

Original article

Regulation of allergen-induced bone marrow eosinophilopoiesis: role of CD4⁺ and CD8⁺ T cells

Background: The mechanisms of the distant stimulation of the bone marrow (BM) after airway allergen exposure remain largely obscure. T cells have been implicated in allergic airway inflammation but their role in allergen-induced BM eosinophilopoiesis is poorly understood. The aim of this study was to determine the role of CD4⁺ and CD8⁺ T cells in allergen-induced BM eosinophilopoiesis.

Methods: Ovalbumin (OVA)-sensitized wild type (WT), CD4 knockout (CD4^{-/-}) and CD8 knockout (CD8^{-/-}) mice were exposed intranasally to OVA or saline. Bromo-deoxyuridine (BrdU) was used to label newly produced cells. Bone marrow, blood and bronchoalveolar lavage (BAL) were sampled 24 h after the final exposure. Immunostaining for newly produced eosinophils (i.e. BrdU⁺/MBP⁺) and BM eosinophil progenitor [CD34⁺/CD45⁺/interleukin-5 (IL-5)Ralph⁺] cells was performed.

Results: The number of newly produced BM eosinophils (BrdU⁺/MBP⁺ cells) was significantly reduced in allergen exposed CD4^{-/-} or CD8^{-/-} mice compared with allergen exposed WT mice, which was followed by a subsequent decrease in newly produced blood and airway eosinophils. Furthermore, BM eosinophil progenitors were significantly reduced in allergen exposed CD4^{-/-} and CD8^{-/-} mice compared with WT mice. Finally, serum IL-5 and Bronchoalveolar lavage fluid eotaxin-2 levels were abolished in allergen exposed CD4^{-/-} mice to levels seen in saline exposed WT mice.

Conclusions: These data suggests that both CD4⁺ and CD8⁺ T cells have a regulatory role in allergen-induced BM eosinophilopoiesis, whereas CD4⁺ T cells are obligatory for allergen-induced airway eosinophilia. The subsequent traffic of eosinophils to the airways is likely to be at least partly regulated by a CD4⁺ T-cell-dependent local airway eotaxin-2 production.

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Allergic inflammation is associated with the enhanced infiltration of eosinophils in affected tissues. There is considerable evidence in animals and man that the bone marrow (BM) plays an integral role in allergic inflammation (1–4). Increasing evidence shows that BM progenitor cells proliferate and differentiate in response to airway allergen exposure, which in turn leads to persistent increase in number of eosinophils (5–7). However, the detailed mechanisms of the distant stimulation of the BM after airway allergen exposure remain unknown. Although the nature of BM activation after allergen-challenge is unknown, several pathways have been implicated, including stimulation of resident BM cells, release of allergen-induced hematopoietic growth factors and cell trafficking (4, 8–12). It has been hypothesized that airway allergen exposure leads to greater number of cells in BM expressing interleukin-5 (IL-5), which is a key factor involved in the regeneration of eosinophils (7, 13–15). Furthermore, the eosinophilia in a murine model of asthma has recently been suggested to be a

result of two distinct hematopoietic regulatory mechanisms (16). The increased number of eosinophil progenitors found within the BM in response to airway allergen exposure has been shown to be T-cell-independent, as T-cell-deficient mice responded to airway allergen exposure with increased numbers of eosinophil progenitors. However, the increased number of BM eosinophil progenitors in these allergen sensitized T-cell-deficient mice was not associated with terminal differentiation of these progenitors to eosinophils. Furthermore, the role of different T-cell subpopulations in allergen-induced BM responses has not been previously determined.

The aim of the present study was to determine the role of CD4⁺ and CD8⁺ T cells in allergen-induced BM eosinophilopoiesis and the following airway eosinophilia. To do this, we used a mouse model of allergen-induced inflammation. OVA-sensitized wild type (WT) (C57BL/6), CD4^{-/-} (CD4^{-/-}) and CD8-deficient (CD8^{-/-}) mice were exposed intranasally to OVA or phosphate buffer saline (PBS) on five consecutive days. A thymidine analog

[5-bromo-2'-deoxyuridine (BrdU); Roche Diagnostics, GmbH; Mannheim, Germany] was used to label newly produced cells during the exposure period. Bone marrow, blood and bronchoalveolar lavage (BAL) were sampled 24 h after the final exposure. Differential cell counts, immunostaining for newly produced (BrdU⁺) and major basic protein (MBP⁺) eosinophils were performed and BM cells were stained for eosinophil-committed progenitor cells analysed by flow cytometry. Finally, to evaluate migration responses in the knockout strains, co-expression of very late antigen-4 (VLA-4, also known as $\alpha 4 \beta 1$ integrin) and CC chemokine receptor-3 (CCR3) on eosinophil progenitors was assessed and IL-5 and eotaxin-2 levels was evaluated in serum and airways, respectively.

Methods

Mice

Gene knockout mice lacking either CD4⁺ cells (C57BL/6 J CD4^{tm1Knnw}) or CD8⁺ cells (C57BL/6 CD8a^{tm1Mak}) were purchased from Jackson Laboratories (Bar Harbor, ME, USA) and control C57BL/6 mice were purchased from Taconic (Ry, Denmark). All mice were provided with food and water *ad libitum* and housed in specific pathogen free animal facilities. The Ethical Committee for animal studies in Göteborg, Sweden approved this study.

Allergen sensitization and exposure

Mice, 8- to 12-week-old were sensitized twice, 5 days apart by intraperitoneal (i.p.) injection (0.5 ml) of 8 μ g chicken OVA (Sigma-Aldrich, St Louis, MO, USA) bound to 4 mg aluminium hydroxide (Sigma-Aldrich) in PBS. Eight days after the last sensitization the mice were briefly anesthetized using Isoflurane (Apoteksbolaget AB, Stockholm, Sweden) and exposed intranasally to 10 μ g of OVA or PBS. The exposures were performed on five consecutive days.

Labeling of newly produced cells

Bromo-deoxyuridine was used to label newly produced cells and was administered at a dose of 1 mg in 0.25 ml of PBS i.p. injection on two occasions, 6–7 h apart on days 1 and 3 of exposure.

Sample collection and processing

Samples were collected 24 h after the final OVA exposure. The animals were euthanized with a mixture of xylazine (130 mg/kg) and ketamine (670 mg/kg, both from Apoteksbolaget AB). First blood was obtained by puncture of the heart right ventricle. Secondly, BAL was performed through the tracheal cannula by instillation of 0.5 ml PBS twice. Finally, BM cells were harvested by excising one femur that was cut at the epiphysis and flushed with 2 ml PBS.

Blood. Two hundred microliters of blood was mixed with 800 μ l of 2 mM EDTA (Sigma-Aldrich) in PBS, and red blood cells were lysed in 0.1% potassium bicarbonate and 0.83% ammonium chloride for 15 min at room temperature (RT). White blood cells (WBC) were resuspended in PBS containing 0.03% bovine serum albumin (BSA, Sigma-Aldrich). For measurement of cytokines, the

remaining volume of blood was centrifuged at 800 g for 15 min at 4°C and stored at –80°C until further analysis.

Bronchoalveolar lavage fluid and bone marrow. Bronchoalveolar lavage fluid (BALF) and BM samples were centrifuged at 300 g for 10 min at 4°C. The cells were resuspended with 0.03% BSA in PBS. Bronchoalveolar lavage fluid supernatant was collected for chemokine measurement and stored at –80°C until analysis. The total cell numbers in blood, BM, and BALF was determined using standard hematological procedures. Bone marrow, BALF and blood cytopins were prepared and stained according to the May–Grünwald–Giemsa method for differential cell counts. Cell differentiation was determined by counting 300–500 cells using a light microscope (Zeiss Axioplan 2). The cells were identified using standard morphological criteria.

Double immunostaining for nuclear BrdU and MBP eosinophils

Newly produced cells (BrdU⁺) were immunostained together with MBP, an eosinophil granule protein expressed early in eosinophil-lineage committed cells as well as in mature eosinophils (17, 18). Cytospin preparations were fixed in 2% formaldehyde for 10 min and incubated with 10% rabbit serum (DAKO Cytomation, Glostrup, Denmark) to avoid unspecific binding. Cytospin samples were incubated with a monoclonal rat anti-mouse MBP antibody (kind gift from Drs Nancy and James Lee, Mayo Clinic, Scottsdale, AZ, USA) for 1 h followed by a 45 min incubation with alkaline phosphatase-conjugated rabbit F(ab')₂ anti-rat IgG secondary antibody (Southern Biotechnology Associates Inc, Birmingham, AL, USA). Bound antibodies were visualized with Vector Red Alkaline Phosphatase Substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA). Samples were fixed for a second time overnight in 4% paraformaldehyde. The next day, samples were treated with 0.1% trypsin (Sigma-Aldrich) at 37°C for 15 min followed by 4 M HCl for 15 min and Holmes Borate buffer (pH 8.5) for 10 min. Endogenous peroxidase was blocked with glucose oxidase solution (PBS supplemented with 0.0064% sodium azide, 0.18% glucose, 0.1% saponin and 1.55 units of glucose oxidase/ml PBS) (all from Sigma) preheated to 37°C for 30 min. Bromo-deoxyuridine labeled cells were detected using a Fluorescein isothiocyanate (FITC) conjugated rat anti-mouse BrdU monoclonal antibody (clone BU1/75, Harlan-Sera Lab, Loughborough, UK), followed by horse radish peroxidase conjugated Rabbit F(ab')₂ anti FITC secondary antibody (DAKO-Cytomation) and visualized with DAB Substrate-Chromogen System (DAKO Cytomation). Mayer's Hematoxylin (Sigma) was used for counterstaining. Cells were determined by counting 400 cells using a Zeiss Axioplan 2 light microscope.

Flow cytometry analysis of CD34⁺/CD45⁺/IL-5R α ⁺ progenitor cells

Bone marrow cells were collected and pretreated with 2% mouse serum (DAKO) for 15 min to prevent nonspecific binding and thereafter stained with the following antibodies; Phycoerythrin (PE)-labeled anti-IL-5R α (clone 558488, BD Bioscience, Erembodegen, Belgium), Alexa Fluor 647 anti-CD34 (clone RAM 34; eBioscience, San Diego, CA, USA), Allophycocyanin-Cyanine dye 7 (APC-Cy7) labeled anti-CD45 (clone 30-F115, BD Bioscience), FITC labeled anti-CCR3 (clone 83101; R&D systems, Abingdon, UK), and biotinylated rat anti-mouse VLA-4 (clone 9C10, BD Bioscience) followed by Peridinin-chlorophyll-protein (PerCP) conjugated Streptavidin (BD Bioscience), or an appropriate

isotype control antibody in saturated concentrations. The cells were incubated for 30 min at 4°C with antibodies or isotype control, followed by two washing steps. Finally, the samples were fixed in BD Stabilizing Fixative (BD Biosciences, San Jose, CA, USA) and stored at 4°C until flow cytometric analysis on the FACS Aria flow cytometer (BD, Mountain View, CA, USA). Ten thousand cells were computed in list mode and analyzed using FlowJo Software (Tree Star, Inc, Ashland, OR, USA).

Measurement of serum and BALF IL-5 and eotaxin-2 in BALF

Mouse IL-5 levels in serum and BALF were measured using murine IL-5 ELISA kit (R&D Systems). Eotaxin-2 levels in BALF were assessed by murine eotaxin-2 DuoSet ELISA kit (R&D Systems). The detection limit was 3.9 pg/ml for IL-5 and 8 pg/ml for eotaxin-2, respectively.

Statistical analysis

All data are expressed as mean $\pm$ SEM. Statistical analysis was carried out using a nonparametric analysis of variance (Kruskal–Wallis test) to determine the variance among more than two groups. If significant variance was found, an unpaired two-group test (Mann–Whitney *U*-test) was used to determine significant differences between individual groups. $P < 0.05$ was considered statistically significant.

Results

Bone marrow

Allergen exposure induced a significant increase in BM eosinophils in CD4^{−/−}, CD8^{−/−}, and WT mice when compared with saline exposed mice ($9 \pm 1\%$, $14 \pm 1\%$ and $20 \pm 2\%$ vs $5 \pm 1\%$, $6 \pm 1\%$, and $5 \pm 1\%$ of total cells; $P < 0.05$, $P < 0.01$, and $P < 0.01$). In contrast, BM eosinophils were significantly reduced in OVA exposed CD4^{−/−} or CD8^{−/−} mice compared with OVA exposed WT mice ($9 \pm 1\%$ and $14 \pm 1\%$ vs $20 \pm 2\%$ of total cells; $P < 0.05$ and $P < 0.01$ Fig. 1A).

Newly produced eosinophils (BrdU⁺/MBP⁺ cells) in BM after allergen exposure. The number of BrdU⁺/MBP⁺ BM cells in the CD4^{−/−}, CD8^{−/−}, and WT mice increased compared with the sensitized but saline exposed mice ($3.7 \pm 0.5\%$, $3.0 \pm 0.9\%$, and $8.4 \pm 1.5\%$ vs $1.9 \pm 0.5\%$, $1.5 \pm 0.4\%$ and $2.2 \pm 0.3\%$ of total cells; $P < 0.01$ and $P < 0.05$, Fig. 1C). In addition, BrdU⁺/MBP⁺ BM cells decreased in the allergen-challenged CD4^{−/−} and CD8^{−/−} mice compared with WT mice ($3.7 \pm 0.5\%$ and $3.0 \pm 0.9\%$ vs $8.4 \pm 1.5\%$ of total cells; $P < 0.01$, Fig. 1C). Notably, allergen exposed CD4^{−/−} mice did not exhibit a significant increase in BrdU[−]/MBP⁺ cells when compared with the saline exposed CD4^{−/−} mice (Fig. 1D). BrdU[−]/MBP⁺ cells also decreased in the allergen-challenged CD4^{−/−} and CD8^{−/−} mice compared with WT mice ($6.8 \pm 0.6\%$ and $12.5 \pm 0.9\%$ vs $24.1 \pm 2.3\%$ of total cells; $P < 0.01$, Fig. 1D).

CD34⁺/CD45⁺/IL-5Ralpha⁺ cells in BM. The number of CD34⁺/CD45⁺/IL-5Ralpha⁺ BM cells in allergen sensitized and exposed CD4^{−/−} and CD8^{−/−} mice was less than in WT mice (5.2 ± 0.4 and $9.2 \pm 0.9\%$ vs $15.1 \pm 1.4\%$ of total cells; $P < 0.05$, Fig. 2B). Furthermore, the majority ($> 88\%$) of the CD34⁺/CD45⁺/IL-5Ralpha⁺ BM cells in all groups of animal co-expressed VLA-4 (Fig. 2B). A significant decrease was found in the number of BM CD34⁺/CD45⁺/IL-5Ralpha⁺/CCR3⁺ cells in allergen exposed CD4^{−/−} mice compared with WT mice ($1.4 \pm 0.07\%$ vs $3.4 \pm 0.32\%$ of total cells; $P < 0.05$), whereas the CD8^{−/−} mice showed a similar amount of CD34⁺/CD45⁺/IL-5Ralpha⁺/CCR3⁺ BM cells compared with WT mice (Fig. 2B). As might be expected, CD4^{−/−} mice showed a decrease in CCR3 MFI compared with WT mice (1771 ± 196 vs 3537 ± 352 ; $P < 0.05$). Finally, the majority of CD34⁺/CD45⁺/IL-5Ralpha⁺/CCR3⁺ also express VLA-4 (data not shown).

Serum and BALF IL-5 levels

Allergen exposure increased IL-5 in the serum of WT and CD8^{−/−} mice compared with saline challenged mice (Fig. 3). Notably, there were no detectable serum IL-5 in the allergen exposed CD4^{−/−} mice. In contrast, there were no differences in BALF IL-5 between either of the allergen exposed groups of animals. However, allergen exposed CD8^{−/−} mice showed a significant increase in BALF IL-5 compared with saline exposed CD8^{−/−} mice (Table 1).

Blood

Allergen exposure led to an increase in blood eosinophils in CD4^{−/−}, CD8^{−/−}, and WT mice compared with saline exposed mice (0.42 ± 0.06 , 1.05 ± 0.20 , and 1.93 ± 0.28 vs 0.16 ± 0.08 , 0.52 ± 0.12 , and $0.54 \pm 0.17 \times 10^5$ /ml WBC; $P < 0.05$, $P < 0.01$, and $P < 0.01$, Fig. 4A). Furthermore, the number of blood eosinophils was significantly reduced in allergen exposed CD4^{−/−} or CD8^{−/−} mice compared with allergen exposed WT mice (0.42 ± 0.06 and 1.05 ± 0.20 vs $1.93 \pm 0.28 \times 10^5$ /ml WBC; $P < 0.001$ and $P < 0.05$, Fig. 4A).

Newly produced eosinophils (BrdU⁺/MBP⁺ cells) in blood following allergen exposure. Allergen exposure did not increase the number of newly produced blood eosinophils in the CD4^{−/−} and CD8^{−/−} group when compared with saline exposed mice (0.24 ± 0.04 and 0.61 ± 0.09 vs 0.15 ± 0.06 , $0.32 \pm 0.07 \times 10^5$ /ml WBC, Fig. 4B). By contrast, the allergen exposed WT mice exhibited a significant increase in BrdU⁺/MBP⁺ compared with saline exposed mice (1.38 ± 0.15 vs $0.37 \pm 0.11 \times 10^5$ /ml WBC; Fig. 4B; $P < 0.05$). More importantly, BrdU⁺/MBP⁺ blood cells decreased in the allergen exposed CD4^{−/−} and CD8^{−/−} mice compared

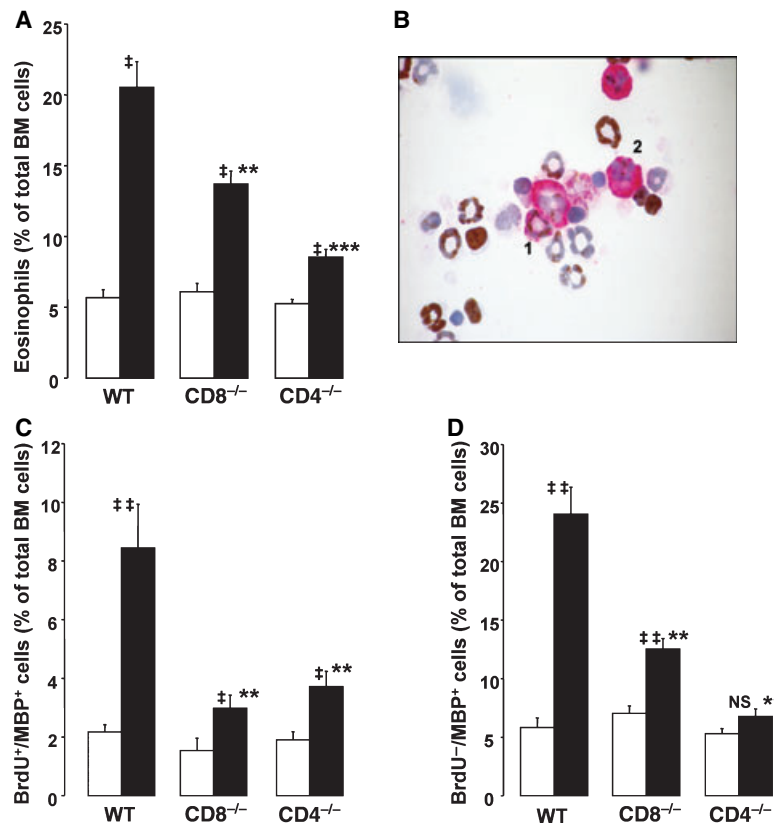


Figure 1. Bone marrow (BM) eosinophils in OVA sensitized/exposed (filled bars) and OVA-sensitized saline exposed (open bars) wild type (WT), CD8^{-/-} and CD4^{-/-} mice (A) May-Grünwald-Giemsa stained eosinophils (C) BrdU⁺/MBP⁺ eosinophils and (D) BrdU⁻/MBP⁺ eosinophils. Data are shown as mean (±SEM) ($n = 5-14$). ‡ $P < 0.05$; ‡‡ $P < 0.01$ increased compared with saline group * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$ decreased compared with WT group; Mann-Whitney U -test. (B) Microscopic image (original magnification: $\times 1000$) of BM cells stained positively for BrdU and MBP. Cells with nuclear brown staining together with red staining in the cytoplasm were counted as BrdU⁺/MBP⁺ eosinophils. (1) BrdU⁺/MBP⁺ eosinophil; (2) BrdU⁻/MBP⁺ eosinophil.

with allergen exposed WT mice (0.24 ± 0.04 and 0.61 ± 0.09 vs $1.38 \pm 0.15 \times 10^5/\text{ml}$ WBC; $P < 0.01$ and $P < 0.05$, Fig. 4B). Allergen exposed CD4^{-/-}, CD8^{-/-}, and WT mice exhibited a significantly increased number of BrdU⁻/MBP⁺ cells compared with PBS exposed mice (0.16 ± 0.03 , 0.74 ± 0.14 , and 1.60 ± 0.15 vs 0.05 ± 0.02 , 0.23 ± 0.11 , and $0.34 \pm 0.20 \times 10^5/\text{ml}$ WBC; Fig. 4C; $P < 0.05$). Furthermore, the BrdU⁻/MBP⁺ cells also showed a decrease in the allergen-challenged CD4^{-/-} and CD8^{-/-} mice compared with WT mice (0.16 ± 0.03 and 0.74 ± 0.14 vs $1.60 \pm 0.15 \times 10^5/\text{ml}$ WBC; $P < 0.01$ and $P < 0.05$, Fig. 4C).

Bronchoalveolar lavage

Allergen exposure induced a significant increase in BAL eosinophils in CD8^{-/-} and WT mice compared with saline exposed mice (32 ± 3 and 58 ± 8 vs 0.15 ± 0.05 and $0.08 \pm 0.03 \times 10^4/\text{ml}$ BAL; $P < 0.01$ and $P < 0.01$, Fig. 5A). However, no increase in BAL eosinophils was observed in the CD4^{-/-} mice when compared with the allergen exposed mice (Fig. 5A). Moreover, BAL eosin-

ophils were significantly reduced in both allergen exposed CD4^{-/-} and CD8^{-/-} mice compared with allergen exposed WT mice (0.10 ± 0.05 and 32 ± 3 vs $58 \pm 8 \times 10^4/\text{ml}$ BAL; $P < 0.005$ and $P < 0.05$, Fig. 5A).

Newly produced eosinophils (BrdU⁺/MBP⁺ cells) in BAL following allergen exposure. The newly produced eosinophils (i.e. BrdU⁺/MBP⁺) in BAL were reduced in the allergen-challenged CD4^{-/-} and CD8^{-/-} mice compared with WT mice (0.07 ± 0.04 and 14 ± 1 vs $38 \pm 8 \times 10^4/\text{ml}$ BAL; $P < 0.01$ and $P < 0.05$, Fig. 5B). In addition, the BrdU⁻/MBP⁺ cells in BAL were also significantly reduced in the allergen-challenged CD4^{-/-} and CD8^{-/-} mice compared with allergen-challenged WT mice (0.07 ± 0.05 and 15 ± 2 vs $29 \pm 8 \times 10^4/\text{ml}$ BAL; $P < 0.01$ and $P < 0.05$, Fig. 5B).

BALF eotaxin-2

Allergen-sensitized and exposed WT and CD8^{-/-} mice showed a similar expression of eotaxin-2 in BALF. However, allergen exposed CD4^{-/-} mice exhibited a

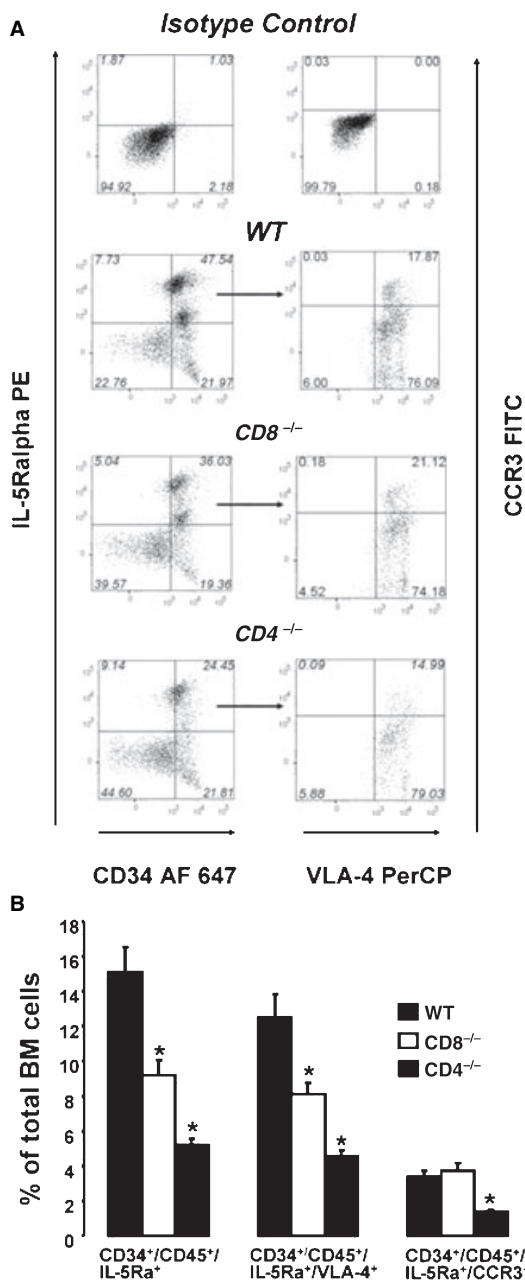


Figure 2. Expression of eosinophil progenitors in bone marrow (BM) of OVA sensitized/exposed wild type (WT), CD8^{-/-} and CD4^{-/-} mice. BM cells were stained with anti-CD45 APC-Cy7, anti-CD34 AF 647, anti-IL-5Ralpha PE, anti-VLA-4 PercP and anti-CCR3 FITC and flow analysis was performed on cells gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. (A) CD45⁺ cells were gated and analysis was performed on their CD34 and IL-5Ralpha expression. CD34⁺/IL-5Ralpha⁺ cells (upper right quadrant) was gated and analysis of VLA-4 and CCR3 expression was also performed. (B) BM CD34⁺/CD45⁺/IL-5Ralpha⁺, CD34⁺/CD45⁺/IL-5Ralpha⁺/VLA-4⁺, and CD34⁺/CD45⁺/IL-5Ralpha⁺/CCR3⁺ cells presented as percentage of BM total cells. Data are shown as mean (+SEM) ($n = 3-4$). * $P < 0.05$ decreased compared with WT group; Mann-Whitney U -test.

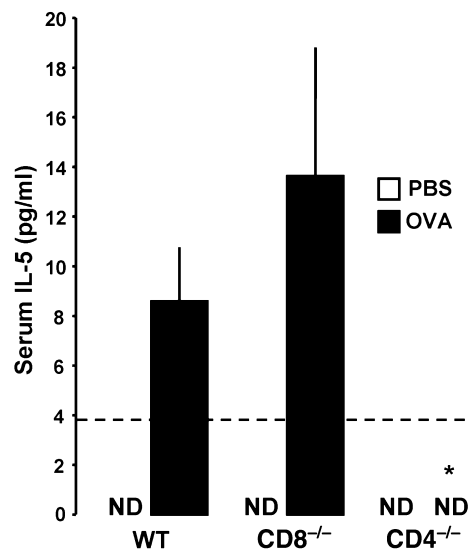


Figure 3. IL-5 in serum of OVA sensitized/exposed (filled bars) and OVA sensitized saline exposed (open bars) WT, CD8^{-/-} and CD4^{-/-} mice. Data are shown as mean (+SEM) ($n = 5$). * $P < 0.05$ compared with WT group. ND, not detectable, dotted line represent the detection limit for serum IL-5; Mann-Whitney U -test.

significant decrease in BALF eotaxin-2 compared with WT mice (0.67 ± 0.02 vs 3.74 ± 0.72 ng/ml BALF; $P < 0.01$), similar to that observed in the saline exposed WT mice (Fig. 6).

Discussion

In the present study, we show that both CD4⁺ and CD8⁺ T cells are involved in the regulation of allergen-induced BM eosinophilopoiesis. Allergen exposure caused a significant increase in BM eosinophilia in the CD4^{-/-} and CD8^{-/-} mice compared with saline exposed mice but significantly lower levels when compared with allergen exposed WT mice. The reduced BM eosinophilia in the CD4^{-/-} and CD8^{-/-} mice was accompanied by a subsequent decrease in both blood and airway eosinophilia when compared with WT mice.

The observed allergen-induced BM eosinophilia in all allergen exposed groups was paralleled with an increase of the newly produced MBP⁺ eosinophils (i.e. BrdU⁺/MBP⁺ cells) compared with saline exposed mice. However, it is important to note that a significant reduction in newly produced eosinophils was found in both knockout strains when compared with allergen-challenged WT mice. The fact that CD4⁺ as well as CD8⁺ T cells are involved in the *in vivo* regulation of newly produced eosinophils in the BM in response to allergen is a novel finding. It has previously been shown that T-cell-deficient (Balb/c nude) mice respond to airway allergen exposure by an increase in the number of eosinophil progenitor

Table 1. IL-5 levels (pg/ml) in BALF

Mouse strain	WT	CD8 ^{-/-}	CD4 ^{-/-}
Exposure			
Saline	40 (13)	32 (8)	30 (8)
OVA	66 (24)	97 (26)*	55 (14)

Bronchoalveolar lavage fluid (BALF) interleukin-5 (IL-5) levels in OVA-sensitized saline or OVA exposed wild type (WT), CD8^{-/-}, and CD4^{-/-} mice.

* $P < 0.05$ increased compared with phosphate buffer saline exposed CD8^{-/-} mice. Data are shown as mean (SEM) ($n = 5-7$); Mann-Whitney U -test.

cells, evaluated by *in vitro* eosinophil colony forming units (CFUs) in BM cells (16). The elevation of eosinophil CFUs was followed by an absence of BM and peripheral blood eosinophilia, suggesting that the initial activation of eosinophil progenitor cells in the BM is independent of either T cells or IL-5, whereas the full differentiation of eosinophil progenitors to phenotypically mature eosinophils is T-cell-dependent (16, 19). Our present study suggests that allergen-induced BM eosinophilopoiesis occurs in the absence of CD4⁺ T-cell-dependent production of IL-5, as CD4^{-/-} mice showed an increase in newly produced BM eosinophils (i.e. BrdU⁺/MBP⁺ cells) following allergen exposure. Importantly, this induced eosinophilopoiesis occurred in absence of detectable IL-5 levels in serum, which is viewed as a mirror of what is occurring in the BM (7, 20).

The BrdU⁺/MBP⁺ cell population represents the eosinophils that are most unlikely to be produced during the allergen exposure period as they have not undergone a recent mitosis. A significant reduction in allergen-induced BM BrdU⁺/MBP⁺ cells was found in both CD8^{-/-} and CD4^{-/-} mice compared with the WT mice, further arguing for a reduced maturation capacity towards the eosinophil phenotype. Importantly, we were not able to detect a significant increase in the BrdU⁺/MBP⁺ cells in the allergen exposed CD4^{-/-} mice compared with the saline exposed CD4^{-/-} mice, which further suggests that CD4⁺ T cells and putatively their production of IL-5 are required for the differentiation and full maturation of eosinophils.

To further evaluate the role of T cells in the detailed maturation of progenitors to a more mature eosinophil phenotype, we performed an immunostaining for CD34⁺/CD45⁺/IL-5Ralpha⁺ cells, reflecting progenitors committed to the eosinophil lineage (21-23). A significant decrease in the number of CD34⁺/CD45⁺/IL-5Ralpha⁺ cells was observed in BM of allergen-challenged CD4^{-/-} as well as in CD8^{-/-} mice compared with allergen-challenged WT mice which further support the hypothesis that both CD4⁺ and CD8⁺ T cells influence the final eosinophil maturation. It has previously been shown that treatment with anti-IL-5 ab inhibits early stage eosinophil maturation of BM CD34⁺ cells of allergen sensitized and exposed mice (24). Additionally, a clinical study has recently shown that anti-IL-5 treatment led to an arrest in maturation of BM eosinophils without a decrease in BM

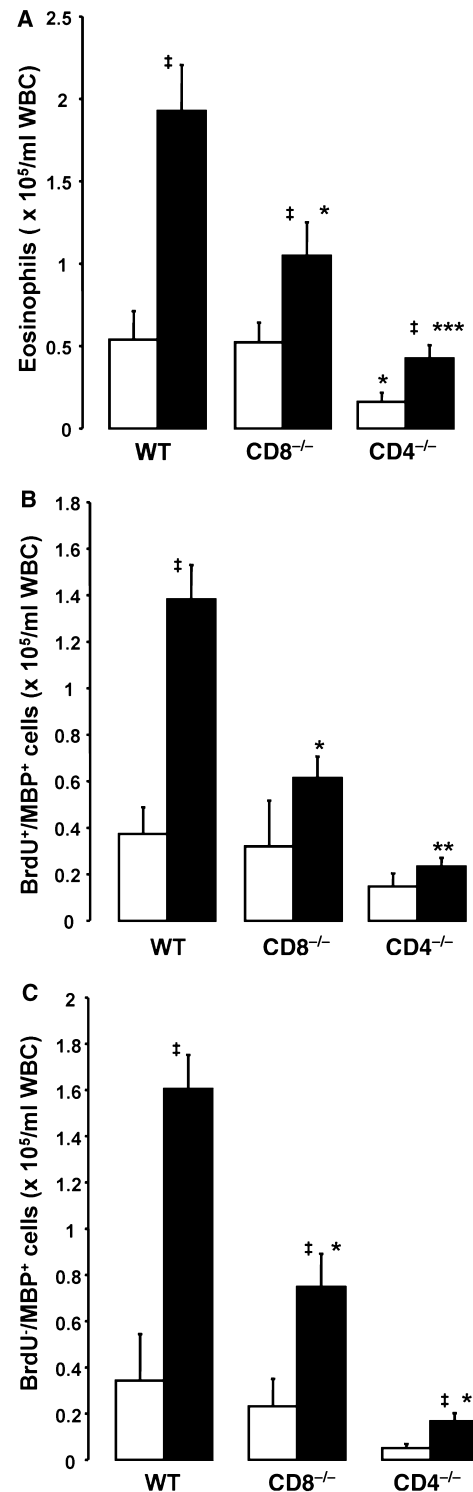


Figure 4. Blood eosinophils in OVA sensitized/exposed (filled bars) and OVA sensitized saline exposed (open bars) wild type (WT), CD8^{-/-} and CD4^{-/-} mice (A) May-Grünwald-Giemsa stained eosinophils, (B) BrdU⁺/MBP⁺ eosinophils, and (C) BrdU⁻/MBP⁺ eosinophils. Data are shown as mean (+ SEM) ($n = 5-14$). ‡ $P < 0.05$, increased compared with saline group * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ decreased compared with WT group; Mann-Whitney U -test.

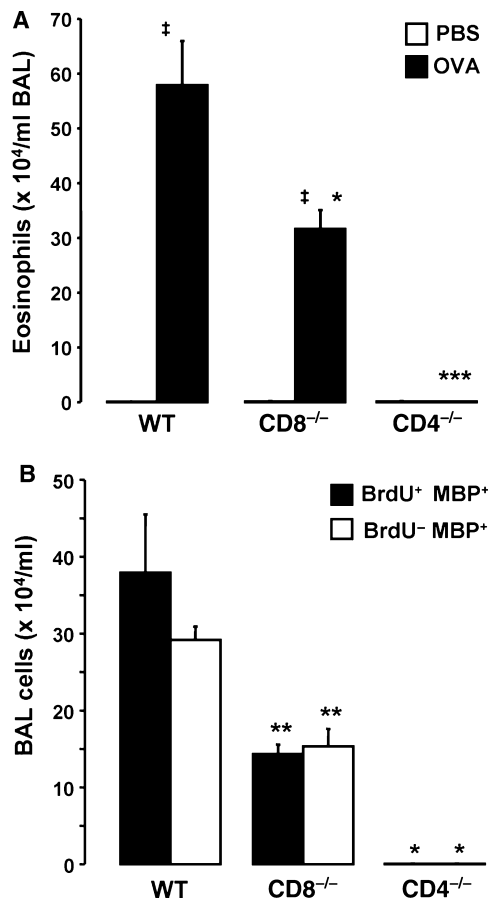


Figure 5. Bronchoalveolar lavage (BAL) eosinophils in OVA sensitized/exposed (filled bars) and OVA sensitized saline exposed (open bars) wild type (WT), CD8^{-/-}, and CD4^{-/-} mice. (A) May-Grünwald-Giemsa stained eosinophils, (B) BrdU⁺/MBP⁺ eosinophils, and BrdU⁻/MBP⁺ eosinophils. Data are shown as mean (+SEM) ($n = 5-14$). [‡] $P < 0.01$, increased compared with saline group. ^{*} $P < 0.05$; ^{**} $P < 0.01$; ^{***} $P < 0.001$ decreased compared with WT group; Mann-Whitney *U*-test.

or blood CD34⁺ progenitor cells (25). The discrepancy between the anti-IL-5 clinical study and our present study could be that the turnover of BM eosinophilopoiesis may differ between the two species. It could also be speculated that the dose of anti-IL-5 antibody used in the clinical study was insufficient to suppress systemic IL-5 to levels affecting progenitor cell populations. Importantly, in the present study allergen-challenged CD4^{-/-} mice showed the most prominent decrease in eosinophil progenitors of the two knockout strains compared with WT mice, which further correlated with the absence of systemic IL-5. However, the finding that also CD8^{-/-} mice have a reduced number of BM eosinophil progenitors without differences in serum IL-5 compared with allergen-challenged WT mice is in agreement with a previous report suggesting that in the presence of IL-5, CD8⁺ T cells seem to be required for the maintenance of

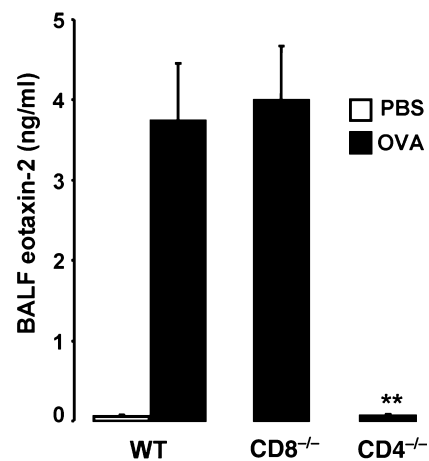


Figure 6. Bronchoalveolar lavage fluid (BALF) eotaxin-2 in OVA sensitized/exposed (filled bars) and OVA-sensitized saline exposed (open bar) WT, CD8^{-/-}, and CD4^{-/-} mice. Data are shown as mean (+SEM) ($n = 5$). ^{**} $P < 0.01$ compared with WT group; Mann-Whitney *U*-test.

eosinophil production in the BM (15), which further could explain the decrease in blood and airway eosinophils in CD8^{-/-} compared with WT mice.

In order to determine, if there is any defect in the release of eosinophil progenitors from the BM in the different knockout strains, co-expression of CCR3 on progenitors was assessed. BM eosinophil progenitors co-expressing CCR3 (i.e. CD34⁺/CD45⁺/IL5Ralpha⁺/CCR3⁺ cells) showed no change in allergen-challenged CD8^{-/-} mice compared with WT mice, whereas CD4^{-/-} mice showed a clear reduction (Fig. 2B). The finding that CD4^{-/-} mice express less CCR3 was not surprising, as it has previously been shown that T helper 2 cytokines, including IL-5 upregulates CCR3 expression on eosinophil progenitors (21). Furthermore, the reduction in CCR3 expression on eosinophil progenitors in the CD4^{-/-} mice most likely explains the greater reduction in blood eosinophils as compared with the CD8^{-/-} mice.

There has been significant therapeutic focus on the role of eosinophil specific adhesion molecules, particularly the β 1 integrin, VLA-4, which is a ligand for the integrin vascular cell adhesion molecule-1 and that mediate the transit of eosinophils across endothelial and epithelial barriers. The importance of VLA-4 for the recruitment of eosinophils has been suggested from several animal studies (26, 27). However, a recent study suggests that VLA-4 may not play a major role in allergen-induced airway responses seen in asthma (28). Our data in the present study suggest that the reduction in airway and blood eosinophilia in the CD8^{-/-} and CD4^{-/-} mice may not be due to a reduced co-expression of VLA-4 on eosinophil progenitors, as most of the progenitors in all groups of animal co-expressed this adhesion molecule, which further support the above study.

It has previously been shown that a reduction in allergen-induced airway eosinophilia occurs in both CD4^{-/-} and CD8^{-/-} mice compared with WT mice (29–31). However, in the present study we clearly show that the reduction in both blood and airway eosinophils is mirrored by an initial reduction in allergen-induced BM eosinophilia in both knockout strains. Furthermore, we were unable to identify differences in BALF IL-5 levels between any of the allergen exposed groups of animals, suggesting that factors other than local airway IL-5 regulate airway eosinophil recruitment (7, 20). It is interesting to note that the prominent eosinophil chemoattractant eotaxin-2 (32) was reduced in the airways of allergen exposed CD4^{-/-} mice to similar numbers of saline exposed WT mice, suggesting that the establishment of an allergen-induced eotaxin-2 gradient in airways is dependent upon CD4⁺ T lymphocytes.

In conclusion, our study suggests that both CD4⁺ and CD8⁺ T cells have a regulatory role in allergen-induced BM eosinophilopoiesis. In particular, CD4⁺ T cells may regulate the final maturation of eosinophils in the BM after airway allergen exposure. The subsequent traffic of eosinophils to the airways is likely to be at least partly regulated by a CD4⁺ T-cell-dependent local airway eotaxin-2 production.

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