Published in final edited form as:

Stem Cells. 2009 December ; 27(12): 3074–3081. doi:10.1002/stem.222.

CXCR2 Mediates the Recruitment of Endothelial Progenitor Cells During Allergic Airways Remodeling

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Abstract

Airway remodeling is a central feature of asthma and includes the formation of new peribronchial blood vessels, which is termed angiogenesis. In a number of disease models, bone marrow-derived endothelial progenitor cells (EPCs) have been shown to contribute to the angiogenic response. In this study we set out to determine whether EPCs were recruited into the lungs in a model of allergic airways disease and to identify the factors regulating EPC trafficking in this model. We observed a significant increase in the number of peribronchial blood vessels at day 24, during the acute inflammatory phase of the model. This angiogenic response was associated with an increase in the quantity of EPCs recoverable from the lung. These EPCs formed colonies after 21 days in culture and were shown to express CD31, von Willebrand factor, and vascular endothelial growth factor (VEGF) receptor 2, but were negative for CD45 and CD14. The influx in EPCs was associated with a significant increase in the proangiogenic factors VEGF-A and the CXCR2 ligands, CXCL1 and CXCL2. However, we show directly that, while the CXCL1 and CXCL2 chemokines can recruit EPCs into the lungs of allergen-sensitized mice, VEGF-A was ineffective in this respect. Further, the blockade of CXCR2 significantly reduced EPC numbers in the lungs after allergen exposure and led to a decrease in the numbers of peribronchial blood vessels after allergen challenge with no effect on inflammation. The data presented here provide in vivo evidence that CXCR2 is critical for both EPC recruitment and the angiogenic response in this model of allergic inflammation of the airways.

Keywords

CXC chemokines; Progenitor cell; Asthma; Angiogenesis

Introduction

Asthma is a chronic disease of the airways that leads to structural changes of the lungs, termed remodeling, that are associated with angiogenesis. The factors and mechanisms driving this angiogenic response have not been explored in the context of this disease. While tissue resident mature endothelial cells are involved in angiogenesis, it is thought that bone marrow-derived endothelial progenitor cells (EPCs) may also contribute to this process [1, 2]. Thus in models of ischemia or inflammation it has been shown that EPCs mobilized from

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Author contributions: C.P.J.: Performance of experiments; S.C.P.: Collection and assembly of data; C.P.J.: Collection and assembly of data, figure creation; C.M.L., S.C.P., S.M.R., and C.P.J.: Conception and design, manuscript writing.

Disclosure of Potential Conflicts of Interest The authors indicate no potential conflicts of interest.

the bone marrow are recruited to tissues, where they can proliferate and facilitate the formation of new blood vessels [3, 4]. A recent study has shown that asthmatic patients exhibit elevated numbers of circulating EPCs, which correlates with increased numbers of peribronchial blood vessels [5]. However, the contribution of EPCs to angiogenesis induced in asthmatic patients is not known. Previous studies have reported that EPCs express CXCR2 and vascular endothelial growth factor (VEGF) receptor 2 in both humans and rodents [6, 7], suggesting that these may be likely candidate receptors mediating EPC recruitment. VEGF-A is a potent proangiogenic factor, and the levels of VEGF-A in the bronchoalveolar lavage fluid (BALF) of asthmatic patients have been correlated with the extent of angiogenesis [8–12]. Overexpression of VEGF-A in the lungs has also been shown to induce inflammation, mucus hyperplasia, edema, and airway hyper-reactivity [13, 14]. CXCR2 and its ligands, CXCL1 and CXCL2 (also known in the mouse as KC and MIP-2, respectively), have been shown to promote angiogenesis in vivo and in vitro [15–19]. Furthermore, it has been reported that CXCR2 is essential for EPC recruitment after arterial injury [6]. The role of CXCR2 and its ligands in the development of angiogenesis associated with allergic airways disease has not previously been investigated.

This study shows that CXCR2 and its ligands, CXCL1 and CXCL2, are essential for the recruitment of EPCs to the lungs of allergen-challenged and allergen-sensitized mice. We have shown that angiogenesis occurs in the early stages of the disease and is further increased during progression of airway remodeling. Importantly, we have demonstrated, for the first time, that treatment with anti-CXCR2 antibody abrogates angiogenesis around the airways after allergen challenge.

Materials and Methods

Reagents

General laboratory reagents were purchased from either Life Technologies (Rockville, MD, <http://www.lifetech.com>) or Sigma-Genosys (Cambridge, U.K., http://www.sigmaaldrich.com/Brands/Sigma_Genosys.html). CXCL1, CXCL2, and VEGF-A were obtained from PeproTech (Rocky Hill, NJ, <http://www.peprotech.com>).

Allergen-Induced Airways Inflammation and Remodeling

Female BALB/c mice (6–8 weeks old) were housed in filter cup-boards on a 12-hour light/dark cycle and received food and water ad libitum. Mice were sensitized using ovalbumin (OVA; Sigma-Genosys) at 0.01 mg/mouse in 0.2 mL alum (Alu-Gel-S; Serva Electrophoresis, Heidelberg, Germany, <http://www.serva.de>) intra-peritoneally on days 0 and 12. Control mice received the same volume of saline in alum. Groups were challenged daily with 5% OVA (aerosolized for 20 min) between days 18 and 23 for the acute phase. Chronic inflammation was induced by challenging mice three times per week with 5% OVA up to day 54 [20]. Mice were euthanized by exsanguination under terminal anesthesia at 24 hours after the last OVA exposure. Alum controls from days 24, 35, and 55 were pooled in a single group (alum) as there were no significant differences between control groups. Leukocyte numbers were assessed in lung tissue digest and BALF as previously described [21]. In selected experiments, CXCL1 and CXCL2 or VEGF-A were given intranasally (30 μ g/kg) at day 18 in OVA- or alum-sensitized mice, and levels were analyzed on day 19. The effect of CXCR2 on EPC recruitment to the lungs was investigated in the acute model of allergic airway inflammation by treating mice with an intravenous neutralizing anti-CXCR2 antibody (50 μ g/mice; R&D Systems Inc., Minneapolis, MN, <http://www.rndsystems.com>) on days 18 and 21. Control mice received the same dose of control IgG. Mice were euthanized on day 24 or left to rest for 6 days and euthanized on day 30. The optimal

antibody dose was established previously by quantification of neutrophil recruitment after CXCL1 instillation [22].

Immunohistochemistry

Lung paraffin sections (5 μm) were immunostained with rabbit anti-human von Willebrand Factor (vWF) (1:200, A0082; DAKO, Glostrup, Denmark, <http://www.dako.com>) to reveal blood vessels. This antibody has been shown to cross-react with murine vWF [2]. The number of peribronchial vessels/ mm^2 were counted in at least four airways per section.

EPC Assay

The procedure for purification of CD34+ cells (expressed in both hematopoietic progenitor cells and endothelial progenitor cells) from lungs has been adapted from elsewhere [23]. Mice were euthanized by exsanguination under terminal anesthesia at 24 hours after the last OVA exposure. In preliminary experiments, 1 mL PBS was flushed through the heart to eliminate blood contamination in the lung. The largest lobe from the lungs was minced and digested with 0.15 mg/ml collagenase type D and 25 $\mu\text{g}/\text{ml}$ DNase type I for 30 min at 37°C. Cell suspensions were prepared by filtration through a 100- μm Falcon 2360 nylon cell strainer (Becton, Dickinson and Company, Franklin Lakes, NJ, <http://www.bd.com>) and separated on discontinuous Percoll gradient. Mononuclear cells ($n = 10^6$) were plated in endothelial basal medium (EBM-2) supplemented with VEGF (50 ng/ml) and 17% FCS (Cambrex BioScience Walkersville, Inc.) on a fibronectin-coated dish (10 $\mu\text{g}/\text{mL}$). EBM-2 media will not support the growth of hematopoietic stem cells.

In selected experiments, blood was collected and red cells were lysed. Numbers of nucleated cells were counted, and 10^6 cells were plated using the same method as for lungs. EPCs were also recovered from bone marrow at the same time points as from lungs, and EPC colonies from all sources were scored on day 21 on an inverted microscope by morphology as described previously [4, 24].

EPC Immunophenotyping

EPCs were cultured on chamber slides as above, and on day 21 cells were fixed in acetone for 5 minutes, blocked with 10% donkey serum, and incubated for 1 hour with primary or isotype control antibody at room temperature. Cells were incubated with secondary antibody for 30 minutes. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI; Sigma-Genosys). We used primary antibodies against CD31, CD14, CD45, CD115, VEGFR2 (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml), or vWF (DAKO). The secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA, <http://www.jacksonimmuno.com>). EPC colonies were also stained with *Griffonia simplicifolia* lectin (GS-lectin) and analyzed for uptake of acetylated-low density lipoprotein (Ac-LDL). Briefly, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled Ac-LDL was added to EPC media (5 $\mu\text{g}/\text{ml}$) and incubated for 4 hours. Colonies were then washed in PBS and fixed with 2% paraformaldehyde before incubation with Alexa488-conjugated GS-lectin (10 $\mu\text{g}/\text{ml}$ in PBS) for 60 minutes as described previously [4].

Chemokine Analysis

Lung homogenates (50 mg/mL) were measured for chemokine levels. Paired antibodies for murine CXCL1, CXCL2, stromal cell-derived factor-1 (SDF-1), and VEGF (R&D Systems) were used in standardized sandwich ELISAs according to the manufacturer protocols. Levels of CXCR4 in bone-marrow EPCs were determined with cytometry in CD34/VEGFR2+ cells as described previously [24].

Statistical Analysis

Data were analyzed with Prism 4 for Windows (GraphPad Software Inc, San Diego, CA, <http://www.graphpad.com>), with the use of Kruskal-Wallis and Mann-Whitney tests.

Results

Acute and Prolonged OVA Challenge Leads to Peribronchial Angiogenesis

We have previously developed a model of prolonged allergen challenge of the airways that incorporates many of the characteristic features of airway remodeling in asthmatics, including increased bronchiolar subepithelial deposition of collagen, increased mucus production, and airway smooth muscle cell proliferation [20]. To evaluate angiogenesis in this model, paraffin-embedded lung sections from acute and prolonged allergen-challenged mice were immunostained for vWF as a marker for endothelial cells. A significant increase in the number of peribronchial blood vessels was observed during acute inflammation (day 24) (Fig. 1B, 1D), and this number increased further during prolonged OVA challenge (days 35 and 55) (Fig. 1C, 1D) when compared with alum controls ($p = .0164$ on day 24; 0.0002 and 0.0003 on days 35 and 55, respectively). In addition, the elevated numbers of peribronchial blood vessels were sustained even in the absence of continued antigen exposure for at least another 25 days after the establishment of airway remodeling in comparison to alum controls ($p = .0042$; data not shown). The increase in the number of vessels in this model is accompanied by an increase in the number of inflammatory cells as described previously by our group [20].

Acute Allergen Challenge Leads to an Increase in EPC Numbers in the Lungs

To evaluate the recruitment of EPCs to the lungs after acute and prolonged exposure to allergen, mononuclear cells were isolated from lungs at the indicated time points and cultured in endothelial colony-specific media. After 21 days, endothelial progenitor-derived colonies were scored. Figure 2A shows that EPC numbers were significantly increased during the acute phase of inflammation (day 24) and at the early stages of the remodeling phase (day 35) when compared to alum controls. However, EPC numbers returned to basal levels at day 55 after vascular remodeling had been established. EPC colonies were further immunostained as described in Materials and Methods. EPCs were positive for vWF, CD31, and VEGFR2 (Fig. 2B). These colonies were also stained positively with GS-lectin and took up Ac-LDL (Fig. 2C), indicating that these cells belong to an endothelial cell lineage and do not express hematopoietic markers; thus, they are distinct to the early outgrowth monocytic EPCs [4]. In addition, Figure 2D (right panel) shows that EPC colonies were negative for CD14 and CD45 (hematopoietic colonies were used as positive controls (Fig. 2D, left and middle panels)).

Effect of CXCR2 Ligands and VEGF-A on EPC Recruitment to the Lungs After Allergen Challenge

To evaluate whether the proangiogenic factors CXCL1, CXCL2, and VEGF-A were contributing to EPC recruitment into the lungs, we first measured the levels of CXCL1, CXCL2, and VEGF-A in lung tissue after acute and prolonged allergen exposure. Acute allergen challenge (day 24) led to a significant increase in CXCL1, CXCL2, and VEGF-A levels in the lung tissue when compared to alum controls. Although levels of these proangiogenic factors were maintained at day 35, they had returned to basal levels by day 55 (Fig. 3A–3C).

We next determined the direct effect of CXCR2 ligands and VEGF-A on EPC recruitment to the lungs of OVA-sensitized mice. To do so, mice were sensitized with OVA or alum on days 0 and 12 and then were given CXCL1, CXCL2, or VEGF-A (30 $\mu\text{g/kg}$) intranasally on

day 18; EPC numbers in the lungs were analyzed on day 19. CXCL1 and CXCL2 are potent neutrophil chemoattractants and, as shown in Figure 4A, these chemokines stimulated neutrophil recruitment to a similar extent irrespective of whether the mice were alum-treated or OVA-sensitized. In contrast, we found that, whereas the chemokines did not promote EPC recruitment into the alum-treated or naive mice (Fig. 4 and data not shown), there was a robust recruitment of EPCs when CXCL1 and CXCL2 were administered to mice sensitized peripherally with OVA. These data suggest that prior antigen exposure is required for chemokines to stimulate EPC recruitment in the lung. However, OVA sensitization did not change the absolute numbers of EPCs in the bone marrow or the percentage of EPC-expressing CXCR2 (alum, $89.5 \pm 1.7\%$; OVA, $85.25 \pm 1.9\%$). Surprisingly, instillation of VEGF-A did not stimulate EPC recruitment into the lungs of control or sensitized mice (Fig. 4B).

Administration of Anti-CXCR2 Antibody Decreased EPC Recruitment to the Lungs Induced by Allergen Challenge

Having established that CXC chemokines could stimulate EPC recruitment into the lungs of OVA-sensitized mice, we next investigated whether blocking CXCR2 would affect EPC recruitment into the lungs in a model of allergic airway disease. The first step in EPC trafficking to the lungs is their mobilization from the bone marrow into the blood; we therefore investigated the effect of CXCR2 blockade on this process. Figure 5C shows that animals sensitized and challenged with OVA exhibited higher numbers of circulating EPCs 24 h after allergen challenge when compared to the controls. However, the blockade of CXCR2 did not affect the increase in circulating numbers of EPCs. In contrast, blockade of CXCR2 caused a significant inhibition of EPC numbers in lungs at both day 24 and day 30 in comparison to Ig-treated mice (Fig. 5A, 5B). These data suggest that, although CXC chemokines play a critical role in the recruitment of EPCs from the blood into the lungs, an alternative factor mediates their mobilization from the bone marrow after allergen challenge.

Effect of CXCR2 Blockade on Peribronchial Angiogenesis After Allergen Challenge

Because the influx of EPCs is temporally related to the increase in the numbers of blood vessels around the airways (Figs. 1 and 2), we next determined the impact of blocking CXCR2 on the formation of new blood vessels in this model. As shown in Figure 6A, treatment with a neutralizing antibody to CXCR2 resulted in a significant reduction in the number of blood vessels around the airways when compared with mice treated with isotype control monoclonal antibody (Fig. 6A, 6B). In addition, administration of the CXCR2-blocking monoclonal antibody had no effect on the total number of leukocytes recruited into the lungs (Fig. 6C, 6D), indicating that in this model CXCR2 does not play a critical role in orchestrating the inflammatory response (predominantly eosinophils (Fig. 6E) and TH2 cells (Fig. 6F)).

The extent of angiogenesis occurring in the lungs is modulated by locally generated proangiogenic factors, notably VEGF-A, which is known to stimulate both the proliferation and the survival of mature endothelial cells. In addition, studies have shown that SDF-1 α and VEGF-A are able to reduce EPC apoptosis [25–27]. Therefore, we also measured the levels of VEGF-A and SDF-1 α in the lungs of anti-CXCR2-treated and isotype control groups. Anti-CXCR2 antibody treatment led to a significant decrease in levels of SDF-1 α ($p = .033$) (Fig. 6G), which may also contribute to the antiangiogenic activity of the CXCR2-blocking monoclonal antibody. Interestingly, levels of VEGF-A were not significantly altered by blockade of CXCR2 (data not shown), indicating that the angiogenic response could be inhibited despite enhanced expression of VEGF in the tissue.

Taken together, our data show that CXCL1 and CXCL2 are the main factors that induce EPC recruitment to the lungs in response to allergen challenge. In addition, we have shown that the blockade of CXCR2 is able to reduce the recruitment of EPCs to the lungs and the formation of new blood vessels around the airways.

Discussion

In this study we have investigated the role of CXCR2 in mediating EPC recruitment and the formation of new blood vessels in a model of allergic airway disease. We have shown that CXCR2 is central in the recruitment of EPCs to the lungs after allergen challenge and is critical for the formation of new peribronchial blood vessels. This is the first time that this chemokine axis has been implicated in EPC recruitment and neovascularization during allergic inflammation *in vivo*.

The formation of new blood vessels is a characteristic feature of chronic inflammatory diseases, including asthma. Indeed, in asthmatic patients the degree of bronchial vascularity is thought to contribute to airflow limitation [11, 28–31]. Furthermore, increased expression of proangiogenic mediators and their receptors has been correlated with disease severity and accelerated decline in lung function [32, 33]. We have developed a model of prolonged airway allergen challenge in the mouse that mimics typical features of airway remodeling, including increased collagen deposition, mucus production, and airway smooth muscle cell proliferation [20]. Here we show that, in addition to these structural changes, airway remodeling is associated with angiogenesis in this model. Moreover, the formation of new peribronchial blood vessels is an early event during airway remodeling, being significant at day 24 and increasing further after prolonged allergen challenge.

Previously, the formation of new blood vessels was thought to result exclusively from the proliferation, migration, and remodeling of fully differentiated endothelial cells from preexisting blood vessels. In recent years, however, the contribution of bone marrow–derived EPCs to this process has been described [34–36]. Whereas EPCs were first described by Asahara et al. as CD34+/VEGFR2+ bone marrow–derived cells, more recently two subsets of these progenitors have been defined [4, 37]. With the use of a clonal assay, it has been shown that endothelial progenitors that give rise to colonies after 5 days in culture are closely related to the monocytic lineage expressing CD14 and CD45. These cells, sometimes referred to as “early outgrowth” EPCs, do not have the capacity to form vessels *in vitro* or *in vivo* but may facilitate angiogenesis by secreting proangiogenic cytokines. In contrast, the endothelial progenitors that give rise to colonies after 21 days in culture (previously referred to as “late outgrowth” EPCs) are uniquely able to differentiate into mature endothelial cells that have the capacity to form tubules *in vitro* [4, 24, 37–39]. A recent study reported that asthmatic patients had higher numbers of circulating early outgrowth EPCs in comparison to normal subjects [5]. In addition, these investigators showed that acute allergic inflammation of the airways induced by OVA leads to increased numbers of Sca+, c-kit+, and VEGFR2+ cells in the lungs and blood of allergic mice [5]; however, these cells were not characterized in terms of their ability to form early or late outgrowth colonies. In this study, we have demonstrated, for the first time, that late outgrowth EPCs, which are CD45–/CD14–/CD115–/VEGFR2+/vWF+/CD31+, are recruited to lungs after allergen challenge. Examining the kinetics of this event revealed that EPC recruitment occurred during the early phase of the model (by day 24) and persisted up to 35 days. However, these cells were not detected at the late time points of the model of allergic airway inflammation, suggesting these cells have distinct kinetics of recruitment to the lungs.

ELR+ CXC chemokines (CXC chemokines that contain a Glu-Leu-Arg motif), such as CXCL1 and CXCL2, are potent neutrophil chemoattractants that have also been

demonstrated to be important proangiogenic factors in both tumor and ischemia reperfusion models [16, 19, 40–43]. Intriguingly, we observed increased levels of CXCL1 and CXCL2 in lungs on both day 24 and day 35 in the mice challenged with allergen, concomitant with EPC recruitment. As it has previously been reported that EPCs express CXCR2 and that this chemokine receptor plays an important role in EPC recruitment into tissue in other diseases [6], we investigated the role of this chemokine receptor axis in EPC recruitment to the lungs of OVA-sensitized mice. In our first experiments we found that when the CXC chemokines were administered intranasally to naive mice, there was a robust recruitment of neutrophils but not EPCs (data not shown). We therefore examined whether EPC recruitment was affected by the sensitization process. Chemokines were administered by intranasal instillation to OVA- and sham-sensitized mice. Intriguingly, we found that while neutrophil recruitment to the lungs was not affected by the peripheral sensitization, EPC recruitment into the lungs in response to these chemokines was critically dependent on this process. Thus EPCs were only recruited to the lungs of mice that had been sensitized to OVA. This suggests that, while CXCL1/2 are critical for EPC recruitment into the lungs, other mechanisms regulate EPC trafficking within the circulation. Similarly, Abbott et al. have shown that overexpression of SDF-1 α in the heart does not stimulate progenitor cell recruitment to this tissue in naive mice but significantly increases the number of progenitor cells recruited after myocardial infarction [44], suggesting that recruitment of elevated numbers of EPCs is disease-dependent.

Trafficking of EPCs from the bone marrow to the lung requires their mobilization from the bone marrow into the blood and subsequent recruitment from the blood to the tissue. We show here that allergen challenge leads to an increase in the numbers of circulating EPCs, suggesting that these cells are being mobilized from the bone marrow in response to allergen challenge. However, blockade of CXCR2 did not affect this mobilization, suggesting that distinct factors are involved in this process. In contrast, we show that neutralizing CXCR2 reduces the recruitment of EPCs from the blood into the lungs of allergen-challenged mice, while the influx of inflammatory cells (such as eosinophils and Th2 cells) was not affected by CXCR2 blockade. Neutrophils are not a major component of the inflammatory response in this model, and this finding is consistent with our previous work where we identified the role of specific chemokines in promoting the inflammatory response in this model [45, 46]. Blockade of CXCR2 did reduce the extent of angiogenesis evident at day 24 and day 30. It is interesting to note that angiogenesis was significantly reduced in the absence of a reduction of the overall inflammatory response, suggesting that distinct factors and mechanisms regulate these parallel processes.

VEGF-A is known as a potent proangiogenic regulator, and it is capable of stimulating the proliferation and survival of mature endothelial cell and EPC chemotaxis in vivo and in vitro [8, 47]. Increased levels of VEGF-A have been found in BALF from asthmatic patients, and it has been reported that this factor contributes to tissue edema through its effect on vascular permeability in asthmatics [9, 13, 48]. We have shown that, despite increased levels of VEGF-A being detected in lung homogenates after allergen exposure, EPC recruitment to the lungs was independent of VEGF-A because instillation of this growth factor in alum- or OVA-sensitized mice did not lead to EPC recruitment to the lungs. Nevertheless, we can not exclude the effects of VEGF-A in mobilization of EPCs from the bone marrow to the blood [8] or in promoting and sustaining formation of vessels within the lungs or induction of other features of airway remodeling as described by Lee et al. [14]. It is interesting to note that, when CXCR2 was neutralized in this study, there was no impact on the elevated levels of VEGF-A observed in the lungs in response to allergen challenge, although there was a robust inhibition of the angiogenic response, suggesting that the CXCR2/CXC chemokine axis is critical in driving this response.

The angiogenic potential of CXC chemokines has long been recognized [16, 17, 19, 40–43]. Whereas previous studies have focused on the role of these chemokines in promoting vessel formation, a recent study showed that CXCR2 was required for EPC recruitment to sites of arterial injury [6]. In this study we show that CXC chemokines can recruit EPCs into the lungs of OVA-sensitized mice and that CXCR2 is critical for both EPC recruitment and angiogenesis in a model of allergic airway disease. Importantly, angiogenesis is abrogated despite elevated levels of VEGF-A and a robust inflammatory response. In the future it would be interesting to assess the relative role of CXCR2 on EPCs and mature endothelial cells with respect to initiating and maintaining the angiogenic response during allergic airway inflammation.

In addition to integrating to newly formed vessels, EPCs can also act in a paracrine manner by expressing several growth factors such as VEGF and SDF-1 α to further enhance vessel growth [27, 49]. Indeed, our data show that the blockade of CXCR2 and EPC recruitment leads to a concomitant decrease in levels of SDF-1 α , which could also affect the survival and proliferation of resident endothelial cells or EPC.

This is the first study to describe a critical and novel role for CXCR2 and its ligands in mediating the recruitment of EPCs to the lungs and subsequent peribronchial neovascularization after allergen exposure. These data show that CXCR2 and its ligands can specifically regulate neovascularization. Delineation of these mechanisms could lead to novel therapeutic strategies to block neovascularization observed in asthmatic patients.

Acknowledgments

This study was supported by grants awarded by the British Heart Foundation (project grant: PG05092) and Wellcome Trust (057704). C.M.L. is a Wellcome Senior Research Fellow. C.P.J. was funded by CNPQ.

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Figure 1.

Acute and prolonged ovalbumin (OVA) challenge leads to peribronchial angiogenesis. Representative photomicrographs of immunostaining for von Willebrand factor of lung sections from alum controls (A) and OVA-challenged mice up to days 24 (B) or 55 (C). Magnification $\times 400$. (D) Bars represent mean $\pm$ SEM of the number of blood vessels per square millimeter from alum- and OVA-treated mice at indicated time points. Data derived from 4–6 mice.

Figure 2.

Acute allergen challenge leads to an increase of EPCs in the lungs. **(A)** Bars represent mean $\pm$ SEM of the numbers of EPCs at 21 days of culture, as described in Materials and Methods. * represents $p < .05$ compared to alum controls. Photomicrograph represents lung EPC colonies in bright-field; magnification $\times 50$ (inset: $\times 400$). **(B)** Representative photomicrographs from EPCs immunostained for CD31-Alexa 488/DAPI; vWF-Alexa 564/DAPI, and VEGFR2/Alexa 564/DAPI. **(C)** Representative photomicrographs from EPC colonies positive for Ac-LDL uptake and GS-lectin. **(D)** Representative photomicrographs from hematopoietic colonies (left and middle) or EPCs (right) immunostained for CD45- and CD14-positive monocytes. Data derived from 8–12 mice. Abbreviations: Ac-LDL, acetylated low-density lipoprotein; DAPI, 4',6-diamidino-2-phenylindole; EPC, endothelial progenitor cell; GS-lectin, *Griffonia simplicifolia* isolectin; vWF, von Willebrand factor.

Figure 3.

Acute allergen challenge leads to increased levels of CXCR2 ligands and VEGF-A in lung homogenates. CXCL1, CXCL2, and VEGF-A levels in lung tissue homogenates were measured in samples from alum controls or samples from mice challenged with OVA at the indicated time points. Data are expressed as individual values and median. * represents $p < .05$ compared to alum controls. Data derived from 6 mice. Abbreviations: OVA, ovalbumin; VEGF, vascular endothelial growth factor.

Figure 4.

CXCL1 in association with CXCL2 is able to induce EPC recruitment to the lungs of sensitized mice. Mice were sensitized with alum or OVA (intraperitoneal) on days 0 and 12. On day 18 mice were challenged with CXCL1 in association with CXCL2, VEGF-A, or PBS intranasally. **(A)** Data represent mean $\pm$ SEM of neutrophils $\times 10^5$ per mL bronchoalveolar lavage fluid. **(B)** Data represent mean $\pm$ SEM of the numbers of EPCs per lung. $n = 4-6$ mice. * represents $p < .05$ compared to PBS-instilled mice. Abbreviations: EPC, endothelial progenitor cell; PBS, phosphate-buffered saline; OVA, ovalbumin; VEGF, vascular endothelial growth factor.

Figure 5.

Administration of anti-CXCR2 decreased EPC recruitment to the lungs induced by allergen challenge. Mice were sensitized with alum or OVA (intraperitoneal) on days 0 and 12, and challenged with OVA aerosol between days 18 and 23. Selected mice were treated with anti-CXCR2 antibody or isotype controls on days 18 and 21. (A) EPC quantities in the lungs were analyzed 24 hours after the last allergen challenge. Animals were left to rest for another 6 days and EPCs from lung (B) and blood (C) were analyzed at day 30. Bars represent mean $\pm$ SEM of the numbers of EPCs. $n = 4-9$ mice. * represents $p < .05$ compared to alum controls. ** represents $p < .05$ compared to OVA-isotype-treated mice. Abbreviations: EPC, endothelial progenitor cell; OVA, ovalbumin.

Figure 6.

Effect of CXCR2 blockade on peribronchial angiogenesis after allergen challenge. (A) Representative photomicrographs of immunostaining for von Willebrand factor of lung sections from alum- and OVA-challenged mice treated with isotype control or anti-CXCR2. (B) Bars represent mean $\pm$ SEM of vessels/mm² immunostained for von Willebrand factor. (C, D) Total number of cells recovered from lung digests and bronchoalveolar lavage fluid, respectively. (E, F) Numbers of eosinophils (SiglecF-positive cells) and Th2 cells (CD3+CD4+T1ST2+ cells) in lung digests. (G) Bars represent mean $\pm$ SEM SDF-1 α levels measured in lung tissue homogenates. * represents $p < .05$ compared to alum controls. ** represents $p < .05$ compared to OVA-isotype-treated mice. Data derived from 4-6 mice. Abbreviations: EPC, endothelial progenitor cell; OVA, ovalbumin; SDF-1 α , stromal cell-derived factor-1 α .