

Chronic allergen challenge induces pulmonary extramedullary hematopoiesis

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ABSTRACT

Allergic inflammation is associated with increased generation and trafficking of inflammatory cells, especially eosinophils, to sites of inflammation. The effect of acute versus chronic airway allergen challenge on hematopoietic activity in the bone marrow (BM) and lungs was investigated using murine models of allergic airway inflammation. Acute allergen challenge induced proliferation of BM cells and significantly increased generation of eosinophil, but not multipotent, granulocyte-macrophage (GM), or B-lymphocyte progenitor cells. However, no hematopoietic activity was observed in the lungs. With chronic challenge, BM cells failed to proliferate, but exhibited increased capacity to generate multipotent as well as eosinophil, GM, and B-lymphocyte progenitors. In addition, increased generation of eosinophil- and GM-specific progenitors was observed in the lungs. Although no differences were observed in their ability to roll on BM endothelium *in vitro* or *in vivo*, CD34-enriched hematopoietic/stem progenitor cells (HSPCs) from chronic-, but not acute-, challenged mice demonstrated reduced migration across BM endothelial cells associated with decreased CXCR4 expression. Overall, these studies demonstrate that chronic allergen exposure can alter BM homing due to decreased transendothelial migration enabling noninteracting HSPCs to egress out of the BM and recruit to sites of inflammation such as the airways, resulting in extramedullary hematopoiesis.

KEYWORDS allergic airway inflammation, extramedullary hematopoiesis, hematopoiesis, hematopoietic/stem progenitor cells, transendothelial migration

Allergic airway inflammation is a complex disease that involves generation, mobilization, trafficking, and recruitment of multiple inflammatory cells, including T-helper type 2 (Th2) cells, eosinophils, neutrophils, and natural killer (NK) cells to the airways [1, 2]. Amongst these, eosinophils play a prominent proinflammatory role in airway allergic inflammation [3], contributing to several of the clinical features of allergic asthma [4, 5]. Clinical studies as well as murine models of allergic airway inflammation have shown up-regulation of bone marrow (BM) eosinophils and their progenitors [6–9]. In addition to the BM, increased numbers of mature eosinophils as well as eosinophil progenitors

have been reported within the bronchial mucosa [10] as well as in induced sputum from allergic subjects with asthma [11]. Recent studies in allergen-challenged mice also demonstrated increased levels of CD34⁺45⁺IL-5R α ⁺ cells in lung tissue associated with elevated levels of interleukin (IL)-5 and eotaxin in the bronchoalveolar lavage fluid (BALF) that can promote *in situ* differentiation of these cells [12]. Likewise, previous studies from our laboratory have demonstrated that constitutive overexpression of IL-5 in IL-5 transgenic mice induces generation of lineage-specific progenitors, including eosinophil progenitors, in the spleen, suggesting that in diseases associated with chronic elevated IL-5 levels such as asthma, mature inflammatory cells, including eosinophils, can potentially be generated outside of the BM at extramedullary sites [13]. Overall, these studies suggest that committed progenitors can migrate to extramedullary sites and potentially undergo differentiation into mature cells, although the factors

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enabling trafficking of these progenitor cells out of the BM milieu to these sites are poorly understood.

In addition to the recruitment of inflammatory cells, persistent allergic asthma is also associated with structural changes in the airways leading to airway remodeling. In mice, chronic and repetitive allergen challenge leads to airway remodeling, which is attributed to the accumulation of elevated levels of inflammatory mediators, such as IL-5, IL-13, transforming growth factor (TGF)- β , matrix metalloproteinases (MMPs) [1, 14], and factor X [15], amongst others, in the lungs, with eosinophils, fibroblasts, epithelial cells, and macrophages being the major cellular source for these mediators. These studies clearly demonstrate that, in addition to eosinophils, the generation, maturation, and recruitment of other inflammatory leukocytes are also an important consideration during chronic airway allergic inflammation. Although most of the studies discussed above are focused on the generation of eosinophil-specific progenitor cells, the effect of allergen challenge on the generation of progenitors of other lineages in the BM or their egress to sites of inflammation including the airways during sustained and repetitive allergen exposure remains poorly understood. In the present study, we have evaluated the effect of short-term (acute) versus chronic allergen challenge over a 3-month period on the generation of inflammatory cell progenitors in the BM as well as in the airways (BALF) in a murine model of ovalbumin (OVA)-induced airway allergic inflammation.

METHODS

Cell lines

STR-12, a murine BM-derived endothelial cell (EC) line provided by Dr. Masanobu Kobayashi, Hokkaido School of Medicine, Sapporo, Japan [16], was cultured in RPMI supplemented with 10% fetal calf serum (FCS).

Sensitization and allergen challenge

Female BALB/c mice (7 to 8 weeks) were anesthetized by inhalation with 5% isoflurane and sensitized with OVA (Grade V; Sigma Chemical, St Louis, MO) in alum by subcutaneous injections (25 μ g/mouse) on days 0, 7, 14, and 21 and then challenged with OVA (20 μ g in phosphate-buffered saline [PBS]/mouse) by intranasal route on days 23, 25, and 28, followed by additional biweekly challenges for 8 weeks, which is known to induce airway remodeling in mice [17]. Age- and gender-matched BALB/c mice

that were sensitized and challenged with PBS instead of OVA served as controls. Mice were evaluated on day 29 (acute allergen challenge) or day 85 (chronic allergen challenge). In all cases, mice were euthanized by CO₂ asphyxiation 24 hours after the last challenge. The care and maintenance of mice during performance of these studies was in accordance with institutional guidelines.

Collection of BALF and BM

BALF was collected immediately after euthanasia by lavage using 2 washings, each with 0.5 mL of sterile PBS, that were pooled. An aliquot was used to determine total cell counts as well as differential cell counts from cytocentrifuged slides stained with Diff-Quik based on morphologic and histologic criteria. Remaining cells were centrifuged and resuspended in RPMI. BM was collected by flushing the femurs with a syringe using sterile RPMI (0.5 to 0.75 mL) and suspending it uniformly in the same medium. BALF and BM cell suspensions were maintained on ice until ready for assay.

Long-term BM culture (LTBMC)

Freshly isolated BM cells were cultured for 8 weeks in MyeloCult M5300 medium (StemCell Technologies, Vancouver, BC, Canada) as described previously [18]. During this period, primitive hematopoietic stem cells (HSCs) undergo proliferation and nonadherent cells in these cultures, which include mature cells as well as their hematopoietic progenitors at different stages of differentiation, are indicative of hematopoietic activity. At the end of each week, the nonadherent cells were collected by aspiration and counted in a hemocytometer. The adherent layer was evaluated under a Leica DM IRB/E inverted microscope (Leica Microsystems, Wetzlar, Germany) at a magnification of 20 $\times$. Images were captured with a Hamamatsu C4742-95 digital camera (Hamamatsu, Japan).

Assay for colony-forming units (CFU)

BALF and BM cells were plated (1×10^4 cells/mL) on methylcellulose medium (MethoCult 3434 medium, StemCell Technologies) supplemented with stem cell factor (SCF), IL-3, IL-6, and erythropoietin (Epo) to evaluate multipotential progenitor cells as recommended by the manufacturer. To evaluate lineage-specific progenitors, BM cells were cultured in MethoCult 3234 (StemCell Technologies) containing granulocyte-macrophage colony-stimulating factor (GM-CSF) (10 ng/mL) for granulocyte

macrophage (CFU-GM), IL-5 (50 ng/mL) for eosinophil (CFU-Eos), or IL-7 (10 ng/mL) for B-lymphoid progenitors at 1×10^4 , 5×10^5 , and 5×10^4 cells/mL, respectively, as described in our previous study [18]. Cultures were observed under an inverted microscope and colonies were identified as described in the Atlas of Human Hematopoietic Colonies published by StemCell Technologies (http://www.stemcell.com/technical/28405_methocult%20M.pdf). The number of colonies were counted in situ and expressed as CFU/number of cells plated.

In vitro laminar flow assay

The interaction of BM cells from control as well as acute and chronic allergen-exposed mice with STR-12 BM-derived ECs was assessed in an in vitro parallel-plate laminar flow chamber as described in our previous studies [19, 20]. Briefly, confluent cultures of STR-12 cells on poly-L-lysine-coated glass coverslips were treated with murine tumor necrosis factor (TNF)- α (50 ng/mL; BD Biosciences, San Jose, CA) for 6 hours at 37°C before exposure to flow conditions (wall shear stress ~ 1.0 dyn/cm²) by perfusing warm medium (RPMI) through a constant-infusion syringe pump (Harvard Apparatus, Holliston, MA). BM cell suspensions (5 mL, 2×10^5 cells/mL) were perfused into the flow chamber for a period of 5 minutes. The interaction of the injected cells with STR-12-coated coverslips was observed using a Leitz Wetzlar inverted microscope as previously described [19, 20]. The images were recorded for subsequent offline analysis to manually determine the number of interacting cells. Cells demonstrating multiple discrete interruptions in flow and moving more slowly were considered as rolling cells and the results were expressed as the number of rolling cells per minute.

Enrichment of murine hematopoietic/stem progenitor cells (HSPCs)

For migration studies and evaluation of CXCR4 expression, HSPCs in the BM were enriched using a kit (StemSep Mouse Progenitor Enrichment Cocktail; catalog no. 13056, StemCell Technologies) and analyzed for CD34 expression by flow cytometry as described in our previous study [18]. This procedure routinely led to a 3-fold enrichment of CD34⁺ cells (data not shown). Due to the low recovery after enrichment, BM recovered from individual mice within the allergen-challenged and control groups were pooled prior to enrichment of HSPCs.

Migration assay

Migration of enriched HSPCs across STR-12 BM ECs in response to stromal cell-derived factor (SDF)-1, a chemoattractant produced by the BM stroma, was assessed as previously described [18]. Briefly, enriched HSPCs from pooled BM of OVA-challenged or control mice were added to the upper wells of Transwell clusters (Costar, Boston, MA) containing a confluent layer of STR-12 cells. Cells that migrate to the lower wells in response to SDF-1 (50 nM) were counted and percent migration was determined based on the total number of cells added to the upper well of the Transwell clusters. Results are expressed as percent migration assuming migration of HSPCs from control mice to be 100%.

Flow cytometry

Expression of CD34 and IL-5R α (CD125) by BALF cells from acute and chronic allergen-challenged and respective control mice was determined using rat anti-mouse CD34-Alexa 647 and rat anti-mouse CD125-PE (phycoerythrin) primary conjugated antibodies (both from BD Biosciences). Rat IgG2a-Alexa 647 and rat IgG1-PE were used as controls. All reagents were used at a concentration of 10 μ g/mL. Red cells in BALF were lysed with deionized water and remaining cells were incubated with 1 μ g of TruStain fcX Fc blocking antibody (BioLegend, San Diego, CA) for 15 minutes prior to incubation with the antibodies. Results are expressed as percent cells positive for CD34 and CD125. Surface expression of CXCR4, the SDF-1 receptor, on enriched HSPCs isolated from pooled BM of chronic allergen-challenged or control mice was determined using a rabbit polyclonal CXCR4 antibody (4 μ g/10⁶ cells) against murine CXCR4 (ProSci, Poway, CA) in the presence of isotype-matched control antibodies followed by fluorescein isothiocyanate (FITC)-conjugated anti-rabbit immunoglobulin G (IgG) (Sigma Chemical, St Louis, MO) on a FACSCanto II or FACScan flow cytometer equipped with FlowJo or CellQuest Pro flow cytometry analysis software (BD Biosciences). Antibody incubations were carried out at 4°C for 30 minutes. The number of enriched HSPCs that were positive for CXCR4 from allergen-challenged and control mice was determined. Results are expressed as percent CXCR4 expression by OVA-challenged HSPCs assuming expression by control HSPCs to be 100%.

Trafficking in BM microcirculation

Trafficking of BM cells in BM microvessels in the skulls of anesthetized BALB/c mice was evaluated

by intravital microscopy (IVM) as described previously [21]. Briefly, 5- to 6-week-old naïve BALB/c were anesthetized and an incision made midline in the scalp exposing the frontoparietal skull ensuring that the bone tissue remains intact. A plastic ring was inserted in the incision to keep the incised skin apart and in place allowing for application of sterile saline to prevent drying of the tissue. The mouse was placed on a Plexiglas stage equipped with a stereo tatic holder (David Kopf Instruments, Tujunga, CA) to immobilize the head. BM cells collected from acute and chronic allergen-challenged mice as well as corresponding control mice were labeled with carboxyfluorescein succinimidyl ester (CFSE) and infused into the anesthetized BALB/c mice via the jugular vein. Interaction of the labeled cells with the BM microvascular endothelium in the skull was visualized by stroboscopic epi-illumination using a Xenon lamp and all images were recorded using StreamPix digital video recording software (NorPix, Montreal, Quebec, Canada) for off-line analysis as described previously for trafficking in lung microvessels [20]. Cells visibly interacting with the BM microvascular endothelium and passing at a slower rate than the main blood stream were considered as rolling cells and were quantitated by manually counting the number of rolling cells passing through a reference point in a vessel segment (200 μ m) for a certain duration of time and expressed as the number of rolling cells per minute. Rolling in 5 to 8 microvessels were analyzed per mouse on an average.

Statistical analysis

Results are expressed as the mean $\pm$ SE. Statistical significance was determined by 2-tailed unpaired Student's *t* test. A *P* value < .05 was considered as significant.

RESULTS

Chronic allergen challenge is associated with persistent airway eosinophilia

Sensitization followed by acute allergen challenge resulted in a significant increase in the number of eosinophils recovered from the BALF compared to control mice that were sensitized and challenged with PBS (Figure 1). Chronic OVA challenge up to 12 weeks resulted in sustained airway eosinophilia. Although the actual number of eosinophils in the BALF of chronic allergen-challenged mice was lower than that observed in acute allergen-challenged mice (as noted in previous studies as well [22]), eosinophil

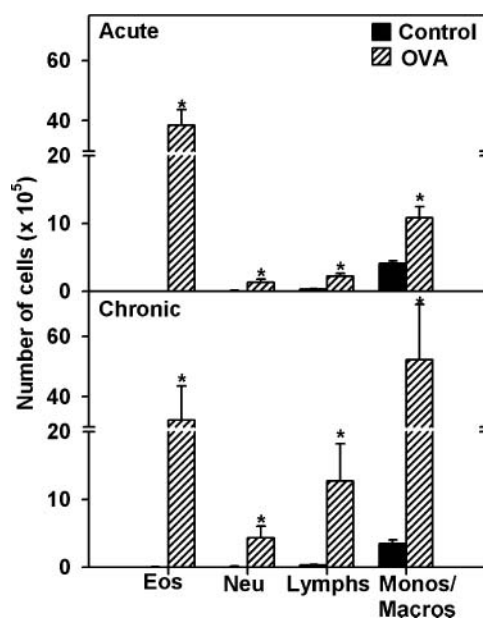


FIGURE 1 Chronic allergen challenge is associated with sustained BALF eosinophilia. BALB/c mice ($n = 10$ – 11 mice/group) were sensitized and challenged with OVA in alum up to 4 or 12 weeks. Control mice received PBS instead of OVA. Differential cell counts in BALF were evaluated by microscopic evaluation of cytocentrifuged slides stained with Diff-Quik and expressed as mean $\pm$ SE of the number of cells $\times 10^5$. **P* < .05 versus respective control.

counts remained significantly elevated compared to control mice. In addition, acute allergen challenge induced an influx of monocytes/macrophages to the airways, which was further enhanced following chronic allergen challenge. Similarly, the small but significant increase in the number of neutrophils and lymphocytes that was observed in BALF of acute OVA-challenged mice compared to control mice was further elevated after chronic challenge.

Effect of allergen challenge on hematopoietic activity in the BM

Since the BM is the major hematopoietic tissue in adult mice, the effect of repeated allergen challenge on the generation, maintenance, and proliferation of HSPCs in the BM was analyzed. LTBMCs were initiated using BM cells freshly isolated from OVA-challenged and control mice. These cultures serve as an experimental *in vitro* model of hematopoiesis, since they contain hematopoietic elements at various stages of differentiation as well as a supportive stromal microenvironment. Although there was no significant difference in the total number of cells recovered from the BM of allergen-challenged and control mice

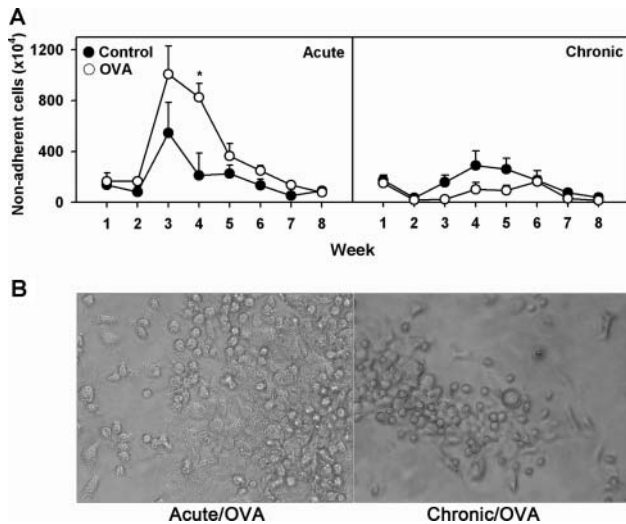


FIGURE 2 BM cells of chronic allergen-exposed mice exhibit diminished proliferation. **(A)** Nonadherent cells withdrawn from LTBM of acute and chronic allergen-challenged and control mice ($n = 9-10/\text{group}$) during weekly feedings were counted and expressed as mean $\pm$ SE $\times 10^4$. **(B)** Photomicrographs of the adherent layer in LTBM of representative acute and chronic allergen-challenged mice at 4 to 5 weeks of culture are shown. Magnification $20\times$. * $P < .05$ versus control.

(53.6 ± 2.1 versus $62.0 \pm 4.49 \times 10^6$ cells after acute challenge and 50.9 ± 7.9 versus $34.9 \pm 8.1 \times 10^6$ cells after chronic challenge, respectively, $P > .05$), considerable differences were observed between the 2 groups in the proliferation of BM cells and formation of an adherent layer in LTBM. BM cells from acute allergen-challenged mice exhibited increased proliferation assessed by the number of nonadherent cells recovered between 3 and 5 weeks ($P < .05$ at 4 weeks of culture) compared to control BM cultures (Figure 2A, left panel). In contrast, BM cells from mice exposed to chronic allergen challenge exhibited no difference in the number of nonadherent cells recovered from LTBM compared to control mice (Figure 2A, right panel). Further, this was associated with the lack of formation of a well-organized hematopoiesis-supportive adherent layer. The adherent layer formed in LTBM of chronic allergen-challenged mice appeared more "patchy" (Figure 2B, right panel) compared to the more uniform confluent adherent layer in cultures from acute allergen-challenged mice (Figure 2B, left panel).

To evaluate the potential of HSPCs in the BM of allergen-exposed mice to generate multipotent progenitor cells and to differentiate into lineage-specific colonies of myeloid (CFU-Eos and CFU-GM) or lymphoid (CFU-B) origin, CFU assays were performed in the presence of specific cytokines using sin-

gle cell suspensions of the BM from OVA-challenged and control mice. In the case of cultures from acute allergen-challenged mice, a significant increase was observed in generation of eosinophil progenitors, but not multipotent, GM or B-lymphoid progenitors compared to control mice (Figure 3). Chronic allergen exposure resulted in sustained increase in generation of eosinophil progenitor cells in the BM but also significantly induced the capacity for generation of multipotent progenitor cells as well as CFU-GM and CFU-B compared to cultures from corresponding control mice (Figure 3). These data suggest that acute allergen exposure induces HSPCs to differentiate predominantly into colonies of eosinophilic lineage in the BM, whereas chronic allergen exposure enhances the potential of HSPCs to differentiate into committed cells of myeloid (CFU-Eos and CFU-GM) as well as lymphoid (CFU-B) lineage in the presence of specific cytokines.

Chronic allergen challenge results in increased presence of myeloid progenitors in the airways

There is increasing evidence for the presence of eosinophil progenitors in the bronchial mucosa and sputum of allergic subjects with asthma [10, 11] as well as in the lung tissue of acute allergen-challenged mice [12]. We investigated the effects of acute versus chronic allergen exposure on hematopoietic activity in the lung by evaluating the BALF for the presence of multipotent as well as lineage-specific progenitor cells using CFU assays (Figure 4). Acute allergen exposure did not indicate the presence of multipotent or GM-specific progenitor cells in the BALF (Figure 4A, upper and lower panels). In contrast, chronic allergen exposure was found to induce the capacity for generation of GM progenitor cells in the airways compared to corresponding controls (Figure 4A, lower panel), whereas the increase in multipotent progenitors was not found to be statistically significant. Since extremely low numbers ($\sim 1-2$ CFU/ 5×10^5 cells) of eosinophil progenitors (CFU-Eos) were detected when BALF cells from acute and chronic allergen-exposed mice were cultured in IL-5, the occurrence of eosinophil progenitors (CD34⁺/CD125⁺) in the airways was evaluated by flow cytometry. A small increase in the number of eosinophil progenitors was observed in the BALF of acute allergen-challenged mice compared to control mice (Figure 4B). On the other hand, BALF of chronic-allergen-challenged mice demonstrated a significant increase in the number of CD34⁺/CD125⁺ cells present. No B lymphoid-specific colonies were

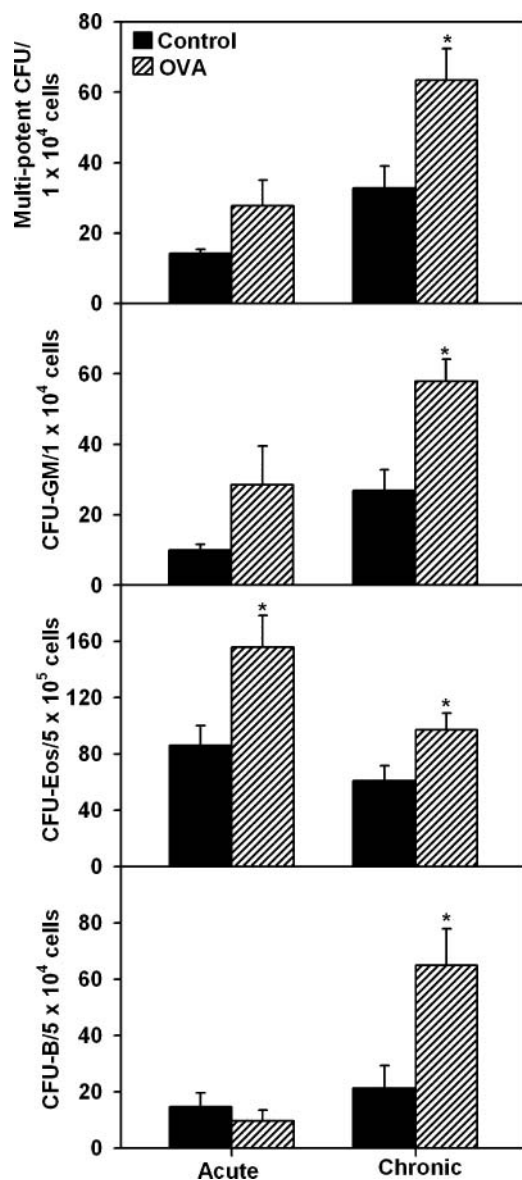


FIGURE 3 Effect of allergen challenge on the generation of lineage specific progenitors in the BM. Freshly isolated BM cells from acute and chronic allergen-challenged as well as respective control mice ($n = 9-10/\text{group}$) were cultured in media supplemented with SCF, IL-3, IL-6, and Epo to evaluate the presence of multipotent progenitor cells or in media containing GM-CSF, IL-5, or IL-7 for presence of GM, eosinophil, or B-lymphocyte progenitors, respectively. Results are expressed as mean CFU $\pm$ SE/number of cells plated in each case. * $P < .05$ versus respective control.

detected in BALF cell cultures from acute or chronic allergen-challenged mice (data not shown).

Overall, these data suggest that acute allergen challenge largely results in activation of BM hematopoiesis involving proliferation of BM cells

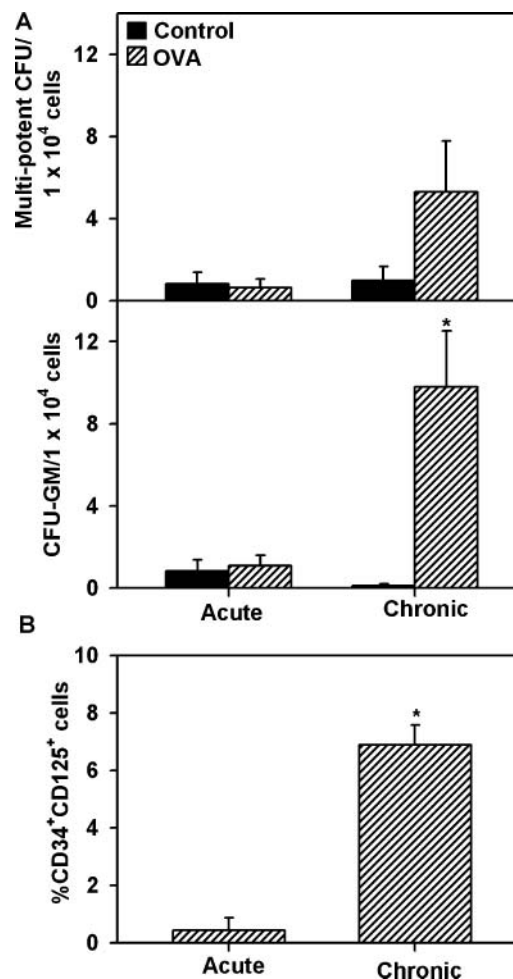


FIGURE 4 Chronic allergen challenge induces generation of GM and eosinophil progenitors in the airways. (A) Cells collected from the BALF of acute and chronic allergen-challenged as well as control mice ($n = 9-10/\text{group}$) were cultured in media supplemented with specific cytokines to evaluate the presence of multipotent and lineage specific progenitor cells. Results are expressed as mean CFU $\pm$ SE/number of cells plated in each case. (B) BALF cells of allergen-challenged ($n = 6$ mice each for acute and chronic) and control mice (pooled BALF from 6 mice) were analyzed by flow cytometry for expression of CD34 and CD125 to identify eosinophil progenitors. Results are expressed as percent CD34⁺CD125⁺ cells. Percent cells positive for CD34 and CD125 was less than 0.05 in control mice. * $P < .05$ versus percent CD34⁺CD125⁺ cells in acute mice.

(Figure 2A, left panel) and generation of eosinophil-specific progenitors (Figure 3) with minimal hematopoietic activity in the lungs (Figure 4A and B). With chronic allergen exposure, not only was there a continued generation of eosinophil-specific colonies, but also an induction of the generation of multipotent, B lymphocyte- and GM-specific

progenitors in the BM (Figure 3). More importantly, there is initiation of hematopoietic activity in the airways with an increased presence of GM and eosinophil progenitor cells in the BALF (Figure 4A, lower panel, and B).

Allergen challenge does not alter rolling of BM cells on BM endothelium

To determine whether allergen exposure alters the interaction of BM cells with the vascular endothelium thus affecting cell trafficking, rolling of BM cells from acute and chronic allergen-challenged as well as respective control mice on BMECs was investigated using a flow chamber assay (Figure 5A). No difference was observed in rolling of BM cells from acute or chronic allergen-challenged mice compared to their respective controls, suggesting that allergen challenge does not affect rolling of BM cells. These studies were further confirmed *in vivo* under physiological conditions of blood flow by IVM, where BM cells from acute or chronic allergen-challenged mice infused into the BM microcirculation of the skull of naïve mice demonstrated rolling similar to corresponding control BM cells (Figure 5B and C).

Chronic allergen challenge inhibits migration of HSPCs

Since isolation of lineage specific progenitors in sufficient numbers is technically challenging, CD34⁺-enriched HPSCs from BM were used in the following studies. Migration of CD34⁺-enriched HPSCs of control and allergen-challenged mice across BMECs in response to SDF-1, a chemoattractant that plays a key role in repopulation of the BM, was evaluated. Chronic allergen-exposure was found to significantly decrease the ability of HSPCs to transmigrate in response to SDF-1 by >2.5-fold, whereas HSPCs from mice exposed to acute allergen challenge exhibited only a modest decrease in migration that was not statistically significant compared to corresponding control cells (Figure 6A). More importantly, the decrease in migration of HSPCs from chronic allergen-exposed mice was associated with a substantial decrease in expression of CXCR4, the SDF-1 receptor, compared to control cells (Figure 6B).

DISCUSSION

Late-phase allergic airway responses involve Th2 cell activation and elaboration of cytokines and chemokines, which in turn regulate the generation and maturation of proinflammatory leukocytes,

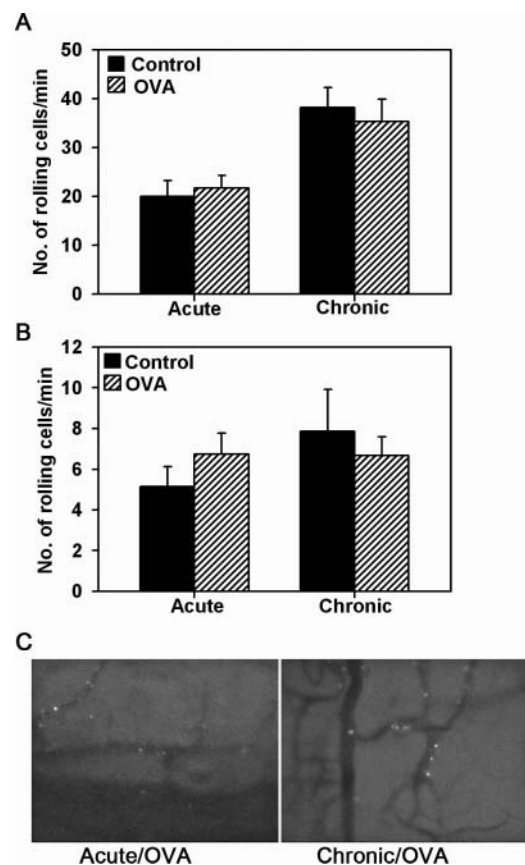


FIGURE 5 Allergen challenge does not alter rolling of BM cells on BM endothelium. (A) Single-cell suspensions of BM from allergen-challenged and control mice ($n = 6$ mice/group) were perfused into a laminar flow chamber and their ability to roll on STR-12 EC-coated coverslips under conditions of flow was evaluated. The assay was run in duplicate and the results are expressed as mean $\pm$ SE of the number of rolling cells per minute. (B) The interaction of CFSE-labeled BM cells from acute and chronic allergen-challenged as well as control mice with BM microvascular endothelium in the skulls of naïve mice was evaluated by IVM ($n = 5-8$ microvessels/mouse, 3-4 mice/group). Representative photomicrographs of CFSE-labeled BM cells from acute (left) and chronic (right) allergen-challenged within BM microvessels at a magnification of 10 $\times$ are shown.

predominantly eosinophils, in the BM and their recruitment into sites of inflammation [1, 23]. The allergic response is also associated with airway hyperreactivity (AHR) and during chronic allergic airway inflammation including asthma, there is structurally and functionally abnormal remodeling of the airways [24], mediated in part by the persistent Th2 inflammatory responses [25, 26]. Airway remodeling is also mediated by factors such as TGF- β , MMPs,

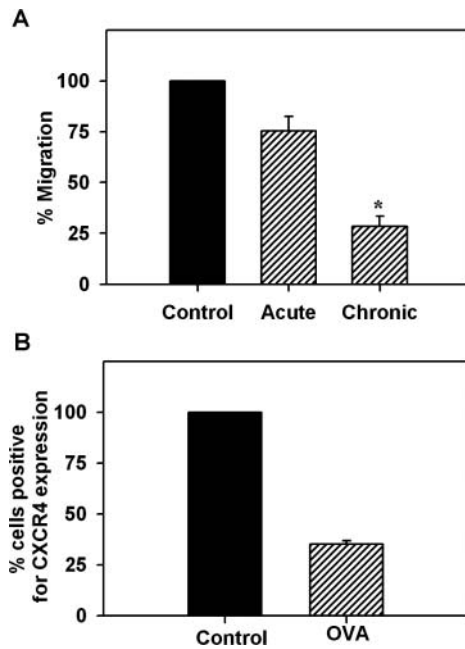


FIGURE 6 Exposure to chronic allergen challenge inhibits transendothelial migration and expression of CXCR4 by HSPCs. (A) Migration of enriched HSPCs from pooled BM of allergen-challenged and control mice ($n = 5$ mice/group for acute and 10 mice/group for chronic) across STR-12 EC layers in response to SDF-1 (lower well) was assessed by performing migration assays in Transwell clusters. Results are expressed as percent migration assuming the migration of HSPCs from control mice to be 100%. (B) Enriched HSPCs isolated from pooled BM of chronic allergen-challenged or control mice ($n = 8$ mice/group) were analyzed for surface expression of CXCR4 by flow cytometry. Results are expressed as percent CXCR4 expression by OVA-challenged HSPCs assuming expression by control HSPCs to be 100%. * $P < .05$ versus control.

and factor X released by inflammatory cells such as eosinophils, epithelial cells, and macrophages in the lungs [15, 22, 27, 28].

As shown in animal models [8, 9] and atopic asthmatic individuals [6, 7], the BM is activated during episodes of allergen challenge, demonstrating increased numbers of mature eosinophils as well as IL-5-sensitive eosinophilic progenitor cells. Although most of these studies are focused on the generation of eosinophil-specific progenitors, the effect of chronic allergen challenge on the generation of other inflammatory cells that contribute to the overall pathogenesis of allergen-induced airway inflammation in addition to eosinophil, which might be a more relevant model for studying symptomatic disease, is not com-

pletely clear. We investigated the effects of acute versus chronic allergen challenge on the generation of multipotent and lineage-specific progenitor cells in the BM and lungs in a murine model. Allergen challenge up to 12 weeks resulted in significant BALF eosinophilia (Figure 1). Although airway eosinophilia in response to acute allergen challenge was associated with BM proliferation (Figure 2A, left panel), persistent allergen exposure up to 12 weeks resulted in a loss of the ability of BM cells to proliferate (Figure 2A, right panel). This may in part be due to the formation of a nonconfluent "patchy" adherent layer in LTBM (Figure 2B, right panel), as opposed to a more uniform and confluent adherent layer observed in LTBM of acute allergen-challenged mice (Figure 2B, left panel). Stromal cells contribute to the formation of niches for HSPCs and support their proliferation and differentiation [29] through the release of important hematopoietic growth factors that in turn provide cues for multiplication, differentiation, and maturation of these cells [30]. One example is SDF-1, which plays a role in chemotaxis, survival, and proliferation of colony-forming progenitor cells [30] as well as in the regulation of trafficking of HSPCs and their homing/retention in the BM [31]. Therefore, an impaired/abnormal stromal cell layer is likely to affect HSC proliferation.

In consistence with previous studies [8], BALF eosinophilia in response to short-term allergen challenge was associated with significantly increased capacity to generate eosinophil-specific progenitors (CFU-Eos) in the BM (Figure 3). However, no significant differences in the generation of multipotent, GM (CFU-GM), or lymphoid (CFU-B) progenitors were observed. Interestingly, with exposure to chronic allergen challenge, the capacity of BM HSPCs to generate multipotent progenitor cells was induced. In addition, not only was there a continued increase in generation of CFU-Eos, but also the potential to differentiate into CFU-GM and CFU-B was observed in the presence of lineage-specific cytokines compared to control HSPCs (Figure 3). The decreased proliferative ability (Figure 2A, right panel) but increased capacity to generate multipotent and lineage specific progenitors by chronic allergen-challenged BM cells may be driven by factors such as TGF- β 1, which is elevated during chronic allergic airway inflammation [1] and has been shown to inhibit the proliferation of primitive murine hematopoietic cells [32] and function as a critical regulator of HSC homeostasis in vivo [33]. Elevated TGF- β 1 levels may disrupt this homeostasis, causing the switch from the proliferative to a more multipotent nature for HSCs in the presence of elevated levels of colony-stimulating factors and cytokines (IL-5 and IL-4)

associated with chronic allergic inflammation. However, additional studies are required to confirm this.

Increased generation of eosinophilic as well as GM and B-lymphoid progenitors in the BM during chronic inflammation appears to be in accordance with the overall spectrum of allergic airway disease where late-phase allergic airway responses involve inflammation associated predominantly with airway eosinophilia and AHR, whereas chronic continuous allergen exposure induces not only eosinophilic inflammation but also airway remodeling that is driven by cellular factors released by cells of monocyte/macrophage-lineage such as TGF- β [22], MMPs [28], and the β -galactoside-binding lectin galectin-3 [34] in addition to eosinophils. Further, alveolar macrophages and alveolar macrophage-derived cytokines have also been shown to be associated with airway remodeling in OVA-challenged rats [35]. Studies have also shown that chronic continuous allergen exposure in mice results in the development of local inhalational tolerance associated with a persistence of B cells in the BALF and hilar lymph nodes and suggest a novel regulatory role for regional B cells in the establishment of tolerance in chronic allergic airway inflammation by conversion of CD4⁺CD25⁻ T-effector cells into functionally suppressive CD4⁺CD25⁺Foxp3⁺ T-regulatory cells [36]. Although we did not specifically evaluate B-cell recruitment to the airways in our studies, total lymphocytes (B and T cells) in the BALF of chronic allergen-challenged mice were higher than lymphocytes in the BALF of acute allergen-challenged mice (Figure 1).

The presence of eosinophil progenitors (CD34⁺/CD125⁺) in the bronchial mucosa [10] and induced sputum [11] of allergic subjects with asthma suggests that progenitor cells can migrate to the lungs and potentially undergo differentiation into mature cells during conditions of chronic airway inflammation. In the present study, short-term allergen exposure did not result in multipotent or lineage-specific colony formation (CFU-Eos, CFU-GM, or CFU-B) in the presence of specific cytokines in the airways (BALF) (Figure 4A). However, analysis of BALF cells from acute allergen-challenged mice by flow cytometry indicated the presence of a small percentage of CD34⁺/CD125⁺ cells (Figure 4B). These findings are in consistence with previous studies in acute allergen-challenged mice demonstrating CD34⁺CD45⁺CD125⁺ cells in lung tissue of allergen-challenged mice [12]. However, it is important to note that in the present study BALF was used for enumeration of CD34⁺/CD125⁺ eosinophil progenitors cells, as opposed to lung tissue cells (extracted by enzymatic digestion of

whole lung tissue) in the previous study, which would include recruited eosinophilic progenitors lodged in the lung tissue [12] and probably be present in larger numbers than in the BALF. It is highly likely that extramedullary proliferation and differentiation of eosinophil progenitors needs to take place within the pulmonary tissue under the control of specific cytokines and chemokines. In fact, there is considerable evidence to support this [37–39]. More importantly, chronic allergen exposure indicated the presence of a significantly larger number of CD34⁺/CD125⁺ eosinophil progenitors in the BALF compared to acute allergen challenge. Overall, these studies demonstrate increased presence of eosinophil progenitors in the airways relative to the extent of allergen exposure (acute versus chronic). Of additional significance is the observation that chronic allergen exposure up to 12 weeks results in increased presence of not only eosinophil but also GM progenitors in the BALF (Figure 4A). Since GM-specific progenitors give rise to granulocytes (eosinophils and neutrophils) and monocytes/macrophages [40], upon maturation in the airways, they can contribute to chronic inflammation, particularly those features driven by cellular factors released by inflammatory cells of GM lineage such as macrophages.

The previous demonstration of eosinophil progenitors (CD34⁺/IL-5R α ⁺) in the bronchial mucosa [10] and induced sputum [11] of allergic subjects with asthma and our current findings of GM and eosinophil progenitors in the BALF support the concept that during episodes of allergic inflammation, committed progenitor cells or HSPCs can traffic out of the BM milieu and migrate to sites of inflammation such as the lungs and potentially differentiate into mature cells in the presence of specific cytokines produced during conditions of chronic airway inflammation [41]. Recent studies have shown that migrating HSPCs contribute to the continuous restoration of specialized hematopoietic cells that reside in peripheral tissues and upon exposure to a stimulus proliferate locally within extramedullary tissues to generate innate immune effector cells [42]. Presence of progenitor cells in the airways in response to chronic allergen challenge described in our studies (Figure 4A and B) may be due to altered trafficking or homing of HSPCs, leading to their inability to be retained in the BM, allowing them to potentially emigrate to these sites. The effect of allergen challenge on BM cell rolling, a critical first step in the process of trafficking and migration was investigated on BMECs and under physiological conditions within BM microvessels (Figure 5). These studies demonstrated that allergen challenge does not alter rolling of BM cells. However, HSPC migration was affected (Figure 6). Homing

of HSPCs to the BM involves SDF-1-mediated migration and recruitment via interaction with HSPC-expressed CXCR4 [43]. SDF-1, constitutively expressed and produced by stromal cells and BMECs, induces chemotaxis of both committed and primitive hematopoietic progenitors [44]. Therefore, in order for progenitor cells to be able to traffic out of the BM to sites of inflammation, one would anticipate decreased interactions between HSPCs and BMECs or a disruption of the SDF-1/CXCR4 axis. Studies in asthmatic subjects have shown that there is a down-regulation in CXCR4 intensity on BM CD34⁺ cells as well as a reduction in SDF-1 α levels after allergen inhalation [45]. In the present study, CD34-enriched cells from chronic, but not acute, allergen-challenged mice exhibited significantly decreased migration across BMECs in response to SDF-1 and also expressed substantially lower levels of CXCR4 compared to cells from control mice (Figure 6A and B). Accordingly, decreased migration across BMECs of the BM sinusoidal vessel by CD34-enriched cells from chronic allergen-challenged mice may alter their ability to be retained in the BM, enabling them to migrate to the lungs and airways where they could proliferate and/or mature. Studies have shown that Th2 cytokines IL-4, IL-5, and IL-13, all of which are elevated during chronic allergic airway inflammation, can inhibit CXCR4 expression by eosinophils [46] and monocytes [47]. It is possible that a similar effect is exerted by these cytokines on progenitor cells of eosinophil and macrophage lineages in the BM in response to chronic allergen exposure (Figure 3), altering their ability to home to the BM and enabling their egress out of the BM milieu to extramedullary sites to yield mature inflammatory cells that are responsible for the persistent airway inflammation associated with chronic allergen exposure.

The BM responses as well as the response at extramedullary sites, i.e., airways/lung, in the present study appear to be specific to airway inflammation, since evaluation of the spleen did reveal any evidence of extramedullary hematopoiesis (CFU-Eos, CFU-B, or CFU-GM) (data not shown). In a previous study we demonstrated that sustained exposure of mice to nicotine induced significant hematopoiesis in the spleen, with only a modest effect on the BM [18]. Further, in a recent study, chronic inflammation was found to result in mobilization of developing B cells to the blood and peripheral lymphoid sites where exposure to antigens and inflammatory agents are more common [48]. Therefore, although chronic inflammation can lead to extramedullary hematopoiesis, it is likely that the target organ is more specific to the factors inducing the inflammation.

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