The Vasoreparative Function of Myeloid Angiogenic Cells Is Impaired in Diabetes Through the Induction of IL1β

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ABSTRACT

Myeloid angiogenic cells (MACs) promote revascularization through the paracrine release of angiogenic factors and have been harnessed as therapeutic cells for many ischemic diseases. However, their proangiogenic properties have been suggested to be diminished in diabetes. This study investigates how the diabetic milieu affects the immunophenotype and function of MACs. Both MACs isolated from diabetic conditions and healthy cells exposed to a diabetic environment were used to determine the potential of MACs as a cell therapy for diabetic-related ischemia. MACs were isolated from human peripheral blood and characterized alongside proinflammatory macrophages M (LPS + IFNγ) and proangiogenic macrophages M (IL4). Functional changes in MACs in response to high-D-glucose were assessed using an in vitro 3D-tubulogenesis assay. Phenotypic changes were determined by gene and protein expression analysis. Additionally, MACs from type 1 diabetic (T1D) patients and corresponding controls were isolated and characterized. Our evidence demonstrates MACs identity as a distinct macrophage subtype that shares M2 proangiogenic characteristics, but can be distinguished by CD163^{hi} expression. High-D-glucose treatment significantly reduced MACs proangiogenic capacity, which was associated with a significant increase in IL1β mRNA and protein expression. Inhibition of IL1β abrogated the antiangiogenic effect induced by high-D-glucose. IL1β was also significantly upregulated in MACs isolated from T1D patients with microvascular complications compared to T1D patients without microvascular complications or nondiabetic volunteers. This study demonstrates that Type 1 diabetes and diabetic-like conditions impair the proangiogenic and regenerative capacity of MACs, and this response is mediated by IL-1β. STEM CELLS 2018;36:834–843

SIGNIFICANCE STATEMENT

Myeloid angiogenic cells (MACs) can be isolated from peripheral blood and injected into patients to assist blood vessel repair in ischemic diseases. Feasibility and efficacy for this approach has been demonstrated in myocardial infarction where delivery of MACs reduced damage and contributed to regeneration of damaged tissue. Some studies suggest that the regenerative potential of MACs in diabetic patients is significantly impaired. Since this patient group suffers serious vascular degenerative disease, the potential to use MACs for cell therapy may be hampered. Furthermore, there is concern that once injected into the diabetic environment, MACs can change phenotype and could potentially worsen the ischemia. This study provides strong evidence that MACs are less reparative and more inflammatory in the diabetic environment. Importantly, we identified IL1β as a key mediator for this change. This information is useful to devise strategies to restore MACs functionality in diabetic patients.

INTRODUCTION

Cell therapy is emerging as a promising option for ischemic diseases. Several clinical trials have been designed to evaluate the potential of various therapeutic cells to induce vascular regeneration and reperfusion of ischemic tissues. Preliminary results have shown cytotherapy to be safe, tolerable, and feasible although clinical efficacy has been inconsistent [1]. Myeloid angiogenic cells (MACs), isolated from the mononuclear cell (MNC) fraction of human peripheral blood, are also known as circulating angiogenic cells (CACs) [2] and we have previously characterized them in detail [3–5]. MACs have shown promising results in preclinical models for critical

limb ischemia [6, 7], acute myocardial infarction [8], and pulmonary arterial hypertension [9]. In addition, we have previously demonstrated the potential use of MACs as a cell therapy in a model of retinal ischemia, where MACs promoted retinal microvascular repair by IL8-mediated transactivation of VEGFR2 [4].

The majority of clinical trials for cell therapies have used cells sorted from the MNC fraction of peripheral blood or bone marrow, containing a heterogeneous mixture of hematopoietic cells. While trials including ASTAMI [10], TOPCARE-AMI [11], REPAIR-AMI [12], and TCT-STAMI [13] have demonstrated safety, there are differences in endpoint readouts such as left ventricular ejection fraction, which have been attributed to the cell isolation procedures [14]. In addition, a recent meta-analysis of clinical trials of autologous cell therapy for critical limb ischemia concluded that cytotherapies derived from peripheral blood-MNCs, such as MACs, outperformed bone marrow-MNCs, and mesenchymal stem cells [15]. Proof-of concept studies have demonstrated therapeutic efficacy for MAC-like cells in patients with critical limb ischemia [16]. MAC-like cells are currently being tested clinically for acute myocardial infarction [17], and pulmonary arterial hypertension [18], underscoring the potential of MACs for cell therapy to treat various ischemic diseases. Tissue ischemia is prevalent in diabetes and patients could benefit from an autologous cell therapy that promotes vascular repair. Unfortunately, a recent report on the MOBILE trial suggested the effectiveness of an autologous cell therapy for critical limb ischemia is limited to nondiabetic patients [19]. In addition, there is accumulating evidence to suggest that MACs are dysfunctional in diabetes. When compared to nondiabetic controls, patients with both type 1 [20] and type 2 [21] diabetes show reduced MACs in their peripheral circulation. There is also evidence suggesting impaired mobilization of MACs and blunted vascular repair capacity in cells isolated from patients with diabetes [22]. It has been suggested that this dysfunction could be due to a phenotype switch from proangiogenic to antiangiogenic [23], although the precise underlying mechanisms remain unknown. Nevertheless, the importance of the diabetic milieu on MACs vaso-reparative functionality, in the context of either autologous or allogeneic treatment regimes, remains paramount in the development of these cells as a safe and effective cell therapy.

This study was designed to elucidate molecular mechanisms underpinning MACs dysfunction under diabetic conditions. In particular, we report that MACs exposed to high D-glucose (HDG) exhibit a significant M2 to M1 phenotypic shift, which is also demonstrated in cells isolated from patients with diabetes. This diabetes-related molecular phenotype in MACs is associated with a diminished proangiogenic capacity which occurs concomitantly with upregulation of proinflammatory cytokines. We have identified IL1β as a key factor driving the loss of MACs vasoreparative properties under diabetic conditions.

MATERIALS AND METHODS

Cell Isolation and Culture

Human peripheral blood was collected from healthy volunteers aged 18–50 years under ethical approval granted by Queen's University Belfast School of Medicine Research Ethics Committee. In addition, 13 human peripheral blood samples were received under ethical approval from the Galway University Hospital Research Ethics Committee. This included samples from five nondiabetic healthy controls and eight patients with type 1 diabetes mellitus. Nondiabetic controls were volunteers recruited concurrently to diabetic patients, and deemed healthy following completion of a health questionnaire to ensure exclusion of other diseases.

MACs were isolated as previously described [4]. Cells were cultured in EBM-2 MV medium (Lonza) supplemented with 10% fetal bovine serum (FBS). MACs were used in experiments at day 7–9. For in vitro high glucose experiments, MACs were randomly assigned to three treatment groups: 25 mM HDG in addition to 5 mM D-glucose already present in control media (HDG), 25 mM high L-glucose (HLG) as an osmolality control (HLG), MACs media containing 5 mM D-glucose (Control). At least three independent isolations of MACs were used for each experiment.

Differentiated macrophages were obtained from CD14+ monocytes sorted from MNCs using CD14 microbeads (Miltenyi Biotec) cultured on FBS-coated dishes for 7 days in RPMI-1640 with L-glutamine, 20% Hyclone FBS, and 100 ng/ml M-CSF at a density of $1.5 \times 10^5/cm^2$. To polarize macrophages, cell culture medium was replaced with RPMI-1640 with L-glutamine, 5% FBS and the following stimulus for a further 18 hours: 100 ng/ml LPS and 20 ng/ml IFNγ for M1 proinflammatory macrophages M(LPS + IFNγ); and 20 ng/ml IL4 for M2 proangiogenic macrophages M(IL4) [24]. Human endothelial colony forming cells (ECFCs) were isolated from umbilical cord blood under ethical approval and following well-established protocols [25, 26]. All cells used tested negative for mycoplasma contamination.

Data Analysis with GEO2R

Four datasets (GSE5099, GSE35449, GSE36537, and GSE18686) were assessed by GEO2R. Gene lists were created for each dataset comparing M1 and M2 macrophage populations. An adjusted p value of $<.05$ was used to select for differentially expressed genes. Genes with a fold change of ≤-2 and ≥2 were assigned to a list of genes differentially expressed in M1 and M2 macrophages, respectively.

RNA Extraction and qRT-PCR

Total RNA was extracted using the MiRNeasy Micro Kit (Qiagen). cDNA was synthesized using High-Capacity RNA-to-cDNA kit (Life Technologies). Quantitative RT-PCR was carried out in 10 μl reactions containing 5 μl of 2x Maxima SYBR Green qPCR Mastermix (Thermo Scientific), 2 μl of 1:10 cDNA dilution and 0.5 μM of gene specific primers for 50 cycles (including denaturation at 94°C for 10 seconds, annealing at 58°C for 10 seconds, and extension at 72°C for 10 seconds) in a Light-Cycler 480 (Roche), in triplicate. Primer sequences and efficiency are shown in Supporting Information Table S1. Gene expression was determined relative to RPL11 housekeeping gene using $2^{(-\Delta CT)}$, and fold change was calculated using $2^{(-\Delta\Delta CT)}$.

Flow Cytometry

At least 5×10^5 cells were prepared per sample. Cells were filtered through a 30 μm cell strainer and resuspended in 100 μl of staining buffer. Cells were incubated with antibodies against CD14-FITC, CD16-e450, CD163-PE, and ICAM1-Pacific

Blue (eBioscience) for 30 minutes at 4°C. Cells were then washed in phosphate-buffered saline and resuspended in 500 μl of flow cytometry staining buffer. Cell surface marker analysis was carried out on the Attune Acoustic Focusing Flow Cytometer (Life Technologies). At least 20,000 events were acquired for each sample and analyzed using FlowJo. Respective immunoglobulin IgG isotype controls were used to establish accurate gating strategy for data analysis.

In Vitro Tubulogenesis Assay

In coculture experiments, cells were labeled with PKH fluorescent cell linker kit (Sigma). ECFCs were mixed with either MACs, M1 macrophages, or M2 macrophages, in a 3:1 ratio and resuspended in a 2:3 mix of EGM-2 medium (without Quots) supplemented with 5% FBS and growth factor-reduced phenol red-free Matrigel (BD Biosciences). Aliquots of 50 μl were spotted in triplicate onto a 24-well plate. After polymerization, spots were covered with medium. Formation of tubules was assessed 24 and 72 hours later. Four images were captured per spot on a confocal scanning microscope (Nikon), and vascular tubes analyzed using ImageJ software.

Conditioned media (CM) from glucose-treated MAC cultures was collected and used to grow ECFCs resuspended in Matrigel. An additional control of unconditioned media plus glucose treatments was also used. ECFCs were labeled with Calcein AM green dye (Life Technologies). Another set of experiments was carried out to assess the effects of IL1β on the angiogenic potential of MACs in the presence of HDG. IL1β was blocked in MAC cultures alongside 4-day HDG treatments with addition of human IL1β monoclonal Ab [10 μg/ml] (MAB201, R&D Systems). An IgG control was also used, monoclonal mouse Ab IgG1 (MAB002, R&D Systems). Recombinant human IL1β [100 ng/ml] (201-LB-025, R&D Systems) was also added to MAC cultures for 4 days, after which CM was collected and used in a tube formation assay.

Protein Extraction and Western Blotting

Protein was extracted by lysing cells in RIPA buffer containing protease and phosphatase inhibitor cocktail (Thermo Scientific). For Western blotting, 25 μg of protein was diluted in Laemmli buffer containing beta-mercaptoethanol and loaded onto 15% SDS gels. Following electrophoresis, proteins were transferred onto PVDF membrane and then blocked for 1 hour in Tris-buffered saline with 0.05% Tween20 containing 4% Blotto solution (Thermo Scientific). Membranes were incubated with human IL1β antibody (MAB201, R&D Systems) or human Caspase-1 antibody (MAB6215, R&D Systems) overnight at 4°C. The next day, membranes were washed in TBS-T and horseradish peroxidase-conjugated secondary antibody (Advansta) was added. Blots were developed using Immobilon chemilluminescent HRP substrate (Millipore) and the G:BOX Chemi XX6 (Syngene). PVDF membranes were stripped and reprobed with a rabbit polyclonal antibody to RPL11 (Thermo-Fisher Scientific).

Statistical Analysis

All data are expressed as mean ± SEM. Statistical significance was calculated using the one way analysis of variance with Bonferroni's Multiple Comparison test on GraphPad Prism software. Statistically significant values had a p value of $< .05$.

RESULTS

MACs Differ from M1 and M2 Macrophages By High CD163 Expression

Based upon molecular profiling using transcriptomics and immunophenotyping, we have previously shown human MACs to be monocytic in nature with some M2 macrophage characteristics [4]. Using unbiased transcriptomics analysis of microarray datasets for human M1 and M2 macrophages generated by four independent studies following similar methodology; we have assessed how distinct these cells are from MACs. Gene lists of differentially expressed mRNAs between M1 and M2 macrophages where identified from four GEO DataSets: GSE5099, GSE35449, GSE36537, GSE18686. Statistical assessment for genes enriched by at least twofold, with an adjusted p-value of $< .05$, produced two lists for each dataset of genes highly expressed in M1 and M2 macrophages, respectively. Comparison of gene signatures from 19 samples in four datasets in a Venn diagram was used to identify the common differentially upregulated genes in M1 and M2 macrophages respectively (Fig. 1A). To determine the relationship of these genes to MACs, their expression profiles were assessed in the MACs transcriptome. MACs showed a dissimilar gene expression profile to M1 macrophages with only 9 out of 38 M1 macrophage genes expressed by MACs. On the other hand, the MAC gene signature was very similar to that of M2 macrophages with MACs expressing five out of the seven major M2 macrophage markers: RAMP1, F13A1, CCL13, PDGFC, and IL17RB. This is also evident by an overlay of gene expression profiles comparing M1 and M2 markers in three sets of MACs versus CD14+ monocytes (Supporting Information Fig. S1).

Microarray results were validated by quantitative real-time qRT-PCR (Fig. 1B) and this also demonstrated a high degree of similarity between M2 macrophages and MACs. For example, MACs and M2 macrophages exhibit low expression of M1 markers: IL1β, ICAM1, IDO1, and PTGS2, and high expression of M2 markers: CD163, CD206, CD204, and PDGFC. Although similar, MACs can be clearly distinguished from M2 macrophages based upon their ~15-fold higher expression of CD163. In addition, MACs differ from M2 macrophages by their significantly lower expression of TGM2, CD209, CCL13, CLEC4A and higher expression of IL10 (Supporting Information Fig. S2). In agreement with qPCR results, flow cytometry cell surface immunophenotyping clearly distinguished MACs from M1 and M2 macrophage populations based upon expression of CD163^{hi} ($>$95%) and ICAM1^{neg} ($<$1%) (Fig. 1C). In addition, CD16 also distinguishes MACs from M2 macrophages with 2 and 40% cell surface expression respectively (Supporting Information Fig. S3).

To assess the functional angiogenic capacity of MACs versus M1 and M2 macrophages, we used the in vitro 3D Matrigel tube formation assay. A coculture approach was used with ECFCs mixed with M1 macrophages, M2 macrophages, or MACs. ECFCs were prelabeled green, and M1 macrophages, M2 macrophages, or MACs prelabeled red and this allowed for unambiguous cell identification and accurate tubulogenesis quantification. M1 macrophages failed to induce ECFC tube formation to the same level as M2 macrophages and MACs (Fig. 1D). Quantification of ECFC tube area indicated that both M2 macrophages and MACs significantly increased ECFC
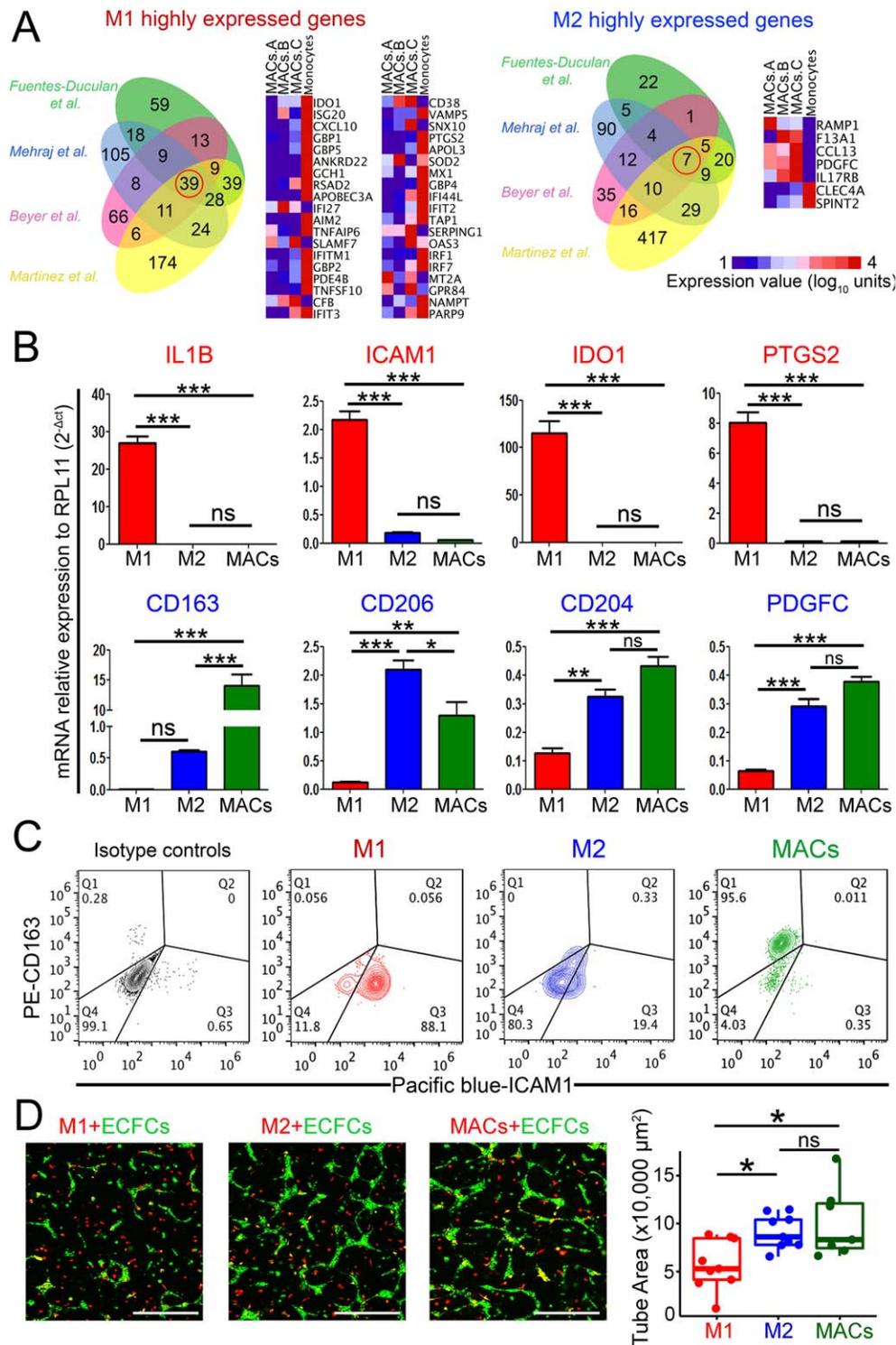
Figure 1. MACs are proangiogenic cells with a distinct gene signature. **(A):** Venn diagram comparison between four datasets of M1 and M2 macrophage gene profiles. Heat maps of the common highly expressed M1 and M2 markers demonstrated that MACs gene signature is similar to M2 macrophages, whereas expression of M1 macrophage markers is weak/low. **(B):** qRT-PCR data confirms MACs weak/low expression of M1 macrophage markers IL1β, ICAM1, IDO1, and PTGS2 in red; while MACs have a high expression of M2 macrophage markers CD163, CD206, CD204, and PDGFC in blue. $n = 4$; mean $\pm$ SEM. One-way analysis of variance (ANOVA): p value not significant (ns), *, $p < .05$; **, $p < .01$; and ***, $p < .001$. **(C):** Cell surface immunophenotyping by flow cytometry for M1 macrophages, M2 macrophages, and MACs. Double stained isotype control was used for gating and shown as black contour plot. Red contour plot show M1 macrophages, blue contour plot show M2 macrophages, and green contour plot show MACs. Percentage is shown in each of four quadrants. $n = 3$. **(D):** Representative images of red-labeled M1 macrophages, M2 macrophages, and MACs cocultured with green-labeled ECFCs in 3D Matrigel Tubulogenesis assay. Scale bars: 500 μm. Quantification and statistical analysis of tube area. $n = 3$; mean $\pm$ SEM. One-way ANOVA: p value not significant (ns), *, $p < .05$. Abbreviations: ECFCs, endothelial colony forming cells; MACs, myeloid angiogenic cells.
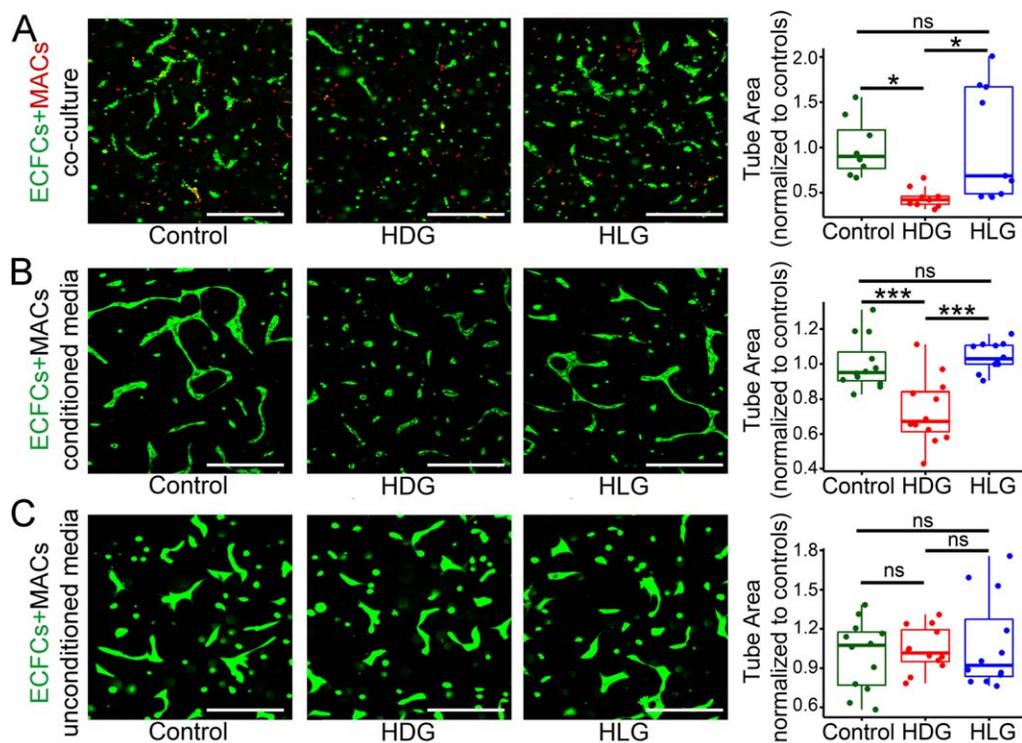
Figure 2. MACs angiogenic potential is affected by high glucose conditions. **(A):** Using a PKH kit, ECFCs were prelabeled green and MACs in red before coculturing them in a 3D Matrigel Tubulogenesis assay. Representative images are shown of cocultures of ECFCs and MACs treated with control, HDG, and HLG. Quantification and statistical analysis of tube area normalized to control. $n = 8$–11; mean $\pm$ SEM. One-way analysis of variance (ANOVA): p value not significant (ns) and *, $p < .05$. **(B):** Representative images of Calcein-green-labeled ECFCs grown in MAC-conditioned media taken from control, HDG, and HLG treated MACs. Quantification and statistical analysis of tube area normalized to control. $n = 11$–12; mean $\pm$ SEM. One-way ANOVA: p value not significant (ns) and ***, $p < .001$. **(C):** Representative images of Calcein-green-labeled ECFCs grown in MACs unconditioned media treated with control, HDG, and HLG. Quantification and statistical analysis of tube area normalized to control. $n = 12$; mean $\pm$ SEM. One-way ANOVA: p value not significant (ns). All scale bars: 500 µm. Abbreviations: ECFCs, endothelial colony forming cells; HDG, high D-glucose; HLG, high L-glucose; MACs, myeloid angiogenic cells.

tubulogenesis compared to M1 macrophages ($p < .05$). Direct comparison between M2 macrophages and MACs showed no significant difference in ECFC tube area confirming similar proangiogenic properties. Colocalization assessment between cells highlighted a closer physical association of MACs with ECFC tubes, where 62% of MACs colocalized with ECFC tubes when compared to M1 macrophages (31%) and M2 macrophages (32%) ($p < .001$) (Supporting Information Fig. S4).

MACs Proangiogenic Capacity Is Reduced in HDG Conditions

To investigate the effects of high glucose on MACs proangiogenic potential, the 3D Matrigel tubulogenesis assay was used. To mimic the diabetic effects of hyperglycemia in vitro, MACs were exposed to 30 mM D-glucose (HDG) for 4 days, and then mixed with ECFCs in the Matrigel tubulogenesis assay. Endothelial tubule formation was significantly reduced in the HDG group compared to control and HLG (osmolality control group) ($p < .05$) (Fig. 2A). Since we have previously shown that MACs promote tube formation by paracrine means [4], the CM of MACs which had been exposed to HDG was also used in the Matrigel tubulogenesis assay, and this showed a significant reduction in ECFC tube formation compared to controls ($p < .001$) (Fig. 2B). To rule out the effects of residual HDG present in the MACs-CM, a third set of

experiments used unconditioned media treated with HDG (Fig. 2C). ECFC tube formation between control, HDG, and HLG unconditioned media showed no significant difference, confirming that the antiangiogenic effect seen in MACs-CM following exposure to HDG is due to phenotypic changes in MACs and resultant secretome.

MACs Proangiogenic Phenotype Shifts Toward a Proinflammatory Phenotype in HDG Conditions

Since HDG treatment induced a significant reduction in the capacity of both MACs and their secreted factors to promote ECFC tube formation in vitro, the phenotype of MACs was assessed by qRT-PCR following exposure to HDG conditions. MACs treated with HDG for 4 days showed a significant change in their gene expression profile, demonstrated by a significant increase in expression of proinflammatory transcripts IL1β, IL6, and IL1α (Fig. 3A), and a significant reduction in expression of proangiogenic and anti-inflammatory markers CD163 and CD204 (Fig. 3B). CD206 expression remained the same in all experimental groups.

Protein expression of IL1β and caspase-1 were determined by Western blot in MACs following a 4 day exposure to HDG treatment and 24 hours stimulation with LPS. Representative image from four independent experiments shows pro-IL1β levels increased with HDG treatment, while no change was
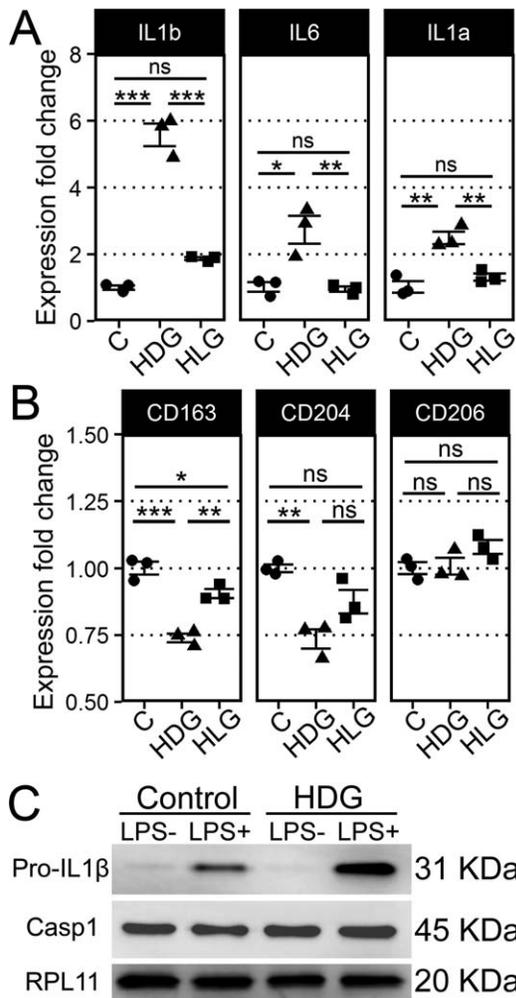
Figure 3. HDG treatment shifts MACs toward a proinflammatory phenotype. **(A):** qRT-PCR of MACs treated in HDG for 4 days resulted in an increase mRNA expression of inflammatory markers IL1β, IL6, and IL1α. Circles represent control, triangles HDG, and squares HLG. $n = 3$; mean ± SEM. One-way analysis of variance (ANOVA): p value is not significant (ns), *, $p < .05$; **, $p < .01$; ***, $p < .001$. **(B):** Assessment of M2 macrophage markers CD163, CD204, and CD206 by qRT-PCR in MACs treated with HDG for 4 days. $n = 3$; mean ± SEM. One-way ANOVA: p value is not significant, *, $p < .05$; **, $p < .01$; and ***, $p < .001$. **(C):** Western blotting for pro-IL1β and caspase1 in MAC whole cell lysates. MACs were exposed to HDG for 4 days and stimulated with LPS for 24 hours. RPL11 expression was used as the loading control. Representative images shown of $n = 4$. Abbreviations: C, control; HDG, high D-glucose; HLG, high L-glucose; MACs, myeloid angiogenic cells.

observed in levels of caspase-1 (Fig. 3C, Supporting Information Fig. S5).

Antiangiogenic Effects Following HDG Exposure in MACs Is Mediated By IL1β

To determine if IL1β was responsible for the antiangiogenic effects of MACs exposed to HDG, a neutralizing antibody against IL1β was added to the HDG MAC cultures, and tested in the 3D tubulogenesis assay (Fig. 4). In agreement with previous results, HDG treatment caused a 22% reduction in ECFC tube formation when compared to controls ($p < .01$). Interestingly, this effect was significantly abrogated by addition of a
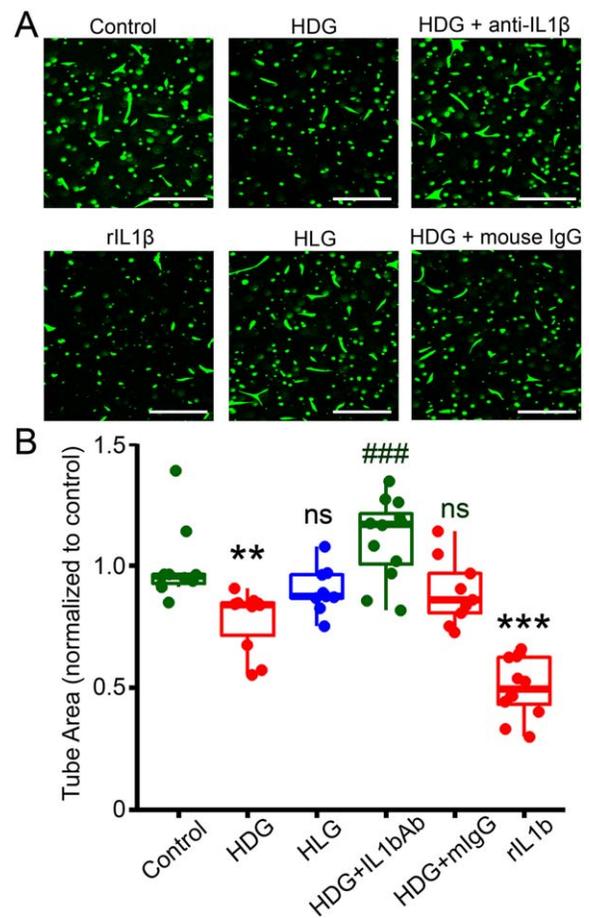
Figure 4. Inhibition of IL1β in high glucose conditions restores proangiogenic capacity of myeloid angiogenic cell (MAC)-conditioned media (CM). **(A):** Representative images of Calcein green-labeled endothelial colony forming cells grown in MAC-CM in 3D Matrigel Tubulogenesis assay. Groups include: control; HDG; HLG; HDG plus addition of IL1β neutralizing antibody; addition of recombinant IL1β (rIL1β); HLG; HDG plus addition of normal mouse IgG. Scale bars: 500 μm. **(B):** Quantification and statistical analysis of tube area normalized to control shown as a box plot. $n = 9$–12; mean ± SEM. One-way analysis of variance: p value is not significant versus control (ns in black), p value is not significant versus HDG (ns in green), **, $p < .01$ versus control, ***, $p < .001$ versus control, ###, $p < .001$ versus HDG. To facilitate data visualization, tube area ≥ 1 is shown in green, < 1 in red, and osmotic control in blue. Abbreviations: HDG, high D-glucose; HLG, high L-glucose.

neutralizing antibody against IL1β in the presence of HDG, resulting in restoration of ECFC tube formation to within ~100% of the control, compared to HDG treatment alone ($p < .001$). A mouse IgG1 isotype control was used to exclude off-target effects of the neutralizing antibody; it had no significant effect on HDG treatment. Addition of recombinant IL1β as a positive control demonstrated a pronounced antiangiogenic effect ($p < .001$). These data suggest that IL1β mediates the HDG-induced impairment of angiogenesis induction in MACs.

Diabetes Induces a MACs Phenotypic Switch in Patients with Type 1 Diabetes and Microvascular Complications

To apply our in vitro-based findings to the clinical setting, MACs were isolated from patients with type 1 diabetes and
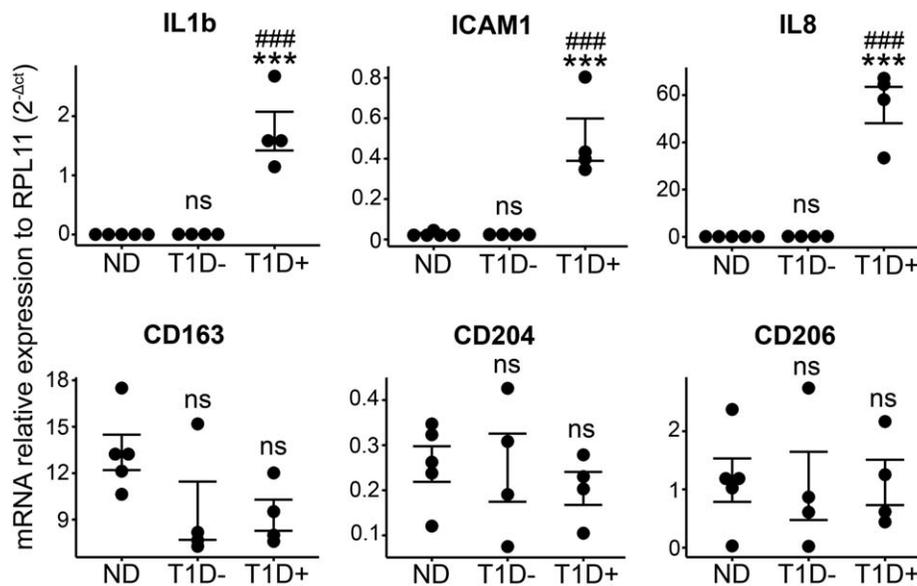
Figure 5. MACs isolated from patients with type 1 diabetes and microvascular complications adopt M1 macrophage characteristics. qRT-PCR analysis of MACs isolated from three patient groups: nondiabetic healthy volunteer (ND) $n = 5$; type 1 diabetic without microvascular complications (T1D–) $n = 4$; type 1 diabetic with microvascular complications (T1D+) $n = 4$; mean ± SEM. One-way analysis of variance: p value is not significant (ns) versus ND, ***, $p < .001$ versus ND, ###, $p < .001$ versus T1D–. Abbreviation: MACs, myeloid angiogenic cells.

assessed for changes in gene expression by qRT-PCR (Fig. 5). MACs from three patient groups were isolated and compared: nondiabetic healthy volunteers (ND); patients with type 1 diabetes mellitus and no microvascular complications (T1D–); and patients with type 1 diabetes mellitus and microvascular complications (T1D+). Details of patient characteristics including age, gender, HbA1c, and diabetes duration are summarized in Supporting Information Table S2. Diabetic microvascular complications included diabetic retinopathy and diabetic nephropathy. Gene expression analysis between MACs from nondiabetic volunteers and patients with type 1 diabetes without microvascular complications showed no significant difference in any of the M1 and M2 markers assessed. Interestingly, MACs isolated from patients with type 1 diabetes with microvascular complications showed a significant upregulation in their expression of M1 markers including IL1β, ICAM1, and IL8 when compared to nondiabetic volunteers and patients with type 1 diabetes without microvascular complications. Other inflammatory cytokines IL1α and IL6 were also significantly upregulated (Supporting Information Fig. S6). Conversely, no change in expression was observed in M2 markers CD163, CD204, CD206 (Fig. 5) and macrophage marker CD68 (Supporting Information Fig. S6) among the groups. Furthermore, assessment of gene microarray data (GEO43950) in proangiogenic CD34+ cells isolated from patients with diabetes, akin to MACs, also identified marked upregulation of IL1β in the patient group with diabetic complications (Supporting Information Fig. S7). These results suggest that MACs isolated from type 1 diabetic patients with microvascular complications adopt a more proinflammatory phenotype which is associated with the development of microvascular complications.

DISCUSSION

A cell therapy using MACs offers a new strategy to treat ischemic disease. The mode of action for these cells is based on their proangiogenic properties and their capacity to act as reservoirs of paracrine vasoactive factors that facilitate revascularization [27]. We and others have demonstrated that MACs produce a conditioned media which is different in composition and superior in proangiogenic properties to their basal endothelial media; this excludes the possibility that they are simply growth factor up-takers and carriers [4, 27]. Previously published data from our group has shown that MACs share properties with M2-activated macrophages and that they can promote vascular repair when delivered into the ischemic retina [4]. Likewise, there is also preclinical evidence to show that M2 polarized macrophages promote tissue repair in a model of retinal ischemia [28]. While phenotypic and functional similarity have been shown between MACs and M2-activated macrophages in our own lab [4] and by others [29], this current study further characterized and distinguished MACs from the M2-activated macrophage population (Fig. 1). Here, we have demonstrated that MACs are similar to, but distinct from M2-activated macrophages. This is based on a range of analyses, but is particularly evident based on the uniquely high expression of CD163 in MACs, both at the transcript (Fig. 1B) and protein level (Fig. 1C). The scavenger receptor CD163 is involved in the clearance of hemoglobin, and associated with anti-inflammatory signals [30]. However, evidence also suggests other roles for CD163 such as driving endothelial differentiation through FGF-2 signaling [31]. Interestingly, a report showed a high correlation between CD163 and VEGF expression in Hodgkin lymphoma [32].

We have shown previously that MACs promote angiogenesis in mature endothelial cells through secretion of paracrine factors [4, 5]. Here, we have used ECFCs, as a recognized subtype of endothelial progenitors [2], because they are an ideal human endothelial test bed for investigating angiogenic responses with enhanced sensitivity. Nevertheless, the intrinsic highly angiogenic capacity of ECFCs when compared to

other endothelial cells needs to be acknowledged when interpreting results. While MACs and M2-activated macrophages enhanced tube formation in ECFCs in a 3D Matrigel model of angiogenesis to a similar level, MACs displayed a higher level of colocalization with ECFCs compared to M2-activated macrophages (Fig. 1D), suggesting that MACs have closer engagement with tube forming cells as part of their supportive role. MACs behavior when cocultured with endothelial cells suggests a potential role for them acting as perivascular macrophages, supporting the endothelium that makes up the vasculature, in part, by sharing close immune-vascular interactions [33]. This phenomenon is under further investigation.

Other myeloid cells involved in angiogenesis include circulating monocytes, Tie-2 expressing monocytes [7], myeloid-derived suppressor cells, and tumor-associated macrophages (TAMs) [34]. In general, these endogenous myeloid cells are proangiogenic and like MACs, they have potential to be used to achieve therapeutic angiogenesis, although it should be noted that TAMs are mainly associated with tumor sites where they are recruited to secrete factors that promote tumor angiogenesis. In the case of MACs, the current study suggests that their development for autologous cell therapy should be approached with caution since there is evidence to suggest that they are dysfunctional in the context of diabetes. Our data provides evidence to confirm that the proangiogenic and possibly tissue reparative properties of MACs are impaired under diabetic conditions (Fig. 2) and is in agreement with previous studies [22, 23]. We focused on simple but consistent in vitro models to elucidate molecular and phenotypic changes in MACs caused by the diabetic milieu, since it has been already reported that diabetic MACs lose proreparative properties when tested in preclinical in vivo models. First, MAC-like cells isolated from diabetic peripheral blood failed to repair ischemic vasculature in a mouse model of ischemic retinopathy, when compared to control cells from nondiabetic donors [22]. Second, MAC-like cells derived from diabetic mice, displayed an altered phenotype due to the hypoxic and inflammatory micro-environment associated with the diabetic milieu. These diabetic cells did not promote vascular repair of murine ischemic limbs, and they displayed reduced survival and proliferation, compared to nondiabetic cells [23].

The current study provides insights into the molecular mechanisms behind the angiogenic switch shown by MACs in diabetic conditions and it has identified the proinflammatory cytokine IL1β as a major mediator in reducing the proangiogenic properties of MACs when exposed to high glucose conditions. On the other hand, there is evidence that IL1β stimulates angiogenesis after ischemia [35, 36]; however, it is suggested that IL1β effect on angiogenesis is dependent on the dose and the target cell type. IL1β was also markedly upregulated in MACs isolated from type 1 diabetic patients with complications (Fig. 5). This is in agreement with previous reports showing that high glucose exposure induces expression of IL1β in macrophages and circulating monocytes [37]. Using MACs under similar conditions, we have also shown an upregulation of IL1β, along with other inflammatory markers at transcript level (Fig. 3A), and an increase in expression of pro-IL1β in protein expression (Fig. 3C). In addition, macrophages taken from biopsy specimens of type 2 diabetic wounds show a higher expression of IL1β and a lower level of pro-tissue repair cytokines when compared to nonactivated

blood monocyte-derived macrophages [38]. A similar finding is observed in this study where there is significant reduction in CD163 and CD204 expression in tissue-reparative MACs under HDG treatment (Fig. 3B).

Thrombospondin-1 (THBS1) has been described to be upregulated in diabetic MACs [39], and a recent study showed that THBS1 regulates IL1β in macrophages [40]. Therefore, further studies on the role of THBS1 in diabetic MACs are warranted. Nitric oxide (NO)-related mechanisms have also been suggested to be impaired in MACs under high glucose conditions [41, 42]. These reported NO changes in MACs under diabetes might also play a role in the defective cellular responses [43] and alterations in secretome composition. Similarly, the matricellular protein osteopontin has been reported to enhance the tissue reparative capacity of diabetic MACs [44], and studies like these are critical for the development of autologous cell therapies where both cells and the delivery site are affected by diabetes.

While the diabetic milieu influences negatively on MAC pro-vasoreparative properties, here we show evidence to demonstrate that IL1β modulation can be used as a therapeutic strategy to restore MAC functionality (Fig. 4). This is of clinical relevance because a recent clinical trial testing the anti-inflammatory therapy with canakinumab (antibody targeting IL1β) for atherosclerotic disease demonstrated that canakinumab at a dose of 150 mg every 3 months protected patients from recurrent cardiovascular events [45]. Although more research is needed to define cellular and molecular pathways for inflammation impacting angiogenesis, evidence shown in this study suggest that modulation of IL1β is a realistic clinical target for vascular repair therapies.

Although a limitation of this study was the small sample size for human blood samples (five controls and eight diabetic patients), there was no statistical significant difference in relation to age and gender among the three experimental groups. Interestingly, an important finding was that MACs have a comparable phenotype when isolated from nondiabetic volunteers and type 1 diabetic patients without microvascular complications, but then show a shift in their phenotype toward a more proinflammatory state when isolated from patients with type 1 diabetes with microvascular complications (Fig. 5). Similarly, in a study of iPS cells isolated from patients with long standing type 1 diabetes, increased DNA damage was observed only in the complications group when compared to no complications and healthy control groups [46], and this suggests that patients who have not developed complications after comparable duration of diabetes may have protective factors. In relation to this, circulating progenitor cell (CPC) levels in peripheral blood are significantly higher in patients with type 1 diabetes without cardiovascular disease and nephropathy when compared to patients with type 1 diabetes with such complications [47]. These CPCs might represent the "endogenous" MACs that neutralize the adverse effects of diabetes in the vasculature. As such, it has recently been suggested that diabetes impairs the release of reparative "endogenous" MACs/CACs from the bone marrow [48].

A study of gene expression profiling of human CD34+ cells isolated from patients with diabetes, with and without microvascular complications, also showed upregulation of IL1β in the patient groups with diabetes compared to controls without diabetes [49]. IL1β expression was increased further

in cells isolated from patients with diabetes and complications. Although MACs are not CD34+, they were first isolated and cultured from CD34+ sorted MNC [50], and such isolates have been shown to contain a high proportion of circulating monocytic cells [51].

CONCLUSION

This study has highlighted that the proangiogenic properties of MACs are lost when exposed to high glucose conditions and when isolated from patients with Type 1 diabetes with microvascular complications. With respect to this, caution must be taken before delivering these cells as an autologous cell therapy into a diabetic microenvironment. Our results have demonstrated for the first time that the high glucose driven antiangiogenic effect on MACs is due to a shift toward a proinflammatory phenotype, and identified IL1β to be a key mediator in this process. These results lay the foundation for further investigations exploring strategies to restore diabetic MACs vasoreparative function.

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AUTHOR CONTRIBUTIONS

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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