and hyper-responsiveness during the progression of asthma (9). Many studies have described the critical role of mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) signaling pathways in the progression/suppression of allergic asthma responses (10, 11). NF-κB is an important transcription factor contributing to the expression of various pro-inflammatory genes. Increased ERK/NF-κB activity has been documented in airway epithelial cells and alveolar macrophages of asthmatic patients (12). Inhalation of corticosteroids in combination with long-acting β2-agonists or leukotriene modulators can commonly be administrated to relieve asthmatic symptoms (13). Because of the possibility of serious side effects (14, 15), new therapeutic approaches are needed.

Along with conventional therapeutic strategies, regenerative medicine is substantial to treat diseases with no accessible or effective treatments (16). For instance, transplantation of different cell lineages alone or in

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combination with growth factors and cytokines can help the host tissue to restore, but not completely, physiological function (17, 18). Among several cells that exist inside tissues, stem cells are expected to possess the unique regenerative potential for dealing with several diseases (19). C-kit+ cells are one of the stem cell subgroups with several regenerative capacities. It has been indicated that activation of the C-kit, a stem cell factor (SCF) receptor, can contribute to indispensable functions of effectors and signaling proteins inside human cells (20). For instance, SCF/C-Kit signaling has been delineated to have an essential role in the modulation of phenotype acquisition of microglia and intensity of immune responses (21-24). Transplantation of human C-Kit+/SSEA4+ retinal progenitor cells has been shown to diminish the frustration of microglia and inflammation intensity in rats and mice with improved vision rate (25). In line with this statement, Ellison and colleagues showed that the adult heart encompasses numerous endogenous c-kit+ cardiac stem cells that could repair the injured myocardium during several pathologies (26). These cells exhibit the typical characteristics of stem cells such as clonogenicity, self-renewal, with prominent capacity committed into cardiomyocytes, smooth muscle, and endothelial cells (27, 28). To the best of our knowledge, few reports are highlighting the c-kit+ progenitor cells during different inflammatory conditions. Here, we proposed to evaluate the effect of intratracheally administered rat bone marrow-derived c-kit+ cells on the ovalbumin-induced sensitized model of male rats. Two weeks after implementation of these cells, circulatory CD4+ and CD8+ T-cell counts and pulmonary ERK/NF-κB signaling pathway were assessed. In addition, the probability of differentiation of these cells was appraised.

Materials and Methods

Animal ethics

All experimental procedures followed guidelines of the care and use of laboratory animals (NIH Publication No. 85-23, revised 1996) and were confirmed by the Animal Care Committee of Tabriz University of Medical Sciences (No: IR.TBZMED.VCR.REC.1397.404).

Experimental groups

In this study, fifty male rats (weighing 200–250 g, 8–9 weeks old) were enrolled in the study. These animals were purchased from the animal house of Tabriz University of Medical Sciences and kept in standard condition and permitted to access chewing food and water ad libitum. Fourteen days after accommodation, 40 rats were randomly selected and divided into the following groups; healthy rats receiving 50 µl of phosphate-buffered saline (PBS) intratracheally (C group); sensitized rats receiving 50 µl of PBS intratracheally (S group); sensitized rats receiving 50 µl of PBS intratracheally containing $3 \times 10^5$ C-kit− cells (S+C-kit− group), and sensitized rats receiving 50 µl of PBS intratracheally containing $3 \times 10^5$ C-kit+ cells (S+C-kit+ group). The remaining rats were used for extraction of C-kit+ and C-kit− cells.

Induction of asthma

The asthma model of the rat was established according to the previously used method (29, 30). Rats were sensitized by injection of 1 mg ovalbumin (OVA; Sigma-Aldrich, USA) mixed with 200 mg aluminum hydroxide (Sigma, Chemical Ltd, UK) dissolved in saline intraperitoneally on the first and 8th days. From the 14th day, animals were kept in a suitable whole-body inhalation exposure chamber with dimensions of $30 \times 20 \times 20$ cm$^3$ and exposed to 4% OVA aerosol produced by a nebulizer (CX3; Omron Co., Netherlands) for 5 min daily for 18±1 days (3 rats per each inhalation). The control group was challenged with normal saline instead of OVA. On the 33rd day, PBS, PBS containing C-kit− and C-kit+ cells were injected into the trachea through a cervical incision in S, S+C-kit−, and S+C-kit+ groups, respectively. All animals were humanely euthanized two weeks later by cervical dislocation after a high-dose injection of xylazine and ketamine.

Magnetic-activated cell sorting (MACS)

To isolate bone marrow-derived C-kit+ cells, rats were humanely euthanized by cervical dislocation after high-dose injection of xylazine and ketamine. Femurs were completely removed and medullary cells flushed out by PBS solution containing 2% fetal bovine serum (FBS; Gibco). The mononuclear cells were isolated by Ficoll (Sigma-Aldrich) gradient centrifugation at 400×g for 20 min. For the MACS procedure, harvested marrow mononuclear cells were incubated with 1% FBS for 30 min at 4 °C. After that, cells were exposed to anti-human C-kit microbead (Catalog no. 130-091-224; Miltenyi Biotec) and cells passed through the LS column (Miltenyi Biotec) to isolate the c-kit+ and c-kit− cells (31).

Cell labeling

Both c-Kit− and c-Kit+ cells were labeled using 20 µM Cell TrackerTM CM-Dil at 37 °C for 40 min followed by three-time PBS washes (32). In this study, 50 µl PBS containing $3 \times 10^5$ of positive and negative C-kit− cells were used for injection per rat.

Flow cytometric analysis of systemic CD4+ and CD8+ lymphocytes

The percentage of systemic CD4+ and CD8+ lymphocytes was measured before and after C-kit cell injection. For this purpose, blood cells were diluted with PBS solution (1:1) and mononuclear cells were isolated using Ficoll solution as above-mentioned. Then, cells were incubated with FITC-conjugated mouse anti-rat CD4 (eBioscience) and CD8 (eBioscience) for 30 min at 4 °C. Finally, cells were analyzed using the BD FACSCalibur flow cytometry system and FlowJo software (ver. 7.6.1).

Western blotting

The right lungs of each group were homogenized using lysis buffer and then the samples were centrifuged at 4 °C for 10 min at 10,000 rpm. The supernatant containing protein was determined by Bradford’s method. Equal amounts of protein were resolved by 10% sodium dodecyl sulfate-polyacylamide gel electrophoresis (SDS-PAGE) and transferred to Polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). The membranes were washed with TBST buffer and blocked with 5% skimmed milk for 1 hr. After that, membranes were incubated with primary antibodies anti-β-actin (Catalog No: sc-47778), ERK 1/2 (Catalog No: sc-292838), p-ERK 1/2 (Catalog No: sc-16981-R), NF-kB p65 (Catalog No: ab16636) at 4 °C for
16–18 hr. Finally, the membrane was washed with TBST buffer and detected with an HRP-conjugated secondary antibody (Catalog No: sc-2357). The bands were visualized by enhanced chemiluminescence (ECL advanced reagents kit, Product Booklet, RPN2135).

**Immunofluorescence imaging (IF)**

Fourteen days after injection of Dil-labeled cells, 5-6 μm thick cryo-sections were prepared from lung tissues. The samples were incubated with anti-cytokeratin 19 antibody for 1 hr. After three-time PBS washes, the samples were exposed to an appropriate FITC-labeled secondary antibody for 1 hr at room temperature. The nuclei were stained using DAPI (4',6-diamidino-2-phenylindol). Here, we monitored the existence of Dil+/cytokeratin-19+ cells (dual red/green stained cells), indicating the differentiation of transplanted cells into the pneumocyte-like cells (33).

**Data analysis**

Results were displayed as mean±SEM and analyzed by one-way ANOVA with Tukey–Kramer post hoc test. Statistical P-values less than 0.05 were considered significant.

**Results**

**Transplanted C-Kit+ cells were engrafted into pulmonary tissue and differentiated into epithelial cells**

To check whether intra-tracheal administration of labeled C-kit- and C-Kit+ cells can lead to successful homing into the pulmonary tissues, we performed an IF analysis. Data showed red-colored C-kit- and C-Kit+ cells inside the lung parenchymal 14 days after intratracheal administration. In S + C-kit- and S + C-Kit+ groups, these cells were randomly distributed. It seems that the number of labeled cells was higher in the S+C-Kit+ group compared with the S+C-kit- group. These data showed that intratracheal administration of C-kit- and C-Kit+ cells led to successful recruitment into the pulmonary niche after 14 days in a rat model of asthma. We also performed an IF analysis based on Cytokeratin-19 staining. This factor is a typical pneumocytes protein marker. We found that C-kit cells had the potential to express Cytokeratin-19 fourteen days after transplantation into the rat model of asthma. Based on our data, the number of green-colored cells (Double green/red-stained cells) was high in the S+C-Kit+ group compared with S+C-kit- rats, showing the prominent trans-differentiation capacity of C-Kit+ cells compared with the C-kit- subsets. As expected, labeled cells were not detectable in either sensitized (S) or control (C) groups. These data showed that C-kit+ progenitor cells can acquire a pneumocyte-like phenotype inside the asthmatic niche which can help the host lung to restore the function of resident pneumocytes (Figure 1).

**Administration of C-kit+ cells changed systemic levels of CD4+ and CD8+ lymphocytes in sensitized rats**

Flow cytometry results demonstrated that the percentage of CD4+ cells in the sensitized group was decreased (60 ± 4.9 vs 29.4 ± 0.1%) as compared with group C. According to our data, transplantation of C-kit- and C-Kit+ cells via intratracheal route increased the systemic number of CD4+ cells in comparison with the asthmatic profile (34.8 ± 5.2 and 43.9 ± 6.3 vs 29.4 ± 0.1%, respectively). These changes were more evident in sensitized rats that received C-Kit+ cells (P<0.001). In contrast to dynamic changes of CD4+ cells, the blood levels of CD8+ cells were diminished after the onset.
of asthmatic changes compared with the control rats (28.7 ± 6.3 vs 43.7 ± 7.5%, *P* < 0.01). The transplantation of both C-kit− and C-Kit+ cells reduced the abnormal elevation of CD8+ cells almost to near-to-control levels (39.4 ± 8.2 and 29.5 ± 6.5 vs 43.7 ± 7.5%, respectively). These data showed that transplantation of C-Kit+ cells in sensitized rats can modulate the abnormal lymphocyte counts. Although C-kit− can alter the number of lymphocyte subsets under asthmatic conditions, these changes were less in sensitized rats that received C-Kit+ cells (*P* < 0.05, Figure 2).

**C-kit+ cells suppressed ERK/NF-κB activity in sensitized rats**

Proteomic analysis revealed alteration of factors related to the ERK/NF-κB signaling pathway. Data showed an increased p-ERK/ERK ratio in sensitized rats (S) compared with the control (C) group (*P* < 0.001). We noted that administration of C-kit+ but not C-Kit− decreased abnormal phosphorylation of ERK effector compared with the sensitized group (*P* < 0.05). Despite reduction of ERK phosphorylation in S+C-Kit and S+C-Kit+ groups, the p-ERK/ERK ratio was higher compared with the normal condition (Figure 3).

Along with these changes, it was notified that promotion of asthmatic conditions can alter protein levels of NF-κB inside the lungs. Consistent with the phosphorylation of ERK, the levels of NF-κB were increased in the lungs of the S group compared with the control rats (*P* < 0.05). We showed that administration of C-Kit+ cells can reduce synthesis of NF-κB compared with the S group (*P* < 0.05). Unlike the therapeutic effects of C-Kit+ cells, C-Kit− cells did not change NF-κB compared with the S group (Figure 4). These data showed that promotion of asthmatic changes can alter specific signaling transduction pathways such as ERK/NF-κB axis which can lead to the inflammatory response. The application of specific progenitor types such as C-Kit+ cells reduced protein synthesis of NF-κB and diminished the p-ERK/ERK ratio, leading to suppression of inflammatory response in asthmatic lungs.

**Discussion**

The global increase of asthma and the absence of completely effective modalities increase the need to invent

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**Figure 2.** Flow cytometric analysis of rat peripheral mononuclear cells in different groups (A and B). The cells were stained with a panel of antibodies, including CD4+ and CD8+. The percentage of mononuclear cells expressing each marker is expressed as mean±SEM. C (control group), S (sensitized animals with ovalbumin), S+c-kit- (sensitized animals received c-kit cells), S+c-kit+ (sensitized animals received c-kit+ cells).

**Figure 3.** Protein level ratios of p-ERK/ERK in the lung tissues of C (control group), S (sensitized animals with ovalbumin), S+c-kit- (sensitized animals received c-kit cells), and S+c-kit+ (sensitized animals received c-kit+ cells) groups. Statistical differences between different groups vs control: +++; *P* < 0.001, ++; *P* < 0.01. Statistical differences between S+c-kit vs group S: **; *P* < 0.01. Statistical differences between S+c-kit+ and S+c-kit groups: #; *P* < 0.05. Bars represent the mean±SEM.

**Figure 4.** Protein level of NF-κB in the lung tissues of C (control group), S (sensitized animals with ovalbumin), S+c-kit- (sensitized animals received c-kit cells), and S+c-kit+ (sensitized animals received c-kit+ cells). Statistical differences between different groups vs control: +; *P* < 0.05. Statistical differences between S+c-kit vs group S: *; *P* < 0.05. Statistical differences between S+c-kit+ and S+c-kit- groups: #; *P* < 0.05. Bars represent the mean±SEM.
新治疗策略。以前，特异性前体细胞类型如C-Kit⁺细胞的存在已经被记录在肺部组织中。然而，这些细胞在哮喘肺的动态治疗中具体的治疗作用还是未明（34, 35）。一些研究认为，肺部C-Kit⁺细胞能够参与组织稳态的重建（36-40）。在目前的研究中，通过检测C-Kit⁺和C-Kit⁻细胞的ERK/NF-κB信号通路，我们检测到了未受刺激的哮喘模型。我们还监测了C-Kit⁺细胞在系统性CD4⁺和CD8⁺淋巴细胞中的变化。是否移植C-Kit⁺和C-Kit⁻细胞能恢复肺部的炎症功能被通过检测细胞分化以及表达模式（Cytokeratin-19）。

在这里，我们发现CD4⁺淋巴细胞数量在哮喘相关的改变中与肺部脱细胞细胞的表达有关（41-43）。根据我们的数据，哮喘相关改变后CD4⁺和CD8⁺淋巴细胞的百分比增加了，这可能与炎症相关（44）。与我们的数据一致，之前的研究表明，C-Kit⁺细胞能够影响哮喘模型的Th1/2平衡，病理特征，以及细胞的分泌功能（47）。我们还假设CD8⁺细胞能够参与哮喘相关的逆转（35, 45, 46）。哮喘性肺部C-Kit⁺细胞的表型移动与抗原特异性联系不大，但是能抑制支气管炎症和气道高反应性（44）。与我们的数据一致，之前的研究表明，C-Kit⁺细胞的移植能够抑制哮喘相关的病理变化（44, 45, 46）。这些数据与我们的研究相一致，表明C-Kit⁺细胞在哮喘相关的条件中可能有治疗潜力（47）。

结论

在本次研究中，我们在哮喘模型中观察到C-Kit⁺细胞向肺部脱细胞细胞的表型移动，通过检测Cytokeratin-19。

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作者贡献

FM和RK设计了研究，RR提取了C-kit⁺和C-kit⁻细胞；FM，MA，和HH为数据的收集做出了贡献；FM和RK分析了数据；FM和RK共同撰写并审阅了文章。所有作者都阅读并批准了最终的版本。

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利益冲突

作者声明：没有利益冲突。

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