Underlying chronic inflammation alters the profile and mechanisms of acute neutrophil recruitment

Running title: Acute responses in chronically inflamed tissues

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Abstract

Chronically inflamed tissues exhibit altered characteristics including persistent populations of inflammatory leukocytes and remodeling of the vascular network. As the majority of studies of leukocyte recruitment have been carried out in normal healthy tissues the impact of underlying chronic inflammation on ongoing leukocyte recruitment is, however, largely unknown. Here we investigate the profile and mechanisms of acute inflammatory responses in chronically inflamed and angiogenic tissues, and consider the implications for chronic inflammatory disorders.

We have developed a novel model of chronic ischemia of the mouse cremaster that is characterized by a persistent population of monocyte-derived cells (MDCs), and capillary angiogenesis. These tissues also exhibit elevated acute neutrophil recruitment in response to locally administered inflammatory stimuli. We determined that $GR1^{low}$ MDCs, which are widely considered to perform anti-inflammatory and reparative functions, amplified acute inflammatory reactions via generation of additional pro-inflammatory signals, changing the both profile and magnitude of the tissue response.

Similar vascular and inflammatory responses, including activation of MDCs by transient ischemia/reperfusion, were observed in mouse hind-limbs subjected to chronic ischemia. This response demonstrates relevance of the findings to peripheral arterial disease (PAD) in which patients experience transient exercise induced ischemia known as claudication.

These findings demonstrate that chronically inflamed tissues exhibit an altered profile and mechanisms of acute inflammatory responses and identify tissue resident MDCs as potential therapeutic targets.

Keywords: Acute inflammation, chronic inflammation, ischemia, neutrophil, monocyte, macrophage, angiogenesis.

Introduction

Leukocyte extravasation is a key feature of inflammation and forms a critical component of host defense and tissue repair in response to injury and infection. Extensive investigation of the mechanisms of leukocyte diapedesis both *in vitro* and *in vivo* has unraveled many of the molecular and cellular pathways that mediate this crucial physiological process. [1,2] In addition to critical protective and regenerative roles, excessive or inappropriate inflammation can contribute to the development and progression of acute and chronic disorders as diverse as tumor growth [3], multiple sclerosis [4], arthritis [5], atherosclerosis [6], acute ischemia reperfusion injury [7] and chronic ischemia associated with peripheral arterial disease (PAD) [8] or myocardial infarction [9].

Chronically inflamed tissues exhibit biochemical and environmental abnormalities including hypoxia, extracellular matrix deposition, angiogenesis and by definition the presence of persistent populations of leukocyte infiltrates and/or ongoing recruitment of inflammatory cells. Chronic ischemia of myocardial or skeletal muscle induces recruitment of both neutrophils and monocytes, with the latter differentiating into macrophages. The functions, properties and phenotype of tissue macrophages has been the subject of intense investigations and these cells are broadly accepted to initially exhibit pro-inflammatory, proteolytic and phagocytic functions, followed by roles supporting angiogenesis and tissue repair [9-13].Tissue macrophages regulate the recruitment of immune cells, and thus perturbation of resident populations is likely to influence inflammatory responses within the tissue [9,14,15].

Much of our understanding of the mechanisms and dynamics of leukocyte recruitment stem from *in vivo* investigations carried out in physiologically normal tissues such as the mesentery, dermis or cremaster [14,16,17]. However, the findings of such works cannot be precisely extrapolated to events within the complex environment of chronically inflamed tissues. Direct investigation of mechanisms of leukocyte infiltration in chronic inflammatory conditions have been constrained by the fact that many experimental disease models do not allow easy visual access to the local vasculature, limiting analysis of vessel structures and dynamic inflammatory responses. The aim of this study was to employ a novel model of chronic ischemia, inflammation and angiogenesis in the mouse cremaster muscle as a means of investigating how the cellular, molecular and vascular changes associated with chronic inflammation influence the profile, dynamics and mechanisms of acute inflammatory responses.

We observed that in chronically inflamed post-ischemic (PI) tissues large populations of pro-angiogenic $GR1^{low}$ monocyte derived cells (MDCs) substantially elevate acute neutrophil recruitment through generation of pro-inflammatory mediators in response to acute pharmacological or physiological stimulation. These findings demonstrate that in chronically inflamed tissues both the mechanisms and magnitude of acute inflammatory responses are altered, potentially exacerbating and prolonging tissue inflammation and adversely affecting healing. These findings have important implications for the interpretation of data obtained in healthy tissue models and the treatment of chronic inflammatory disorders.

Methods

An expanded methods section is available in the online data supplement.

Animals

WT C57BL/6 mice were purchased from Charles River Laboratories (UK). Heterozygous mice in which the gene for GFP has been knocked into the CX3CR1 locus (*CX3CR1-GFP*) [18], or the lysozyme-M locus (*LysM-GFP*) [19], resulting in GFP expression in monocytes and neutrophils respectively, were used. All animal studies were approved of and were performed within the UK Home Office Regulations on Animal Experimentation and our Institute's internal Ethical Review Process.

Chronic ischemia

Ischemia and angiogenesis were induced in the mouse cremaster or hind-limb by cauterization and/or excision of the main arteriole and vein perfusing the tissue [20,21].

Acute inflammatory responses

Acute inflammation of the cremaster or hind-limb muscles was induced by intrascrotal (i.s.), intramuscular (i.m.) or topical application of inflammatory stimuli.

Immunofluorescence confocal microscopy

Confocal microscopy was employed to analyze the extent and characteristics of vascularization and the frequency, distribution and morphology of different leukocyte subsets.

Flow cytometry

Flow cytometry was used to analyze the frequency and phenotype of leukocyte populations in blood or enzymatically digested tissues, or to purify specific leukocyte subsets.

Cell transfer model

CX3CR1-GFP^{pos} cells were purified from PI tissues and injected into naïve hind-limb muscles. Acute ischemia reperfusion (IR) of the hind-limb was induced by transient occlusion of the femoral artery and vein. Tissue leukocyte populations were then analyzed by flow cytometry.

Cytokine/Chemokine array

The profile of cytokines and chemokines in muscle homogenates was analyzed using an R&D mouse chemokine/cytokine array according to manufacturer's instructions.

Quantitative PCR

Expression various inflammatory mediators was quantified by qPCR analysis of leukocyte subsets purified from digested cremasters.

Statistical analysis

Results are presented as mean ± s.e.m. Statistical significance was assessed by the Student T-test or by one-way analysis of variance (ANOVA) with the Newman-Keuls multiple comparison test. P values below 0.05 were considered significant.

Results

Prolonged ischemia induces acute and chronic inflammatory responses and angiogenesis in the cremaster

Chronic ischemia was induced in murine cremaster muscles by cauterization of the main vessels supplying the tissue. Intravenous (i.v.) fluorescent microbeads and the hypoxia probe Pimonidazole demonstrated that this procedure induces partial ischemia and hypoxia (Supporting figure S1A and S1B). The presence of neutrophils, monocytes and macrophages in sham and post-ischemic (PI) tissues was investigated using LysM-GFP and CX3CR1-GFP mice [18,19], in conjunction with locally applied fluorescent dextran-TRITC (dex-TRITC, i.s.) to visualize phagocytic cells. Sham tissues exhibit low numbers of neutrophils and a pronounced infiltration at 1 day PI which has resolved by 7 days PI (Figure 1A-i and B). A population of CX3CR1-GFP^{pos} cells is present in naïve and sham tissues, and is significantly elevated at 1 day PI, with this elevation persisting at 7 days PI. Clodronate liposomes were administered intravenously to deplete circulating monocytes before surgery, or 40 hours after surgery. This procedure abolished the elevation in tissue resident CX3CR1-GFP^{pos} cells at day 7, confirming that this population derives from circulating monocytes rather than *in situ* proliferation of the homeostatic tissue resident population (Figure 1A-ii and B). Of note, intravenous liposomes do not pass through the endothelial barrier and do not affect tissue resident cells (not shown). Cells with a high level of dex-TRITC uptake but no CX3CR1-GFP expression (dex-TRITC^{pos}/CX3CR1-GFP^{neg}) were seen throughout the tissues and were particularly prevalent in perivascular locations. The frequency of these cells did not change after induction of ischemia (Figure 1A-iii and B). These cells are morphologically similar to perivascular macrophages described previously [14].

The phenotype of CX3CR1-GFP^{pos} cells was analyzed by flow cytometry (Supporting figure S2 for full gating strategies). GR1 expression (also known as Ly6G/Ly6C) detected with the RB6-8C5 monoclonal antibody, along with CX3CR1-GFP expression in monocytes, was used to identify neutrophils and the two major monocyte subsets (CX3CR1-GFP^{pos}/GR1^{high} or GR1^{low}) (Figure 1C). PI cremasters have elevated numbers of CX3CR1-GFP^{pos} cells, initially CX3CR1-GFP^{pos}/GR1^{high}, but at later times CX3CR1-GFP^{pos}/GR1^{low} are predominant (Figure 1C and D).

In order to determine whether CX3CR1-GFP^{pos}/GR1^{high} cells downregulate GR1 *in situ*, or if there is a later recruitment of CX3CR1-GFP^{pos}/GR1^{low} cells we used a model in which intravenously delivered fluorescent microspheres (MSP) are phagocytosed by, and so selectively label, GR1^{low} monocytes in the circulation, without affecting recruitment to inflamed tissues [22-24]. MSP were administered 48 h prior to surgery. At the time of surgery 1.7%±0.8 GR1^{high} monocytes were MSP^{pos}, and this had fallen to 0.8%±0.4 by 7 days post-surgery. However, GR1^{low} monocytes were 7.9%±0.47 and 3.0%±0.9 MSP^{pos} at these time points, demonstrating that throughout the post-surgical period ~80% of MSP^{pos} monocytes in the circulation were GR1^{low} (Figure 1E and supplementary figure S2-ii). The low frequency of GR1^{high}/MSP^{pos} cells was comparable in the blood and the 7D PI tissues, while the frequency of GR1^{low}/MSP^{pos} cells was significantly lower in the tissue than in the circulation (Figure 1F and supplementary figure S2-ii). These data indicate that CX3CR1-

GFP^{pos}/GR1^{low} cells are not recruited to the tissues in substantial numbers, and *in situ* down-regulation of GR1 is the most likely explanation for the phenotypic differences in CX3CR1-GFP^{pos} cells observed between 1D and 7D PI tissues.

CX3CR1-GFP^{pos}/GR1^{low} cells also increased expression of the macrophage marker F4-80 at 1 day PI and this was further elevated at the 7 days PI. Dex-TRITC^{pos}/CX3CR1-GFP^{neg} cells also expressed F4-80, confirming their macrophage lineage (Figure 1G and supporting figure S2-i and iii). CX3CR1-GFP^{pos}/GR1^{low} cells showed no change in F4-80 expression between 1D and 7D (RFI 3.7±1.5 and 5.0±2.3). CX3CR1-GFP^{pos} cell morphology was altered between 1 and 7 days PI (Supporting figure S1C). Of note, T-cells and NK cells have been reported to express CX3CR1 [18,25], but very few CD3 expressing T-cells or CD335 expressing NK cells were seen in 7D PI tissues, further indicating that this population is of a monocyte/macrophage lineage (supporting figure S2-iv).

The effect of chronic ischemia and inflammation on the cremasteric vasculature was investigated. Sham tissues exhibit an organized vascular tree in which capillary networks efficiently deliver oxygenated blood to the regions between arterioles and venules, while in 7 days PI tissues the capillary networks appeared denser and more disorganized (Figure 1H and Supporting figure S1D for more examples). The majority (> 90%) of vessels in both the sham and 7 days PI tissues had a functional lumen (Supporting figure S1E i-iii).

PI tissues had increased frequency of small diameter capillary like vessels but no changes in the frequency or gross morphology of larger diameter venules or arterioles were detected (Figure 1I). Depletion of circulating monocytes with intravenous Clodronate liposomes prior to surgery and subsequent prevention of recruitment to PI tissues inhibited the angiogenic response to chronic ischemia (Figure 1J).

Collectively these data demonstrate that an influx of neutrophils and GR1^{high} monocytes from the blood occurs rapidly after induction of ischemia and ongoing recruitment of GR1^{high} monocytes may also occur. Following phenotypic changes *in situ* GR1^{low} MDCs with an elongated morphology and elevated F4-80 expression persist in the tissue for a prolonged period of time after the initial injury. This population of MDCs supports proliferation of small diameter capillary-like vessels with a functional lumen. Thus the cremaster exhibits a similar profile of inflammatory and angiogenic responses to that seen in other animal models of chronic ischemia [26,27], but is significantly more amenable to high resolution imaging

Chronically inflamed post-ischemic tissues exhibit elevated acute neutrophil recruitment

A primary aim of this study was to compare the profile and mechanisms of acute inflammatory responses in chronically inflamed ischemic tissues with those of physiologically normal tissues. 7 days PI cremasters of LysM-GFP^{pos} mice were acutely stimulated with IL-1β, TNF, LPS, LTB_4 or saline, and neutrophil infiltration was quantified. PI tissues exhibited a substantially greater neutrophil influx than sham tissues in each stimulus group (Figure 2A and B).

Intravital confocal microscopy was employed to investigate the route of neutrophil extravasation. PECAM-1 labelled Sham or PI tissues were exteriorized, LTB_4 was topically applied and the inflammatory response was imaged. These videos show extravasation from

post capillary venules, but not capillaries, in both sham and PI tissues (Figure 2C and Videos V1 and V2). The vascular location of luminally adherent neutrophils prior to extravasation demonstrated that little adhesion occurs in vessels of < 10μm diameter, and that, as in sham tissues, post-capillary venules are the primary site of adhesion in PI tissues, with significantly higher levels of luminal adhesion than in sham tissues (Figure 2D). Elevated levels of endothelial ICAM-1 expression was seen in stimulated PI tissues, suggesting that endothelial activation contributes to elevated neutrophil adhesion and recruitment (Figure 2E and Supporting Figure S1F).

As neovascularisation did not account for elevated neutrophil influx we investigated the possibility that the increased responses were related to the abundant population of monocyte derived cells (MDCs) present. Sham or PI cremasters of CX3CR1-GFP mice were stimulated with IL-1β or LPS, and fluorescently labelled with antibodies against PECAM-1 and the neutrophil marker S100A9. A correlation between areas with abundant CX3CR1-GFP^{pos} cells and high levels of neutrophil recruitment was seen (Figure 2F and Supporting figure S1G). When tissue resident monocytes and macrophages were depleted by locally applied Clodronate liposomes (i.s.) on day 6, after influx of blood monocytes and vessel proliferation had taken place (Figure 2G), IL-1β or LPS induced neutrophil recruitment was significantly reduced (Figure 2H).

These data demonstrate that in PI tissues morphologically normal post-capillary venules exhibit greater levels of stimulation dependent ICAM-1 expression, luminal neutrophil adhesion and extravasation. An association between MDCs in chronically inflamed tissues and elevated acute inflammatory responses is also seen.

Acute stimulation of GR1^{low} MDCs induces generation of pro-inflammatory signals

We hypothesized that acute stimulation of MDCs produces additional inflammatory mediators, amplifying the original stimulus and elevating neutrophil recruitment. Thus it should be possible to identify known neutrophil stimuli which are: i) elevated by LPS stimulation, ii) higher in stimulated PI tissues than stimulated sham tissues, and iii) are reduced if the tissues are monocyte/macrophage depleted prior to stimulation. The profile of inflammatory mediators generated following acute LPS stimulation of sham, PI and tissues depleted of monocytes/macrophages was analyzed in whole tissue homogenates using a chemokine/cytokine array. Examples of unstimulated and LPS treated PI tissue blots and the intensity values for all mediators are shown (Figure 3A and Supporting table T1 for complete data). Using the criteria described above several candidate mediators were identified; IL-β, CCL3, CCL5, CXCL1, CXCL2 and TNF (Figure 3B).

Expression of the candidate mediators specifically by CX3CR1-GFP^{pos} and dex-TRITC^{pos}/CX3CR1-GFP^{neg} cells purified from control and LPS stimulated PI tissues was analyzed by qPCR (Supporting figure S2-iii for gating). Expression in unstimulated cells was normalized to 1, and values from LPS stimulated cells of each phenotype are expressed as fold difference (Figure 3C) [28]. IL-1β, CCL3 and CCL5 mRNA was upregulated in both cell types, while CXCL2 and TNF were upregulated only in CX3CR1-GFP^{pos} cells. CXCL1 expression was not upregulated by either of the cell populations examined (not shown). The majority (~70%) of CX3CR1-GFP^{pos} cells in 7 days PI tissues exhibit a GR1^{low} phenotype, but specific analysis of the GR1^{low} population demonstrated that the more abundant GR1^{low}

subset upregulate TNF transcription to a similar degree and thus make a significant contribution to the pro-inflammatory response in these tissues (Figure 3D). In order to illustrate the relative inflammatory contribution of CX3CR1-GFP^{pos} and dex-TRITC^{pos}/CX3CR1-GFP^{neg} cells we multiplied the frequency of each cell type (Figure 1B) by the mRNA level of each mediator to generate a measure of the total mRNA generation in LPS stimulated 7 day PI tissues (Figure 1E). The functional role of mediators generated by tissue resident cells was confirmed by inhibition of neutrophil recruitment by co-application of neutralizing antibodies against CCL5, CXCL2 or TNF with the LPS stimulus. Anti-CCL3 had no inhibitory effect (Figure 3F).

Collectively these data show that CX3CR1-GFP^{pos}/GR1^{low} MDCs contribute to the elevated neutrophil recruitment by altering the profile and magnitude of secondary signal generation. These mechanisms have been summarized in Figure 6.

Ischemic hind-limb muscles exhibit a similar vascular and inflammatory profile

The mouse femoral artery occlusion model is widely used in the study of PAD and was employed here to examine the relationship between chronic ischemia, angiogenesis, MDCs and acute neutrophil recruitment in a clinically relevant pathophysiological model. Induction of chronic ischemia induced an increase in the capillary:muscle fiber ratio by 14 days PI (Figure 4A and B). Elevated numbers of CX3CR1-GFP^{pos} cells were seen in PI muscles at 7 and 14 days PI (Figure 4C and F). A similar pattern of GR1^{high} cells at early time points, with GR1^{low} cells predominant at 7 and 14 days PI was also seen (Figure 4D). Importantly LPS (i.m.) stimulated neutrophil recruitment was significantly elevated in 7 and 14 days PI tissues (Figure 4E and F). Thus similar vascular and inflammatory responses are seen in the ischemic hind limb and the cremaster.

GR1^{low} MDCs amplify the response to acute ischemia and reperfusion

PAD patients commonly experience exercise induced pain known as claudication as a result of insufficient perfusion and oxygenation of tissues [29]. To further investigate the clinical relevance of pro-inflammatory MDCs in chronically inflamed tissues we used a model of acute IR of the hind limb intended to mimic transient exercise-induced ischemia, in conjunction with cell transfer of MDCs.

CX3CR1-GFP^{pos} cells were isolated from 7 days PI cremasters and injected into the hind-limb muscle of naive CX3CR1-GFP mice. Acute ischemia (60 minutes) and reperfusion (120 minutes) of the hind-limb was induced, and the leukocyte profile of the tissues analyzed by flow cytometry (Supporting figure S3 for cell transfer method). Cell transfer approximately doubled the total number of CX3CR1-GFP^{pos} cells present in the muscle (Figure 5A). Whilst IR or cell transfer alone did not induce neutrophil recruitment, the combination of cell transfer and IR did induce significant neutrophil recruitment (Figure 5B). The profile of inflammatory mediators in these tissues was analyzed by chemokine/cytokine array (Supporting table T2 for complete data), and several neutrophil stimuli were elevated in response to cell transfer, IR, or cell transfer and IR combined (Figure 5C).

These data indicate that tissue resident MDCs from chronically inflamed tissues have the potential to respond to transient IR by the release and/or *de novo* generation of inflammatory mediators, thus inducing neutrophil recruitment in response to otherwise sub-threshold IR events.

Discussion

The mechanisms governing leukocyte extravasation have been extensively studied. In the majority of *in vivo* models employed acute inflammatory responses are induced in otherwise healthy tissues [14,16,17], however, in many clinical conditions chronic inflammation and attendant tissue abnormalities are a prominent feature, and it is likely that the responses in these tissues will be mediated by very different mechanisms. Here we have investigated the mechanisms governing acute leukocyte extravasation in chronically inflamed and angiogenic tissues as compared to physiologically normal conditions. The findings report on a population of ischemia and angiogenesis associated cells derived from circulating monocytes, which can amplify modest inflammatory insults through generation of additional signals. This renders the tissues highly sensitive to acute inflammatory stimuli, and may affect disease progression and the resolution of chronic inflammation.

In a novel model of permanent occlusion of the main vessels supplying the mouse cremaster, chronic partial ischemia and hypoxia were induced, triggering acute recruitment of neutrophils and $GR1^{high}$ monocytes. A population of $GR1^{low}$ MDCs then persists for at least 7 days. Other populations of tissue resident macrophages were identified by their ability to phagocytose dex-TRITC, F4-80 expression and were morphologically similar to previously identified perivascular macrophages [14]. This abundance of this population was not altered in ischemic tissues.

These biphasic populations of $GR1^{high}$ and $GR1^{low}$ monocytes have previously been reported [24,26,27,30]. Some reports show that $GR1^{high}$ monocytes down regulate GR1 *in situ* [24], while others report a second wave of CX3CL1 dependent recruitment of $GR1^{low}$ monocytes from the blood subsequently occurs [26,27]. In our model depletion of circulating monocytes 40 h after surgery, by which time an initial influx of monocytes to the tissue had occurred, inhibited the increase in tissue resident cells at day 7, suggesting that there is ongoing recruitment for some time after the initial injury. Specific labelling of $GR1^{low}$ monocytes in the circulation indicated that, in this model, recruitment of $GR1^{low}$ monocytes does not play a major role, although we cannot totally exclude the possibility that the subset of $MSP^{pos}/Gr1^{low}$ subset represent a less transmigratory subset than those which are not labelled with the fluorescent microspheres. Taken together these data suggest that ongoing recruitment of $GR1^{high}$ monocytes followed by GR1 downregulation *in situ* is likely to occur.

In line with previous studies PI cremasters exhibited monocyte dependent proliferation of capillary-like vessels [26,27,30]. Many animal models of chronic inflammation and angiogenesis require histological sectioning e.g. arthritic joints or large skeletal muscles, or highly specialized techniques such as those employed in studies of inflammatory responses in plaque associated *vasa vasorum* [31]. Permanent ligation of the cremasteric branch of the rat hypogastric artery has previously been shown to elevate the frequency of 2^{nd} and 3^{rd} order arterioles after 6 weeks, presumably by the process of collateral artery remodeling,

but no information inflammatory responses was reported in this study [32,33]. This model provides a powerful tool for high resolution imaging of inflammatory and angiogenic responses, and it is anticipated that this system will be of value to future studies of the mechanisms associated with inflammatory angiogenesis.

While the link between chronic ischemia and inflammation is well established, to our knowledge, few studies have directly investigated how underlying chronic inflammation affects responses to acute inflammatory insults. In the current work PI tissues consistently showed greater neutrophil recruitment than sham tissues and this excessive recruitment of neutrophils could contribute to tissue damage [34], and support ongoing angiogenesis [35-37]. Several possible explanations for this elevated recruitment were considered, including (i) that the vasculature in PI tissues was structurally compromised allowing more neutrophils to transmigrate, (ii) that the increased vascularity of the PI tissues provided more opportunities for neutrophil egress, or (iii) that the normal mechanisms of leukocyte recruitment are altered and/or highly activated in the PI tissues.

At the level of individual vessels we did not observe any overt abnormalities in terms of PECAM-1 or VE-cadherin expression in either the capillaries or larger diameter post-capillary venules and arterioles. Analysis of the site of luminal adhesion preceding extravasation, in conjunction with *in vivo* imaging, confirmed that pre-existing post-capillary venules rather than the capillary neovasculature support extravasation in PI tissues. These venules exhibited greater stimulus dependent neutrophil adhesion and endothelial ICAM-1 expression than in sham tissues, indicating that enhanced activation of the venular endothelium may be contributing to elevated neutrophil recruitment [1,2] .

Post-capillary venules are the primary site of leukocyte extravasation and so it is perhaps not surprising that in the already highly vascularized skeletal muscle used in this model the capillary neovasculature did not act as a key route for diapedesis. In other pathophysiological scenarios in which the affected tissues are not well vascularized in the non-disease state e.g. arthritic joints or arterial walls, angiogenesis may indeed provide a novel route of leukocyte or plasma protein entry [31,38-42].

Having determined that post-capillary venules are highly activated in PI tissues, we investigated a possible link with the abundant ischemia associated MDCs, the distribution of which correlated with areas of high neutrophil recruitment. Depletion of tissue resident monocytes and macrophages prior to acute stimulation reduced subsequent neutrophil recruitment. Perivascular macrophages identified in the dermis and cremaster have been shown to support neutrophil recruitment through the generation of inflammatory mediators [14], but the numbers and hence likely contribution of this population is not altered between sham and PI tissues in this model, and the CX3CR1-GFP^{pos} cells are more numerous in chronically inflamed tissues thus generating a greater proportion of pro-inflammatory signals. Taken together, our findings suggest that ischemia and angiogenesis associated MDCs support elevated neutrophil recruitment in PI tissues.

GR1^{high} monocytes have been reported to generate inflammatory mediators such as IL-1β, TNF, MCP-1 and perform a phagocytic and proteolytic role in the early response to injury [24,27]. In contrast unstimulated GR1^{low} cells express anti-inflammatory signals such as IL-10 and TGFβ, and are important in tissue repair and revascularization [9,24]. So while GR1^{low} MDCs exhibit anti-inflammatory and reparative characteristics under resting

conditions, we have associated activation of these cells with generation of a range of pro-inflammatory signals and elevated acute neutrophil recruitment. Neutralizing antibodies against some of these signals confirmed their functional role in LPS stimulated neutrophil recruitment. TNF and IL-1β stimulate neutrophil recruitment via direct activation of neutrophils and increased endothelial ICAM-1 expression [43,44] and CXCL2, CCL3 and CCL5 have been shown to recruit both neutrophils and monocytes [45], although we saw no effect of CCL3 blockade. These data demonstrate that in chronically inflamed tissues the response to an acute inflammatory stimulus is not only substantially elevated, but that the profile of secondary signals generated is altered.

To investigate the broader clinical relevance of these findings we employed the murine femoral artery occlusion model, which is widely used in the study of PAD despite some limitations when comparing an acute vessel occlusion animal model with the progressive arterial stenosis seen in patients [20,21]. The muscle of the lower hind limb exhibited a similar profile of capillary angiogenesis, tissue resident CX3CR1-GFP^{pos} cells and enhanced neutrophil recruitment to the cremaster. PAD patients often experience exercise induced pain known as Claudication resulting from transient ischemia/hypoxia of the affected limbs which are unable to meet the increased oxygen demand during exercise [29].

By injecting PI tissue derived MDCs into naive limbs, which were then subjected to transient IR we demonstrated that introduction of CX3CR1-GFP^{pos} cells from chronically inflamed tissues rendered the tissues sensitive to an IR stimulus that on its own was insufficient to induce a neutrophil migration response, possibly as a result of abundant collateral arteries present in C57BL6 mice [46]. This increased sensitivity to an apparently mild IR stimulus suggests that the transferred cells are phenotypically different and more responsive to IR than the homeostatic CX3CR1-GFP^{pos} tissue resident population.

The present study demonstrates that abundant GR1^{low} MDCs in chronically inflamed tissues, which are phenotypically distinct from homeostatic tissue macrophage populations, significantly alter the profile and magnitude of responses to a wide range of pharmacological or physiological stimuli, rendering the tissue highly sensitive to acute inflammatory stimuli. These changes may in turn affect the severity, progression and duration of chronic inflammation, and these observations highlight the risks of extrapolating data obtained in studies of healthy tissues to pathological scenarios. Furthermore, our results suggest the real possibility of uncoupling beneficial effects of macrophages in terms of tissue revascularization and reperfusion [10,47,48] from potentially harmful pro-inflammatory effects. Collectively the present study highlights the need for a better understanding of the profile, dynamics and mechanisms of inflammatory responses within chronically inflamed tissues and strongly suggests that such studies may open opportunities for therapeutic interventions that could break the cycle of inflammation without exerting significant adverse effects on the host's normal immune or tissue repair responses.

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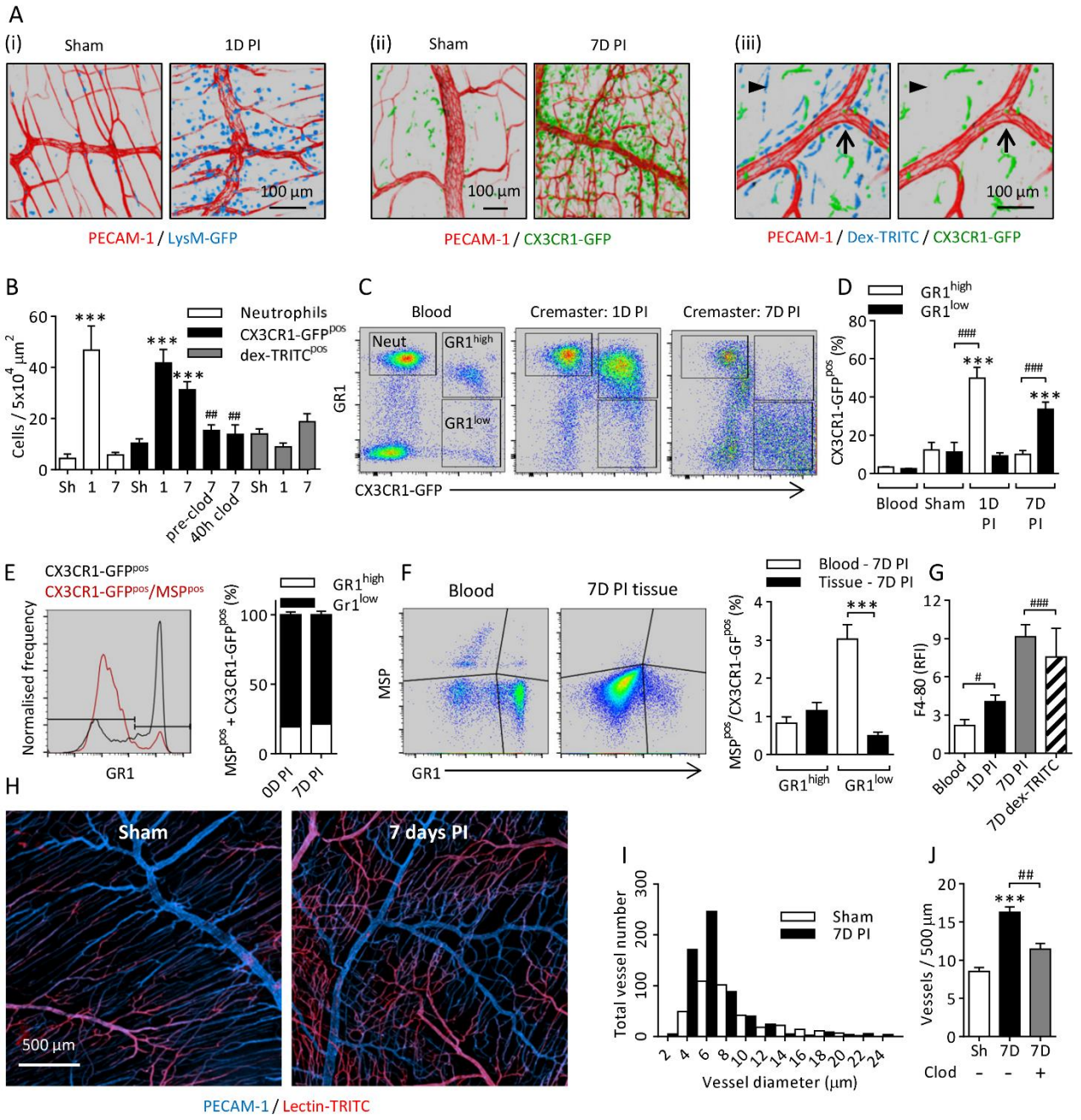

Figure 1. Prolonged ischemia induces acute and chronic inflammatory responses and angiogenesis Chronic ischemia was induced in cremaster muscles by cauterizing the primary vessels perfusing the tissue. (A) Leukocyte populations were analyzed in sham tissues and at 1 or 7 days PI using (i) LysM-GFP and (ii) CX3CR1-GFP mice to visualize neutrophils and monocytes respectively. (iii) Topical application (i.s.) of fluorescent dextran-TRITC (dex-TRITC) was used to visualize phagocytic cells in CX3CR-1GFP^{pos} mice. Image shows dex-TRITC^{pos}/CX3CR1-GFP^{neg} cells in perivascular (arrow) and non-perivascular (arrowhead) locations in naïve tissues. Vasculature was fluorescently labelled with anti-PECAM-1 antibody. (B) Quantification of neutrophils, CX3CR1-GFP^{pos}, and CX3CR1-GFP^{neg}/dex-TRITC^{pos} cells in sham (sh), 1 or 7 days PI tissues. In some cases circulating monocytes were depleted with Clodronate liposomes (i.v.) pre- or 40 hours post-surgery. (C) Blood and cremasters were collected from naïve and 1 or 7 days PI mice. Cremasters

were enzymatically digested and cells fluorescently labelled with antibodies against CD45 and GR1 (clone RB6-8C5) and DAPI to identify live leukocytes (Supporting figure S2-i). Examples of leukocyte populations from blood and digested cremasteric tissues are shown. (D) Frequency of CX3CR1-GFP^{pos}/GR1^{high} or GR1^{low} cells as a proportion of total CD45^{pos} leukocytes. (E) Analysis of blood post i.v. injection of fluorescent MSP. Histogram showing frequency of GR1^{high} and GR1^{low} monocytes in the circulation (black line) and the normalized frequency of MSP labelled monocytes. Graph GR1^{high} or GR1^{low} expression amongst all CX3CR1-GFP^{pos}/MSP^{pos} cells. (F) Example scatters and quantification of MSP labelling of CX3CR1-GFP^{pos} cells in blood and 7D PI tissue digest after. Graph shows frequency of MSP^{pos} GR1^{high} or GR1^{low} cells in blood and tissues 7D PI. (Supporting figure S2-ii for full gating strategy) (G) F4-80 expression on CX3CR1-GFP^{pos}/GR1^{low} and dex-TRITC^{pos} cells in the blood and cremaster digests at 1 or 7 days PI quantified relative to an isotype control (Supporting figure S2-I and iii). (H) Examples of sham or 7 days PI tissues labelled with topical anti-PECAM-1 antibody to visualize venules and capillaries, with less intense labelling of arterioles, and lectin-TRITC (i.v.) to visualize arterial vessels and capillaries, with less intense labelling of venules. (I) Frequency histogram of vessel diameters in 12 images of sham and PI tissues. (J) Circulating monocytes were depleted with Clodronate liposomes (i.v.) prior to surgery. The frequency of vessels was quantified in sham, PI and monocyte depleted 7 days PI tissues. N = 5-15 animals per group. Data is presented as mean ± SEM and statistically significant (T-test) differences between sham and PI groups or blood and tissues are indicated by asterisks: *P<0.05, **P<0.01, and ***P<0.001. Differences between control and monocyte depleted groups, cell subsets or time points are indicated by hash symbols: #P<0.05, ##P<0.01, and ###P<0.001.

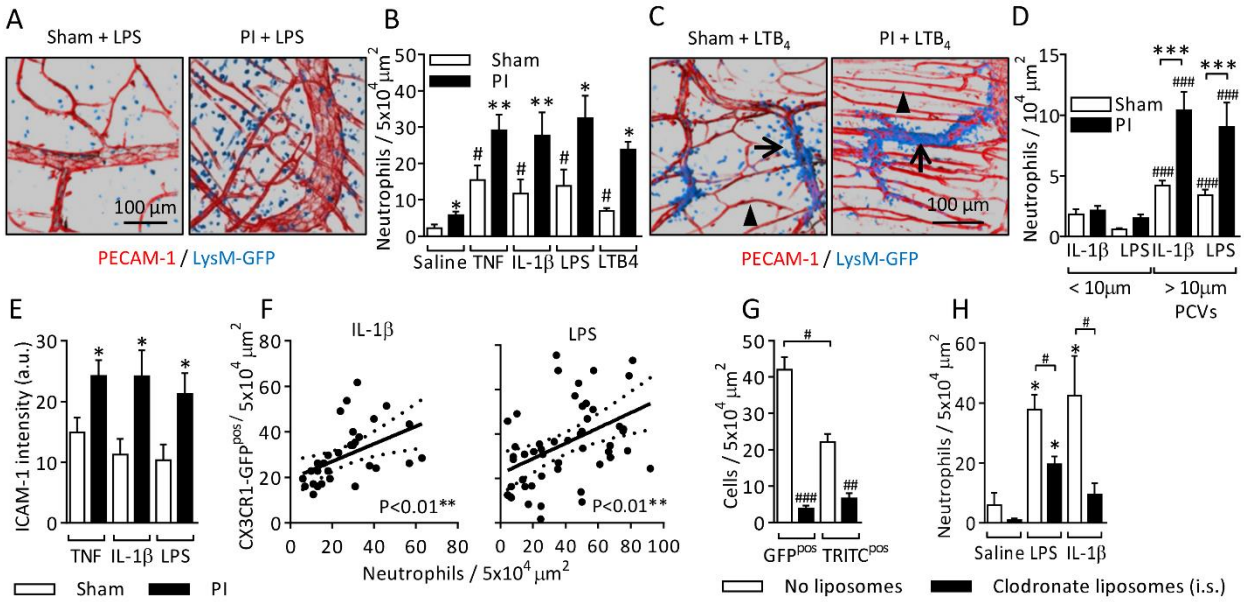

Figure 2. Post ischemic tissues exhibit elevated acute neutrophil recruitment. Chronic ischemia was induced in the cremasters of LysM-GFP mice which have GFP positive neutrophils. After 7 days the tissues were fluorescently labelled with antibodies against PECAM-1 (i.s.) and stimulated with TNF (300 ng), IL-1β (50 ng), LPS (300 ng) or LTB_4 (100 nM) (i.s. 4 hours) before imaging. (A) Examples of LPS stimulated sham and PI tissues. (B) Quantification of neutrophil extravasation. (C) Still images from supporting videos V1 and V2 showing the neutrophil extravasation in in sham and PI tissues after stimulation with topical LTB_4 (100 nM). Examples of post-capillary venules (arrows) and capillaries (arrowheads) are indicated. (D) Quantification of luminal neutrophil adhesion in small diameter capillary-like vessels (< 10 µm) and larger post-capillary venules (PCVs > 10um) 2 hours after stimulation with TNF, IL-1β or LPS (i.s.). Data is normalized to luminal surface area. (E) Endothelial ICAM-1 labelling intensity in post capillary venules in sham or 7 days PI tissues. (Supporting figure S1G for images). (F) At 7 days PI CX3CR1-GFP tissues were acutely stimulated with IL-1β (50 ng) or LPS (300 ng) (i.s. 4 hours), collected and fluorescently labelled with antibodies against PECAM-1 and the neutrophil marker S100A9. Correlation of spatial distribution CX3CR1-GFP^{pos} cells and neutrophils in $5x10^4$ μm^2 regions of IL-1β (left panel) or LPS (right panel) stimulated tissues (Supporting figure S1H for example image). (G) Depletion of CX3CR1-GFP^{pos} and CX3CR1-GFP^{neg}/dex-$TRITC^{pos}$ cells by local application (i.s.) of Clodronate liposomes on days 6-8 PI. (H) LPS or IL-1β induced neutrophil recruitment in control or monocyte/macrophage depleted tissues. N = 6-12 animals per group. Data is presented as mean ± SEM and statistically significant (T-test) differences between sham and PI groups or correlations are indicated by asterisks: *P<0.05, **P<0.01, and ***P<0.001. Differences between saline and stimulated, vessel size groups, or control and Clodronate depleted groups are indicated by hash symbols: #P<0.05, ##P<0.01 and ###P<0.001.

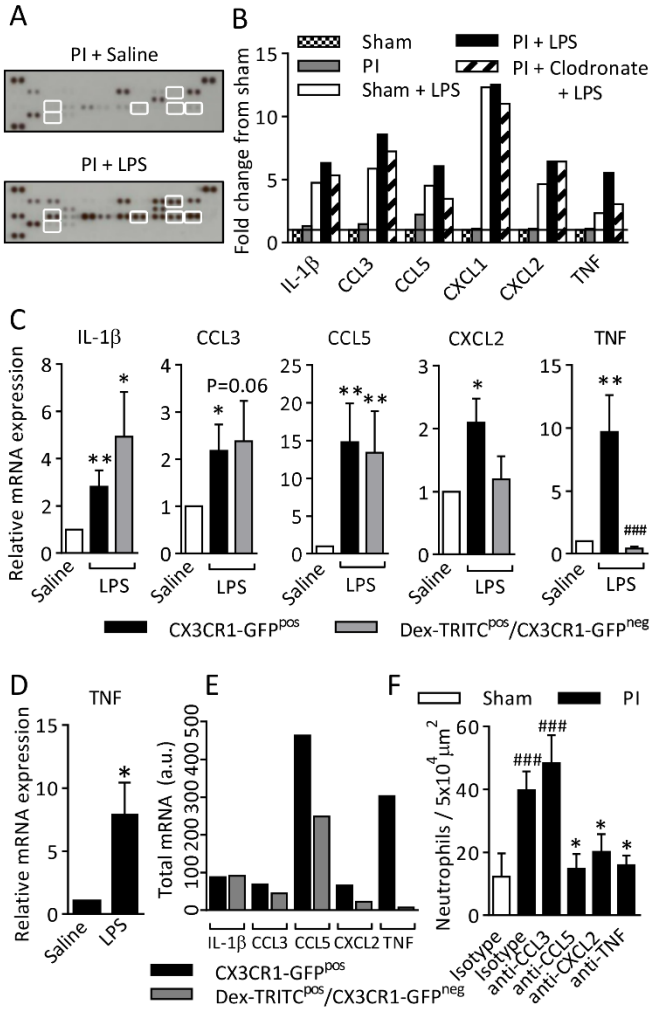

Figure 3. Acute LPS stimulation of GR1^{low} MDCs induces generation of pro-inflammatory signals. Chronic ischemia was induced in the cremasters of WT mice. Some tissues were monocyte/macrophage depleted with Clodronate liposomes (i.s.) and circulating neutrophils were depleted with anti-Ly6G antibody (clone 1A8). On day 7 cremasters were acutely stimulated with LPS (300ng i.s., 4h) or saline. Tissues were collected, homogenized and analyzed using a chemokine/cytokine array immunoblot. (A) Examples of an unstimulated and LPS stimulated blot. Highlighted spots are the mediators shown in B. (B) Intensity data was analyzed for mediators which conformed to certain parameters: i) Known to recruit neutrophils ii) Elevated by LPS stimulation iii) Higher levels in PI compared to sham tissues iv) reduced in monocyte/macrophage depleted tissues. Data is normalized to the fold difference from unstimulated sham tissues. N = 2 animals pooled per blot, 2 blots (n=4 animals) per treatment group. (Supporting table T1 for complete data set) (C) 7 days PI cremasters from CX3CR1-GFP mice were stimulated with LPS or saline and phagocytic cells were labelled with TRITC-dextran (i.s.) *in vivo*. Tissues were enzymatically digested and labelled with anti-CD45 and DAPI to identify live leukocytes. CD45^{pos}/DAPI^{neg}/CX3CR1-GFP^{pos} cells (which have a variable level of dex-TRITC intensity), and DAPI^{neg}/CD45^{pos}/dex-TRITC^{pos}/CX3CR1-GFP^{neg} cells were purified by FACS (see supporting figure S2 for full gating strategy). Purified cell populations were analyzed by qPCR for IL-1β, CCL3, CCL5, CXCL2 and TNF mRNA. Data was normalized to GAPDH and is shown as the fold difference of LPS stimulated cells (black and grey bars) compared to unstimulated samples of each cell type (white bars). (D) The CX3CR1-GFP^{pos}/GR1^{low}

population was selectively purified from saline and LPS stimulated tissues and TNF mRNA quantified. (E) The frequency of dex-TRITC^{pos}/CX3CR1-GFP^{neg} and CX3CR1-GFP^{pos} cells (from Figure 1B) was multiplied by the expression level of each mediator to visualise the total inflammatory contribution of each cell population. F) Sham or 7 days PI tissues were treated with blocking antibodies against CCL3, CCL5, CXCL2 or TNF (10-20 µg) co-administered with LPS (300 ng, 4h i.s.). Tissues were collected and labelled with fluorescent antibodies against PECAM-1 and S100A9 and neutrophil extravasation was quantified. N = 3-11 animals per group. Data is presented as mean ± SEM and statistically significant (T-test) differences between saline and LPS stimulated groups, or isotype control and blocking antibody groups, are indicated by asterisks: *P<0.05, **P<0.01, and ***P<0.001. Differences between sham and PI tissues or between CX3CR1-GFP^{pos} cells and dex-TRITC^{pos}/CX3CR1-GFP^{neg} are indicated by hash symbols: #P<0.05.

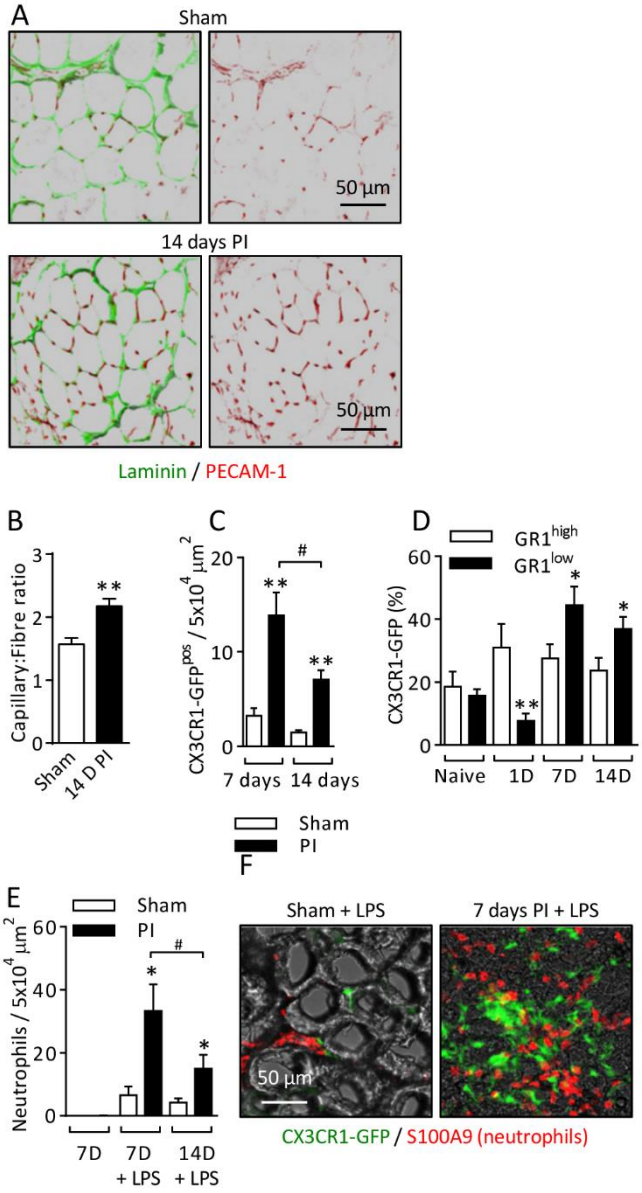

Figure 4. Ischemic hind-limb muscles exhibit a similar vascular and inflammatory profile. Chronic ischemia was induced in the hind limbs of WT, CX3CR1-GFP or LysM-GFP mice by occlusion of the femoral artery and vein. The anterior tibalis muscles from sham or PI limbs were collected at 7 or 14 days after surgery and either examined as whole mount muscles or fixed and sectioned for labelling with fluorescent antibodies against Laminin, PECAM-1 or S100A9 (A) Example images of capillaries (PECAM-1) and muscle fibers (laminin) in sham and 14 days PI tissue sections. (B) Quantification of the capillary:fiber ratio in sham and 14 days PI. (C) The frequency of CX3CR1-GFP^{pos} cells was quantified in whole mount tissues at 7 and 14 days PI. (D) Muscles were collected from naïve and 1, 7 or 14 days PI mice, enzymatically digested and fluorescently labelled with antibodies against CD45 and GR1 (clone RB6-8C5) and DAPI to identify live leukocytes (supporting figure S2 for full gating strategy). The frequency of CX3CR1-GFP^{pos}/GR1^{high} or GR1^{low} cells as a proportion of total CD45^{pos} leukocytes is shown. (E) In experiments using LysM-GFP mice PI or sham anterior tibalis muscles were injected with LPS (100 ng, 4h) at 7 or 14 days PI. Neutrophil recruitment was quantified in whole mount tissues. (F) Example images of

CX3CR1-GFP^{pos} cells and S100A9^{pos} neutrophils in LPS stimulated sham and 7 days PI tissue sections. N = 6-8 animals per group. Data is presented as mean ± SEM and statistically significant (T-test) differences between sham and PI groups are indicated by asterisks: *P<0.05, **P<0.01, and ***P<0.001. Differences between time points are indicated by hash symbols: #P<0.05.

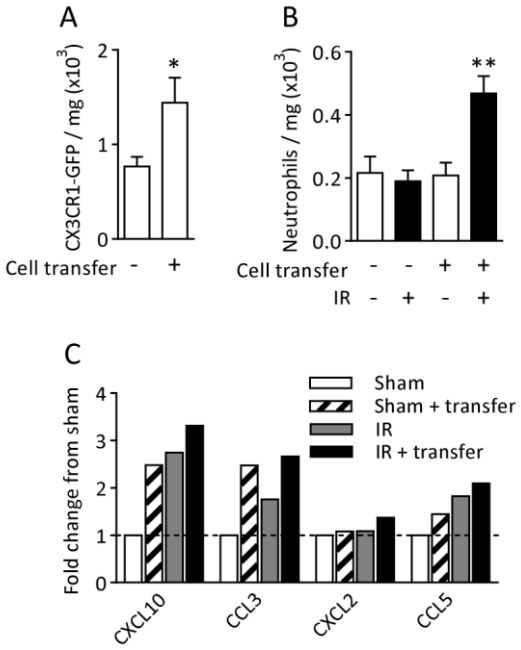

Figure 5. GR1^{low} MDCs amplify the response to acute ischemia and reperfusion. (A) CX3CR1-GFP^{pos} cells were quantified in naïve tissues and those which received cell transfer. (B) Quantification of neutrophils in naïve, cell transfer and cell transfer with IR tissues. (C) Quantification of chemokines and cytokines in whole tissue homogenates (supporting table T2 for complete data set). Values are shown as the fold difference from sham. N = 3-6 animals per group in flow cytometry and pooled tissues from 6 animals in cytokine/chemokine array. Data is presented as mean ± SEM and statistically significant (T-test) differences between groups are indicated by asterisks: *P<0.05, **P<0.01.

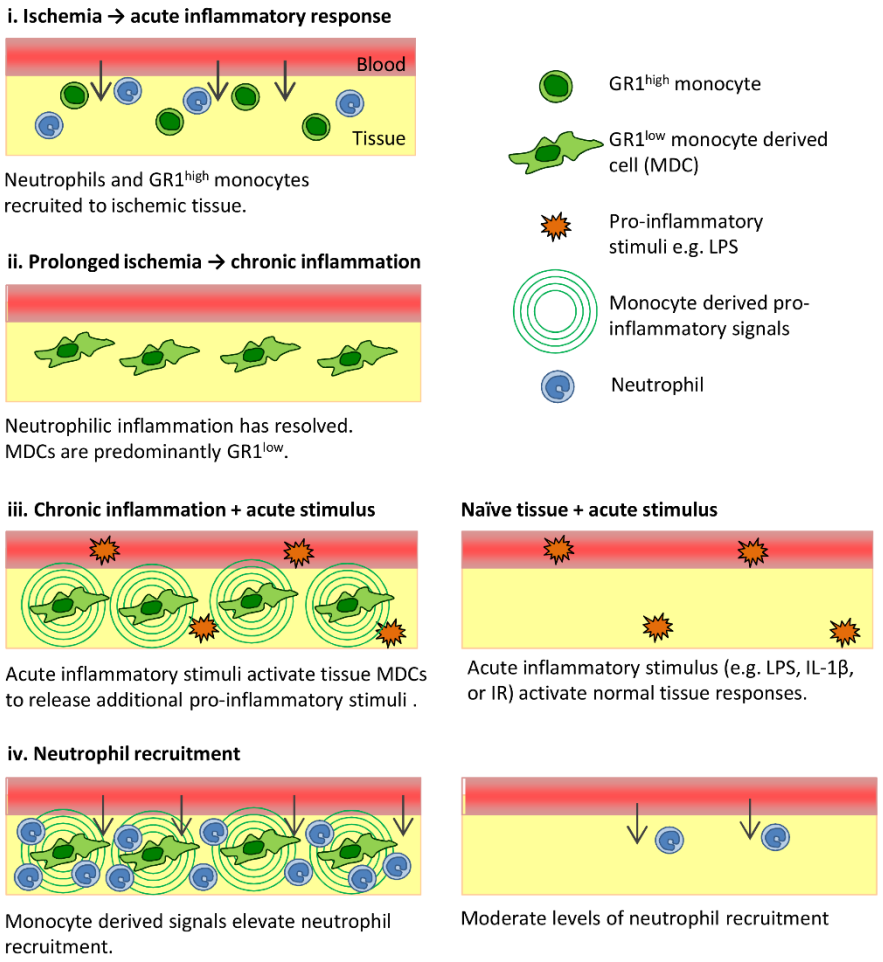

Figure 6. Schematic illustration of the interaction of acute and chronic inflammatory responses. (i) Induction of chronic partial ischemia induces a rapid recruitment of neutrophils and GR1^{high} monocytes from the circulation. (ii) 7 days after the induction of ischemia the neutrophilic inflammation has resolved, but an elevated population of GR1^{low} MDCs persist in the tissue. (iii) These GR1^{low} MDCs are highly sensitive to a range of pharmacological or physiological stimuli (e.g. LPS or ischemia reperfusion) and respond by generating a range additional of pro-inflammatory stimuli. (iv) These secondary signals act on the endothelium to upregulate ICAM-1 expression, which supports luminal neutrophil adhesion, and/or directly activate neutrophil chemotaxis. Naive tissues exposed to the same exogenous stimuli lack this additional source of secondary mediators, resulting in lower overall tissue activation.