

# New Production of Eosinophils and the Corresponding Th1/Th2 Balance in the Lungs after Allergen Exposure in BALB/c and C57BL/6 Mice

Y. Lu\*, M. Sjöstrand\*, C. Malmhäll\*, M. Rådinger\*, P. Jeurink\*,†, J. Lötvalld\* & A. Bossios\*

\*Krefting Research Centre, Department of Internal Medicine, Institute of Medicine, The Sahlgrenska Academy, University of Gothenburg, Gothenburg, Sweden; and  
†Danone Research-Centre for Specialized Nutrition, Immunology Group, Wageningen, The Netherlands

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Correspondence to: A. Bossios, MD, PhD, Krefting Research Centre, University of Gothenburg, Box 480, SE-405 30 Göteborg, Sweden. E-mail: apostolos.bossios@gu.se

## Abstract

Allergic asthma is associated with eosinophilic inflammation in the airways. Animal models commonly used to elucidate allergic inflammation mechanisms include BALB/c and C57BL/6 mice. Our aim was to evaluate lung eosinophilia and the corresponding Th1/Th2 balance in the two strains after allergen exposure. BALB/c and C57BL/6 mice were subjected to ovalbumin-induced allergic airway inflammation using BrdU to label newly produced cells. The numbers of new eosinophils were evaluated by differential cell count and immunocytochemistry (MBP<sup>+</sup>BrdU<sup>+</sup>). Proliferation rate of lung eosinophils was measured by analysis of CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup> cells by FACS. Distribution of newly produced eosinophils in the lung and the Th1/Th2 (CD4<sup>+</sup>T-bet<sup>+</sup>/CD4<sup>+</sup>GATA-3<sup>+</sup>) balance was evaluated by immunohistochemistry. Allergen challenge with ovalbumin induced comparable eosinophilia in bone marrow (BM), blood and lung tissue in both strains of mice compared to phosphate-buffered saline controls, which was confirmed by immunocytochemistry. There was a small increase in the number of lung MBP<sup>+</sup>BrdU<sup>+</sup> eosinophils in C57BL/6 mice compared to BALB/c mice, which suggests a basal increase in this strain following sensitization. While there was no difference in eosinophilic proliferation in the lung, the distribution of the newly produced eosinophils differs between the two strains. BALB/c mice showed staining primarily around vessels and airways, whereas C57BL/6 mice showed a more even distribution in the lung tissue. No difference in the Th1/Th2 balance was observed between two strains. This study shows that there is a difference in the distribution of eosinophils in the lung between the C57BL/6 and BALB/c mice, but no difference in eosinophil production or Th1/Th2 balance.

## Introduction

Allergic asthma is associated with inflammatory changes in the airways, both in residual cells (epithelial, fibroblasts, smooth muscle cells) and in increased numbers of inflammatory cells such as lymphocytes, neutrophils, dendritic cells and mast cells, with the most striking increase being in the number of eosinophils [1]. Eosinophilic inflammation is the characteristic that is best associated with allergic asthma. Several studies have documented a relationship between the degree of eosinophilia in sputum, bronchial biopsies and blood with asthma severity and exacerbations [2–4]. Furthermore, recent studies have shown that eosinophils are strong

immunoregulatory cells and not just 'end-stage', terminally differentiated, non-replicating cells [5, 6]. It has been shown recently in a mouse model of allergic inflammation, that in a T helper 2 (Th2)-like environment with a dominance of IL-5, a continuous production of eosinophils occurs [7], further supporting the T helper 1 (Th1)/Th2 hypothesis. However, the T cell diversity has been expanded to several sub-populations, including T-regulatory cells and T helper 17 (Th17) cells, suggesting that the overall picture is more complicated [8].

Animal studies have been valuable for elucidating the cellular and molecular response necessary for allergic airway inflammation. Mouse models have been particularly useful, because of the many mouse-specific tools available

to dissect mechanisms of allergic inflammation [9]. Studies of airway allergic inflammation primarily use two mouse strains, BALB/c and C57BL/6 mice. Both strains show highly elevated airway eosinophils after allergen exposure [10, 11]. While some reports suggest C57BL/6 mice exhibit a more Th1-like immune response after allergen exposure [12, 13], and BALB/c, a more Th2-like response [12–14], contradicting results also exist [15, 16].

The aims of this study were mainly to determine the eosinophilic inflammation of using these two common mouse strains for studying allergic asthma mechanisms by determining the *de novo* production and terminal distribution of lung eosinophils after allergen exposure and second to verify the established balance between Th1/Th2 lymphocytes.

A standard ovalbumin exposure protocol was used, together with the administration of BrdU (5-bromo-2'-deoxyurine) to determine which cells have undergone mitosis after allergen exposure. Two different BrdU regimes were used to examine the labelling efficiency of new eosinophils. The number and distribution of eosinophils and newly produced eosinophils were compared by differential cell counts, ICC, FACS and immunohistochemical (IHC) staining. Finally IHC staining was used to assess the CD4<sup>+</sup>T-bet<sup>+</sup> and CD4<sup>+</sup>GATA-3<sup>+</sup> cells to reveal the Th1/Th2 balance.

## Materials and methods

**Animals.** This study was approved by the Animal Ethics Committee in Gothenburg, Sweden. Male BALB/c and C57BL/6 mice which were 5–6 weeks old were purchased from Taconic (Ry, Denmark). All mice were kept under standard animal housing conditions and provided with food and water *ad libitum*.

**Allergen sensitization and allergen exposure.** Mice were sensitized twice at an interval of 5 days by intraperitoneal (i.p.) injection of 0.5 ml alum-precipitated antigen containing 8 µg chicken ovalbumin (Sigma-Aldrich, St Louis, MO, USA) bound to 4 mg aluminium hydroxide (Sigma-Aldrich) in phosphate-buffered saline (PBS). Eight days after the second sensitization, each strain was divided in two groups. Both groups were briefly anesthetized using isoflurane (Baxter, Deerfield, IL, USA) followed by the exposure group receiving an intranasal (i.n.) administration of 100 µg OVA in 25 µl PBS on five consecutive days, while the control group received only PBS. PBS was used on both occasions in one additional group of mice, as a model for non-sensitized, naïve animals (Fig. 1).

**Evaluation of newly produced inflammatory cells.** All mice were given 4 mg BrdU (Roche Diagnostics Scandinavia AB, Bromma, Sweden) but with two different administration schedules. In the first schedule

## ANIMALS

### BALB/c or C57BL/6 mice 5–6 weeks old, n = 5–8/group

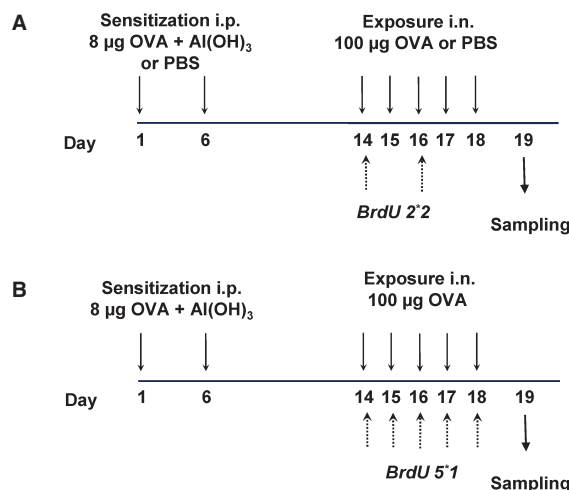


**Figure 1** Illustration of the sensitization and the BrdU administration protocols in our study. Four groups of animals were totally included. (A) Naïve animals; phosphate-buffered saline (PBS) used both at sensitization and exposure. Sensitized animals; sensitization was done by OVA and exposure by PBS. Exposed animals; sensitization and exposure were done with OVA. Additionally, animals were given BrdU 2\*2 times to mark the newly produced cells. (B) A final group was included of exposed animals, where BrdU was administered 5\*1 times to evaluate the effect of BrdU administration.

(BrdU2\*2), BrdU was given in a dose of 1 mg in 0.25 ml PBS by i.p. injection on two occasions, 7 h apart on days 1 and 3 of allergen exposure. Allergen exposure was performed 1 h after the first BrdU injection. In the second schedule (BrdU5\*1), BrdU was given in a dose of 0.8 mg in 0.2 ml PBS by i.p. injection once a day just after allergen exposure, on days 1–5 of allergen exposure.

**Sample collection and processing.** Samples were collected 24 h after the final OVA exposure. The animals were deeply anesthetized with a mixture of xylazine (130 mg/kg; Rompun®, Bayer, Germany) and ketamine (670 mg/kg; Ketalar®, Parke-Davis, UK). First, animals were killed by puncturing the right heart ventricle and blood was collected. Second, the mice were tracheostomized and bronchoalveolar lavage (BAL) was performed with 2 × 1 ml PBS through the tracheal cannula to wash away airway lumen inflammatory cells and the lungs were perfused and removed to harvest parenchymal inflammatory lung cells. The left lobe was filled with Tissue-Tek® (Sakura Finetek Europe B.V., 2382 AT, Zoeterwoude, The Netherlands) and then immediately frozen in liquid nitrogen. The right lobes were stored in Hanks balanced salt solution (HBSS) (Sigma-Aldrich) on ice. Third, BM cells were harvested by excising one femur which was cut at the epiphysis and flushed with 3 ml HBSS.

**Blood.** Blood (200  $\mu$ l) was added to 2 mM EDTA (800  $\mu$ l) (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 room temperature (RT). White blood cells (WBC) were resuspended in 1% fetal calf serum (FCS) (Sigma-Aldrich) in PBS, counted in a standard hemocytometer and cytopspins prepared, either for staining with May-Grünwald-Giemsa for differential counts or for ICC.

**Bone marrow.** BM samples were washed, and the number of cells was counted as performed for the blood.

**Lung tissue.** The right lobes of the lung were weighed and pushed by a plunger through a cell strainer with a 100- $\mu$ m nylon mesh (BD Biosciences, Erembodegem, Belgium). The strainer was washed with 5 ml of PBS supplemented with 1% FCS, and the cell solution was passed through a 40- $\mu$ m nylon mesh. The cells were washed as mentioned above and diluted in PBS supplemented with 1% FCS. The numbers of cells were counted and cytopspins prepared, either for staining with May-Grünwald-Giemsa for differential counts or for ICC. Lung cells were saved for FACS analysis.

Cell differentiation in blood, BM and lungs was determined by counting 300–500 cells using a light microscope (Zeiss Axioplan 2, Carl Zeiss, 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.

**Detection of BrdU<sup>+</sup> MBP<sup>+</sup> in BM, blood and lung cells.** Newly produced cells (BrdU<sup>+</sup>) were immunostained with MBP (major basic protein), an eosinophil granule protein expressed early in eosinophil-lineage committed cells as well as in mature eosinophils. (Kind gift from Dr James Lee, Mayo Clinic, Scottsdale, AZ, USA) On day 1, cytospin preparations were fixed in 2% formaldehyde for 10 min and incubated with 10% rabbit serum (DAKO Corporation, Glostrup, Denmark) to block unspecific binding. Cytospin samples were incubated with a rat anti-mouse MBP monoclonal antibody for 1 h followed by 45 min incubation with a secondary antibody, alkaline phosphatase (AP)-conjugated rabbit F(ab')<sub>2</sub> anti-rat IgG. Bound antibodies were visualized with Liquid Permanent Red Chromogen (LPR) (DAKO). Samples were fixed for a second time overnight in 4% paraformaldehyde. On day 2, 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 preheated glucose oxidase (37 °C for 30 min) by incubation for 30 min. BrdU labelled cells were detected using a fluorescein isothiocyanate (FITC) labelled rat anti-mouse BrdU monoclonal antibody (clone BU1/75; Harlan-Sera Lab, Loughborough, UK), followed by horseradish peroxidase (HRP)-conjugated rabbit anti-FITC ab

(DAKO) and visualized using liquid 3, 3'-diaminobenzidine (DAB) substrate chromogen system (DAKO). Mayer's hematoxylin (Sigma-Aldrich) was used for counterstaining. The distribution of the cells was determined by using a light microscope (Zeiss Axioplan 2) at the magnification of  $\times 400$  and a digital camera (Canon Power Shoot G6, Canon Inc., Tokyo, Japan).

**Flow cytometry analysis of CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup> cells.** Lung cells were collected and pretreated with 2% mouse serum (DAKO) for 15 min to prevent unspecific binding and thereafter stained with the following antibodies: FITC anti-mouse CD45 (clone 30-F11; BD Biosciences), phycoerythrin (PE)-labelled CCR3 (clone 83101; R&D, Abington, UK) and 7-amino-actinomycin (7AAD) (BD Biosciences) or a matching isotype control antibody. The cells were incubated for 30 min at 4 °C with the antibodies or isotype controls then fixed and permeabilized with BD Cytotfix/Cytoperm™, followed by two washes and stored at 4 °C overnight for the intracellular staining with BrdU on the second day. The BrdU staining was performed according to the BrdU Flow kit Staining Protocol in Instruction Manual from BrdU Flow kits (BD Pharmingen™, BD Biosciences). Finally, flow cytometer analysis was carried out using a BD FACSAria™ flow cytometer (BD Biosciences, San Jose, CA, USA). Ten thousand cells were computed in list mode and analysed using the FlowJo Software® (Tri star Inc, Ashland, OR, USA). BrdU incorporation and intensity were estimated by rMFI (relative mean fluorescence intensity equals MFI of monoclonal antibody/MFI of corresponding isotype control) [8].

**Lung histology.** Eosinophils: The left lung lobe was embedded in Tissue-Tek® O.C.T. Compound (Miles, Inc., Elkhart, IN, USA) and placed in a container of 2-methylbutane (Sigma-Aldrich) which was then dipped in liquid nitrogen. Frozen samples were stored at –80 °C until sliced into 5- $\mu$ m sections with a cryostat Leica CM1900 UV-kryostat® (Leica Microsystems Nussloch GmbH, Nussloch, Germany). Sections were stained, as described previously, to detect BrdU<sup>+</sup> eosinophils. The stained samples were assessed in a blinded fashion under a microscope Axioplan 2 (Carl Zeiss Jena GmbH) at the magnification of  $\times 400$ . A semi-quantitative grading system was used to score the extent of inflammation in the histological sections. The eosinophilic inflammatory infiltrate of four compartments (perivascular tissue, alveolar lung tissue, central airways and peripheral airways) was analysed. Inflammation accumulation was graded as 1 for absent or representing very few cells, 2 for an intermediate number of cells or one cell layer, and 3 for 3 or more than 3 layers of cells [17].

**Th1 and Th2 lymphocytes.** Tissue sections of 5  $\mu$ m were fixed with 2% formaldehyde for 10 min, rinsed in PBS and incubated with 10% Donkey serum (Jackson

ImmunoResearch Laboratories, inc., West Grove, PA, USA). Samples were incubated with a rat anti-mouse CD4 monoclonal antibody (clone RM4-5; BD Pharmingen™) for 1 h followed by 45 min incubation with a secondary antibody, donkey anti-rat IgG-AP (Jackson ImmunoResearch Laboratories, inc.). Bound antibodies were visualized with LPR. Samples were fixed for a second time 20 min in 2% formaldehyde, rinsed in tris-buffered saline (TBS) and then subjected to heat-induced antigen retrieval using target retrieval solution pH 6 (DakoCytomation, Glostrup, Denmark). Endogenous peroxidase was inhibited as described previously. All washings between steps were performed using TBS with or without Tween 20. For T-bet staining, blocking of unspecific binding sites was performed by incubating slides with normal horse blocking serum (ImmPRESS™ REAGENT; Vector Laboratories, Inc., Burlingame, CA, USA). Primary antibodies for detection of T-bet (Rabbit anti-mouse T-bet, clone H-210; Santa Cruz Biotechnology Inc.) were applied followed by a secondary antibody anti-rabbit Ig-HRP (ImmPRESS™ REAGENT; Vector Laboratories, Inc.). Bound antibodies were visualized by DAB, and Mayer's hematoxylin was used for counter-staining.

For GATA-3 staining, blocking endogenous biotin was performed by using the Avidin/Biotin Blocking kit (Vector Laboratories, Inc.). Biotinylated primary antibody was prepared by incubating GATA-3 ab (mouse anti-GATA-3, clone L50-823; BD Pharmingen™) and biotinylation reagent (DakoCytomation ARK™ (Animal Research kit), Dako Cytomation Inc., Carpinteria, CA, USA peroxidase for mouse primary antibodies) for 15 min followed by adding blocking reagent for 5 min to block the unspecific staining, the mixture solution was applied to the samples for 20 min incubation followed by streptavidin-HRP for 20 min. Antibody detection and counter-staining were carried out as described previously.

The stained samples were assessed in a blinded fashion under an Axioplan 2 microscope (Carl Zeiss Jena GmbH) at a magnification of  $\times 1000$ . To facilitate counting, a graticule was applied to the ocular. CD4<sup>+</sup>T-bet<sup>+</sup> and CD4<sup>+</sup>GATA-3<sup>+</sup> cells were evaluated on a 4-point scale (0 = 0–2 cells; 1 = 3–6 cells; 3 = 7–10 cells; 4  $\geq$  10 cells per field) [18]. Positive cells were counted in four representative fields (a perivascular tissue, an alveolar lung tissue, a central airway and a peripheral airway). Th1/Th2 balance was expressed as CD4<sup>+</sup>T-bet<sup>+</sup>/CD4<sup>+</sup>GATA-3<sup>+</sup> ratio [19].

### Data analysis

All data were expressed as mean  $\pm$  SEM (standard error of the mean). Statistical analysis was carried out using a non-parametric analysis of variance. The Mann–Whitney *U* test was used to determine significant differences

between the individual groups. A *P* value  $<0.05$  was considered statistically significant.

## Results

### Systemic eosinophilia after allergen exposure

The number of eosinophils was significantly increased in OVA-sensitized- and OVA-exposed BALB/c and C57BL/6 mice compared to the control OVA-sensitized-PBS-exposed groups in BM, blood and lung (Fig. 2). However, there was no significant difference in eosinophil numbers between the two strains in any compartment after OVA exposure, although in BALB/c mice, there was a trend towards more eosinophils in the blood.

### Newly produced eosinophils in BM, blood and lungs after OVA exposure

The majority of the eosinophils in all compartments were BrdU-positive (MBP<sup>+</sup>BrdU<sup>+</sup>) after OVA exposure in both BALB/c (BM 81.8%, blood 79.9%, lung 86.8%) and C57BL/6 (BM 87.5%, blood 88.5%, lung 79.5%) mice. The MBP<sup>+</sup>BrdU<sup>+</sup> eosinophils were not significantly different in BM, blood and lung between the two strains. Comparison of the BrdU-negative (MBP<sup>+</sup>BrdU<sup>-</sup>) eosinophils revealed no significant difference between the two strains in BM and blood (Fig. 3A, B). However, a statistically significant increase of MBP<sup>+</sup>BrdU<sup>-</sup> eosinophils was observed in lung tissue of C57BL/6 mice compared to BALB/c mice after allergen exposure (*P* = 0.034) (Fig. 3C). Finally, no difference was observed between the two mice strains in the production of non-eosinophilic cells (MBP<sup>-</sup>BrdU<sup>+</sup>) in BM, blood or lung.

### Influx and distribution of eosinophils in the airways and lungs evaluated by FACS analysis and histological examination

#### Flow cytometric analysis for CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup>

The numbers of CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>-</sup> (Fig. 4A) and CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup> (Fig. 4B) cells did not differ between the two mouse strains. While a significant increase of the CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup> cell population in the lungs was evident in both mouse strains after OVA sensitization/exposure with the two BrdU protocols compared to the controls (Data not shown). Importantly, there was no difference between the eosinophil proliferation rates in two strains as shown by the BrdU intensity (rMFI) (Fig. 4C). The BrdU dose administered did not have any effect on either the number of newly produced eosinophils (Fig. 4A, B) or the uptake of BrdU (Fig. 4C, D). BrdU did not induce any inflammation in the lungs as evaluated by the enumeration of neutrophils after injection with either BrdU protocol (BALB/c: BrdU2\*2,  $10.9 \pm 3.0$ ; BrdU5\*1,  $11.4 \pm 2.8$ ;

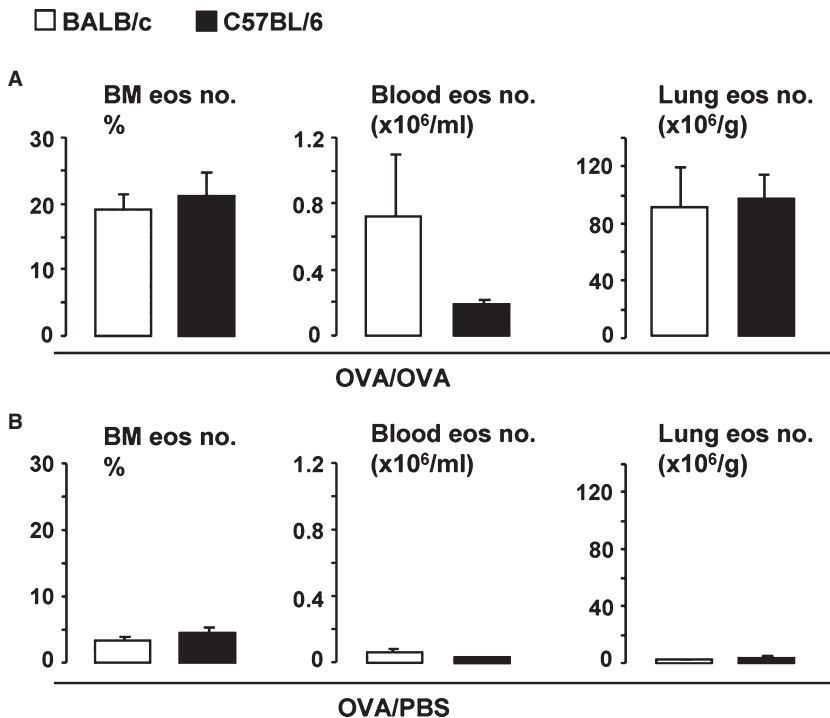


Figure 2 OVA-sensitized BALB/c and C57BL/6 mice exposed to either (A) OVA or (B) phosphate-buffered saline (PBS) for 5 consecutive days, 24 h after the last OVA exposure. Eosinophils were stained with May-Grünwald-Giemsa, BM is expressed as a percentage of total number of cells and blood and lung tissue is expressed as total number of eosinophils  $\times 10^6$  and  $10^6/\text{g}$ , respectively. Values are expressed as mean  $\pm$  SEM,  $n = 6$  mice/group.

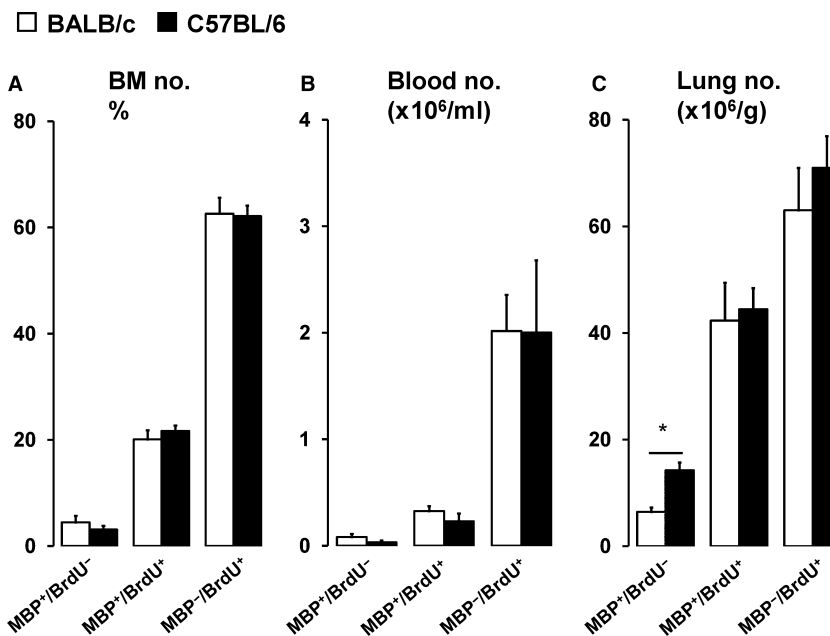


Figure 3 Newly produced eosinophils from OVA-sensitized- and OVA-exposed BALB/c and C57BL/6 mice treated with BrdU 2\*2, 24 h after the last OVA exposure. Cells were stained with anti-MBP and anti-BrdU, showing eosinophils produced before allergen exposure (MBP<sup>+</sup>BrDU<sup>-</sup>) and eosinophils produced during allergen exposure (MBP<sup>+</sup>BrDU<sup>+</sup>) and a non-eosinophilic cell population produced after allergen exposure (MBP<sup>-</sup>BrDU<sup>+</sup>) in (A) BM expressed as a percentage of total cell number, (B) blood and (C) lung tissue expressed as total number of eosinophils  $\times 10^6$  and  $10^6/\text{g}$ , respectively. Data are shown as mean  $\pm$  SEM,  $n = 6-10$  mice/group, \* $P = 0.034$ , comparing BALB/c and C57BL/6 mice.

C57BL/6: BrdU2\*2,  $6.4 \pm 1.1$ ; BrdU5\*1,  $6.8 \pm 1.5$ ;  $\times 10^6/\text{g}$  lung tissue, neutrophils.).

#### Lung histology

To evaluate the distribution of MBP<sup>+</sup>BrDU<sup>+</sup> eosinophils in the lungs, the OVA/OVA and BrdU 2\*2 mice were examined histologically (Fig. 5A). Four different lung compartments were evaluated: perivascular tissue

(Fig. 5B), alveolar lung tissue (Fig. 5C), central airways (Fig. 5D) and peripheral airways (Fig. 5E). In BALB/c mice, the majority of newly produced eosinophils were found around vessels and airways, while in C57BL/6 mice, the majority was homing around vessels as well as in the lung tissue (Table 1). C57BL/6 mice had significantly more newly produced eosinophils in alveolar lung tissue ( $P = 0.006$ ) and peripheral airways ( $P = 0.017$ ) compared to BALB/c mice.

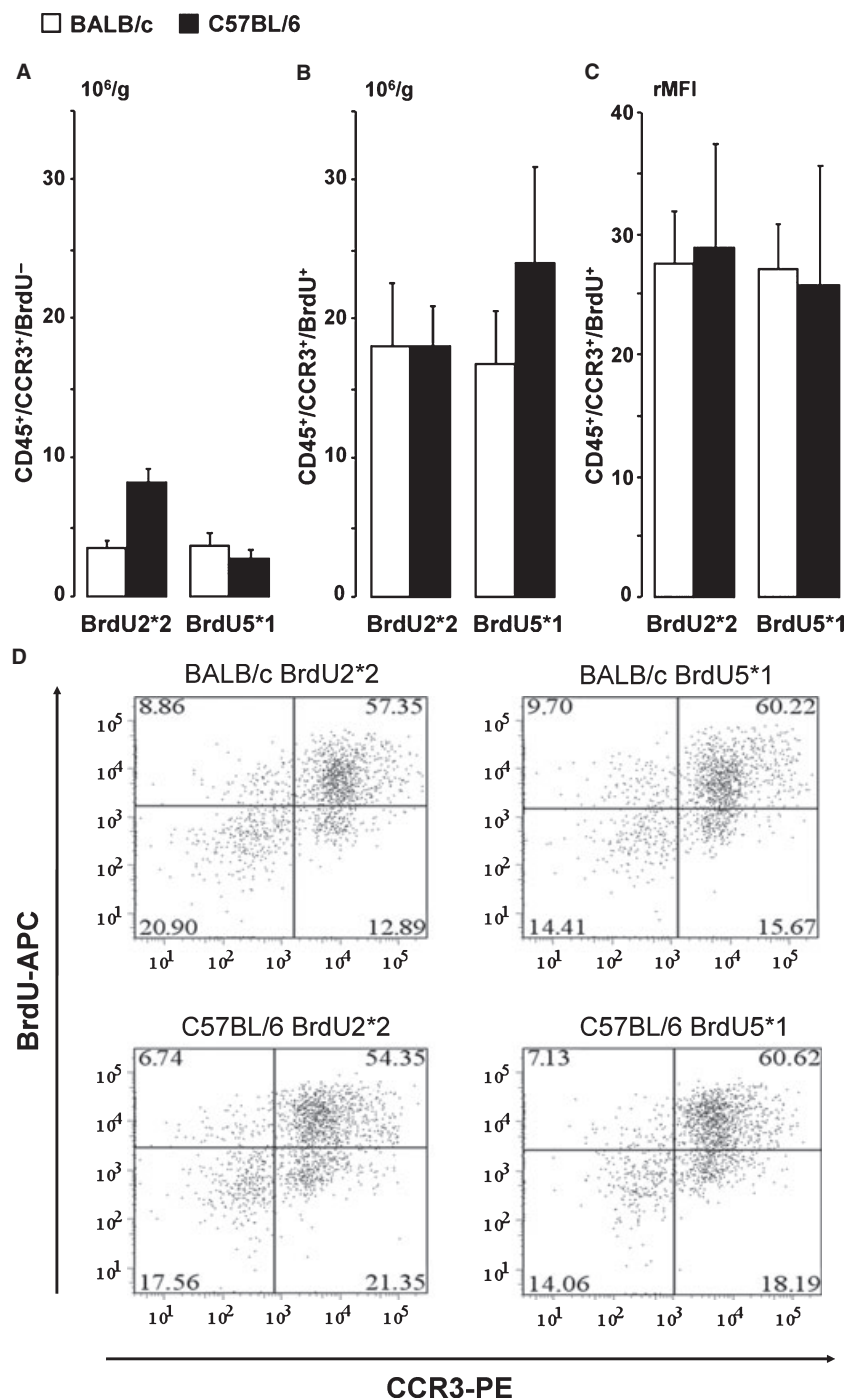


**Figure 4** BALB/c and C57BL/6 mice were sensitized and exposed with OVA, 24 h after the last OVA exposure, lung cells were stained with anti-CD45, anti-CCR3 and anti-BrdU. Live cells were gated based on 7-AAD exclusion, leucocytes and granulocytes were gated based on their forward and side scatter characteristics. In this population, CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>-</sup> represents 'old' eosinophils produced before allergen exposure (A) and CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup> represents the 'new' eosinophils produced during allergen exposure (B). The uptake of BrdU shows indirectly the proliferation rate of eosinophils that was estimated by the rMFI (C). To determine labelling efficiency two different BrdU administrations were given, BrdU 2\*2 and BrdU 5\*1, details of two BrdU administration are described in the Materials and methods. Representative dot plots of mice lung cells expressing CCR3 and BrdU (D). Cells were gated as described previously and leukocytes for expression of CD45. Data are shown as mean  $\pm$  SEM,  $n = 5$  mice/group.

### Th1/Th2 balance in the lungs

To evaluate the role of the Th1 or Th2 phenotype in eosinophils production, CD4<sup>+</sup>T-bet<sup>+</sup> and CD4<sup>+</sup>GATA-3<sup>+</sup> lymphocytes were calculated, respectively, by IHC in the lungs of OVA/OVA, OVA/PBS and PBS/PBS and BrdU 2\*2 mice. Figure 6A shows positive cells in alveolar lung tissue after OVA sensitization and exposure. The Th1/Th2 balance were expressed as CD4<sup>+</sup>T-bet<sup>+</sup>/CD4<sup>+</sup>

GATA-3<sup>+</sup> ratio. No significant differences were found between the two strains in any group (OVA/OVA  $P = 0.115$ , OVA/PBS  $P = 0.053$  and PBS/PBS  $P = 0.23$ ).

### Eosinophilia in the lung tissue of naïve (PBS/PBS) mice

Finally, to determine if there was any difference at baseline between the two mouse strains, we evaluated the

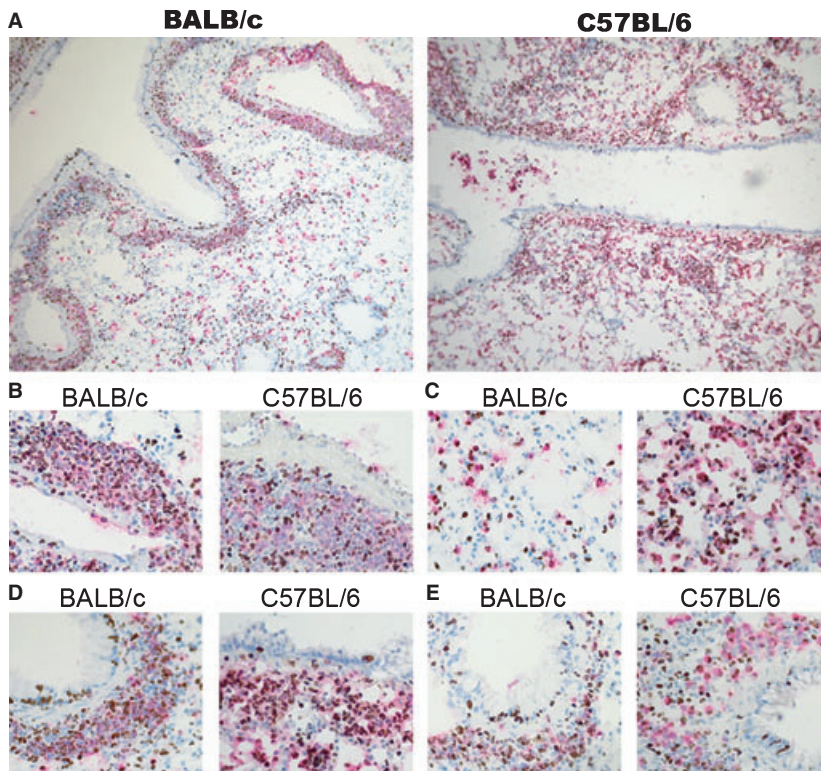


**Figure 5** Lung histology from OVA-sensitized- and OVA-exposed BALB/c and C57BL/6 mice treated with BrdU 2\*2, to evaluate the tissue distribution of newly produced eosinophils (MBP<sup>+</sup>BrdU<sup>+</sup>). Immunohistochemical (IHC) staining of MBP<sup>+</sup> (red) and BrdU<sup>+</sup> (brown). Representative photomicrographs (×100 magnification) from (A) BALB/c and C57BL/6 mice showed the distribution of the newly produced eosinophils. Higher magnification (×200) photomicrographs expose eosinophils in (B) perivascular tissue, (C) alveolar lung tissue, (D) central airways and (E) peripheral airways.

**Table 1** Grading of lung histology for the newly produced eosinophils in BALB/c and C57BL/6 mice.

| Mouse strain | Perivascular tissue | Alveolar lung tissue | Central airways | Peripheral airways |
|--------------|---------------------|----------------------|-----------------|--------------------|
| BALB/c       | 3.00 ± 0.00         | 1.97 ± 0.16          | 3.00 ± 0.0      | 2.19 ± 0.15        |
| C57BL/6      | 3.00 ± 0.00         | 3.00 ± 0.00*         | 2.8 ± 0.12      | 2.85 ± 0.10**      |

Lung sections were sampled 24 h after the last OVA exposure from BALB/c and C57BL/6 mice which were OVA-sensitized and OVA-exposed. Lung histology was used to evaluate MBP<sup>+</sup>BrdU<sup>+</sup> newly produced eosinophils. A semi-quantitative grading system was used to score the extent of inflammation in the histological sections in four compartments: perivascular tissue, alveolar lung tissue, central airways and peripheral airways. Inflammation accumulation was graded as 1 for absent or representing very few cells, 2 for an intermediate number of cells or one cell layer, and 3 for 3 or more layers of cells. The histological sections were graded in a blinded fashion. Data are expressed as mean severity scores ± SEM,  $n = 6$  mice/group, \* $P = 0.006$ , \*\* $P = 0.017$ .

lung eosinophilia in mice sensitized and exposed with PBS, modelling the naïve mice. Giemsa staining showed practically no eosinophilia in both strains (BALB/c:  $0.4 \pm 0.08\%$ , C57BL/6:  $0.5 \pm 0.12\%$ ). This was also confirmed by histology where MBP<sup>+</sup>BrdU<sup>+</sup> staining in lung revealed rare double positive cells (Fig. 7).

## Discussion

The present study compared the pattern of allergic inflammation in two commonly used mouse strains, BALB/c and C57BL/6, by measuring the differences in OVA-induced eosinophilia. It was found that OVA exposure induced similar eosinophilic inflammation in both strains in all compartments investigated (BM, blood and lung). While there were no differences in total or newly

produced eosinophils between the two strains, C57BL/6 mice had slightly more MBP<sup>+</sup>BrdU<sup>+</sup> eosinophils in the lungs, suggesting that these cells had been produced before the first allergen exposure. A comparison of different administrations of BrdU revealed no major differences in the incorporation of BrdU in cells. This suggests that the influx and proliferation rate of newly produced eosinophils is equal in both strains. However, the distribution patterns are different. Both mouse strains showed a strong accumulation of eosinophils in perivascular and central airway tissue, while in the alveolar lung tissue and peripheral airways, the eosinophil numbers were significantly greater in C57BL/6 mice compared to BALB/c mice. Furthermore, both mouse strains showed no eosinophilic inflammation without allergen sensitization and exposure. Finally, evaluation of Th1/Th2

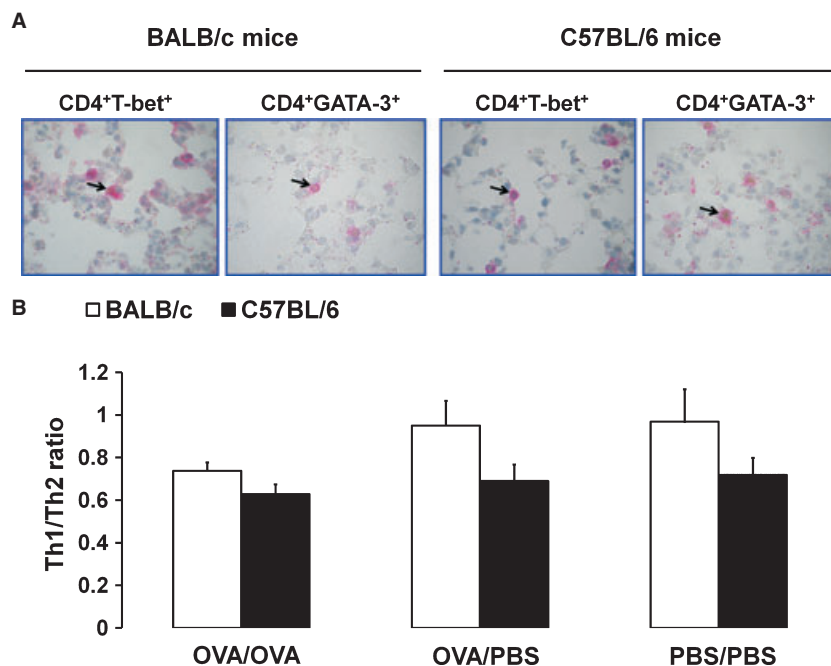


**Figure 6** Lung histology from phosphate-buffered saline (PBS)- or OVA-sensitized- and exposed BALB/c and C57BL/6 mice treated with BrdU 2\*2, to evaluate the expression of Th1 (CD4<sup>+</sup>T-bet<sup>+</sup>) and Th2 (CD4<sup>+</sup>GATA-3<sup>+</sup>) cells. (A) Representative photomicrographs (×1000 magnification) from alveolar lung tissue of OVA-sensitized- and OVA-exposed BALB/c and C57BL/6 mice. CD4<sup>+</sup> shows as a red staining and T-bet<sup>+</sup> or GATA-3<sup>+</sup> shows as a brown nuclear staining. (B) Th1/Th2 balance expressed as their ratio in naïve; PBS/PBS, sensitized; OVA/PBS and exposed; OVA/OVA, BALB/c and C57BL/6 mice.

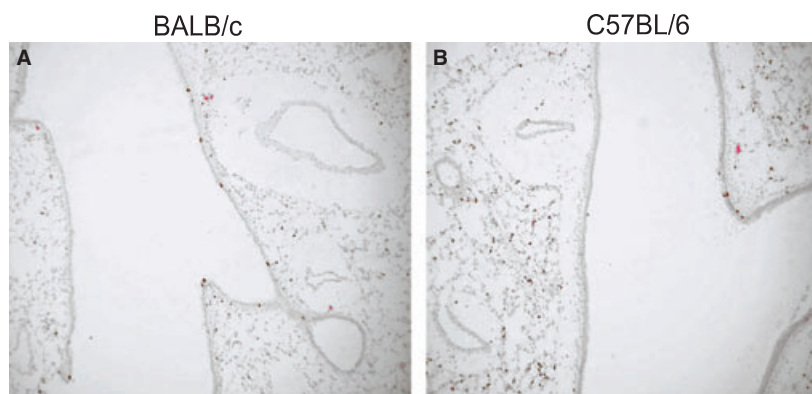


**Figure 7** Lung histology from naïve phosphate-buffered saline (PBS) sensitized and exposed BALB/c and C57BL/6 mice treated with BrdU 2\*2, to evaluate the tissue distribution of newly produced eosinophils (MBP<sup>+</sup>BrdU<sup>+</sup>). Immunohistochemical (IHC) staining of MBP<sup>+</sup> (red) and BrdU<sup>+</sup> (brown). Representative photomicrographs (×100 magnification) from (A) BALB/c and (B) C57BL/6 mice show some newly produced BrdU<sup>+</sup> cells but only some rare MBP<sup>+</sup> BrdU<sup>+</sup> cells.

balance in lung revealed no differences between the two mouse strains. Overall, the above findings show that both BALB/c and C57BL/6 mice acquire an eosinophilic inflammation, but the distribution of the newly produced eosinophils in the lung differs. In addition, this eosinophilic inflammation seems to be dependent of the Th1/Th2 balance in the lungs.

The genetic differences between the two mouse strains, BALB/c and C57BL/6, could influence the mechanisms of immune response and subsequent outcomes. It has been indicated that BALB/c mice have a higher degree of airway responsiveness to methacholine than C57BL/6 during allergic exposure, suggesting a more airway orientated inflammation [13, 20, 21]. Furthermore, it has been suggested that C57BL/6 mice have a weaker inflammatory immune response in the lungs mediated by allergen-independent inflammation [12, 14, 16]. The current study argues that the immune response is similar in the two strains. These results differ from a

previous report by Fukunaga *et al.* [13] found that increased eosinophilic inflammation in BALB/c mice compared to C57BL/6 following OVA exposure. In comparison, a recent article by van Hove *et al.* [22] found that airway eosinophilia was more pronounced in the C57BL/6 strain. However, BAL cells were analysed in both studies, which do not necessarily reflect the eosinophilic lung inflammation.

Newly produced eosinophils were evaluated using a specific eosinophilic marker detecting MBP together with the thymidine analogue BrdU. There was no significant difference in the number of newly produced eosinophils (MBP<sup>+</sup>BrdU<sup>+</sup>) in BM, blood or lungs between the different mouse strains. However, eosinophils produced before allergen exposure (MBP<sup>+</sup>BrdU<sup>-</sup>) seemed to be increased in the lungs of the C57BL/6 mice. A recent report showing a higher rate of apoptosis in eosinophils recovered from the lungs of BALB/c mice compared to C57BL/6 [23] could be a possible explanation for this observation.

Interestingly, there was also no difference in the production of cells other than eosinophils (MBP<sup>-</sup>BrdU<sup>+</sup>) between the two strains.

Eosinophil proliferation and distribution in the lungs was analysed. The cells were stained with CD45<sup>+</sup>CCR3<sup>+</sup>BrdU<sup>+</sup> to identify eosinophils by flow cytometric analysis but also to determine the presence of newly produced eosinophils in the lungs of the different mouse strains used. This analysis can determine different proliferation rates in the lungs and airways of either strains or accelerated *in situ* maturation. Eosinophils originate from CD34<sup>+</sup> progenitor cells which increased in the lungs after allergen exposure, suggesting an *in situ* proliferation of eosinophils [24]. However, we could not detect any difference between the mouse strains in the proliferation rate of CD45<sup>+</sup>CCR3<sup>+</sup> BrdU<sup>+</sup> cells.

We have shown that newly produced eosinophils are not equally distributed in the two mouse strains used in this study. C57BL/6 mice had significantly more newly produced eosinophils distributed in the alveolar lung tissue and around peripheral airways than BALB/c mice (Fig. 5). In BALB/c mice, the newly produced eosinophils accumulated around central airways (Fig. 5). In both strains, a prominent amount of eosinophils were found in the perivascular tissue. These results show different homing of newly produced eosinophils in the lungs of the two strains and suggest that BALB/c mice have a more airway-orientated inflammation than C57BL/6 mice. Therefore, future studies of distribution of newly produced eosinophils using these two strains may produce different results.

A possible explanation for the previously mentioned differences can be searched in the complexity of the local milieu during airway inflammation. First, the small airways and the lung parenchyma can produce many Th2 cytokines, chemokines and mediators involved in initiation and perpetuation of the inflammatory process [25]. It is generally believed that once eosinophils leave the circulation and migrate into tissue sites, they do not recirculate. The survival is dependent on local production of cytokines that prevent eosinophil apoptosis. Second, selective recruitment of eosinophils to specific tissue sites is effectively regulated by unique patterns of cytokines that activate endothelial cells and induce tissue-resident cells to produce eosinophil-active chemokines and other chemoattractants to facilitate their preferential migration. [26] Based on mouse studies, IL-5, IL-13, CD44 (a receptor for hyaluronic acid) and CCR3-active chemokines are implicated in lung homing of eosinophils [27, 28]. Both eotaxin-1/CCL11 and eotaxin-2/CCL24 are important for recruitment of eosinophils to perivascular and peribronchial tissue [29]. Third, the discrepant results could lie in the differing genetic backgrounds of the two strains [30]. Overall, the different distribution of newly produced eosinophils can be correlated with the local unique

milieu of cytokines, chemokines and chemoattractants in different in-situ part of the lungs, as well as based on the genetic backgrounds.

Several reports have shown that BALB/c exhibited Th2-like immune responses, while C57BL/6 displayed a Th1-like diathesis, although opposite responses have also been observed [15, 16, 31]. Interestingly, our results show that there is no difference in the Th1/Th2 balance between the two strains, revealing the existence of CD4 cells expressing the one or the other phenotype. Our results are based on the expression of the representative transcription factors T-bet for Th1 and GATA-3 for Th2, showing commitment to respective phenotype. However, our data do not give us information about the functional status of above cells i.e. cytokine production can differ because of activation status of some cells. Therefore, our data can partly explain the reported inconsistency about the Th1 or Th2 character of C57BL/6 or BALB/c mice. A possible explanation can be that in each specific stimulus, the one or the other population can predominate and characterize eventually the final outcome.

To examine the incorporation of the BrdU, two different protocols with the same total dosage were used. Previous studies have shown that the time needed for BrdU to reach detectable levels after a single injection is about 10 min. Rapid DNA labelling continues for 40 min followed by persistent low level incorporation for 4–8 h [32]. This short duration of activity is often countered by applying BrdU several times during a single day or over several consecutive days [33–35]. This ensures that large numbers of cells are labelled. To minimize the potential problems with toxicity [36, 37], studies have been performed titrating BrdU to determine the lowest dose of BrdU that results in visible labelling. The current data shows no difference between the two different BrdU administration schedules in labelling efficiency of the newly produced inflammatory cells or in unspecific inflammation, as assessed by neutrophil numbers.

In conclusion, this study, examining the two most common mouse models utilized in allergic inflammation, showed that OVA exposure caused significant eosinophilic production in both strains using two different BrdU protocols. The eosinophil distribution, however, was significantly different between the strains. Interestingly, there was no difference in the expressed Th1/Th2 balance between the two mouse strains. Enhanced understanding of the underlying mechanisms of eosinophilic inflammation can contribute to improved therapeutic approaches in allergic inflammation diseases in the future.

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