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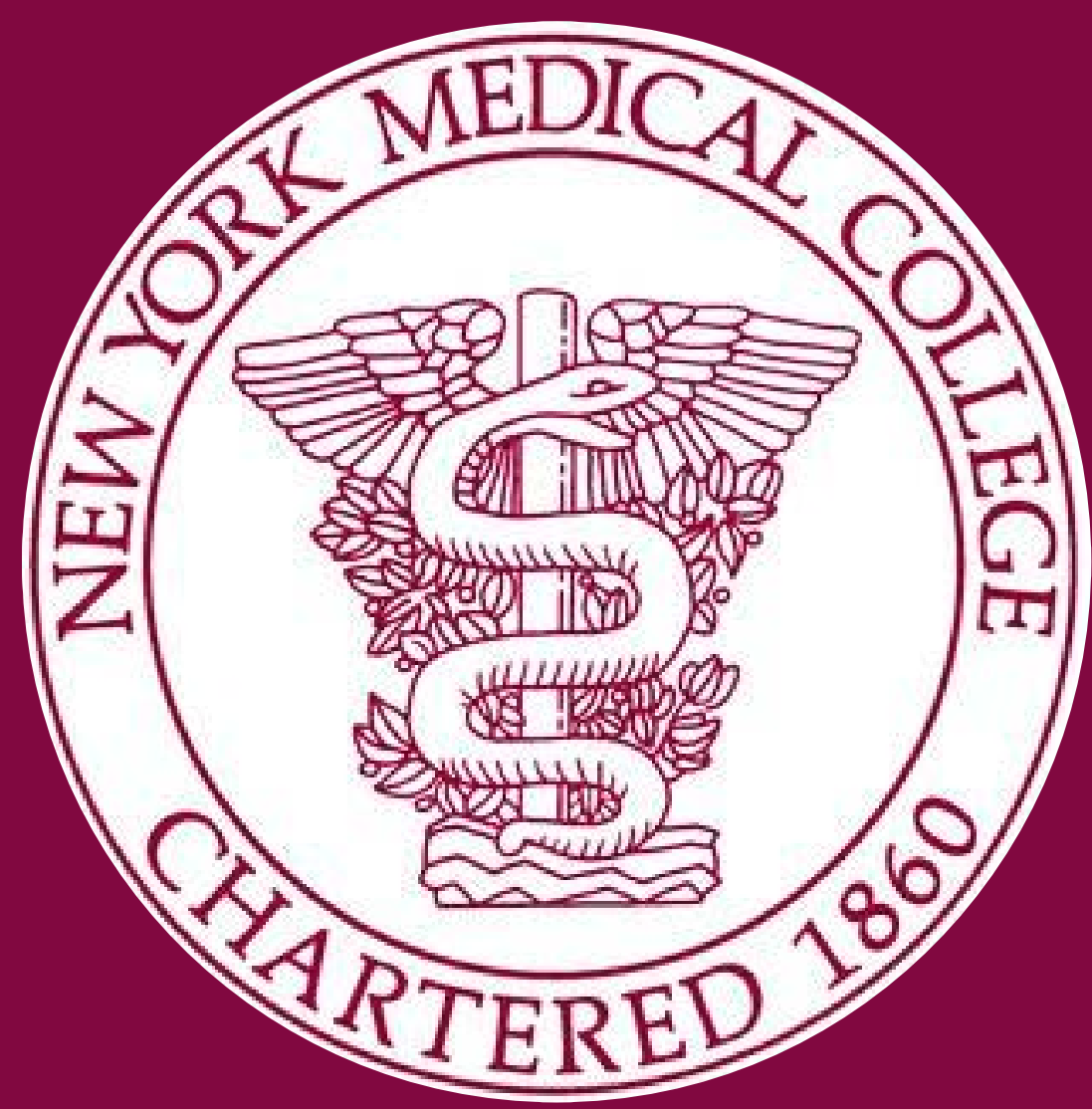
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Role of Inflammation in 20-HETE Regulation of Ischemia-Induced Angiogenesis

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Abstract

Objective: 20-Hydroxyeicosatetraenoic acid (20-HETE), an important bioactive lipid metabolite, has recently been identified to be a novel contributor of angiogenesis secondary to ischemia. Moreover, an inflammatory response is required for the initiation of ischemic angiogenesis, in response to ischemic tissue injury. The goal of this study is to investigate the role of inflammation in 20-HETE regulation of ischemia-induced angiogenesis.

Methods: We first established a mouse hind limb ischemia model for immunocompetent Balb/C mice and immunodeficient NOD-SCID mice by femoral artery ligation. Groups of Balb/C and NOD-SCID mice were administered a 20-HETE synthesis inhibitor, DDMS, or saline as a solvent control. Laser Doppler perfusion imaging (LDPI) was used to visualize and quantify blood perfusion on days 0, 1, 3, 7, 14, and 21 post ligation, confirmed by microvessel density analysis. LC/MS/MS analysis was performed on day 3 post ligation on ischemic and non-ischemic control gracilis muscles to measure 20-HETE levels. Additionally, an antibody to lymphocyte antigen 6 complex (Ly6G/C) was administered to neutralize the infiltration of neutrophils, macrophages, and monocytes. 20-HETE levels were again measured on day 3 post ligation in these mice.

Results: Quantification of the compensatory blood perfusion recovery post ischemia by LDPI showed that immunocompetent Balb/C control mice demonstrated a normal course of the compensatory angiogenic response while NOD-SCID immunodeficient mice showed a significantly decreased response. Additionally, DDMS was shown to inhibit the compensatory response in Balb/C mice, while no inhibitory effect was observed in immunodeficient NOD-SCID mice. This observation is confirmed by a marked decrease in microvessel density in SCID mice (1.5±0.2) post ischemia compared to immunocompetent Balb/C mice (2.65±0.32). As expected, ischemia markedly increased 20-HETE levels in the ischemic gracilis muscle of Balb/C mice by 6-fold (6±2 in non-ischemic vs 27±5 pg/mg in ischemic), while levels in NOD-SCID mice showed no change between the ischemic and non-ischemic control. Lastly, Balb/C mice that were treated with Ly6G/C neutralizing antibody exhibited significantly decreased 20-HETE levels in their ischemic gracilis muscle compared to the non-ischemic control.

Conclusion: Inflammation may be an essential contributor in 20-HETE regulation of the ischemia-induced angiogenic response.

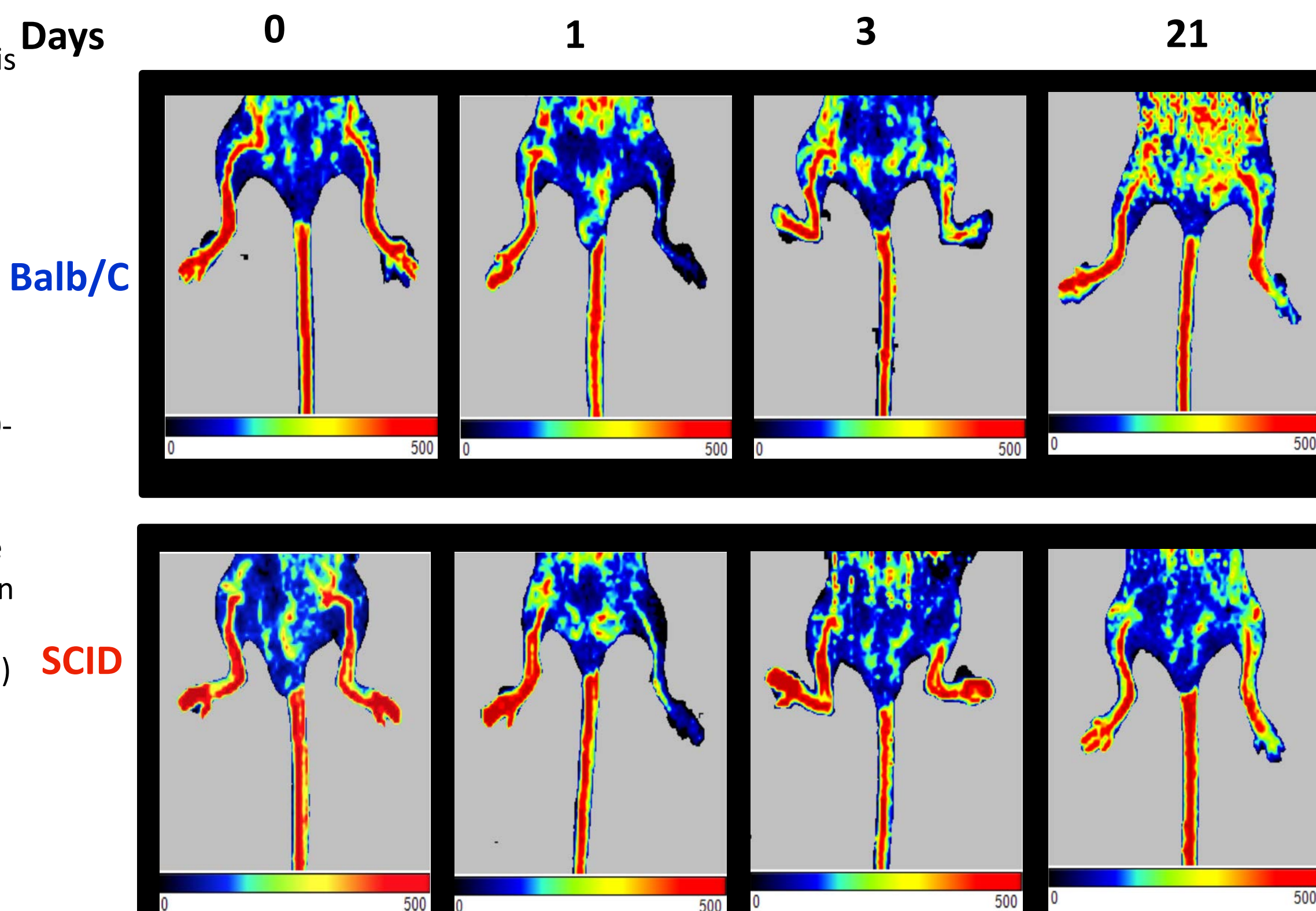


Figure 1A: Representative Laser Doppler Perfusion Imaging Scans for Balb/C and SCID mice are shown from days 0, 1, 3, and 21 post ligation. Blood perfusion was used as one of the indexes of compensatory angiogenesis. **Results: Immunodeficient NOD-SCID mice display a decreased compensatory blood perfusion recovery compared to immunocompetent Balb/C mice.**

Results

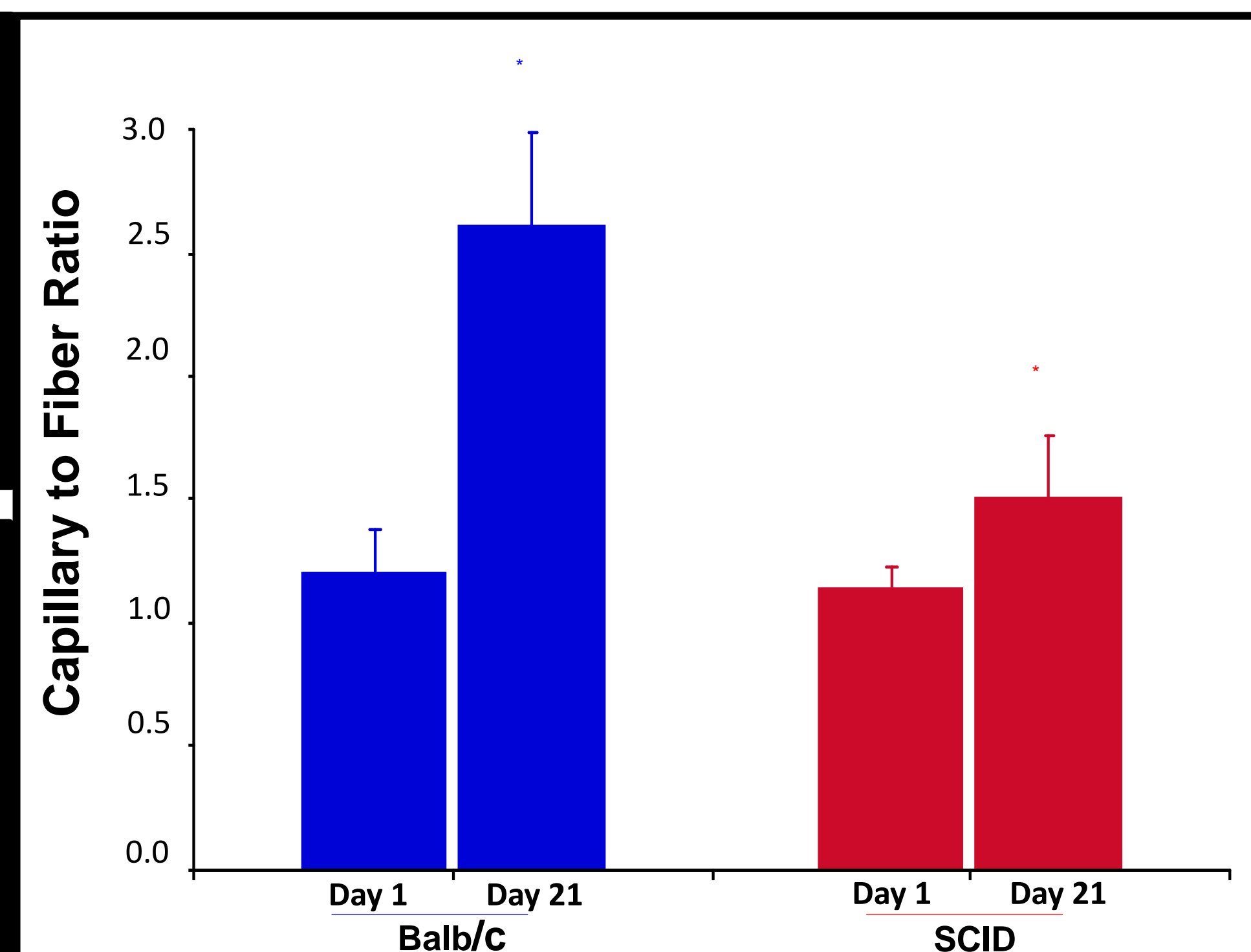


Figure 1C: Microvessel density (MVD) analysis of Balb/C and SCID non-ischemic and ischemic limbs. Gracilis muscles were excised from ischemic and nonischemic limbs at the end of the 21 day period, and were sectioned and stained with CD31 and tomato lectin. The number of muscle fibers and capillaries were counted in each field. **Results: Balb/C mice have a significantly higher MVD than SCID mice, consistent with a decreased angiogenic response in SCID mice.**

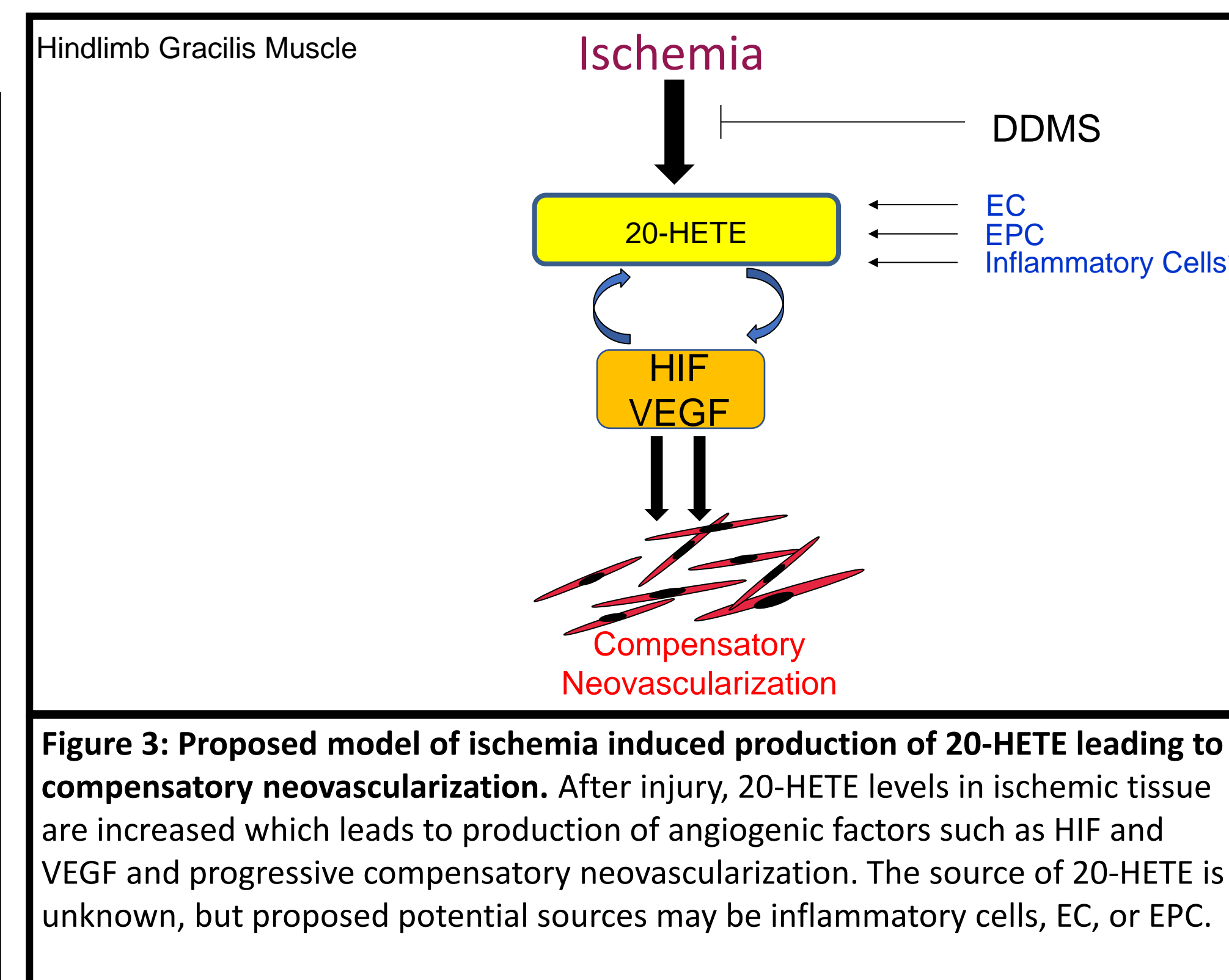


Figure 3: Proposed model of ischemia induced production of 20-HETE leading to compensatory neovascularization. After injury, 20-HETE levels in ischemic tissue are increased which leads to production of angiogenic factors such as HIF and VEGF and progressive compensatory neovascularization. The source of 20-HETE is unknown, but proposed potential sources may be inflammatory cells, EC, or EPC.

Introduction

20-HETE is an arachidonic acid derived eicosanoid, mainly synthesized by the enzyme cytochrome P450 (CYP450) 4A and 4F. Previous studies by our group (Chen et al, 2014; Guo et al, 2011; Guo et al, 2007) have demonstrated that 20-HETE regulates both endothelial cell (EC) and endothelial progenitor cell (EPC) functions that are associated with angiogenesis.

Our recent publication (Chen et al, 2016) further demonstrated that **20-HETE is a novel contributor of ischemia-induced angiogenesis in vivo** based on the following two important findings: 1) Pharmacological 20-HETE interference significantly inhibited the compensatory angiogenesis secondary to ischemia; and 2) ischemia markedly stimulated the production of 20-HETE in the hindlimb gracilis muscle where angiogenesis is taken place. The precise cellular origin of the increased 20-HETE and the molecular mechanism underlying 20-HETE regulation in ischemia-induced angiogenesis remain unknown.

After ischemic injury, inflammatory cytokines are quickly produced and immune cells are recruited to the site of injury. The first inflammatory cells to arrive at the site of injury are neutrophils, which then recruit macrophages through the release of additional cytokines (Parham, 2014). Thus, inflammatory cells play an important role in mediating ischemic angiogenesis.

In the current study, we aim to determine the role of inflammation in 20-HETE regulation of ischemia-induced angiogenesis. We hypothesized that the **inflammatory response may contribute to increased 20-HETE that regulates ischemia-induced angiogenesis in vivo.**

Methods

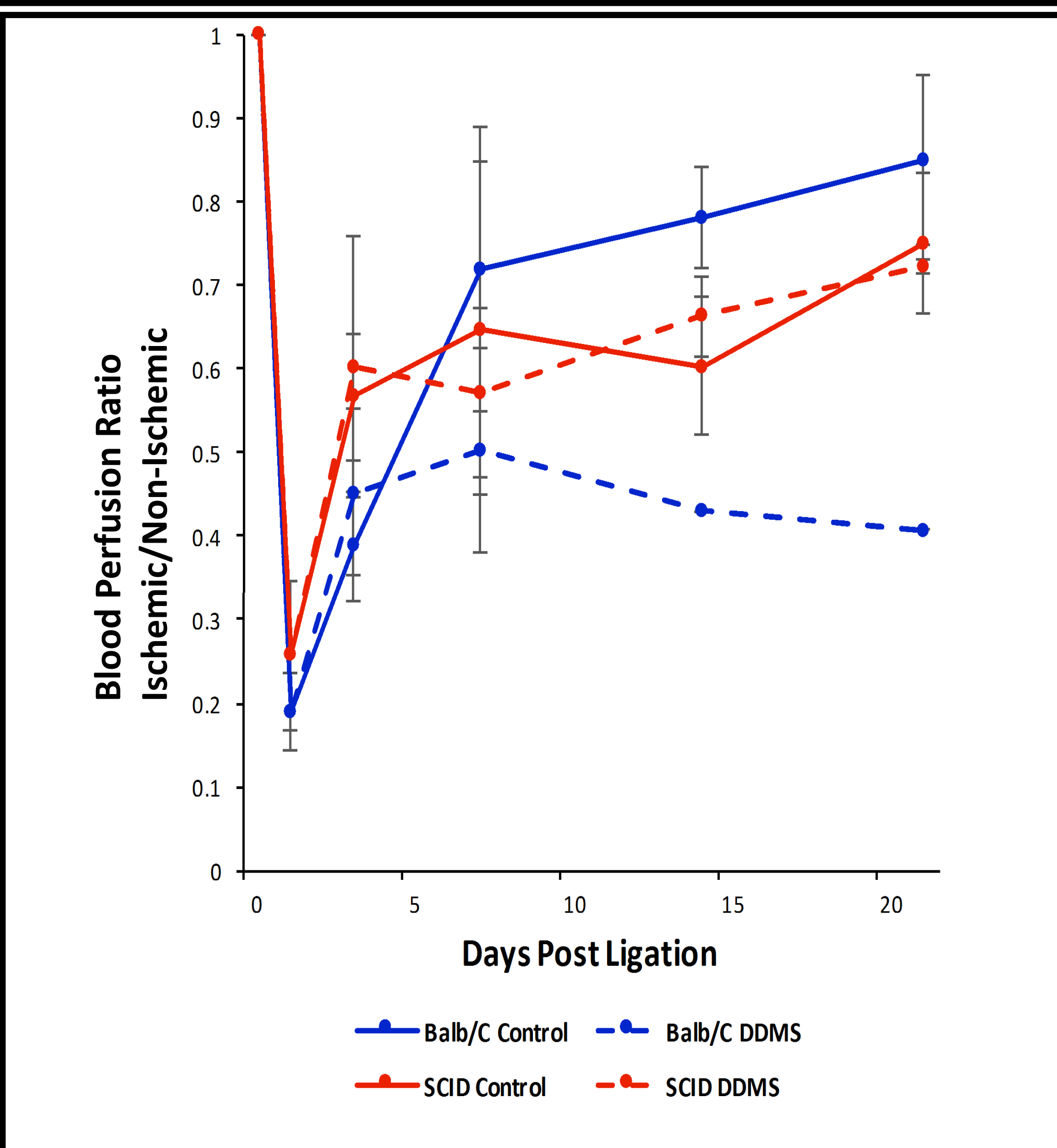
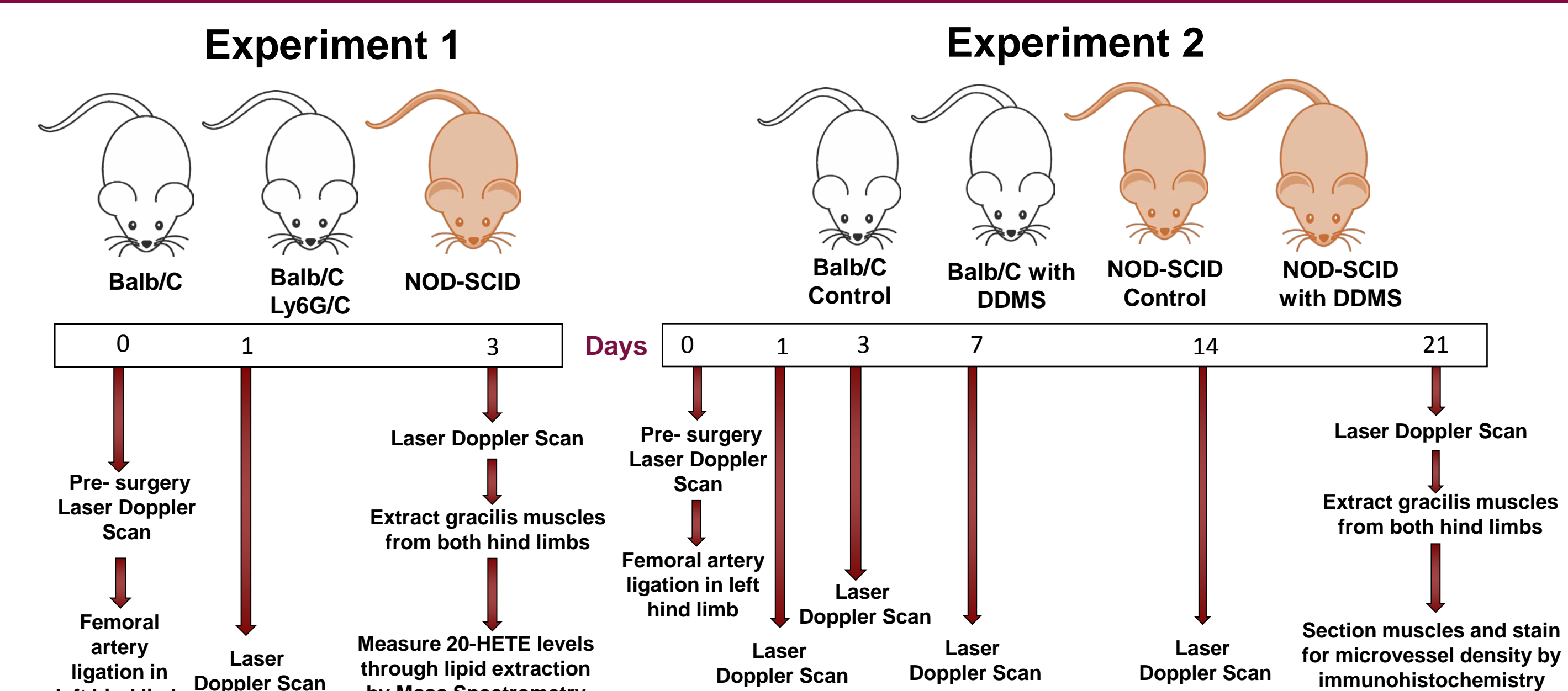


Figure 1B: Quantitation of recovery of blood perfusion secondary to ischemia. Blood perfusion for days 0, 1, 3, 7, 14, and 21 were quantified and plotted for Balb/C control, Balb/C DDMS, SCID control, and SCID DDMS groups. **Results: The Balb/C controls underwent normal courses of compensatory angiogenesis as we have previously published, whereas SCID controls showed a significantly decreased compensatory response. As previously demonstrated, DDMS decreased compensatory blood perfusion in Balb/C mice. Interestingly, we see no effect of DDMS on blood perfusion post-ischemia in NOD-SCID mice. On the third day after ligation, SCID mice seem to have an increase in blood flow, but the delta of the SCID curve is skewed by this increase. Without day 3 data, there is no change in blood flow.**

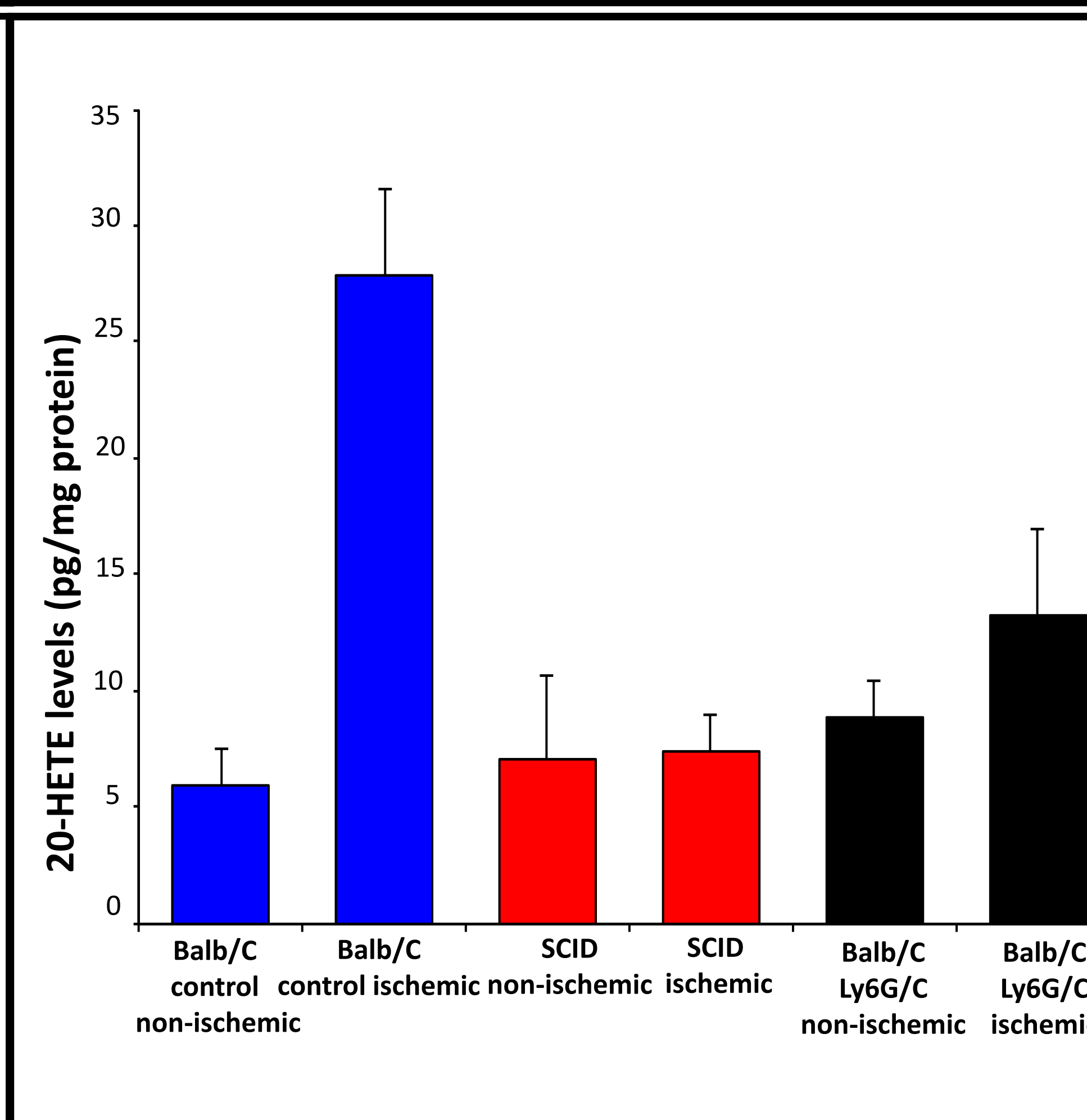


Figure 2: Comparison of 20-HETE levels in hind limb gracilis muscles of Balb/C, SCID, and Ly6G/C treated Balb/C mice before and after ischemia. Femoral artery ligations were performed in Balb/c, SCID, and Balb/c mice treated with 0.5mg of Ly6G/C antibody (i.p.). Ly6G/C targets and depletes neutrophils, macrophages, and monocytes. On day 3 post-ligation, gracilis muscles were excised from both the ischemic and non-ischemic hind limbs of Balb/C and SCID mice and were quantified for 20-HETE levels by LC/MS/MS analysis. 20-HETE levels were normalized to total protein. **Results: Ischemic muscles in Balb/C mice have a 6-fold increase in 20-HETE compared to contralateral non-ischemic muscles, while SCID mice have no difference in 20-HETE levels between ischemic and non-ischemic muscles. Balb/C treated with Ly6G/C antibody showed decreased 20-HETE production post ligation in the ischemic limb. 20-HETE levels in Ly6G/C treated Balb/C mice were closer to basal levels of 20-HETE production.**

Summary

1. Immunodeficient NOD-SCID mice show a decreased compensatory blood perfusion recovery as compared to immunocompetent Balb/C mice, indicating that the presence of immunity may lead to a more significant and prolonged compensatory angiogenic response.
 2. Furthermore, 20-HETE synthesis inhibitor, DDMS decreased compensatory response in Balb/C mice, but had no such effect on NOD-SCID mice.
 3. Consistently, Balb/C mice show an increased MVD on day 21 in the ischemic limb gracilis muscle compared to immunodeficient NOD-SCID mice, supporting a decrease angiogenic response in this immunodeficient model.
- Taken together, these data strongly support a potential role of immunity in 20-HETE regulated ischemic angiogenesis.**
4. NOD-SCID mice show virtually no difference in 20-HETE production post ischemia when compared to Balb/c mice.
 5. Targeted depletion of neutrophils, macrophages, and monocytes using Ly6G/C antibody in immunocompetent Balb/C mice results in a marked lower 20-HETE levels post ischemia.
- These findings implicate that inflammatory cells may contribute to increased 20-HETE production during compensatory angiogenesis.**

Conclusion

Inflammation may be an essential contributor in 20-HETE regulation of ischemia-induced angiogenic response.

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