

Allergen-induced traffic of bone marrow eosinophils, neutrophils and lymphocytes to airways

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We evaluated whether bone marrow (BM) inflammatory cells have capacity to traffic into the airways following allergen exposure in a mouse model of allergen-induced airway inflammation. We also evaluated the effect of IL-5 overexpression on (i) the production of eosinophils in BM, (ii) the accumulation of eosinophils, neutrophils and lymphocytes in blood and airways and (iii) the changes in CD34⁺ cell numbers in BM, blood and airways. Bromodeoxyuridine (BrdU) was used to label cells produced during the exposure period. Furthermore, CD3 splenocytes were adoptively transferred to investigate the BM inflammatory response. Allergen exposure induced traffic of BM eosinophils, neutrophils and lymphocytes to the airways and increased the number of BrdU⁺ eosinophils, neutrophils, lymphocytes and CD34⁺ cells in BALf. IL-5 overexpression enhanced the eosinophilopoiesis and increased the presence of BrdU⁺ eosinophils and CD34⁺ cells in airways and enhanced the number of CD34⁺ cells in BM and blood after allergen exposure. Adoptive transfer of CD3 lymphocytes overexpressing IL-5 caused increased BM eosinophilia. In conclusion, allergen exposure induces traffic of not only newly produced eosinophils but also newly produced neutrophils and lymphocytes into the airways.

Key words: Inflammation / CD3⁺lymphocytes / CD34⁺cells / NJ.1638

Received	24/2/04
Revised	7/7/04
Accepted	17/8/04

1 Introduction

Airway inflammation in asthma is dominated by eosinophils. It has been suggested that the eosinophils cause tissue damage and bronchial hyperresponsiveness (BHR) [1–3]. The number of neutrophils also increases within asthmatic airways and these cells have been associated with BHR and airway wall remodeling [4]. Activated T lymphocytes contribute to allergic airway inflammation via the release of pro-inflammatory cytokines and chemokines [5]. IL-5 is an important cytokine in eosinophilopoiesis since it regulates the differentiation of CD34⁺ cells to eosinophils [6, 7], as well as activates [8] and prolongs the survival of eosinophils [9].

Despite the recent debate about local eosinophil production within airways [10–15], cells produced and recruited from the bone marrow (BM) are considered to be the major contributors of allergen-induced airway inflammation [15–21]. However, to date there is no direct evidence of traffic of BM cells into the airways in response to airway allergen exposure.

The aim of this study was to evaluate the effect of IL-5 overexpression on (i) the production of eosinophils in BM, (ii) the accumulation of eosinophils, neutrophils and lymphocytes in blood and airways in response to allergen exposure and (iii) the changes in CD34⁺ cell numbers in BM, blood and airways after allergen exposure. Furthermore, we examined whether there is any traffic of eosinophils, neutrophils and lymphocytes into airways following allergen exposure. To test this, we adoptively transferred BM cells to sensitized wild-type mice (C57BL/6) 21 h prior to allergen exposure. In addition, we adoptively transferred CD3⁺ splenocytes from IL-5-transgenic (NJ.1638) and wild-type mice to sensitized wild-type mice to evaluate whether the BM eosinophilic response was affected.

[DOI 10.1002/eji.200425043]

Abbreviations: BALf: Bronchoalveolar lavage fluid BrdU: 5-Bromo-2'-deoxyuridine i.n.: Intranasal(ly) MBP: Major basic protein CD3^{IL-5}: CD3 lymphocytes from NJ.1638 mice SCID-bg: SCID-beige

2 Results

2.1 Effect of allergen sensitization and exposure

The effect of allergen exposure and overexpression of IL-5 was determined using May-Grünwald-Giemsa staining for differential cell counts and immunocytochemistry (ICC) for detecting eosinophils, neutrophils and lymphocytes produced during the exposure period (newly produced, BrdU⁺) as well as CD34⁺ cells.

2.2 Eosinophils and IL-5

In wild-type mice, exposure to OVA (10 or 100 µg) caused an increase in the absolute number of BALf and blood eosinophils and in the relative number of BM eosinophils as compared to PBS exposure ($p < 0.005$; Table 1). The relative number of BM immature eosinophils was increased after each allergen exposure, whereas the higher dose also increased the number of mature eosinophils. Exposure to 100 µg OVA further augmented

the number of BM eosinophils when compared to exposure to 10 µg of OVA ($p < 0.05$).

In NJ.1638 mice, exposure to OVA (10 or 100 µg) increased the absolute number of BALf eosinophils, the relative number of BM mature eosinophils and the total number of BM eosinophils ($p < 0.01$; Table 1). However, allergen exposure did not significantly affect the absolute number of blood eosinophils compared to PBS exposure.

In wild-type mice, the concentration of IL-5 in serum, but not in BALf, increased significantly after higher dose of allergen exposure ($p < 0.04$; Table 1). After allergen exposure (10 or 100 µg), the concentration of IL-5 in NJ.1638 mice was significantly increased in BALf but not in serum ($p < 0.0005$).

Compared to wild-type mice, NJ.1638 mice had a significantly higher number of eosinophils in BALf, blood and BM ($p < 0.03$), as well as a higher concentration of IL-5 in both BALf and serum ($p < 0.01$). Allergen-induced changes in other cell types are presented in Table 1.

Table 1. Cell differential counts in bone marrow, BALf, and blood in C57BL/6 and NJ.1638 mice. Concentration of IL-5 in BALf and serum^{a)}

Exposure	PBS		10 µg OVA		100 µg OVA	
	C57BL/6	NJ.1638	C57BL/6	NJ.1638	C57BL/6	NJ.1638
Bone marrow (%)	<i>n</i> =10	<i>n</i> =6	<i>n</i> =15	<i>n</i> =11	<i>n</i> =7	<i>n</i> =6
Immat eosinophils	2.6(2.0)	17.8(1.5)●	5.0(0.4)*	27.4(3.2)●*	7.0(0.9)*○	18.4(0.7)●
Mat eosinophils	3.3(0.3)	20.6(1.8)●	4.5(0.5)	34.6(4.0)●*	5.4(0.8)*	35.1(1.8)●*
Total eosinophils	5.9(0.4)	38.3(2.9)●	9.6(0.7)*	62.0(3.0)●*	12.4(1.5)*○	53.4(1.7)●*
Total cells	16.6(1.8)	12.6(2.5)	16.7(1.7)	13.6(1.8)	17.6(1.8)*	7.7(1.7)
BALf (x10 ⁴ /ml)	<i>n</i> =9	<i>n</i> =9	<i>n</i> =15	<i>n</i> =11	<i>n</i> =10	<i>n</i> =12
Macrophages	5.2(0.7)	3.6(0.6)	8.8(1.2)	13.5(3.7)*	7.0(0.9)	16.9(5.9)*
Neutrophils	0.01(0.004)	0.13(0.09)	1.5(0.7)*	8.1(3.5)*	1.3(0.6)	15.1(3.9)●*
Lymphocytes	0.1(0.02)	0.04(0.01)●	7.3(1.7)*	6.6(0.9)*	3.4(0.6)*	12.6(3.1)●*
Eosinophils	0.01(0.005)	0.1(0.03)●	68.5(14.4)*	220.0(63.6)●*	46.2(8.9)*	322.8(49.0)●*
Total cells	5.3(0.7)	3.9(0.6)	87.0(16.4)*	247.7(69.2)●*	58.0(10.3)*	367.5(58.6)●*
BALf IL-5 (pg/ml)	10.5(0.7)	18.1(4.3)●	11.7(2.8)	144.3(18.9)●*	47.9(31.1)	182.6(32.8)●*
Blood (x10 ⁶ /ml)	<i>n</i> =9	<i>n</i> =6	<i>n</i> =15	<i>n</i> =10	<i>n</i> =5	<i>n</i> =5
Monocytes	0.2(0.03)	2.3(0.6)●	0.4(0.1)	1.4(0.5)●	0.2(0.04)	1.5(0.3)●
Neutrophils	0.2(0.04)	2.9(0.6)●	0.7(0.1)*	1.1(0.2)*	0.7(0.1)*	1.6(0.4)●
Lymphocytes	1.9(0.2)	10.5(2.4)●	2.8(0.4)	5.8(1.3)●	3.2(0.3)*	10.8(2.3)●
Eosinophils	0.02(0.006)	3.2(1.0)●	0.2(0.03)*	3.5(0.8)●	0.1(0.03)*	5.6(0.8)●
Total WBC	2.4(0.3)	19.4(4.1)●	4.0(0.6)	12.0(2.4)●	4.3(0.4)*	19.8(3.9)●
Serum IL-5 (pg/ml)	9.4(0.4)	451.2(61.0)●	12.1(3.0)	475.5(109.1)●	27.3(12.9)*	499.9(79.2)●

^{a)} OVA-sensitized C57BL/6 and NJ.1638 mice were exposed to either PBS, 10 or 100 µg of OVA on 5 consecutive days. Samples were collected 24 h after the last exposure. Data are shown as mean (SEM). ● $p < 0.05$ between C57BL/6 and NJ.1638 mice within the same exposure-group, * $p < 0.05$ as compared to PBS exposure, ○ $p < 0.05$ between 10 and 100 µg of OVA exposure (Mann-Whitney U-test)

2.3 Newly produced (BrdU⁺) cells

A typical example of the ICC staining for nuclear BrdU together with eosin staining in BALf cytopsin is illustrated in Fig. 1C.

In wild-type mice, allergen exposure increased the number of newly produced (BrdU⁺) BALf eosinophils, neutrophils and lymphocytes ($p < 0.005$; Fig. 2A–C) as well as BrdU⁺ BM eosinophils ($p < 0.05$; Fig. 2G) compared to PBS exposure. However, allergen exposure did

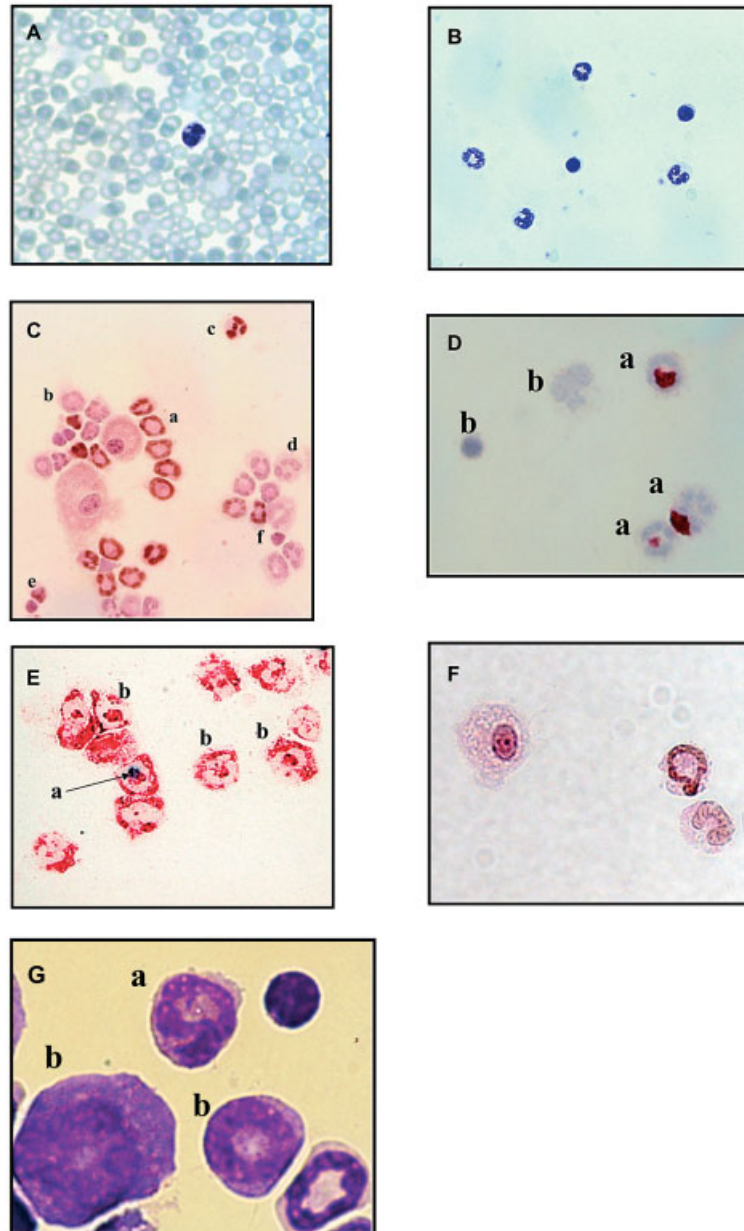


Fig. 1. Photomicrographs of preparations stained with May-Grünwald Giemsa for cell differential counts (A, B, G) or ICC staining (C–F). (A) Blood from the traditional smear preparation and (B) the cytopsin method. (C) ICC staining for nuclear BrdU together with eosin staining on BALf cytopsin. Brown nuclear staining indicates positive staining for BrdU while pink cytoplasmic staining illustrates eosinophilic granulation. (a) shows a newly produced (BrdU⁺) eosinophil, (b) illustrates a non-newly produced (BrdU[−]) eosinophil, (c) BrdU⁺ neutrophil, (d) BrdU[−] neutrophil, (e) BrdU⁺ lymphocyte and (f) BrdU[−] lymphocyte. (D) ICC staining for CD34 antigen in BALf cells. (a) illustrates CD34⁺ cell and (b) CD34[−] cell. (E) ICC staining of CD34 antigen (a) and MBP (b) in BALf cells. (F) A BrdU⁺ eosinophil in BALf taken 43 h after adoptive transfer of BrdU⁺ bone marrow (BM) cells. (G) Mature (a) and immature (b) BM eosinophils.

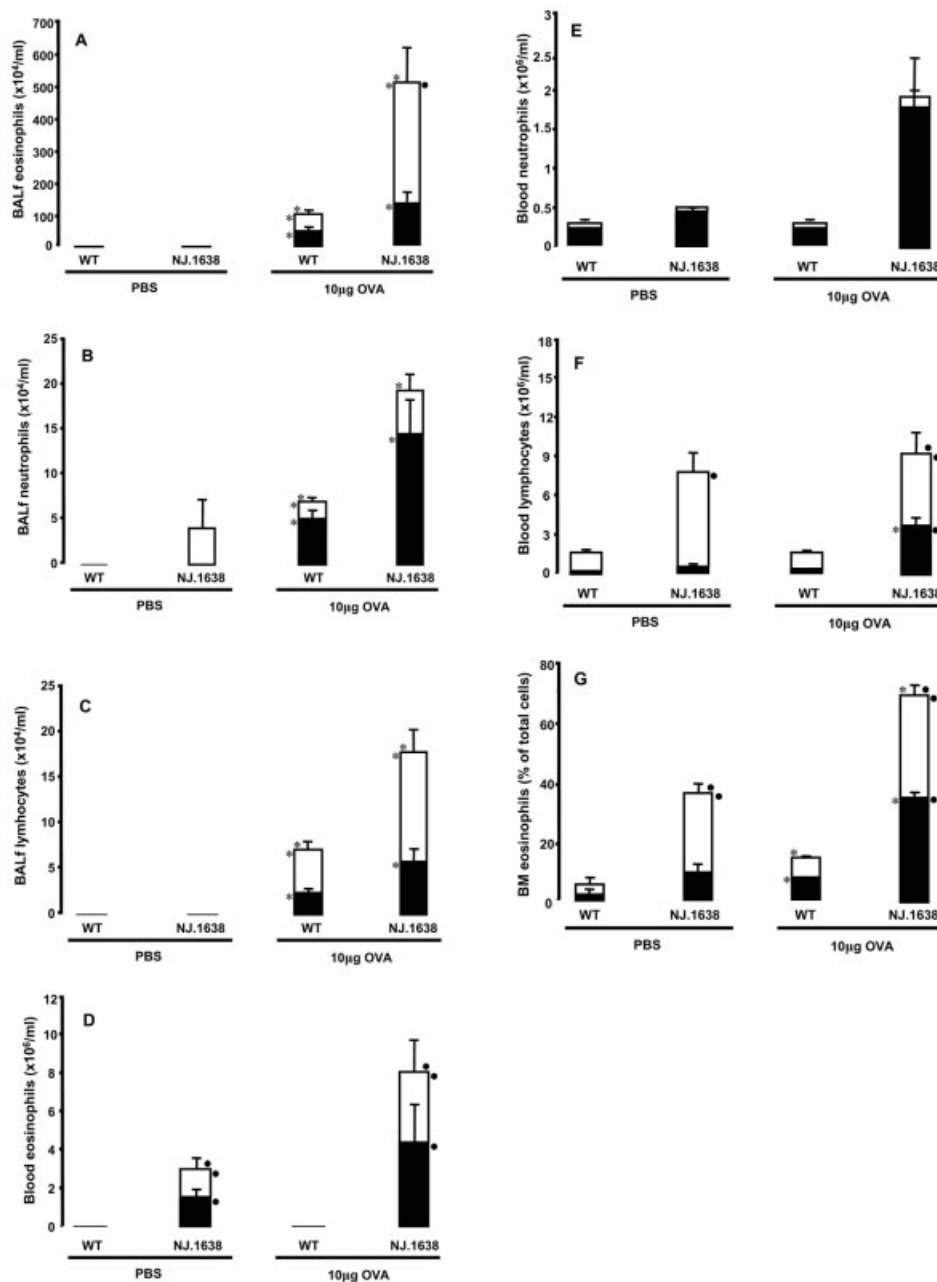


Fig. 2. Number of BALf and blood eosinophils, neutrophils and lymphocytes as well as bone marrow (BM) eosinophils. Number of BrdU⁺ (filled column) and BrdU⁻ (open column) eosinophils, neutrophils and lymphocytes in BALf (A–C), blood (D–F) and BM eosinophils (G) ($n=6-15$) in OVA-sensitized and exposed wild-type (WT, C57BL/6) and IL-5-transgenic mice (NJ.1638). The mice were exposed to 10 µg of OVA or vehicle (PBS) on 5 consecutive days. Data are shown as mean $\pm$ SEM. ● $p < 0.05$ between WT and NJ.1638 for the same exposure, * $p < 0.05$ as compared to vehicle (Mann-Whitney U test).

not alter the number of BrdU⁺ blood eosinophils, neutrophils or lymphocytes (Fig. 2D–F).

In NJ.1638 mice, allergen exposure increased the number of BrdU⁺ eosinophils, neutrophils and lymphocytes in BALf ($p < 0.005$; Fig. 2A–C) as well as BrdU⁺ BM eosinophils ($p < 0.05$; Fig. 2G). In addition, allergen

exposure increased the number of BrdU⁺ lymphocytes, but not eosinophils and neutrophils in the blood of NJ.1638 mice ($p < 0.05$; Fig. 2D–F).

After 5 days of allergen exposure, NJ.1638 mice had significantly higher number of BrdU⁺ eosinophils in blood and BM ($p < 0.0005$; Fig. 2D and G) but not in BALf

(Fig. 2A) as well as a greater number of blood lymphocytes (Fig. 2F) when compared to wild-type mice. After prolonged allergen exposure, NJ.1638 mice also had a significantly higher number of BrdU⁺ eosinophils also in BALf (184 ± 48 and $17 \pm 15 \times 10^4$, $p < 0.03$) as well as in blood and BM (data not shown).

2.4 CD34⁺ cells

A typical example of ICC staining for CD34 antigen in BALf cytopsin is illustrated in Fig. 1D.

In wild-type mice, allergen exposure (10 or 100 μ g) induced an increase in CD34⁺ cells in BALf and blood compared to PBS exposure ($p < 0.02$; Fig. 3A, B). Allergen

exposure did not affect the number of BM CD34⁺ cells (Fig. 3C).

In NJ.1638 mice, allergen exposure induced a dose-dependent increase of BALf CD34⁺ cells ($p < 0.01$; Fig. 3A). Allergen exposure did not alter the number of blood or BM CD34⁺ cells (Fig. 3B, C).

NJ.1638 mice had, irrespective of exposure, a higher number of CD34⁺ cells in blood and BM compared to wild-type mice ($p < 0.03$). Furthermore, these mice had a higher number of BALf CD34⁺ cells after the higher dose of allergen exposure ($p < 0.01$).

2.5 CD34⁺/MBP⁺ cells

CD34 antigen and major basic protein (MBP) in BALf cells were detected in order to identify CD34⁺ cells committed to the eosinophilic lineage [11].

A typical example of ICC staining for CD34 antigen and MBP in BALf cytopsin is illustrated in Fig. 1E.

BALf CD34⁺ cells in both wild-type and NJ.1638 mice were MBP⁺ (Fig. 4). Few MBP⁺CD34⁺ cells were found in one allergen exposed wild-type mouse.

2.6 Adoptive transfer of bone marrow cells

The question whether BM eosinophils, neutrophils and lymphocytes have the capacity to traffic into the airways following airway allergen exposure was evaluated using an adoptive transfer model of BM cells.

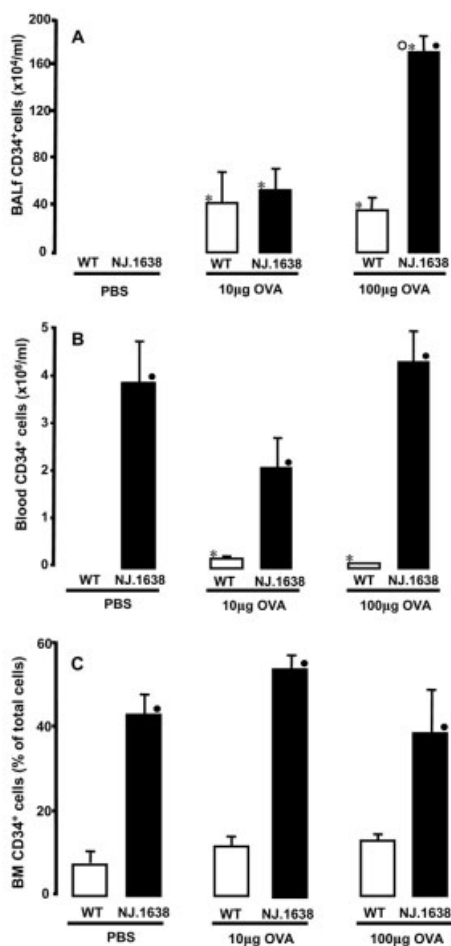


Fig. 3. Number of CD34⁺ cells in BALf (A), blood (B) and bone marrow (BM)(C). OVA-sensitized WT (C57BL/6) and IL-5-transgenic mice (NJ.1638) ($n=4-6$) were exposed to either 10 or 100 μ g of OVA or vehicle on 5 consecutive days. Data are shown as mean $\pm$ SEM. * $p < 0.05$ between WT and NJ.1638 mice for the same exposure, * $p < 0.05$ as compared to vehicle, $\circ p < 0.05$ between 10 and 100 μ g OVA exposure (Mann-Whitney U test).

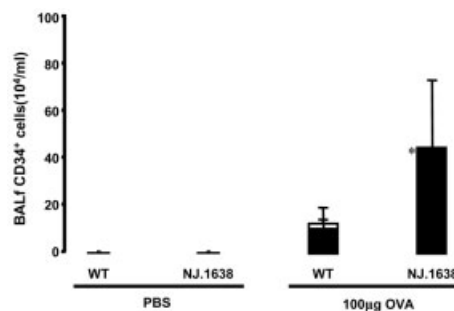


Fig. 4. Number of CD34⁺ cells in BALf in OVA-sensitized WT and IL-5-transgenic mice (NJ.1638) ($n=4-5$). Filled column shows CD34⁺ cells expressing MBP and open column CD34⁺ cells not expressing MBP. The animals were exposed to 100 μ g of OVA or vehicle on 5 consecutive days. Data are shown as mean $\pm$ SEM. * $p < 0.05$ as compared to vehicle (Mann-Whitney U test).

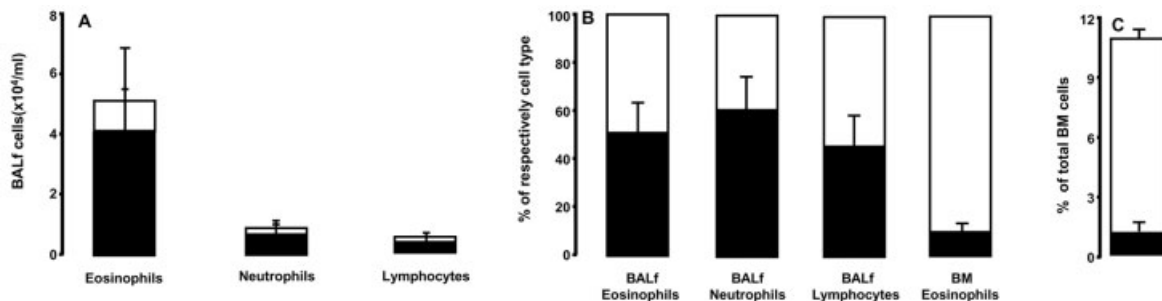


Fig. 5. Absolute (A) and relative number (B) BALF eosinophils, neutrophils and lymphocytes in recipient mice ($n=6$). The filled column shows BrdU⁺ cells and open column BrdU⁻ cells. Relative number of eosinophils in BM of recipient mice (C).

After adoptive transfer of 10^7 BM cells (60% of these BM cells were BrdU⁺), BrdU⁺ eosinophils, neutrophils and lymphocytes were detected in BALF after allergen challenge in five out of six recipient mice (Fig. 1F, 5A). In BALF of recipient mice $54(\pm 13)\%$ of the eosinophils were BrdU⁺, $64.0(\pm 15.2)\%$ of the neutrophils were BrdU⁺ and $45.7(\pm 14.3)\%$ of the lymphocytes were BrdU⁺ (Fig. 5B). BrdU⁺ eosinophils were also found in BM (Fig. 5C) and blood in all recipient mice.

2.7 Adoptive transfer of CD3⁺ lymphocytes to C57BL/6 mice

The role of CD3⁺ lymphocytes in BM inflammatory response was evaluated using an adoptive transfer model of CD3⁺ lymphocytes.

A typical example of May-Grünwald Giemsa staining is illustrated in Fig. 1G.

Adoptive transfer of 10^7 CD3⁺ lymphocytes from sensitized wild-type mice did not affect the BM eosinophil numbers in sensitized and allergen-exposed recipient mice (Fig. 6A). However, transfer of 10^7 CD3⁺ splenocytes from NJ.1638 mice (CD3^{IL-5}) increased the relative number of total and mature BM eosinophils when compared to the transfer of CD3 lymphocytes from wild-type mice (CD3^{WT}) or an injection of PBS ($p<0.03$, Fig. 6A). Transfer of either CD3 or CD3^{IL-5} significantly increased the levels of IL-5 in serum in the recipient mice when compared to injection of PBS ($p<0.02$; Fig. 6B).

2.8 Bone marrow eosinophils in SCID-beige mice

Naive C.B-17/Gbms Tac-SCID-beige (bg) mice have reduced number of BM eosinophils compared to naive C57BL/6 wild-type mice ($2.0\pm 0.4\%$ vs. $5.4\pm 1.2\%$, $p=0.01$; Fig. 7).

3 Discussion

We have shown that allergen exposure increased the number of newly produced (BrdU⁺) eosinophils, neutrophils, lymphocytes and CD34⁺ cells in BALF as well as newly produced eosinophils in BM. The presence of

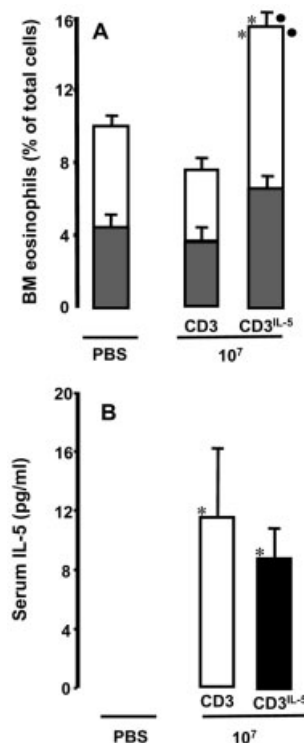


Fig. 6. (A) Relative number of BM eosinophils and (B) serum IL-5 concentration in OVA-sensitized and exposed WT (C57BL/6) mice after adoptive transfer of 10^7 CD3⁺ splenocytes from OVA-sensitized and PBS-exposed WT (CD3^{WT}) or NJ.1638 (CD3^{IL-5}) mice ($n=4-6$). Filled column shows immature and open column mature eosinophils. Data are shown as mean $\pm$ SEM. $\bullet p<0.05$ compared to adoptive transfer of CD3^{WT}, $\ast p<0.05$ compared to PBS injection (Mann-Whitney U test).

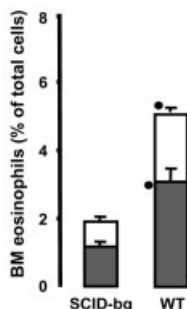


Fig. 7. Relative number of bone marrow (BM) eosinophils in naive WT (C57BL/6) and SCID-bg mice at baseline ($n=6$). The filled column shows immature and open column mature eosinophils. Data are shown as mean $\pm$ SEM. $\bullet p < 0.05$ as compared to naive SCID-bg mice (Mann-Whitney U test).

BrdU⁺ eosinophils in BALf, blood and BM is enhanced in mice overexpressing IL-5 in CD3⁺ lymphocytes, compared to wild-type mice. Furthermore, IL-5 overexpression increased the number of CD34⁺ cells in BALf, blood and BM. Adoptive transfer of IL-5 overexpressing CD3⁺ lymphocytes increased the number of BM eosinophils in wild-type mice exposed to allergen. Most importantly, we have shown that adoptively transferred BM eosinophils, neutrophils and lymphocytes traffic into the airways in response to an airway allergen exposure.

It has previously been shown that a majority of granulocytes found in BALf after airway allergen exposure are newly produced [19]. In this study, allergen exposure was found to increase the number of BrdU⁺ eosinophils as well as BrdU⁺ neutrophils and lymphocytes in BALf, showing that allergen exposure induces the production of inflammatory cells in the airways.

IL-5 overexpression in CD3⁺ lymphocytes (NJ.1638 mice) increased the allergen-induced airway, blood, and BM eosinophilia, confirming a previous study showing that mice overexpressing IL-5 generally or in tissues such as the airway epithelium have increased eosinophilia when compared to wild-type mice [22–24]. We found that IL-5 overexpression significantly increased the presence of BrdU⁺ eosinophils and CD34⁺ cells in airways, blood, and BM. The accumulation of BrdU⁺ eosinophils in the airways was most obvious when the allergen exposure was prolonged to 6 days (BALf taken 8 days after the first allergen exposure). After 5 days of allergen exposure, a higher number of BrdU⁺ eosinophils was present in BALf in the IL-5-transgenic mice compared to wild-type mice. This can be explained by the fact that IL-5-transgenic mice have a larger pool of eosinophils within the BM and blood prior to the initiation of allergen exposure, and that these previously produced cells (BrdU⁺) are recruited into the airways immediately after allergen exposure. Most

importantly, IL-5 overexpression was also associated with an increase in the number of CD34⁺ cells in all compartments, which can probably be explained by CD34⁺ cells trafficking to airways from the BM via the blood. Most likely, this increase in CD34⁺ cells *in vivo* is closely related to the enhanced eosinophilopoiesis induced by IL-5, in that IL-5 stimulates CD34⁺ cells to commit to the eosinophil lineage. The CD34 antigen is considered as a stage-, rather than a lineage-specific, leukocyte differentiation antigen and has been shown to be expressed at highest density on earlier progenitors, with the expression slowing down as the cells mature. The CD34 antigen is lost in terminally differentiated cells [25]. The presence of CD34⁺ cells in the BALf in the present experiment can be explained by the fact that the CD34 antigen had not been fully shed from recently matured eosinophils. It is important to note that almost all CD34⁺ cells in BALf express the eosinophil granule MBP, showing commitment to the eosinophilic lineage [11]. In IL-5-transgenic mice, all CD34⁺ cells found stained positive for MBP. In wild-type mice, CD34⁺ cells without MBP-staining were found in one allergen-exposed mouse. The increased number of CD34⁺/MBP⁺ cells in the BALf after allergen exposure in IL-5-transgenic mice suggests enhancement of eosinophilopoiesis, which is further supported by the increase of BrdU⁺ eosinophils.

Adoptive transfer of BrdU⁺ BM cells in this study shows, for the first time, that not only BM eosinophils but also neutrophils and lymphocytes have the capacity to traffic from blood into airways in response to allergen exposure. Approximately 20% of the total cells found in the BALf of the recipient mice were eosinophils originating from the BM of the donor mice, which amounted to 54% of all BALf eosinophils. Furthermore, approximately 1% of the BM eosinophils were BrdU⁺. These data, therefore, show that BM eosinophils have the capacity to contribute extensively to allergen-induced airway inflammation. There was only a slight increase in neutrophils and lymphocytes in the airways in this model of airway allergic inflammation, where eosinophils dominated. Our finding that BM-derived neutrophils and lymphocytes reach the airways is of great interest, since both of these cell types have been implicated in asthma and allergic inflammation [4, 5, 26–29].

The finding of a high number of CD34⁺ cells in BALf after exposure to allergen, which was further increased by IL-5 overexpression, could imply that these cells have the capacity to mature and perhaps proliferate within the airways. In fact, we have recently shown that BALf-derived CD34⁺ cells in this model have the capacity to produce eosinophil colonies when cultured *in vitro*, and it was estimated that 10% of airway eosinophils may mature locally in the airways [15]. Experiments using human tissue have shown that nasal explants stimulated with allergen or

IL-5 have reduced tissue CD34 expression as well as increased expression of MBP, suggesting that eosinophil maturation may occur in local tissue [11]. It is also known that allergic patients have an increased number of CD34⁺ cells expressing IL-5R α in both the airways and the BM, and it is therefore likely that these cells can differentiate into eosinophils in the BM before progressing to the airways [10, 11, 30]. Together with our present study, the evidence therefore argues that, while maturation of eosinophils occurs both in BM as well as in airways, the majority of airway eosinophils have matured in the BM.

One possible signaling mechanism between the airways and the BM could be the traffic of activated and IL-5-producing T lymphocytes from airways to BM after exposure to allergen [31]. In the present study we found that adoptive transfer of sensitized CD3^{IL-5} lymphocytes increased BM eosinophilia after exposure to allergen compared to transfer of CD3^{WT} lymphocytes. Importantly, IL-5 in serum was increased in recipients of both CD3^{IL-5} and CD3^{WT} lymphocytes as compared to PBS injection, although there was no difference in the IL-5 concentration between the recipient groups. This suggests that the level of IL-5 in serum 7 days after the adoptive transfer is not directly related to the enhanced BM eosinophilic response to allergen exposure, although the concentration in the BM microenvironment may differ. It is also possible that CD3⁺ lymphocytes may release other cytokines in the BM microenvironment, thereby enhancing the BM eosinophilopoiesis. The role of T lymphocytes in eosinophilopoiesis is further confirmed by our data, which reveal that SCID-bg mice, which are known to have greatly reduced numbers of CD3⁺ lymphocytes [32], also have fewer eosinophils in the BM (approximately 1%) compared to wild-type mice (approximately 5% in C57BL/6) at baseline. It should also be noted that T cell-deficient mice (nude mice) respond with a reduced increase in BM eosinophilia after parasite infestation [33]. However, CD3 lymphocytes may not be absolutely required, since some BM eosinophilia can be induced in the nude mice [33], and some eosinophils are found in the SCID-bg mice.

This study shows that allergen exposure leads to an increase in the number of newly produced eosinophils, neutrophils and lymphocytes in the airways, and we have demonstrated by means of adoptive transfer of BM cells that these cells can traffic from blood to the airways. CD3⁺ lymphocytes probably regulate BM eosinophilopoiesis *in vivo*, both because adoptive transfer of CD3⁺ lymphocytes increases the number of BM eosinophils, and mice with few CD3⁺ lymphocytes (SCID-bg mice) have few BM eosinophils. Overexpression of IL-5 in CD3⁺ lymphocytes enhances the allergen induced BM eosinophilopoiesis, the recruitment of BrdU⁺ eosinophils to the airways,

and the number of CD34⁺ cells in both BM and airways committed to the eosinophilic lineage. Taken together, in line with our previous results [15], this study argues that a majority of inflammatory cells traffic from BM to airways after exposure to allergen, although local maturation of eosinophils may also contribute to some extent.

4 Materials and methods

4.1 Animals

This study was approved by the Animal Ethics Committee at Göteborg University, Sweden. C57BL/6 mice were purchased from Banting-Kingman Universal AB (Sollentuna, Sweden). NJ.1638 male mice were obtained from Dr. James J. Lee (Mayo Clinic, Scottsdale, AZ) and maintained in a heterozygous fashion by back-crossing to C57BL/6 (Møllegaard-Bommice A/S Ry, Denmark). Smear preparations were made from blood obtained from the tail of the offspring. The preparations were stained with May-Grünwald-Giemsa and the relative number of eosinophils was counted. Mice with more than 25% eosinophils in their blood were considered to have incorporated the IL-5 transgene. C.B-17/Gbms Tac-SCID-bg male mice were purchased from Møllegaard-Bommice. NJ.1638 and C.B-17/Gbms Tac-SCID-bg mice were housed in special pathogen-free animal facilities. All mice were 5–8 weeks old and were provided with food and water *ad libitum*.

4.2 Effect of allergen sensitization and exposure

4.2.1 Allergen sensitization and exposure protocol

Wild-type (C57BL/6) and IL-5-transgenic mice (NJ.1638) were sensitized twice, 5 days apart, by intraperitoneal (i.p.) injections of 0.5 ml aluminum-precipitated antigen containing 8 μ g of ovalbumin (OVA, Sigma-Aldrich Sweden AB, St Louis, MO) bound to 4 mg of aluminum hydroxide (Sigma-Aldrich) in phosphate-buffered saline (PBS). Eight days after the second sensitization, the animals were briefly anesthetized using CO₂, and exposed intranasally (i.n.) to OVA (10 or 100 μ g in 50 μ l of PBS) or to PBS only. This procedure was performed on 5 consecutive days.

4.2.2 In vivo protocol for evaluation of newly produced (BrdU⁺) cells

5-Bromo-2'-deoxyuridine (BrdU, Roche Diagnostics GmbH, Mannheim, Germany) is a thymidine analogue, which is incorporated into the DNA during mitosis [34]. NJ.1638 and wild-type mice (PBS and 10 μ g OVA exposure groups) received an intravenous (i.v.) injection of 1 mg BrdU in 0.25 ml PBS on days 1 and 3 of exposure, at the same time as allergen exposure and again 6 h later.

4.2.3 Prolonged allergen exposure protocol

NJ.1638 and wild-type mice were sensitized exposed and received BrdU as described above. After the fifth exposure, the mice had 2 days of rest before the sixth exposure to allergen.

4.3 Sample processing

4.3.1 Sample collection

All samples were collected 24 h after the final exposure. The animals were euthanized with a mixture of xylazine (130 mg/kg, Rompun®, Bayer AB, Sweden) and ketamine (670 mg/kg, Ketalar®, Pfizer AB, Täby, Sweden). First, blood was obtained by puncturing of the right ventricle. Secondly, bronchoalveolar lavage (BAL) was performed through the tracheal cannula by an initial instillation of 0.25 and thereafter 0.20 ml of PBS. Finally, BM was harvested. One femur was excised, cut at the epiphyses and flushed with 2 ml of PBS.

4.3.2 Blood

Two hundred microliters of blood was mixed with 800 µl of 2 mM EDTA (Sigma-Aldrich) in PBS, and red blood cells (RBC) were lysed in 0.1% potassium bicarbonate and 0.83% ammonium chloride for 15 min at 4°C. White blood cells (WBC) were resuspended in PBS containing 0.03% BSA (Sigma-Aldrich). In order to measure IL-5 in serum (murine IL-5 ELISA kit, R&D Systems, Inc, Abingdon, UK) the remaining volume of blood was centrifuged at 3,000 rpm for 10 min at 4°C.

4.3.3 Bronchoalveolar lavage fluid and bone marrow

BALF and BM samples were centrifuged at 1,000 rpm for 10 min at 4°C. Supernatant was aspirated and the cells were resuspended with 0.03% BSA in PBS. BALF supernatant was later used for IL-5 measurement.

The total cell numbers in blood, BALF, and BM were determined using standard hematological procedures. Cytospins of blood (Fig. 1B, compared with blood smear, Fig. 1A), BALF, and BM were prepared and stained with May-Grünwald-Giemsa for differential cell counts. Cell differentiation was determined by counting 300–500 cells using a light microscope (Zeiss Axioplan 2, Carl Zeiss, Jena, Germany). The cells were identified using standard morphological criteria, and BM mature and immature eosinophils were determined by nuclear morphology, cell size and cytoplasmic granulation [22].

4.4 Immunocytochemistry (ICC)

Cytospin preparations were fixed in 2% paraformaldehyde for 10 min. All incubations were performed at room temperature unless otherwise stated. The preparations were

evaluated blinded and 400–500 cells were counted under a light microscope.

4.5 Newly produced (BrdU⁺) cells

Cytospin preparations of blood, BALF, and BM were subjected to digestion in 0.1% trypsin and 0.1% CaCl₂ in PBS at 37°C for 15 min, incubated in 4 M HCl for 15 min to denature DNA followed by neutralization in Holmes Borate-Borax buffer (1.24% H₃BO₃ in distilled H₂O, pH 8.5) for 10 min. Peroxidase blocking was performed for 1 h (DAKO Corporation, Glostrup, Denmark). Nonspecific binding was blocked with normal rabbit serum (DAKO). The samples were incubated with a rat anti-BrdU monoclonal antibody (mAb) (Clone BU1/75, no. MAS 250p, Harlan-Sera Lab Limited, Loughborough, UK) or isotype control (rat Ig2a, Clone R35-95, BD Biosciences Europe, Erembodegem, Belgium) followed by incubation with rabbit F(ab')₂ anti-rat Ig-horse-radish peroxidase (S6130-05, Southern Biotechnology Associates, Birmingham, AL) and normal mouse serum (DAKO). Bound antibodies were visualized by 3,3'-diaminobenzidine-chromogen system (DAKO). The preparations were stained with eosin followed by counterstaining with Mayer's hematoxylin (DAKO). Cells with nuclear brown staining together with red cytoplasmic staining were counted as BrdU⁺ eosinophils. BrdU⁺ neutrophils, lymphocytes and macrophages could also be detected. BrdU⁺ slides were also stained. No false positive staining was detected.

4.6 CD34⁺ cells

Endogenous biotin was blocked with DAKO Biotin Blocking system in cytospin preparations of BALF, blood, and BM. The preparations were incubated with a biotinylated rat anti-mouse CD34 mAb (clone RAM34, BD Biosciences) or isotype control (biotinylated Rat IgG2a, BD Biosciences) followed by incubation with streptavidin alkaline-phosphatase (DAKO). Bound antibodies were visualized by Vector Red alkaline phosphatase substrate kit (Vector Laboratories Inc. Burlingame, CA) and Mayer's hematoxylin (DAKO) was used for counterstaining.

4.7 CD34⁺ and MBP⁺ cells

Detection of CD34 antigen and MBP in BALF cells was performed in two steps. After incubation with the rat anti-mouse CD34 mAb, the preparations were incubated with streptavidin-β-galactosidase (Roche). Bound antibodies were visualized with β-Gal staining set (Roche). For MBP detection, saponin (Sigma-Aldrich) was added to all antibody dilutions. After blocking endogenous biotin and unspecific staining with swine serum (DAKO), we stained for MBP using a rabbit anti-mouse MBP polyclonal antibody (a gift from Dr. James J. Lee, Mayo Clinic, Scottsdale, AZ) at 37°C. The preparations were then incubated with a biotinylated F(ab')₂

swine anti-rabbit IgG antibody (DAKO) and mouse serum (DAKO), followed by incubation with streptavidin alkaline-phosphatase (DAKO). Bound antibodies were visualized by Vector Red alkaline phosphatase substrate kit (Vector Laboratories Inc).

4.8 Adoptive transfer of bone marrow cells

BM, containing approximately 60% BrdU⁺ cells from OVA-sensitized and -exposed NJ.1638 mice, was injected i.v. in a dose of 10⁷ cells in 0.35 ml of PBS into sensitized NJ.1638 littermates, 8 days after their second sensitization. The recipient mice were challenged i.n. to 10 µg OVA 22 h after the adoptive transfer. Samples of BALf, blood, and BM from the recipient mice were taken 21 h after allergen exposure.

For detection of BrdU⁺ cells in the recipient mice the mAb BrdU antibody was biotinylated and extravidin-horseradish peroxidase (DAKO) was used as a second step. All other steps in the protocol were the same as those described for immunodetection of BrdU⁺ cells.

4.9 Adoptive transfer of CD3 lymphocytes to C57BL/6 mice

Donor mice, NJ.1638 and parallel wild-type (C57BL/6) were OVA-sensitized and PBS-exposed as described above. Recipient wild-type mice were OVA-sensitized and exposed to 100 µg of OVA. Two days before the first exposure, the recipient mice received i.p. injection of CD3 lymphocytes from either wild-type (CD3^{WT}) or NJ.1638 mice (CD3^{IL-5}) obtained from donor mice in 10⁷ cells/0.5 ml of PBS. The dose of CD3 lymphocytes proved to be the most suitable in a preliminary study (data not shown). Mice injected with PBS only served as controls.

4.10 Preparation of CD3⁺ lymphocytes

Spleens were collected from the donor mice 24 h after the final exposure. Shortly, the spleens were homogenized and RBC were lysed. Spleen cells were labeled with hamster-anti-mouse biotinylated mAb directed to CD3 lymphocytes (CD3ε clone145-2C11, BD Biosciences). After washing, streptavidin magnetic microbeads (MACS, Miltenyi Biotec GmbH, Germany) were added, in accordance with the manufacturer's instructions. CD3⁺ lymphocytes were enriched over a magnetic field, diluted in PBS and injected i.p. into recipient mice.

The purity of the enriched CD3⁺ lymphocyte fractions was analyzed by FACS and was shown to be approximately 80% in both fractions.

When using a positive selection of CD3⁺ lymphocytes it is possible that the antibody *per se* can activate the cells. The release of IL-5 from both positive (described above) and

negative selection (by using MACS Pan T cell isolating kit according to the manufacturer's instructions, Miltenyi Biotec GmbH) of CD3^{IL-5} and CD3^{WT} lymphocytes was evaluated *in vitro*. Positive selection of CD3 lymphocytes did not affect the release of IL-5 compared to negative selection (data not shown).

4.11 Bone marrow eosinophils in SCID-bg mice

Bone marrow samples were taken from naive C.B-17/Gbms Tac-SCID-bg and were prepared as previously described.

4.12 Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was carried out using nonparametric analysis of variance (Kruskal-Wallis test) to evaluate the difference between more than two groups. If a significant variance was found, the Mann-Whitney U-test was performed. *p* < 0.05 was considered statistically significant.

Acknowledgements: Funding sources: The Swedish Heart and Lung Foundation, the Swedish Research Council and the Vårdal Foundation. Prof. Jan Lötvall received financial assistance from the Krefling Foundation against Asthma Allergy.

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