Monocytes Are Recruited From Venules During Arteriogenesis in the Murine Spinotrapezius Ligation Model

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Objective—Chronic arterial occlusion results in arteriogenesis of collateral blood vessels. This process has been shown to be dependent on the recruitment of growth-promoting macrophages to remodeling collaterals. However, the potential role of venules in monocyte recruitment during microvascular arteriogenesis is not well demonstrated. First, we aim to document that arteriogenesis occurs in the mouse spinotrapezius ligation model. Then, we investigate the temporal and spatial distribution, as well as proliferation, of monocytes/macrophages recruited to collateral arterioles in response to elevated fluid shear stress.

Approach and Results—Laser speckle flowmetry confirmed a postligation increase in blood velocity within collateral arterioles but not within venules. After 72 hours post ligation, collateral arteriole diameters were increased, proliferating cells were identified in vessel walls of shear-activated collaterals, and perivascular CD206+ macrophages demonstrated proliferation. A 5-ethyl-2′-deoxyuridine assay identified proliferation. CD68+CD206+ cells around collaterals were increased 96%, whereas CX3CR1+/GFP+ cells were increased 126% in ligated versus sham groups after 72 hours. CX3CR1+/GFP+ cells were predominately venules after 6 hours after ligation; and CX3CR1+/GFP+ cells shifted from venules to arteriole associated between 6 and 72 hours after surgery exclusively in ligated muscle. We report accumulation and extravasation of adhered CX3CR1+/GFP+ cells in and from venules, but not from arterioles, after ligation.

Conclusions—Our results demonstrate that arteriogenesis occurs in the murine spinotrapezius ligation model and implicate postcapillary venules as the site of tissue entry for circulating monocytes. Local proliferation of macrophages is also documented. These data open up questions about the role of arteriole–venule communication during monocyte recruitment. (Arterioscler Thromb Vasc Biol. 2014;34:00-00.)

Key Words: macrophages • monocytes

Arteriogenesis is the process by which collateral arterioles remodel and undergo diameter expansion in response to increased luminal shear stress. This occurs in arterioles running collateral to an occluded artery as a result of decreased downstream pressure. Macrophages that accumulate around collateral vessels undergoing arteriogenesis are known to be crucial to the remodeling process because of their secretion of numerous cytokines and growth factors. These influence further cell recruitment to the site of remodeling, breakdown, and deposit of extracellular matrix and cell proliferation within the growing collateral.

The recruitment of circulating monocytes during arteriogenesis has been implicated by numerous studies. Heil et al demonstrated that increasing circulating monocyte numbers enhances arteriogenesis, whereas depleting them diminishes arteriogenesis. Impaired arteriogenesis in chemokine (C-C motif) receptor 2 (CCR2) knockout mice was also demonstrated by this group and Nickerson et al. showed this defect to be dependent on bone marrow–derived cells. However, no previous study has simultaneously examined arteriolar and venular recruitment of these cells during arteriogenesis. Furthermore, the spatial and temporal dynamics of this process have not been well established in the setting of microvascular flow alterations because of occlusion.

Although leukocyte transmigration into tissues is known to occur through postcapillary venules generally, Schaper et al documented adherence of monocytes to the collateral endothelium, as well as monocyte penetration into the subintimal space, during arteriogenesis of canine coronary collaterals. In the setting of murine microvascular arteriogenesis, where no neointima formation takes place, it is unknown how these cells arrive to sites of arteriolar remodeling. Here, extravasation from arterioles versus extravasation from venules has been open to debate. Likewise, the potential proliferation and expansion of tissue-resident macrophages have remained unexplored.

This study first sought to confirm that arteriogenesis occurs in collateral arterioles of the murine spinotrapezius muscle...
after feeder arteriole ligation. The second goal was to examine a time course of monocyte recruitment to collateral arterioles during this process to determine whether these cells extravasate from arterioles or venules. Third, we sought to determine whether a local population of macrophages reside in this muscle, and if so, whether ligation surgery resulted in their expansion. Finally, we sought to determine whether local proliferation of macrophages occurs in this model. A better understanding of how these cells arrive in the tissue could affect future treatments for promoting arteriogenesis.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Ligation Increases Blood Velocity and Vessel Diameters in Collateral Arterioles

Feeder arteriole ligation increased blood velocities in collateral arterioles but not in venules (Figure 1B and 1D). Sham surgery had no detectable effect on blood velocities in either microvessel type (data not shown).

Speckle intensities normalized for both background and microvessel radii were similar for collateral arterioles and draining venules before ligation. However, only collateral arterioles experienced an 86% increase in normalized speckle intensity over radius at 30 minutes after ligation. Normalized speckle intensity over radius for collateral arterioles was still elevated at 72 hours after ligation and had decreased only an insignificant amount from the previous time point (Figure 1D).

External collateral arteriole diameters 72 hours after ligation were significantly increased when compared with both unoperated and 72-hour sham-operated muscles. No difference was detected in venule diameters between groups. Venule diameters were significantly larger than arteriole diameters in each group (Figure 1E). No significant change from unoperated or sham was detected in arteriole diameter for postligation time points before 72 hours (data not shown).

Macrophage and Mast Cell Populations Reside in the Unoperated Spinotrapezius

Examination of unoperated spinotrapezius revealed few resident CX3C chemokine receptor 1 (CX3CR1)+/CD45+ cells, and no difference was detected in the number of these cells associated with arterioles versus venules (Figure 2A and 2D). Similar numbers of CD68+/CD206+ resident macrophages were observed residing in the perivascular spaces of collateral arterioles and venules (Figure 2B and 2D).
CD68<sup>+CD206<sup>- cells (indicated along microvessels by arrows in Figure 2B) were observed to be associated with both arterioles and venules. These cells appeared larger than CD68<sup>+CD206<sup>+ cells, were fewer in number than CD206<sup>+ cells, often had evident granules at ×600 magnification, and stained much brighter than most CD206<sup>+ cells for CD68 (Figure 2B). Staining with conjugated Avidin confirmed these to be mast cells<sup>11 (Figure 2C).

**Figure 2.** Macrophages and mast cells reside in the unoperated spinotrapezius. A, Example field of view (FOV) from unoperated muscle showing CX3C chemokine receptor 1 (CX3CR1<sup>+/GFP</sup>) cells (arrowheads) in an NG2-DsRed hemizygote with DsRed expression evident in arteriolar smooth muscle, pericytes, and glial cells. CX3CR1<sup>+/GFP</sup> cell counts were low in unoperated muscles with no difference in numbers surrounding arterioles compared with venules (V). White dashed line, venule outline. B, Example FOV from unoperated wild-type muscle showing resident CD68<sup>-</sup> (blue) CD206<sup>-</sup> (green) macrophages and CD68<sup>+</sup> mast cells exhibiting granules (arrows). Equal numbers of macrophages were associated with both vessel types. C, Staining with conjugated Avidin confirmed the presence and positioning of mast cells. Scale bars, 50 μm. D, No significant differences were detected between microvessel types for associated resident cell populations. Red bars, arteriole; blue bars, venule. The number of vessel-associated cells were counted along a single arteriole or venule stretched across a 640 μm FOV. n=12 FOVs from 3 mice per group. A indicates arteriole; and GFP, green fluorescent protein.

**CD68<sup>+</sup>CD206<sup>+</sup> Macrophages Are Increased in Ligated Muscle**

Ligation surgery significantly increased macrophages associated with both collateral arterioles (96% increase versus sham) and venules (59% increase versus sham) after 72 hours. Conversely, sham surgery induced no significant change in the number of macrophages associated with microvessels after 72 hours when compared with unoperated muscles (Figure 3A and 3B).

**Figure 3.** Macrophage and mast cell recruitment is increased in ligated vs sham muscles. A, CD68<sup>-</sup> (blue) CD206<sup>-</sup> (green) macrophages and CD68<sup>-</sup> mast cells (arrows) surrounding smooth muscle actin<sup>+</sup> (red) microvasculature in sham and ligated muscles at 72 hours postligation surgery. Arrowheads, mast cells associated with microvessels. Scale bars, 50 μm. B, Vessel-associated CD68<sup>+</sup>CD206<sup>+</sup> macrophages were not increased significantly from unoperated for either collateral arterioles (red bars) or venules (V; blue bars) in sham muscles after 72 hours. In contrast, CD68<sup>+</sup>CD206<sup>+</sup> macrophages associated with both arterioles (A) and V in ligated muscles were significantly increased compared with sham surgery. C, A-associated mast cells were significantly increased in the ligated group compared with muscles within the sham group. Also, significantly more mast cells were associated with A (red bars) compared with V (blue bars) in the ligated group. *P<0.05 between vessel types within surgery group. ^P<0.05 increase between surgery treatments for the indicated group. n=12 fields of view from 3 mice per group.
**Figure 4.** Differential recruitment of CX3C chemokine receptor 1 (CX3CR1)(+/GFP) cells is evident in ligated vs sham surgery muscles. 

**A**, CX3CR1(+/GFP) (green) cell recruitment around isolectin (blue) stained vasculature (with arterioles exhibiting NG2-DSRED) in ligated and sham surgery muscles for each time point. White dashed line, venule (V) outline. Scale bars, 50 μm. 

**B–D**, Red, arteriole (A); blue, V. Outlined bars, sham surgery; solid bars, ligation surgery. *P*<0.05 between indicated groups; ^P*<0.05 increase from the previous time point; vP*<0.05 decrease from the previous time point; #comparing A and V cell counts, *P*<0.05 between surgery treatments within the indicated time. n=12 fields of view (FOVs) from 3 mice per group. For comparison of unoperated values, see data in Figure 2D. The number of vessel-associated cells were counted along a single A or V stretched across a 640 μm FOV. 

**B**, CX3CR1(+/GFP) cells were primarily associated with venules 6 hours after ligation in both surgery treatments. Associations with A increased significantly by 24 hours within both surgery treatments, and numbers associated with A at 24 hours were significantly higher in the ligated group vs the sham. Significantly more GFP+ cells were present in ligated muscles vs sham at both 24 and 72 hours after surgery. GFP+ cells associated with V decreased significantly in the sham group at 24 hours and the ligated group at 72 hours. 

**C**, CX3CR1(+/GFP lo) cells were primarily associated with venules at 6 hours after surgery in both treatment groups. Associations with A increased significantly by 24 hours in both treatments, and numbers associated with A at 24 hours were significantly higher in the ligated group vs the sham. Significantly more of these cells were present in ligated muscles vs sham at 24 hours. Cell counts associated with V increased significantly by 24 hours in the ligated group before decreasing significantly by 72 hours. This change in V-associated numbers did not occur in the sham group. Note that no significant differences existed between any groups within the 72 hour time point. 

**D**, CX3CR1(+/GFP hi) cells were primarily associated with venules at 6 hours after surgery in both surgery treatments. Cell counts increased significantly between 6 and 24 hours in the ligated group only. At 24 hours, significantly more cells were associated with A in the ligated group vs the sham group. Cell numbers associated with V decreased by 24 hours in the sham group but not in the ligated group. CX3CR1(+/GFP hi) cells associated with arterioles increased between 24 and 72 hours in the ligated group but not in the sham. These cells were preferentially associated with A at 72 hours in only the ligated group. Significantly more of these cells were present in ligated muscles vs sham at both 24 and 72 hours. GFP indicates green fluorescent protein.
Mast Cells Are Increased in Ligated Muscle
Ligation surgery significantly increased mast cell numbers associated with arterioles after 72 hours when compared with both unoperated and sham. Mast cells in ligated muscles were preferentially associated with arterioles (Figure 3A and 3C). Sham surgery did not induce a significant increase in the number of mast cells after 72 hours when compared with unoperated muscle.
CX3CR1(GFP) Cells Are Recruited From Venules During Arteriogenic Remodeling

After 6 hours, in both sham and ligated groups, the number of CX3CR1(GFP) cells present had increased greatly from the unoperated group. Most of these were associated with venules. Significantly higher numbers of both CX3CR1(GFP hi) cells and CX3CR1(GFP lo) cells were associated with venules when compared with arterioles for both
surgery treatments. Numbers did not differ significantly between surgery treatments.

At 24 hours after ligation, fields of view (FOVs) from ligated animals contained significantly greater numbers of both CX3CR1(+/GFP lo) and CX3CR1(+/GFP hi) cells associated with microvessels. Within the ligated group, CX3CR1(+/GFP) cells associated with arterioles had risen significantly from 6 hours after ligation (up 274%). Both CX3CR1(+/GFP lo) cells and CX3CR1(+/GFP) cells associated with collateral arterioles had increased significantly from 6 hours in the ligated group. In addition, there was a significant increase from 6 hours (≤39%) in CX3CR1(+/GFP) cells associated with venules in the ligated group. There was no significant difference in associated cell numbers between microvessel types at 24 hours after ligation. CX3CR1(+/GFP) cells associated with collateral arterioles in the sham-operated group had increased significantly from 6 hours (≤194%). This increase was because of an accumulation of CX3CR1(+/GFP lo) cells. CX3CR1(+/GFP) cells associated with venules in the sham-operated group dropped significantly from the 6-hour time point. This was because of a decrease in CX3CR1(+/GFP hi) cells. This did not occur in the ligated group.

After 72 hours, FOVs from ligated animals contained significantly greater numbers of CX3CR1(+/GFP lo) cells associated with microvessels versus sham. These cells were now commonly seen encircling the entire circumference of the smooth muscle layer of collateral arterioles in ligated tissue. The sham group experienced no significant changes in cell numbers associated with either collateral arterioles or venules versus the 24-hour time point. In the ligated group, CX3CR1(+/GFP) cells associated with venules decreased significantly from 24 hours. Also, CX3CR1(+/GFP hi) cells associated with collateral arterioles increased significantly from 24 hours in the ligated group. This resulted in a significantly higher number of CX3CR1(+/GFP lo) cells being associated with arterioles versus venules in the ligated group. CX3CR1(+/GFP) cells associated with collateral arterioles were increased 126% in ligated versus sham groups. There was no significant difference in vessel-specific associations for CX3CR1(+/GFP lo) cells at 72 hours after ligation. Example confocal FOVs are shown in Figure 4A. Graphical representations of changes over time in cell/microvessel association counts are shown in Figure 4B and 4D.

**CCR2(+/RFP) Cells Exhibit Impaired Migration to Remodeling Collaterals**

Examination of 3 (+/CX3CR1-GFP) animals at 72 hours after ligation revealed a significant 84% increase in CX3CR1(+/GFP) cells associated with venules when compared with (+/CX3CR1-GFP) animals at 72 hours after ligation. Arteriole-associated CX3CR1(+/GFP) cell counts did not differ from those in (+/CX3CR1-GFP) animals (Figure IA in the online-only Data Supplement). However, in stark contrast to what was observed in (+/CX3CR1-GFP) animals, CX3CR1(+/GFP) cells in (+/CX3CR1-GFP)/(+/CCR2-RFP) animals were never observed to have encircled the smooth muscle layers of collateral arterioles (Figure IB in the online-only Data Supplement compared with ligated 72-hour panel in Figure 4A). The few CX3CR1(+/GFP) cells that had colocalized to the arteriolar smooth muscle still exhibited CCR2-RFP expression (Figure IB in the online-only Data Supplement).

**Only Venular Lumina Exhibit Accumulation of Adhered Monocytes After Ligation**

Adhered CX3CR1(+/GFP) cells were present in venular lumina at every time point examined as were adhered isolecitin+ leukocytes (Figure 5A–5D). Some double-positive cells were observed in venular lumina. CX3CR1(+/GFP) cells could clearly be seen extravasating from venules at the 6-hour time point (Figure 5A).

Adhered CX3CR1(+/GFP) cells in arteriolar lumina were exceedingly rare at every time point examined. We observed only 2 of these cells total in all x600 FOVs examined at sites of CX3CR1(+/GFP) cell recruitment (30+ FOVs total from 23 animals with every time point represented). Two other examples were observed, which were not in regions exhibiting recruitment to the tissue. They exhibited a round morphology and were all GFP+. The single example observed in (+/CX3CR1-GFP)/(+/CCR2-RFP) tissue had no detectable CCR2-RFP.

We were unable to detect isolecitin+ adhered leukocytes within arteriolar lumina at any time point. We also detected no CCR2(+/RFP) adhered cells within arteriolar lumina of (+/CX3CR1-GFP)/(+/CCR2-RFP) animals. These specimens revealed that the vast majority of CX3CR1(+/GFP) cells present exhibited CCR2-RFP expression (Figure 5D).

For individual CX3CR1(+/GFP) cells present at 24 hours after ligation in (+/CX3CR1-GFP)/(+/CCR2-RFP) animals, there was a strong positive correlation (correlation coefficient, 0.814; P=2.1×10−12) between CX3CR1-GFP and CCR2-RFP mean pixel intensity (Figure 5E).

We examined collateral arterioles in gracilis adductor muscles from 4 mice 24 hours after femoral artery ligation. Single monocytes were again rarely seen in arteriolar lumina. We found no evidence of CX3CR1(+/GFP) cell accumulation in collaterals arterioles, which exhibited CX3CR1(+/GFP) cell recruitment. CX3CR1(+/GFP) cells were observed to be adhered in nearby venules (Figure 5F).

**5-Ethynyl-2′-Deoxyuridine Cell Proliferation Assays Verify Collateral Remodeling**

5-Ethynyl-2′-deoxyuridine (EdU)+ nuclei, indicating that cells had entered S-phase, were associated with both microvessel types in muscles examined at 24 and 72 hours after ligation. Positive nuclei associated with microvessels at 24 hours after ligation sat primarily in the perivascular spaces of venules (Figure 6A). This difference was significant, with venules exhibiting 94% more EdU+ nuclei in their perivascular spaces (Figure 6H). Positive nuclei within arteriolar vessel walls were rare at this time point. EdU+ nuclei in the perivascular spaces of arterioles were more prevalent after 72 versus 24 hours. In addition, EdU+ nuclei within the vessel walls of collateral arterioles had increased dramatically by 72 hours after ligation. Extensive EdU staining was present in the endothelial cells of the intima at this time. These changes
resulted in a significant 435% increase in EdU+ nuclei associated with collateral arterioles between 24 and 72 hours after ligation (Figure 6B–6D, 6H). Collateral arterioles in unoperated contralateral muscles did not possess these EdU+ nuclei within the cells of their walls and did not exhibit the increase in positive nuclei within perivascular cells (not shown). We found EdU+ nuclei within CD206+ cells at both time points examined. These were more common at 72 hours after ligation, especially around remodeling arterioles (Figure 6C and 6D). However, most EdU+ nuclei in the perivascular spaces of microvessels resided in cells, which did not express either marker. We also observed EdU+ nuclei within mast cells stained with conjugated Avidin although these were rare (Figure 6G).

**Discussion**

With this study, we sought to determine whether arteriogenesis takes place in collateral arterioles of the murine spinotrapezius after feeder arteriole ligation. It has previously been shown that vascular networks in spinotrapezi of C57BL/6 mice contain multiple native collateral,12,13 and that they can undergo remodeling.14,15 Increased fluid shear stress is known to be the determinate stimulus, which initiates arteriogenesis in arteries running collateral to the occlusion.16 Our results demonstrate that ligation increased the blood velocity in collateral arterioles, and that this increase was accompanied by commensurate diameter increases in these arterioles after 72 hours. As we did not observe an increase in luminal diameter, the external diameter increase observed at this early time point may be the result of thickening of the collateral walls because of edema associated with cell proliferation.16 Arteriogenesis in this model is further confirmed by recruitment of CX3CR1+/GFP+ monocytes to activated collateral arterioles, which encircle the smooth muscle layer, and by the presence of proliferating cells in the vessel walls of these collaterals after 72 hours.

We sought to determine whether monocytes recruited to collateral arterioles during microvascular arteriogenesis in the murine spinotrapezius extravasate from shear-activated arterioles or nearby paired venules. In (+/CX3CR1-GFP)/(+/NG2-DsRed.T1) mice, the following observations collectively suggest that monocytes involved in this model extravasate from venules: the significant association of CX3CR1+/GFP+ cells with venules at 6 hours after ligation, the shift in CX3CR1+/GFP hi cells from venule to arteriole associated over the time course examined, the significant reduction in venular-associated CX3CR1+/GFP+ cells between 24 and 72 hours after ligation, and the lack of detectable monocyte adhesion in arteriolar lumina at any time point. Our observations in (+/CX3CR1-GFP)/(+/CCR2-RFP) mice at 72 hours after ligation further support this conclusion. Because monocytes were clearly able to extravasate in these animals, the increased numbers of monocytes associated with venules and lack of monocyte colocalization with the medial layer of collateral arterioles demonstrate that these cells are originating from venules. Furthermore, the apparent deficit in chemotaxis of CCR2+/RFP+ monocytes implicates the importance of CCL2/CCR2 signaling to monocyte migration between the 2 microvessel types. Heil et al6 have previously shown that CCL2-driven chemotaxis of monocytes is abolished in cells from CCR2−/− mice. Studies in murine models cannot exclude the possibility of monocyte transmigration through the arteriolar endothelium into the subintimal space in other species, which exhibit neointima formation during arteriogenesis.9,16

We observed adherent leukocytes accumulated in venular lumina at every time point examined. In contrast, we were unable to detect an accumulation of adhered leukocytes in arteriolar lumina for any time point examined. In addition, at high magnification, we observed many instances at the 6-hour time point in which CX3CR1+/GFP+ cells were clearly extravasating from the venule as evident by their orientation and distribution in the tissue relative to the microvessels. Our examination of gracilis adductor collateral arterioles at 24 hours after ligation also revealed a lack of leukocyte accumulation in these vessels; adhered CX3CR1+/GFP+ cells were again common in venules. This suggests that recruitment occurs similarly in the femoral artery ligation model. Although we are unable to observe real-time dynamics of cell recruitment in these models, the evidence acquired in harvested tissues across different time points suggests that recruited monocytes do not normally arrive via extravasation from activated collateral arterioles. This may be, in part, because of the highly elevated fluid shear stress present in these vessels after feeder ligation. However, the findings of Ley and Gaehghten57 in similarly sized microvessels of the rat mesentery suggest that differences in shear may not explain this phenomenon. They found that in stark, contrast to the consistent leukocyte rolling observed on venular endothelium, practically no lymphocytes rolled on arteriolar endothelium regardless of wall shear rate. This was hypothesized to be attributed to differences between the arteriolar and the venular endothelium because previous studies had already shown preferential expression of selectins on venular endothelium. Jung and Ley58 later demonstrated differences in selectin and ICAM-1 expression between arteriolar and venular endothelium in mouse cremaster, after the activation by tumor necrosis factor-α, which were consistent with rolling and adhesion taking place preferentially in venules. We are unaware of any published study that compares adhesion molecule expression between venular and arteriolar endothelium in the setting of microvascular arteriogenesis. Additional studies are needed to address this question.

Given the significantly greater CX3CR1+/GFP+ cell recruitment in ligated muscle when compared with sham-operated at 24 hours, it would seem that the presence of shear-activated arterioles in close proximity to venules increases leukocyte recruitment from these venules. Previous studies have suggested that communication between venules and closely paired arterioles regulates capillary perfusion and that this communication is altered during inflammation.19 The mechanisms of this signaling are largely unknown and may involve factors originating from leukocytes, as well as the vessels themselves. Other studies have provided evidence to support roles for both reactive oxygen species20 and angiotensin II in activating venules to recruit inflammatory cells. Whether
these potent signaling molecules are involved in communication between shear stress–activated arterioles and venules in muscle remain to be seen.

The presence of CD206+ cells undergoing DNA synthesis suggests that these cells are proliferating locally. These are most often residing in a perivascular position around venules and arterioles at both 24 and 72 hours after ligation. We observed significantly more EdU+ nuclei in the perivascular spaces of venules at 24 hours after ligation, suggesting that some local proliferation may be associated with the inflammatory response induced by surgery. Many studies have indicated that some tissue-resident macrophage populations are capable of self-renewal by local proliferation.10 If proliferation of tissue-resident perivascular macrophages were contributing significantly to the accumulation around collateral arterioles evident at 72 hours after ligation, we would expect to observe more CD206+ macrophages with EdU+ nuclei associated with collateral arterioles at the 24-hour time point. We reained CX3CR1-GFP heterozygote muscles harvested 72 hours after ligation for CD206 after initial imaging for GFP was completed. We observed faint CD206 staining on more rounded areas in CX3CR1(+/GFP) cell accumulation around remodeling collateral arterioles (Figure III in the online-only Data Supplement). Because much of the GFP was lost while permeabilizing the tissue to deliver antibody, we were unable to confirm whether this staining was on recruited CX3CR1(+/GFP) cells. Cote et al22 concluded that both infiltrating and resident cells contribute to macrophage accumulation after muscle injury. Epelman et al23 found that both recruited monocytes and local proliferation contributed to cardiac macrophage populations during inflammation. On the basis of our observations during this study, we hypothesize that CD206 macrophages in our model are derived from both local proliferation and recruited monocytes.

Although local proliferation of macrophages is occurring in our model, it seems that monocyte-derived cells may be more integral to early remodeling of the collateral wall, because CX3CR1(+/GFP) cells, but not CD206+ cells, were observed to encircle the entire circumferences of collateral medial layers tightly. This is consistent with numerous previous studies demonstrating the influence that circulating monocytes have on arteriogenesis.5,24 Interestingly, local proliferation of CD206+ macrophages around collateral arterioles seemed to be increased at 72 hours after ligation, after recruited CX3CR1(+/GFP) cells had colocalized to the medial layers of these vessels. Clearly, more studies are needed to address questions about the contributions of specific macrophage populations to arteriogenesis.

We observed striking differences in the distributions of CX3CR1(+/GFP) cells between ligated and sham-operated treatment groups. In contrast to the ligated group, the sham group displayed a significant drop in CX3CR1(+/GFP) cells from 6 to 24 hours after ligation. This was attributable to a large decrease in CX3CR1(+/GFP hi) cells within the sham group during this time. Because recruitment was nearly identical between treatments at 6 hours, this suggests that fewer recruited cells were retained near the microvessels in the sham group at 24 hours. In addition, we observed a steady accumulation of CX3CR1(+/GFP hi) cells around collateral arterioles in the ligated group, which was completely absent in the sham group. These results suggest that collateral arterioles in the sham group were not shear-activated as would be expected. Thus, recruited monocytes in the sham group migrated to other areas without being retained by collateral arterioles. For instance, after ligation surgery, we commonly saw an accumulation of CX3CR1(+/GFP) cells on the dorsal face of the muscle where the fascia layer was disturbed.

Although our thresholding method to designate GFP+ cells was relatively arbitrary in comparison with gating methods for flow cytometry, it nevertheless enabled the detection of 2 distinct populations of recruited CX3CR1(+/GFP) cells, which seem to behave differently in the setting of microvascular arteriogenesis. CX3CR1(+/GFP lo) cell accumulation peaked by the 24-hour time point and then trended down in both surgery treatment groups. The patterns of recruitment observed for these cells over the examined time course also varied little between venules and collateral arterioles. In contrast, CX3CR1(+/GFP hi) cells steadily accumulated around shear-activated collateral arterioles between 6 and 72 hours, whereas nearby venules saw a sharp decline in the number of these cells between 24 and 72 hours. CX3CR1(+/GFP hi) cells but not CX3CR1(+/GFP lo) cells were preferentially associated with activated collateral arterioles 72 hours after ligation. This is consistent with the findings of Butoi et al,25 which demonstrated that monocytes upregulate CX3CR1 expression during interactions with smooth muscle cells and indicate that the CX3CR1(+/GFP hi) population is the one more relevant to the remodeling of activated collateral arterioles. The importance of CX3CR1 to both arteriolar and venular remodeling has previously been demonstrated by Meisner et al,26 who documented a diminished remodeling response in both microvessel types for chimeras reconstituted with knockout bone marrow.

On the basis of the work of Geissman et al,27 it seems that most of the CX3CR1(+/GFP) monocytes we document extravasating from venules at 24 hours after ligation are likely of the CX3CR1(+/GFP) CCR2+Gr1+ subset. The vast majority of cells we designated CX3CR1(+/GFP hi) in (+/CX3CR1-GFP)/(+/-CCR2-RFP) animals exhibited CCR2-RFP expression and CCR2-RFP expression correlated positively to CX3CR1-GFP expression in these cells at 24 hours after ligation. At 72 hours after ligation, CX3CR1(+/GFP hi) cells in these animals, which had colocalized with the smooth muscle layer of collateral arterioles and adopted an elongated morphology, still exhibited CCR2-RFP expression. It is not known what happens to CX3CR1 expression in monocytes over time once they enter the tissue after extravasation in this setting. It may be that the observed recruitment patterns are simply the result of differential chemokine receptor expression in these cells, which make CX3CR1(+/GFP hi) cells more likely to home to the remodeling collateral arterioles. Alternatively, some extravasated CX3CR1(+/GFP lo) cells could be upregulating their CX3CR1 expression between the examined time points.
Troidl et al. recently documented the recruitment of CD68+CD163− (M1) and CD68+CD163+ (M2) macrophages to collateral arterioles undergoing arteriogenesis in the murine hindlimb and provided evidence for both classically activated (M1) and nonclassically activated (M2) macrophages playing determinate roles during the remodeling process. The current paradigm is that M1 macrophages play an inflammatory role during arteriogenesis to increase cell recruitment, whereas M2 macrophages secrete factors to aid in structural remodeling and cell proliferation within the collateral vessel itself. We used CD206 as our marker for nonclassically activated (M2) macrophages. However, with the exception of mast cells, virtually all CD68+ cells in murine spinotrapezius were also CD206+ in the unoperated group and after both surgery treatments. This highlights the inadequacy of using a single marker when attempting to determine macrophage activation states. It is becoming increasingly clear that these designations sometimes have limited the use in describing activation states present in in vivo processes, where ongoing cell recruitment and tissue remodeling likely result in macrophages being present at various stages of phenotype switching.

We found mast cell numbers to be increased around collateral arterioles at 72 hours after ligation. Mast cells have previously been implicated as contributors to arteriogenesis, and we have previously noted an apparent increase in mast cells associated with collateral arterioles after ligation,31 and we have previously implicated as contributors to arteriogenesis. Local proliferation of mast cells in skin was previously been implicated as contributors to arteriogenesis to increase cell recruitment, whereas M2 macrophages secrete factors to aid in structural remodeling and cell proliferation within the collateral vessel itself.4 We used CD206 as our marker for nonclassically activated (M2) macrophages. However, with the exception of mast cells, virtually all CD68+ cells in murine spinotrapezius were also CD206+ in the unoperated group and after both surgery treatments. This highlights the inadequacy of using a single marker when attempting to determine macrophage activation states.5 It is becoming increasingly clear that these designations sometimes have limited the use in describing activation states present in in vivo processes,30 where ongoing cell recruitment and tissue remodeling likely result in macrophages being present at various stages of phenotype switching.

We found mast cell numbers to be increased around collateral arterioles at 72 hours after ligation. Mast cells have previously been implicated as contributors to arteriogenesis,31 and we have previously noted an apparent increase in their numbers in thrombin-treated muscles exhibiting postligation capillary arterialization.32 Mast cells are known to modulate inflammatory responses and to contribute to breakdown and remodeling of the extracellular matrix.33 These functions are consistent with the observed increase in mast cells associated with collateral arterioles after ligation treatment. Local proliferation of mast cells in skin was recently documented in a canine model of dermatitis.34 Our EdU proliferation assay results suggest that these cells are capable of local proliferation in skeletal muscle. However, the rarity of observed mast cells with EdU+ nuclei suggests that the population expansion apparent at 72 hours after ligation is mainly attributable to recruitment rather than local proliferation.

This study clearly demonstrates that arteriogenesis occurs in the murine spinotrapezius ligation model and highlights the resolution with which this model allows examination of recruited cell populations involved in the remodeling process. Our study is the first to offer direct evidence of monocyte recruitment from venules to collateral arterioles during microvascular arteriogenesis. Our results support the growing consensus that cells derived from circulating monocytes are integral to arteriogenesis and suggest that manipulations designed to increase monocyte adhesion and extravasation during microvascular arteriogenesis should target venules.

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References
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**Significance**

The study presented here examines the underlying role of venules when compared with that of arterioles in macrophage and mast cell recruitment during vascular network remodeling. Whereas previous studies have focused nearly exclusively on the remodeling collateral arteries themselves; our article underscores the contribution of postcapillary venules in the setting of microvascular arteriogenesis. Using the murine spinotrapezius artery ligation model, we show that changes in blood velocity in collateral arteries after ligation stimulate increased monocyte extravasation predominantly from postcapillary venules. Our data on monocyte and macrophage recruitment during arteriogenesis in the muscle implicate a new mode of arteriole–venule cross-talk. We hope that this article will serve as a platform for new studies examining the molecular signaling that underpins the spatial and temporal dynamics of recruited cell populations during collateral vessel remodeling.
Monocytes Are Recruited From Venules During Arteriogenesis in the Murine Spinotrapezius Ligation Model

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Supplemental Figure I.  A. CX3CR1(+/GFP) cells associated with venules in 2D confocal volume renders at 72 hours post-ligation were increased 84% in (+/CCR2-RFP)/(+/CX3CR1-GFP) animals compared to (+/CX3CR1-GFP) animals while arteriole-associated counts were similar. Red indicates arteriole; blue indicates venule. + indicates p < 0.05 increase in associated cells between groups. n=12 FOVs from 3 animals per group. The number of vessel associated cells were counted along a single arteriole or venule stretched across a 640 micron FOV. B. In contrast to (+/CX3CR1-GFP) animals, CX3CR1(+/GFP) cells in (+/CCR2-RFP)/(+/CX3CR1-GFP) animals had not surrounded collateral mural cell layers. We did not observe a single collateral arteriole segment in 3 ligated animals that exhibited normal wrapping by CX3CR1(+/GFP) cells at 72 hours post-ligation as displayed by CCR2 wild type animals (shown in Figure 4A and Supplemental Figure III). “A” designates arteriole; “V” designates venule; white dashed line indicates venule outline. Scale bar indicates 50 µm.
**Supplemental Figure II.**

Examination of gracilis adductor collateral arterioles at 24 hours following femoral ligation suggests similar CX3CR1\(^{+/GFP}\) cell recruitment in this murine model. Gracilis adductor collateral arterioles exhibited no accumulation of adhered monocytes in arteriolar lumina at sites of CX3CR1\(^{+/GFP}\) cell recruitment. CX3CR1\(^{+/GFP}\) cells could be seen to be adhered within nearby venules (arrows). “A” designates arteriole; “V” designates venule; white dashed line indicates venule outline. Scale bar indicates 50 µm.
Supplemental Figure III.

A-B. CD206 staining is detectable in regions of CX3CR1\(^{+/GFP}\) cell recruitment to remodeling collateral arterioles at 72 hours post-ligation. “A” designates arteriole; “V” designates venule. All scale bars indicate 50 µm. **A.** \((+/CX3CR1-GFP)\) spinotrapezius at 72 hours post-ligation with perfused lectin exhibiting CX3CR1\(^{+/GFP}\) cells encircling the collateral arteriole mural cell layer. **B.** The same field of view with superfused lectin exhibiting CD206 staining in the region of CX3CR1\(^{+/GFP}\) cell recruitment (example location indicated by dashed line). Most CX3CR1-GFP is lost during permeabilization to deliver CD206 antibody.
SUPPLEMENTAL MATERIAL

Detailed Methods

Experimental Animals

We utilized mice on C57BL/6 background as these are known to possess consistent pre-existing collateral arterioles in their spinotrapezius(1). Homozygous CX3CR1-GFP mice(2) on C57BL/6 background were a kind gift of Dr. Klaus Ley (La Jolla Institute for Allergy and Immunology). Hemizygous STOCK Tg(Cspg4-DsRed.T1)1Aikik/J mice were purchased from Jackson Laboratory (Bar Harbor, ME). These strains were bred together to generate (+/CX3CR1-GFP)/(+/Cspg4-DsRed.T1) male offspring which were used to examine a time course of GFP+ cell recruitment. CX3CR1-GFP allowed visualization of monocytes/macrophages, while Cspg4-DsRed.T1 allowed visualization of arteriolar smooth muscle which aided in distinguishing arterioles from venules(3). Male (+/CX3CR1-GFP) littermates were used to compare CX3CR1-GFP and CD206 staining. Homozygous B6.129(Cg)-Ccr2tm2.1Ifc/J mice were purchased from Jackson Laboratory. These were bred to homozygous CX3CR1-GFP mice to generate (+/CX3CR1-GFP)/(+/CCR2-RFP) male offspring which were used to evaluate CCR2 expression by recruited CX3CR1+ cells and to evaluate monocyte recruitment to collateral arterioles in a setting of decreased CCR2. Microvessel lumina in all CX3CR1-GFP mice were examined for leukocyte adhesion following ligation surgery. Male C57BL/6J mice were purchased from Jackson Laboratory for proliferation assays and to examine CD68+CD206+ macrophages as well as mast cells. All mice were 12-24 weeks of age at time of manipulation with treatment and control groups age-matched within experiments.

Spinotrapezius Ligation Surgery and Harvest

All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. Mice were anesthetized with an i.p. injection of ketamine/xyloazine/atropine (60/40.2 mg/kg). Ligation surgeries were performed as previously described(4) (Figure 1A). Briefly, a small incision was made on the dorsum above the lateral edge of the right spinotrapezius at the edge of the fat pad. The fascia was separated from the top of the muscle and the fat pad moved before isolating an anatomically reproducible feeding arteriole entering the muscle from below. This feeding arteriole was ligated with 10-0 nonabsorbable suture in two places and cut. The fat pad and fascia were moved back into position and the skin was closed with 8-0 nonabsorbable suture. Sham surgeries were comprised of the same steps except ligatures were not tied and the feeder arteriole was not cut.

For the CX3CR1-GFP cell-quantification study, 3 animals were examined at baseline and surgery was performed on 6 groups of 3 animals each (18 total). For the CD68/CD206 cell and mast cell quantification study, 3 mice were examined at baseline and surgery was performed on two groups of 3 animals each (6 total). Two (+/CX3CR1-GFP)/(+/CCR2-RFP) animals underwent ligation surgery to evaluate CCR2-RFP+ cells at 24 hours post-ligation. Three (+/CX3CR1-GFP)/(+/CCR2-RFP) animals underwent ligation surgery to allow evaluation of GFP+ cell recruitment in a setting of decreased CCR2. Eight wild type animals (4 per group) underwent ligation surgery to examine cell proliferation at 24 and 72 hours post-ligation.

To allow visualization of vascular endothelium in mice with fluorescent reporter proteins, anesthetized mice were administered an intra-jugular injection of labeled isoleciton (IB4-Alexa
Fluor 647 or 568; Life Technologies, Carlsbad, CA) which was circulated for 10 minutes. This consistently resulted in robust staining of arteriolar and capillary endothelium but not venular endothelium. Faint staining was present on venules which allowed for their identification in confocal volume renders. Anesthetized mice were euthanized in a CO₂ chamber. The vasculature was immediately flushed with an intracardiac infusion of adenosine (70 mg/L) in Ringer’s solution followed by an intracardiac infusion of 4% paraformaldehyde in phosphate buffered saline (PBS). Muscles were harvested un-operated or at 6, 24, or 72 hours post-surgery and whole-mounted.

**Immunohistochemistry**

Muscles were permeabilized with 0.1% saponin in PBS, blocked with 5% normal mouse serum, and superfused with a combination of isolectin (IB4 Alexa Fluor 647 or 568, Life Technologies, Carlsbad, CA), anti-alpha smooth muscle actin (clone IA4 Cy2 or Cy3, Sigma-Aldrich, St. Louis, MO, 1:300 dilution) to label vascular smooth muscle cells, and/or anti-CD68 (clone FA-11 Alexa Fluor 647, AbD Serotec/Bio-Rad, Hercules, CA) to label macrophages and mast cells, and anti-CD206 (clone MR5D3 Alexa Fluor-488, AbD Serotec/Bio-Rad, Hercules, CA) to label macrophages. Previous studies have documented the presence of both classically and alternatively-activated macrophages near growing collateral arterioles undergoing arteriogenesis(5). Our motivation for co-staining CD206 and CD68 is that CD206 has been shown to label alternatively activated macrophages(6), while CD68 has been shown to label all macrophages (both classically and alternatively activated). Avidin conjugated to Alexa Fluor 488 (Life Technologies, Carlsbad, CA, 1:200 dilution) was used to stain mast cells. Muscles were whole-mounted with coverslips on gelatin-coated slides using a 50:50 glycerol/PBS solution.

**Femoral Ligation and Gracillis Harvest**

All surgical procedures were approved by the Institutional Animal Care and Use Committee at the University of Virginia. (+/CX3CR1-GFP)/(+/Cspg4-DsRed.T1) mice were anesthetized (i.p. 60 mg/kg ketamine, 4 mg/kg xylazine, and 0.02 mg/kg atropine), depilated, and prepped for aseptic surgery. On the left leg, an incision was made directly above and along the femoral artery, and a window of skin was dissected free and retracted directly above the superficial adductor muscles. The femoral artery was gently dissected from the femoral vein and nerve between the bifurcation of the superior epigastric artery and popliteal artery. Two 6.0 silk sutures were placed underneath the femoral artery immediately distal to the epigastric artery and tied. The femoral artery was then severed between ligatures and the skin incision sutured. See Meisner et al.(7) for more information on this ligation scheme.

**5-ethynyl-2’-deoxyuridine (EdU) Cell Proliferation Assay**

While typically used in cell culture, past studies have demonstrated effective use of EdU staining in vivo(8). Proliferative cells were labeled with the Click-it™ EdU imaging kit (Life Technologies, Carlsbad, CA). All reagents used were prepared according to the manufacturer’s protocol. Four mice per time point received an intraperitoneal injection of EdU at 100 mg/kg. Mice were euthanized four hours later. Once muscles were harvested, they went under the following protocol at room temperature: (1) wash in PBS for 10 minutes, (2) permeabilization overnight at 4 degrees Celsius in 0.1% saponin in PBS, (3) wash in 3% Bovine Serum Albumin (BSA) in PBS for 15 minutes twice, (4) incubate in the Click-it™ reaction cocktail (containing reaction buffer, CuSO₄, Alexa Fluor® 488 or 647 Azide, and reaction buffer additive) for 30 minutes protected from light, (6) wash in 3% BSA in PBS for 30 minutes twice. Muscles were then superfused with antibodies and whole-mounted as above.
Laser Speckle Flowmetry for *in Vivo* Blood Velocity Quantification

Laser speckle images were recorded intravitally for 3 mice of the group harvested at 72 hours post-ligation to confirm ligation-induced blood velocity increases in collateral arterioles. The dorsal skin was reflected back from the incision made during ligation and spinotrapezii were kept hydrated with warmed Ringer’s solution and illuminated with a beam expanded 4.5 mW 650 nm laser (Thorlabs, Newton, NJ)(9). The raw laser speckle patterns were recorded using Nikon Elements software (Nikon Instruments Inc., Melville, NY) and a Photometrics CoolSNAP HQ2 camera (Photometrics, Tucson, AZ) mounted to a Nikon Eclipse 80i microscope (Nikon Instruments Inc., Melville, NY) equipped with a 2X dry objective. Speckle patterns were recorded at 3 time points: pre-ligation, 30 minutes post-ligation, and 72 hours post-ligation.

Confocal Imaging in Excised Tissue

Digital images of whole-mount spinotrapezii were acquired using confocal microscopy (Nikon Instruments Incorporated, Melville, NY; Model TE200-E2; 10X, 20X and 60X objectives). Collateral arterioles connecting to redundant feeder arterioles, as well as nearby venules, were examined for recruited leukocytes or EdU+ cell nuclei. Muscles used for laser speckle capture were completely imaged using a 10X dry objective. This allowed montages to be compiled for comparison to laser speckle index maps for the purpose of confirming microvessel type in speckle index maps. For monocyte/macrophage quantification, 3-4 different fields of view (FOVs) per muscle containing a collateral arteriole and draining venule with monocytes/macrophages evident were located manually (This was not always possible in baseline muscle when quantifying CX3CR1(+/GFP) cells. For these muscles, fields without CX3CR1(+/GFP) cells were included as necessary to achieve the minimum number). FOVs with minimal 2-dimensional overlap between arterioles and venules were given preference so that associations of cells with a particular microvessel type could be observed. Full-thickness Z-stack (2 µm step size) volume renders of these FOVs were generated using a 20X oil immersion objective. When microvessels were located superficially enough in the tissue to image through their entire depth, full-thickness Z-stacks (1 µm step size) were generated of these vessel pairs using a 60X oil immersion lens. These were examined for adhered leukocytes within microvessel lumina. 1-3 of these were collected per muscle.

Image Analysis

Raw laser speckle captures were converted to laser speckle flow index maps using a MATLAB algorithm. These were then normalized to median background intensity and analyzed in ImageJ as described by Meisner *et al.*(9). Draining venules exhibiting measurable speckle patterns were much larger than collateral arterioles and their normalized speckle intensities (NSI) higher at baseline. These venules had a larger average diameter than those in the FOVs used for monocyte/macrophage quantification. To minimize the contribution of vessel diameter on midline blood velocity, we normalized NSI values by dividing by the vessel radius in µm for comparisons between microvessel types.

100X whole-muscle confocal montages used for comparison to laser speckle flow index maps were compiled for muscles at 72 hours using Adobe Photoshop (Adobe Systems Inc., San Jose, CA). These montages were used to locate vessel segments where speckle intensity measurements were taken. NG2-DsRed expression was used to identify microvessel type as it has been shown that NG2 expression is present in arteriole smooth muscle cells, but absent from venule smooth muscle cells(3). 200X confocal images taken at these locations were used to measure vessel diameters for which laser speckle flowmetry was performed.
200X confocal Z-stack volume renders were used for cell association quantification. We found that isolectin staining is brighter on arteriolar endothelium than venular endothelium. This, along with vessel morphology and DsRed expression in arteriolar smooth muscle, allowed for microvessel identification in fluorescent reporter mice. In wild type mice, vessel identity was determined by smooth muscle cell morphology as visualized by anti-alpha smooth muscle actin staining (3). Vessel-associated CX3CR1^{+/GFP} or CD68^{+}CD206^{+} cells were defined as cells falling within the 2-dimensional area of the vessel in question and within 50 µm of the vessel border. The number of vessel associated cells were counted along a single arteriole or venule that spanned the 640 micron wide field FOV. We gave preference to FOVs with visible separation between arterioles and venules but regions of close proximity/overlap still existed in most fields. Note that due to the 2-dimensional nature of the images, close arterioles-venule proximity, and arteriole-venule overlap in some regions most images contained some cells which were counted as being associated with both microvessel types. The same images used for CX3CR1^{+/GFP} cell/vessel association counts were also used to quantify cell numbers and distributions for cells exhibiting different levels of CX3CR1-GFP expression. A threshold at which only the brightest CX3CR1^{+/GFP} cells (CX3CR1^{+/GFP hi}) were visible was applied to all images. CX3CR1^{+/GFP hi} cell/vessel associations were counted as described above. These counts were subtracted from the total CX3CR1^{+/GFP} cell/vessel association counts to generate counts for cells with lower CX3CR1-GFP expression (CX3CR1^{+/GFP lo}).

ImageJ (NIH, Bethesda, MD) imaging software was used to quantify vessel diameters and mean pixel intensities. Diameter measurements were taken for both arterioles and venules in each 200X field FOV. These were calculated by averaging measurements taken approximately every 200 µm across the entire vessel within each FOV while ignoring small branches terminating to capillaries. CX3CR1^{+/GFP} cell counts and green channel thresholding to designate CX3CR1^{+/GFP hi} cells were performed in Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA).

CCR2-RFP expression by CX3CR1^{+/GFP} cells was evaluated by separating the color channels in 600X FOVs taken in regions of close proximity between venules and collateral arterioles where CX3CR1^{+/GFP} could be seen extravasating from venules. FOVs were divided into four equal quadrants. From each quadrant, 3 cells exhibiting the highest GFP expression and 3 cells exhibiting the lowest GFP expression were selected. Individual color channels were converted to black and white and the median grey value of both red and green signal was measured for each cell.

Full-thickness Z-stack volume renders (1 um step size) of microvessel lumina were examined at 600X for CD68+, CX3CR1^{+/GFP}, isolectin+, and CCR2^{+/RFP} cells as appropriate.

**Statistical Analysis**

Laser speckle flowmetry data were analyzed by Two Way Repeated Measures Analysis of Variance (ANOVA) with multiple comparisons by the Holm-Sidak method. Diameter, cell quantification, and proliferation data were analyzed by Two Way ANOVA with multiple comparisons by the Holm-Sidak method. Some data sets required log10 or rank transformation to achieve normality prior to analyses. CX3CR1-GFP/CCR2-RFP mean pixel intensities were analyzed by Pearson Product-Moment Correlation. Significance was assigned at p<0.05. Raw data are presented as mean +/- standard error.
Supplemental References


