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A Biomaterial Model to Assess the Effects of Age in Vascularization

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Aging · Biomaterial · Macrophages · Vasculogenesis

Abstract
As humans age, there is an increased risk for developing age-associated diseases. Many of these diseases, such as cardiovascular disease, involve dysfunction in the vasculature. Cardiovascular disease stems from endothelial cell dysfunction and reduction in vascularization. Macrophages, prominent innate immune cells involved in orchestrating inflammation and wound healing, have a significant influence on vascularization. While much recent work has investigated the crosstalk between endothelial cells and macrophages, it is still not well defined. The interactions between the cell types are even less understood in specific disease states such as advanced age. Understanding how age influences macrophage/endothelial cell interaction is essential for understanding cardiovascular disease development in the elderly. In the polyethylene glycol (PEG)-based hydrogel system, we model the effects of age on vascularization by encapsulating endothelial cells, pericytes, and human donor macrophages. We created a biomaterial model system in which macrophages, either from young (<35 years old) or old (>65 years old) donors, interact with the modeled vasculature, termed microvessels. Confocal image analysis of vessel density, vessel length, and branch points were used to quantify microvessel growth depending on the age of the macrophage donor. Alongside this, soluble factor secretion and gene expression were evaluated using ELISA and NanoString to showcase biological mechanisms based on the age of each donor. Endothelial cells cultured with macrophages from old donors have reduced microvessel density. There also is reduced soluble factor secretion by the macrophages from old donors, which likely influenced microvessel growth. Altogether, we establish our PEG-based hydrogel vascular model as a system to evaluate patient-specific cell function as well as proposed mechanisms for how age influences microvessels.

Introduction

In 2019, there were 703 million people 65 years or over in the world. That number is projected to double to 1.5 billion by 2050 [World Population Ageing, 2019]. Aging is correlated with a lower capacity for regeneration, leading to health complications such as chronic wounds and increased susceptibility to infection [Wicke et al., 2009; Kline and Bowdish, 2016]. This increase in the elderly population presents a critical need to improve our understanding of the mechanisms of aging to better address the future increase in demand for medical care [Yazdanyar

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and Newman, 2009; Dall et al., 2013]. In advanced age, the immune system is observed to exist in a chronic, low-grade inflammatory state termed inflamm-aging [Franceschi et al., 2000a]. When the immune system remains in a low-grade inflammatory state, the ability to orchestrate the full wound healing cascade is reduced [Olivieri et al., 2018; Moura et al., 2019]. Contrary to the adaptive immune system, which is far more investigated in the context of age [Nikolich-Žugich, 2018], the innate immune system was initially thought to not be as impacted by aging [Franceschi et al., 2000b]. Recent work has characterized the effects of age on functions of the innate immune system such as reduced phagocytosis, altered phenotype, and reduced cytokine production which all contribute to the loss of functionality in aging [Hearps et al., 2012; Albright et al., 2016; Hall et al., 2016; Pence and Yarbro, 2018]. While progress has been made, the impact of aging on the innate immune response still requires more investigation, particularly in macrophage interactions with vasculature.

Macrophages exist either as differentiated monocytes or as resident in tissues throughout the body [Rees, 2010]. Macrophages coordinate the wound healing response by first releasing inflammatory factors and clearing debris. They then change phenotype to promote healing through secretion of cytokines which stimulate cell growth and extracellular matrix (ECM) repair [Minutti et al., 2017; Kim et al., 2019]. Critically, macrophages support blood vessel development following injury and during tissue repair [Fantin et al., 2010; Godwin et al., 2013]. It has been shown extensively that macrophages from aged patients have a lower capability to fully orchestrate the immune response [Albright et al., 2016; Olivieri et al., 2018; Coppari et al., 2021]. In the perpetual inflammatory conditions of macrophages associated with aging, wound healing and the growth of new vasculature are reduced [Nucera et al., 2011; Moore et al., 2017, 2018; Moore and West, 2019]. This contributes to health complications such as atherosclerosis and hypertension [Widlansky et al., 2003; James et al., 2006; Banarjee et al., 2018; Donato et al., 2018].

Vascular diseases are associated with the function of endothelial cells, the cells that line the blood vessels [Widlansky et al., 2003; Krüger-Genge et al., 2019]. It has been shown that the crosstalk between macrophages and endothelial cells is dynamic and can either incite or prevent vascular disease associated with endothelial dysfunction [Auffray et al., 2007; Fantin et al., 2010; Corliss et al., 2016; Hernandez and Iruela-Arispe, 2020]. However, the exact mechanisms of endothelial-macrophage crosstalk require further investigation with explicit consideration of age. To study these effects, immune cells must be in co-culture with endothelial cells in vitro or ex vivo, or via an in vivo model [Irimia and Wang, 2018; Silberman et al., 2021].

Here, we establish an all-human biomaterial platform that compares the effects of age on macrophage-influenced vascularization in a polyethylene glycol (PEG) hydrogel. Modeled vasculature is kept consistent between groups by using endothelial cells and pericytes. The effects of age are investigated by encapsulating endothelial cells and pericytes with macrophages from human donors younger than 35 or older than 65 years. With this system, we introduce a model system to assess the effects of age on vascular formation. Using this model system, we quantify changes in vessel formation, the differences in soluble factor secretion, and gene expression to investigate how age influences macrophage interactions with endothelial cells.

Materials and Methods

Cell Culture

All cell culture used in this work was approved through University of Florida Institutional Review Board IRB IRB202101975. Human Umbilical Vein Endothelial Cells (Lonza C2519A) were cultured in EGM-2 media (Lonza CC-3162) and passaged according to the manufacturer's protocol. Cells were used at passage 4 or 5.

Patient peripheral blood mononuclear cells were ordered from Hemacare, and monocytes were obtained by adhesion for 24 h in RPMI-1640 supplemented with 2 mML-glutamine (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), 0.1 mM sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), 50 μM 2-mercaptoethanol (Gibco), and 10% fetal bovine serum (Atlanta Biologicals). After 24 h, cells were washed twice with RPMI-1640 to remove a majority of the non-adherent cells and cultured for an additional 5 days in the culture media described above supplemented with 20 ng/mL Monocyte-Colony Stimulating Factor (M-CSF) [Rozner et al., 2009]. Monocytes were ordered from healthy male donors of European ancestry younger than 35 or older than 65 years (Table 1).

Human Brain Vascular Pericytes (ScienCell 1200) were cultured in pericyte media (ScienCell 1201) with pericyte growth supplement. Cell culture dishes were coated with 2 μg/mL poly-lactic acid 24 h before plating with pericytes.

Hydrogel Preparation

PQ (GGGPQGIWGQGK) peptide and RGDS (Arg-Gly-Asp-Ser) were conjugated to acryl-polyethylene glycol (PEG)-succinimidyl valerate (SVA) as previously described [Mann et al., 2001; Weber et al., 2006; Culver et al., 2012; Singh et al., 2014; Moore et al., 2017, 2018] to render the hydrogel cell-adhesive and enzyme-degradable (confirmed by MALDI). The gel was formed with 3.5% PEG-PQ-PEG and 3.5 mM PEG-RGDS in HEPES buffered saline

Table 1. Cell types and donor information

Cell type	Donor age, years	Self-identified ancestry	Biological sex
"Old" macrophage	65, 65, 68	European	Male
"Young" macrophage	22, 28, 21	European	Male

with 1.5% triethanolamine, 10 μM eosin-y, and 0.35% N-vinyl pyrrolidone. The cells at a ratio of 5:1:1 endothelial cells, pericytes, and monocytes were added to the polymer solution and 5 μL pipetted onto a Poly-Dimethyl Siloxane (PDMS) slab in between two 380-μm thick PDMS spacers. The droplet was then covered by a methacrylated cover slip and exposed to white light for 60 s. The coverslip with the attached PEG hydrogel was placed in a 24-well plate and filled with 1 mL of EGM-2 media.

PDMS was prepared using SYLGARD 182 A and B at a 10:1 ratio, desiccated for 1 h, then baked at 65°C overnight. Coverslips were methacrylated by first cleaning with piranha solution (4:1 ratio of hydrogen peroxide and sulfuric acid) for 1 h, then rinsed with 200 proof ethanol, 190 proof ethanol, and 140 proof ethanol successively. The cover slips were then incubated in TMPSA to methacrylate for 3 days on a rocker at room temperature. They were then rinsed with 140 proof ethanol and wiped dry with Kimwipes and stored at 4°C under Argon protected from light.

The encapsulated cells were incubated at 37°C in EGM-2 media for 7 days, the media was changed after 24 h, then every 48 h after that. On day 7, the gels were either fixed for imaging or digested for RNA or soluble factor isolation for NanoString® and ELISA, respectively.

Immunocytochemistry

Cells were fixed using 4% paraformaldehyde at room temperature for 45 min, followed by rinsing with 1× tris buffered saline (TBS). Cells in hydrogels were permeabilized with 0.25% Triton-X in TBS for 45 min and rinsed with TBS, then blocked with 5% donkey serum in phosphate buffered saline (PBS) overnight at 4°C. Cells in hydrogels were rinsed for 5 min the following day and primary stained with CD31 rabbit anti-human at 1:200 dilution in 0.5% donkey serum in PBS for 24 h at 4°C. Gels were rinsed the next day 6 times in 0.01% Tween and PBS, with only TBS as the final rinse for 1.5 h per rinse. The cells were stained with Alexa Fluor 555, donkey anti-rabbit, to label CD31 on endothelial cells at 1:200 dilution in 0.5% donkey serum in PBS for 48 h.

Samples were imaged on the Zeiss LSM 980 Confocal Microscope. Ten 70-μm thickness Z-stacks were taken per gel with 3 gels per donor and $n = 3$ donors per condition. Orthogonal projection process using Zeiss Blue software was used to generate a single 2D image of each Z stack. Vessel density was quantified using ImageJ Vessel analysis package on the orthogonal projection images. Vessel length was quantified using ImageJ measurement tool and the average length of each microvessel in an image was recorded. The microvessel length for each image in a gel was averaged then included as a data point for statistical analysis. Statistics are discussed in the Statistics section. Branch points were defined as any intersection of 2 or more individual microvessels. They were counted under blind review by hand, recorded for each Z stack image, then averaged for each gel.

NanoString® Gene Expression Analysis

Hydrogels were broken down in 50 μL of 25 mg/mL collagenase at 37°C for 30 min. The collagenase, polymer, cell solution was added to Qiagen RNeasy Plus Micro Kit for RNA isolation. RNA was stored at −80°C until use. Purity and concentration of RNA was assessed using BioTek Synergy HT plate reader with an accepted purity (260/280) between 1.9 and 2.1. All RNA solutions were diluted to 30 ng/mL RNA as directed by NanoString® protocol.

The gene expression analysis for $n = 3$ biologically independent samples was conducted with the nCounter® Myeloid Innate Immunity V2 Panel (XT-CSO-MIP1-12, NanoString Technologies). Based on mRNA quantification, 50 ng of mRNA per sample was added to a barcoded probe set reagent and hybridized for 20 h at 65°C according to the manufacturer's protocol. NanoString data were processed with the nSolver 4.0 software kit according to the manufacturer's protocol. Differentially expressed genes ($p < 0.05$ and positive fold change) for each sample group were used for further analysis. Differential gene expression analyses of each gene were performed using nSolver NanoString software. Pathway analysis was conducted from the annotated gene set global significance score (calculated as the square root of the mean squared t-statistics of genes, informed by the gene expression analysis) [Wang et al., 2016; Bhattacharya et al., 2021]. Similar to ELISA, these data are used to evaluate the gene expression profile of the elderly patient cells compared to younger controls to highlight the gene expression differences between the populations.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was used to quantify the following inflammatory soluble factors in the system: TNF-α, IL-6, IL-8, GM-CSF, INF-γ, and MCAF (MyBioSource Cat# MBS590064). In order to release the secreted soluble factors from inside the hydrogel, the soluble media was saved on day 7, and 25 mg/mL collagenase was added to the hydrogels for 30 min at 37°C. The collagenase and digested gels were added back to their conditioned media. The samples were stored at −80°C. For each sample $n = 3$ there were 3 experimental replicates and 2 technical replicates run on different plates. A standard curve for each factor was generated using 3 standards at 1:2, 1:4, and 1:32 dilution per manufacturer recommendation.

Statistics

For imaging, we had an $n = 3$ for 2 groups: Old: HUVECs, HPs, and macrophages from donors older than 65 years, and Young: HUVECs, HPs, and macrophages from donors younger than 35 years. Differences between these groups were compared using a Student's t-test with $p < 0.05$ defined as significant using GraphPad Prism 9.

Gene expression: Pathway analysis was conducted from the annotated gene set global significance score (calculated as the square root of the mean squared t-statistics of genes, informed by the gene

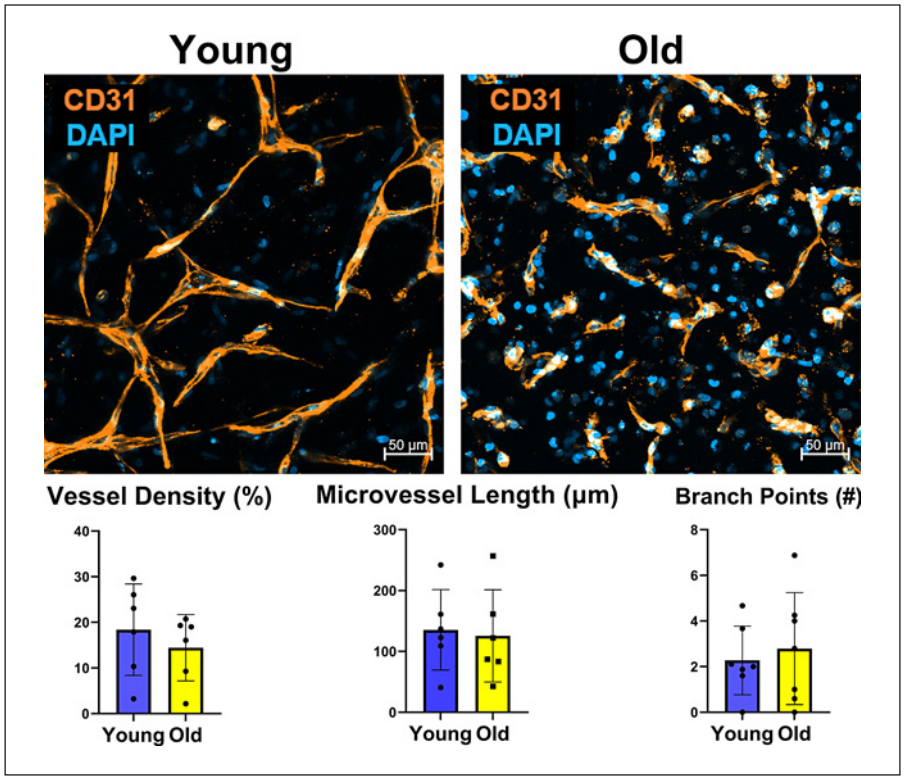

Fig. 1. Confocal images of endothelial cell (CD31, orange) forming microvessels inside polyethylene glycol-based 3D hydrogels in co-culture with endothelial cells, pericytes, and donor macrophages (Young <35 years old, Old >65 years old). Vessel density percentage was quantified using ImageJ's Vessel Analysis Package (p = 0.46). Average vessel length was quantified using ImageJ's measurement tool (p = 0.81). Branch points were counted by hand with a single blind counter (p = 0.64). All 3 methods compared Old versus Young data using a Student's t-test, statistical significance at $p < 0.05$. Scale bars, 50 µm.

expression analysis). Post-hoc correction using Benjamini-Yekutieli.

Soluble factor array: $n = 3$ for 2 groups Old and Young, each sample had 2 technical replicates that were averaged before analysis per manufacturer's recommendation. Differences between soluble factor concentration analyzed by Student's t-test with $p < 0.05$ defined as significant using GraphPad Prism 9.

Results

Macrophages from Old Donors Microvessel Density and Length in PEG Hydrogels

After encapsulation, endothelial cells, pericytes, and human donor macrophages were cultured for 7 days. Samples were then fixed, stained, and imaged for microvessel quantification. Endothelial cells were stained for CD31. Figure 1 shows representative images of microvessel formation encapsulated with macrophages from Young or Old donors in PEG hydrogels. We quantified microvessel formation through vessel density, vessel length, and branch points using ImageJ. Endothelial cells cultured with macrophages of Young donors had on average higher vessel density (Young 18.39 ± 9.1%, Old 14.45 ± 6.6%, p = 0.46). However, between the groups there was minimal difference in the average length of microvessels (Young 135.4 ± 60.4 µm, Old 125.6 ± 69.1 µm, p = 0.82). Also, endothelial cells with macrophages from Old donors had more branch points (Young 2.27 branch points ± 1.4, Old 2.79 branch points ± 2.3, p = 0.64). This indicates that not only do macrophages influence endothelial cell growth in this system, but the age of the macrophage donor also correlates with decreased vessel density.

Age of Macrophage Donor Alters Gene Expression

Gene expression of all cells encapsulated in the hydrogel was evaluated between the 2 experimental groups and compared using NanoString Innate Immunity V2 panel. Figure 2a shows that most genes have varied expression depending on the macrophage donor. The x-axis of this plot is generated by comparing the amount of mRNA associated with a gene between the Young and Old groups. The value on the x-axis is calculated by taking the average gene expression in the Young group and dividing it by the average gene expression of that gene in the Old group, then calculating the log2 of that value. Negative values mean that the gene is downregulated in the Young group, positive means the gene is upregulated in the Young group. Depending on the distribution of the samples, a p

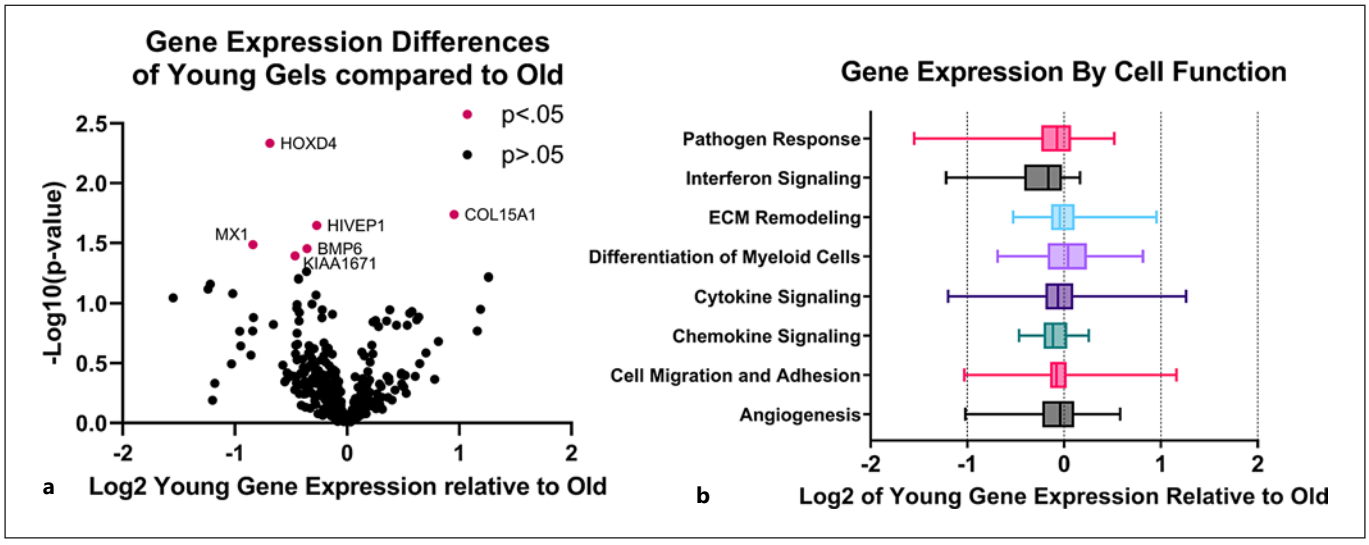

Fig. 2. Gene expression data comparing the mRNA content tri-culture with macrophages encapsulated in the PEG-based hydrogel. **a** Volcano plot of every gene in the NanoString Myeloid Innate Immunity Panel. Genes in pink signify $p < 0.05$. **b** Box and whisker plot showcasing general trends of gene expression of genes associated with the labelled cellular processes. Dark center line in each box represents the median, the box represents the middle 50% of genes, and error bars represent the min and max fold change expression. Some cellular processes such as cytokine and interferon signaling are notably downregulated.

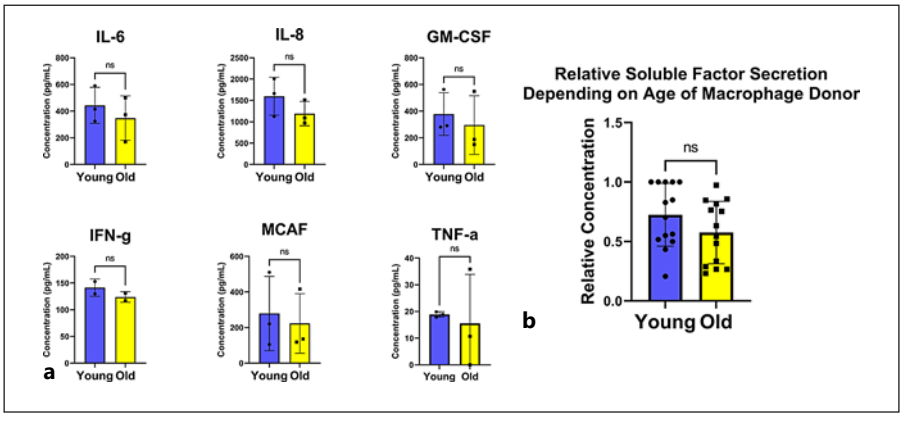

Fig. 3 a Enzyme-linked immunosorbent assay (ELISA) from MyBioSource Inflammatory Cytokine array was used to characterize the macrophage response in the hydrogel depending on the age of the macrophage donors. **b** Relative soluble factor secretion comparing Young and Old. $n = 3$ donors per group. Comparisons between Old and Young were made using a Student's *t*-test. Significance at $p < 0.05$.

value is generated for each gene. The –log10 of each *p* value is calculated, then used for the *y*-value of the gene in the plot. This means values with a lower *p* value (more statistically significant results) will be higher on the *y*-axis, thus creating the volcano plot. Notably though, few genes have a $p < 0.05$, but trends in gene-associated cell processes can still be investigated. Of the significant genes, *HOXD4*, *MX1*, *HIVEP1*, *BMP6*, and *KIRAA1671* all are significantly downregulated in Young compared to Old. *COL15A1*, however is upregulated in Young compared to Old.

To generate the plot in Figure 2b, all genes were categorized into their corresponding cell function as established in the NanoString panel. All the genes associated with a cell function were compared based on their expression in the Young system relative to the Old. The boxes in each group represent the distribution of the middle 50% of genes, and the dark line in each box represents the median expression. Genes associated with cell processes such as cytokine signaling are shown to be downregulated in the cells in tri-culture with the young donor macrophages. Other notable processes affected by age of mac-

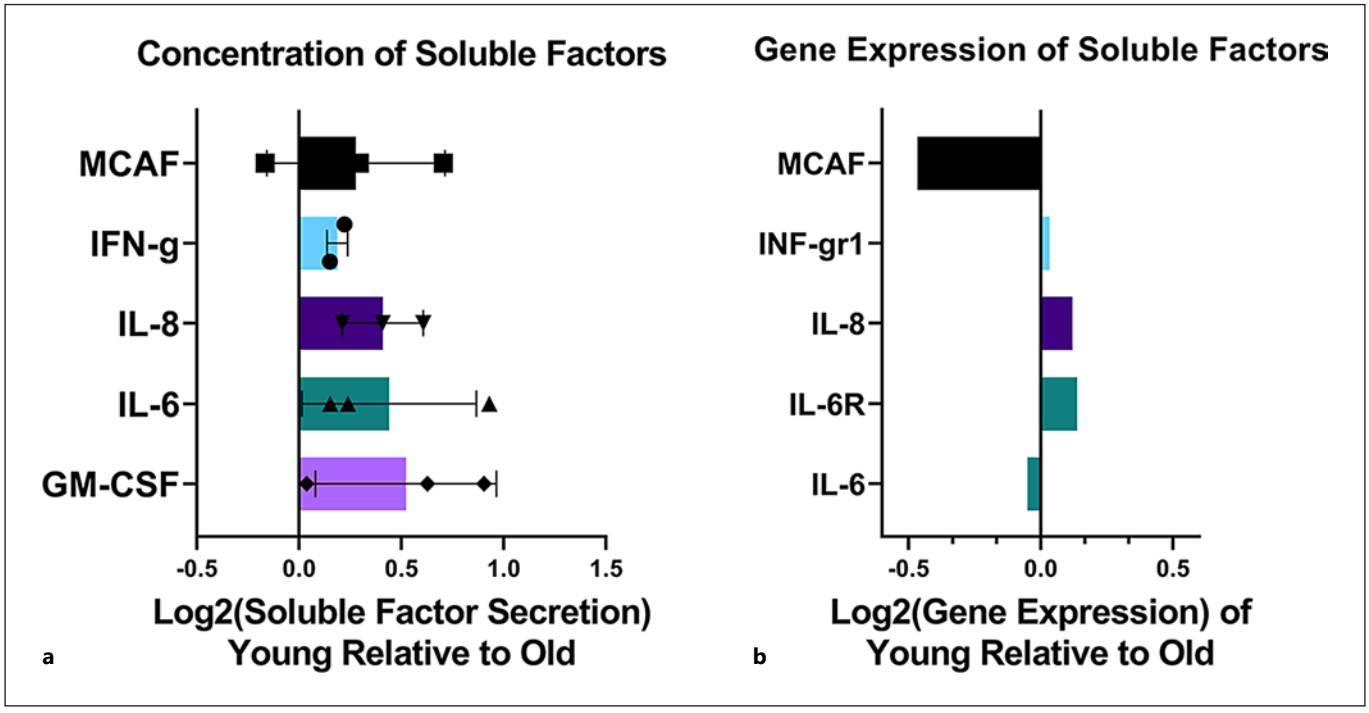

Fig. 4. a Relative concentration of soluble factors from Figure 3 compared to the relative gene expression of the genes associated with these soluble factors (**b**).

rophage donor are decreasing interferon signaling, angiogenesis, and cell migration and adhesion expression in Young. On the other hand, genes associated with the differentiation of myeloid cells are slightly downregulated on average in the Old samples.

Macrophages from Old Donors Have Reduced Cytokine Secretion

Following tri-culture with different donor macrophages, we lysed the hydrogel to analyze the soluble factor expression profile of the encapsulated cells. Concentration of interferon-γ (IFN-γ), monocyte chemotactic-activating factor (MCAF also known as MCP-1), interleukin (IL)-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNFa) were all measured and compared between hydrogels with macrophages from Young or Old donors. No individual cytokine showed a statistically significant change on its own, however, overall cytokine secretion from the Old group was lower than from the Young. IL8 and IFN-γ had more significant differences in concentration than most with the difference between means ± standard error of the mean of IL8 at 407 ± 303.9 pg/mL and IFN-γ at 17.49 ± 13.47 pg/mL.

Figure 3b compares the overall cytokine expression between the Young and Old groups. To account for every cytokine equally, the concentrations of each sample for each cytokine were normalized to the sample with the highest concentration. The difference in total cytokine secretion between Young and Old had a p value of 0.15. Samples with macrophages from young donors had on average a relative concentration of 0.725 ± 0.3 pg/mL compared to 0.576 ± 0.3 pg/mL for samples with macrophages with old donors.

Soluble Factor Secretion Trends Do Not Match mRNA Expression of Associated Genes

Figure 4A plots the log2 of the cytokine secretion in samples with macrophages from Young donors relative to sample with macrophages from Old donors. On average, there is a higher concentration of cytokines from samples with macrophages from Young donors. TNFα was left out of this analysis, as its concentration was below a level where relative results are accurately obtainable. These observations are compared with Figure 4B, where the expression of genes associated with production of these cytokines are compared to their concentration. All soluble factors on average are at a higher concentration

in the hydrogels with macrophages from young donors. The gene expression, however, shows minimal increase in the mRNA associated with these genes present. Notably, MCAF has an inverse relationship between its relative gene expression and concentration. There is not an observed correlation between gene expression of the soluble factor and its concentration.

Discussion

In this work, we demonstrate the effects of macrophage donor age on endothelial cell microvessel formation in a 3D hydrogel system. Looking at the visual changes of the endothelial cells, we connect the increase in vessel density and length of ECs to the source of the macrophages. Endothelial cells cultured with macrophages from donors older than 65 had reduced vessel density than endothelial cells cultured with macrophages from donors younger than 35. The average microvessel length was slightly higher in the Young samples, but not statistically significant enough to make any claims with this data. The number of branch points was higher in Old samples compared to Young. One potential reason is that the lower average length of the microvessels results in a higher density of branch points in a microvessel system. For the image data, it is difficult to discern significant trends in the endothelial cell growth. The variance in donor samples, lack of stimulating factors, and cell ratio all contributed to clouding any observable changes in microvessel morphology [Pereira et al., 2010; Wang et al., 2017].

In related studies, we studied the effects of polarization on endothelial cell vascularization in 3D hydrogel coculture of primary mouse macrophages and endothelial cells [Moore et al., 2018]. We found that LPS/IFNγ stimulated, M1 macrophages reduced microvessel volume significantly compared to M0 or M2 stimulation [Moore et al., 2018]. Similarly, Graney et al. [2020] investigated the effects of macrophage phenotype on vascularization in transwell coculture. Macrophages were stimulated towards M1, M2a, M2c, and M2f to characterize endothelial cell behavior with varied macrophage phenotypes. They observed a significant shift in the gene expression of endothelial cells depending on the macrophage phenotype. The results obtained from the experiments in this study are more muted compared to the macrophage-endothelial cell coculture experiments referenced. The significant difference between our results and those studies is the use of stimulating factors [Spiller et al., 2014; Moore et al., 2017, 2018; Graney et al., 2020; Wang et al., 2021]. In our study, all macrophages are cultured the same way and are from human donors. This both makes the comparisons more physiologically relevant, but also more variable depending on the donor. The experimental design of this model development based initial estimates on methods used in the literature of $n = 3$ donors per group [Spiller et al., 2014]. Although there were not many statistically significant differences, we are still interested in the trends that introduce new elements of age influence on macrophage-endothelial interactions.

Commonly, when stimulatory factors are used to artificially induce a macrophage phenotype, a concentration higher than what would happen physiologically is used [Ferraro et al., 2017; Orecchioni et al., 2019; Sommerfeld et al., 2019; Whitaker et al., 2021]. This ensures that enough of the macrophage population is affected but decreases the physiological relevance of the model. In our PEG tri-culture hydrogel, the macrophages used were not stimulated towards M1 or M2. This isolates the effects of age on macrophages in their influence on microvessel growth, soluble factor secretion, and gene expression.

To evaluate what cellular processes differ with age, gene expression of tri-culture was measured. In these experiments, the mRNA assessed is from all 3 cell types, and the differences in donor age are assumed to be associated with the variation in gene expression. If the gene expression difference is from the endothelial cells or pericytes, we assume that variation is caused by interactions with the different sources of macrophages in culture. Figure 2a displays every gene in the panel and their varying expression levels depending on the age of the macrophage donor. There are 6 genes significantly differentially expressed depending on the donor age of the macrophages in the tri-culture. *HOXD4* is the most significantly affected gene between the groups and is downregulated in Young samples compared to Old. *HOXD4* belongs to the homeobox family of genes which regulate development of multicellular organisms [Krumlauf, 1994]. In the context of vascularization, these genes are understandably significant in the growth of new vasculature as nearly every cell in the human body is at most 200 μm away from a blood vessel [Radisic et al., 2005]. HOXD4 in particular is associated with perturbations in vasculogenesis when its function is decreased in zebrafish [Amali et al., 2013]. The fact that HOXD4 is more highly expressed in Old samples is interesting considering there was less vasculature growth.

Similarly, BMP6 was downregulated in Young samples. The proteins associated with BMP6, TGFβ, and

SMAD have signaling processes that guide endothelial cells. This is done through crosstalk between bone morphogenic proteins and vascular endothelial growth factor receptor 2 (VEGFR2) [Pulkkinen et al., 2021]. BMP6 is suggested to be pro-angiogenic which again theoretically is inversely related to the observations made in Figure 1 [David et al., 2009; Pardali and Dijke, 2012]. These trends appear to hold true for all of the significantly downregulated genes in Young samples.

COL15A1 is the single gene significantly upregulated in Young samples compared to Old. *COL15A1* codes for type XV collagen, which with cleavage of the C-terminal of NC1 domain produces restin [Li et al., 2000]. Restin is associated with inhibiting endothelial cell migration and exhibits anti-angiogenic properties [John et al., 2005; Walia et al., 2015]. Despite *COL15A1's* associating with anti-angiogenesis, Young samples had increased microvessel density despite the upregulation of *COL15A1*. Diving deeper, Rygh et al. [2014] use *COL15A1* knockout mice to indicate that knockout of *COL15A1* does alter microvessel function. They state that collagen XV, and COL15A1 by extension, have important roles in the maintenance of vessel wall integrity. The observations made in Figure 1 support this claim, since Young samples have higher microvessel density. Endothelial cells likely have reduced collagen XV production when in coculture with macrophages from old donors. The production of collagen XV may be influenced by age and should be investigated further to see how exactly macrophages influence collagen XV production in endothelial cells.

Figure 2b sorts the genes investigated in the panel into groups that correspond to the cell function they are associated with. From the trends shown in Figure 2b, many processes associated with endothelial vessel growth are altered depending on the source of the macrophages. One major mechanism macrophages use to influence in vessel growth is the secretion of cytokines [Rymo et al., 2011; DeFalco et al., 2014; Hsu et al., 2015]. Interestingly, the gene expressions of genes associated with cytokine secretion are downregulated in the gels with macrophages from young donors. This directly contradicts Figure 3b, where cytokine concentration is higher in samples with macrophages from young donors. Age-induced reduction of cytokine secretion is seen in the literature, but the causes behind it are still unclear [Rea et al., 2018]. Figure 4 directly pairs cytokine concentration to the corresponding gene expression of Young samples compared to Old. With the information available, we suggest that this is a result of an age-induced decrease in cytokine production capability. However, much is still unknown as well about tying gene expression to protein production. The answer may lie in investigating age influence on post-translational modifications (PTMs). Battle et al. [2015] concluded that the PTMs are most strongly correlated with what causes a gene to be transcribed. Specifically in age, Santos and Lindner [2017] reviewed PTMs and how they correlate with age and age associated disease. They stated that PTM dysregulation is associated with aging, largely through the presence of metabolites and free radicals [Kikis Elise and Gidalevitz, 2010; Gorisse et al., 2016]. One example is the increased level of reactive oxygen species in age, which increases the number of free radicals present and disrupts amino acid side chains [Zhang et al., 2016].

Prolonged inflammatory conditions are commonly associated with reduced endothelial cell growth [Moore et al., 2018; Graney et al., 2020; Hernandez and Iruela-Arispe, 2020]. However, inflammatory soluble factors such as IFN-γ, MCAF, and GM-CSF are all at a higher concentration in the samples with macrophages from young donors. Alongside this, on short time scales, pro-inflammatory macrophages have been shown to support microvessel growth [Spiller et al., 2014]. This difference in functionality could be from the fact that macrophages from young donors are more able to produce cytokines compared to macrophages from old donors as seen in Figure 3b and in literature [Rea et al., 2018]. Given that cytokines play a large part in macrophage-endothelial cell cross talk, differences in cytokine concentration would affect microvessel growth [Hernandez and Iruela-Arispe, 2020].

From these findings it is clear that the processes involving vascularization are interconnected, yet sometimes contradictory. In this preliminary study, we investigated microvessel growth in relation to the source of macrophages co-culture in the context of gene expression and soluble factor analysis. We identified several established biological mechanisms associated with age such as reduction in cytokine secretion and variances in mRNA presence for genes associated with vascularization. Altogether, we conclude that there is likely a reduction in the efficiency of gene expression to protein synthesis in macrophages from older donors, and this decreases the ability for macrophages from older donors to support microvessel growth. The functions identified all have potential to uncover more about the aging process and vascularization. The model system introduced demonstrates ability to interrogate donor macrophage influence on blood vessel development in vitro. We also acknowledge that this preliminary work only looks at the variable

of age and controlling for donor self-identified ancestry and biological sex. Future work will seek to address this limitation and utilize this model system to investigate the contributions of ancestry and biological sex.

Statement of Ethics

For the use of our cells, we were granted approval from the University of Florida Institutional Review Board via IRB IRB202101975 for the human endothelial cells and monocyte/macrophages used in this work.

Conflict of Interest Statement

No conflict of interest declared.

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Author Contributions

Justin Silberman: Data collection and analysis, manuscript writing, design of experiments, interpretation of the data. Erika Moore: Manuscript editing, conception of the work, interpretation of the data. Talia Abbate: Data analysis. Jessica Boehlein: Data analysis.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

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