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The role of $CXCR_2$ in systemic neovascularization of the mouse lung

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Sánchez J, Moldobaeva A, McClintock J, Jenkins J, Wagner E. The role of $CXCR_2$ in systemic neovascularization of the mouse lung. *J Appl Physiol* 103: 594–599, 2007. First published June 7, 2007; doi:10.1152/japplphysiol.00037.2007.—We previously showed increased expression of the ELR+, CXC chemokines in the lung after left pulmonary artery obstruction. These chemokines have been shown in other systems to bind their G protein-coupled receptor, $CXCR_2$, and promote systemic endothelial cell proliferation, migration, and capillary tube formation. In the present study, we blocked $CXCR_2$ in vivo using a neutralizing antibody and also studied mice that were homozygous null for $CXCR_2$. To estimate the extent of neovascularization in this model, we measured systemic blood flow to the left lung 14 days after left pulmonary artery ligation (LPAL). We found blood flow significantly reduced (67% decrease) with neutralizing antibody treatment compared with controls. However, blood flow was not altered in the $CXCR_2$-deficient mice compared with wild-type controls after LPAL. To test for ligand availability, we measured macrophage inflammatory protein (MIP)-2 in lung homogenates after LPAL, because this is the predominant CXC chemokine previously shown to be increased after LPAL (22). MIP-2 protein was two- to fourfold higher in the left lung relative to the right lung in all treatment groups 4 h after LPAL and this increase did not differ among groups. We speculate that the $CXCR_2$-deficient mice have compensatory mechanisms that mitigate their lack of gene expression and conclude that $CXCR_2$ contributes to chemokine-induced systemic angiogenesis after pulmonary artery obstruction.

angiogenesis; chemokines; macrophage inflammatory protein-2

ANGIOGENESIS IN THE LUNG involves predominantly the systemic vasculature and becomes prominent in several pathologic states where chronic inflammation prevails, such as cystic fibrosis (4), asthma (13), and chronic pulmonary thromboembolic disease (19). Pulmonary ischemia resulting from chronic pulmonary thromboembolism or other forms of pulmonary artery obstruction leads to proliferation of the systemic circulation within and surrounding the lung (5, 6, 9, 29). Left pulmonary artery ligation (LPAL) in experimental animals creates a unique model of angiogenesis, which allows investigation into mechanisms of lung neovascularization. In mice, intercostal artery proliferation and perfusion of the lung develop within 4–5 days after LPAL (15, 28). Determination of the growth factors responsible for systemic neovascularization of the lung is essential and these likely differ from other organs due to the normoxic conditions of the lung. A growing body of evidence demonstrates the prevalence of the glutamic acid-leucine-arginine (ELR+) CXC chemokines in the lung in association with neovascularization (2, 3, 25). In human tissue, the ELR+ chemokines have been shown to promote neovascularization through binding G protein-coupled receptors $CXCR_1$ and $CXCR_2$ and promoting systemic endothelial cell proliferation and migration (12, 21, 26). In mice, relatively little is known regarding the function of $CXCR_1$ since its expression was only recently confirmed (7, 8, 16). The three ELR+ CXC chemokines that have been shown to function through the binding of $CXCR_2$ in mice are macrophage inflammatory protein-2 (MIP-2; CXCL2), keratinocyte-derived chemokine (KC; CXCL1), and lipopolysaccharide-induced chemokine (LIX; CXCL5). Our laboratory has confirmed increased expression of the ELR+ CXC chemokines in the proangiogenic left lung after LPAL relative to the normal right lung with levels of MIP-2 predominating (14, 22). Using in vitro techniques, we also showed that mouse arterial endothelial cells express $CXCR_2$ and that MIP-2 elicits endothelial cell proliferation and migration in a dose-dependent manner (17). Thus it was the goal of the present study to confirm in vivo the importance of $CXCR_2$ to the overall process of lung angiogenesis developed after LPAL.

METHODS

General Surgical Methods

LPAL. Our protocol was approved by the Johns Hopkins Animal Care and Use Committee. Male mice, aged 5–6 wk, of several strains were studied: C57Bl/6 mice (total studied, $n = 38$ mice) and BALB/c mice ($n = 24$ mice) purchased from Charles River (Wilmington, MA) and $CXCR_2$ (−/−)-deficient mice [knockout (KO)] on a BALB/c background ($n = 16$ mice, Jackson Laboratories, Bar Harbor, ME). Mice were anesthetized (2% isoflurane in oxygen), intubated, and ventilated at 120 breath/min, 0.2 ml/breath with the anesthetic-gas mixture. As previously described, this model included: left lateral thoracotomy, left pulmonary artery ligation (6–0 silk suture), and closure of the thoracotomy with suture while the animal was placed on positive end-expiratory pressure [1 cmH_2O; (14, 15, 22, 27)]. Lidocaine (2%) was applied for analgesia and the skin incision was closed using methyl acrylamide adhesive. The animal was removed from the ventilator, extubated, and allowed to recover.

Neovascularization assessed by blood flow. To determine the extent of neovascularization, systemic blood flow to the left lung was measured after LPAL in mice using radiolabeled microspheres. Diethylenetriamine pentaacetic acid (DTPA)-coated polystyrene microspheres (10 μm diameter; Kisker Products, Steinfurt, Germany) were bound to Technetium-99m radioligand (Cardinal Health; Dublin, OH). Based on previous observations, we made blood flow measurements 14 days after LPAL when a systemic neovasculature arising from intercostal arteries was clearly established (27). Mice were anesthetized, ventilated as described above, the common carotid artery was cannulated (PE-10), and the tip was advanced to a point just within the thorax. Microspheres (150,000; stock = 1.5 million spheres/ml) were infused (0.1 ml at 0.04 ml/min: Harvard Apparatus, Holliston, MA). Mice were killed by exsanguination, and the left lung was excised. Gamma emissions from lodged radiolabeled micro-

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spheres were immediately counted in the Hidex Triathler (Bioscan, Washington, DC). Left lung activity was normalized to whole body activity counted in a Capintec counter (Capintec Products, Ramsey, NJ), which had been calibrated to the Bioscan instrument. Systemic perfusion of the left lung was expressed as percent of total measured blood flow (carcass + all organs), i.e., percent of cardiac output.

Experimental protocols. A total of 78 mice were studied, applying three experimental protocols: blood flow assessment, lung MIP-2 protein determination, and endothelial cell harvest and culture for measurement of $CXCR_1$/$CXCR_2$ expression.

Blood flow groups. Neovascularization was assessed by blood flow determination in eight experimental groups of mice (see Table 1). Systemic blood flow to the left lung 14 days after LPAL was determined in control *group 1*: C57Bl/6 mice; *group 2*: C57Bl/6 mice administered a $CXCR_2$-neutralizing antibody extensively tested and kindly provided by Dr. Robert Strieter, University of Virginia. The treatment protocol for this group required intraperitoneal delivery of the neutralizing antibody (0.5 ml ip goat anti-mouse serum) 24 h prior to LPAL as well as immediately after surgery and every 48 h after LPAL until blood flow determination at 14 days. This treatment protocol was based on previous studies examining angiogenesis in mouse models of lung cancer (11, 30). A serum control *group 3*: C57Bl/6 mice treated with intraperitoneal injection of goat serum (Sigma-Aldrich, St. Louis, MO) since the neutralizing antibody was raised in goats and using the same volume and time points as *group 2*. This group served as a control for the $CXCR_2$-neutralizing antibody.

Since $CXCR_2$ KO mice are commercially available only in BALB/c mice, *group 4*: BALB/c mice, received no treatments and were compared with *group 5*: $CXCR_2$ KO mice. $CXCR_2$-neutralizing antibody treatment and goat serum controls were repeated in the BALB/c strain as *group 6*: BALB/c mice administered a $CXCR_2$-neutralizing antibody and *group 7*: BALB/c mice treated with goat serum. *Groups 6* and *7* were treated according to the same protocol as *groups 2* and *3*, respectively (described above).

Group 8: C57Bl/6 mice were evaluated 2 days after LPAL and served as an experimental control group since we showed previously that a functional neovasculature does not develop until 5 days after LPAL. Consequently, blood flow measured in this group represents an experimental background.

MIP-2 protein evaluation. Changes in lung MIP-2 protein were evaluated in additional mice 4 h after LPAL. Lung samples were collected from naive C57Bl/6 mice ($n = 3$) and C57Bl/6 mice after LPAL ($n = 6$), with $CXCR_2$-neutralizing antibody ($n = 4$), goat serum control ($n = 4$), and $CXCR_2$ KO ($n = 5$). Four hours after LPAL, anesthetized mice were killed (cervical dislocation), and the upper third of the left lung and the right lung were dissected and excised. Lung samples were weighed, homogenized (Fastprep Bio101; Thermo Savant, Holbrook, NY), and aliquoted for ELISA and BCA assays. The samples were processed according to the Quantikine MIP-2 ELISA kit (R&D Systems, Minneapolis, MN). Total protein measurements were made according to the BCA protein assay kit (Pierce, Rockford, IL).

Table 1. *Groups evaluated for left lung blood flow 14 days after LPAL*

Group #	Strain	Treatment	*n*
1	C57Bl/6	None	5
2	C57Bl/6	$CXCR_2$-neutralizing antibody	4
3	C57Bl/6	Goat serum	4
4	BALB/c	None	9
5	BALB/c	$CXCR_2$ (−/−)	8
6	BALB/c	$CXCR_2$-neutralizing antibody	7
7	BALB/c	Goat serum	5
8	C57Bl/6	None (2 days LPAL)	5

LPAL, left pulmonary artery ligation.

Isolation of mouse aortic endothelial cells. The aortas from C57Bl/6, BALB/c, and $CXCR_2$ KO mice ($n = 3$ mice/strain) were dissected and placed with the intima side down on Matrigel-coated 35 mm tissue culture dishes. After 4–6 days, endothelial cells that had migrated were replated to a T-25 flask coated with gelatin. Cells were cultured in DMEM supplemented with 20% FCS, 15 μg/ml ECGS, 100 μg/ml penicillin-streptomycin, 0.25 μg/ml amphotericin B, and 0.1 mM MEM with nonessential amino acids. Endothelial cell phenotypes were confirmed by immunostaining techniques for platelet endothelial cell adhesion molecule (PECAM), von Willebrand factor (vWF), and uptake of acetylated low-density lipoprotein (Dil-ac-LDL). Only cell populations with positive staining were used for further experiments. All experiments were carried out using endothelial cells between passages 2 and 8.

Endothelial cell mRNA $CXCR_1$/$CXCR_2$ expression by quantitative real-time RT-PCR. To confirm the presence of $CXCR_1$ and $CXCR_2$ mRNA in aortic endothelial cells, we performed quantitative real-time RT-PCR.Total RNA was extracted from endothelial cells (5×10^6 cells) using RNeasy Mini Kit (Qiagen, Valencia, CA) with standard procedures. Total RNA was also extracted from lung tissue as a positive control. Total RNA (2 μg) from aortic endothelial cells ($n = 3$ isolations) was used for first-strand cDNA synthesis with random hexamer primers and a first-strand cDNA synthesis kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's protocol. The primers used for amplification were as follows, for $CXCR_1$: CCG TCA TGG ATG TCT ACG TG and CAG CAG CAG GAT ACC ACT GA; and for $CXCR_2$: GGT GGG GAG TTC GTG TAG AA and CGA GGT GCT AGG ATT TGA GC. PCR reactions were performed with QuantiTect SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and IQ5 Multicolor real-time PCR Detection System (Bio-Rad Laboratories, CA), using 1 μl of cDNA as the template in each 25-μl reaction mixture. The absolute copy number of mRNA of interest was determined by interpolation of the standard curve with the threshold cycle value of each sample. The melting curve protocol was performed following the RT-PCR and revealed a single clean melting peak for all samples tested. Data were normalized to the quantity of β-actin mRNA in individual samples.

Endothelial cell $CXCR_1$/$CXCR_2$ expression by flow cytometry. Endothelial cells were washed, detached (2 mM EDTA/PBS), and resuspended (PBS with 0.2% BSA). After washing, cells were incubated with primary human anti-$CXCR_1$ (0.2 μg/ml) and anti-$CXCR_2$ (1.0 μg/ml) monoclonal antibodies (Abcam, Cambridge, MA), washed, incubated with Alexa-488 mouse anti-human antibody (Molecular Probes, Eugene, OR), and analyzed. Mouse isotype IgG served as a negative control. Since antibodies against mouse $CXCR_1$ are not commercially available, we used antibodies raised against human $CXCR_1$ and $CXCR_2$. Monoclonal antibody against human $CXCR_1$ shows no cross-reactivity with human $CXCR_2$, and $CXCR_2$ antibody does not cross-react with human $CXCR_1$. Human $CXCR_2$ shares 71% identical amino acids with mouse $CXCR_2$ and human $CXCR_1$ shares 65% identity with mouse $CXCR_1$. Cells were evaluated for $CXCR_1$/$CXCR_2$ surface expression using the FACS Calibur (Becton Dickinson, Franklin Lakes, NJ).

Statistics. All data are presented as the means ± SE. Primary outcome variables (blood flow, MIP-2 protein, and CXC receptor intensity) were not normally distributed (Shapiro-Wilk test for normality) and, consequently, the data were log transformed to assume a normal distribution. Changes in blood flow, MIP-2 protein, and CXC receptor intensity were evaluated by one-way ANOVA. Relevant within-group comparisons were made using either Student's *t*-test for unpaired data (blood flow) or multiple comparisons using Fisher's test for least significant differences (left lung/right lung protein, receptor fluorescence intensity). A P value ≤ 0.05 was accepted as significant.

RESULTS

Neovascularization Assessed by Blood Flow

Results of the effects of $CXCR_2$ inhibition on systemic neovascularization of the lung after LPAL are presented in Fig. 1, *A–C*. Overall, neovascularization was significantly different among groups ($P = 0.019$). Left lung blood flow in control *group 1* C57Bl/6 mice was comparable to our previously published values and averaged 2.1 ± 0.5% of total blood flow or cardiac output (14). Control serum-treated mice (*group 3*; 2.2 ± 0. 5%) did not differ from mice without treatment (*group 1*), 14 days after LPAL. However, as shown in Fig. 1*A*, mice treated with the $CXCR_2$-neutralizing antibody demonstrated a significant reduction in blood flow to the left lung 14 days after LPAL (0.7 ± 0.3%; $P = 0.025$). To determine an experimental zero flow in *group 8*, mice ($n = 5$) were evaluated 2 days after LPAL when we previously established there was no neovasculature (15, 28). The values obtained for these control experiments (0.20 ± 0.06%) are considerably lower than after antibody treatment. Thus the neutralizing antibody, although effectively decreasing blood flow, did not completely inhibit neovascularization.

Figure 1*B* shows the results of the evaluation of blood flow to the left lung in wild-type BALB/c mice (*group 4*) and $CXCR_2$ KO mice (*group 5*). Average blood flow to the left lung in the BALB/c wild-type mice was somewhat lower than that seen in the C57Bl/6 mice and no difference was observed in the $CXCR_2$ KO mice ($P = 0.757$).

Figure 1*C* shows the results of BALB/c mice after LPAL that had been treated with control serum (*group 7*) or the $CXCR_2$ neutralizing antibody (*group 6*). Similar to what had been seen in the C57Bl/6 mice, the neutralizing antibody significantly reduced angiogenesis as determined by blood flow to the left lung after LPAL ($P = 0.026$).

A comparison of the results of Fig. 1A with Fig. 1C suggests an overall strain-dependent difference in response to LPAL. Since within C57Bl/6 mice, control *group 1* was not different from serum control *group 3* ($P = 0.87$) and within BALB/c mice, control *group 4* was not different from serum control *group 7* ($P = 0.97$), the control groups of each strain were combined to form larger groups. A comparison of the combined group of C57Bl/6 controls (*groups 1* and *3*) with the combined BALB/c control groups (*groups 4* and *7*) demonstrates that neovascularization assessed by blood flow was significantly greater in the C57Bl/6 mice compared with the BALB/c mice, thereby suggesting a strain-dependent difference in responsiveness ($P = 0.030$).

MIP-2 Protein

To confirm that the marked decrease in vascularization was not related to decreased ligand production, we measured MIP-2 levels in lung homogenates 4 h after LPAL. Absolute MIP-2 protein harvested from left and right lungs of naive C57Bl/6 mice was very low (0.2 ± 0.1 pg/mg of total lung protein). As shown previously and confirmed here, MIP-2 protein in left

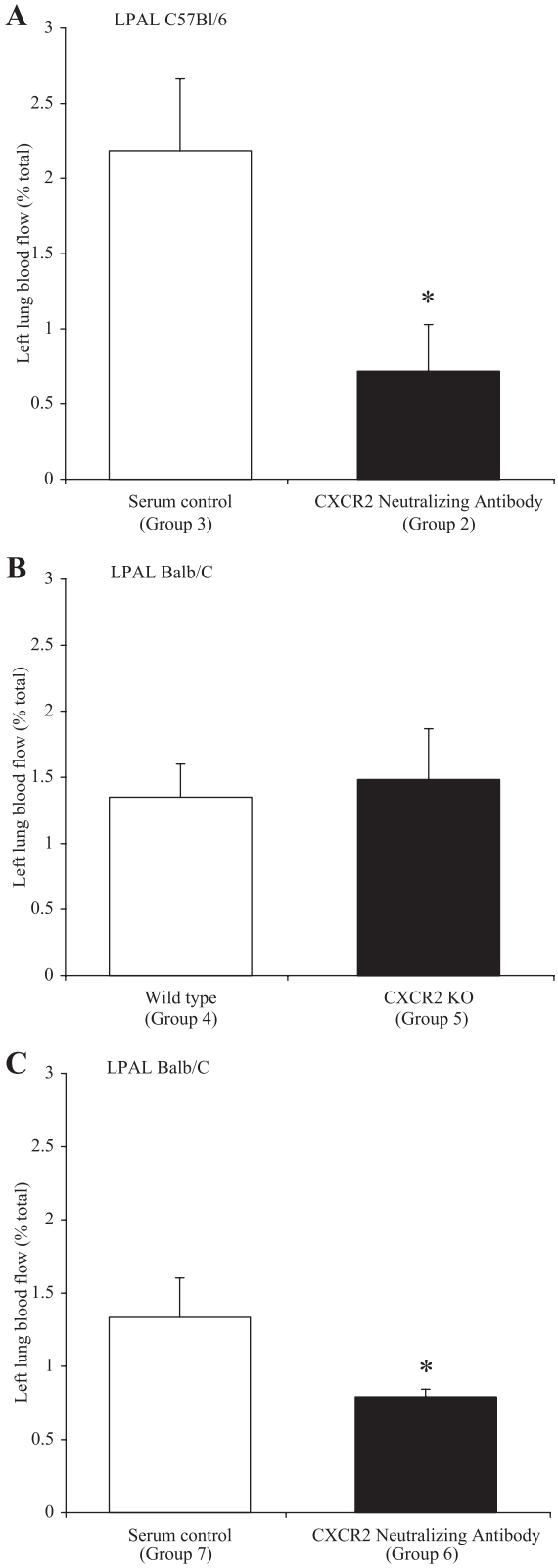

Fig. 1. Average left lung blood flow as a percent (%) total blood flow, 14 days after left pulmonary artery ligation (LPAL). *A*: compares serum-treated control C57Bl/6 mice (*group 3*; $n = 4$) with mice treated every 48 h with a $CXCR_2$-neutralizing antibody (*group 2*; $n = 4$; $*P = 0.025$). *B*: compares wild-type BALB/c mice (*group 4*; $n = 9$) with $CXCR_2$ knock out (KO) mice (*group 5*; $n = 8$). No difference in blood flow to the left lung was observed between the two groups. *C*: shows blood flow to the left lung after LPAL in serum treated BALB/c mice (*group 7*; $n = 5$) with mice treated with $CXCR_2$-neutralizing antibody (*group 6*; $n = 7$). A significant reduction in blood flow to the left lung was observed 14 days after LPAL ($*P = 0.026$).

lungs after LPAL (*group 1*; 156 ± 19 pg/mg of total lung protein) was significantly greater than paired right lungs (47 ± 10 pg/mg of total lung protein; $P = 0.003$). Left lung MIP-2 in *groups 3*, *2*, and *5* averaged 94 ± 25, 208 ± 143, and 81 ± 33 pg/mg, respectively ($P > 0.05$). Figure 2 shows the group results of average fold change in MIP-2 protein in the left lung/total protein compared with right lung/total protein in C57Bl/6 mice and the $CXCR_2$ KO mice. An average two- to fourfold increase in MIP-2 protein in the left lung of all experimental groups after LPAL is shown compared with paired right lungs. These changes were statistically indistinguishable across the experimental groups ($P = 0.41$).

Endothelial Cell $CXCR_1$/$CXCR_2$ Expression

We first confirmed the presence of both $CXCR_1$ and $CXCR_2$ mRNA in aortic endothelial cells. Copy numbers of $CXCR_1$ mRNA normalized to β-actin averaged $1.08^{-3} \pm 0.25^{-3}$. In these aortic endothelial cells from C57Bl/6 mice, $CXCR_2$ mRNA expression was significantly less than $CXCR_1$ expression, averaging $3.3^{-4} \pm 0.075^{-4}$ ($P < 0.05$). Thus $CXCR_1$ showed an average 3.4-fold greater mRNA expression than $CXCR_2$. To explore one potential explanation for the lack of inhibition of blood flow in the $CXCR_2$ KO mice, cell surface receptor expression was evaluated in primary culture of C57Bl/6, BALB/c, and $CXCR_2$ KO mouse aortic endothelial cells. Figure 3 shows the results of $CXCR_1$ and $CXCR_2$ expression. In mouse aortic endothelial cells cultured from each different group, both $CXCR_1$ and $CXCR_2$ expression was evident and showed differences across groups ($P < 0.0001$). In endothelial cells from C57Bl/6 mice and $CXCR_2$ KO mice, expression of $CXCR_1$ was significantly greater than $CXCR_2$ ($P < 0.01$). Although a similar average result was observed in cells from BALB/c mice, the difference failed to reach statistical significance ($P = 0.058$). Average $CXCR_1$ expression was increased in cells from $CXCR_2$ KO mice compared with expression in background control BALB/c cells ($P < 0.01$). As expected, $CXCR_2$ expression was very low in the $CXCR_2$ KO ($P < 0.01$ compared with BALB/c).

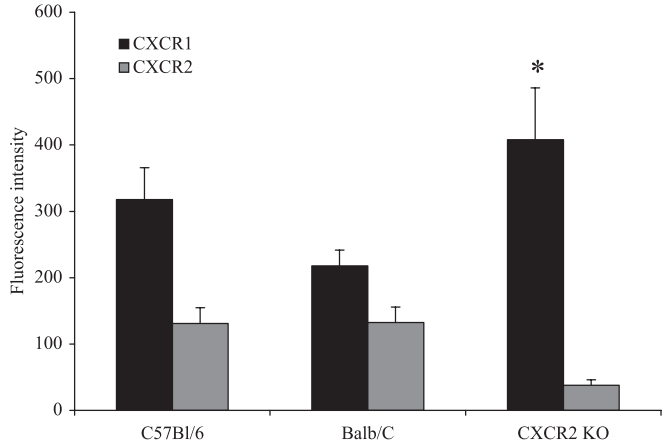

Fig. 3. Comparison of $CXCR_1$ and $CXCR_2$ levels on cultured arterial endothelial cells, as assessed by fluorescence intensity in mouse strains. $CXCR_1$ expression is greater in $CXCR_2$-deficient mice than in wild-type BALB/c mice (*$P < 0.01$).

DISCUSSION

The ELR+ CXC chemokines have been shown to be potent proangiogenic growth factors in human tissue and in a variety of experimental models (1, 2, 10, 21). These chemokines are released by several different inflammatory cell types as well as epithelial cells, and in human tissue their action is initiated by binding the G protein-coupled receptors $CXCR_1$/$CXCR_2$ (23, 24). Earlier work suggested that mice lack $CXCR_1$, and the proangiogenic mouse ELR+ CXC chemokines (MIP-2, KC, and LIX) function through $CXCR_2$ binding (18). The purpose of the present study was to determine whether $CXCR_2$ is essential for systemic angiogenesis in the mouse lung after complete left pulmonary artery obstruction. We showed previously that LPAL results in a robust systemic neovascularization of the ischemic lung by intercostal arteries within 5 days after LPAL (15, 28). We also showed by gene array analysis, mRNA, and protein quantification, that the mouse CXC chemokines MIP-2, KC, and LIX are upregulated early after pulmonary ischemia (22). Thus, to confirm that these growth factors, binding to their receptors, are essential for lung neovascularization, we used a $CXCR_2$-neutralizing antibody and measured blood flow to the left lung 14 days after LPAL. We showed previously that at this time point after LPAL, a stable neovasculature is established that perfuses the left lung parenchyma (15, 27). Our present results demonstrate significant

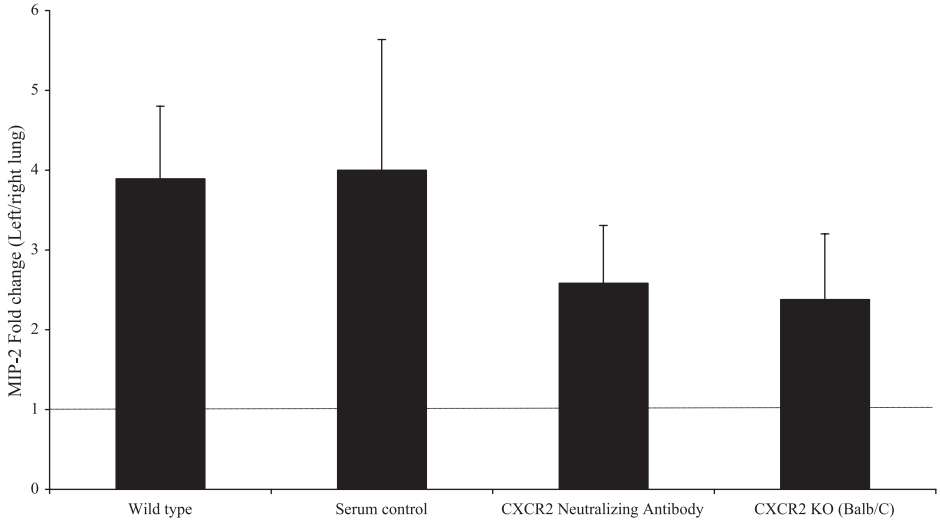

Fig. 2. Average fold change in macrophage inflammatory protein-2 (MIP-2) expression in the left lung after LPAL relative to normal right lung protein expression. No difference in MIP-2 expression was observed across treatment groups.

attenuation of blood flow after $CXCR_2$-neutralizing antibody treatment. These results are consistent with a large body of literature demonstrating the overall importance of this receptor and the effectiveness of this neutralizing antibody (1, 3, 11). Systemic blood flow to the ischemic left lung in C57Bl/6 mice was decreased by an average 67%. This represents a substantial inhibition of neovascularization and confirms the importance of $CXCR_2$ in new vessel growth. Although the effectiveness of antibody treatment appeared to be more limited in BALB/c mice (39% decrease), the absolute magnitude of blood flow after treatment was approximately the same in the two strains. The increase in blood flow 14 days after LPAL was significantly different between C57Bl/6 mice compared with BALB/c mice, demonstrating a strain-dependent difference in angiogenic potential. Thus, if $CXCR_2$ is blocked, the response in the two strains is equivalent. An important role for $CXCR_2$ in the growth of new vessels is confirmed and these results are also consistent with the decreased $CXCR_2$ expression in BALB/c mice endothelium (Fig. 3).

We followed an antibody treatment protocol extensively validated and previously shown to be effective in blocking angiogenesis in mouse models of bronchiolitis obliterans and non-small cell lung cancer (1, 2, 3). The positive response observed as an attenuation in blood flow confirms that the neutralizing antibody reached the site of proliferating intercostal blood vessels and was effective. However, the observation that the neutralizing antibody did not completely inhibit neovascularization could be related to inadequate dosing in this in vivo model as well as the likelihood that other growth factors contribute to the complex process of angiogenesis.

An important series of control experiments was to confirm that proangiogenic agonists were not decreased due to treatment protocols. We measured MIP-2 protein levels in the left lung 4 h after LPAL as representative of the CXC chemokines since this protein was previously shown to be the most highly expressed of the ELR+ CXC chemokines after LPAL (22). We again confirmed increased left lung/right protein expression of MIP-2 and the fold change did not vary among treatment groups (Fig. 2). These results suggested that the levels of CXC ligand, as represented by MIP-2, did not contribute to the reduction in blood flow observed after neutralizing antibody treatment. Additionally, the level of MIP-2 was not different in the C57Bl/6 mice compared with the BALB/c $CXCR_2$ KO, suggesting differences in ligand production were not responsible for strain-dependent differences in angiogenesis. Thus we conclude that the neutralizing antibody reached recovering intercostal arteries and prevented neovascularization despite a proliferative/migratory stimulus from lung chemokines.

Given the results using the $CXCR_2$-neutralizing antibody, we were perplexed by the lack of attenuation in angiogenesis in the $CXCR_2$-deficient mice as assessed by blood flow 14 days after LPAL. The overall hypothesis tested in this study was that $CXCR_2$ is essential for maximal angiogenesis. Thus we fully expected to obtain similar results in antibody-treated mice and $CXCR_2$-deficient mice. The $CXCR_2$-deficient mice used in this study are commercially available, homozygous for the $Il8rb^{tmMwm}$ targeted mutation, and are raised on a BALB/c background. Previous work by Strieter and colleagues (1, 11) showed consistent inhibitory results with both $CXCR_2$-neutralizing antibody and $CXCR_2$-deficient mice. We questioned whether potential compensatory mechanisms exist in the $CXCR_2$-deficient mice that might allow angiogenesis to take place during pulmonary ischemia, circumventing the normal process. With the assessment of the wide range of phenotypic alterations reported on these specific mice (http://jaxmice.jax.org/strain/002724.html), several factors might contribute to a compensatory mechanism in this model. Additionally, since $CXCR_1$ has been shown in human endothelium to contribute to the proangiogenic phenotype, we questioned earlier reports that stated that rodents lack $CXCR_1$ (18). Several recent studies show evidence for $CXCR_1$ in mouse tissue (7, 8, 16). Therefore, we harvested endothelium from each of the mouse strains studied and evaluated $CXCR_1$/$CXCR_2$ receptor expression in primary culture mouse arterial endothelial cells. We first confirmed the presence of mRNA for both $CXCR_1$ and $CXCR_2$ using real-time RT-PCR in mouse endothelial cells from C57Bl/6 mice. $CXCR_1$ showed an average 3.4-fold greater mRNA expression than $CXCR_2$. Using human antibodies raised against $CXCR_1$ and $CXCR_2$, we further evaluated differences in cell surface expression of these two receptors. In endothelial cells from both C57Bl/6 and BALB/c mice, measurement of fluorescence intensity representing cell surface receptor levels, showed the presence of both receptors with the signal for $CXCR_1$ predominating in all cells. Interestingly, Salcedo and colleagues (20) showed differential expression of these chemokine receptors in different types of human endothelial cell subtypes, and in human dermal microvascular endothelial cells, approximately equivalent levels of $CXCR_1$ and $CXCR_2$ as we report (average fluorescent intensity = 261 and 156 fluorescent units respectively). In the present study, $CXCR_1$ was increased significantly in endothelial cells from $CXCR_2$ KO mice compared with cells from wild-type BALB/c mice (Fig. 3). Based on these results, while acknowledging the limitations imposed by the use of human antibodies, we suggest that $CXCR_1$ expression may have been increased in the $CXCR_2$ KO mice, which contributed to endothelial cell signaling and sustained levels of angiogenesis. This confirmation of $CXCR_1$ on mouse arterial endothelial cells provides a potential explanation for the lack of complete effectiveness of the $CXCR_2$-neutralizing antibody. Previously, it was shown that this neutralizing antibody did not cross-react with human $CXCR_1$ nor did it prevent binding of IL-8 with $CXCR_1$ in human cells (1). A recent study by Fan and colleagues (7) suggested that another chemokine ligand of $CXCR_1$, CXCL6, plays a critical role in angiogenesis. However, in the present model, gene array profiling did not indicate a change in this chemokine after LPAL (22). Further clarification of the proangiogenic role of both chemokine receptors is needed and definitive answers await the availability of specific antibodies targeted to mouse $CXCR_1$ for in vivo use.

It should be noted that the neovascularization that occurs in this mouse model is different from other mammals where bronchial arterial neovascularization predominates. Because mice lack a developed subcarinal systemic vasculature, after LPAL, intercostal arteries play the major role in perfusing the ischemic lung (15). However, case reports in human subjects with chronic pulmonary embolism demonstrate growth of systemic vessels into ischemic lung from bronchial vessels as well as intercostal, mammary, and phrenic arteries (19). Thus, in mice and humans, systemic vessels in proximity to ischemic lung tissue appear to be primed to respond in situations of pulmonary ischemia. In this mouse model, we believe a che-

motactic gradient is provided by growth factors within the ischemic left lung, which is directly apposed to systemic vessels within the chest wall. Although we have not specifically prevented left lung association with the thoracic wall to test this hypothesis, it is the only explanation we can provide for the growth of intercostal vessels into the ischemic lung that lacks pulmonary perfusion. As noted previously, we see no intercostal vessel attachments in sham-operated mice (thoracotomy but no LPAL).

In summary, we showed that treatment of mice with a neutralizing antibody to $CXCR_2$ results in an attenuation of neovascularization after LPAL. We confirmed that ligand production, assessed by MIP-2 levels, was not limiting or altered despite differences in the absolute level of neovascularization. Using commercially available antibodies, we confirm the presence of both $CXCR_1$ and $CXCR_2$ in primary cultured mouse arterial endothelial cells. Mice with the genetic deletion of $CXCR_2$ may have other compensatory mechanisms that allow for the essential function of new vessel growth and proliferation.

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GRANTS

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