Vascular Stem/Progenitor Cell Migration Induced by Smooth Muscle Cell-Derived Chemokine (C-C motif) Ligand 2 and Chemokine (C-X-C motif) Ligand 1 Contributes to Neointima Formation

Running title: Vascular stem cell migration

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Keywords: vascular stem/progenitor cells, smooth muscle cells, CCL2, CXCL1, migration

ABSTRACT

Recent studies have shown that Sca-1$^+$ (stem cell antigen-1) stem/progenitor cells within blood vessel walls may contribute to neointima formation, but the mechanism behind their recruitment has not been explored. In this work Sca-1$^+$ progenitor cells were cultivated from mouse vein graft tissue and found to exhibit increased migration when co-cultured with SMCs or when treated with SMC-derived conditioned medium. This migration was associated with elevated levels of chemokines, CCL2 (chemokine (C-C motif) ligand 2) and CXCL1 (chemokine (C-X-C motif) ligand 1), and their corresponding receptors on Sca-1$^+$ progenitors, CCR2 (chemokine (C-C motif) receptor 2) and CXCR2 (chemokine (C-X-C motif) receptor 2), which were also up-regulated following SMC conditioned medium treatment. Knockdown of either receptor in Sca-1$^+$ progenitors significantly inhibited cell migration. The GTPases Cdc42 and Rac1 were activated by both CCL2 and CXCL1 stimulation and p38 phosphorylation was increased. However, only Rac1 inhibition significantly reduced migration and p38 phosphorylation. After Sca-1$^+$ progenitors labeled with GFP were applied to the adventitial side of wire-injured mouse femoral arteries, a large proportion of GFP-Sca-1$^+$-cells were observed in neointimal lesions, and a marked increase in neointimal lesion formation was seen 1 week post-operation. Interestingly, Sca-1$^+$ progenitor migration from the adventitia to the neointima was abrogated and neointima formation diminished in a wire injury model using CCL2$^{-/-}$ mice. These findings suggest vascular stem/progenitor cell migration from the adventitia to the neointima can be induced

by SMC release of chemokines which act via CCR2/Rac1/p38 and CXCR2/Rac1/p38 signaling pathways.

INTRODUCTION

Smooth muscle cells (SMCs) are well established as a key cell type that can contribute to the pathology of vascular diseases such as atherosclerosis and post-angioplasty restenosis[1-3]. Increasing evidence suggests that during vascular injury, SMCs in the media layer of the vessel undergo dedifferentiation from a quiescent/contractile phenotype to an active/synthetic phenotype and contribute to neointima formation through increased cell proliferation and migration[4, 5]. During this process, SMCs secrete a variety of chemokines such as chemokine (C-C motif) ligand 2 (CCL2) and (C-X-C motif) ligand 1 (CXCL1)[6]. These chemokines are largely responsible for recruitment of inflammatory cells[7, 8]. For example, after vascular injury, CCL2 is released by SMCs in a hypercholesterolemia mouse model and simultaneously chemokine (C-C motif) receptor 2 (CCR2) is up-regulated on monocytes, which leads to over-recruitment of leukocytes to lesion sites[9, 10]. In $ApoE^{-/-}$ mice CXCL1 presented by the lesion-prone endothelium triggers monocyte recruitment in a process dependent on $\alpha_4\beta_1$ integrin[11]. In the late stage of atherosclerotic lesion development, CXCL1 is also important for macrophage accumulation via leukocyte expression of chemokine (C-C motif) receptor 2 (CXCR2)[12]. Additionally, neutralization of CXCL1 reduces endothelial chemotaxis *in vitro* and delays endothelial recovery after arterial injury *in vivo* in a CXCR2-dependent manner[13]. However, little is known about the roles of CCL2 and CXCL1 secreted by SMCs in recruitment of other cell types, e.g. vascular resident stem/progenitor cells, into the intima in response to injury.

Recently, studies from several laboratories have identified the presence of a range of multipotent and lineage-restricted stem/progenitor cells in the adventitia of the vessel wall. These cells possess high potential to differentiate into many cell lineages, including endothelial and smooth muscle cells, adipocytes and osteoblasts[14-16]. Progenitor cells residing in the adventitia are positive for the stem/progenitor markers stem cell antigen-1 (Sca-1) and c-kit[17]. In our recent studies, we have shown that Sca-1^+ cells can migrate towards sirolimus, a drug used to coat stents[18], and differentiate into SMCs *in vitro* and in an *ex vivo* model of decellularized vessels[17, 19]. However, it is unknown whether chemokines released from SMCs in response to injury play a role in attracting vascular stem cells. The aims of this study were to investigate whether local SMC-released chemokines can induce adventitial-derived resident progenitor cell migration and to elucidate the mechanism behind progenitor cell promotion of neointima formation after vessel injury. In the present report, we demonstrate that SMCs can induce Sca-1^+ progenitor cell migration upon the release of CXCL1 and CCL2, which in turn activate the Rac1/p38 MAPK signaling pathways via the receptors CXCR2 and CCR2 respectively. Importantly, we demonstrate that CCL2-deficent mice display reduced Sca-1^+ cell migration and neointima formation in response to vessel injury.

MATERIALS AND METHODS

Mouse vascular progenitor cell culture

The vena cava of a C57BL/6J mouse was isografted to the carotid artery of an isogenic mouse or GFP C57BL/6J mouse. After 2 weeks graft tissues were harvested and explanted in gelatin-coated flasks. Culture was in complete stem cell culture medium.

Cell Sorting

Vascular progenitor cells (VPCs) were sorted with anti-Sca-1 immunomagnetic microbeads and then selected using a magnetic cell separator. Sca-1^{+} vascular progenitor cell populations were expanded for up to 5 population doublings.

Statistical Analysis

Data for this study are presented as the mean $\pm$ standard error of the mean (S.E.M.) of at least three separate experiments. Analysis was performed using Graphpad Prism V.6 (GraphPad Software, San Diego CA) using analysis of variance (one way ANOVA) followed by Dunnett's multiple comparison tests. Significance was considered when $p<0.05$.

Detailed Methods are included in the Online Data Supplemental.

RESULTS

Characterization of vascular stem/progenitor cells (VPCs)

Previously published works by our group identified a population of progenitor cells within pathological vessels from a mouse vein graft model[19, 20]. As in this previous work, 2 weeks after implantation of vein grafts, a large heterogeneous population of cells was found to migrate from the adventitia of vessels and contributed to neointima formation[17]. Cells were isolated from the adventitia and used to derive clones of single cells. Immunofluorescent staining showed that cells from this cloned population express progenitor cell marker Sca-1 (stem cell antigen-1) (Supplemental Figure 1A, B), but express very few smooth muscle cell or endothelial cell markers (Supplemental Figure 1C, D). In order to purify a larger pool of Sca-1^+ progenitor cells from the initially heterogeneous adventitial population anti-Sca-1 immunomagnetic microbeads were used. Approximately 15% of total cells collected using this method was identified as Sca-1^+ progenitor cells after sorting (Data not shown). Sca-1^+ cells derived from clones and a mixed population of Sca-1^+ progenitor cells derived by magnetic sorting were further characterized by a qPCR array showing gene expression of chemokines and chemokine receptors, further details are described in the following results. Our hypothesis is that Sca-1^+ progenitors may contribute to the pathology of atherosclerosis, potentially via their expression of chemokines and receptors.

SMC-conditioned medium induced VPC migration

To investigate whether SMCs influence the migratory capacity of vascular progenitor cells, a transwell migration assay was performed. Increasing numbers of SMCs were seeded on the

base of the lower chamber while fixed numbers of VPCs were seeded on the 8.0 μm pore membrane of the upper chamber. Using an increasing ratio of VPCs to SMCs (from 1:0 to 1:5), and allowing an overnight incubation, we found that the migration of VPCs increased as the number of SMCs increased and this reached its peak when the VPC:SMC ratio was 1:2 (Figure 1A). Next, SMC-conditioned medium was collected from SMC cultured overnight in serum free medium and used for migration assays. Addition of SMC-conditioned medium consistently stimulated VPC migration in both transwell and wound healing assays, when compared to serum free medium. (Figure 1B, C).

Time lapse microscopy was used to track the speed and the persistence of single progenitor cells during their migration when treated with SMC conditioned medium in an overnight incubation. These results showed that SMC-conditioned medium augmented directional persistence of VPC migration, while migrational velocity remained unaltered between the treatments (Figure 1D, E, F and supplemental online Video 1 (control)，supplemental online Video 2 (SMC conditioned medium)). Use of a BrdU cell proliferation assay confirmed that this effect was not due to proliferation (Supplemental Figure 2A). qPCR analysis revealed that the mRNA levels of SMC markers (calponin and α-SMA) and endothelial marker (CD31) in VPCs were not significantly changed during the experiment (Supplemental Figure 2B, C, D). VPCs also maintained progenitor characteristics, as the progenitor marker Sca-1 was not significantly changed when compared to the control (Supplemental Figure 2E). Additionally, immunofluorescence staining for paxillin, vinculin and phosphorylated FAK showed that relocation of cytoskeleton-related proteins was increased (Supplemental Figure

3A). Taken together, the above results support the notion that both co-culture with SMC and SMC-derived conditioned medium can induce VPC migration.

SMC-released CCL2 and CXCL1 induced VPC migration

To further investigate which mediators released by SMCs were responsible for the conditioned medium induced VPC migration, we performed a chemokine multi-ELISA array. This revealed that CXCL1 and CCL2 levels were markedly increased in SMC-conditioned medium when compared to serum free medium (Figure 2A). Murine CCL2 or CXCL1 Quantikine kits established that the amounts of CCL2 and CXCL1 in the conditioned medium were 4.63±1.29 ng/ml and CXCL1 3.08±0.99 ng/ml, respectively (Figure 2B, C). While the level of CCL5 was also up-regulated in comparison to the control, subsequent transwell migration assays found that it did not affect VPC migration (Supplemental Figure 4A, B). To confirm the roles of CCL2 and CXCL1 in mediating VPC migration, we performed transwell and wound healing assays using exogenous mouse recombinant CCL2 and CXCL1 proteins, respectively. VPC migration was enhanced by CCL2 and CXCL1 treatment and peaked at 5 ng/ml (a 6 fold increase in CCL2 and a 2 fold increase in CXCL1 treated cells) (Figure 2D, E). These were similar concentrations of CCL2 and CXCL1 to those measured in SMC-derived conditioned medium. That a 5ng/ml CCL2 or CXCL1 treatment enhanced VPC migration was confirmed by wound healing assay (Figure 2F, G). Silencing of CCL2 or CXCL1 in the SMCs using siRNAs resulted in significant down-regulation of CCL2 and CXCL1 at both the mRNA and protein levels (Figure 2H, I, J, K). Importantly, CCL2 or CXCL1 depleted SMCs failed to stimulate VPC migration in transwell assays (Figure 2L,

M). To further investigate the relationship between CCL2 and CXCL1 in SMC conditioned medium induced VPC migration，CCL2 and/or CXCL1 concentrations were depleted using their corresponding neutralizing antibodies. The antibodies were found to be effective and selective as they significantly reduced the availability of their target chemokine while they had no effect on the other chemokine studied. Depletion of either CCL2 or CXCL1 did not result in a compensatory release of the other chemokine and there appeared to be no interactions between the two antibodies (supplemental Figure 5A, B). Having confirmed the effectiveness of the neutralizing antibodies they were used to study the role of CCL2 and CXCL1 in VPC migration. Depletion of either CCL2 or CXCL1 in SMC conditioned medium inhibited migration of VPCs by nearly 50%. However, simultaneous depletion of CCL2 and CXCL1 in SMC conditioned medium did not further reduce this migration level (supplemental Figure 5C, D). Finally, incubation with various concentrations of CCL2 or CXCL1 did not stimulate VPCs autocrine production of the other chemokine (supplemental Figure 5E, F). Taken together, these results suggest that though CCL2 and CXCL1 derived from SMCs play an important role in the induction of VPC migration, their interaction is neither cumulative nor redundant but both are required for migration to be induced.

VPC migration is mediated by CCR2 and CXCR2

Each chemokine needs to interact with its corresponding receptor in order to exert an effect. We investigated the chemokine receptor profile of VPCs by performing a qPCR-array of a mixed population of Sca-1^+ progenitor cells derived by magnetic sorting and Sca-1^+cells

derived from a clone. We demonstrated that VPCs possess a variety of chemokine receptors (Figure 3A, Supplemental Table 2) and importantly, CCR2 and CXCR2, the corresponding receptors of CCL2 and CXCL1. Both mRNA and protein level expression of these receptors was significantly up-regulated after treatment with SMC conditioned medium (Figure 3B, C, D, E). To silence CCR2 and CXCR2 in VPCs at the gene level, VPCs were infected with CCR2 or CXCR2 lentiviral shRNA while a null shRNA was also introduced into VPCs as a control. The cells were selected for 4-5 days using neomycin and maintained in complete culture medium. Subsequent transwell migration experiments with CCR2 or CXCR2 silencing resulted in a significant decrease in VPC migration (Figure 3F). In addition, pretreatment with antagonists of CCR2 or CXCR2 led to down-regulation of VPC migration in response to chemokines (Supplemental Figure 6A, B). These findings suggest that CCL2 and CXCL1 released from SMCs induce VPC migration via interaction with their corresponding receptors CCR2 and CXCR2.

CCL2 and CXCL1 induced VPCs migration through the Rac1-p38 pathways

The Rho GTPase family members Rac1 and Cdc42 have been implicated as important regulators of cell migration[21]. To investigate whether Cdc42 and Rac1 are involved in VPC migration, we measured the level of GTP-Cdc42 and GTP-Rac1 using pull down assays. Stimulation of VPCs with mouse recombinant CCL2 or CXCL1 resulted in both Cdc42 and Rac1 activation (Figure 4A, B). Treatment with either ML141 (an inhibitor of Cdc42) or NSC23766 (an inhibitor of Rac1) resulted in a significant reduction in VPC migration induced by CCL2 and CXCL1 as compared to control (Figure 4C, D).

p38 MAPK has been identified as the down-stream signal transducer of active forms of Cdc42 and Rac1 and the p38 signaling pathway can induce rearrangements of the cytoskeleton that regulate cell migration[22, 23]. A time course using western blotting analysis showed p38 phosphorylation occurred in VPCs after treatment with SMC conditioned medium for 20 minutes (Figure 4E). p38 phosphorylation was up-regulated in response to mouse recombinant CCL2 and CXCL1 treatment (Figure 4F), and suppressed in response to conditioned medium derived from CCL2 siRNA or CXCL1 siRNA depleted SMCs (Figure 4G). p38 phosphorylation was also suppressed in VPCs where CCR2 or CXCR2 had been knocked down using the corresponding shRNA (Figure 4H). Pretreatment of VPCs with CCR2 or CXCR2 antagonists consistently reduced the p38 phosphorylation (Supplemental Figure 6C, D). Importantly, p38 phosphorylation was significantly reduced in VPCs pretreated with NSC23766 but not ML141 (Figure 4I, J). Finally, migration of VPCs was reduced in the presence of SB203580 (an inhibitor of the p38-MAPK signaling pathway) (Figure 4K). These indicate Rac1 regulates CCL2 and CXCL1 induced VPC migration via the p38 signaling pathway. RhoA, as another important small GTPase has also been reported to regulate the cell migration process[21], however, pretreatment of VPCs with C3 transferase (an inhibitor of RhoA), did not alter VPC migration or p38 phosphorylation (Supplemental Figure 7). We therefore conclude that CCL2 and CXCL1 induce VPC migration by activating GTPase Rac1 and p38 signaling pathways.

Lack of CCL2 inhibits Sca-1$^+$ cell migration and neointima formation

SMCs can be described as activated and switched into the synthetic phenotype when they are cultured *in vitro*[24] and under these conditions they constitutively release CCL2 and CXCL1. To study chemokine release in *in vivo* conditions, a mouse femoral artery wire injury model was used to assess whether native SMCs only produce chemokines which attract VPC migration after cell injury. Co-immunofluorescence staining of α-SMA and CCL2 or CXCL1 showed that in intact vessels, SMCs are quiescent and do not produce CCL2 or CXCL1. However, once SMCs are injured they release increasing levels of CCL2 and CXCL1 (both at a short time point (6 hours) and a long time point (2 weeks) (Supplemental Figure 8). These chemokines could be potent and persistent attractants for VPC migration. To verify this, GFP-Sca-1$^+$-VPCs, additionally labeled using a Qtracker® 655 Cell Labeling Kit, were seeded on the adventitia of vessels which were injured. *En face* confocal microscopy revealed that 72 hours after injury the number of migrated cells found on the intimal side of the vessel wall was significantly lower in CCL2$^{-/-}$ mice when compared to WT mice (Figure 5A, Supplemental Figure 10A). CCL2$^{-/-}$ mice were identified by genotyping mice and measuring CCL2 levels in peripheral blood (Supplemental Figure 9A, B). Quantification based on either GFP-Sca-1$^+$-VPCs or Qtracker® showed similar results (Figure 5B, Supplemental Figure 10B). Sca-1 immunofluorescence staining in sections of injured arteries 2 weeks post injury, showed that GFP-Sca-1$^+$-VPCs remained Sca-1 positive after 2 weeks *in vivo* but that fewer migrated into the intimal side to contribute to neointima formation in CCL2$^{-/-}$ mice compared to the WT mice (Figure 5C, D, E). These results

suggest a role for CCL2 in VPC migration from the adventitia to the intima where they may contribute to neointima formation.

To further investigate the role of CCL2 in VPC neointima formation, wire-injured femoral arteries were seeded with VPCs or PBS in the adventitia. HE stained sections showed neointima formation in the vessel at 1 or 2 weeks after wire injury (Figure 6A, B). Quantification of the data indicated that neointimal lesions were significantly increased in vessels seeded with VPCs. In the 2 week post injury group, the difference in ratio of neointima to media with VPCs or PBS was higher than that in the 1 week group. However, neointimal lesions in $CCL2^{-/-}$ mice were decreased in vessels with either VPCs seeded in the adventitia or PBS. There is a significant reduction in neointimal lesions area of vessels with VPC seeding in $CCL2^{-/-}$ mice when compared to the WT mice at 2 weeks.

To characterize cells contributing to neointima formation, immunofluorescence staining of α-SMA and Sca-1 was used, which showed that the cells in the neointima of PBS treated vessels were approximately 70% $\alpha\text{-SMA}^{+}$ and 10% Sca-1^{+} 2 weeks after injury (Supplemental Figure 11). Staining of α-SMA and CCR2 in Figure 6 revealed that cell numbers in neointima of VPC seeded vessels were increased, though fewer were $\alpha\text{-SMA}^{+}$. These results indicate that in the PBS treated vessels, most cells contributing to neointima formation are $\alpha\text{-SMA}^{+}$. These cells are either from media SMC migration and proliferation or from progenitor cell differentiation. When Sca-1^{+} VPC were seeded in the adventitia, the ratio of $\alpha\text{-SMA}^{+}$ cells was significantly decreased which implies that VPCs possess high

potential to migrate and proliferate. Over a short time most cells maintained their progenitor cell characteristics, though several had differentiated into SMCs (Figure 6C, E). CCR2 was more highly expressed on the cells in the neointima of VPC-seeded vessels (Figure 6D, F). These data suggest that VPCs applied to the adventitia significantly increase neointima formation after vessel injury, largely through their migration though with a lesser effect due to their differentiation into SMCs. Furthermore, lack of CCL2 markedly inhibits the effect of VPCs on neointima formation.

Role of CCL2 released from non-bone marrow tissue

CCL2 is not only released from bone marrow cells but also from SMCs, endothelial cells and other tissues[9, 25]. To investigate which source of CCL2 plays the most important role in neointima formation due to VPC migration chimeric mouse models were created. Wild-type bone marrow was transplanted into an irradiated $CCL2^{-/-}$ mouse to form a CCL2 chimera. Conversely, a wild-type chimeric mouse model was prepared by transplanting $CCL2^{-/-}$ mouse bone marrow cells into an irradiated wild-type mouse. The levels of CCL2 in serum of peripheral blood were measured using a murine CCL2 ELISA kit. In the wild-type chimeric mouse model, the level of CCL2 in peripheral blood was increased despite transplantation with $CCL2^{-/-}$ bone marrow when compared to a non-transplanted wild-type mouse control, indicating that CCL2 is mostly released from non-bone marrow tissue. In contrast, the level of CCL2 was increased in $CCL2^{-/-}$ mice with transplanted wild-type bone marrow cells, but to a much lesser extent, indicating that only a small proportion of CCL2 is released from bone marrow derived cells (Figure 7A). Data from HE stained sections showed that neointima

lesion area correlated with the CCL2 level in blood (Figure 7B, C). These results support the observations that CCL2 induced VPC migration contributes to neointima formation.

CXCL1 plays a role in Sca-1 positive cell migration *in vivo*

Previous studies have demonstrated that CXCL1 can induce endothelial cell migration and tube formation *in vitro*[26] and also acts as an angiogenic factor to promote tumor growth[27]. To further investigate the role of CXCL1 in induction of VPC migration *in vivo*, VPCs were mixed with matrigel containing either PBS or mouse recombinant CXCL1 and injected subcutaneously. After 2 weeks, the number of cells that migrated into the matrigel was significantly increased in the CXCL1 group when compared to PBS controls (Supplemental Figure 12A). Immunofluorescence staining of Sca-1, CD31 and α-SMA showed that most of the migrated cells in the matrigel plug were Sca-1 positive, whilst some were CD31 positive, and only very few were α-SMA positive (Supplemental Figure 12B). In addition, CXCL1 or control siRNA within the pluronic gel was delivered to the adventitial side of wire injured vessels to assess the effect of local CXCL1 knockdown on Sca-1^+ VPC migration *in vivo*. Successful *in vivo* knockdown of CXCL1 mRNA level in injured femoral arteries was confirmed after 6 days using real time quantitative PCR (Supplemental Figure 13A). Furthermore, *En face* confocal microscopy revealed that 72 hours after seeding GFP-Sca-1^+VPC in the adventitia, the number of migrated cells found on the intimal side of the vessel wall was lower in CXCL1 siRNA treated vessels compared to the control siRNA (supplemental Figure 13B). These results indicate the important role of CXCL1 in Sca-1^+ cells migration *in vivo*.

DISCUSSION

Restenosis is still the main complication that exacerbates the outcome of coronary artery disease after percutaneous coronary intervention[28-30]. Smooth muscle cell proliferation and migration are suggested to be important factors in development of neointimal hyperplasia and restenosis[31]. In the present study, we identify a new mechanism of smooth muscle accumulation in neointimal lesions after vascular injury, in which vascular stem/progenitor cells migrate from the adventitia to the intima. We demonstrate that proliferating SMCs can release several chemokines, including CXCL1 and CCL2, which have a role in attracting these vascular progenitors. Single cell tracking experiments indicate that cells are migrate directionally and efficiently but not randomly. Importantly, perivascular application of GFP-Sca-1^{+}-VPC to injured arteries significantly enhanced neointimal lesion formation via progenitor migration. This effect is diminished by CCL2 knockout. We provide the first evidence that the SMC-produced chemokine CCL2 is crucial for vascular progenitor migration from the adventitia to the intima where these cells contribute to lesion formation.

After endothelial injury, an inflammatory response occurs in the vessel wall, and chemokines are released by both mononuclear cells and SMCs[32, 33]. Using multiple chemokine ELISA we demonstrated that several chemokines were up-regulated in cultured SMCs and amongst them, CXCL1, CCL2 and CCL5 were secreted at the highest levels. To further confirm the effects of these chemokines, we stimulated stem cells with exogenous mouse recombinant proteins and found that CCL2 and CXCL1 significantly induced vascular progenitor migration. Previous studies have reported that CCL5 mediates trafficking and homing of T

cells, monocytes, basophils and eosinophils[34-37]. However, it does not appear to play such a role in induction of progenitor migration, indicating that vascular stem cells may selectively or specifically respond to certain chemokines.

Chemokine receptors are expressed on many different cell types, such as granulocytes, monocytes, mast cells, T cells, and endothelial cells[38, 39], but no prior study has shown the presence or absence of chemokine receptors on vascular progenitors. We used a qPCR array to profile the expression of chemokines, cytokines, and their receptors in different vessel wall cell lines. The results showed the presence of most cytokines and chemokine receptors in a mixed population of Sca-1^+ progenitor cells derived by magnetic sorting and one clone of Sca-1^+ progenitor cells and although SMCs display a higher expression level of different chemokine receptors. This distinct chemokine receptor profile marks a distinction between mature SMCs and progenitors. Expression of CCL2 and CXCL1 was much higher on SMCs compared to other cells, which was similar to our finding at the protein level of CCL2 and CXCL1 in SMCs as measured in the ELISA array. As many reports have demonstrated, CCL2/CCR2 and CXCL1/CXCR2 signal transduction mediates recruitment of neutrophils, monocytes or macrophages into inflammatory sites during progression of different kinds of disease[12, 40-43]. In our study, we found that both CCR2 and CXCR2 were up-regulated on VPCs after treatment with SMC-derived chemokines. The ability of VPCs to migrate after CCR2 and CXCR2 genes were permanently silenced by shRNA was significantly reduced. This indicates that SMC-released CCL2 and CXCL1 activate their corresponding receptors on vascular stem/progenitor cells.

In order to elucidate the mechanism of cell migration we studied the role of the Rho GTPase family. Rac1, Cdc42 and RhoA, as the main Rho GTPase family members, regulate the formation of lamellipodia, filipodia and focal adhesions, respectively[44]. In the present study, we found both Cdc42 and Rac1 in VPCs were activated by CCL2 and CXCL1 and inhibition of either Cdc42 or Rac1 impaired VPC migration in response to CCL2 or CXCL1. Cytokines such as IL-4 and TNF-α can activate stress-activated pathways leading to phosphorylation of p38 MAPK which is dependent on Rac1 and Cdc42[45, 46]. In our study, p38 phosphorylation was up-regulated in response to mouse recombinant CCL2/CXCL1, and p38 phosphorylation was suppressed by CCL2/CXCL1 knockdown or CCR2/CXCR2 inhibition. These results suggest that progenitor migration is induced through a p38 MAPK signaling pathway. Importantly, we also found that p38 phosphorylation was markedly down-regulated by Rac1 inhibition but not inhibition of Cdc42 or RhoA, suggesting that progenitor migration is induced via a Rac1/p38 signaling pathway. Although the migration assay showed the participation of Cdc42 in SMC induced progenitor migration, it may through an as yet unidentified signaling pathway.

As the above results show that CCL2 and CXCL1 contribute to VPC migration equally, we wanted to further investigate possible interactions between the two chemokines. Depletion of each chemokine was found to partially inhibit VPCs migration, but treatment with each chemokine individually failed to stimulate VPCs to release the other. After loss of one chemokine, the induction of VPCs migration is not compensated by the other chemokine. Simultaneous depletion of both chemokines did not further inhibit VPC migration when

compared to inhibition of a single chemokine. The effects of CCL2 and CXCL1 in the mediation of VPCs migration were found to be neither cumulative nor redundant. However, there may also be additional factors in SMC conditioned medium which play a role in inducing VPC migration, as migration was not completely abolished on depletion of both chemokines. We have shown that both chemokines induce VPC migration through the Rac1/p38 signaling pathway but act through their own receptors. Previous studies[47, 48] have shown that chemokine receptors can form co-receptors to mediate their effects, thus further studies need to be performed to determine whether CCR2 and CXCR2 form a co-receptor to induce VPC migration.

Reports using different animal models have shown that CCL2 plays an important role in neointimal hyperplasia[49-51], for example CCL2 enhances SMC migration, proliferation and invasion to remodel vessels[52, 53]. VPCs have also previously been identified as playing a role in neointima formation[17, 19]. In the present study, we demonstrated that migrating progenitors are an important cellular component of CCL2 mediated neointima formation. We found CCL2 not only enhanced VPC migration from the adventitia to the intima in a short time period but also accelerated neointima formation by inducing VPC migration. We also identified that the majority of cells contributing to neointima formation are not smooth muscle cell marker positive but express a stem cell marker (Sca-1), which indicates that though some VPCs participate in neointima formation and differentitate into SMCs, most of them retain progenitor cell characteristics as they migrate into the neointima.

The expression of CCR2 in neointimal cells suggests that the participtation of VPCs in neointima formation may be dependent on CCL2-driven recruitment. Vande et al demonstrated the main source of CCL2 is bone marrow-derived monocytes[54]. In our study, through use of chimeric mouse models, we found that after bone marrow transplant, CCL2 can be detected in the peripheral blood of a $CCL2^{-/-}$ chimeric mouse (bone marrow was from WT mouse), where it was originially undetectable. This confirms the findings of previous studies[54]. However, in a WT chimeric mouse (bone marrow was from $CCL2^{-/-}$ mouse), the levels of CCL2 were markedly up-regulated and showed an even higher increase compared to the $CCL2^{-/-}$ chimeric mouse. This indicates that CCL2 from non-bone marrow tissues (e.g. SMC) contributes more to the global CCL2 levels in the peripheral blood than bone marrow. Quantitative data from neointimal lesions further confirms that CCL2 from non-bone marrow tissues-induces progenitor migration which contributes to neointima formation. Taken together, CCL2 released from non-bone marrow tissues (e.g. SMC) accelerates neointima formation through induction of progenitor migration from adventitia to neointima.

It was believed that neointimal formation in response to endothelial injury was mainly due to cell accumulation via recruiting inflammatory cells[55, 56] and SMCs[57, 58] from the media. In this study, we utilised an accelerated model of *in vivo* neointima formation by inducing endothelial injury. From the data generated in non-VPC seeded groups, we found that during the short time period of 1-2 weeks very few cells contributed to the spontaneous development of neointimal lesions. In contrast, an obvious neointima was found in VPC seeded groups, which suggested that the neointima formation is largely generated by VPCs

migration in the early stage of the model. These findings have two implications. First, the cells accumulating in neointimal lesions may be derived from migration/proliferation of vascular stem cells, which can differentiate into SMC-like cells, although mononuclear cells and medial SMCs contribute to the process. To obtain quantative data on how many cells in neointimal lesions are derived from progenitor cells, lineage tracing for progenitor cells in animal models would be essential. Secondly, a new treatment strategy for restenosis could be considered. Current treatments used in clinical are based on inhibition of cell proliferation[59, 60], e.g. sirolimus. A recent report from our group suggests that sirolimus can enhance vascular stem/progenitor cell differentiation into SMCs, but inhibits endothelial differentiation[18]. Our current findings that large numbers of stem/progenitor cells are recruited to the intima in response to CCL2 and CXCL1 provide a potential to direct cell differentiation into the endothelial lineage. In other words, a high number of stem cells exist during neointima formation, which could differentiate into endothelial cells if a new drug coated stent could harness their potential and directed them specifically.

SUMMARY

Taken together, these results may provide crucial answers as to why restenosis and delayed re-endothelialization persist after angioplasty and stenting, which may be fundamental for developing new drugs that can improve the long-term outcome of patients.

ACKNOWLEDGEMENTS

This study was supported by grants from the British Heart Foundation (RG/14/6/31144) and the Oak Foundation. B.Y. was supported by the China Scholarship Council (No.201206010064).

DISCLOSURE OF POTEINTIAL CONFLICTS OF INTEREST

None

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Figure Legends

Figure 1. **SMC conditioned medium can induce VPCs migration**. **A**. Chemotaxis of vascular progenitor cells (5×10^4 cells/well) across 8.0 μm transwells toward an increasing number of SMCs after 18 hours was documented after 0.1% crystal violet staining. Serum free culture medium was used as control for all migration experiments, n=5. Scale bars, 100μm. **B, C.** Migration of vascular progenitor cells in response to SMC conditioned medium. Chemotaxis index was defined by average of 9 fields of view and was presented as fold increase compared to the control, n=5. Scale bars, 100μm. **D, E, F** Vascular progenitor cells were incubated with or without SMC conditioned medium for 20 hours. Time lapse microscopy was performed to observe single cell movement. Trajectory plots of migrating VPC are shown in (**D**), 100 tracked single cells in each group are displayed. Quantification analysis of the speed (**E**) and persistence (**F**) of VPC were performed in 3 independent experiments. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. ns, P>0.05. ctrl, control, serum free medium. CM, SMC conditioned medium.

Figure 2. **CCL2 and CXCL1 released from SMC conditioned medium induced VPCs migration. A.** The identification of chemokines in SMC conditioned medium using a Chemokine Multiarray ELISA kit. The graph indicates the absorbance of each chemokine at 450nm. n=3. **B, C.** The concentrations of CCL2 and CXCL1 in SMC conditioned medium were quantified by the murine CCL2 or CXCL1 Quantikine ELISA kit. n=3. **D, E.** Changes in vascular progenitor cells migration in response to a gradient of CCL2 or CXCL1 in serum free culture medium were evaluated using a transwell assay. n=5. Scale bars, 50μm. **F, G.**

Wound healing assay was performed on vascular progenitor cells treated with CCL2 (5ng/ml), CXCL1 (5ng/ml) or vehicle control. Graphs are shown as fold increase relative to controls. Scale bars, 100μm. SMCs were transfected either with control noncoding small interfering RNA (siRNA), CCL2 siRNA (300nM) or CXCL1 siRNA (100nM) to knockdown corresponding mRNA. The real-time quantitative PCR and Quantikine ELISA kit showed the folds decrease in mRNA (**H, J**) and protein (**I, K**) levels in CCL2 or CXCL1 siRNA transfected SMCs and their conditioned medium respectively. n=3. **L, M.** Transwell assay was performed on vascular progenitor cells migrating towards SMC (transfected either with noncoding siRNA, CCL2 siRNA or CXCL1 siRNA) conditioned medium. n=5. Scale bars, 50μm. All graphs are shown as mean ± SEM. **p<0.01, ***p<0.001. NCsi, noncoding siRNA. siCCL2, CCL2 siRNA. siCXCL1, CXCL1 siRNA. ctrl, control, serum free medium. CM, SMC conditioned medium.

Figure 3. **SMC conditioned medium induced VPC migration by up-regulating expression of CCR2 and CXCR2. A.** Cluster grams show the expression of 84 chemokine receptors, chemokine and other chemotactic cytokines on the untreated smooth muscle cells, clones and Sca-1^+ VPCs individually. A row in the cluster represents a cell line, and relative level of expression for a gene is arranged in column. The magnitude of gene expression increases from green to red. The dendrogram of the different cell lines clustering is displayed aside and describes the degree of relatedness among different cell lines. Quantification was performed with three different batches of each cell line. The vascular progenitor cells were treated with or without SMC conditioned medium for 18 hours, untreated cells served as

controls. n=3. The mRNA (**B, D**) and cell-surface protein (**C, E**) expression of CCR2 or CXCR2 was confirmed using real-time quantitative PCR and flow cytometry. n=3. **F.** Vascular progenitor cells were infected with lentiviral short hairpin RNA (shRNA) (noncoding shRNA, CCR2 shRNA or CXCR2 shRNA) for ablation of CCR2 or CXCR2 before migration toward SMC conditioned medium. The noncoding shRNA served as the control. n=3. Scale bars, 100μm. All graphs are shown as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001. NCsh, noncoding shRNA. shCCR2, CCR2 shRNA. shCXCR2, CXCR2 shRNA. ctrl, control, serum free medium. CM, SMC conditioned medium.

Figure 4. **SMC induces VPCs migration via activated GTPase Cdc42 and Rac1 and p38 signaling pathway. A, B.** Pull down assays were performed on VPCs treated with either CCL2 or CXCL1 for 5 minutes. The transwell assay was performed on VPCs that were pretreated with either vehicle, DMSO or ML141 (20 μM) (**C**), NSC23766 (50 μM) (**D**), SB203580 (10μM) (**K**) for 1 hour before migration toward either SMC conditioned medium or recombinant CCL2, CXCL1. Scale bars, 100μm. The quantification of transwell assay shows as the fold of changes compared with cell migrated toward each treatment pre-treated with vehicle or DMSO. Western blotting was performed on SMC conditioned medium (**E**), CCL2 or CXCL1 (**F**), SMCs (transfected with CCL2 siRNA or CXCL1 siRNA) conditioned medium (**G**)-treated VPCs for the detection of p-p38 and t-p38. **H.** Cell lysates from CCR2 shRNA or CXCR2 shRNA transfected VPCs cultured in the SMC conditioned medium were harvested for the detection of the p-p38 and t-p38. Untreated cells served as the controls. **I, J.** After pre-treated with ML141, NSC23766 for 1 hour, VPC were stimulated by SMC

conditioned medium or CCL2, CXCL1 before cell lysates were harvested for detection of the p-p38 and t-p38. All the blots shown are representative of 3 separate experiments. All graphs are shown as mean ± SEM. n=3. *p<0.05, **p<0.01, ***p<0.001. NCsi, noncoding siRNA. siCCL2, CCL2 siRNA. siCXCL1, CXCL1 siRNA. NCsh, noncoding shRNA. shCCR2, CCR2 shRNA. shCXCR2, CXCR2 shRNA. ctrl, control, serum free medium. CM, SMC conditioned medium. DMSO, dimethyl sulfoxide. vehicle, sterile distilled water. p-p38, phosphorylated p38. t-p38, total p38.

Figure 5. **Lack of CCL2 inhibits Sca-1$^+$ cell migration *in vivo*. A.** Using a mouse femoral artery wire injury model, GFP-Sca-1$^+$ vascular progenitor cells (GFP-Sca-1$^+$-VPC) (1x10^6) were seeded in the adventitia of each injured vessel. *En face* staining shows the cells were migrated to the intima side of the vessels 72hrs post injury of WT and CCL2$^{-/-}$ mice. Scale bars, 25μm. **B.** The percentage of GFP-Sca-1$^+$-VPC within respective DAPI$^+$ populations in each view was quantified. **C.** The femoral arteries sections from WT and CCL2$^{-/-}$ mice 2 weeks post injury were prepared for immunofluorescent Sca-1 staining. Scale bars, 50μm. **D, E.** The graphs show the percentage of GFP-Sca-1$^+$-VPC or Sca-1$^+$ cells within the DAPI$^+$ cells in the neointima (white dotted line indicates internal elastin, the neointima area was surrounded by the line). Representative images and graphs shown as mean ± SEM of n=8 mice/group. **P<0.01, ***P<0.001. WT, wild type.

Figure 6. **Lack of CCL2 reduces neointima formation and inhibits Sca-1$^+$ cells migration and differentiation into SMCs A.** Animals were euthanized at indicated time points after injury, and the femoral arteries were fixed in 4% phosphate-buffered (pH=7.2) formaldehyde, embedded in paraffin, sectioned in 5 μm, and stained with hematoxylin–eosin. Scale bars, 50μm. **B.** The ratio of neointima (the area between arrows) to media was quantified as shown in the graph. **C, D.** Vessel sections were also prepared for immunofluorescent α-SMA and CCR2 staining 2 weeks post wire injury (white dotted line indicates internal elastin, and the above is neointima area). Scale bars, 50μm. **E, F.** Quantification of the percentage of positively stained cells within the neointima was shown in graphs as mean ± SEM of n=8 mice/group. **P<0.01, ***P<0.001. WT, wild type. PBS, phosphate buffered saline. VPC, vascular progenitor cell.

Figure 7. **CCL2 released from peripheral tissue plays an important role in contribution of the neointima formation A.** Using chimeric mice model, the CCL2 in peripheral blood of indicated mice was subjected to a CCL2 Quantikine ELISA kit. The vessels sections of indicated mice at various time points were stained with hematoxylin–eosin, and the neointima area (the area between arrows) was shown in the represented images (**B)** Scale bars, 50μm. , and quantified in the graphs (**C**) shown as mean ± SEM of n=5 mice/group. *p<0.05, **p<0.01, ***p<0.001. WT+CCL2$^{-/-}$ BMT，wild type mice of which bone marrow was transplanted from CCL2$^{-/-}$ mice. CCL2$^{-/-}$+WT BMT, CCL2$^{-/-}$ mice of which bone marrow was transplanted from wild type mice.

SUPPLEMENATAL MATERIAL

Vascular Stem/Progenitor Cell Migration Induced by SMC-derived CCL2 and CXCL1 Contributes to Neointima Formation

Running title: Vascular stem cell migration

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SUPPLEMENTAL MATERIAL AND METHODS

Mice

All procedures were performed according to protocols approved by the Institutional Committee for Use and Care of Laboratory Animals. The CCL2 deficient mice (B6.129S4-*Ccl2*^{tm1Rol}/J) and GFP mice (C57BL/6-Tg(UBC-GFP)30Scha/J) on the C57BL/6J background were purchased from The Jackson Laboratory (Bar Harbour, Maine, USA). C57BL/6J mice (as WT mice) were purchased from Harlan, UK. Genotyping for CCL2$^{+/-}$ and CCL2$^{-/-}$ mice was performed using standard PCR according to a protocol provided by The Jackson Laboratory. The following primers were used: oIMR7415 (CCL2$^{-/-}$ forward) 5'- GCC AGA GGC CAC TTG TGT AG-3', oIMR9219 (CCL2$^{+/+}$ forward) 5'- TGA CAG TCC CCA GAG TCA CA' and oIMR9220 (common reverse) 5'- TCA TTG GGA TCA TCT TGC TG -3'.

Mouse vascular progenitor cell culture

Mouse vascular progenitor cells were derived from the outgrowth of adventitial tissues of vein grafts as previously described[1]. Briefly, the vena cava from a C57BL/6J mouse was

isografted between two ends of the carotid artery of an isogenic mouse or GFP C57BL/6J mouse. After 2 weeks graft tissues were carefully harvested and cut into pieces and explanted on 0.04% gelatin (0.04% of 2% Solution Type B from Bovine Skin, Sigma)-coated flasks. Culture was in complete stem cell culture medium comprised of Dulbecco's Modified Eagle's Medium (ATCC, Rockville, Massachusetts, USA) supplemented with 10% ES Cell Qualified Fetal Bovine Serum (EmbryoMax, Millipore), 10 ng/ml leukemia inhibitory factor, 0.1 mM β-mercaptoethanol, 100U/ml penicillin/streptomycin and 2mM L-glutamine(Life Technologies). Graft tissues were incubated at 37℃，5% CO_2 for 3 days. The cells that grew out from the graft tissue were dissociated from the flasks using 0.05% trypsin-EDTA (Life Technologies) and were passaged every other day at a 1 to 3 ratio.

Isolation of clones and characterization

Single cell clones were isolated by serial dilution of vascular progenitor cells, as described on the Corning Incorporated website[2]. Briefly, 96 wells (two 48-well plates) were labeled from rows A to H and columns 1 to 12. 200μl of culture medium was added to each of the wells except well A1. 400μl cell suspension was then added to A1, and 200μl was transferred from A1 to B1. These 1:2 dilutions were repeated down the column until well H1, discarding 200μl from H1. 200μl was then transferred from the wells in the first column (A1 to H1) to those in the second column (A2 to H2). These 1:2 dilutions were repeated across the whole 96 wells, discarding 200μl from each well in the last column. Plates were then incubated at 37℃， in a 5% CO_2 incubator. On the next day, wells that contained only a single cell were marked and medium was changed every other day. 7 to 10 days later, the single colony could

be sub-cultured successively into a single well of a 24-well plate, a 12-well plate, or a T25 flask. The number of clones that were successfully expanded was recorded. Clones were characterized using conventional PCR and immunofluorescent staining.

Mouse vascular smooth muscle cell culture

Mouse vascular smooth muscle cells were isolated from the aortas of C57BL/6J mice as described previously[3]. In brief, the murine aortas were removed from the aortic arch to the thoracic aorta and washed within DMEM. The intima and inner two thirds of the media were carefully dissected from the aortas and cut into pieces, then placed onto a gelatin-coated (0.04%) plastic flask. The cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Gibico 10270) and 100U/ml penicillin/streptomycin. Cells were incubated at 37℃ in a humidified atmosphere of 5% CO_2. The medium was changed every 2 days. The purity of SMCs was routinely confirmed by immunostaining with α-SMA, SM-22 α and SM-MHCII antibodies. Smooth muscle cell conditional medium was collected from the supernatant of serum free medium, in which cells had been cultured overnight, by centrifuging 1500rpm, 15mins, at 4℃.

Antibodies

Primary antibodies were mouse anti-Cdc42, mouse anti-Rac1 (Millipore 17-441 | Rac1/Cdc42 Activation Assay Kit), rabbit anti- Phospho-p38 MAPK (Thr180/Tyr182) (Cell signaling 4511), rabbit anti-p38 MAPK (Cell signaling 9212), rat anti-Sca-1/ Ly6A/E (abcam 51317), mouse anti-Actin, α-Smooth Muscle-Cy3 (α-SMA) (Sigma C6198), rabbit anti-

calponin (abcam ab46794), rabbit anti-CCR2 (abcam ab21667), rabbit anti-vinculin (abcam ab73412), rabbit anti-paxillin (abcam ab32084) and goat anti-PECAM-1(Santa Cruz sc1506), goat anti-VE-cadherin (Santa Cruz sc-6458), mouse anti-CCL2 (R&D AF-479-SP), mouse anti-CXCL1 (R&D AF-453-SP). Secondary antibodies for immunostaining were anti-mouse Alexa Fluor 546, anti-rabbit Alexa Fluor 546 and anti-rat Alexa546 and were purchased from Invitrogen. Cells were also counterstained with Alexa Fluor® 488 Phalloidin (Life technologies A12379) and 10 μg/ml 4', 6-diamidino-2-phenylindole (DAPI). Secondary antibodies for Western Blotting were purchased from Dako.

Recombinant Proteins, Antagonists, siRNA and shRNA

Recombinant Murine JE/CCL2 and Recombinant Murine CXCL1 were obtained from Peprotech and added to DMEM medium at indicated concentrations. CCR2 antagonist ($C_{28}H_{34}F_3N_5O_4S$) and CXCR2 antagonist, Cpd-19 ($C_{18}H_{21}N_3O_4$) were purchased from Calbiochem. Rac1 inhibitor NSC23766 ($C_{24}H_{38}Cl_3N_7$) and Cdc42 inhibitor ML141 ($C_{22}H_{21}N_3O_3S$) were purchased from Calbiochem. Rho Inhibitor I (CT04, active site is C3 transferase) was purchased from Cytoskeleton. P38 inhibitor SB203580 ($C_{12}H_{16}FN_3OS$), was purchased from Merck Millipore. CCL2 siRNA (m) and Control siRNA (fluorescein Conjuagate)-A were purchased from Santa Cruz Biotechnology. Silencer® Select mouse CXCL1 siRNA and Negative Control siRNA was purchased from Ambion. The CCR2 and CXCR2 Mission shRNA Bacterial Glycerol Stocks were purchased from Sigma Aldrich. The shRNA Non-Targeting (NT) vector, SHC002 was used as a negative control.

Cell Sorting

As described in previous studies[4], heterogeneous vascular progenitor cells which grew out from vein grafts were sorted by magnetic cell sorting kits (MACS) with anti-Sca-1 immunomagnetic microbeads (Miltenyi Biotec, GmbH, Bergisch Gladbach, Germany). Briefly, the cells were incubated with the antibody-conjugated/coated microbeads at 4℃ and then selected using a magnetic cell separator (Miltenyi Biotec). Sca-1 positive vascular progenitor cell populations were expanded for up to 5 population doublings.

Immunofluorescence Staining

Cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma) and blocked with 5% normal donkey serum (Dako) for 1 hour at room temperature. Incubation of cells with primary antibodies was performed at 4℃ overnight, followed by incubation with secondary antibodies (Corresponding fluorescent-conjugated IgG antibodies were used as secondary antibodies (Invitrogen)) for 45 mins at 37℃. Then cells were counterstained with DAPI (Sigma) for 5 mins at room temperature and mounted with fluorescent mounting media (Dako). Images were acquired using an Olympus IX81 microscope and Volocity software (PerkinElmer).

Transwell Chemotaxis Assay

Migration assays were performed using transwell inserts with 8.0 μm pore membrane filters (Corning). Vascular progenitor cells were loaded onto the upper chamber at $5x10^4$ cells in serum free media, while the bottom chamber contained SMC conditional medium or serum

free medium with indicated concentrations of recombinant murine CCL2 or CXCL1. Serum free medium served as negative control. After an overnight incubation, non-migrating cells on the upper side of the filters were carefully washed and removed using a swab. Vascular progenitor cells on the underside of the membrane were fixed with 4% PFA for 10 mins followed by 0.1% crystal violet solution staining at room temperature for 15 mins. Data was expressed as the fold of migrated vascular progenitor cells compared to the control in 9 fields of each insert (at 20x). For experiments which involved inhibitors, vascular progenitor cells were pre-treated with the respective inhibitors before transferring to transwells.

Scratch-wound Assay

Vascular progenitor cells were seeded in a 12-well plate. Upon reaching complete confluency, a straight scratch was made using a pipette tip to stimulate a 'wound' through the middle of each well. The wells were gently washed with PBS to remove cell debris prior to treatment with SMC conditional medium or serum free medium with or without (control) recombinant murine CCL2 or CXCL1. After an overnight incubation, the migration of vascular progenitor cells into the “wound” area was quantified as the mean number of the cells which migrated into the “wound” in 5 random fields of each well using a phase contrast microscope(at 10x).

Time-lapse Microscopy

Cells were seeded onto 6-well plates and held with or without SMC conditioned media at 37˚C and 5% CO_2 on the stage of a fully motorized, multi-field time-lapse microscope

(Eclipse TE 2000-E; Nikon) with a charge-coupled device camera (ORCA; Hamamatsu Photonics). Bright-field images were acquired for 20 hours with pictures taken at 5-min intervals using the 10x objective. Images were acquired using Volocity software (PerkinElmer), and cells were tracked using ImageJ software (National Institute of Health). Analysis of cell speed and persistence was performed using the Chemotaxis plug in from Integrated BioDiagnostics.

RT-PCR

Total RNA was isolated from vascular progenitor cells using a QIAGEN RNeasy Mini kit according to the manufacturer's instructions. 1 μg RNA was reverse transcribed into cDNA with random primers using the MMLV reverse transcriptase kit (Promega). PCR was carried out on 50ng cDNA to amplify the mouse CCL2 receptor CCR2 gene using primers:

Forward 5'- TCCACTCTACTCCCTGGTATTC-3';

Reverse 5' - TGGCCAAGTTGAGCAGATAG - 3'.

The murine CXCL1 receptor CXCR2 gene primers:

Forward 5'-TCTGGCCCTGCCCATCTTAATTCT-3';

Reverse 5'- AAAGTCTGAGGCAGGATACGCAGT -3'.

The PCR products were analyzed on 2% agarose gels and assessed using a BioSpectrum AC Imaging System and Vision- WorksLS software.

Quantitative Real Time Polymerase Chain Reaction (qPCR)

The methods described above were used for RNA extraction and cDNA reverse transcription. Real time RT-PCR was performed using 20ng of cDNA per sample with a SYBR Green Master Mix in a 20 μl reaction. Ct values were measured using the Eppendorf Mastercycler ep Realplex and GAPDH was used as an endogenous control to normalize the amounts of RNA in each sample. The sequences of chemokine CCL2 primer sets are:

Forward 5'-AGTAGGCTGGAGAGCTACAA-3',

Reverse 5'-GTATGTCTGGACCCATTCCTTC-3',

The sequences of chemokine CXCL1 primer sets are:

Forward 5'-GCTGGGATTCACCTCAAGAA-3',

Reverse 5'-TGGCTATGACTTCGGTTTGG-3',

Western Blot Analysis

Harvested vascular progenitor cells were lysed with lysis buffer (25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, 1%Triton X-100 plus protease inhibitors(Roche), PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche)) and proteins were sequentially measured using the Bradford method. 40 μg of protein lysate was applied to 4-12% Bis-Tris Protein gels (NuPAPE, Novex) before being transferred to a nitrocellulose membrane (Amersham Biosciences), followed by a standard western blotting procedure.

CCL2 and CXCL1 gene knockdown

Gene suppression of CCL2 or/and CXCL1 in mouse smooth muscle cells was carried out using the Basic SMC Nucleofector® Kit (Lonza) with CCL2 siRNA (Santa Cruz Biotechnology) or/and CXCL1 siRNA (ambion) as the manufacturers described. Control siRNA (FITC Conjugate)-A (Santa Cruz Biotechnology) or Negative control siRNA (ambion) was used as a negative control. After gene ablation, the level of down regulation of total RNA or proteins was assessed using real time qPCR or ELISA analysis, respectively.

CCR2 and CXCR2 gene knockdown

Gene ablation of CCR2 or/and CXCR2 was carried out using short hairpin RNA (shRNA) lentiviral plasmid transfer as previously described[5, 6]. A non-targeting vector (SHC002) was used as a negative control. Total gene and protein levels were assessed using qPCR and flow cytometry analysis after gene ablation.

FACS analysis

Vascular progenitor cells were treated with/without SMC conditioned media, and incubated with antibodies (mouse anti-CXCR2 PE-conjugated antibody (R&D), rabbit anti-CCR2 (abcam) or mouse IgG PE (BD biosciences) for 30 mins on ice before analysis by FACS in order to test the change of CXCR2 or CCR2 receptor expression levels. Data analysis was carried out using FlowJo software.

Femoral artery injury

Mice were anesthetized with (ketamine and medetomidine hydrochloride) and the surgical procedure was similar to that described previously[7]. Both of the femoral arteries of each mouse were injured by inserting a 0.25 mm guide wire (CROSS-IT 100XT, HI-TORQUE) 4-mm length from one of the distal muscle branches to femoral artery. One artery was seeded with Sca-1^+ vascular progenitor cells ($1x10^6$ cells) within 25 μl Matrigel® Basement Membrane Matrix (Corning), and the other injured artery served as a control. Arteries were harvested at day 7 or day 14 and used to prepare frozen or paraffin sections.

For the siRNA knockdown experiments *in vivo*, either 5 μg CXCL1 or Negative Control siRNA dissolved in 30% pluronic Gel-127 was perivascularly delivered to the femoral arteries immediately after wire injury. After 3 days, GFP-Sca1^+ vascular progenitor cells ($1x10^6$ cells) within 25 μl Matrigel® Basement Membrane Matrix were seeded on the adventitia of both siRNA treated femoral arteries. Arteries were harvested for either RNA extraction or *en face* staining after a further 72 hours.

***En face* staining**

En face staining was used for quantification of reendothelializaiton. Briefly, femoral arteries were fixed with 4% paraformaldehyde, then permeabilized and blocked with a solution of 0.5% Triton X-100 and 5% donkey serum in PBS. Incubation of vessels with primary antibodies was performed at 4℃ overnight, followed by incubation with corresponding fluorescent-conjugated secondary antibodies (Invitrogen) or IgG as negative control for 2 hours at room temperature. Then nuclei were stained with DAPI for 5 mins at room temperature before

vessel segments were mounted with the endothelium face up on a glass slide. Images were taken using a Leica SP5 confocal microscope and assessed using LAS AF lite software

Creation of chimeric mice

The procedure used for creating chimeric mice was similar to previously described[8]. In brief, bone marrow transplantation was carried out on the $CCL2^{+/+}$ mice and $CCL2^{-/-}$ mice separately. Bone marrow cells were obtained from the femurs and tibias of either $CCL2^{+/+}$ or $CCL2^{-/-}$ mice (donors) and injected ($1x10^7$cells in 0.2ml) into the tail veins of the 6-8 week old $CCL2^{-/-}$ mice or $CCL2^{+/+}$ mice (recipients) which received lethal irradiation (950 Rads) before. Femoral artery injury was performed 3 weeks after bone marrow transplantation.

Rac1/Cdc42 GTPase activation assay

The activation assay of GTP-bound Rac1/Cdc42 was carried out according to the manufacturer's instructions (Rac1/Cdc42 Activation Assay Kit (Upstate, Millipore)). In addition to the solutions from the kit, PAK-PBD beads from cytoskeleton, Protease Inhibitor Cocktail (Sigma-Aldrich) and PhosSTOP Phosphatase Inhibitor Cocktail Tablets (Roche) were also used. Briefly, cells were stimulated with the indicated treatments then placed on ice and scraped within 1x MLB (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Igepal CA-630, 10 mM MgCl2, 1mM EDTA and 2% glycerol), and samples were centrifuged for 10 mins. Aliquots were collected for total Rac1 or Cdc42 (input) and GTPγS (positive) and GDP (negative) control analysis. Lysates were rotated with 20μg of PAK-PBD agarose beads at

4 ℃ for 1 hour. The agarose pellet was washed twice and re-suspended in 25μl SDS sample buffer. Samples were separated by 4–12% Bis-Tris gels, transferred to nitrocellulose membranes, and blotted for Rac1 or Cdc42 antibodies (Upstate, Millipore).

RT^2 Profiler^{TM} PCR Arrays for Mouse Chemokines and Receptors

Total RNA (0.5 μg) (extracted from either mouse peritoneal macrophages, vascular smooth muscle cells, Sca-1$^+$ vascular progenitor cells or one cloned colony of vascular progenitor cells cultured *in vitro)* was reverse transcribed to cDNA and used to screen mouse chemokine receptors using quantitative real-time PCR arrays according to the manufacturer's instructions (Qiagen PAMM-022Z). Reactions were performed in an Eppendorf® Mastercycler® ep Realplex model 4S. Acquired data were analyzed using the $2^{-(\text{average}\Delta CT)}$ method to determine the expression level of each transcript normalized to the expression level of housekeeping gene controls.

SUPPLEMENTAL REFERNCES

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2. Corning Incorporated Life Sciences. Cell Cloing by Serial Dilution in 96 Well Plates Protocol. Available at http://www.level.com.tw/html/ezcatfiles/vipweb20/img/img/34963/3-2Single_cell_cloning_protocol.pdf (http://www.level.com.tw/html/ezcatfiles/vipweb20/img/img/34963/3-2Single_cell_cloning_protocol.pdf)
   Accessed March 28, 2016
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Supplement Figure Legend

Figure 1. **Isolation of clones and characterization.** Single cells were obtained by serial dilution, approximately 3% to 5% of cells formed clones that could be gradually expanded. **A,** Two cells (in red circles) from the same clone colony are shown in the represented image. **B, C, D.** Clone colonies were characterized using immunofluorescence staining for progenitor cell marker (Sca-1), SMC markers (SM-MHCII, Calponin) or endothelial cell markers (CD31，CD144). Scale bars, 100μm. SM-MHCII, smooth muscle cell myosin heavy chain class II.

Figure 2. **SMC-CM has no effect on VPCs proliferation and differentiation.**

A. Evaluation of vascular progenitor cell proliferation in response to SMC conditioned medium, CXCL1or CCL2 treatments compared to an untreated control after 18 hours was by BrdU incorporation assay. **B, C, D, E.** Total RNA from vascular progenitor cells was harvested and subjected to qPCR for analysis of SMC markers (α-SMA, SM-MHCII), endothelial cells marker (CD31) and progenitor cells marker (Sca-1) expression. All graphs are shown as mean ± SEM of n=3. ns, P>0.05. ctrl, control, serum free medium. CM, SMC conditioned medium.

Figure 3. **SMC conditioned medium increases paxillin, vinculin and phosphorylated FAK expression. A.** Vascular progenitor cells were treated with SMC conditioned medium for 5 minutes before immunofluorescence staining for paxillin, vinculin and phosphorylated

FAK. Scale bars, 25μm. ctrl, control, serum free medium. CM, SMC conditioned medium. p-FAK, phosphorylated FAK.

Figure 4. **CCL5 has no effect on VPCs migration. A.** The transwell assay was performed on vascular progenitor cells that migrated toward either serum free medium (control) or serum free media containing murine recombinant CCL5 (5ng/ml, 50ng/ml). Transwell migration assays show VPCs migrated towards serum free medium with or without mouse recombinant CCL5 (5ng/ml, 50ng/ml). Scale bars, 100μm. **B.** The graph is shown as mean ± SEM of n=3. ns, P>0.05.

Figure 5. **The effects of CCL2 and CXCL1 are neither cumulative nor redundant in mediating VPC migration. A-D.** The SMC conditioned medium was treated with either neutralizing antibodies for CCL2 and/or CXCL1 or the relevant control IgG for 1 hour at 37℃ before pull-down of chemokines combined with antibodies using protein G beads. The supernatant was collected and used to measure the concentrations of CCL2 (**A**) and CXCL1 (**B**) using corresponding ELISA kits. The transwell assay (**C, D**) was performed on VPCs that migrated toward SMC conditioned medium (positive control) or SMC conditioned medium after depletion of chemokines with IgG, CCL2 and/or CXCL1 neutralizing antibodies pull down. Scale bars, 100μm. **E, F.** The VPCs were treated with either CXCL1 or CCL2 at indicated concentrations overnight followed by measurement of concentrations of CCL2 and CXCL1 using ELISA. The graphs are shown as mean±SEM of n=3, **p<0.01, ***p<0.001, ns, P>0.05.

Figure 6. **Antagonists of CCR2 or CXCR2 inhibited SMC-mediated VPC migration via the p38 signaling pathway. A, B**. The transwell assay was performed on vascular progenitor cells that were pre-treated with DMSO, antagonists of CCR2 or CXCR2 1 hour before migrating toward SMC conditioned medium. Scale bars, 100 μm. **C, D**. Western blotting was performed on vascular progenitor cell lysates (40 μg per condition) to detect phosphorylation of p38 and total p38. Untreated cells served as the control. The quantification is represented as mean ± SEM of n=3. ***p<0.001. ctrl, control, serum free medium. CM, SMC conditioned medium. DMSO, Dimethyl sulfoxide. CCR2 Anta, CCR2 antagonist. CXCR2 Anta, CXCR2 antagonist.

Figure 7. **The RhoA signalling pathway is not involved in the SMC induced VPCs migration. A.** After pre-treatment with either vehicle or C3 transferase (2 μg/ml) for 2 hours, VPC were stimulated with SMC conditioned medium or recombinant CCL2 or CXCL1 before cell lysates were harvested for western blotting to detect the p-p38 and t-p38. **B.** The transwell assay was performed on VPCs that were pre-treated with either vehicle or C3 transferase (an inhibitor of RhoA) (2 μg/ml) for 2 hours before migration toward either SMC conditioned medium or recombinant CCL2 or CXCL1. Scale bars, 100 μm. Quantification is represented as mean ± SEM of n=3. ns, P>0.05. CM, SMC conditioned medium. vehicle, sterile distilled water.

Figure 8. **CCL2 and CXCL1 are expressed on wire-injured vessels *in vivo*.** Femoral arteries of C57BL/6J mice were harvested after wire injury or from uninjured animals. Frozen sections of intact vessels or vessels 6 hours or 2 weeks after wire injury were co-stained with immunofluorescent markers either CCL2 (**A, B, C**) or CXCL1(**D, E, F**) and α-SMA. n=4 mice/group. Scale bars, 50μm.

Figure 9. **Identify the CCL2$^{-/-}$ mouse genotype and CCL2 level in the peripheral blood.** **A.** To expand CCL2$^{-/-}$ mice number, CCL2$^{-/-}$ mice were crossed with CCL2$^{+/+}$. A piece of ear tissue from each mouse was used to genotype offspring. The image shows a heterozygote (CCL2$^{+/-}$) with bands at 287bp and 179bp and a mutant (CCL2$^{-/-}$) with only one band at 179bp. **B.** Peripheral blood was collected from the vena cava before euthanasia of WT or CCL2$^{-/-}$ mice and CCL2 level was measured using a murine CCL2 Quantikine ELISA kit from serum of each sample. The graph is shown as mean ± SEM of n=11 mice/group, ***p<0.001.

Figure 10. **Lack of CCL2 inhibits Sca-1^{+} vascular progenitor cell migration *in vivo*.**

A. Sca-1^{+} vascular progenitor cells (1x10^{6}), which were labeled with Q-tracker to locate them as they migrated, were seeded in the adventitia of each injured femoral artery. 72hrs post injury, the vessels were harvested. *En face* staining shows that cells migrated to the intima side of the vessels of WT and CCL2$^{-/-}$ mice. Scale bars, 25μm. **B.** The ratio of the fluorescence intensity (Qtracker:DAPI) is represented in the graphs. The graph is shown as mean ± SEM of n=8 mice/group, **p<0.01.

Figure 11. **Characterization of cells in the neointima. A.** Femoral arteries without seeding VPC in the adventitia were harvested 2 weeks after wire injury from C57BL/6J mice and stained with Sca-1 and α-SMA (white dotted line indicates internal elastin, and the circled part is neointima area). Scale bars, 50μm. **B.** The ratios of positively stained cells with each marker in the neointima area are quantified and shown in the graph.

Figure 12. **CXCL1 can induce Sca-1 positive cell migration *in vivo*.** Using a matrigel plug assay, matrigel including PBS or mouse recombinant CXCL1 was injected subcutaneously into C57BL/6J mice. The matrigel plugs were harvested 2 weeks later and the frozen sections were stained with either (A) hematoxylin–eosin (Scale bars, 100μm) or (B) immunofluorescent markers (Sca-1, CD31, α-SMA) (Scale bars, 100μm). The numbers of cells migrated into the matrigel (quantification from bottom images in A) or positively stained with each marker are quantified and shown in the graphs. Graphs are shown as mean ±SEM of n=8, **P<0.01. vehicle, sterile distilled water.

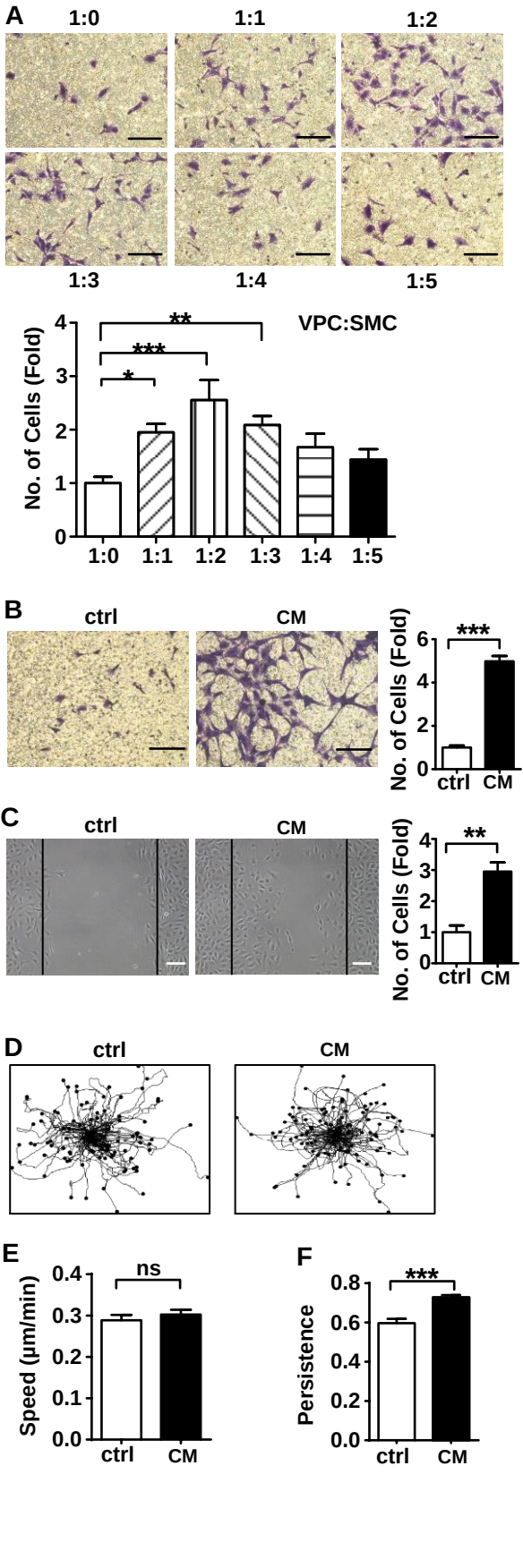
Figure 13. **CXCL1 small interfering RNA inhibits Sca-1$^+$ cells migration *in vivo*.**

A, Quantification of CXCL1 mRNA level 6 days after perivascular application of CXCL1 or negative control siRNA dissolved in pluronic gel by qPCR. Graphs are shown as mean± SEM of n=4, **P<0.01. **B.** Representative images of *En face* staining show the cells migrated to the intima side of the vessels 72hrs post seeding GFP-Sca-1$^+$-VPC ($1x10^6$) in the adventitia of femoral arteries treated with CXCL1 or control siRNA. Scale bars, 25μm.

Figure 14. **Schematic illustration of the role of CCL2 and CXCL1 released from SMCs in enhancing VPCs chemotaxis.** SMCs release CCL2 and CXCL1 into the medium. When VPCs are treated with this SMC-CM, these chemokines bind to their corresponding receptors CCR2 and CXCR2 on the VPCs. The GTPase Rac1 and Cdc42 become activated and then p38 is phosphorylated via Rac1, finally leading to increased VPC migration. SMC-CM also induces expression of cytoskeleton related proteins paxillin, vinculin and phosphorylated FAK, which may also activate the Rac1 or Cdc42 signaling pathway.

Graphical Abstract. **Schematic illustration of the role of CCL2 and CXCL1 released from SMCs in enhancing VPCs chemotaxis.** SMCs release CCL2 and CXCL1 into the medium. When VPCs are treated with this SMC conditioned medium, these chemokines bind to their corresponding receptors CCR2 and CXCR2 on the VPCs. The GTPase Rac1 become activated and then p38 is phosphorylated via Rac1, finally leading to increased VPC migration. SMC conditioned medium also induces expression of cytoskeleton related proteins paxillin, vinculin and phosphorylated FAK, which may also activate the Rac1 signaling pathway.

Figure 1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No. of Cells (Fold)
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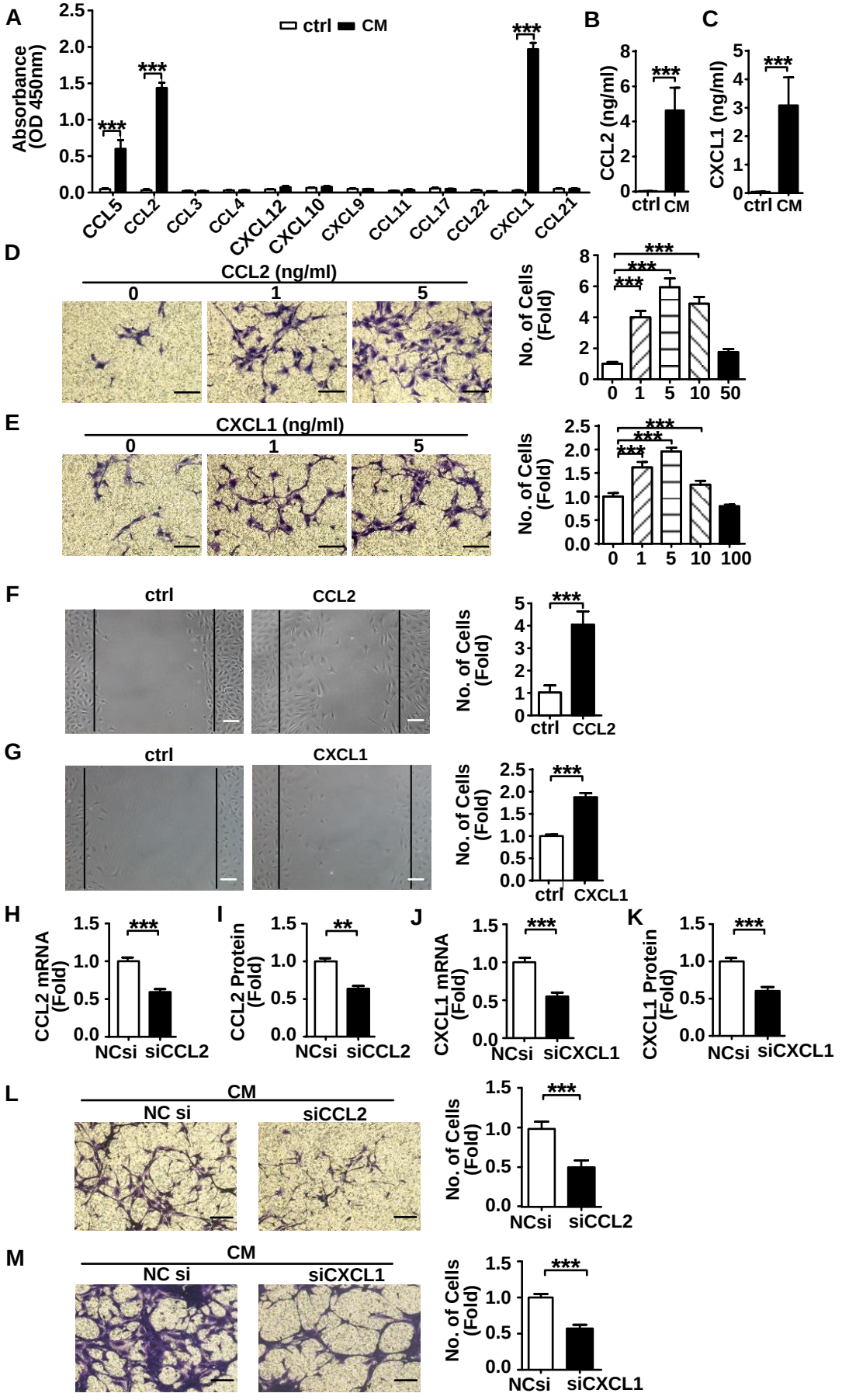
D
ctrl
CM
E
Speed (μm/min)
ns
F
Persistence
***

Figure 2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ctrl
CM
C
CXCL1 (ng/ml)
ctrl
CM
D
CCL2 (ng/ml)
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1
5
No. of Cells (Fold)
0
1
5
10
50
E
CXCL1 (ng/ml)
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1
5
No. of Cells (Fold)
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1
5
10
100
F
ctrl
CCL2
No. of Cells (Fold)
ctrl
CCL2
G
ctrl
CXCL1
No. of Cells (Fold)
ctrl
CXCL1
H
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NCsi
siCCL2
I
CCL2 Protein (Fold)
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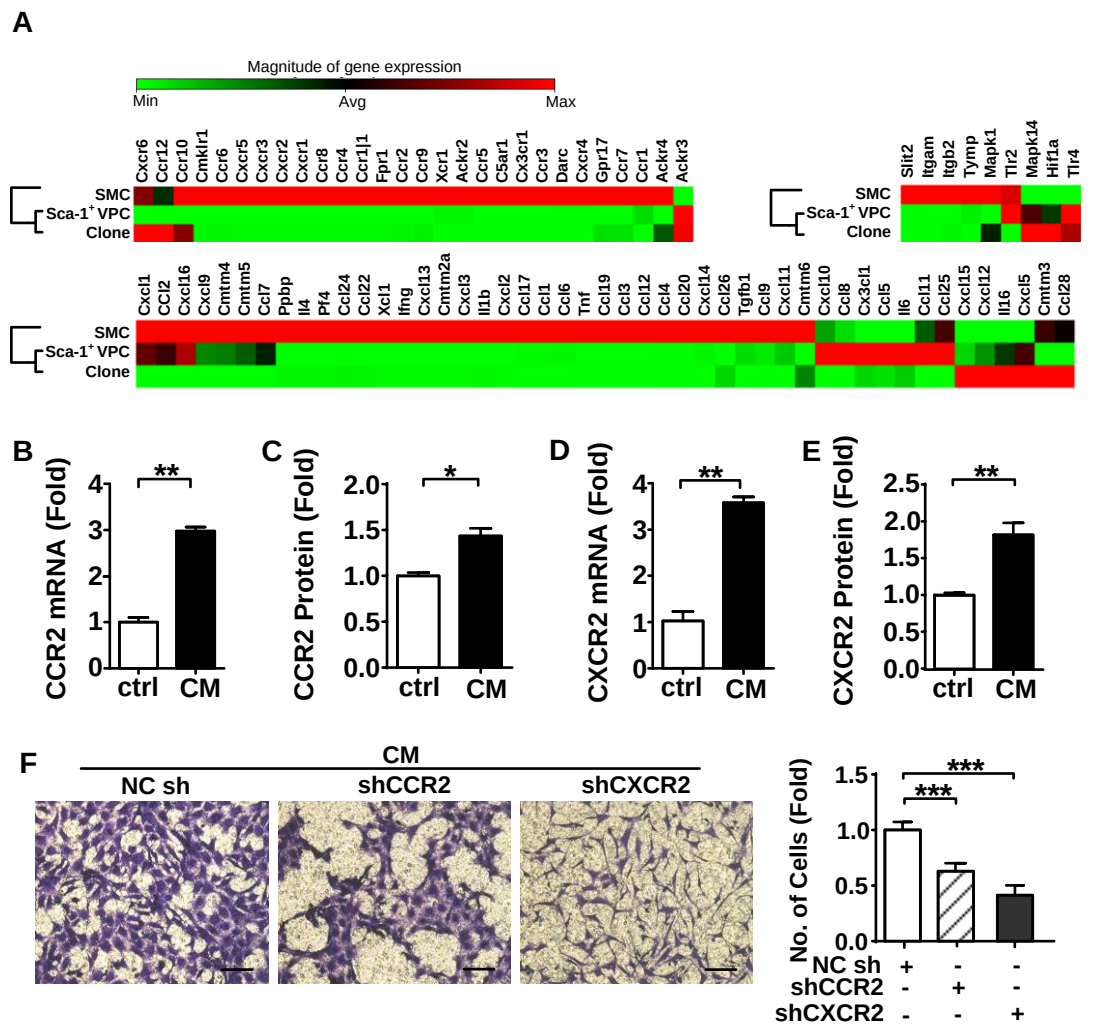
Figure 3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Avg
Max
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Slit2 Itgam Itgb2 Tymp Mapk1 Tlr2 Mapk14 Hif1a Tlr4
SMC
Sca-1+ VPC
Clone
Cxcl1 CCl2 Cxcl16 Cxcl9 Cmtm4 Cmtm5 Ccl7 Ppbp Il4 Pf4 Ccl24 Ccl22 Xcl1 Ifng Cxcl13 Cmtm2a Cxcl3 Il1b Cxcl2 Ccl17 Ccl1 Ccl6 Tnf Ccl19 Ccl3 Ccl12 Ccl4 Ccl20 Cxcl14 Ccl26 Tgfb1 Ccl9 Cxcl11 Cmtm6 Cxcl10 Ccl8 Cx3cl1 Ccl5 Il6 Ccl11 Ccl25 Cxcl15 Cxcl12 Il16 Cxcl5 Cmtm3 Ccl28

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C
CCR2 Protein (Fold)
*
ctrl
CM

D
CXCR2 mRNA (Fold)
**
ctrl
CM

E
CXCR2 Protein (Fold)
**
ctrl
CM

F
CM
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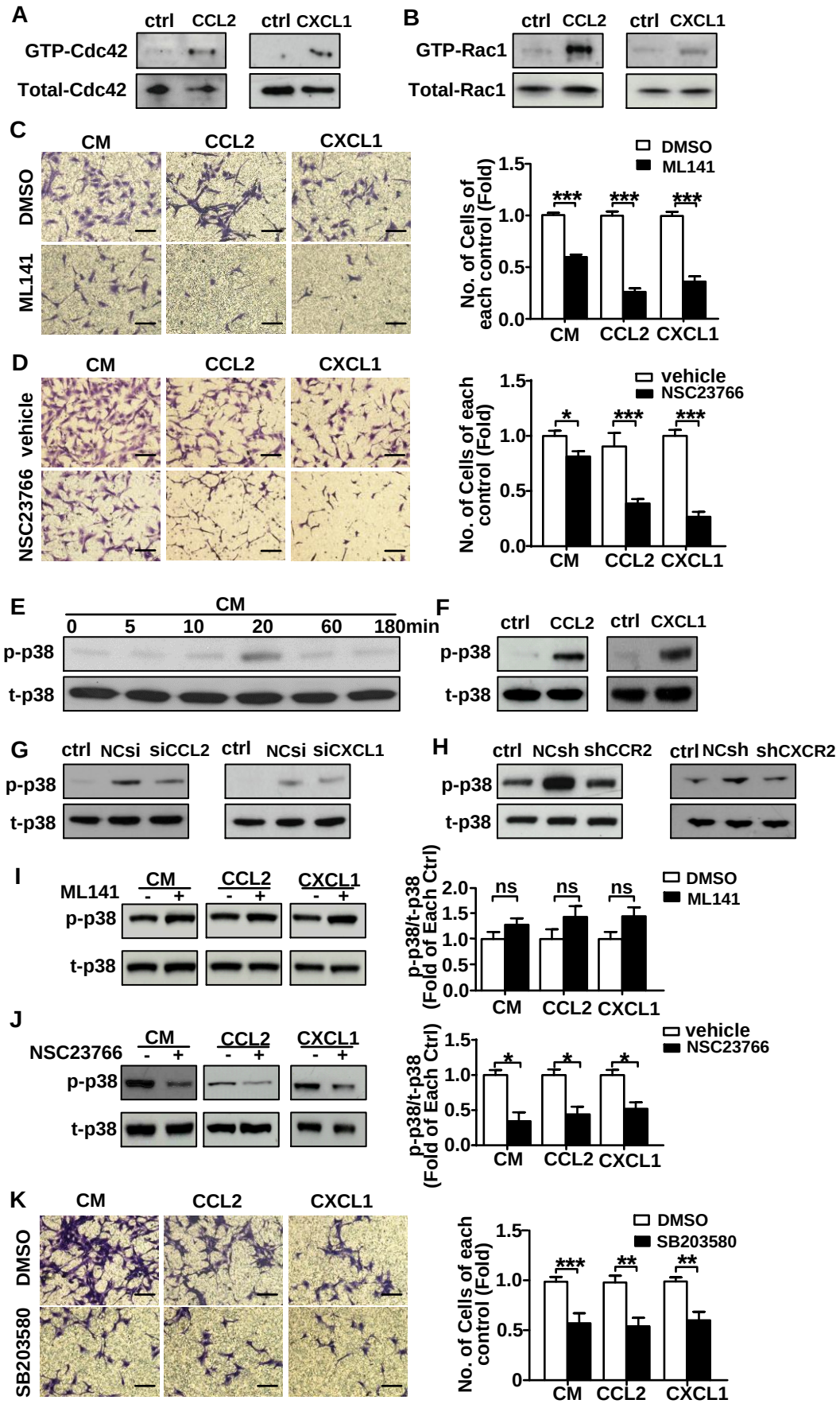
shCCR2 - + -
shCXCR2 - - +

Figure 4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ctrl
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Total-Rac1
C
CM
CCL2
CXCL1
DMSO
ML141
No. of Cells of each control (Fold)
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ML141
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***
***
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CCL2
CXCL1
D
CM
CCL2
CXCL1
vehicle
NSC23766
No. of Cells of each control (Fold)
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NSC23766
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***
***
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CXCL1
E
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10
20
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180min
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t-p38
F
ctrl
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ctrl
CXCL1
p-p38
t-p38
G
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H
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ctrl
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t-p38
I
ML141
CM
CCL2
CXCL1
- +
- +
- +
p-p38
t-p38
p-p38/t-p38 (Fold of Each Ctrl)
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ns
ns
DMSO
ML141
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CCL2
CXCL1
J
NSC23766
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*
*
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vehicle
NSC23766
CM
CCL2
CXCL1
K
CM
CCL2
CXCL1
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No. of Cells of each control (Fold)
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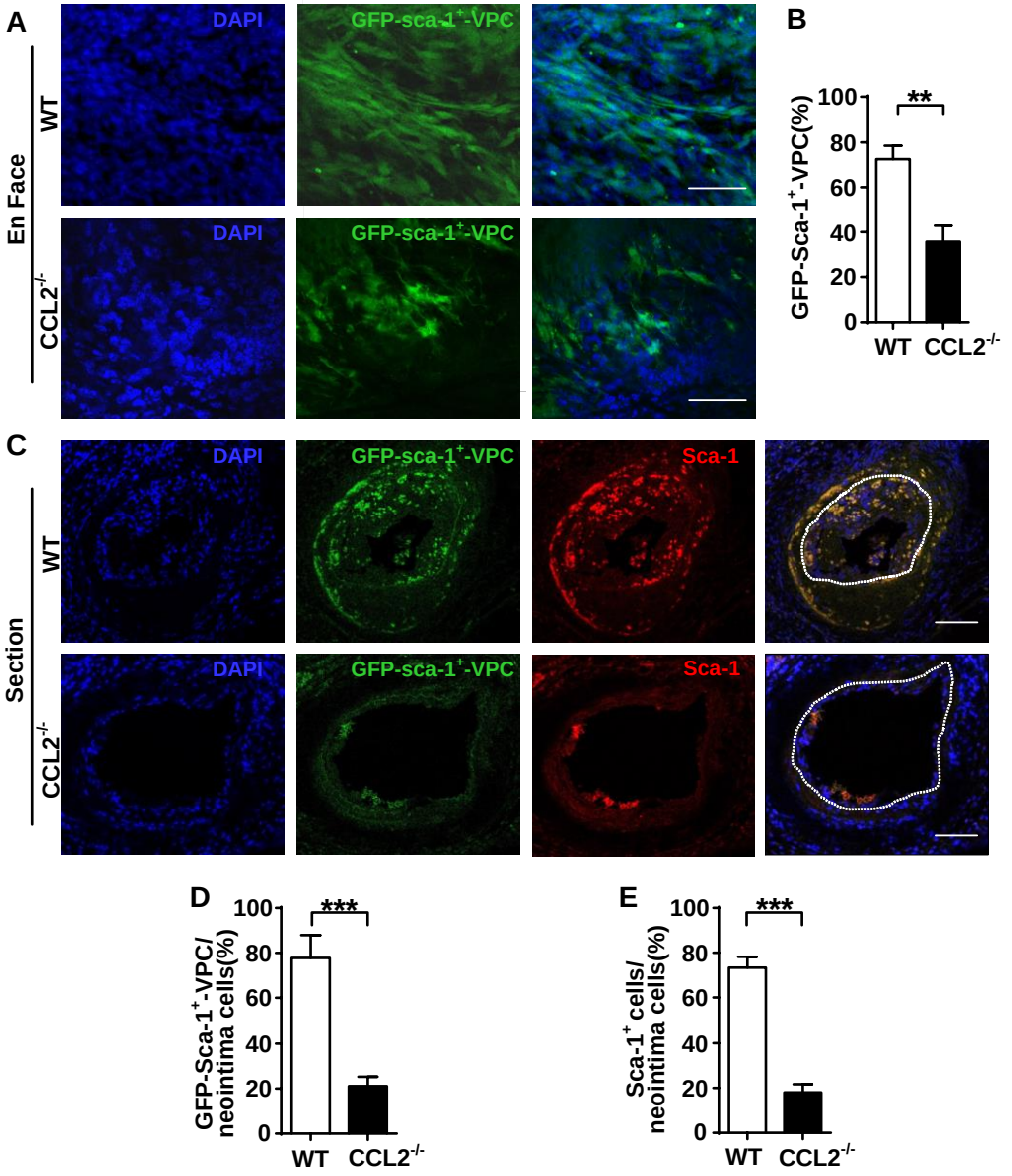
SB203580
***
**
**
CM
CCL2
CXCL1

Figure 5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D
GFP-Sca-1+-VPC/ neointima cells(%)
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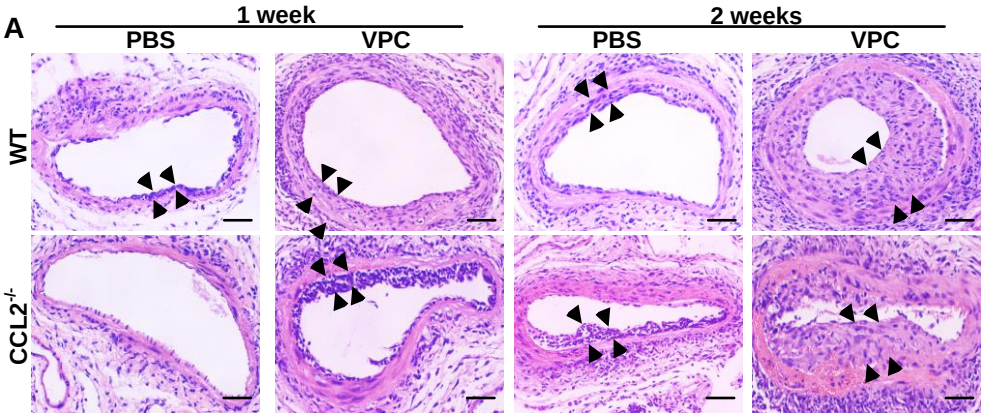
WT
CCL2-/-

Figure 6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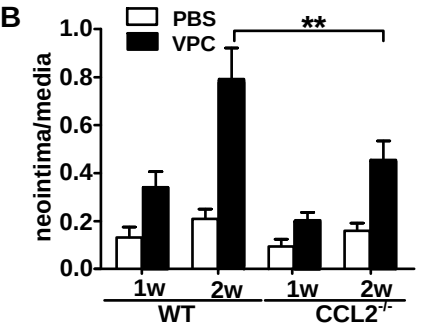
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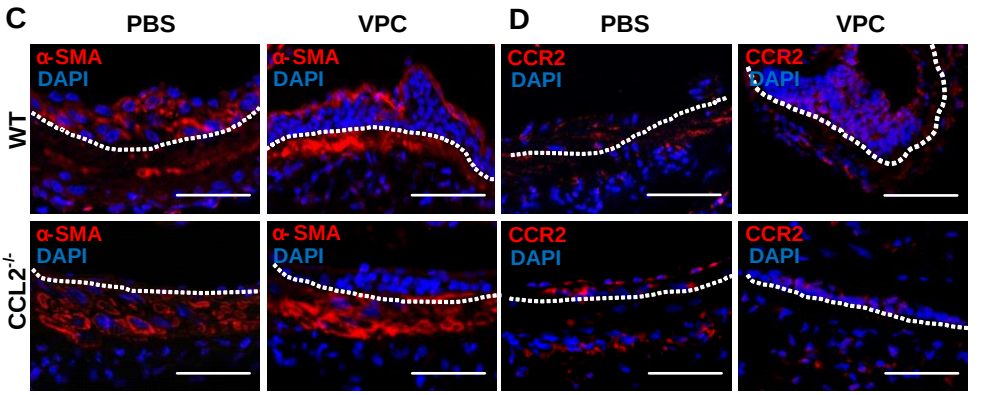
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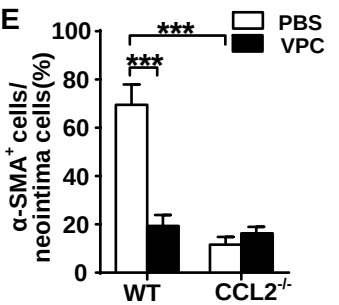
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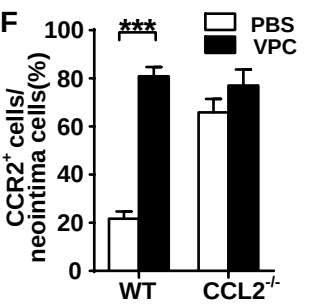
WT
CCL2-/-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VPC
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CCR2+ cells/ neointima cells(%)
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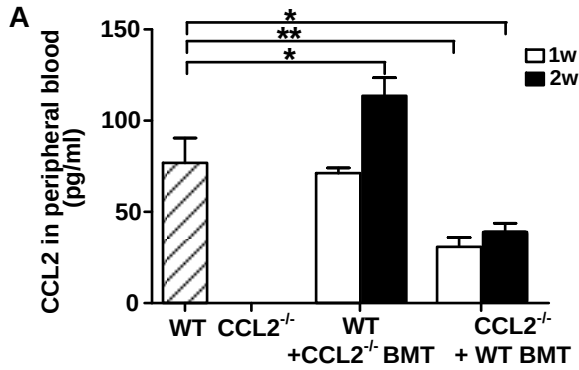
WT
CCL2-/-

Figure 7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CCL2 in peripheral blood (pg/ml)
150
100
50
0
*
**
*
1w
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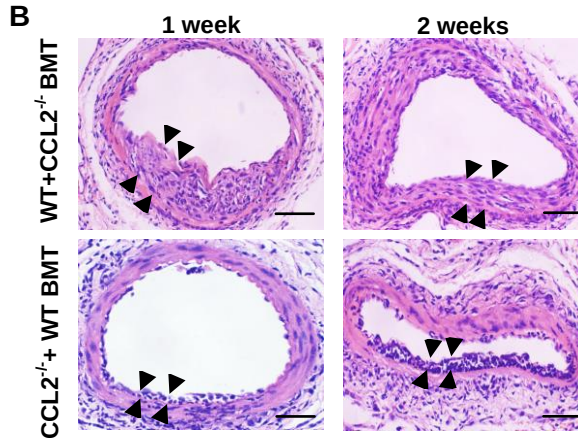
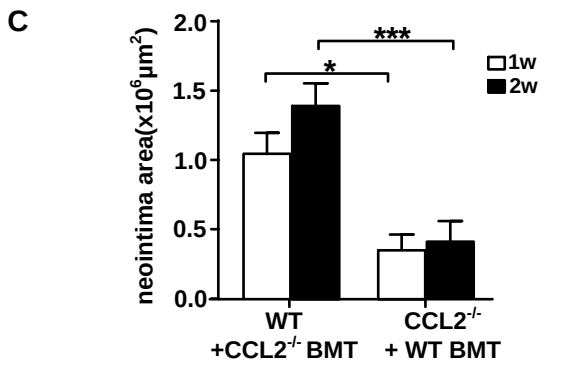
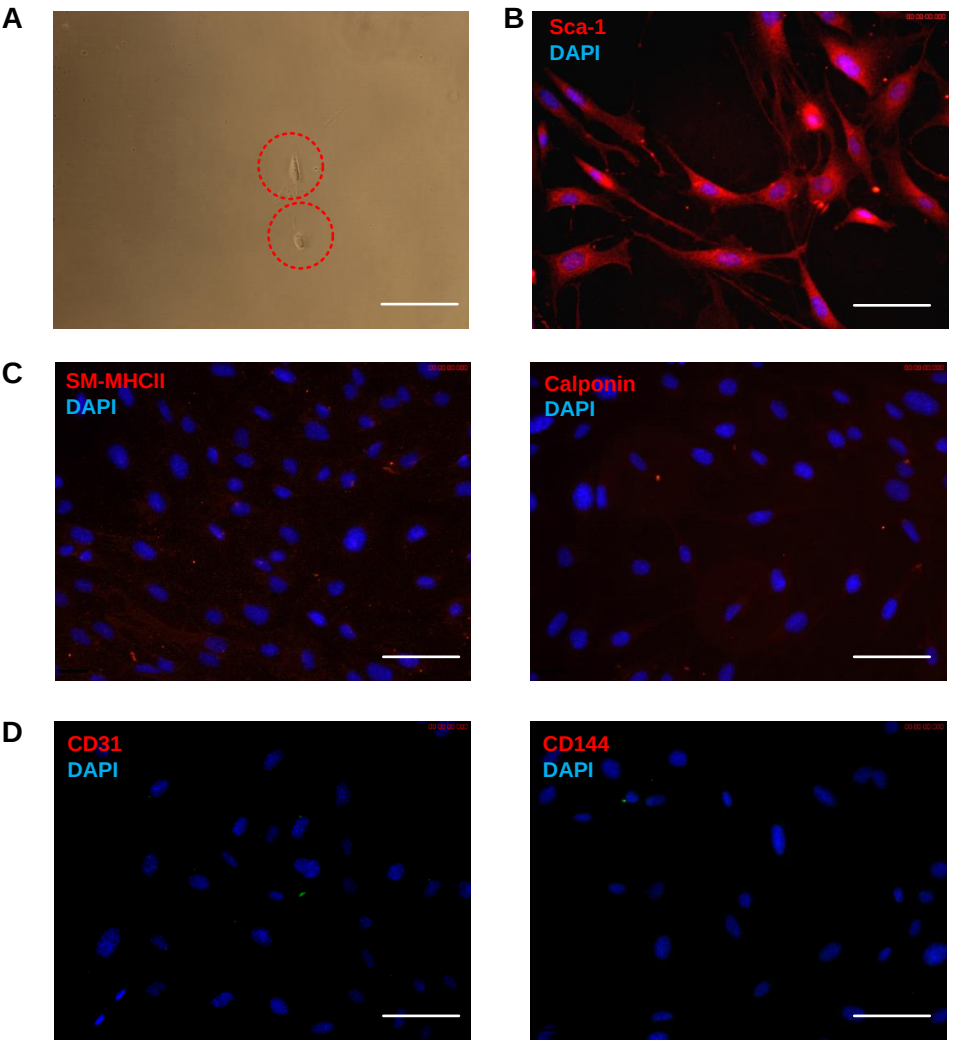
WT
CCL2-/-
WT +CCL2-/- BMT
CCL2-/- + WT BMT

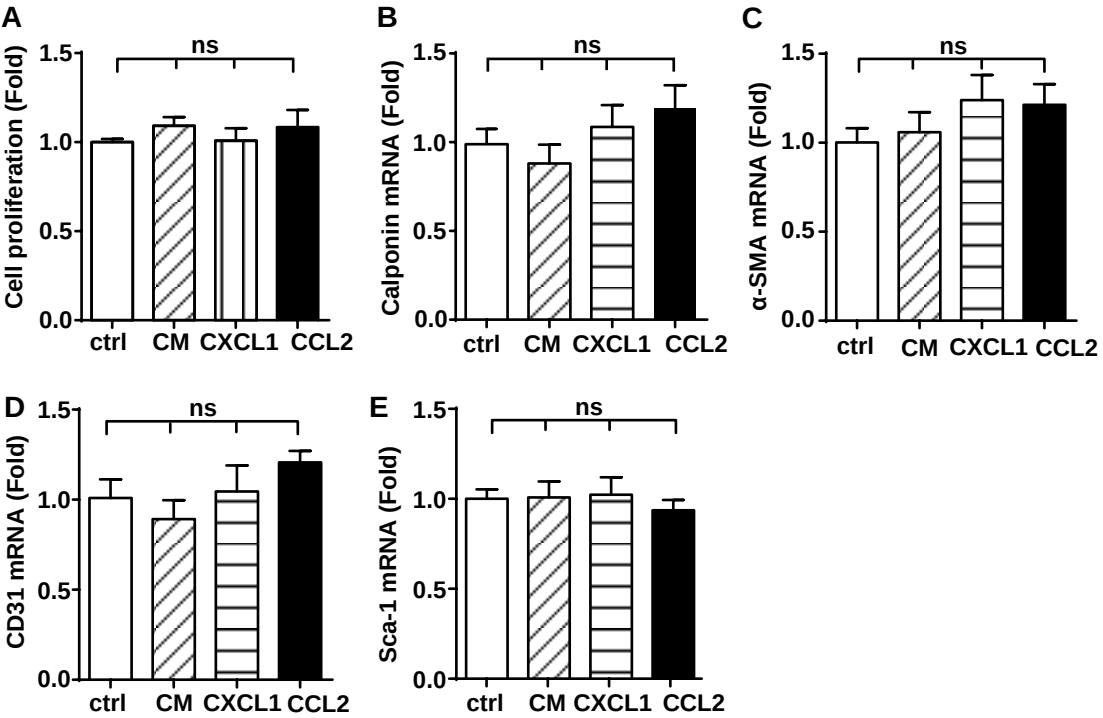

B
1 week
2 weeks
WT+CCL2-/- BMT
CCL2-/-+ WT BMT


C
neointima area(x10^6µm^2)
2.0
1.5
1.0
0.5
0.0
***
*
1w
2w
WT +CCL2-/- BMT
CCL2-/- + WT BMT

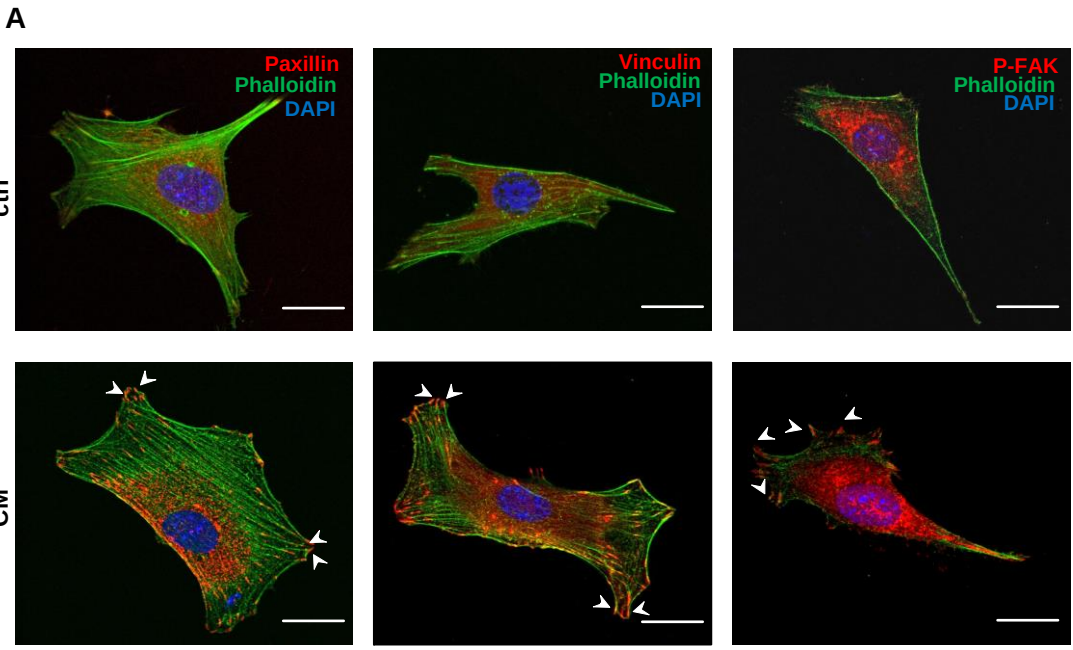
Supplemental Figure 1



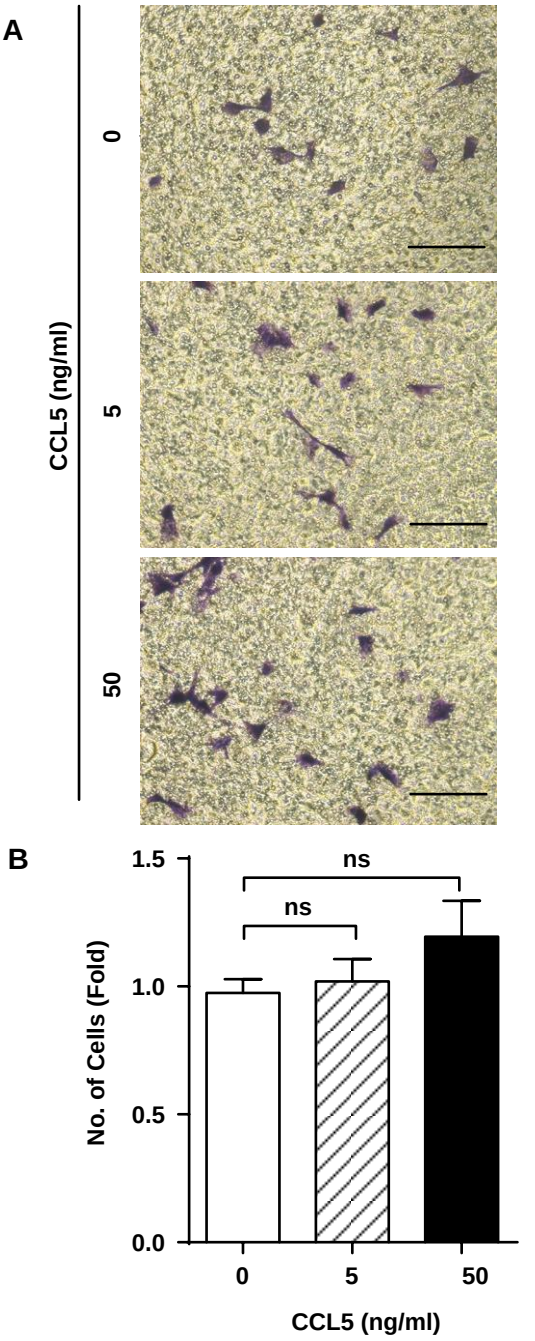
Supplemental Figure 2



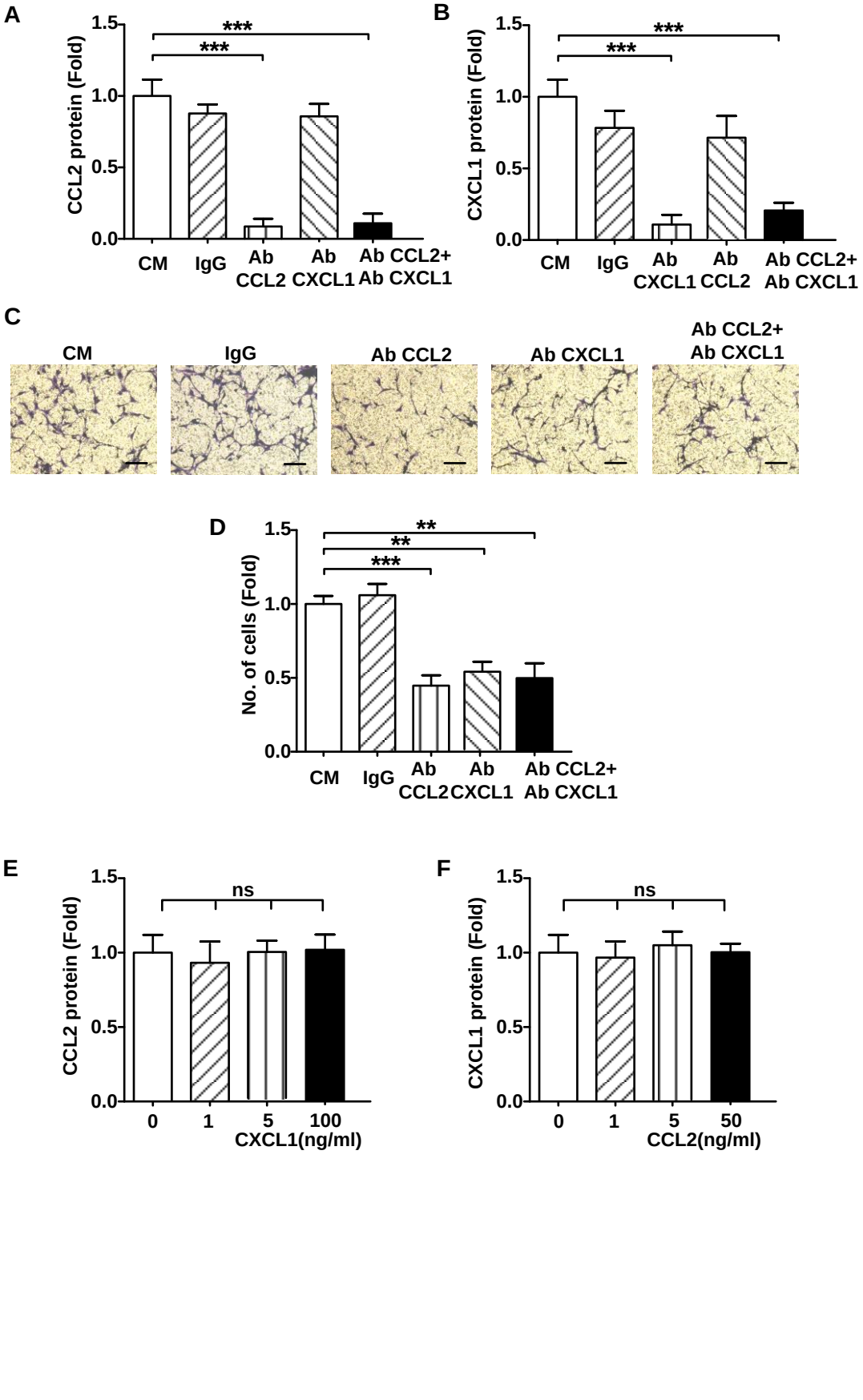
Supplemental Figure 3



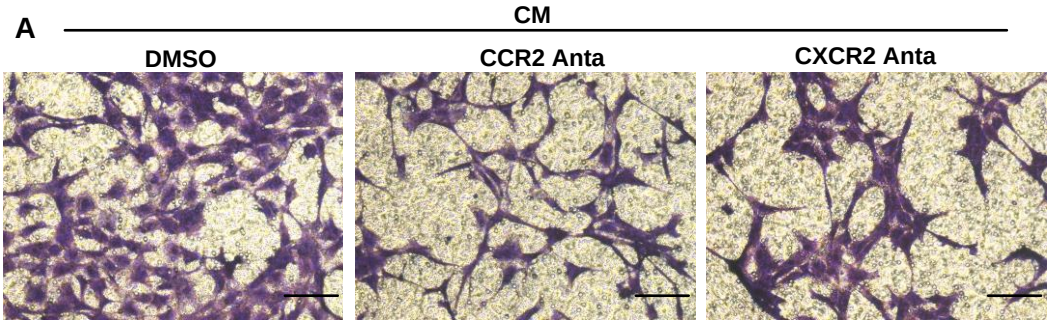
Supplemental Figure 4

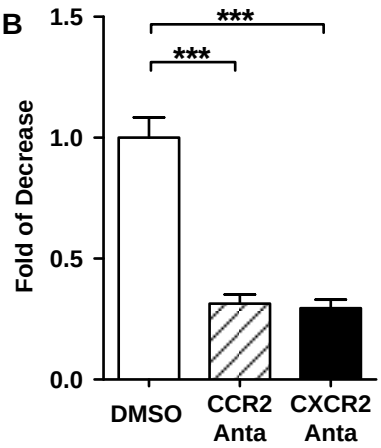


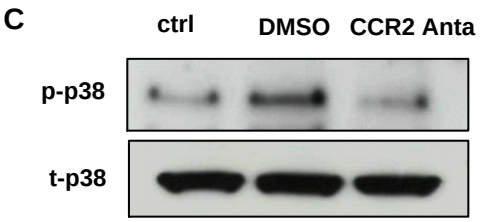
Supplemental Figure 5

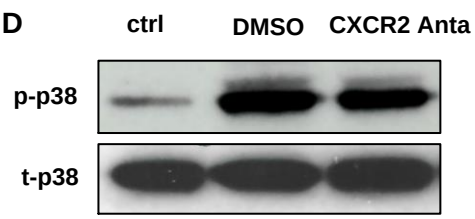


Supplemental Figure 6

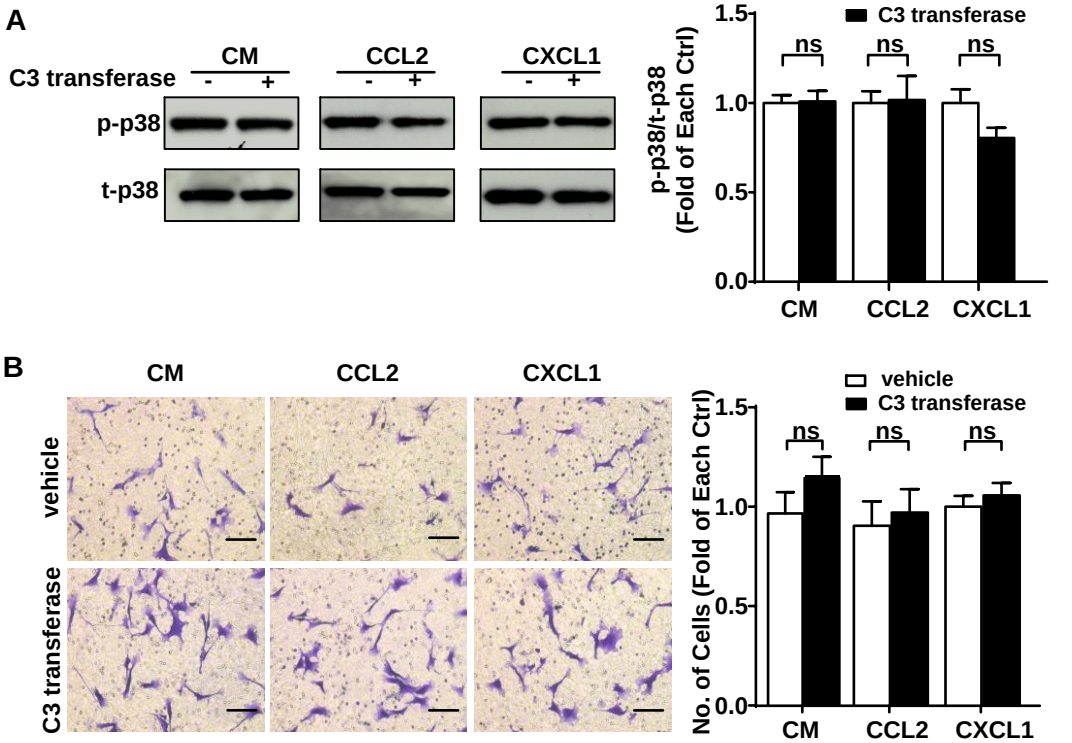




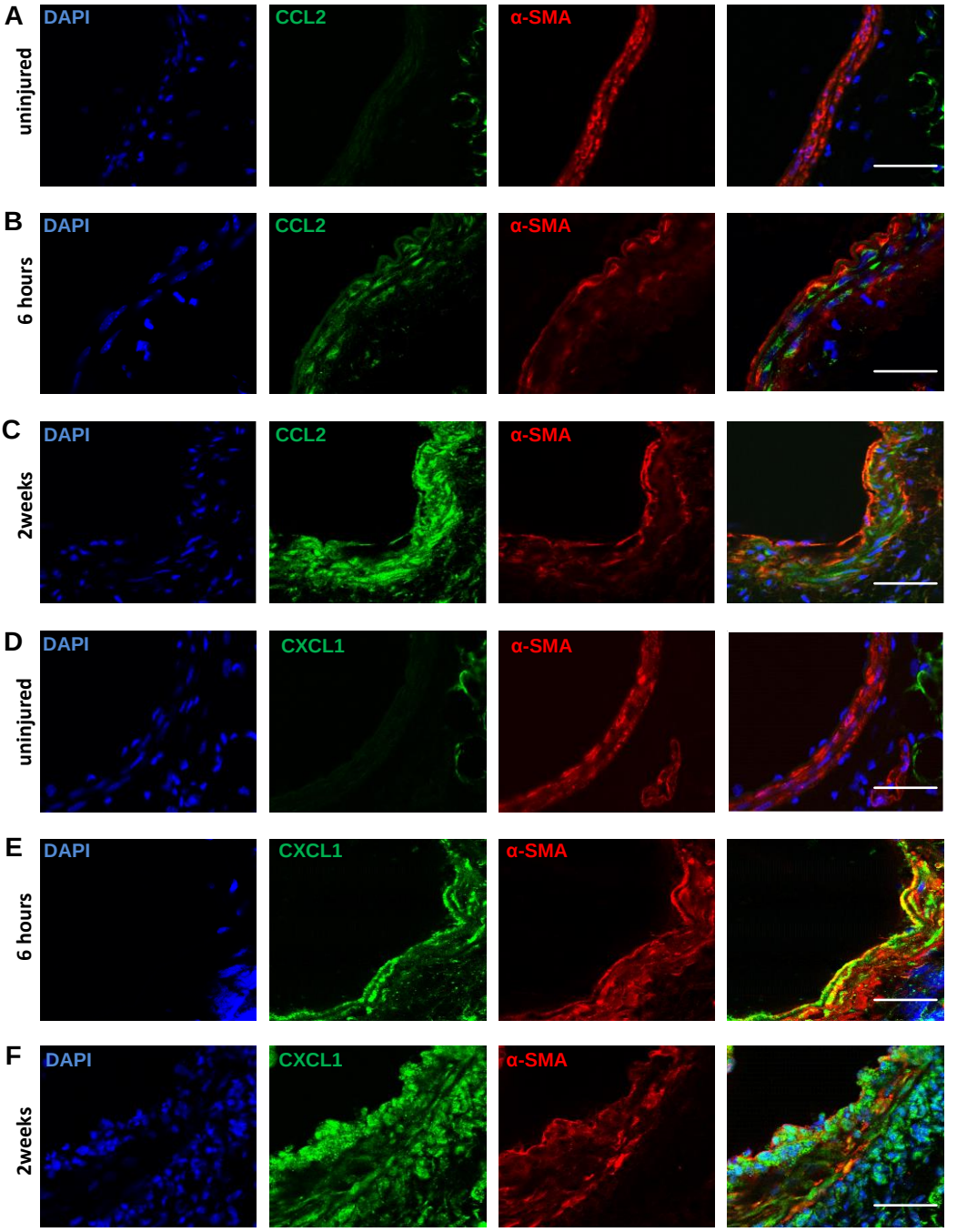




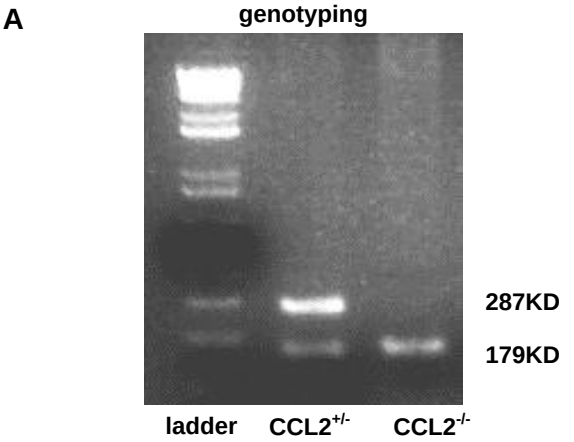
Supplemental Figure 7

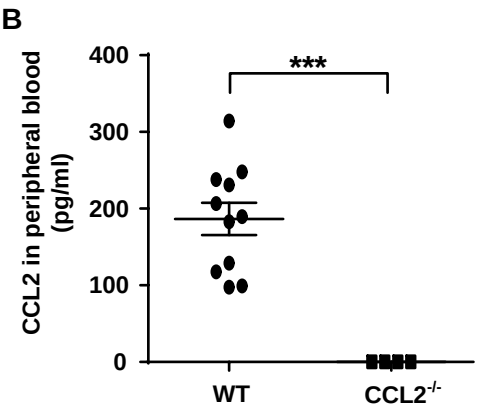


Supplemental Figure 8

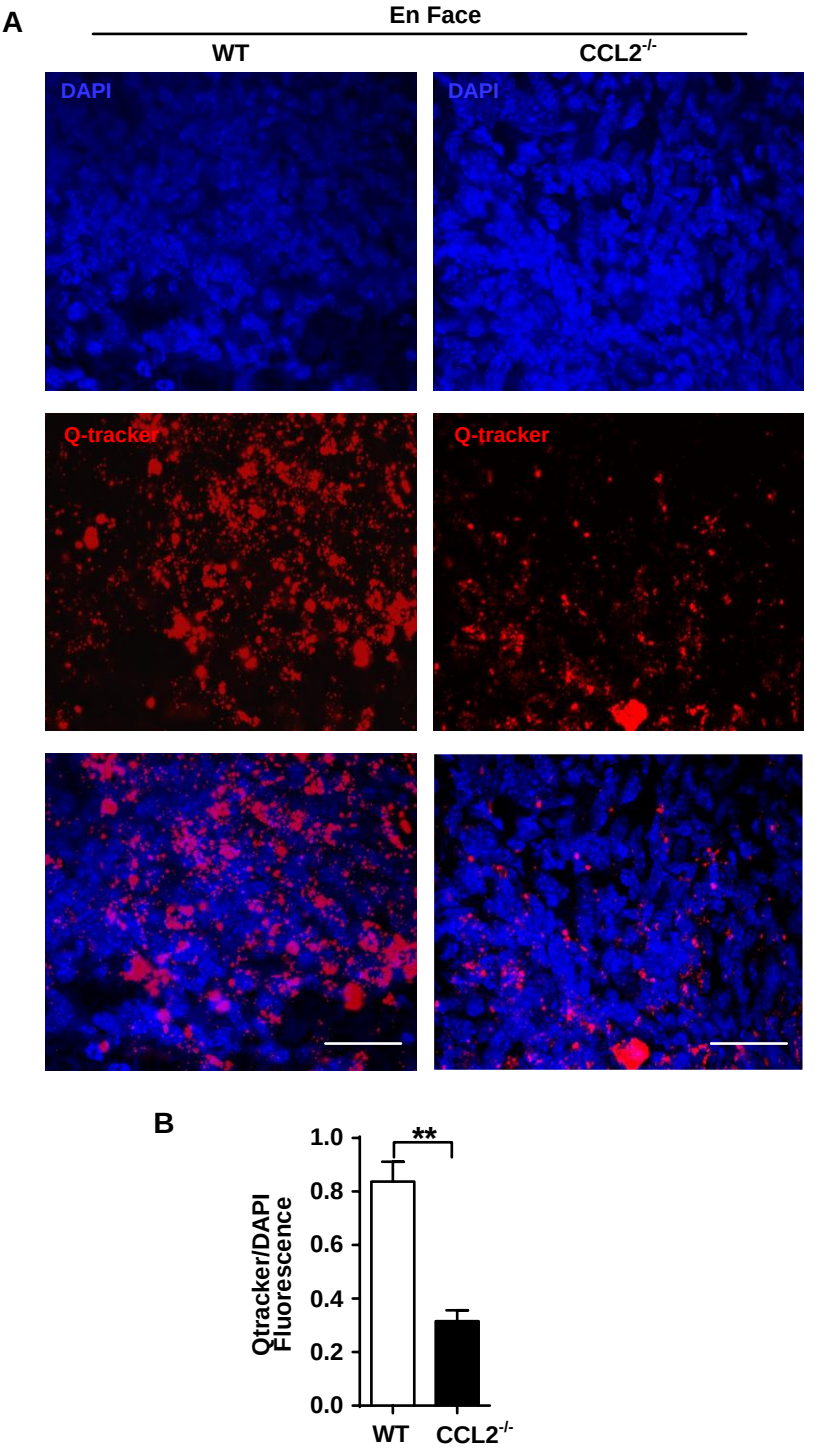


Supplemental Figure 9

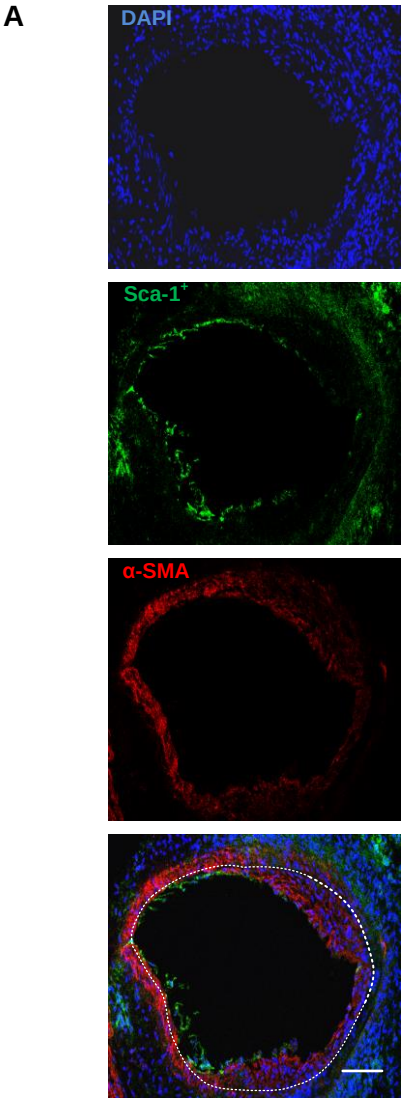




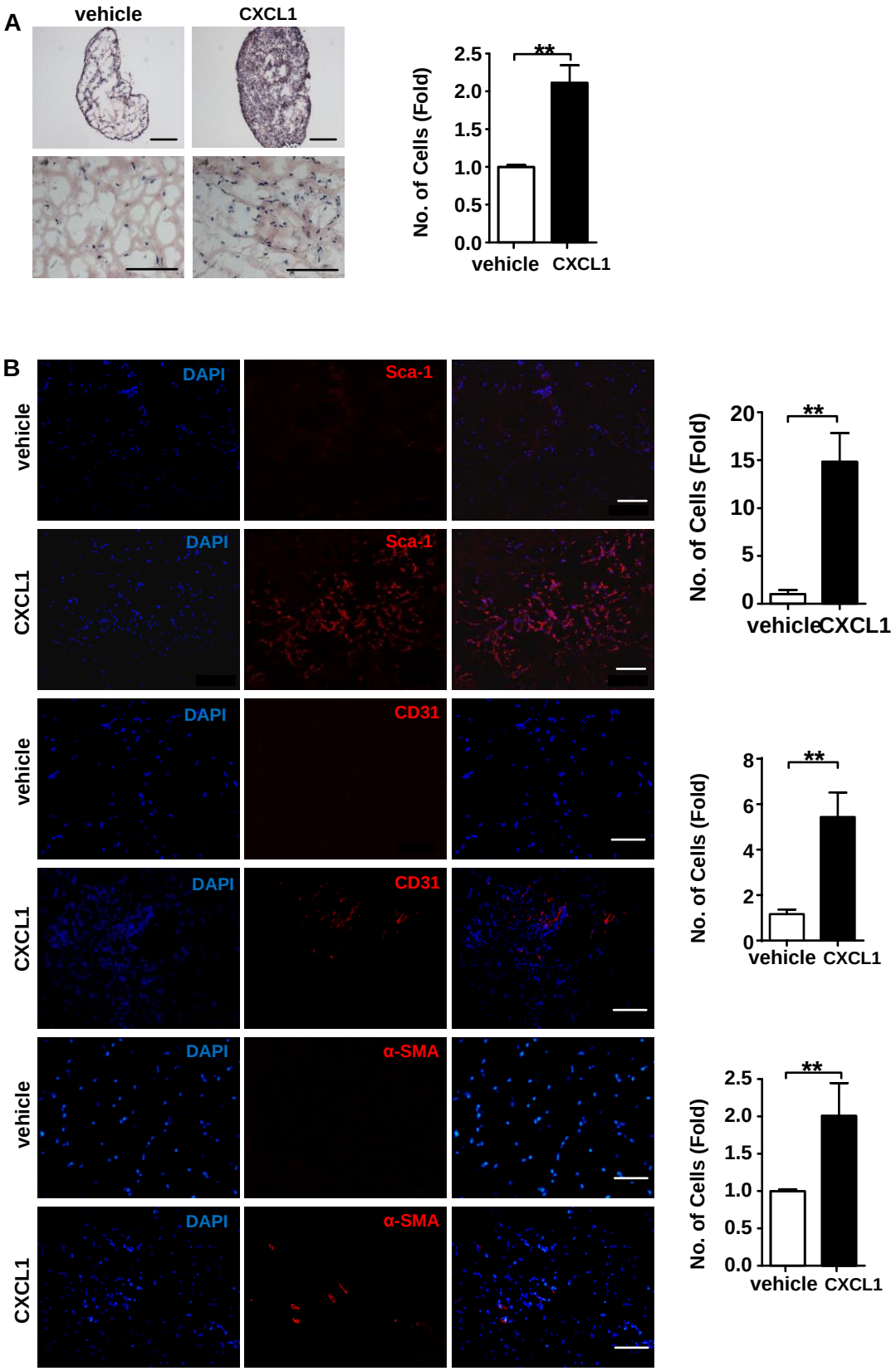
Supplemental Figure 10



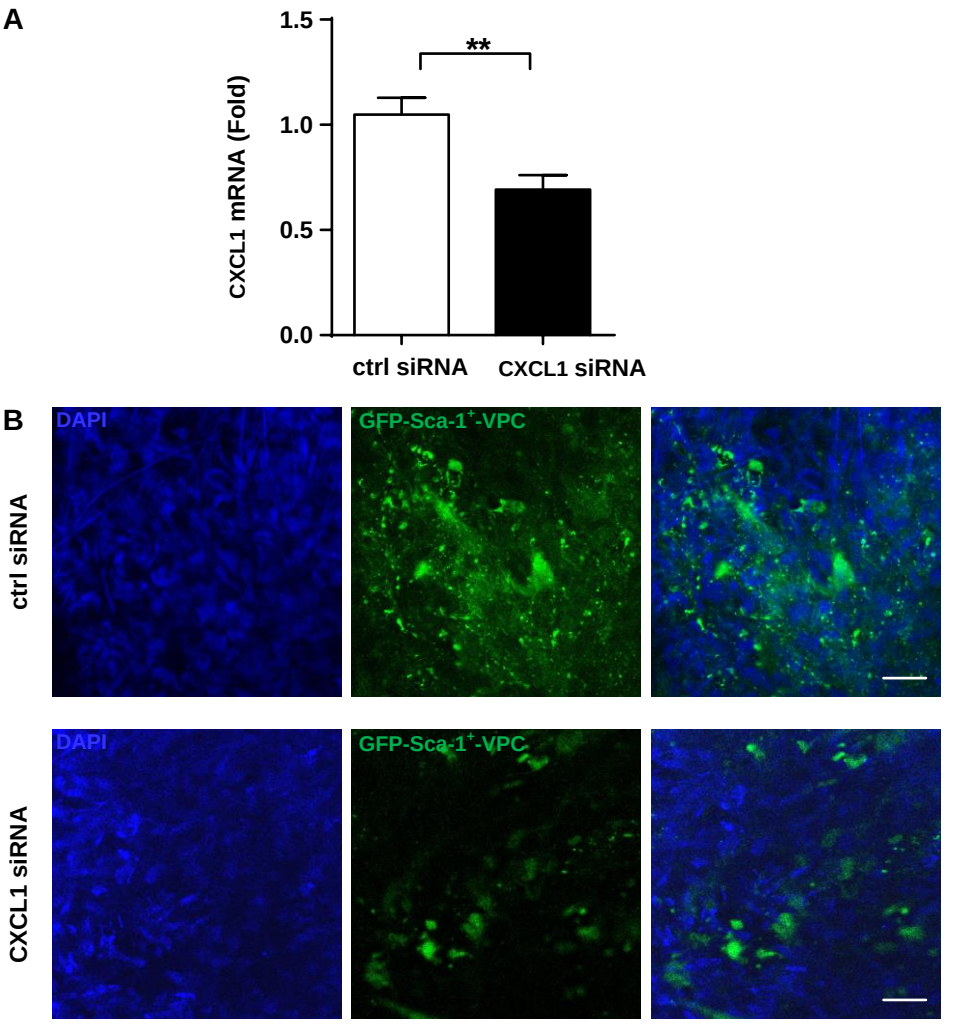
Supplemental Figure 11



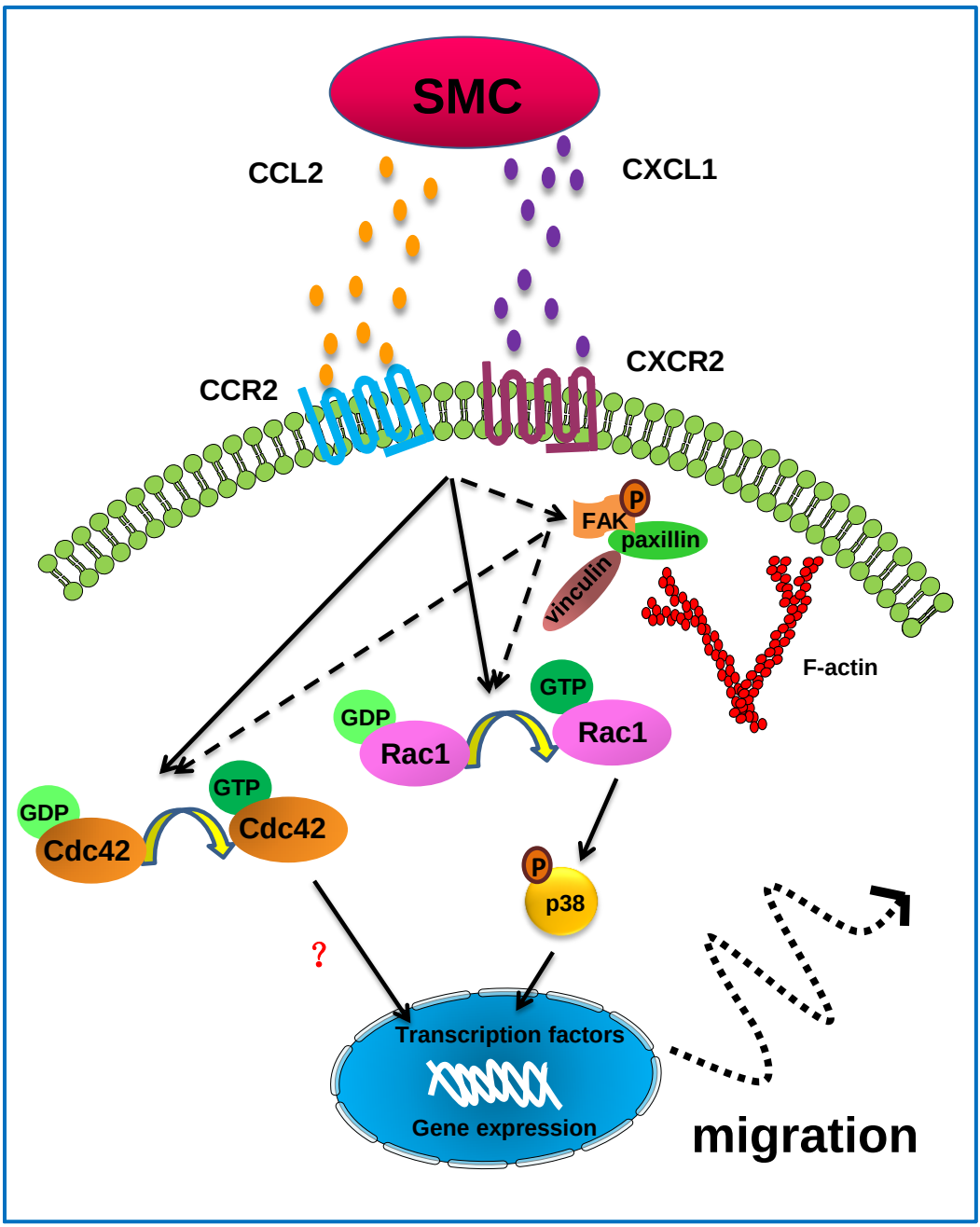
Supplemental Figure 12



Supplemental Figure 13



Supplemental Figure 14



Supplemental Table 1

Gene expressions of chemokines and chemotatic cytokines in different cell lines

($2^{-avg\Delta ct}$)($x10^{-4}$)

Refseq	Symbol	Description	SMC	Sca-1^{+} VPC	clone
NM_011329	Ccl1	Chemokine (C-C motif) ligand 1	7.16	0.83	0.8
NM_011330	Ccl11	Chemokine (C-C motif) ligand 11	7.16	15.81	3.14
NM_011331	Ccl12	Chemokine (C-C motif) ligand 12	560.56	0.42	0.46
NM_011332	Ccl17	Chemokine (C-C motif) ligand 17	175.55	2.95	2.05
NM_011888	Ccl19	Chemokine (C-C motif) ligand 19	7.16	1.6	1.6
NM_011333	Ccl2	Chemokine (C-C motif) ligand 2	11916.83	7981.14	1677.83
NM_016960	Ccl20	Chemokine (C-C motif) ligand 20	309.91	1.21	0.67
NM_009137	Ccl22	Chemokine (C-C motif) ligand 22	7.16	0.45	0.58
NM_019577	Ccl24	Chemokine (C-C motif) ligand 24	7.16	0.47	0.61
NM_009138	Ccl25	Chemokine (C-C motif) ligand 25	100.48	116.09	73.64
NM_001013412	Ccl26	Chemokine (C-C motif) ligand 26	7.16	0.62	1.15
NM_020279	Ccl28	Chemokine (C-C motif) ligand 28	7.16	6.88	7.42
NM_011337	Ccl3	Chemokine (C-C motif) ligand 3	600.79	0.42	0.5
NM_013652	Ccl4	Chemokine (C-C motif) ligand 4	191.44	1.17	0.79
NM_013653	Ccl5	Chemokine (C-C motif) ligand 5	26.1	263.02	38.74
NM_009139	Ccl6	Chemokine (C-C motif) ligand 6	617.68	6.35	0.89
NM_013654	Ccl7	Chemokine (C-C motif) ligand 7	25105.44	13329.91	4055.64
NM_021443	Ccl8	Chemokine (C-C motif) ligand 8	34.43	417.52	7.98
NM_011338	Ccl9	Chemokine (C-C motif) ligand 9	156.03	9.6	3.76
NM_027022	Cmtm2a	CKLF-like MARVEL transmembrane domain containing 2A	7.16	0.42	0.46
NM_024217	Cmtm3	CKLF-like MARVEL transmembrane domain containing 3	1464.01	1350.28	1536.8
NM_153582	Cmtm4	CKLF-like MARVEL transmembrane domain containing 4	193.44	91.29	56.33
NM_026066	Cmtm5	CKLF-like MARVEL transmembrane domain containing 5	7.26	2.62	0.49

NM_026036	Cmtm6	CKLF-like MARVEL transmembrane domain containing 6	158.21	52.8	78.02
NM_009142	Cx3cl1	Chemokine (C-X3-C motif) ligand 1	82.47	111.61	84.59
NM_008176	Cxcl1	Chemokine (C-X-C motif) ligand 1	5598.06	4179.3	965.89
NM_021274	Cxcl10	Chemokine (C-X-C motif) ligand 10	24.1	95.39	8.59
NM_019494	Cxcl11	Chemokine (C-X-C motif) ligand 11	7.16	1.13	0.46
NM_021704	Cxcl12	Chemokine (C-X-C motif) ligand 12	1222.58	3211.53	10752.46
NM_018866	Cxcl13	Chemokine (C-X-C motif) ligand 13	7.16	0.42	0.46
NM_019568	Cxcl14	Chemokine (C-X-C motif) ligand 14	233.25	8.33	5.32
NM_011339	Cxcl15	Chemokine (C-X-C motif) ligand 15	9.82	44.19	450.6
NM_023158	Cxcl16	Chemokine (C-X-C motif) ligand 16	264.24	249.41	175.14
NM_009140	Cxcl2	Chemokine (C-X-C motif) ligand 2	133.04	3.22	3.83
NM_203320	Cxcl3	Chemokine (C-X-C motif) ligand 3	29.56	0.54	0.75
NM_009141	Cxcl5	Chemokine (C-X-C motif) ligand 5	1351.84	22058.48	34058.15
NM_008599	Cxcl9	Chemokine (C-X-C motif) ligand 9	8.4	2.33	0.46
NM_008337	Ifng	Interferon gamma	7.16	0.42	0.46
NM_010551	Il16	Interleukin 16	7.57	95.17	231.64
NM_008361	Il1b	Interleukin 1 beta	34.79	0.74	1.02
NM_021283	Il4	Interleukin 4	7.16	0.54	0.63
NM_031168	Il6	Interleukin 6	183	2128.63	366.85
NM_019932	Pf4	Platelet factor 4	149.16	1.26	2.99
NM_023785	Ppbp	Pro-platelet basic protein	7.16	2.65	2.72
NM_011577	Tgfb1	Transforming growth factor, beta 1	386.87	221.69	210.7
NM_013693	Tnf	Tumor necrosis factor	42.1	0.42	0.46
NM_008510	Xcl1	Chemokine (C motif) ligand 1	7.16	0.42	0.46

Supplemental Table 2

Gene expressions of chemokines and chemotatic cytokines receptors in different cell lines

($2^{-avg\Delta ct}$)($x10^{-4}$)

Refseq	Symbol	Description	SMC	Sca-1^{+} VPC	clone
NM_007577	C5ar1	Complement component 5a receptor 1	118.66	0.42	0.46
NM_021609	Ackr2	Chemokine binding protein 2	7.16	0.65	0.59
NM_009912	Ccr1	Chemokine (C-C motif) receptor 1	30.5	3.99	2.13
NM_007721	Ccr10	Chemokine (C-C motif) receptor 10	7.16	3.11	6.23
NM_007718	Ccr1l1	Chemokine (C-C motif) receptor 1-like 1	7.16	0.42	0.46
NM_009915	Ccr2	Chemokine (C-C motif) receptor 2	7.29	0.42	0.51
NM_009914	Ccr3	Chemokine (C-C motif) receptor 3	45.75	0.42	0.46
NM_009916	Ccr4	Chemokine (C-C motif) receptor 4	7.16	0.42	0.46
NM_009917	Ccr5	Chemokine (C-C motif) receptor 5	126.74	0.42	0.47
NM_009835	Ccr6	Chemokine (C-C motif) receptor 6	7.16	0.42	0.48
NM_007719	Ccr7	Chemokine (C-C motif) receptor 7	7.16	0.45	0.78
NM_007720	Ccr8	Chemokine (C-C motif) receptor 8	7.16	0.42	0.46
NM_009913	Ccr9	Chemokine (C-C motif) receptor 9	7.16	0.88	1.03
NM_145700	Ackr4	Chemokine (C-C motif) receptor-like 1	7.16	2.02	3.72
NM_017466	Ccrl2	Chemokine (C-C motif) receptor-like 2	7.16	4.99	10.26
NM_008153	Cmklr1	Chemokine-like receptor 1	29.67	0.42	0.72
NM_009987	Cx3cr1	Chemokine (C-X3-C) receptor 1	80.77	0.42	0.46
NM_178241	Cxcr1	Chemokine (C-X-C motif) receptor 1	7.16	0.42	0.46
NM_009909	Cxcr2	Chemokine (C-X-C motif) receptor 2	7.16	0.42	0.46
NM_009910	Cxcr3	Chemokine (C-X-C motif) receptor 3	7.16	0.42	0.46
NM_009911	Cxcr4	Chemokine (C-X-C motif) receptor 4	63.59	0.59	0.46
NM_007551	Cxcr5	Chemokine (C-X-C motif) receptor 5	7.16	0.42	0.46
NM_030712	Cxcr6	Chemokine (C-X-C motif) receptor 6	7.16	1.31	9.02
NM_007722	Ackr3	Chemokine (C-X-C motif) receptor 7	145.08	795.5	779.13

NM_010045	Darc	Duffy blood group, chemokine receptor	9.42	0.48	0.48
NM_013521	Fpr1	Formyl peptide receptor 1	33.72	0.91	1.16
NM_001025381	Gpr17	G protein-coupled receptor 17	7.16	0.87	1.24
NM_011798	Xcr1	Chemokine (C motif) receptor 1	7.16	0.54	0.46

Supplemental Figure 3

Gene expressions of other chemotaxis in different cell lines
($2^{-avg\Delta ct}$)($x10^{-4}$)

Refseq	Symbol	Description	SMC	Sca-1^{+}VPC	clone
NM_010431	Hif1a	Hypoxia inducible factor 1, alpha subunit	1209.93	2109.05	3514.36
NM_008401	Itgam	Integrin alpha M	89	0.42	0.46
NM_008404	Itgb2	Integrin beta 2	226.87	15.13	17.62
NM_011949	Mapk1	Mitogen-activated protein kinase 1	910.62	526.05	694.12
NM_011951	Mapk14	Mitogen-activated protein kinase 14	159.87	191.66	208.76
NM_178804	Slit2	Slit homolog 2 (Drosophila)	1105.68	276.74	276.1
NM_011905	Tlr2	Toll-like receptor 2	59.33	61.21	34.59
NM_021297	Tlr4	Toll-like receptor 4	50.76	100.13	91.92
NM_138302	Tymp	Thymidine phosphorylase	7.16	0.73	0.55