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Ager Deletion Enhances Ischemic Muscle Inflammation, Angiogenesis, and Blood Flow Recovery in Diabetic Mice

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Abstract

Objective—Diabetic subjects are at higher risk of ischemic peripheral vascular disease (PVD). We tested the hypothesis that advanced glycation end products (AGEs) and their receptor (RAGE) block neoangiogenesis and blood flow recovery after hind limb ischemia induced by femoral artery ligation (FAL) through modulation of immune/inflammatory mechanisms.

Approach and Results—Wild type (WT) mice rendered diabetic with streptozotocin and subjected to unilateral FAL displayed increased accumulation and expression of AGEs and RAGE in ischemic muscle. In diabetic WT mice, FAL attenuated neoangiogenesis and impaired blood flow recovery, in parallel with reduced macrophage content in ischemic muscle and suppression of early inflammatory gene expression, including chemokine (C-C motif) ligand 2 (*Ccl2*) and early growth response gene 1 (*Egr1*) versus non-diabetic mice. Deletion of *Ager* or transgenic expression of *Glo1* (reduces AGEs) restored adaptive inflammation, neoangiogenesis and blood flow recovery in diabetic mice. In diabetes, deletion of *Ager* increased circulating Ly6C^{hi} monocytes and augmented macrophage infiltration into ischemic muscle tissue after FAL. *In vitro*, macrophages grown in high glucose display inflammation that is skewed to expression of tissue damage versus tissue repair gene expression. Further, macrophages grown in high versus low glucose demonstrate blunted macrophage-endothelial cell interactions. In both settings, these adverse effects of high glucose were reversed by *Ager* deletion in macrophages.

Conclusions—These findings indicate that RAGE attenuates adaptive inflammation in hind limb ischemia; underscore microenvironment-specific functions for RAGE in inflammation in tissue repair versus damage; and illustrate that AGE/RAGE antagonism may fill a critical gap in diabetic PVD.

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Disclosures

None.

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Keywords

angiogenesis; diabetes; receptor; monocyte; inflammation

Introduction

Subjects with diabetes are at higher risk of developing ischemic vascular disease, including peripheral artery disease (PAD). PAD results from an obstruction of blood flow in the peripheral arteries. Compromised peripheral blood supply leads to tissue ischemia (lack of circulation) and tissue hypoxia (lack of oxygen). Neoangiogenesis, the formation of new capillaries from pre-existing vessels, occurs in response to an ischemic/hypoxic insult. Inflammation is a requisite process for ischemia/hypoxia-induced neoangiogenesis. However, the ability to develop functional new vessels is significantly lower in diabetic patients with PAD vs. age-matched control subjects.[1–3] This may lead to the severe course of limb ischemia often observed in diabetic patients, in which PAD may result in foot ulceration, impaired wound healing, lower extremity amputation and mortality.[4–7] Treatments to date have not been fully effective at improving blood flow through neoangiogenesis in PAD. Therefore, identification of new therapeutic targets is important for drug discovery for the treatment of PAD, especially in diabetes.

Hyperglycemia, a hallmark of diabetes, leads to the formation of advanced glycation end products (AGEs), a heterogeneous class of post-translationally modified proteins. AGEs form, in part, through the precursor methylglyoxal (MG) in subjects with diabetes.[8] Glyoxalase 1 (GLO1) is a principal enzyme responsible for detoxifying MG and regulating AGE levels.[9] However, in diabetes, elevated levels of glucose may render GLO1 insufficient to block AGE accumulation, thereby resulting in increased AGE levels. The receptor for AGE (RAGE) is a member of the immunoglobulin superfamily of cell surface molecules present on multiple cell types, usually expressed at low levels in homeostasis and to increased degrees in diabetic tissues and at sites of stress or injury. Increased accumulation of AGEs and enhanced activation of RAGE contribute to the pathogenesis of diabetic complications.[10–15] In human subjects, RAGE is highly expressed in peripheral occlusive vascular disease lesions.[16] In experimental models, earlier studies showed that RAGE plays central roles in the injury response to global hypoxia and ischemia/reperfusion (I/R) injury.[17–20]

Here, we hypothesized that deficiency of *Ager* (gene encoding RAGE) or overexpression of *Glo1* in hind limb ischemia would restore blood flow recovery and neoangiogenesis in diabetic mice. To address this concept, we used a well-established mouse model of hind limb ischemia (a preclinical model of PAD)[21] to assess the role of RAGE in both the immediate response to ischemic stress, and the long-term effects of this receptor on tissue remodeling and neoangiogenesis in both the non-diabetic and diabetic states. In addition, we explored the role of RAGE ligands, AGEs, by examining the impact of overexpression of *Glo1*[22] in mice undergoing hind limb ischemia and we probed the underlying mechanisms using *in vivo*, *ex vivo*, and *in vitro* analyses of peripheral blood and muscle tissues. Collectively, our data reveal that the AGE-RAGE axis blunts neoangiogenesis and blood flow recovery, at

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least in part through an impingement on adaptive immune and vascular cell responses consequent to hind limb ischemia, which may be restored by deletion of *Ager* or over-expression of *Glo1*. These data contribute to an emerging paradigm in which RAGE-condition-dependent pro- versus anti-inflammatory responses may prevail depending on the unique stress and distinct cues within the discrete microenvironment.

Materials and Methods

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

Results

RAGE impairs blood flow recovery and angiogenesis after hind limb ischemia

To establish if RAGE modulates responses to hind limb ischemia, we began by detecting RAGE expression in the leg muscles of streptozotocin-induced diabetic and non-diabetic WT mice on day 5 after femoral artery ligation (FAL). RAGE protein was significantly higher in the ischemic muscles of diabetic WT mice compared to non-ischemic muscles of diabetic WT mice ($p<0.0001$; Figure 1A). A significant increase in RAGE protein was also observed in the ischemic muscles of non-diabetic WT mice compared to the non-ischemic muscles of non-diabetic WT mice ($p<0.05$; Figure 1A). Furthermore, the expression of RAGE protein in the ischemic muscles of diabetic WT mice was significantly higher than that observed in the ischemic muscle of non-diabetic WT mice ($p<0.01$; Figure 1A). These observations indicate that hind limb ischemia induced increased expression of RAGE in ischemic muscles, especially in diabetic WT mice, and suggested potential roles for RAGE in the response to hind limb ischemia. To address this premise, we tested the effect of deletion of *Ager* on vascular repair responses in the presence or absence of diabetes.

We performed FAL and began by assessing the role of RAGE in blood flow recovery. Laser Doppler Perfusion Imaging revealed that restoration of perfusion was significantly lower in diabetic WT mice compared with non-diabetic WT mice on day 28 after FAL ($p<0.05$, Figure 1B). In contrast, significantly higher blood flow was observed in both diabetic and non-diabetic $Ager^{-/-}$ mice compared to their respective controls 28 days after FAL ($p<0.01$, Figure 1B). Of note, induction of diabetes by streptozotocin resulted in expected rises in levels of blood glucose and there were no differences based on *Ager* genotype (Table I in the online-only Data Supplement).

To assess the effects of diabetes and RAGE on neoangiogenesis in response to hind limb ischemia, histological assessment of capillary density was performed in WT mice and in mice devoid of *Ager* on day 28 post-FAL. As shown in Figure 1C, diabetic WT mice displayed significantly lower capillary density than non-diabetic mice on day 28 post-ligation ($p<0.01$). In contrast, mice devoid of *Ager* displayed significantly higher capillary density on day 28 in both the non-diabetic and diabetic states vs. respective WT mice ($p<0.05$ and $p<0.01$, Figure 1C).

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RAGE impairs gene expression of inflammatory and angiogenic mediators and macrophage infiltration after hind limb ischemia, especially in diabetic mice

Prompted by these observations, we sought to identify the genes impacted by diabetes and RAGE in hind limb ischemia and we profiled genes essential to neoangiogenesis using a commercial angiogenesis RT2 profiler™ PCR array. Among the 84 genes tested, Table II, III and IV in the online-only Data Supplement illustrate some of the findings on skeletal muscle RNA of WT and *Ager*$^{-/-}$ mice on day 3, 5 and 7 after FAL in the absence and presence of diabetes compared to that of non-diabetic WT-sham controls. A significant theme that emerged from this analysis was that prominent gene expression changes related to inflammation were observed among the groups, and that these changes were driven, at least in part, by RAGE. After FAL, particularly in non-diabetic and diabetic *Ager*$^{-/-}$ mice, marked increases in chemokines, inflammatory and matrix metalloproteinase genes were observed compared to non-diabetic and diabetic WT mice. Levels of key chemokine [*Ccl2*, chemokine (C-X-C motif) ligand *(Cxcl)2* (*Gro-β*), and *Cxcl5* (*Ena-78*)] remain elevated in *Ager*$^{-/-}$ mice vs. respective WT mice in the non-diabetic and diabetic states on day 7, as well as on days 3 & 5. At the later time point, day 7, higher expression of growth and pro-angiogenic factors was observed in *Ager*$^{-/-}$ vs. WT mice in both the diabetic and non-diabetic states. Transcripts for *Angpt2*, hepatocyte growth factor (*Hgf*), midkine (*Mdk*), placental growth factor (*Pgf*), sphingosine kinase 1 (*Sphk1*), transforming growth factor beta (*Tgfb*) and thrombospondin-1 (*Thbs1*) were higher in *Ager*$^{-/-}$ ischemic tissues. Although we did not observe any differences in hypoxia-inducible factor-1 (*Hif1)α* mRNA transcripts on days 3 and 5, on day 7, the *Ager*$^{-/-}$ groups displayed higher levels of *Hif1α* mRNA transcripts vs. the WT groups. Levels of Integrin β chain, β3 precursor (*Itgβ3*) and platelet/endothelial cell adhesion molecule 1 (*Pecam1/Cd31*) transcripts were higher in both groups of *Ager*$^{-/-}$ mice on day 7. Note that no significant differences in *Vegf a, b* or *c* were observed by diabetes status or by the expression or not of *Ager*.

We performed real-time quantitative PCR to confirm the key findings and focused on inflammatory genes given the striking increase in the *Ager*-deficient tissues vs. the WT and the profound importance of inflammatory mechanisms in facilitating vascular and tissue repair after FAL. We found that in the ischemic muscle tissue, significantly lower levels of *Ccl2* mRNA transcripts were observed in WT diabetic mice vs. non-diabetic WT mice on day 7 post-ligation ($p<0.001$, Figure 2A). In mice devoid of *Ager*, higher *Ccl2* transcripts vs. WT mice on day 7 were observed in the skeletal muscle tissue in both the non-diabetic and diabetic state after FAL ($p<0.001$, Figure 2A). These data suggest that RAGE suppresses chemokine gene expression in the microenvironment of hind limb ischemia.

Based on our previous findings that *Egr1* is a principal regulator of *Ccl2* in hypoxia/ischemia,[17, 23] we assessed *Egr1* mRNA in the ischemic muscles vs. sham. Compared to WT non-diabetic mice, levels of *Egr1* mRNA transcripts were significantly lower in diabetic WT mice muscle tissues on day 5 post-ligation ($p<0.05$, Figure 2B). Moreover, *Egr1* mRNA transcripts on day 5 post-ligation were significantly higher in *Ager*$^{-/-}$ vs. respective WT mice in the absence and presence of diabetes ($p<0.001$, Figure 2B). However, by day 28 post-ligation, mRNA levels of *Egr1* and *Ccl2* were overall much lower in both WT and *Ager*$^{-/-}$ mice, in the non-diabetic and diabetic state and in sham vs. ischemia, thereby

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indicating that the expression of mediators linked to the inflammatory response was significantly attenuated in all groups by this time point (Figure I in the online-only Data Supplement). Collectively, these data suggest that RAGE attenuates hypoxia-ischemia-dependent inflammation and mechanisms to recruit immune cells in the response to hind limb ischemia.

CCL2 has been recognized as one of the key chemokines that regulates migration and infiltration of monocytes macrophages,[24] and macrophages have been recognized as critical regulators of neoangiogenesis.[25–27] We thus evaluated the numbers of infiltrating macrophages into the ischemic muscle on day 7 post-FAL by quantifying CD68+ macrophages in this tissue. In line with gene expression data in the muscle tissue, diabetic ischemic muscle tissue displayed lower numbers of CD68+ macrophages per unit area compared to non-diabetic tissue in WT mice ($p<0.05$, Figure 2C). However, deficiency of *Ager* in the non-diabetic and especially diabetic ischemic muscles resulted in significantly higher numbers of CD68+ cells/unit area compared to that observed in respective WT mice ($p<0.05$ and $p<0.01$, respectively, Figure 2C). These data suggest that RAGE reduces macrophage content in ischemic muscle, in parallel with impaired angiogenesis and blood flow recovery.

AGEs are increased in diabetic, ischemic tissue

We next sought to identify putative RAGE ligands accounting for these effects in the ischemic muscles of diabetic and non-diabetic WT mice by using an antibody against carboxymethyl lysine (CML), one of the major structures of AGEs highly prevalent *in vivo*. Two key patterns were evident from the results of this experiment; first, a significant increase in CML modified protein was noted in WT diabetic muscle sham tissues at baseline compared to non-diabetic WT mice ($p<0.001$; Figure 3A). Second, in WT non-diabetic or diabetic mice, hind limb ischemia itself resulted in a significant increase in CML modified protein in muscle tissue vs. sham treatment ($p<0.01$ and $p<0.05$, respectively; Figure 3A). Since glyoxalase-1 (GLO1) is a principal detoxifying enzyme for AGE precursor methylglyoxal (MG) controlling AGE levels, we tested levels of *Glo1* gene expression in hind limb muscle tissues. Levels of *Glo1* mRNA were significantly reduced by FAL in hind limb ischemia vs. sham treatments in both the non-diabetic and diabetic state ($p<0.05$ in both cases; Figure 3B), but were significantly higher in respective *Ager* deficient tissue at baseline and on day 3 post-ligation compared to WT tissue in both the non-diabetic and diabetic state ($p<0.05$ in both cases, Figure 3B). Western blotting revealed no statistically significant differences in levels of GLO1 protein in the muscle tissues among these groups of mice (Figure II-A in the online-only Data Supplement).

As it is established that AGEs are generated in diabetes, hypoxia/reoxygenation and ischemia/reperfusion injury,[17, 20] we measured levels of CML protein in the ischemic and non-ischemic muscle tissues of diabetic and non-diabetic transgenic (Tg) mice overexpressing *Glo1*. We found that levels of CML modified protein were significantly lower in Tg(*Glo1*) mice compared to respective WT mice on day 5 post-ligation in diabetes ($p<0.05$, Figure 3A) and at baseline in the diabetic state ($p<0.05$, Figure 3A), although the levels of CML modified protein were undetectable at baseline in both non-diabetic WT and

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Tg(*Glo1*) mice (Figure 3A). These data validated that transgenic mice expressing *Glo1* reduced AGE content in the muscle tissue, especially in diabetes and post-FAL and provided a key strategy to test the premise that AGEs contributed mechanistically to impaired neoangiogenesis and blood flow recovery in FAL.

Finally, we examined levels of a distinct RAGE ligand, S100B, in the skeletal muscle of the mice under study. No significant differences were observed among any of the mouse groups when considering surgical condition, diabetes vs. non-diabetes or genotype (Figure II – B in the online-only Data Supplement). These data suggested that the AGE ligands, not S100B, were more likely to contribute to the RAGE-dependent effects in both non-diabetic and diabetic hind limb ischemia.

Overexpression of *Glo1* restores diabetes- and ischemia-induced impairment of blood flow recovery, neoangiogenesis, macrophage infiltration and inflammatory mediator gene expression

As our data suggested that both diabetes and ischemia increased AGE levels, we tested the hypothesis that transgenic overexpression of *Glo1* would rescue FAL-subjected mice from suppression of neoangiogenesis, blood flow recovery and inflammation in ischemic muscle tissue, especially in diabetes. Laser Doppler blood flow analyses showed significantly higher ischemic/sham limb blood flow ratio in both sets of Tg(*Glo1*) mice (non-diabetic and diabetic condition), compared to their respective WT controls on day 28 post-ligation ($p<0.01$ and $p<0.001$, respectively; Figure 4A). Consistent with this observation, Tg(*Glo1*) mice displayed significantly higher capillary density in both the non-diabetic and diabetic states compared to WT mice on day 28 post-ligation ($p<0.05$ and $p<0.01$, respectively, Figure 4B). We measured gene expression of inflammatory mediators in the muscle tissue using real-time PCR analysis. Our data reveal that significantly higher *Ccl2* ($p<0.05$ and $p<0.001$, Figure 4C) and *Egr1* ($p<0.001$ in both cases, Figure 4D) transcripts in the skeletal muscle tissue of Tg*(Glo1)* vs. WT mice on day 5 after FAL were noted in both the non-diabetic and diabetic state. These data indicate that reduction of RAGE ligands AGEs by overexpression of *Glo1* restored adaptive inflammatory gene expression in the microenvironment of hind limb ischemia.

Effects of high glucose on gene expression of inflammatory mediators from *Ager*- or *Glo1*-modified macrophages

Based on the observation of increased macrophage content in the ischemic muscle tissues of $Ager^{-/-}$ mice compared to WT mice, especially in diabetes, the inflammatory gene expression of macrophages under high glucose conditions was studied using BMDMs from WT, $Ager^{-/-}$ and Tg(*Glo1*) mice as a model system. Following exposure to high glucose (HG, 25 mM D-glucose), BMDMs from all genotypes cultured in low glucose (LG, 5.5 mM D-glucose) displayed significant time-dependent induction of *Ccl2* (Figure 5A) and *Egr1* (Figure 5B) mRNA transcripts. Maximal induction of transcription of *Ccl2* (Figure 5A) or *Egr1* (Figure 5B) occurred within 2h or 1h of exposure to high glucose, respectively. The extent of high glucose-induced *Ccl2* ($p<0.0001$, Figure 5A) and *Egr1* ($p<0.0001$,, Figure 5B) transcription was significantly higher in $Ager^{-/-}$ or *Glo1* overexpressing BMDMs compared to WT BMDMs.

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We extended the time course to 7 days to test if the effects of HG were sustained and if the impact of HG condition was RAGE-dependent. BMDMs were cultured in LG or HG and real-time quantitative PCR was performed to detect mRNA transcripts for pro- and anti-inflammatory genes. Levels of *Egr1* mRNA were significantly higher in *Ager*$^{-/-}$ BMDMs grown in LG or HG vs. their respective WT controls (p<0.05 in both LG and HG; Figure III-A in the online-only Data Supplement). Levels of *Ccl2* were significantly lower in WT BMDMs grown in HG vs. LG (p<0.01, Figure III-B in the online-only Data Supplement). However, in *Ager*$^{-/-}$ BMDMs, levels of *Ccl2* were higher in the HG vs. LG state and were significantly higher when compared to their respective WT controls (p<0.05 and p<0.0001, respectively; Figure III-B in the online-only Data Supplement. We tested expression of pro-inflammatory mediators in BMDMs and found that levels of *Il1b, Tnfa, Nos2* and *Ccr7* were all higher in the WT HG vs. LG state and compared to WT HG, levels of these markers were all significantly lower in the *Ager*$^{-/-}$ HG state (Figure III-C-D-E-F the online-only Data Supplement). Finally, we tested two anti-inflammatory genes, *Arg1* and *Il10*. Significantly lower levels of *Arg1* and *Il10* mRNA transcripts in HG vs. LG WT BMDMs were noted (p<0.05 and p<0.001, respectively, Figure III-G the online-only Data Supplement). However in *Ager*$^{-/-}$ BMDMs grown in HG, significantly higher levels of *Arg1* and *Il10* were noted compared to the WT HG state (p<0.0001, Figure III-G-H the online-only Data Supplement). Taken together, these data indicate that both HG and RAGE modulate macrophage inflammatory properties, with HG reducing expression of *Egr1* and *Ccl2* in WT but not *Ager*$^{-/-}$ BMDMs. Further, HG increases pro-inflammatory type markers and reduces anti-inflammatory markers, at least in part via RAGE.

Effect of RAGE on high glucose-induced macrophage/endothelial interaction and monocyte inflammation

These data led us to probe the role of RAGE in macrophage-endothelial interactions and in monocyte inflammation, two key factors in the response to tissue injury. We began by investigating whether high glucose conditions modulated the interaction of macrophages with the endothelium and probed the potential role of RAGE by determining the adhesion of fluorescently-labeled WT or *Ager*$^{-/-}$ BMDMs to WT or *Ager*$^{-/-}$ MAEC monolayers, respectively, under LG or HG conditions. Under LG condition, no significant *Ager* genotype-dependent differences in the adhesion ability of BMDMs to MAEC monolayers were noted (Figure 6A). In contrast, when both cell types were cultured in HG, WT BMDM adhesion to WT MAECs was significantly lower compared to that observed in cells cultured in LG (p<0.05, Figure 6A). However, in HG, significantly higher adhesion of *Ager*$^{-/-}$ BMDMs to WT MAECs and to *Ager*$^{-/-}$ MAECs was observed compared with the adhesion of WT BMDMs to WT MAECs or *Ager*$^{-/-}$ MAECs (p<0.001 and p<0.0001, Figure 6A). Taken together, these data indicate that HG mediates reduced WT BMDM adhesion to MAECs and that deletion of *Ager*, particularly in BMDMs, or BMDMs and MAECS, but not MAECs alone, rescued the suppressive effects of HG on BMDM-MAEC adhesion. These data suggested that deletion of *Ager*, especially in the HG state, increased BMDM interaction with ECs.

To address the underlying mechanisms, we first tested circulating levels of MCP1; MCP1 is the protein product of the *Ccl2* gene. Although in diabetic WT mice, significantly higher

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baseline levels were observed compared to non-diabetic mice ($p<0.01$, Figure IV in the online-only Data Supplement), no increases were noted at day 7 or 28 days after FAL in the mice devoid of *Ager* (Figure IV in the online-only Data Supplement). In fact, no significant differences were noted at any time point or condition in the mice devoid of *Ager*. These findings suggested that cell-specific factors, not circulating mediators, might account for the significant functional differences observed in WT or mice devoid of *Ager* after hind limb ischemia. Hence, we examined the $Ly6C^{hi}$ and $Ly6C^{lo}$ monocyte and neutrophil populations.

Prompted by our finding that higher levels of CD68+ macrophages were noted in diabetic or non-diabetic ischemic tissues in mice devoid of *Ager* vs. the WT controls (Figure 2C), we obtained peripheral blood from the mice under study at baseline (day 0), 7 or 28 days after FAL and examined monocyte populations, including $Ly6C^{hi}$ and $Ly6C^{lo}$ subgroups normalized to the overall CD45+ cells (Figure V-A in the online-only Data Supplement). We first analyzed the baseline state and compared these cellular populations across WT vs. $Ager^{-/-}$ mice. In non-diabetes at baseline, there were no significant differences in total monocytes or monocyte subsets comparing WT vs. $Ager^{-/-}$ mice (Figure V-B in the online-only Data Supplement). In diabetes at baseline, significantly higher total monocytes, $\%Ly6C^{hi}$ and $\%Ly6C^{lo}$ monocyte subsets were observed in WT vs. $Ager^{-/-}$ mice ($p<0.01$, $p<0.05$ and $p<0.05$, respectively; Figure IV-B in the online-only Data Supplement).

We examined the total monocytes across the entire time course and within each genotype as well. In the WT mice over the time course and non-diabetic and diabetic conditions, there were no significant differences in total monocytes in any of the groups (Figure 6B). In contrast, in the $Ager^{-/-}$ mice, total monocytes were significantly higher in diabetic vs. non-diabetic mice at baseline ($p<0.01$) and total monocytes were significantly higher on day 28 in diabetic vs. non-diabetic mice devoid of *Ager* ($p<0.0001$). Finally, total monocytes were significantly higher in diabetic $Ager^{-/-}$ mice on day 28 vs. day 7 ($p<0.01$) (Figure 6C).

Next, we examined the monocyte subsets. First, in the WT mice at baseline, the $\%Ly6C^{hi}$ cells was significantly higher in the diabetic vs. non-diabetic mice ($p<0.01$, Figure 6B). In non-diabetic WT mice, there were no significant differences in $\%Ly6C^{hi}$ or $Ly6C^{lo}$ monocytes comparing baseline with day 7 or day 28 after FAL (Figure 6B). In the diabetic state, significantly lower $\%Ly6C^{hi}$ and $\%Ly6C^{lo}$ monocytes were observed at day 7 vs. baseline ($p<0.01$ and $p<0.05$, respectively, Figure 6B). By day 28 in the diabetic WT mice, only the $\%Ly6C^{lo}$ not the $\%Ly6C^{hi}$ population was significantly higher compared to the levels observed at day 7 ($p<0.05$, Figure 6B).

In $Ager^{-/-}$ mice, surprisingly, we found that significantly higher $\%Ly6C^{hi}$ monocytes was observed at baseline in the diabetic vs. the non-diabetic mice devoid of *Ager* ($p<0.0001$, Figure 6C). The $\%Ly6C^{hi}$ population was significantly lower in the diabetic $Ager^{-/-}$ mice on day 7 vs. diabetic baseline ($p<0.01$, Figure 6C) and unlike in the diabetic WT mice, this population had significantly recovered by day 28 (compared to day 7) in the diabetic $Ager^{-/-}$ mice ($p<0.01$, Figure 6C). In the $Ager^{-/-}$ mice, there were no statistically significant differences in the proportion of $Ly6C^{lo}$ cells at any time point or condition (Figure 6C).

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Finally, we examined blood neutrophil content at baseline and found that the only significant difference was in the non-diabetic state, in which non-diabetic *Ager*$^{-/-}$ mice displayed significantly lower neutrophil content vs. the WT mice (p<0.01, Figure V-A, C in the online-only Data Supplement). In contrast, no genotype differences were observed in the diabetic state, thereby strongly suggesting that differences in neutrophils likely did not contribute to *Ager*-dependent effects in the response to hind limb ischemia.

Taken together, these data revealed that significant and surprising patterns emerged in the total monocytes and Ly6C monocyte subsets with respect to RAGE expression as follows: (1) total monocytes and Ly6C^{hi} monocytes were significantly higher at baseline in diabetic vs. non-diabetic *Ager*$^{-/-}$ mice, indicating that deletion of *Ager* not only did not attenuate this inflammatory response, but enhanced it in diabetes; and (2) the %Ly6C^{hi} cells significantly recovered by day 28 vs. day 7 post-FAL only in diabetic mice devoid of *Ager* but not in the WT mice, in parallel with improved blood flow recovery and neoangiogenesis on day 28.

Discussion

Enhancement of neoangiogenesis and the resulting improvement of limb blood flow are key restorative mechanisms in response to ischemia.[28–30] However, the impairment of physiological neoangiogenesis has been implicated in exacerbation of peripheral limb ischemia, especially in diabetes, but the underlying mediating mechanisms have yet to be fully dissected, thereby mitigating the development of effective therapies.[2, 3, 31] We demonstrate here that ischemia-induced neoangiogenesis was significantly impaired by the actions of RAGE ligand AGEs and RAGE, especially in diabetes, and the mechanism in WT mice likely involves multiple factors, including reduced expression of inflammatory mediators, such as *Egr1* and *Ccl2*, that recruit myeloid cells into perturbed tissues; decreased inflammatory macrophage content in ischemic muscles; and downregulation of ischemic muscle matrix metalloproteinases and growth and repair factors. Compared to WT mice undergoing FAL, mice devoid of *Ager* or mice overexpressing *Glo1* displayed restoration of adaptive inflammatory and angiogenic responses to hind limb ischemia.

Previous studies linked AGEs and RAGE to impairment of neoangiogenesis in diabetes but did not elucidate the underlying mechanisms.[32–34] Inhibition of AGE formation by aminoguanidine, an inhibitor of AGE, improved angiographic score, capillary density, and laser Doppler skin-perfusion ratios and restored matrix degradation processes in diabetic mice subjected to hind limb ischemia.[32] Blockade of AGE-RAGE by adenovirus-induced overexpression of soluble RAGE (sRAGE), a decoy receptor for AGE, as well as deficiency of *Ager* restored diabetes-induced impairment of angiogenesis in a matrigel patch model.[33] In addition, intramuscular administration of sRAGE improved angiogenic responses to hind limb ischemia in diabetic mice.[34] It is important to note that testing the effects of sRAGE does not directly point to RAGE-dependent mechanisms, as roles for non-RAGE receptors for the families of RAGE ligands are not ruled out solely by ligand sequestration. Therefore, studies using mice genetically deficient in *Ager* are essential to definitively assign a critical role for RAGE activation in diabetes- and ischemia-induced impaired neoangiogenesis and to pinpoint underlying mechanisms.

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In the present study, we demonstrated the increased expression of CML-AGE and its receptor (RAGE) in the hind limb ischemic vs. sham muscles, in both the non-diabetic and diabetic state, five days post-FAL (Figure 3). This work adds to the body of evidence that even in the absence of diabetes, ischemia increases AGE formation, shown previously in the heart.[35] Recently, it was reported that mice devoid of *Ager* displayed improved angiogenesis after hind limb ischemia and that 21 days post-FAL, levels of CML-AGE in the muscles were higher in diabetes but not lower in the $Ager^{-/-}$ mice (non-ischemic tissues were not tested), whereas levels of RAGE ligand HMGB1 were higher in ischemic vs. non-ischemic muscle, but not affected by deletion of *Ager*.[36] In that work, however, genetically-modified mice or distinct ligand-directed strategies were not employed to probe the potential mechanistic roles of CML-AGE or HMGB1 in hind limb ischemia.

The present experiments, in contrast, addressed mechanistic roles for AGEs in this setting. Given their roles in AGE formation and accumulation, GLO1 and MG have been implicated in the pathogenesis of diabetic complications.[8, 37–39] Several lines of experimental evidence indicate beneficial impact of overexpression of *Glo1* in animal models in diabetes.[40–43] Overexpression of *Glo1* in *Caenorhabditis elegans* decreased hyperglycemia-induced accumulation of AGEs and oxidative stress and enhanced lifespan.[40] Transgenic rats overexpressing human *Glo1* decreased MG-derived AGE formation and reduced retinal, neuroglial, and vascular pathology in diabetes and were resistant to renal ischemia-reperfusion injury.[41, 42] Overexpression of *Glo1* in bone marrow cells in mice restored neovascularization to ischemic hind limbs in diabetes.[43] In the present study, we identified the mechanism by which *Glo1* exerted its beneficial effects. First, however, we confirmed that in our model, overexpression of *Glo1* in mice prevented diabetes-induced increased levels of CML-AGE modified proteins (Figure 3). Further, we demonstrated that *Glo1* prevented increases in CML-AGE modified proteins in ischemia, confirming that *Glo1* plays an important role in the suppression of AGE formation in the vessel wall not only in diabetes, but also in ischemic injury. Second, consistent with our previous observation linking RAGE to down-regulation of *Glo1* in the diabetic kidney,[38] we discovered that *Ager* deficiency leads to higher levels of *Glo1* mRNA in the skeletal muscle tissue after hind limb ischemia in both non-diabetic and diabetic mice, while levels of *Glo1* mRNA were suppressed by RAGE in WT mice after hind limb ischemia. Although we did not find differences in GLO1 protein levels among WT and *Ager* modified mice, we did note overall trends to lower levels of GLO1 in the respective sham vs. FAL mice. Recent evidence suggests regulation of *Glo1* by oxidative stress-related pathways such as NRF[44]; if and to what extent levels of GLO1 protein may be modulated in a high oxidative/inflammatory environment require further study. Third, we provided evidence that Tg(*Glo1*) mice reversed diabetes- and ischemia-induced impaired regulation of inflammatory mediators, *Egr1* and *Ccl2* and angiogenic responses to hind limb ischemia in both non-diabetic and diabetic states. Taken together, these data link RAGE ligand AGEs to impaired inflammatory and angiogenic responses in hind limb ischemia and suggest that upregulation of the *Glo1* pathway may provide a complementary therapeutic target in the prevention of diabetic vascular damage.

Inflammatory cell infiltration early after ischemia is an important trigger for the angiogenic response to tissue ischemia. Impaired muscle regeneration in $Ccl2^{-/-}$ mice suggests an

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important role for macrophages and MCP1 in tissue reparative processes.[45] Consistent with the implications of the current findings, *Ccl2*$^{-/-}$ mice displayed decreased angiogenesis and reduced blood flow recovery after hind limb ischemia vs. WT mice.[45] Furthermore, it was earlier reported that mice devoid of *Egr1* (non-diabetic state) displayed reduced numbers of regenerating arterioles after femoral artery ligation.[46] In both studies, however, the effect of deletion of *Egr1* or *Ccl2* on inflammatory mechanisms in the ischemic tissue was not explored. Here, we demonstrate that RAGE-dependent mechanisms mediate regulation of these two key factors in the ischemic hind limb. In contrast to these findings in FAL and hind limb ischemia, in hyperlipidemic mice, deletion of either *Egr1* or *Ccl2* is atheroprotective.[47, 48] What might begin to explain these apparent conflicting findings?

Our data unveil the intriguing finding that RAGE plays opposing roles in regulating *Egr1* and *Ccl2* expression in acute vs. chronic hypoxia/ischemia, as when *Ager* is deleted in hind limb ischemia, higher macrophage content in the ischemic muscle ensues – and is linked to repair – quite distinct from that observed in chronic atherosclerosis and acute hypoxia in mouse hearts in which *Ager* deletion significantly reduced *Egr1* and/or *Ccl2* transcripts and lesional macrophage content in vascular tissues.[17, 49, 50] As differences in circulating levels of MCP1 did not explain the benefits of *Ager* deletion in hind limb ischemia, we turned to examination of peripheral monocytes and neutrophils in these mice, as our data revealed that muscle tissue levels of CD68+ macrophages were significantly higher in non-diabetic or diabetic *Ager*$^{-/-}$ mice vs. respective WT mice after FAL. These endeavors uncovered the surprising finding that higher Ly6C^{hi} monocytes were found in diabetic vs. non-diabetic mice of both the WT and *Ager*$^{-/-}$ genotypes (Figure 6). In atherosclerosis, this subset of Ly6C^{hi} monocytes facilitates atherosclerosis; in contrast, the present work implicates these cells in tissue repair.[51, 52] Here, in hind limb ischemia, we surmise that the local environment, at least in part driven by RAGE, recruits tissue-reparative cells driven by early but transient upregulation of *Egr1* and *Ccl2*. Critically, in all mouse groups, by day 28 post-FAL, muscle tissue levels of these factors were dramatically lower and did not greatly differ by genotype post-ischemia (Figure I in the online-only Data Supplement).

Examination of the PCR array data (Tables II, III and IV in the online-only Data Supplement) supports the paradigm that in hind limb ischemia, particularly in diabetes, RAGE blunts a pro-inflammatory microenvironment needed for tissue repair. In contrast, both non-diabetic or diabetic mice devoid of *Ager* display higher levels of a number of chemokines and their ligands, particularly *Ccl2* in the ischemic muscle tissue, compared to their WT counterparts. CCL2/MCP1 are ligands for Ly6C^{hi} cells[53–55], thereby contributing to the higher number of macrophages infiltrating the ischemic muscle tissue on day 7 in the *Ager*$^{-/-}$ vs. WT mice (Figure 2C and Tables II, III, IV in the online-only Data Supplement). Interestingly, although levels of *Il1b* were significantly higher in non-diabetic or diabetic *Ager*$^{-/-}$ ischemic muscle vs. respective WT counterparts on day 5 post-FAL, these levels were much lower in the mice devoid of *Ager* by day 7, relative to the WT. In parallel, levels of *Thbs1* (latent activator of *Tgfb1*)[56] were significantly higher in *Ager*$^{-/-}$ ischemic tissue by day 7 post-FAL and by 7, significantly higher levels of tissue-reparative *Tgfb1*, as well as *Angpt2, Hgf, Pgf*, were evident in ischemic tissue devoid of *Ager* vs. the respective WT mice in the non-diabetic and diabetic states. These data mirror the improvement in neoangiogenesis and blood flow recovery observed in the *Ager*$^{-/-}$ vs. WT mice in both the

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diabetic and non-diabetic states and reflect the overall patterns that were observed in isolated BMDMs. In BMDMs devoid of Ager, lower levels of "M1" like pro-inflammatory cytokines and higher levels of "M2" like molecules linked to tissue repair (*Arg1* and *Il10*) were observed in high glucose conditions (Figure III in the online-only Data Supplement). Interestingly, levels of *Vegfa, b* and *c* were not significantly modulated in FAL in the non-diabetic or diabetic ischemic muscles of WT or $Ager^{-/-}$ mice, but levels of other factors such as *Thbs1* and *Tgfb1* were significantly affected by diabetes and deletion of *Ager*. Our findings regarding lack of RAGE-dependent effects on *Vegf* are intriguing and warrant further analyses; it is notable, however, that studies of others in cultured bone marrow cells from 12 months diabetic or non-diabetic mice failed to demonstrate differences in the production of VEGF protein.[57]

In vitro studies also supported inflammatory roles for RAGE in macrophages, as we demonstrated that macrophages grown in high glucose displayed decreased functional potential, that is, these macrophages demonstrated reduced adhesion to WT murine endothelial cells compared to WT macrophages grown in physiological levels of glucose in a RAGE-dependent manner (Figure 6A). Critically, our study also uncovered that simply deleting *Ager* in murine endothelial cells did not rescue the suppressive effects of high glucose on impaired WT macrophage adherence. Rather, only when *Ager* was deleted either in macrophages alone, or in both macrophages and endothelial cells, was the reduced adhesion of macrophages to endothelial cells in high glucose rescued. In future studies, it would thus be useful to determine how conditions mimicking the hyperlipidemic environment might affect macrophage-endothelial adhesion vis-à-vis the RAGE axis. It is notable that Babu and colleagues tested the impact of hyperlipidemia vs. hyperglycemia (type 2 diabetes) stresses on ischemic muscle macrophages isolated 7 days post-FAL.[58] Differential promoter methylation studies revealed that in both settings, significant promoter hypomethylation (increased transcription) of prototypic "M1" macrophages) and hypermethylation (reduced transcription) of anti-inflammatory and pro-angiogenic "M2" macrophages was evident when compared to the WT control mice. Of note, however, the genes altered by hyperlipidemia vs. hyperglycemia were not fully overlapping, suggesting that further in-depth probing of these datasets might uncover factors exhibiting differential responses to these metabolic perturbations.

It is not surprising that the immune/inflammatory system was designed to establish multiple checkpoints to protect against imbalances in pro- vs. anti-inflammatory forces. In the case of the toll receptor family, for example, it was shown that extensive cross-talk between *Tlr4* and *Tlr2* is required in mediating adaptive responses to hind limb ischemia, when tested in non-diabetic mice.[59, 60] The present work adds RAGE to the cadre of genes that exhibit tissue microenvironment-dependent roles in inflammation and tissue regeneration. In this context, a burgeoning body of evidence links RAGE to opposing outcomes in murine models of infection challenge. For example, whereas deletion of *Ager* in *Streptococcus pneumoniae* pneumonia or *Acinetobacter baumanii* sepsis resulted in improved survival and diminished tissue damage,[61, 62] *Ager* deletion in a distinct setting of *Klebsiella pneumoniae* was detrimental to survival and recovery[63]. These considerations underscore the complexities of RAGE in the immune and vascular response to imposed stresses.

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Taken together, the present work suggests unique hypoxia/ischemia-dependent mechanisms in hind limb ischemia that may be rescued by deletion of *Ager* and by specific means to reduce AGE burden. This work underscores the premise that RAGE may dampen tissue-reparative inflammation in hind limb ischemia, thereby blocking adaptive neoangiogenesis and restoration of blood flow. We conclude that deletion of *Ager* re-sets adaptive inflammatory cues in a tissue microenvironment-sensitive manner and surmise that antagonism of RAGE might fill a key therapeutic gap in peripheral arterial disease, particularly in diabetes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

AGEs	Advanced glycation end products

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RAGE	the receptor for AGE
sRAGE	soluble RAGE
***Ager*$^{-/-}$**	gene encoding RAGE
MG	methylglyoxal
GLO1	glyoxalase 1
Tg	transgenic
MG	methylglyoxal
WT	wild type
Egr1	early growth response gene-1
Ccl2	chemokine (C-C motif) ligand 2
MCP-1	formerly referred to as monocyte chemoattractant protein 1
Vegf	vascular endothelial growth factor
Angpt1	angiopoietin-1
Ena78	chemokine (C-X-C motif) ligand *(Cxcl)2 (Gro-β)*, and *Cxcl5*
Hgf	hepatocyte growth factor
Mdk	midkine
Pgf	placental growth factor
Sphk1	sphingosine kinase 1
Tgfb	transforming growth factor beta
Thbs1	thrombospondin-1
Hif1	hypoxia-inducible factor-1
Itgβ3	integrin β chain, β3 precursor
Pecam1/Cd31	platelet/endothelial cell adhesion molecule 1
FAL	femoral artery ligation
CML	carboxymethyl lysine
BMDMs	bone marrow derived macrophages
MAEC	mouse aortic endothelial cell
LG	low glucose

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HG	high glucose
NDM	non-diabetes
DM	diabetes
PAD	peripheral artery disease
PVD	peripheral vascular disease

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Highlights

- The present study demonstrates that ischemia-induced neoangiogenesis was significantly impaired by AGE-RAGE action in WT mice, especially in diabetes, and the mechanisms were traced to blockade of tissue reparative inflammatory responses, including reduction of gene expression of inflammatory/angiogenic mediators and decrease of pro-angiogenic inflammatory macrophage infiltration into ischemic muscles. In contrast, *Ager* deficiency or overexpression of *Glo1* restored physiological inflammatory and angiogenic responses to hind limb ischemia.
- These data challenge the paradigm that RAGE solely triggers tissue-damaging inflammation in stressed tissues and suggest that in hypoxia/ischemia in the hind limb, RAGE attenuates pro-repair inflammatory gene expression and macrophage content in ischemic muscle.
- This work underscores the emerging concept that discrete microenvironment cues may stimulate unique RAGE-dependent responses and suggest that RAGE antagonism may fill a critical gap in the therapeutic arsenal targeting diabetic peripheral vascular disease.

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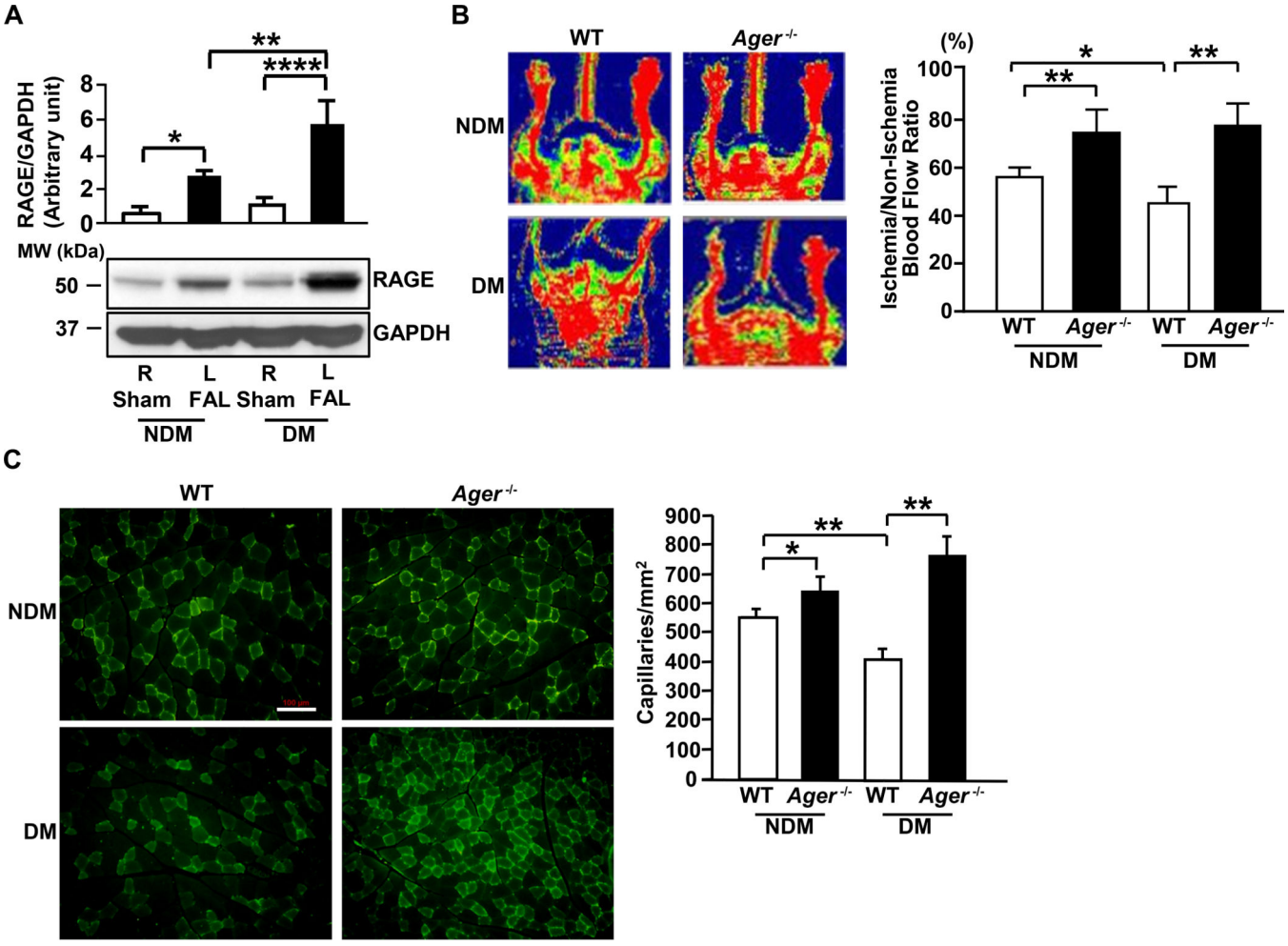

Figure 1. Blood flow recovery and angiogenesis in hind limb ischemia: effect of RAGE

(A) Proteins from the indicated skeletal muscle on day 5 after femoral artery ligation (FAL) were subjected to Western blotting for detection of RAGE and GAPDH (n=3 mice/group). **(B)** Hind limb blood flow was monitored by laser Doppler Perfusion Imaging on day 28 post-ligation (red indicates normal perfusion; blue, reduced blood flow) and quantitative evaluation of blood flow was expressed as a ratio of blood flow in ischemic to sham limb (n=10 mice/group). **(C)** Representative photomicrographs of ischemic muscles from the indicated mice stained with anti-CD31 IgG on day 28 post-ligation (scale bar=100 μm) and quantitative evaluation of capillary density was performed in muscle tissue sections of non-diabetic and diabetic WT vs. *Ager*$^{-/-}$ mice (n=10 mice/group). R denotes right leg/sham control and L denotes left leg/FAL. NDM denotes non-diabetes and DM denotes diabetes. Error bars represent ±SEM. *p<0.05, **p<0.01, ****p<0.0001.

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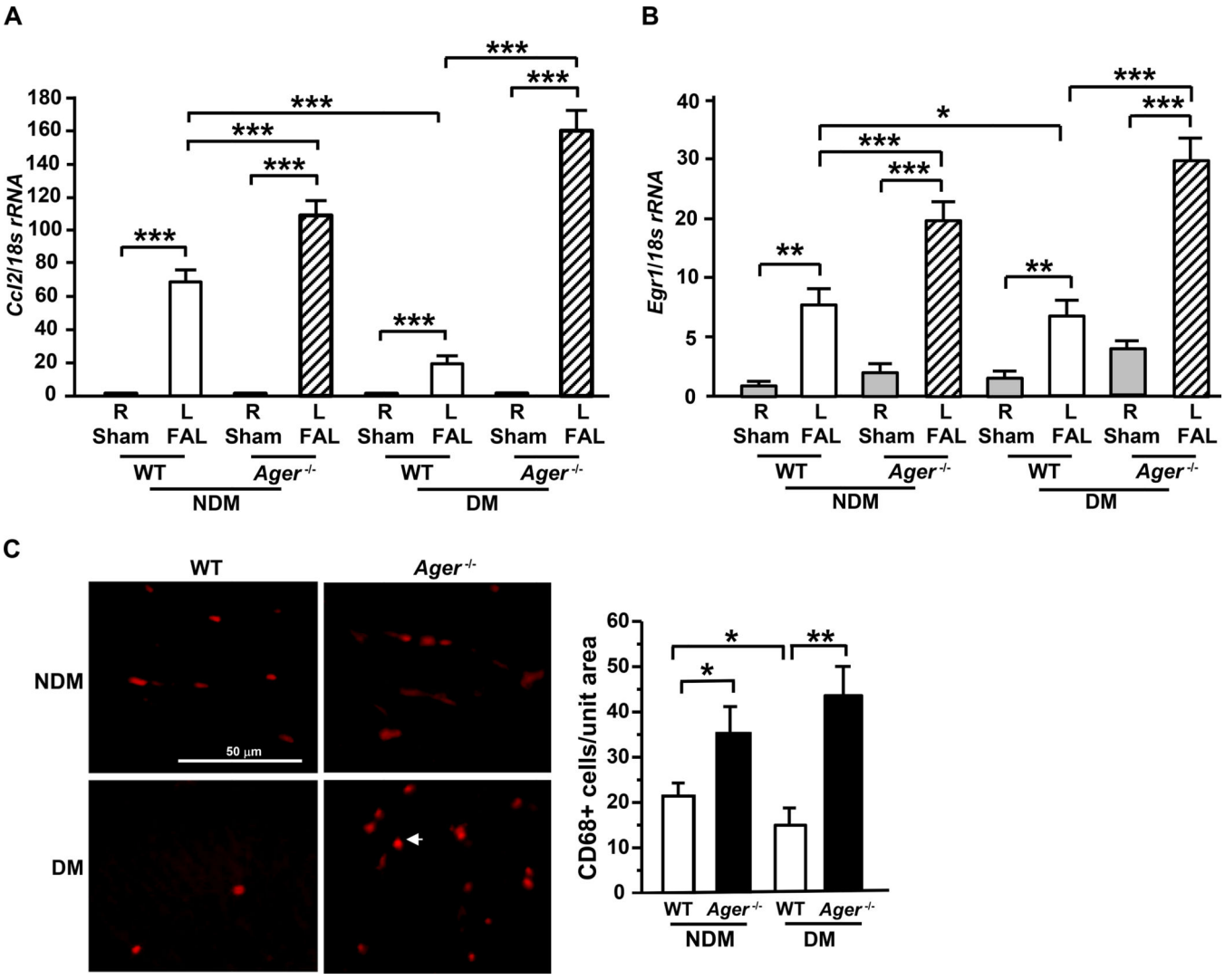

Figure 2. Gene expression and macrophage infiltration in hind limb ischemia: effect of RAGE

(A–B) Total RNA was isolated from the indicated skeletal muscle and subjected to real-time PCR analysis. *Ccl2* mRNA was detected on day 7 after FAL vs. sham (A) and *Egr1* mRNA was detected on day 5 after FAL vs. sham (B), and normalized to 18*s* rRNA (n=5 mice/group). **(C)** Immunostaining with anti-CD68 IgG was performed on skeletal muscle tissue from the indicated mice on day 7 after FAL and the mean number of CD68+ cells/unit area is reported (n=3 mice/group). R denotes right leg/sham control and L denotes left leg/femoral artery ligation (FAL). NDM denotes non-diabetes and DM denotes diabetes. Error bars represent ±SEM. **p*<0.05, ***p*<0.01, ****p*<0.001.

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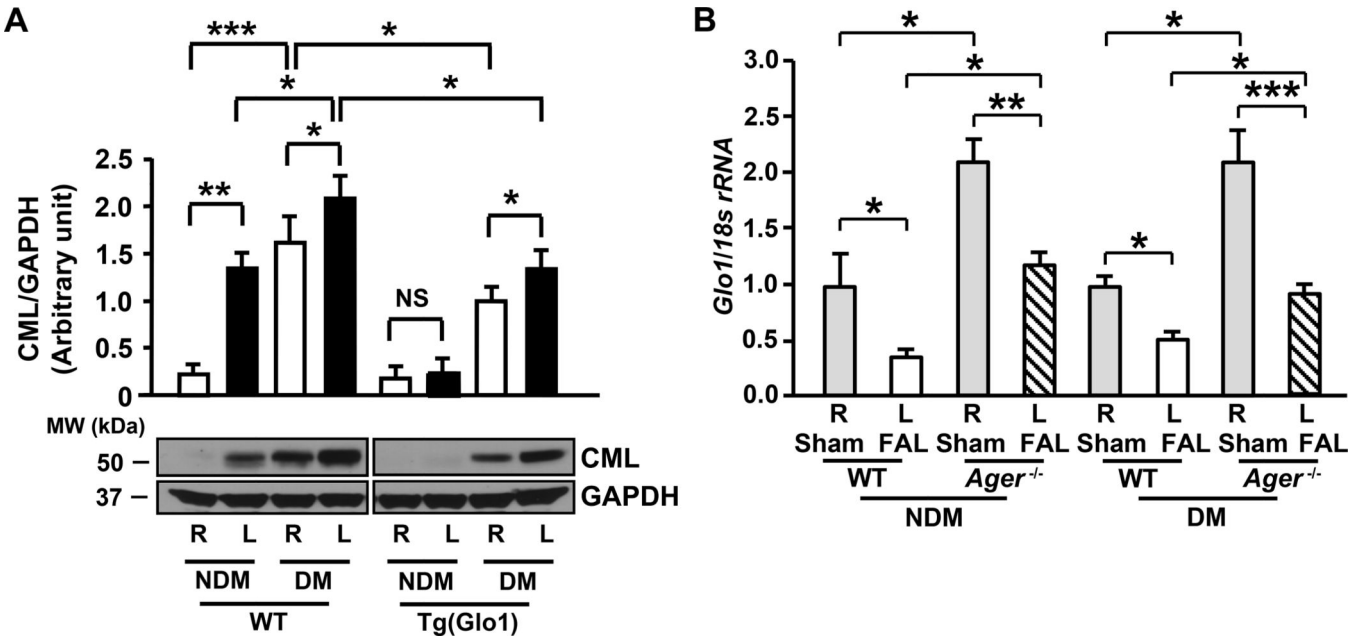

Figure 3. Expression levels of CML-AGEs and *Glo1* in skeletal muscle after hind limb ischemia

(A) Proteins from the indicated skeletal muscles on day 5 after FAL were subjected to Western blotting for detection of carboxymethyl lysine (CML)-AGEs and normalized to GAPDH (n=3 mice/group). **(B)** *Glo1* mRNA transcripts were detected in the indicated skeletal muscles of mice on day 3 after FAL vs. sham by real-time PCR analysis and normalized to 18*s* rRNA (n=3 mice/group). R denotes right leg/sham control and L denotes left leg/femoral artery ligation (FAL). NDM denotes non-diabetes and DM denotes diabetes. Error bars represent ±SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, NS not statistically significant.

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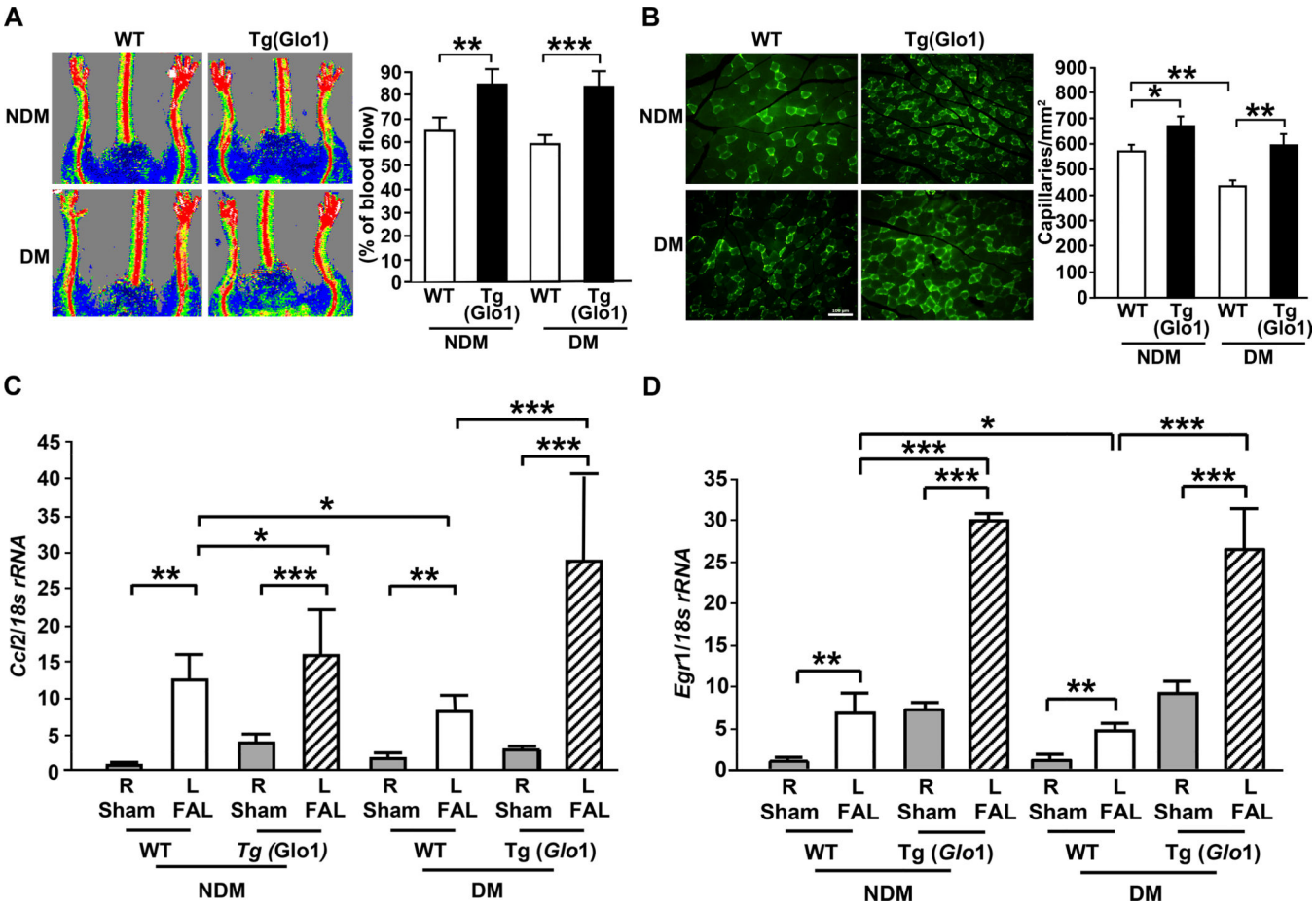

Figure 4. Impact of *Glo1* overexpression on gene expression, inflammatory responses, angiogenesis and blood flow recovery in hind limb ischemia

(A) Hind limb blood flow was monitored by Laser Doppler Perfusion Imaging on day 28 post-ligation (red indicates normal perfusion; blue, reduced blood flow). Quantitative evaluation of blood flow was expressed as a ratio of blood flow in ischemic to sham limb (n=10 mice/group). **(B)** Representative photomicrographs of ischemic muscles from the indicated mice stained with anti-CD31 IgG on day 28 post-ligation (scale bar=100 μm). Quantitative evaluation of capillary density is shown (n=10 mice/group). **(C)** *Ccl2* and **(D)** *Egr1* mRNA transcripts were detected in the indicated skeletal muscles of mice on day 5 after FAL vs. sham by real-time PCR analysis and normalized to 18*s* rRNA (n=5 mice/group). R denotes right leg/sham control and L denotes left leg/femoral artery ligation (FAL). NDM denotes non-diabetes and DM denotes diabetes. Error bars represent ±SEM. **p*<0.05, ***p*<0.01, ****p*<0.001.

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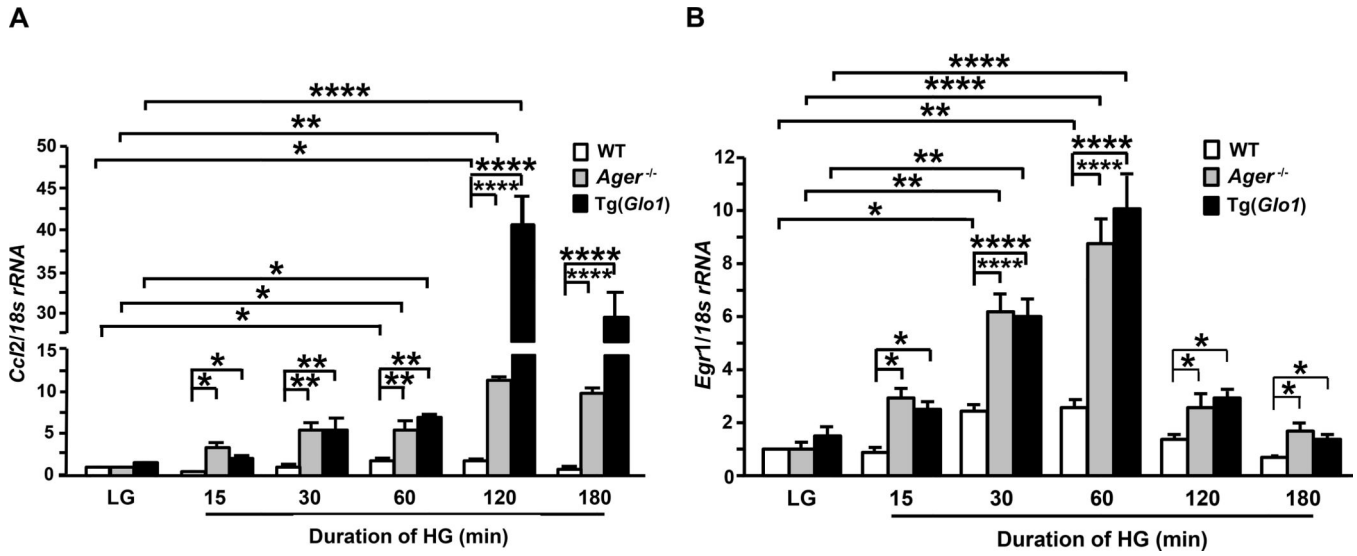

Figure 5. High glucose induces gene expression in *Ager*- or *Glo1*-modified macrophages

(A–B) Bone marrow derived macrophages (BMDMs) were isolated from mice of each genotype and cultured individually in 5.5 mM D-glucose (LG) for 7 days (n=6 WT/LG mice/group and n=4 Tg*Glo1* mice and n=4 $Ager^{-/-}$ mice/group) and exposed to 25 mM D-glucose (HG) (n=4 mice/group) for the indicated time periods. Total RNA was isolated from these cells and subjected to real-time PCR analysis. *Ccl2* **(A)** and *Egr1* **(B)** mRNA transcripts were detected and normalized to 18*s* rRNA. Error bars represent ±SEM. *p<0.05, **p<0.01, ****p<0.0001.

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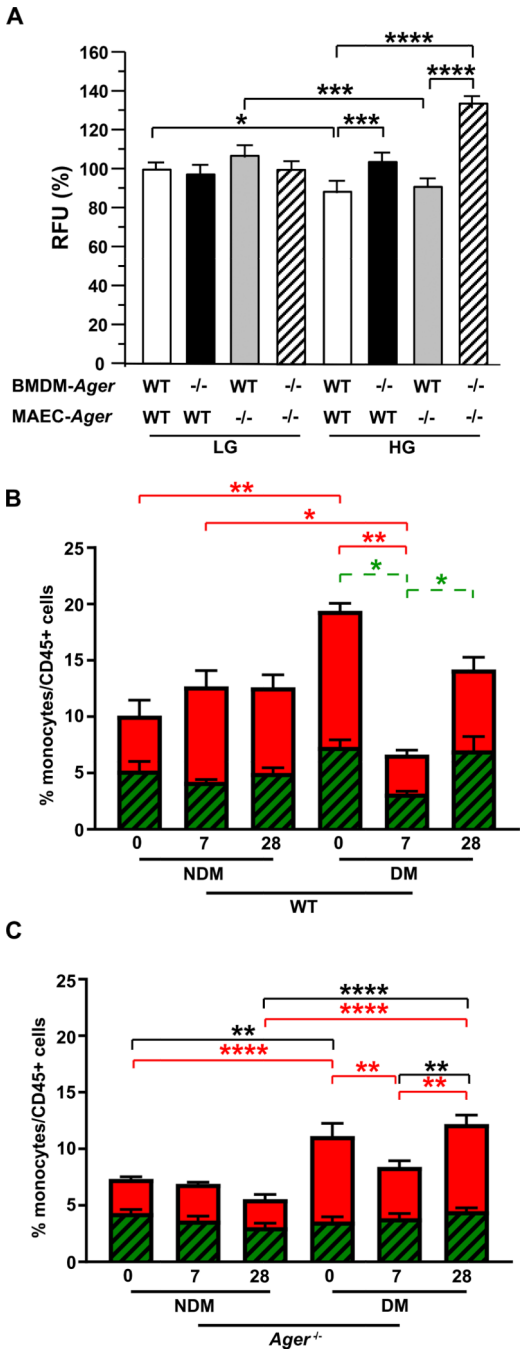

Figure 6. Effect of RAGE on high glucose-induced macrophage/endothelial interaction and circulating monocyte populations

(A) Mouse macrophage-endothelial cell adhesion assay, using BMDMs isolated from mice of n=4 mice per genotype was performed. Leuko Tracker™ labeled WT or *Ager*$^{-/-}$ BMDMs were allowed to attach to WT or *Ager*$^{-/-}$ murine aortic endothelial cell (MAEC) monolayer for 1 h. Adherent cells were lysed and quantified by reading fluorescence with a fluorescence plate reader at 480 nm/520 nm. **(B–C)** Ly6C^{hi} and Ly6C^{lo} monocyte populations were analyzed by flow cytometry in WT (B) and *Ager*$^{-/-}$ (C) mice at baseline (day 0), 7 and 28 days after FAL. NDM denotes non-diabetes and DM denotes diabetes. Red

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bars: Ly6C^{hi} monocytes subpopulation; Green bars: Ly6C^{lo} monocyte subpopulation. N=5–14 per group as follows: WT/NDM, 8; WT/DM, 5; *Ager*$^{-/-}$/NDM, 14; and *Ager*$^{-/-}$/DM, 6. Statistical analysis was performed from the total population (Ly6C^{hi} + Ly6C^{lo}/CD45+ cells) (black); Ly6C^{hi} population (red) and Ly6C^{lo} population (dashed green). Error bars represent ±SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.