Investigating the Roles of Macrophages in Vessel Development Utilizing Poly(Ethylene Glycol) Hydrogels

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Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2018
ABSTRACT

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Abstract

Macrophages, key cells of the immune system, are often present at active sites of angiogenesis. It has been found that macrophages can play a critical role in supporting blood vessel development as the removal of these cells results in impaired vessel development. Because of the supportive role macrophages can play in vessel formation, macrophages can be considered as a novel cell source to support vessel development.

Within the field of tissue engineering, one major limitation towards the development of large scale tissues is the need for vascularization of the tissues to support oxygen and nutrient demands. The need for vascularization combined with the roles of macrophages in vessel development introduce a unique opportunity to utilize cells of the immune system (in this case, macrophages) to support vessel development within tissue engineered constructs. In this thesis, we identify the roles of macrophages in vessel development utilizing a cell-adhesive and proteolytically-degradable poly(ethylene glycol) (PEG)-based hydrogel.

In our initial studies, we introduced the notion that macrophages enhance vessel formation of endothelial cells when both cells are simultaneously encapsulated into the PEG-based hydrogel. We next assessed the macrophage response to the presence of endothelial cells in our PEG-based hydrogel. Macrophages became more spread depending on the density of endothelial cells they were encapsulated with. We found
that a 1:1 ratio of endothelial cells to macrophages resulted in the most spread population of macrophages within the PEG-based hydrogels. Macrophages also closely associated with endothelial cells in a proximity dependent manner; macrophages closest to endothelial cells were more spread than macrophages further away from the endothelial cells. We next classified the types of associations seen between the macrophages and the endothelial cells: macrophages closely associating with endothelial cells and macrophages bridging neighboring endothelial cells. The close association seen between the macrophages and endothelial cells mimics the close contact seen between endothelial cells and support cells. The bridging association seen mimics the cell-chaperoning behavior of macrophages during in vivo vessel formation. It has been seen that macrophages can physically connect two endothelial tip cells, thus acting as the cell-chaperone. The bridging association seen in this work complements the cell-chaperone behavior seen in vivo.

This work also explored the roles of macrophage phenotypes in governing the role of macrophages in vessel formation. Macrophages are highly plastic cells that alter their function based on environmental cues. There are two main paradigms of macrophage phenotypes: M1, pro-inflammatory macrophages, and M2, pro-tissue healing macrophages. This work explored the roles of macrophage phenotypes to vessel formation in the PEG-based hydrogels. M0 and M2 macrophages were found to support vessel development when encapsulated with endothelial cells. M1 macrophages
significantly retarded vessel formation when encapsulated with endothelial cells. The endothelial cell and M2 macrophage co-culture secreted VEGF while the M1 macrophages retarded endothelial cell proliferation.

Due to the diverging effects of macrophage phenotype on vessel formation, we developed of PEG-based hydrogels capable of presenting a stimulating microenvironment to macrophages and endothelial cells. We found that a M2 stimulating hydrogel enhanced vessel formation when endothelial cells and macrophages were encapsulated in the hydrogel.

Overall, this dissertation demonstrates the role of macrophages in supporting vessel formation in PEG-based hydrogels. Findings in this thesis have helped to elucidate the diverging roles of macrophage phenotypes in supporting vascularization of PEG-based hydrogels. Moreover, this work has created PEG-based materials can be manipulated macrophage phenotype. This work highlights the usefulness of macrophages in vessel development and the usefulness of a macrophage-directing platform to enhance vascularization of tissue engineered constructs.
Dedication

“In all thy ways, acknowledge Him and He shall direct thy paths.” Proverbs 3:6.

For Dewayne Junior, Brenda Cheryl and Hattie Lee, thank you for being my giants.
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1. Introduction

1.1 Motivation

Currently, there are over 120,000 people on the waitlist for organ donations in the United States [1]. Each year, more patients are added, while the availability of donated organs remains relatively constant [1]. To meet the need for replacement organs, the field of tissue engineering endeavors to create tissues capable of replacing damaged organs in the body [2].

While there has been success in certain areas of tissue engineering (such as the skin and bladder [3], [4]), oxygen and nutrient demands limit the clinical impact of the field to thin avascular tissues [5]. Large tissues, such as the heart, liver, kidney or lung, are currently unable to be engineered due to oxygen and nutrients demands at distances >200 μm. Large engineered tissues, thus, experience cell death and loss of function. To overcome the current size limitations, vascularization is required to provide oxygen and nutrients. Specifically, microvasculature within the engineered tissue will allow synthesis of larger complex tissues [2], [6], [7].

To meet the need for vascularized tissue engineered constructs, a variety of biomaterials, cell types and culture conditions have been investigated [8] (Figure 1.1). Within the context of cell types for vascularization, endothelial cells are the basis of vessel structures. Support cells (pericytes or smooth muscle cells) are often co-cultured with endothelial cells to stabilize the vessels [9]. This work will investigate the use of
macrophages as a novel support cell source that may actively foster formation of microvascular structures rather than just supporting them to prevent regression.

Figure 1.1. Formation of Vascularized biomaterials. In order to form vascularized tissue constructs vascular cells (typically endothelial cells and support cells) are combined with biomaterials. Ideally, vascularized tissues are formed.

Macrophages, cells of the innate immune system, are inherently present at implants sites and throughout most tissues [10]. Recent in vivo work has also demonstrated macrophages are present at active sites of vessel development [11], [12]. Macrophages are seen as key regulators in enhancing vessel sprouting [11]; they serve as cell-chaperones to guide the anastomosis of tip cells [12]; they secrete pro-angiogenic growth factors [11], [13]; and they have been seen closely aligning with nascent vasculature in a manner similar to support cell association [14]. Thus, macrophages may serve as a novel cell source within the context of vascularized engineered tissues.
Because the in vivo interactions between macrophages and endothelial cells occur in highly complex microenvironments, there is a need to study macrophage-endothelial cell associations within a reductionist system. This system will better define the roles macrophages can play in vessel development within the context of tissue engineering [15]. Moreover, macrophages are known to possess varying functions, typically dictated by their phenotype [16]. It is currently not well known what roles each phenotype plays in governing macrophage influences on endothelial cells [15]. Thus, this proposal also seeks to clarify the roles of macrophage phenotypes with regards to vessel development.

1.2 Tissue engineered microvasculature

Microvasculature development occurs via angiogenesis or vasculogenesis. Angiogenesis is the formation of new blood vessels from a previously established blood vessel [2]. It is a complex process usually initiated in the presence of a stimulus (specifically, angiogenic growth factors such as vascular endothelial growth factor (VEGF) [17]). These growth factors result in the sprouting of endothelial cells. As endothelial cells sprout from the established blood vessel, they secrete matrix metalloproteases (MMPs), particularly MMP-2 and MMP-9, to degrade the surrounding basement membrane and allow migration [18]. Within this process, one endothelial cell will become a tip cell while the endothelial cells that follow are labeled as stalk cells. The tip cell migrates to the area of stimulus and is responsible for the formation of the leading edge of a new capillary [19]. Alternatively, vasculogenesis is the de novo
formation of blood vessels usually from endothelial progenitor cells (EPCs) [20]. The process of vasculogenesis also occurs in response to a given stimulus (typically growth factors) and results in the fusion and organization of endothelial cells into capillary structures. In both processes, mural cells, typically pericytes or smooth muscle cells, are recruited to stabilize the nascent vasculature via focal contacts with the endothelial cells [9] (Figure 1.2).
Figure 1.2 Vasculogenesis and angiogenesis form blood vessels. A) Vasculogenesis begins with endothelial progenitors which undergo lumen formation to form blood vessels. B) Angiogenesis is the formation of blood vessels from an established blood vessel. Vascular sprouting creates new blood vessels. These vessels are stabilized by the recruitment of pericytes which wrap around the endothelial lumen. Image adapted from: Moon et al.[21].

Within the context of tissue engineering, both vasculogenesis and angiogenesis have been considered to encourage the vascularization of tissue engineered constructs. To form microvasculature, endothelial cells and support cells, such as smooth muscle
cells, pericytes and fibroblasts have been co-cultured in biomaterials under varied culture conditions. Over time, spontaneous formation of microvessels can occur in which endothelial cells form lumenized structures and support cells wrap around the nascent vessels. Common cell types that are used for the endothelial cell sources include: human umbilical vein endothelial cells (HUVECs), EPCs and induced pluripotent stem cells (iPSCs) [22]. Support cell sources range from smooth muscle cells, mesenchymal stem cells (MSCs), adipose-derived stem cells (ADSCs), and primary pericytes [22]. More recently, autologous and/or primary cell types have been employed due to clinical relevance [5].

To form microvascular constructs, the cells of interest are cultured within or on biomaterials of interest. There are three main categories of biomaterials: natural, synthetic and hybrid materials. The main purpose of these materials includes providing physical support and serving as a source of biochemical cues to facilitate cell adhesion, cell migration and tissue formation [23].

Common natural materials that have been used include collagen, fibrin, and a reconstituted basement membrane extract known as Matrigel™ [24]. Natural materials have been able to support the formation of microvessels due to their inherent bioactivity [25]. For example, Matrigel is commonly used to assess tubule formation of endothelial cells both via 2D seeding and 3D encapsulation [26] (Figure 1.3). However, there are several limitations to the use of natural materials: rapid clearance once implanted into
the body, immunogenic risk, and inherent batch to batch variability [27]. In the case of Matrigel, each lot is known to vary dramatically and thus the cellular response in culture with the material also varies [28].

**Figure 1.3 Matrigel has been used as a natural material to form vessel networks.** Endothelial cells have been seeded on Matrigel to assess vessel formation in 2D. Endothelial cells have also been encapsulated within Matrigel to assess vessel formation in 3D.

To overcome the challenges posed by natural materials, researchers have explored synthetic materials. Synthetic materials are advantageous as they can provide greater control over degradation kinetics, mechanical properties as well as limited batch to batch variability [22], [29]. Synthetic materials are intrinsically biologically inactive. These materials require bio-activation in order to support cell adhesion. In order to acquire bioactivity, synthetic materials mainly rely on the uncontrolled and passive process of protein adsorption [30]. Protein adsorption occurs at the interface of the synthetic material and biological fluids. Proteins will adsorb onto the surface of the
material in a largely disorderly manner based on ionic interactions, hydrogel bonding, and hydrophobic interactions; this adsorption limits the ability to selectively render a synthetic material bioactive. Hybrid materials are composed of both synthetic and natural components. Hybrid materials introduce biomaterials capable of customizable mechanical and biochemical properties. In particular, hybrid materials take advantage of the natural material properties which usually entails exploiting cell adhesive and cell degradative properties of natural. Hybrid materials also take advantage of synthetic materials which usually entails exploitation of mechanical and structural properties of synthetic materials.

1.2.1 Hydrogels for tissue engineered microvasculature

Within the context of tissue engineered microvasculature, hydrogels are of keen interest due to their tissue-like properties, which includes their high water content and biocompatibility [31]. Hydrogels are comprised of hydrophilic polymers and water. Upon crosslinking, water constitutes more than 90% of the mass in hydrogels [31], [32]. Hydrogels are permeable, allow rapid mass transport and closely mimic soft tissue in vivo conditions [33], [34]. Common hydrogels used include: hyaluronic acid, chondroitin sulfate, alginate, fibrin, collagen, Matrigel, poly(acrylamide) (PAAm), poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) (Figure 1.4). It is of note that there are both natural and synthetic hydrogels. As previously discussed, the same
limitations for both natural and synthetic materials also apply to hydrogels. Thus, work in the field of tissue engineering has explored hybrid hydrogels at length [35].

![Chemical structures of common hydrogels](image)

**Figure 1.4. Chemical structures of common hydrogels.**

Specifically, PEG hydrogels have been extensively investigated for tissue engineering applications. Within our lab, we have developed a PEG bioactive hydrogel system in which peptides are covalently incorporated onto PEG chains, creating PEG-biomolecule composites which are able to form a PEG-based hydrogel [36]–[39] (See Chapter 2). Within this system, we have demonstrated the spontaneous formation of microvasculature following the encapsulation of endothelial cells and pericytes (Figure 1.5). These cells self-assemble into tubule structures in which pericytes wrap around the endothelial cells as they form lumenized structures [36]. Because this hydrogel system supports the formation of microvasculature and allows control over cell-material interactions, this system will be manipulated for this work.
Figure 1.5 Microvascular structures form in bioactive PEG based hydrogels. HUVECs encapsulated with human brain vascular pericytes (HPs) form tubule structures in PEG hydrogels. Day 5. 10% PEG-PQ-PEG, 3.5 mM PEG-RGDS. Green = CD31 (endothelial cell marker), red =αSMA (pericyte marker) and blue =DAPI (nuclear marker). Scale bar = 100 µm.

1.3 Macrophages

As previously mentioned, current standards of generating vascularized tissue engineered constructs rely on endothelial cells and a secondary support cell. Within this work, macrophages are considered as a secondary support cell. Macrophages are cells of the immune system. They are innate immune cells that invade wound sites often after neutrophils. As inferred from the Greek-derivation of the name makros-phagein, macrophages are traditionally known as phagocytes [40]. Macrophages initiate and control the inflammatory response of the body acting as key mediators of the innate immune system [41]. Macrophages are also thought to be the connection between the
innate immune system and the adaptive immune system; they phagocytose debris and present the antigens to B and T cells, thus eliciting a response from the adaptive immune system.

Macrophages are classically thought to be derived from monocytes. However, recent work has demonstrated macrophages are also derived from the yolk sac. These cells are distinct in function from macrophages derived from the bone marrow (see Figure 1.6) [42]. Macrophages from the yolk sac are known as resident macrophages. These cells often populate specific tissues prior to the development of bone-marrow macrophages. Resident macrophages arise from erythro-myeloid progenitors (EMPs) and populate the brain (as microglia), liver (as Kupffer cells) and skin (as Langerhans) [43], [44]. These cells are highly proliferative, maintain their own population in resident tissues (largely independent of any bone marrow macrophages) and orchestrate tissue homeostasis [43].

Bone marrow macrophages arrive on the developmental scene later than resident macrophages. These cells are known as hematopoietic stem cell-derived (HSC-derived) or transitory macrophages; they can populate adult tissues with age (but are largely present in tissues with rapid turnover, such as the intestine) [43], [45]. Studies have compared resident macrophages to bone-marrow macrophages; they have found distinct morphological, phenotypic and proliferative differences between the groups [46]. While more work is required to better understand the varying functions of
macrophages from the yolk sac in comparison to those from the bone-marrow, it is known that resident macrophages involved in organogenesis are fundamentally distinct from bone-marrow macrophages involved in adult wound healing [10], [47]–[49].

![Diagram showing the derivation of macrophages from EMPs and HSCs](image)

**Figure 1.6** Macrophages are derived both from erythro-myeloid progenitors (EMPs) and hematopoietic stem cell (HSCs). Each macrophage cell source produces macrophages that vary in function, phenotype and morphology. Additionally, resident macrophage function is often determined by the tissue environment.

Recent work speculates that resident macrophages first respond to wounds and recruit monocytes which then differentiate into transitory macrophages [50], [51]. Monocytes themselves are heterogeneous [52]; they have been classified according to the following paradigm: lymphocyte antigen 6 complex (Ly6C<sub>high</sub>) inflammatory monocytes, intermediate Ly6C monocytes and Ly6C<sub>low</sub> resident or patrolling monocytes [40], [41], [52], [53]. Certain monocytes are associated with different aspects of a wound. For
example, in spinal-cord repair, Ly6C\textsubscript{low} monocytes remained near the VCAM-1\textsuperscript{*} (vascular cell adhesion molecule-1) structures while Ly6C\textsubscript{high} monocytes invaded sites of tissue injury [54]. Additionally, both Ly6C\textsubscript{low} and Ly6C\textsubscript{high} monocytes were found to patrol endothelial cells for damages through the lymphocyte function-associated antigen 1 (LFA-1) via integrin binding [49]. Unfortunately, it is not clear which monocytes differentiate into certain macrophage phenotypes, but data suggests that each monocyte is capable of differentiating into macrophages of varying phenotypes (Figure 3) [41].

Within the wound environment, macrophages adopt a myriad of roles outside of the scope of phagocytosis. Specifically, these cells adopt the following functions: scavenging, efferocytosis, and promoting repair, extracellular signaling, and angiogenesis [55]. It is important to note that the functions adopted by macrophages are highly dependent on stimulants from the microenvironment [56]. Varying stimulants such as hypoxia, tissue debris and platelet-derived factors can dramatically alter the macrophage response. Nevertheless, macrophages orchestrate the wound bed [40]. They also coordinate the repair process, largely through the release of potent cytokines and proteases [57]. Depending on the environmental cues, macrophages can release a broad repertoire of growth factors. Within the context of a wound environment, macrophages are known to release a host of factors, including tumor necrosis factor alpha (TNF\textalpha), transforming growth factor beta (TGF\beta), interleukin-1 (IL-1), IL-6, platelet-derived growth factor (PDGF), transforming growth factor alpha (TGF\alpha), TGF\beta, and insulin-like
growth factor-1 (IGF-1). Once released, these factors influence surrounding cells in the microenvironment such as endothelial cells, fibroblasts, and alternative stromal cell types [56], [58]. Macrophages also secrete proteases that degrade ECM components, depending on context [10], [57], [59], [60]. For example, macrophages may cleave collagen; collagen fragments then act as chemokines for neighboring fibroblasts [59]. Thus, macrophages have a plethora of functions; they coordinate the responses of the immune system as well as surrounding cell types largely through the secretion of products as well as through phagocytosis.

Macrophages are also involved in tissue repair and development [61]. Within the adult salamander, macrophages are necessary for limb regrowth. Macrophage depletion disrupts healing and inhibits limb regeneration [62]. Macrophages are also often found in developing tissues. During development in mammals, macrophages make up 10-20% of cells found in each tissue [63]. The presence of macrophages is critical for normal tissue development as they affect growth rate, remodeling and organization of the tissues [48], [61], [64]. Within the eye (specifically, within the retina), macrophages are involved in vascular remodeling following the creation of a vascular plexus [63], [65], [66]. Macrophages also regulate lymphomagenesis [67] and are found to play critical roles in tumor progression [40], [68], [69]. In summary, macrophages possess the ability to regulate tissue repair and assist during tissue development. Specific cues alter the roles that macrophages play thus leading to alternate macrophage phenotypes.
1.3.1 Macrophage phenotypes

Within the field, the classifications and descriptions of macrophage phenotypes are somewhat contested. This summary will attempt to explain the ideas behind macrophage phenotypes while also being consistent with what is practiced within the field. Generally, the classification of macrophage phenotypes is often too restrictive [63]. Macrophage phenotypes exist along a broad continuum of overlapping functions. It is thus naïve to only consider two phenotypes and not a multitude of others [70], [71]. Additionally, it is important to note that phenotype state of a macrophage is transient and defined within the context of stimulation. Moreover, it is contested which stimulation contexts exist in vivo versus only created in vitro. With these limitations in mind, macrophage phenotypes can be discussed.

Broadly, there are two proposed extremum phenotype states: M1 classically activated macrophages that are ‘pro-inflammatory’ and M2 alternatively activated macrophages considered ‘pro-tissue healing’ (Figure 1.7). The history of the M1/M2 paradigm, created in the 1990’s, is informed from the T helper cell (T\textsubscript{H}) paradigm [72]. T\textsubscript{H} cells are CD4+ T cells (of the adaptive immune system) that adopt various phenotypes based on stimulation patterns; these cells play a critical role in mounting the adaptive immune response and can direct B cell antibody generation [73]. T\textsubscript{H}1 cells secrete interferon gamma (IFN\textgamma) while T\textsubscript{H}2 secrete IL-4 and IL-13, amongst a host of other cytokines [74]. When generating macrophage phenotypes, researchers observed distinct
differences between the macrophage response to Th1 conditioned media versus Th2 conditioned media which led researchers to affiliate macrophage activation states with each condition- M1 and M2 [71]. The key differences in macrophages from each state are seen in gene expression, surface markers, and cytokine secretion [50]. For example, M2 macrophages express certain surface markers (such as the mannose receptor, MMR/CD206) and M1 macrophages express iNOS (inducible nitric oxide synthase). However, it is important to note, the establishment of the M1/M2 paradigm is largely reliant upon in vitro settings. Thus, there are fundamental issues with the absolute use of M1 versus M2 when applying this classification in vivo. Moreover, the markers that are used to assess M1 versus M2 are not mutually exclusive. M1 macrophages can stain positive for M2 markers and vice versa [71], [72]. Nevertheless, Th1 cells were found to secrete IFNγ and TNFα. These cytokines were found to be the major stimulators for M1 macrophages [41]. Moreover, Th2 cells secrete IL-4 and IL-10 which stimulate the M2 phenotype.
There are also subsets of the M2 macrophage paradigm: M2a (macrophages stimulated by IL-4 and IL-13), M2b macrophages (stimulated simultaneously by IL-1R agonists and Immune Complexes (ICs) such as DNA fragments and TLR agonists), and M2c macrophages (stimulated by IL-10 and TGF-β) [53], [75]. The subsets of the M2 phenotype exist within the same spectrum of the M1 and M2 phenotypes and have been previously reviewed [76]. However, there is little evidence that these distinct subsets exist in vivo [71]. Within the literature, alternative cytokines, such as granulocyte macrophage-colony stimulating factor (GMCSF) and macrophage-colony stimulating factor (MCSF), have also been studied in order to understand how these cytokines
promote M1 or M2 activation; however, researchers are hesitant to consider these alternative cytokines in the traditional M1/M2 classifications [71], [72]. For example, it was proposed that GMCSF stimulate macrophages towards an M1 phenotype and MCSF stimulate macrophages towards an M2 phenotype [77]. However, recent studies have refuted this claim [71]. Moreover, it has recently been recommended that scientists only consider IFN\(\gamma\) and IL-4 for stimulation of macrophages to provide consistency within the field [71].

Within the context of the wound environment, more questions abound as far as what types of activated macrophages are present and the function they play. It has been suggested that M1 macrophages first invade the wound to promote inflammation; M2 macrophages are then present to promote tissue repair [78]. It is not known if M1 macrophages transition to M2 (as shown in Figure 1.7) or if M2 macrophages are subsequently recruited [76]. It is also questioned whether wound macrophages are directly related to the M1/M2 phenotypes [41]. Nevertheless, it is appreciated that the dominance of one phenotype (either M1 or M2) leads to a diseased state [79]. M1 macrophages can exacerbate tissue injury and lead to chronic inflammation, as seen in the spine, liver and within vascular tissues injuries [10]. Conversely, a dominance of M2 macrophages can lead to fibrosis via the activation of myofibroblasts [80].

Finally, because of the translational impact, the difference between activated human and mouse macrophage cell lines must be noted. There are critical differences in
the synthesis of nitric oxide (NO), arginase and iNOS between mouse and human cell lines [16], [81]–[83]. These metabolic differences between each cell line introduced critical discrepancies between human and mouse activated macrophages. Moreover, the use of human and mouse cell lines does not correlate with what has been seen from primary cells and thus, there is now the argument that primary macrophages be utilized for reliable phenotype studies (and subsequent comparisons) [71]. Murine models largely rely upon bone-marrow derived or peritoneal macrophages from the C57BL/6 or Balb/c mouse strains [72]. Primary human macrophages are predominantly derived from human peripheral blood monocytes. Unfortunately, genetic profiling between primary human and mouse macrophages is still largely lacking [72]. However, other aspects of the phenotypes have been established and are conserved between the species, such as surface markers between phenotypes (CD206 for M2, CD86 for M1, etc.) [70]. Thus, it is accepted that both human and mouse primary macrophages adopt activated profiles corresponding to M1 and M2 phenotypes.

Within the field of immunology, there is a need to clarify and organize the discussion surrounding macrophage phenotypes [71]. However, critical aspects of macrophage activation are readily apparent both in vivo and in vitro. Within the context of tissue engineering, there is a need to better understand how phenotypes can be exploited to create a regenerative environment capable to supporting angiogenesis and tissue repair.
1.4 Macrophages within vessel development: in vivo models

Historically, most studies probing the roles of macrophages in angiogenesis have focused primarily on paracrine signaling by exposing endothelial cells to conditioned media from cultured macrophages. Recently, cell-cell interactions between macrophages and endothelial cells have been studied to examine the juxtacrine influences of macrophages. Specifically, this section highlights advances from these models.

The main aspect of macrophage influence on the angiogenic processes is speculated to be via secreted growth factors and enzymes [11], [13], [40], [56], [57], [78]. Activated and mature macrophages can secrete proteases to remodel the surrounding ECM in wound beds to facilitate neovascularization [56], [57]. For example, Kolattukudy’s group developed a transgenic mouse to express monocyte chemoattractant protein-1 (MCP-1) in the heart; macrophages subsequently migrated to the heart, secreted matrix metalloproteinases and created tunnels in the myocardium [84]. These tunnels demonstrate macrophages can remodel the ECM of an ischemic heart to promote endothelialization of the tunnels. The presence of tunnels has also been demonstrated in other groups [85], [86]. Additionally, the Caplice group introduced CX3CR1+ macrophages as the cell source that is responsible for interactions with microvessels within an in vivo Matrigel plug assay following tunneling [86].

Within the context of angiogenesis, macrophages can stimulate neovascularization and enable healing via secretion of pro-angiogenic factors [87], [88].
Initial studies conducted by Unanue’s group within the guinea pig cornea demonstrated that macrophages (and conditioned media from macrophages) can stimulate neovascularization [87]. Macrophages that were activated (either via latex beads in vitro or via thioglycollate in vivo) subsequently stimulated increased neovascularization in the cornea. This study suggests that the activation of macrophages can influence angiogenesis. Additionally, Schaper’s group analyzed monocyte recruitment in a rabbit hindlimb study of femoral occlusion [88]. They found that increased monocyte presence resulted in capillary sprouting and arterial growth. It was speculated, from both of these initial studies, that secreted factors from the macrophages are responsible for the positive effect on vascularization.

Broadly, macrophages can secrete a myriad of factors that have been shown to influence endothelial cells: basic fibroblast growth factor (bFGF), VEGF-A, IL-8, PDGF, IL-6, IL-1, TGF-β, TNF-α and many more [89], [90]. It is known that each of these factors influence endothelial cells [13]. Some factors (such as IL-8) induce the recruitment and proliferation of endothelial cells [91]. Specifically, antibodies against IL-8 blocked the angiogenic activity of conditioned media from stimulated monocytes. TNFα has also been studied within the context of macrophage-mediated angiogenesis [92]. Nuseir’s group studied TNFα within the rat cornea and the chick chorioallantoic membrane (CAM) models. They found that TNFα induced blood vessel formation. Conditioned media from peritoneal macrophages (activated in the presence of thioglycollate)
demonstrated pro-angiogenic activity when incubated with bovine adrenal capillary endothelial cells [92]. When the conditioned media was exposed to anti-TNFα antibodies, the angiogenic effect was significantly reduced. Additionally, alternative factors (such as IFN-γ) are known to inhibit proliferation of endothelial cells [93]. Because the secretory products of macrophages, in particular the roles of pro-angiogenic growth factors, are known to have a tremendous influence on endothelial cells, they have been studied at length elsewhere [57], [94]. Additional reviews have covered the influence of enzymes (for example collagenases), hormones, and lipid secretions of macrophages in governing cellular responses, including endothelial cells [13], [95].

In vivo models have also focused on tissue development to better understand the roles of macrophages within these dynamic environments. Yolk sac macrophages have been studied extensively within the context of vascularization and organogenesis [12], [96]. Fetal testis macrophages were studied within the gonads of mice from E10.5-E13.5 (embryo day) [96]. M2-like macrophages, defined as CD206+, were shown to play a dominating role in vascular reorganization. Specifically, these macrophages were positive for neuropilin-1 (NRP1, a VEGF co-receptor involved in neovascularization) and the endothelial-specific receptor for tyrosine kinase (TIE2). In the presence of a VEGF-stimulated vascular plexus, macrophages invaded the gonad. While these macrophages were not responsible for the initial recruitment of endothelial cells, they
were responsible for vascular pruning and reorganization. Once present in the gonad, the macrophages localized to the vasculature and regulated tissue morphogenesis. This work introduced the notion that macrophages are critically involved in morphogenesis of the gonad through their influence on vessel organization.

Ruhrberg’s group also studied yolk sac derived macrophages but within the context of brain development, in the subventricular zone (SVZ), up to 12.5 days postcoitum [12]. Macrophages rapidly associated with endothelial cells upon endothelial cell ingrowth into the SVZ. Macrophages promoted the formation of the vascular plexus, as the removal of macrophages from the site resulted in reduced vessel connections. Importantly, this work introduced the notion that macrophages act as cell-chaperones for endothelial tip cells. Specifically, macrophages were found at junctions between neighboring endothelial tip cells; the subsequent bridging between the macrophages and the tip cells resulted in the joining or fusion of the tip cells to create a continuous vessel. This work also proposed the notion that VEGF-mediated endothelial tip cell sprouting and macrophage-mediated anastomosis were two separate but affiliated processes. Zebrafish embryos were also used within this work to allow live imaging of the dynamic fusions between endothelial tip cells [12]. Specifically, macrophages were seen fusing endothelial tip cells which supports the notion that macrophage cell-chaperoning behavior is conserved between species.
Betsholtz’s group also studied the communication between microglia (brain macrophages) and endothelial cells within the context of retinal development [11]. Microglial cells were present before the formation of vasculature within the retina. As endothelial cells sprouted and spread into the retina, microglia associated with the endothelial tip cells. Microglia were found between neighboring endothelial tip cells at the ratio of 1:2. Macrophages appear to be involved in sprout anastomosis and acting as guide posts for the endothelial tip cells as the endothelial tip cells fuse with one another. Furthermore, the use of knockout MCSP<sup>op/op</sup> mice (which reduced the number of microglia cells by approximately 4-fold) led to sparser vessel networks and altered vessel orientation. Thus, this work supports the notion that microglia can act as guides for endothelial tip cells and influence higher order aspects of the vascular plexus, such as network alignment.

Within these fetal models, macrophages were from the primitive yolk sac. It is difficult to translate the function of yolk sac cells to the function of bone-marrow derived macrophages. Additionally, TIE2 and NPR1 macrophages are closely associated with tumor vascularization [40], [69]. Thus, the functions of these TIE2+/NPR1+ macrophages may be limited to organogenesis or tumor-angiogenesis environments.

Distinct from fetal models, adult wound models have also been created to assess the pro-angiogenic roles of macrophages in wound repair [97]. Specifically, Kubato’s group made use of the dorsal excision wound model, the decubitus ulcer model and the
ear punch model in mice. Macrophages, found to be the dominant cell recruited to the wound site, closely associated with endothelial cells. Specifically, this work found that macrophages at day 7 from the dorsal excision wound were M2-like and expressed high amounts of MMPs-2, -9 and -13. Moreover, reduction in the number macrophages at the site (via MCSF inhibitor or MCSF antibodies) resulted in decreased blood vessel density at the site. The expression of MMPs suggests that macrophages contribute to vessel repair via matrix remodeling as seen in the myocardial model discussed above [84].

Stainier’s group recently investigated macrophage and endothelial cell interactions in several zebrafish models to ascertain the influence of hypoxia inducible factor-1 alpha (HIF-1α) on vessel plexus formation [98]. Importantly, HIF-1α is a known regulator of VEGF secretion [99], [100] and thus, Stainier’s group is also influencing VEGF as HIF-1α is altered. They found that macrophages migrate and localize to vessels in a HIF-1α dependent manner. Moreover, the knockout of HIF-1α inhibited the formation of vascular plexus in a macrophage dependent manner. This knockout also inhibited macrophages assistance in vessel repair. Lastly, they commented on the fact that most of the macrophages closely associating with the endothelial cells in the WT zebrafish were TNFα+, although the authors were careful to mention the fact that these developmental macrophages differ from the classical definitions of macrophage phenotypes.
A few groups have also developed implant models to assess macrophage response to vessel development within the context of hybrid biomaterials. Botchwey’s group has investigated the role of monocyte recruitment in vascular invasion and arteriogenesis [101], [102]. They synthesized heparin-functionalized PEG hydrogels to deliver stromal derived factor-1α (SDF-1α) within a dorsal skinfold window chamber model [101]. The goal of this work was to promote vascular remodeling by recruiting monocytes to the injury site through the sustained release of SDF-1α. As described earlier, monocytes exist along a spectrum of activation: inflammatory monocytes (IMs, Ly6C<sub>high</sub>) and anti-inflammatory monocytes (AMs, Ly6C<sub>low</sub>). SDF-1α, a CXC family chemokine, was used to mediate the recruitment of immune cells via the CXCR4 receptor; AMs express higher levels of CXCR4 and thus, the sustained release of SDF-1α recruited more AMs to the injury site. The localized recruitment of AMs led to sustained arteriolar remodeling, increased arteriolar diameter, and increased peri-vascular microvessels near the hydrogel. Notably, this work is an example of “immune-regenerative engineering” - the design of biomaterials to advance regeneration through the manipulation of immune cells.

Prior work from our lab utilized the cornea micropocket model in mice to implant bioactive PEGDA (polyethylene glycol diacrylate) hydrogels [14], [103]. Within this work, growth factors, specifically VEGF, PDGFBB, and bFGF were assessed for their chemotactic ability to recruit macrophages and promote vessel development in the
cornea. PEGDA hydrogels were impregnated with growth factors that could diffuse out of the hydrogel to the adjacent limbus following implantation to recruit host cells. Initially, two groups were considered: PDGFBB/bFGF and VEGF alone. When compared to the VEGF alone hydrogels, the PDGFBB/bFGF hydrogels stimulated migration of macrophages. The VEGF alone group did not stimulate the migration of macrophages. Following the influx of the macrophages in the PDGFBB/bFGF group, endothelial cells migrated to the site and formed stable vessel structures. The PDGFBB/bFGF group resulted in significantly greater vessel density, greater vessel invasion into the hydrogel and more advanced structural organization of the vessels that formed. The VEGF only hydrogels, which did not recruit macrophages, induced vasculature that was less organized and contained more endothelial cell sprouts than the PDGFBB/bFGF group. The PDGFBB/bFGF group contained lumenized structures that were more perfused than the VEGF alone groups. Dynamic interactions were also seen between the macrophages and the endothelial cells; at an early time-point (day 4), macrophages interacted with sprouting endothelial tip cells in a manner suggestive of cell-chaperoning behavior (Figure 1.8A). At a later time-point (day 11), macrophages aligned along the nascent vessels in a manner similar to support cells (Figure 1.8B). To highlight the role of the macrophages in the vessel formation, VEGF + MCSF was impregnated into the hydrogel. The MCSF recruited macrophages and resulted in increased VEGF-induced angiogenesis (a nearly 3-fold increase in vessel density) when compared to VEGF alone.
Thus, the recruitment of macrophages can promote vessel formation. In addition, cell-cell interactions between macrophages and endothelial cells can play a role in vessel development.

![Figure 1.8](image)

**Figure 1.8 Macrophages interact with vessels in the murine cornea.** (A) At day 4, macrophages (in green) interact with endothelial cells (in magenta), with tip cell interactions shown by the arrowhead and the asterisk. (B) At day 11, macrophages wrap around nascent vessels. Adapted from: Hsu, et al. [14].

There has been a tremendous amount of in vivo work to probe macrophages and their roles in angiogenesis. Animal models have been used to assess the impact of macrophage presence, phenotype and interactions with endothelial cells during vessel development (Table 1.1) [11], [90]. While these models have demonstrated macrophages can play a critical role in vessel development, the microenvironments in which these interactions were seen are highly complex or abnormal, such as the cornea [104]. Moreover, the functions of yolk sac-derived macrophages (tissue resident macrophages) must be translated to bone-marrow derived macrophages. Thus, there is a need to understand macrophage-endothelial cell interactions within a simplified
microenvironment with cells derived from translatable macrophage sources. To meet this need, several groups have begun to develop in vitro systems to assess the roles of macrophages in vessel development.

Table 1.1 Macrophages within vessel development: in vivo models

<table>
<thead>
<tr>
<th>Key Finding</th>
<th>Animal/Model</th>
<th>Macrophage Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages create tunnels in the myocardium (for neovascularization) [84]</td>
<td>MCP-1 transgenic mouse</td>
<td>Adult macrophages</td>
</tr>
<tr>
<td>Macrophages and conditioned media stimulate neovascularization [87]</td>
<td>Guinea pig cornea</td>
<td>Peritoneal cavity of guinea pig (unstimulated and thioglycollate stimulated)</td>
</tr>
<tr>
<td>Increased presence of macrophages led to increased capillary density (temporally dependent) [88]</td>
<td>Rabbit hind-limb femoral occlusion</td>
<td>Rabbit monocytes and macrophages (LPS stimulated)</td>
</tr>
<tr>
<td>Macrophages are responsible for vascular reorganization and pruning [96]</td>
<td>Fetal testes of mouse</td>
<td>Yolk sac derived macrophages</td>
</tr>
<tr>
<td>Macrophages act as cell-chaperones separate from VEGF-stimulated endothelial cell sprouting [12]</td>
<td>Subventricular zone of fetal mouse</td>
<td>Yolk sac derived tissue resident macrophages</td>
</tr>
<tr>
<td>Microglia (brain macrophages) act as guide post for endothelial tip cells [11]</td>
<td>Retina of fetal mouse</td>
<td>Microglia (tissue resident macrophages)</td>
</tr>
<tr>
<td>AM (anti-inflammatory monocytes) increased the microvessel density surrounding implant site [101]</td>
<td>Dorsal skinfold window chamber on a mouse</td>
<td>Adult monocytes</td>
</tr>
<tr>
<td>Macrophages (recruited by PDGFB/ bFGF) interact with tip cells and behave as pericyte-like cells [14]</td>
<td>Mouse cornea (PEGDA gel implant)</td>
<td>Recruited macrophages (adult)</td>
</tr>
<tr>
<td>Macrophages are responsible for vascular plexus formation, and repair vessel ruptures [98]</td>
<td>Zebrafish</td>
<td>Developmental macrophages</td>
</tr>
</tbody>
</table>

1.5 Macrophages within vessel development: in vitro models

Because macrophage phenotypes are known to play a dominant role in macrophage function, most in vitro work has focused on the modulation of macrophage
phenotypes to understand the roles of macrophages both within an angiogenesis context and also within a biomaterial context.

Few groups have studied macrophage and endothelial cell interactions within in vitro systems. Recently, Kitajewski and colleagues utilized the bead-based capillary sprouting assay to better understand the intercellular dynamics between murine bone-marrow macrophages, human umbilical vein endothelial cells (HUVECs) and human brain vascular pericyte cells (HP) [105]. HUVECs were seeded onto Cytotex beads. The HUVEC-beads were then encapsulated into a fibrin gel. To introduce macrophages into this system, macrophages were encapsulated within the fibrin gel. Finally, fibroblasts (D551s) were then seeded on top of the fibrin gel, forming a confluent 2D layer of cells above the HUVEC-beads + macrophages in the fibrin gel below. Initial culture with the macrophages found that the presence of macrophages increased the number and length of the HUVEC sprouts from the bead (in comparison to HUVECs alone). Macrophage phenotypes were also investigated within this work; M1 macrophages were stimulated with LPS (lipopolysaccharide, a fragment of the membrane of gram-negative bacteria) and IFNγ; M2 macrophages were stimulated with IL-4. M1 macrophages increased the number of sprouts from HUVEC-beads in comparison to non-stimulated (or M0) macrophages and in comparison to M2 macrophages. Next, pericytes (HPs) were added onto the beads with the HUVECs. The HUVEC/HP-beads were then encapsulated in the fibrin gel with macrophages. An additive effect was seen in the number of sprouts: the
HUVEC/HP-beads + macrophages resulted in more sprouts than both the HUVEC/HP and the HUVEC/macrophage groups. Importantly, this work provides evidence that the first stimulating macrophages in 2D towards M1 and then encapsulating toward macrophages with HUVEC-beads can have a beneficial effect on endothelial sprouts. However, it is important to note that recent work in the Liu lab has indicated that culture in fibrin suppresses inflammatory responses to LPS and supports an anti-inflammatory macrophage phenotype [106]. Thus fibrin is capable of altering the M1 macrophage phenotype upon encapsulation. Additionally, multiple cell types (both pericytes and macrophages) can promote endothelial cell sprouts in diverging but complementary manners. It is important to note that this system incorporated multiple species types as the endothelial cells and the pericytes were human and the macrophages were mouse. Moreover, the macrophages did not spread, migrate or directly interact with any of the HUVEC or HPs. Thus, only paracrine influences were considered within this model which may isolate a key element of M1s in vessel formation.

Other groups have also developed systems to utilize macrophages to enhance vascularization [107]. The Spiller group introduced the notion that a sequential presence of macrophage phenotypes would optimize the roles of macrophages in vascularization [51], [108], [109]. Within this work, Spiller and colleagues utilize human monocytes that have been differentiated into macrophages via MCSF stimulation over 5 days. Following
differentiation, the macrophages were stimulated along various phenotypes: M1 via LPS and INF\(\gamma\), M2a via IL-4 and M2c via IL-10. To assess macrophage phenotype influence on endothelial cells, HUVECs network formation was assessed in the presence of conditioned media from each of the phenotypes. In a 2D Matrigel assessment over 18 hr, M2c-conditioned media promoted the greatest number of sprouts as well as the greatest total tube length. On fibrin gels over 4 days in culture, M1 conditioned media lead to loosely connected HUVEC networks while M2a-conditioned media led to an increase fusion of endothelial cell sprouts. This work led to the hypothesis of a progression from M1 to M2a to M2c macrophages; first M1 macrophages promote the recruitment of endothelial cells (via secretion of VEGF, bFGF and TNF\(\alpha\)); second, M2a macrophages promote fusion of the endothelial cells as well as the recruitment of pericytes (via secretion of PDGF); finally, M2c macrophages secrete MMP-9 to enable vascular remodeling.

Spiller and colleagues then attempted to apply this sequential phenotype coordination to enhance vascularization of decellularized bone, which may have utility as a tissue engineering scaffold [109]. Within this work, the group provided direct evidence of in vitro macrophage phenotype switching; macrophages were stimulated toward a certain phenotype for 3 days then switched to an alternative phenotype (M1 to M2, M2 to M1, etc.). Flow cytometry confirmed the ability for macrophages to alter their phenotypes following treatment of soluble factors. To mimic the proposed coordinated
M1 to M2 transition, decellularized bone scaffolds were then coated with IFNγ (for quick release) and functionalized with biotin/streptavidin bound IL-4 (for delayed release). Following subcutaneous implantation, however, it was found that the INFγ only group stimulated the most vessel structures. Nevertheless, this work serves as a proof of concept that the manipulation of macrophage phenotypes can influence vessel response, specifically within the context of vascularized scaffolds.

In contrast to IFNγ-stimulated (M1 stimulated) vessel development, Donners’ group found that M2 macrophages are responsible for promoting angiogenesis [110]. This group combined mouse bone-marrow macrophages and a mouse endothelial cell line for their studies on Matrigel. Using the tube formation assay, they found that only M2a and M2c in co-culture with endothelial cells enhanced tubule formation while M1 stimulated macrophages reduced tubule formation. Moreover, they found that conditioned media from M0 and M1 stimulated macrophages enhanced tubule formation; thus, they speculated that direct cell-cell contact between M1 macrophages and endothelial cells was responsible for inhibition of tubule formation. Importantly, this work considered both paracrine and juxtacrine signaling effects, which may account for the differing results from the prior studies discussed above where only paracrine signaling was possible.

Additionally, work from our lab has also investigated macrophage and endothelial cell communications in vitro [14]. Utilizing collagen gels, HUVECs and
murine bone-marrow macrophages were combined in co-culture conditions. Again, close associations and bridging were seen between the macrophages and endothelial cells. Increased network formation was also observed between the co-culture to HUVECs alone. Furthermore, this work supports the notion that direct cell-cell contact encompasses key aspects of macrophages pro-angiogenic roles. Altogether, many of the in vitro models used to study macrophage and endothelial cell communication or the roles of macrophages in vascularization rely on multiple cell species, natural materials and soluble signaling (Table 1.2). Specifically, work within our lab has found that the mixture of species and/or alternative donors, in particular human bone marrow derived mesenchymal stem cells (MSCs) and human endothelial progenitor cells (EPCs) from another donor, led to complications and rejection of the cell types upon co-culture. In particular, CD45+ cells (immune cells) from the MSC population attacked the EPCs and inhibited network formation [111]. Because many of these in vitro co-culture studies have introduced alternative species and donors, these perspectives represent a limited view of macrophage associations with and influences on endothelial cells.
Because macrophage phenotypes influence the immune response, other groups have investigated design parameters to control macrophage phenotypes with the goal of enhancing biomaterial and host interaction [50], [51], [112]. Specifically, sectors of the field of biomaterials endeavor to design ‘immune-informed’ materials to exploit favorable aspects and avoid adverse aspects of the immune response [15]. The ability to ameliorate a foreign body response, enable rapid host anastomosis and eliminate the fibrotic response could dramatically change the outcomes of implanted devices. These processes are in large part controlled by the macrophage response [113]. Thus, macrophage phenotypes are being studied within the context of biophysical cues,
biochemical cues and interactions with other cells. The overarching goal of this work is to take advantage of favorable aspects of macrophages (specifically control over phenotypes) to enhance biomaterial or tissue engineering outcomes.

A few of the biophysical cues that have been assessed include mechanical cues such as stiffness, pore size, electrospinning for fiber diameter and alignment as well as nano- and microtopography [50], [112], [114]. Ratner’s group uncovered the role of pore size in reducing fibrosis as well as enhancing vascularization of scaffolds [115]. Ratner’s group compared pore size between 34 µm and 120 µm of sphere-templated poly(2-hydroxyethyl methacrylate) (pHEMA) scaffolds. They found more M1 macrophages and decreased M2 macrophages in the 34 µm pore. This increase in M1 macrophages is likely due to the 34 µm pore size inducing aggravated phagocytosis and a foreign body response (FBR) of the macrophages. The 34 µm pore size also resulted in increased vascularization of the scaffold which indicated that M1+M2 macrophage populations are capable of supporting vascularization. Another study from Liu’s group studied limitations on macrophage shape and found that controlled topography in the form of thin 20 µm lines led to the M2 phenotype [116]. Strain-sensitive phenotypes have also been assessed [117]. After seeding on poly-ε-calprolactone strips, macrophages were subjected to cyclic strain for 1 week in culture. At day 1, both M1 and M2 macrophage populations were present. However, by day 7, a dominant shift was made towards the
M2 macrophage phenotype. Thus, it appears that biophysical cues can inform macrophage activation.

Studies considering biochemical cues in macrophage activation have largely been limited to the traditional soluble phenotype stimulators: LPS, IFN$\gamma$, IL-4, Il-13 and IL-10. As seen in prior examples, the adsorption of specific growth factors can induce certain macrophage phenotypes [109], [112]. Additionally, hypoxia has been studied with regards to macrophage phenotype and has demonstrated alterations in gene expression as well as cytokine secretion [118]. The presence of additional cell types, such as lymphocytes, MSCs or muscle cells have also been studied [112], [119]. Specific cell-cell interactions can stimulate macrophage phenotypes towards certain states as seen when M1 macrophages are co-cultured with skeletal muscle progenitor cells [119].

While there has been progress in understanding the impact of macrophage phenotype manipulation on biomaterial-host interactions, there are still many unknown influences and interactions. Most importantly, it is not clear what roles each phenotype plays in host-response and in vascularization of the scaffolds [15]. Many hypotheses have been presented but it difficult to test or compare between different models due to lack of uniformity in cell species, phenotype stimulation profiles and eventual implant site. Because it is appreciated that different tissues, diseases and implants have varying functions, it is also appreciated there is not a clear cut answer in regards to the roles of macrophage phenotypes [63]. Nevertheless, there is a need for a system that enables
both control over macrophage phenotype as well as tunability to better understand the roles with regards to vascularization and eventually biomaterial–host interactions.

1.6 Summary and overview of thesis

Much work has been done to elucidate the angiogenic roles of macrophages. From both in vitro and in vitro work, it is known that macrophages can behave in pro-angiogenic manners both through paracrine signaling (the secretion of growth factors) as well as through juxtacrine interactions (cell-cell interactions with endothelial cells). Within the context of the in vivo work, these interactions were seen in highly complex environments in which organogenesis was simultaneously occurring. While the in vitro work has demonstrated control over macrophage phenotype, the influence of macrophage phenotype on vessel development has led to conflicting results and may further depend on the context of cell-cell signaling. It is hard to begin to enact comparisons between these studies as species, macrophage origin, and surrounding microenvironment all varied dramatically.

While there have been many advances in understanding the roles of macrophages, there is still a need for a simplified system to better clarify the roles of macrophages. To our knowledge, no system currently exists in which macrophage and endothelial cell associations (from the same species) are studied within a reductionist microenvironment. An ideal system would allow control over the ECM biochemical and biomechanical properties, and the ability to study both paracrine and juxtacrine
interactions (Figure 1.9). To address these limitations, my work proposes to utilize our bioactive PEG-based hydrogel system to study macrophage and endothelial cell interactions.

Figure 1.9 Schematic of an in vitro system to study macrophage and endothelial cell interactions. This system would allow explicit control over ECM, the incorporation of alternative cell types to mimic various cell environments, and the assessment of both paracrine and juxtacrine interactions.

This dissertation covers the development of the in vitro macrophage-endothelial cell model and the use of the system to exploit the roles of macrophage phenotypes in vessel development. In chapter 2, synthesis of the PEG-based hydrogel is discussed. Cell encapsulation as well as manipulation of the PEG-based hydrogels to incorporate alternative factors is included. In chapter 3, the characterization of macrophage influences on endothelial cells is discussed, as well as the evaluation of macrophage
associations and influence on vessel formation. Chapter 4 includes the context of macrophage phenotypes in vessel development. Chapter 5 introduces PEG-based hydrogels capable of manipulating macrophage phenotype. Finally, chapter 6 includes concluding remarks and future directions for macrophages in vessel development.
2. Bioactive hydrogels

Within this work, PEG-based hydrogels are used in order to assess macrophage influence on endothelial cells during vessel formation. The context of scaffolds in studies involving macrophages and endothelial cells is included as well as the motivation for PEG-based scaffolds. This chapter will also detail the synthesis of the PEG macromers used to create the bioactive PEG-based hydrogel. This chapter will discuss the protocol to encapsulate cells into the PEG-based hydrogel. PEG hydrogels are used within all subsequent studies as a hydrogel platform to assess macrophage influence on endothelial cells during vessel development.

2.1 PEG hydrogels

As discussed in Chapter 1, a variety of materials have been used to assess macrophages role in vessel development. Matrigel (reconstituted basement membrane), fibrin and collagen substrates are those most commonly used within macrophage-endothelial cell studies [11], [14], [105], [108], [110]. These materials are all naturally-derived, isolated from tissues. These materials form hydrogels that are cell adhesive and that can be degraded by cellular proteases, but offer little ability to control or manipulate these properties. These materials are also plagued by batch-to-batch variability. In order to assess macrophage and endothelial interactions, this work considered the
development of a scaffold consisting of a reductionist microenvironment in which the cell-material interactions are controlled.

Towards that end, we selected PEG-based hydrogels as the biomaterial platform as PEG hydrogels offer a microenvironment in which cell-material interactions are able to be selectively incorporated. PEG hydrogels are advantageous due to their non-toxic, non-inflammatory and non-immunogenic properties (dependent on PEG chain length) [120]–[122], [33]. PEG polymers are inherently resistant to protein adsorption due to their high chain mobility and hydrophilic properties [123]. This resistance to protein adsorption minimizes cell attachment to PEG-based materials (Figure 2.1). Without the adsorption of protein onto the polymer, cells are unable to attach to the PEG polymer. Thus, PEG has been identified as a ‘stealth’ or ‘blank slate’ material as proteins are largely unable to adsorb onto materials coated with or comprised of PEG [122]. As a result of this ‘stealth’ property, PEG chains are non-immunogenic; without the adsorption of protein, PEG constructs are ‘unseen’ in immunological surveillance [122], [124]. PEGDA is a derivative of the PEG chain with the addition of acrylate groups on each end (Figure 2.1). This allows crosslinking of PEGDA into a hydrogel.
Figure 2.1 Chemical structures of (A) Poly(ethylene glycol) (PEG) and (B) Poly(ethylene glycol) diacrylate (PEGDA).

Importantly, peptides and proteins can be covalently immobilized onto the ends of PEG polymers [124]–[126]. This covalent incorporation renders specific aspects of the PEG hydrogel bioactive while the rest of the PEG hydrogel is essentially bioinert. Thus, PEG hydrogels allow selective incorporation of bioactivity via the covalent immobilization of peptides into the PEG hydrogel [126]. To covalently incorporate peptides onto PEG chains, a peptide is reacted with an amine-reactive PEG derivative (such as acrylate-PEG-succinimidyl valerate (SVA)). The N-terminal amine is reacted with the succinimidyl valerate (a N-hydroxysuccinimide ester group) on the PEG derivative. Via amine substitution chemistry, a final product of the reaction is acrylate-PEG-peptide (FIGURE 2.2). The covalent immobilization of peptides and proteins allows for sustained cell-peptide/protein interaction within the PEG hydrogels in comparison to alternative studies that have relied solely on passive peptide/protein adsorption [124], [126].
**Figure 2.2 Chemical reaction of acryl-PEG-peptide.** PEG derivate acrylate-PEG-SVA is reacted with a peptide. Via nucleophilic attack, the peptide displaces the succinimide group to form acryl-PEG-peptide.

To render the PEG hydrogels capable of cell adhesion, RGDS was covalently incorporated into the PEG hydrogels. The peptide RGDS (Arginine-Glycine-Aspartic Acid-Serine), in particular, is derived from fibronectin, an extracellular matrix (ECM) protein [127], [128]. It is important to note that anchorage-dependent cells require cell adhesion for survival. The PEG hydrogels resist protein adsorption and thus without the presence of RGDS, anchorage-dependent cells encapsulated within the PEG hydrogels would not survive [129]. Specifically, RGD is a highly conserved cell adhesion peptide as it has been found within other ECM proteins [130]. RGD also has the ability to bind to a variety of integrins on most types of cells [131]–[133]. RGDS was covalently incorporated into the PEG hydrogel via reaction with the PEG derivative (acrylate-PEG-SVA). The final product of this reaction yields acrylate-PEG-RGDS via amine
substitution (Figure 2.3A). The acrylate-PEG-RGDS is grafted into the PEG hydrogel which allows the cells in the PEG hydrogel to bind to the exposed RGDS.

In order to render the PEG hydrogel sensitive to cell-mediated proteolytic degradation, GGGPQGIWGQGK [134], [35], abbreviated as PQ, a matrix metalloprotease (MMP)-2/9 sensitive peptide is covalently immobilized within the PEG hydrogel. In order for endothelial cells to form vessel networks, migration of the cells is tantamount. Migration requires the ability for the cells to degrade the PEG hydrogels in order to create pathways for cell movement. In particular, endothelial cells secrete MMPs-2/9 in order to cleave ECM in vivo as they undergo angiogenesis or vasculogenesis [135], [26], [40]. The PQ peptide, derived from type I collagen, is sensitive to cleavage in the presence of MMPs-2/9 [125], [35]. Previously work in our group has demonstrated the ability for endothelial cells to secrete MMPs-2/9, cleave the PQ sequence and form vessel structures within the PEG hydrogels [37], [36], [38], [39]. In order to incorporate PQ sequence into the PEG hydrogel, the PQ sequence is reacted with the acrylate-PEG-SVA. However, unlike the grafting of the acrylate-PEG-RGDS, the PQ sequence is flanked on both sides by PEG chains forming acrylate-PEG-PQ-PEG-acrylate (Figure 2.3B) due to reaction with amines on the N-terminus but also on the C-terminal lysine residue. This allows the PEG-PQ-PEG to serve as the ‘backbone’ on the PEG hydrogel. The presence of MMPs-2/9 cleaves the PQ peptide and allows migration of the cells throughout the PEG hydrogel.
Figure 2.3 PEG-macromer synthesis. (A) Formation of cell-adhesive PEG-RGDS. (B) Formation of proteolytically degradable PEG-PQ-PEG.

In order to support polymerization of the PEG hydrogel, acrylate groups are present on the ends of all the PEG macromers created (acrylate-PEG-RGDS and acrylate-PEG-PQ-PEG-acrylate). The acrylate groups participate in the crosslinking of the PEG hydrogel. Specifically, the PEG macromers are dissolved in an aqueous buffer solution. Due to the hydrophilic nature of the PEG chains and the hydrophobic nature of the acrylate groups, the acrylate groups form ‘micelle-like’ centers. As free radicals are generated, propagation readily occurs within the acrylate ‘micelles’ (Figure 2.2). This process allows rapid polymerization of the acrylate centers. Moreover, this process is gentle and allows for cells to be encapsulated within the PEG hydrogel as it undergoes
polymerization. The swollen PEG hydrogel is dominantly composed of water (similar to tissues). The PEG hydrogels can also be altered to attain mechanical stiffness ranges of various tissues [37]. The mechanical properties of the PEG hydrogels can be altered either by adjusting the PEG-PQ-PEG polymer weight per volume percentage or by introducing alternative groups with carbon-carbon double bonds (but) that terminate rather than propagate the free radical polymerization [37].
Figure 2.4 Formation of PEGDA crosslink center. A free radical (originated from Eosin Y, a photoinitiator) initiates the crosslink center and propagates through the acrylate ‘micelle-like’ group. (B) Schematic of the PEGDA hydrogel including crosslinking centers.

The properties discussed as well as previous studies in vessel development support notion that PEG hydrogels serve as an optimal platform to investigate the roles of macrophages in vessel development.
### 2.2 Materials and methods

#### 2.2.1 Synthesis of PEG-macromers (PEG-RGDS, PEG-PQ-PEG)

Synthesis of PEGylated peptides used to form the hydrogel scaffold has been previously described [36], [37], [39], [136] (Figure 2.3). Briefly, GGGPQGWGQK (abbreviated as PQ peptide, substrate for MMP-2 and MMP-9) [137] was synthesized via solid-phase fmoc chemistry using an Apex 396 synthesizer (Aapptec, Louisville, KY). Successful synthesis of the PQ peptide was confirmed via DE-Pro MALDI-MS (Applied Biosystems, Foster City, CA). The PQ peptide was resuspend in MilliQ water at 100 µM. Successful peptide synthesis was observed after dissolving the PQ peptide in α-Cyano-4-hydroxycinnamic acid (HCCA; dilution of 1:5 peptide to matrix) and spotting the peptide using the DE-Pro MALDI-MS.

After confirming synthesis via MALDI, the PQ peptide was conjugated to PEG by reacting the N-terminal amine and the amine on the C-terminal lysine with acrylate-(poly (ethylene glycol) (PEG)-succinimidyl valerate (SVA) (acrylate-PEG-SVA; Laysan Bio Inc., Arab, AL). A 1:2 molar ratio of PQ peptide to acrylate-PEG-SVA was mixed in 20 mM (N-(2-hydroxyethyl)piperazine-N’-(4-butanesulfonic acid)) (HEPBS) buffer with 100 mM NaCl, 2 mM CaCl₂ and 2 mM MgCl₂ at pH 8.5 (referred to as protein conjugation buffer). The pH of this mixture was then titrated to 8.0 and reacted overnight (16 hr) at 4 °C under constant agitation. The final product (acrylate-PEG-PQ-PEG-acrylate) was then dialyzed (6000-8000 molecular weight cut-off (MWCO)
regenerated cellulose; Spectrum Laboratories, Rancho Dominguez, CA), lyophilized and stored at -80°C until use. Conjugation efficiency was assessed using gel permeation chromatography with an evaporative light scattering detector (Polymer Laboratories, Amherst, MA). All batches were stored at -80°C under argon until use.

The cell adhesive peptide RGDS was conjugated to a single PEG derivate to allow grafting into the PEG-PQ-PEG network upon hydrogel formation [36], [136]. Briefly, RGDS (American Peptide Company, Sunnyvale, CA) was combined with acryl-PEG-SVA at a 1.2:1 molar ratio (RGDS:acryl-PEG-SVA) in protein conjugation buffer. The pH of the reaction was again titrated to 8.0 and reacted overnight (16 hr) at 4°C under constant agitation. Following dialysis (3.5 kDa MWCO regenerated cellulose; Spectrum Laboratories) and lyophilization, products were analyzed by gel permeation chromatography detector. All batches were stored at -80°C under argon until use.

2.2.2 PEG hydrogel formation and encapsulation of cells

To form hydrogels, the polymers (2.5% PEG-PQ-PEG and 3.5 mM PEG-RGDS) were dissolved in a HEPES-buffered saline (HBS; 10 mM HEPES and 100 mM NaCl at pH 7.4) with 1.5% triethanolamine (TEOA; Sigma), 10 µM eosin Y and 0.35% (v/v) N-vinylpyrrolidone (NVP; Sigma) at pH 8.3 (Figure 2.5). Cells were then added to the polymer solution at varying cell concentrations (see additional chapters for specific cell densities). A 5 µL droplet of the cell-polymer suspension was then placed on a sterilized Simgacote-modified glass slide (modified to be highly hydrophobic, see protocol below)
between two polydimethylsiloxane (PDMS) spacers (380 µm height). A methacrylate-modified coverslip (see protocol below), bearing groups capable of covalently bonding with the hydrogel, was placed on top of the 5 µL cell polymer droplet, housing the droplet between the two PDMS spacers and the two pieces of glass. Gelation was induced by white light exposure for 40 seconds (Dolan-Jenner, Boxborough, MA; 200 mW/cm²). The Sigmacote-modified slide was removed, leaving the hydrogel covalently bonded to the methacrylate-modified coverslip. This was done for ease of handling of the delicate hydrogel and ease of culturing in well plates. Media was then added to the hydrogels in the wells. All gels were cultured at 37°C in 5% CO₂. Media changes occurred at 16 hr post-encapsulation and subsequently every 48 hr afterwards.

Sigmacote-modified slides were prepared according to the manufacturers’ protocol. Glass slides were rinsed with DI H₂O, dried and then treated with to Sigmacote for 5 min at room temperature (Sigma). The slides were then dried and cleaned with 70% ethanol (Koptec, Radnor, PA). Methacrylate-modified coverslips were prepared by treating the coverslips with 2% 3-(trimethoxysilyl) propyl methacrylate (Sigma) in 95% ethanol (Koptec) for 3 days. Following methacrylate-modification, coverslips were cleaned with 70% ethanol and dried. Before use all glass slides and coverslips were exposed to ultraviolet radiation for 30 min.
2.2.3 Cell adhesion onto PEG hydrogels

To assess cell adhesion, PEGDA hydrogels were formed with PEG-RGDS as previously described without encapsulation of cells into the hydrogel. Hydrogels composed of 5% PEGDA with 3.5 mM PEG-RGDS were formed on the methacrylate-modified slides. The hydrogels were allowed to swell for 16 hr in media at 37°C. The following day, 3,000 mouse pancreatic-derived endothelial cells (MS1s) were seeded on top of the PEGDA/PEG-RGDS hydrogels to verify the PEG-RDGS adhesive properties. PEGDA alone hydrogels were compared to the PEGDA/PEG-RGDS hydrogel. At 4 hr after seeding, phase images were taken to observe cell attachment and spreading on the hydrogels.
2.2.4 Degradation of PEG hydrogels

To assess PEG-PQ-PEG cleavage and verify the degradation of the PEG-based hydrogels, acellular hydrogels were formed using the same protocol as described above, and then hydrogels were exposed to proteolytic enzymes for accelerated degradation testing. Specifically, the hydrogels were allowed to swell overnight in PBS at 37°C in 5% CO₂. To assess cleavage of the PQ peptide, collagenase from clostridium histolyticum (20 µg/mL; Sigma) was incubated with the hydrogels (500 µL per gel). Degradation of the hydrogel was checked for the following 4 hr until no visible hydrogel remained in the well. Absorbance at 280 nm was also recorded to monitor tryptophan in solution indicating cleavage of the PQ peptide (Varian Cary® 50 UV-Vis Spectrophotometer; Varian Inc., Palo Alto, CA). Controls for this study included: a PEG-PQ-PEG hydrogel incubated in PBS alone (to observe any baseline PEG-PQ-PEG not incorporated into the hydrogel) and a well with the collagenase solution (alone to observe a baseline for tryptophan associated with the enzyme).

2.2.5 Mechanical testing of PEG-based hydrogels

To assess mechanical properties of PEG hydrogels, acellular gels were formed as previously described. In order to only assess mechanical properties of the hydrogels and not observe an influence of the underlying solid support, 1 mm thick PDMS spacers were used. The volume of the precursor droplet was increased to 15 µL. PEG hydrogels were made ‘free-floating’ as a Sigmacote-modified glass slide was placed on both the top
and bottom of the precursor droplet. Following polymerization, the hydrogels were moved into well plates and allowed to swell in PBS overnight. Using a RSA III Microstrain Analyzer (TA Instruments, New Castle, DE), compressive testing was performed. A uniaxial compressive strain of 0.001 mm/s was applied to each gel. The compressive modulus was calculated from the slope of linear region of the stress-strain plot.

2.3 Results

2.3.1 Synthesis of PEG-macromers (PEG-RGDS, PEG-PQ-PEG)

Using MALDI-ToF mass spectrometry, the synthesis of the GGGPQGIWGQGK peptide (known as PQ) was verified (Figure 2.6). A dominant peak is seen at 1141 Da, the intended weight of the PQ sequence. Following synthesis of the PQ peptide, the PEG macromer acrylate-PEG-PQ-PEG-acrylate was created using amine substitution reactions. Gel permeation chromatography was performed to assess conjugation of acrylate-PEG-PQ-PEG-acrylate (Figure 2.7A). A comparison was made to acrylate-PEG-SVA. Conjugation indicated increased molecular weight in comparison to the acrylate-PEG-SVA. The RGDS was also conjugated to the acrylate-PEG-SVA. Gel permeation chromatography results are shown in Figure 2.7B. Conjugation efficiency was calculated for each PEG-macromer by the calculating the area under the curve of conjugated peak (left shifted) and the area under unconjugated peak of the same PEG-macromer (this is the peak that overlaps with the acrylate-PEG-SVA peak). The calculation for conjugation
efficiency then represented a fraction; the numerator was the area under the shifted (conjugated peak) while the denominator was the area of the shifted (conjugated peak) in addition to the area of the unconjugated peak. Conjugation efficiency for both PEG-macromers was greater than 90% for all samples used in this work.

Figure 2.6 MALDI-ToF mass spectrometry on GGPQGIWGQGK (PQ) peptide

Figure 2.7 GPC of PEG-macromers. (A) PEG-PQ-PEG in red compared to PEG derivate in black. (B) PEG-RGDS in blue compared to PEG derivate in black.
2.3.2 Cell adhesion to surface of PEG-based hydrogel

PEGDA hydrogels with 3.5 PEG-RGDS were created. Mouse endothelial cells (MS1s) were seeded on the hydrogels to confirm RGDS bioactivity and retained cell adhesivity following conjugation. MS1s adhered to PEGDA+PEG-RGDS hydrogels at 24 hr post-seeding (Figure 2.8A). In comparison, no MS1s were attached to PEGDA only hydrogels (Figure 2.8B).

![Figure 2.8](image)

**Figure 2.8 Endothelial cell adhesion on** (A) **PEGDA+PEG-RGDS hydrogels in comparison to (B) PEGDA hydrogels alone at day 1 in culture. Scale bar = 100µm.**

2.3.3 Degradation of PEG-based hydrogel

After 4 hr of exposure to 20 µg/mL collagenase, with agitation every 30 min, 100% hydrogel degradation was seen. The increase absorbance of the 2.5% PEG-PQ-PEG (Figure 2.9) also validates the cleavage of the PQ peptide and degradation of the hydrogel via peptide cleavage.
Figure 2.9 Collagenase degradation of 2.5% PEG-PQ-PEG hydrogel over 4 hr incubation. For some points, the error bars are shorter than the height of the symbol.

### 2.3.4 Mechanical testing

Compressive moduli of PEG-hydrogels were assessed for 2.5% PEG-PQ-PEG based hydrogels. The compressive modulus of 2.5% PEG-PQ-PEG hydrogels was measured as 4.5 ± 0.8 kPa. The compressive moduli reported are comparable to what has been previously reported for PEG hydrogels of similar polymer concentration [37], [138]. This stiffness regime also matches that for soft tissues such as fat and muscle [139].

### 2.4 Conclusions

This chapter introduced the material synthesis of two PEG-macromers: a cell adhesive PEG-RGDS and a proteolytically sensitive PEG-PQ-PEG. Cell adhesion, cell encapsulation and mechanical properties of the PEG-based hydrogels were also discussed. The cell encapsulation technique will be used in all future studies to assess
macrophage influence on endothelial cells during vessel formation in PEG-based hydrogels.
3. Macrophages influence vessel formation in 3D bioactive hydrogels¹

3.1 Introduction

As discussed in chapter 1, macrophages have been traditionally thought to serve solely in their primary roles as phagocytes and inflammatory agents of the immune system [56], [63], [78], [140]. However, it is known that macrophages play a role in tissue reorganization by promoting angiogenesis and wound healing [40], [53], [76], [84], [141]. Because of the potential to utilize macrophages as a pro-healing cell source, there is great interest in better defining the roles that macrophages can play in tissue repair and angiogenesis [15], [142], [143].

As discussed in chapter 1, recent in vivo studies have begun to highlight key influences of macrophages with regards to angiogenesis and vasculogenesis. In both zebrafish and mouse hindbrain models, macrophages can act as cell-chaperones within vessel networks, facilitating the connections between neighboring endothelial tip cells [12]. Additionally, yolk sac macrophages have been observed associating with nascent vasculature and were shown to be required for vascularization of the testis [96]. In the murine cornea micropocket model, macrophages were shown to promote and stabilize

¹ Parts for this chapter have been adapted from:
vasculature by bridging endothelial tip cells and serving as support cells to nascent vessels [14], [103]. This body of in vivo studies supports the hypothesis that macrophages can play beneficial roles in vessel development. However, the in vivo environments in which these beneficial interactions have been observed were either within abnormal, avascular settings (cornea) or within a developmental context [90]. Specifically, in the cornea micropocket model, vasculature and macrophages are recruited into an immunologically privileged and normally avascular site by growth factors such as MCSF, bFGF/PDGFBB [103], [104]. The use of the cornea micropocket model induces abnormal invasion in order to allow for ease of visualization. This model, however, limits the ability to translate these results to other tissues in the host as the cornea is a site in which macrophage invasion and function is abnormal to compared to non-immunologically privileged tissues [103], [104]. Additionally, the remaining in vivo models have studied macrophages in an embryonic or yolk sac form; these macrophages are derived from alternative progenitors and function differently than adult bone marrow derived macrophages [42]. Thus, it is unknown if the macrophage interactions within these embryonic models readily translate to adult tissue [42], [43], [48], [63], [144]. Moreover, the highly complex tissue morphogenesis occurring simultaneously in the embryonic models limits the ability to isolate the function of macrophage interactions from the context of embryonic development.
To better understand the role of macrophages in vessel formation, in vitro models have been employed. Conditioned media has been used to investigate paracrine signaling between macrophages and endothelial cells [108]. Endothelial cells and other support cells have also been cultured on and within naturally-derived materials such as fibrin, collagen and Matrigel® and subsequently, exposed to conditioned media from macrophages [105], [108], [110]. In these studies, media from macrophages increased the number of endothelial cell sprouts and vessel tube length [105], [108], [110]. While this work supports the notion that factors released from macrophages can enhance vessel development, these studies did not consider the role of cell-cell contacts. One additional study assessed macrophages and endothelial cells on collagen gels to assess cell-cell interactions [14]. That work identified close associations between the macrophages and endothelial cells on the collagen gels in 2D. However, the cells used in this work were from multiple species and the associations seen between the cells were only explored within the context of the collagen microenvironment [14]. Because both the cell-cell interactions and the soluble signals between macrophages and endothelial cells have been shown to play a critical role in vessel development, there is a need to better identify the influence of macrophages on vessel formation as well as the types of interactions macrophages can have with endothelial cells during vessel formation. To address this, we created a highly controlled system in which both paracrine and direct cell-cell interactions between macrophages and endothelial cells can be studied.
In this chapter, we utilized a bioactive PEG-based hydrogel to assess the role of macrophages during vessel formation by encapsulating macrophages with endothelial cells. Through this platform, we were able to isolate the influence macrophages have on endothelial vessel volume, the morphological response of macrophages to endothelial cells, and categorize the types of interactions between the macrophages and endothelial structures. This work demonstrates that macrophages can serve as a novel cell source for regenerative medicine efforts as macrophages promote vessel development within bioactive hydrogels.

3.2 Materials and methods

3.2.1 Cell maintenance

Primary bone marrow derived mouse macrophages were used within this work (C57BL/6 Mouse Bone Marrow Macrophages; Cell Biologics, Chicago, IL). These cells were used at passage 2 and maintained in Macrophage Medium (Cell Biologics). Primary aortic endothelial cells (C57BL/6 Mouse Primary Aortic Endothelial Cells; Cell Biologics) were also used and cultured in Mouse Endothelial Cell Medium (Cell Biologics). These cells were used at passage 3-6. Murine MILE SVEN 1 (MS1; ATCC, Manassas, VA), a C57BL/6-derived endothelial cell line, were cultured in DMEM (Corning, Corning, NY) supplemented with 5% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and 100 IU penicillin and 100 µg/mL streptomycin (Corning). All cells were maintained at 37°C in 5% CO₂.
3.2.2 Hydrogel formation and encapsulation of cells

Formation of hydrogels has previously been detailed in chapter 2. Specifically, macrophages were encapsulated at densities ranging from $4 - 20 \times 10^6$ cells/mL. MS1s were encapsulated at densities ranging from $10 - 24 \times 10^6$ cells/mL. The primary aortic endothelial cells (AECs) were encapsulated at $8 \times 10^6$ cells/mL. Hydrogels were formed on the methacrylate-modified glass coverslips. This was done for ease of handling of the delicate hydrogel and ease of culturing in well plates. MS1 media (DMEM, 5% FBS, 100 IU penicillin and 100 µg/mL streptomycin) or Mouse Endothelial Cell Medium (Cell Biologics) was then added to the hydrogels in the wells. For the macrophage only hydrogels used for viability testing, Macrophage Medium (CellBiologics) was added to the hydrogels. All gels were cultured at 37°C in 5% CO₂. Media changes occurred at 16 hr post-encapsulation and subsequently every 48 hr afterwards.

3.2.3 Viability testing

Macrophages were encapsulated in hydrogels at $6 \times 10^6$ cells/mL. MS1 cells were encapsulated in hydrogels at $15 \times 10^6$ cells/mL and AECs were encapsulated at $8 \times 10^6$ cells/mL. At 24 hr or 72 hr post-encapsulation, media was removed and viability staining was performed. Calcein AM (2 µM; ThermoFisher Scientific, Grand Island, NY) and ethidium homodimer-1 (EthD-1; 4 µM; ThermoFisher Scientific) were added to the hydrogels after two PBS (Corning) washes. Hydrogels were incubated in the viability staining solution for 45 min at room temperature under constant agitation. Following
two PBS rinses, hydrogels were imaged on a Zeiss 510 inverted confocal microscope using a 10X EC Plan-Neofluar objective (NA = 0.30) at excitation/emission wavelength 494/517 nm for calcein AM and 517/617 nm for EthD-1. Manual counts were conducted to ascertain cell viability (>100 cells counted per cell type).

3.2.4 Immunocytochemistry

Gels were fixed with 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA) at day 3 (unless otherwise noted). All staining was conducted in Tris buffered saline (TBS, 0.05M Tris and 0.15M sodium chloride, pH 7.4 ;Corning). Cells in gels were permeabilized with 0.25% triton-X 100 (Sigma), rinsed, and blocked in 10% normal donkey serum (Sigma). Following blocking, gels were rinsed and incubated in primary antibody solutions. For this work, the following antibodies were used: goat anti-mouse iNOS (1:200; ThermoScientific, Rockford, IL), rabbit anti-mouse CD206 (1:40; R&D Systems, Minneapolis, MN), rat anti-mouse CD31 (1:200; BD Biosciences, Franklin, NJ) and rabbit anti-mouse CD11b (1:200; abcam, Cambridge, MA). Simultaneous imaging included: CD31, iNOS and CD206 or CD31 and CD11lb. Following primary incubation, gels were rinsed and incubated with secondary antibodies. The following secondary antibodies were used at a 1:200 dilution: donkey anti-rat Alexa Fluor ® 488, donkey anti-goat Alexa Fluor ® 647, donkey anti-rabbit Alexa Fluor ® 555 (Life Technologies, Carlsbad, CA). Washes were then performed and 2 μM 4’,6-diamidino-2-phenylindole (DAPI; Sigma) was used as a nuclear stain. Gels were imaged using a 20X
plan-apochromat objective (NA = 0.80) on a Zeiss 510 inverted confocal microscope. Stacks of 36 µm were taken with 2 µm thick slices.

3.2.5 Tubule volume analysis

To assess tubule volume within the hydrogel matrices, hydrogels were formed with 8 x 10^6 AECs/mL. Co-culture hydrogels incorporated 8 x 10^6 AECs/mL and 4 x 10^6 macrophages/mL. Cells in hydrogels were cultured for 7 days, then fixed, stained and imaged. IMARIS (Bitplane, Zurich, Switzerland) was used for tubule volume assessment. Specifically, tubule volume was defined as CD31^+ structures of volume > 1400 µm³, as single cell structures were found to be <1400 µm³ (N=4 gels, 14 stacks for each group). Quantification utilized a one-tailed t-test where a p-value less than 0.05 was considered significant. All values are reported as mean ± standard deviation.

3.2.6 Macrophage spreading

To assess macrophage spreading, cell circularity was determined from fluorescent images. Specifically, the CD11b^+, iNOS^+ and CD206^+ staining channels were assessed. The analyze particle macro in ImageJ was employed for each condition (N=4 for each group, each group >75 macrophages). Thresholds and range of particle size were held constant for all analysis. ANOVA followed by Student’s t-test was utilized via GraphPad Prism 7 and p-values less than 0.05 were considered significant. All values are reported as mean ± standard deviation.
For analysis of cell spreading versus distance to nearest endothelial cell, the same circularity procedure was utilized. For distance to nearest endothelial cell, the analyze particle feature in ImageJ was used to outline macrophages. This outline was then overlaid with the CD31+ channel and the DAPI+ channel (N=5 stacks in total, N>100 macrophages). It is important to note that distance to nearest endothelial cell accounted for the x,y direction in the same plane of the macrophage. Distance was measured via the “line selection” tool measuring nucleus (from the DAPI+ channel) from the macrophage outline to nucleus of the endothelial cells in the CD31+ channel. Bins were utilized to assess distance on the scale of cell lengths. ANOVA followed by Student’s t-test were employed for quantification in GraphPad Prism 7; p<0.05 was used for significance. All values are reported as mean ± standard deviation.

3.2.7 Assessment of macrophage-endothelial cell interactions and bridges

Overlay of CD31+ channels and macrophage channels demonstrated macrophages interacting closely with endothelial cells. To quantify macrophage association along endothelial structures, macrophages with circularity <0.5 and less than <5 µm from the nearest endothelial cell were considered (N=16 slices per condition, where each slice is a surface area of 2.0 x 10^5 µm^2). For quantification purposes, bridging macrophages are defined by cells staining positive for macrophage markers (CD11b+, iNOS+ or CD206+) that interact with two adjacent CD31+ structures while the CD31+ structures themselves must not interact or overlap. Both ends of the CD31+ structures
must overlay with macrophage positive staining (N=16 slices per ratio condition, where each slice is a surface area of $2.0 \times 10^5 \text{µm}^2$). Overlay, circularity and distance measurements were conducted in ImageJ. ANOVA followed by Student’s t-test were employed to report statistical significance (*p<0.05 indicates significance, GraphPad Prism 7). All values are reported as mean ± standard deviation.

### 3.2.8 Live imaging

Live imaging of the macrophages and endothelial cells was conducted using the Viva View FL Incubator Microscope (Olympus Corporation, Tokyo, Japan). For these studies, cells were labeled with CellTracker dyes for 24 hr prior to encapsulation following the manufacturer’s protocol. MS1s were labeled with 10 µM CellTracker Green MFDA (Life Technologies) in MS1 media for 16 hr. Macrophages were labeled with 10 µM CellTracker Red CMTPX (Life Technologies) in Macrophage media for 16 hr. Cell morphology was unchanged after incubation with the dyes as assessed via bright field microscopy using a Zeiss Axiovert 135 inverted microscope (Zeiss, Oberkochen, Germany). The gel pre-polymer solution was prepared in the same manner as described previously. For the co-culture gels, macrophages and endothelial cells were combined at a 1:1 ratio with $15 \times 10^6$ cells/mL for each cell type. For the macrophage only gels, macrophages were encapsulated at $15 \times 10^6$ cells/mL. For live imaging, glass-round bottom 35 mm petri dishes (MatTek, Ashland, MA) were used to fit into the Viva View stage. The glass bottom of the petri dish underwent methacrylate-modification as
described above. PDMS spacers were placed directly on to the methacrylate-modified glass-round bottom dish. A 5 µL droplet of the precursor solution was placed between the PDMS spacers. A hydrophobic Sigmacote- modified glass coverslip was placed on top of the droplet. Gelation was induced as previously described. The Sigmacote-modified glass coverslip was removed. The gel was covalently immobilized onto the glass-round bottom dish allowing for ease of handling. At 5 hr post-encapsulation, the media was changed and the samples were loaded into the Viva View incubator. Images were collected every 45 min for macrophage only gels and every 100 min for macrophage and endothelial cell co-culture gels.

3.2.9 Analysis summary

For clarity, the following table summarizes the experiments and analyses conducted within this chapter.

<table>
<thead>
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<th>Experiment</th>
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<td>Macrophages in co-culture with AECs</td>
<td>Macrophages + AECs</td>
<td>Tubule volume (via IMARIS)</td>
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<td>AECs alone as control</td>
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<td>Macrophage circularity compared between culturing conditions</td>
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<td>Circularity of macrophages (via ImageJ)</td>
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<td>Macrophages + MS1s (at ratios: 2:1, 1:1 and 1:2)</td>
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<td>Macrophage circularity versus distance to nearest MS1</td>
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<td>Circularity of macrophages and distance (nucleus to nucleus) (via ImageJ)</td>
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<tr>
<td>Macrophage associations with MS1s</td>
<td>Macrophages + MS1s (at ratios: 2:1, 1:1 and 1:2)</td>
<td>Circularity of macrophages and distance (nucleus to nucleus) (via ImageJ)</td>
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3.3 Results

3.3.1 Characterizing macrophage and endothelial cell responses to encapsulation within PEG-based hydrogels

Macrophage viability was maintained following the encapsulation process and subsequent culturing within the hydrogels (Figure 3.1A). Approximately 85% of the macrophages were viable at 24 hr after encapsulation. After 3 days in culture, macrophage viability was assessed again. The remaining macrophages encapsulated in the hydrogel had a viability of 87% after 3 days of culture in the hydrogel (Figure 3.1B). Within this work, both primary aortic endothelial cells (AECs) and MS1s, the endothelial cell line, were used as C57BL/6 mouse endothelial cell (EC) sources. As seen in Figure 1C and D, AECs and MS1s maintain excellent viability after encapsulation. At 24 hours after encapsulation, approximately 91% of the AECs and 93% of the MS1s were viable.
Figure 3.1 Characterizing the macrophage and endothelial cell response to encapsulation within PEG-based hydrogels. (A) Live/Dead at 24 hr and (B) 3 days demonstrates macrophages survive encapsulation. Scale bar= 50 µm (C) Live/Dead at 24 hours post-encapsulation of AECs and (D) MS1s. Scale bar= 100 µm

3.3.2 Macrophages enhance tubule formation when co-encapsulated with AECs

To assess the influence of macrophages on tubule formation, macrophages and primary aortic endothelial cells (AECs) were encapsulated together, homogenously mixed, in a hydrogel matrix. Tubule volume was assessed to quantify the formation of vessel structures. Macrophages in co-culture with AECs enhanced tubule volume in comparison to AECs alone by nearly four-fold (Figure 3.2A,B). AECs in co-culture with macrophages had an average tubule volume of $1.9 \times 10^6 \pm 0.86 \times 10^6 \, \mu m^3$ (Figure 3.2C). In comparison, AECs alone had an average tubule volume of $0.54 \times 10^6 \pm 0.19 \times 10^6 \, \mu m^3$. 
Figure 3.2 Macrophages enhance tubule volume in co-culture with primary aortic endothelial cells (AECs). (A) AECs alone vs. (B) AECs and macrophages within hydrogels at day 7. EC (CD31), DAPI (nuclear stain). Scale bar= 100 µm. (C) Tubule volume of AECs alone vs. AECs and macrophages. *p<0.03

3.3.3 Macrophage morphology is influenced by co-culture with endothelial cells

To assess macrophage interactions with endothelial tubules, the MS1 cell line was used. MS1s robustly form tubules independent of a secondary cell source allowing investigation of macrophage interactions with ECs under conditions where the vascular structures were not changing between conditions (Figure 3.3). Upon encapsulation with endothelial cells, macrophage morphology changed dramatically, from highly rounded cells in monoculture to highly spread cells in co-culture (Figure 3.4 ABCD). In contrast,
macrophages encapsulated alone in hydrogels remained highly rounded (Figure 3.4). Live imaging confirmed that macrophages in co-culture with MS1 endothelial cells migrated and spread along vessel structures (Figure 3.5). In comparison, macrophages encapsulated alone migrated to a similar degree within the hydrogels but remained in a highly rounded morphology (Figure 3.6). To further probe the macrophage response to endothelial cell co-culture conditions, macrophages and endothelial cells were encapsulated at varying ratios: 2:1 (macrophage to endothelial cell), 1:1 and 1:2. Macrophage circularity was assessed at each of the ratios (Figure 3.7A). Macrophages alone had a circularity of 0.82 ± 0.13; macrophages in co-culture adopted circularities of 0.67 ± 0.19, 0.60 ± 0.22 and 0.55 ± 0.25 at ratios of 2:1, 1:1 and 1:2 (macrophage: EC), respectively (Figure 3.7A). Moreover, 19% of macrophages in 2:1 ratio had a circularity of ≤0.5, indicating highly spread cells, compared to 38% of macrophages in 1:1 ratio and 46% of macrophages in 1:2 ratio (Figure 3.7B). In comparison, 0.91% of the macrophages encapsulated alone had circularity ≤0.5. Representative images and the populational response of macrophages in co-culture are displayed in Figure 3.4A,B,C,D.
Figure 3.3 MS1s form tubules independently of a support cell. Day 3. EC (CD31) and DAPI (nuclear stain). Scale bar = 100 µm
Figure 3.4 Macrophage circularity is influenced by co-culture with endothelial cells. (A) Circularity of macrophages alone (N=110) (B) 2:1 ratio (macrophages:ECs) (N=142) (C) 1:1 ratio (N=132) and (D) 2:1 ratio (N=115). Representative images in each histogram. Day 3, MAC (iNOS, CD206), EC (CD31), DAPI (nuclear stain). Scale Bar=50 µm.
Figure 3.5 Macrophages (red) and MS1s (green) are seen migrating and interacting with one another over a period of 5 hours beginning at 5 hours post-encapsulation. Scale Bar = 25 µm.

Figure 3.6 Macrophages (red) alone remain highly rounded and migrate in the PEG-based hydrogel at day 1 (over a period of 6 hours beginning at 5 hours post-encapsulation). Scale Bar = 100 µm.
Figure 3.7 Circularity of macrophages as a function of culturing conditions. 
(A) Bar graph of macrophage:endothelial cell ratio conditions compared to macrophage only. Bars that do not share a common letter are statistically significant, * p<0.05. (B) Cumulative % of population of macrophages at varying macrophage to endothelial cell ratios.

3.3.4 Macrophage morphology is dependent on distance to nearest EC structure

Because macrophages that were co-encapsulated with higher densities of MS1 endothelial cells were more spread than macrophages alone, we hypothesized that distance to nearest endothelial cell could affect macrophage morphology. To test this hypothesis, we quantified circularity versus distance to nearest endothelial cell. In particular, distance to nearest endothelial cell was measured as the distance from nucleus to nucleus. We found that macrophages closest to vessel structures were less circular and more spread; they have an average circularity of 0.48 ± 0.22 over a distance of 0-5 µm from the nearest structure (Figure 3.8). Macrophage circularity increased as distance from vessel structures increased: 0.60 ± 0.23 for 5-10 µm, 0.69 ± 0.21 for 10-15 µm, 0.77 ± 0.20 for 15-20 µm and 0.83 ± 0.072 for > 20 µm (Figure 3.8). Statistical
significance (p < 0.05) was achieved when comparing the 0-5 µm group to all groups >10-15 µm away from nearest MS1 endothelial cell.

Figure 3.8 Macrophage circularity is dependent on distance to nearest EC structure. A bar graph of macrophage circularity by distance to nearest vessel structure where A, indicates statistical significance compared to (0-5) group and B, indicates statistical significance compared to (5-10) group, * p<0.05.

3.3.5. The two types of interactions between macrophages and endothelial cells depend on co-culture conditions

Two classes of interactions between macrophages and ECs were observed in this work (Figure 3.9A). The most common interaction seen in the work involved macrophages in close association with the outerwall of endothelial cell tubules (Figure 3.9B). The second type of interaction involved macrophages bridging neighboring vessel structures (Figure 3.9C).
To quantify these two interactions, we analyzed total number of macrophages associating with endothelial cells and the total number of macrophages present at bridges between neighboring endothelial cells. Macrophages associating with endothelial cell structures occur more frequently at 1:1 and 2:1 ratios with an average of 5.06 ± 2.91 and 4.38 ± 1.85 interactions per slice while the 1:2 ratio with 2.25 ± 1.94 interactions per slice (Figure 3.9D). The number of macrophages acting as bridges between two adjacent endothelial cell structures occurs more frequently at the 1:1 ratio with 2.0 ± 1.0 interactions per slice and at the 1:2 with 1.6 ± 0.7 interactions than the 2:1 ratio with 0.9 ± 0.8 interactions (Figure 3.9E).
Figure 3.9 The two types of interactions between macrophages and endothelial cells depend on co-culture conditions. (A) Schematic of two main macrophage interactions visualized within this model. (B) Macrophages associating with ECs. (C) Macrophages interacting with sprouting ECs on both sides of the EC sprouts. Day 2, MAC (CD11b, iNOS), EC (CD31), DAPI (nuclear stain). Scale Bars= 20 µm. (D) Macrophage association (set via circularity and proximity thresholds) divided by total macrophages overlapping with EC structures by macrophage:EC ratio. (E) Average number of bridges per ratio. Statistical significance is represented as * p<0.05.

3.4 Discussion

Within this work, macrophages influenced vessel formation. In particular, one of the novel responses initially seen between the macrophages and endothelial cells was the influence macrophages have on vessel formation through the assessment of tubule volume. In particular, macrophages encapsulated with AECs enhanced tubule volume when compared to AECs alone. Other studies have demonstrated macrophages can play
a beneficial role in endothelial cell sprouting and tubule formation [14], [105], [107], [108]. Tattersall et al. demonstrated that mouse macrophages encapsulated in fibrin gel spatially located near HUVECs enhanced endothelial sprouting [105] and Spiller et al. noted that macrophage-conditioned media positively influenced vessel network structure by increasing endothelial sprout, nodes and tube length [15]. These prior studies showed a clear impact of macrophage-derived soluble signals, but could not investigate cell-cell interactions or the simultaneous effect of both soluble signals and cell-cell interactions on endothelial cells and vessel network formation. Within our system, we can simultaneously study macrophage soluble influence on and cell-cell interactions with endothelial cells. Because we demonstrated that macrophages co-encapsulated with AECs resulted in increased tubule volume when compared to AECs alone, we next probed the mechanisms by which macrophages could enhance tubule volume. In particular, we identified specific interactions and associations between macrophages and endothelial cells (Figure 3.9). These results indicated that both cell-cell interactions and paracrine signaling have the ability to influence tubule formation. Macrophages appear to be capable of acting as a secondary support cell source for AECs as they enhanced the formation of tubule structures when in 3D co-culture. Thus, this platform is a novel tool that can be used to further probe the influence of soluble signaling and cell-cell signaling between macrophages and endothelial cells to parse out
the influence of each. The mechanisms by which cell-cell contacts play a role in vessel enhancement can also be studied within this system.

Because of the cell-cell associations observed between macrophages and endothelial cells within this work, we next examined the macrophage response to the endothelial cells within the hydrogels. While the studies with the primary aortic endothelial cells (AECs) identified the supportive role macrophages can have on the volume of newly formed vessels, AEC tubule formation varied significantly with different co-culture settings. In particular, the formation of stable AEC vessel structures was dependent on presence of a secondary cell source. Because it is hypothesized that macrophage interactions with endothelial cells occur independently of VEGF-mediated vessel formation [12], we also wanted to consider the macrophage response to and interactions with vessel structures that developed independently of macrophage co-culture. For the next aspect of this work, the focus then shifted to identify the types of interactions macrophages can have with vascular tubule structures, but where changes in the vascular structures are less marked. Thus, we studied macrophage interactions with MS1s, a mouse endothelial cell line. MS1s are able to form robust tubule structures independent of the presence of support cells (Figure 3.3). The use of MS1s allowed macrophage interactions with endothelial cells to be decoupled from macrophage-induced changes in the vascular structure.
When encapsulated with endothelial cells, macrophages became highly spread within the hydrogel. It is important to note that in a prior study macrophage morphology was not altered as a result of co-culture with endothelial cells when co-cultured within fibrin gels [105]. In that study, macrophages were encapsulated within the fibrin gel. The endothelial cells were adhered onto beads before being suspended in the fibrin gel thus the macrophages were spatially close to the endothelial cells but the macrophages remained immobile and did not migrate. This co-culture arrangement likely limited macrophage proximity to the endothelial cells and thus impaired changes in macrophage morphology as a result of co-culture. Conversely, in our system, macrophages significantly alter their morphology when co-encapsulated with endothelial cells. We also confirmed that this was dependent on proximity to endothelial cells. The presence of endothelial cells increased the range of macrophage circularity indicating enhanced spreading. A maximum ratio of 1:1 (macrophage to endothelial cell) resulted in the most significant increase in macrophage spreading. Thus, for this platform, the influence of endothelial cells on macrophage circularity was limited by density of endothelial cells. We hypothesize this limitation in endothelial cell influence represents an ideal spacing between the macrophages and endothelial cells that permits macrophages to respond to the presence of endothelial cells. In particular, we surmise that growth factor gradients between the macrophages and the endothelial cells result in macrophage chemotaxis and migration towards the gradient, as has been previously
demonstrated within the literature [66], [89], [94], [145], [146]. When endothelial cells are close to macrophages, the macrophages can respond in a proximity dependent manner and alter their circularity. As the number of endothelial cells was lowered, a greater fraction of the macrophages were further away from endothelial cell structures. These macrophages were then less likely to interact and respond to endothelial cell signals and thus displayed circularity similar to the macrophages in monoculture hydrogels. To probe the hypothesis of a critical distance, we assessed macrophage circularity versus distance to nearest endothelial cell. A clear trend revealed macrophages closest to endothelial cells are the most spread of the population of macrophages (Figure 3.8). The response of the macrophages in close proximity to or touching vessel structures introduced the notion that both paracrine signaling and cell-cell associations are responsible for this alteration in macrophage circularity.

We next classified the types of cell-cell interactions seen in our model. Two main classes of interactions were visualized: macrophages associating with endothelial cells in a pericyte-like manner and macrophages bridging neighboring endothelial structures in a cell-chaperoning manner (Figure 3.9A). Both of these interactions are often seen within in vivo models [11], [12], [14], [90], [96]. The recurrence of these interactions further reinforces the likelihood that macrophages play a beneficial role in vessel development via juxtacrine contact with endothelial cells. Specifically, pericyte-like associations are seen via highly spread macrophages in close contact with endothelial cells. This
association is similar to pericyte affiliation to vessel structures seen in other microvascular models [36], [37]. In particular, support cells and pericytes are known to closely affiliate and stabilize nascent vessel structures as the vessel structures develop [9], [147], [148]. Within this work, we demonstrated that not only can macrophages enhance tubule formation but that macrophages also associate with endothelial cells in a manner reminiscent of support cell behavior. This work also suggests that macrophages may be harnessed to enhance vessel formation for regenerative medicine applications.

Broadly, within the context of implanted biomaterial scaffolds, macrophages are naturally some of the first cells to respond to injuries and implants [50], [112]. While particular attention has been given to the deleterious roles macrophages can play in implant rejection and foreign body giant cell formation [56], [78], [149], this work demonstrates macrophages can play a beneficial role in facilitating vessel formation if their activation state can be appropriately controlled.

The second type of macrophage-endothelial cell interaction demonstrated within this work was macrophages bridging endothelial cells structures. The bridging interaction, which occurred more rarely than the pericyte-like associations at least at the time points and conditions examined, was reminiscent of cell-chaperone interactions seen in vivo [12], [14]. In particular this cell chaperoning behavior is described as macrophages orchestrating anastomosis between sprouting endothelial tip cells [12]. In zebrafish and in the mouse hindbrain, macrophages were seen physically joining two
endothelial tip cells [12]. Moreover, when macrophages were removed from sites of active angiogenesis in these models, the number of vessel connections within the network was reduced [11], [12]. This suggests macrophages play a critical role in vessel network organization and in endothelial tip cell fusion [11], [12]. Our current work demonstrated macrophages interacting with endothelial cells in a manner reminiscent of cell-chaperoning behavior within a controlled 3D synthetic hydrogel system. We also probed the influence of macrophage to endothelial cell ratios on the types of interactions that would occur within this system. Prior in vivo models within the mouse retina have speculated a ratio of 1:2 (macrophage to endothelial cell) would result in macrophage associations with endothelial tip cells however the motivation for this ratio was not shown [11]. Moreover, the in vivo mouse retina model only considered microglial cells (macrophages of the brain) within the context of retina development. Within our system, macrophages were more likely to serve as bridges between endothelial structures at a 1:1 ratio (Figure 3.9E). It is reasonable to assume that the conditions probed within our hydrogel system vary from the highly complex retina tissue morphogenesis context used within the in vivo model. Furthermore, microglial cells are known to behave differently from bone marrow derived macrophages due to differences in cell origin and function [45], [48], [61]. Thus the difference in cell ratios for macrophage bridging associations between our in vitro system and the retina in vivo bridging model is not unexpected.
### 3.5 Conclusion

This chapter aimed to better clarify the roles that macrophages can play within vessel formation in a 3D reductionist microenvironment. This research demonstrated the ability for macrophages to enhance tubule formation, respond to the presence of endothelial cells by altering their morphology and associate with endothelial cells in a pericyte-like and bridging manner. The PEG-based hydrogel used in this work offered unique control over the cell microenvironment and allowed focus on the interactions between macrophages and endothelial cells within the context of vessel development. Thus, this chapter has demonstrated macrophages are capable of influencing vessel formation within a 3D PEG-based system. Moreover, this system can be used in future work to understand the role of macrophage phenotypes in vessel development as discussed in chapter 4.
4. M0 and M2 macrophages enhance vascularization of tissue engineering scaffolds

4.1 Introduction

As previously mentioned in chapter 1, macrophages, normally thought of as phagocytic inflammatory cells, can also play an important stimulatory role within vessel development [50], [70], [71], [107]. Recent work has demonstrated that macrophages are often present at sites of angiogenesis, tissue development, and wound repair [48], [61], [63]–[66]. The removal, knockout or inhibition of macrophages from these sites has been shown to impair the resulting vasculature [12], [96], [97]. Additionally, depending on environmental cues, macrophages can alter their characteristics and assume different macrophage phenotypes. There are two main extremum of macrophage phenotypes: M1 macrophages, which are thought to be pro-inflammatory, and M2 macrophages, which are thought to be pro-tissue healing [71]. Thus, depending on the phenotype adopted, macrophages could possibly benefit or retard vessel development [53]. In order to better understand the roles of macrophages on vessel development, we utilized our PEG-based hydrogel to assess the interactions and influences of macrophage phenotypes and endothelial cells during vessel formation.

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1 Portions of this chapter have been adapted from: Moore, E.M., Suresh, V., Ying, G., and West, J.L. M0 and M2 macrophages enhance vascularization of tissue engineering scaffolds. Regenerative Engineering and Translational Science. (In Press).
Recent work has begun to uncover the roles of macrophage phenotypes in vessel development [97], [105], [107], [109], [110]. Within the context of normal development, M2-like macrophages were found to be a critical presence at sites of blood vessel development as the knockout of macrophages from these sites resulted in abnormal vasculature with decreased connectedness and altered architecture [12], [96]. Specifically, in a murine fetal testis model, M2-like macrophages (CD206+ cells) were found localized to nascent vessel structures. These M2-like macrophages were thought to regulate vessel organization both through the controlled secretion of VEGF-A and through vascular surveillance as the absence of these macrophages resulted in irregular vessels [96].

To better dissect the roles of macrophage phenotypes in vessel formation, a number of groups have focused on in vitro studies [11], [14], [66]. These studies have mainly adopted the following progression: initial stimulation of each macrophage phenotype and, subsequent culturing of endothelial cells with either macrophages or their conditioned media on a 2D surface. These studies have unveiled the roles of soluble factors in influencing endothelial tubule formation but have largely neglected the role of cell-cell contact in determining macrophage-endothelial cell communication [105], [108], [110]. In particular, the few co-culture studies either failed to consider 3D systems [110] or failed to note cell interactions between the macrophages and endothelial cells as the macrophages did not spread or migrate within their 3D models.
As noted from prior work in our lab and in other in vivo studies, macrophage-endothelial cell interactions can play a critical role in facilitating the formation of vessel structures [12], [97], [150]. Using a poly(ethylene glycol)(PEG)-based model developed in prior work [36], [39], [138], [150], [151], we sought to assess the influence of macrophage phenotypes on vessel development within a reductionist 3D microenvironment.

### 4.2 Materials and methods

#### 4.2.1 Cell maintenance

As previously described in Chapter 3.2.1, primary bone marrow derived mouse macrophages (C57BL/6 Mouse Bone Marrow Macrophages; Cell Biologics, Chicago, IL) were used at passage 2. Three stimulation profiles were then used in order to alter the macrophage phenotype: M0 macrophages were cultured in DMEM (Corning, Corning, NY) supplemented with fetal bovine serum (10% (v/v), Atlanta Biologicals, Lawrenceville, GA), penicillin (60 µg/mL) and streptomycin (100 µg/mL) (Corning), herein referred to as M0 basal media; M1 macrophages were stimulated in the M0 basal media supplemented with 100 ng/mL of lipopolysaccharide (LPS; Santa Cruz Biotechnology, Dallas, TX) and 10 ng/mL of interferon gamma (IFN-γ; ProSpec, East Brunswick, NJ) (based on prior literature [15], [105], [109]). M2 macrophages were stimulated in the M0 basal media supplemented with 20 ng/mL interleukin-4 (IL-4; ProSpec) and 20 ng/mL interleukin-13 (IL-13; ProSpec) (based on prior literature [15], [105].
After 24 hr of exposure to the stimulation media, the macrophages and the conditioned media of each were collected and used in subsequent experiments.

Two types of mouse endothelial cells were used in this work: AECs and MS1s (see Chapter 3.2.1 for additional details). All cells were maintained at 37°C in 5% CO₂.

4.2.2 Flow cytometry

The flow cytometry protocol utilized in this work has been previously described. Following 24 hr exposure to stimulating media, each macrophage sample was washed with TBS (Corning) and incubated in TruStain fcX™ (BioLegend, San Diego, CA) for 10 min in order to reduce non-specific binding of antibody IgGs to the Fc receptor on the macrophages. The cells were then incubated with the following antibodies for 20 min: FITC (Fluorescein) anti-mouse CD206, Alexa Fluor®647 anti-mouse CD11b, and PE (R-Phycoerthrin) anti-mouse CD86 (BioLegend). Isotype controls were also conducted in tandem: FITC Mouse IgG1, Alexa Fluor® 647 Mouse IgG1, and PE Mouse IgG1 (BioLegend). Following antibody incubation, 1% bovine serum albumin (w/v) (BSA; Sigma) in TBS was added to each sample. The cells were then centrifuged at 500 g for 7 min and resuspended in TBS. 4% paraformaldehyde (Electron Microscopy Services, Hatfield, PA) was then added to each sample for 5 min. The samples were assessed using a BD FACSCanto II system (BD Biosciences, Franklin Lakes, NJ).
4.2.3 Immunocytochemistry

2D cell culture samples were fixed with 4% paraformaldehyde (Electron Microscopy Services) for 20 min at room temperature. All staining solutions were made using TBS (Corning). 0.25% Triton-X 100 was added to the samples for 20 min in order to permeabilize the cells (Sigma). The 2D samples were then rinsed with TBS and incubated in 10% (v/v) normal donkey serum (NDS; Sigma) in TBS for 1 hr in order to block nonspecific binding of the primary and secondary antibodies. After blocking, the 2D samples were incubated overnight in a 1% (v/v) NDS of TBS with the following primary antibodies: rabbit anti-mouse iNOS (1:200; ThermoScientific), goat anti-mouse arginase-1 (1:100; Novus Biologicals, Littleton, CO), rat anti-mouse CD31 (1:200; BD Biosciences), goat anti-mouse CD206 (1:40; R&D Systems, Minneapolis, MN) and rabbit anti-mouse ki67 (1:100; Abcam, Cambridge, MA). The samples were rinsed with TBST (TBS + 0.01% Tween 20; Corning) and then incubated for 1 hr in 1% (v/v) NDS of TBS with the following secondary antibodies (used at a 1:200 dilution): donkey anti-rat Alexa Fluor ® 488, donkey anti-goat Alexa Fluor ® 647, donkey anti-rabbit Alexa Fluor ® 555 (Life Technologies, Carlsbad, CA). The 2D samples were washed and 2 μM DAPI (Sigma) was used as a nuclear stain. The same protocol was utilized for 3D hydrogel samples (see Chapter 3.2.4) with increased fixation and antibody incubation time to allow diffusion through the hydrogel. Additionally, the following antibodies were used for immunocytochemistry with the 3D hydrogels: rat anti-mouse CD31 (1:200;
BD Biosciences) and rabbit anti-mouse ki67 (1:100; Abcam, Cambridge, MA). All samples were imaged as described in Chapter 3.2.4.

4.2.4 Tubule volume analysis

As in prior work [150], tubule volume was assessed using IMARIS software (Bitplane, Zurich, Switzerland). See Section 3.2.5 for additional details. To assess tubule volume within the hydrogel matrices, AEC hydrogels were formed with $8 \times 10^6$ AECs/mL. Co-culture hydrogels incorporated $8 \times 10^6$ AECs/mL and $8 \times 10^6$ macrophages/mL. Cells in hydrogels were cultured for 3 or 7 days, then fixed, stained, and imaged as described above. IMARIS volume rendering was used for tubule volume assessment. Statistical analysis utilized a two-tailed ANOVA followed by Tukey’s HSD post hoc test with an alpha-value of 0.05 to determine statistical significance. All values are reported as mean ± standard deviation with p-values.

4.2.5 Macrophage interactions with endothelial cells

To assess macrophage interactions with CD31$^+$ cells and vessel structures, ImageJ was utilized as previously described [150]. Broadly, masks of each macrophage marker were overlaid with masks of the CD31$^+$ structures and the points of overlap were quantified as interactions. This quantification was conducted on each slice within a single stack. Each macrophage within a stack was labeled and tracked to ensure each cell was counted only once. Specifically, iNOS$^+$, arginase-1$^+$, and/or CD206$^+$ cells were converted into a macrophage mask using the merge channel feature in ImageJ. The
macrophage mask was then overlaid with the corresponding CD31+ mask. The common points of overlay between the CD31+ mask and the macrophage mask were recorded as interactions (n=4 gels, 4 stacks per condition with 18 slices per stack). It is important to note that in the case of the endothelial cells co-encapsulated with M1 macrophages, the CD31+ structures were mainly single cells. All thresholding was held constant per experiment and compared to the secondary antibody control gel. Statistical analysis utilized a two-tailed ANOVA followed by Tukey’s HSD post hoc test with an alpha-value of 0.05 to determine statistical significance. All values are reported as mean ± standard deviation with p-values.

4.2.6 Macrophage phenotype analysis

To quantify the population of macrophages positive for specific markers, Zeiss LSM Image Browser was employed. Each macrophage, defined as all cells DAPI+ and CD31+, were assessed for iNOS, arginase-1 or CD206 positivity. Some cells were iNOS+/Arg-1+, thus, the ratio of iNOS+ to Arg-1+ or CD206+ cells was presented in order to demonstrate shifts in the macrophage phenotype population. Statistical analysis utilized a two-tailed ANOVA followed by Tukey’s HSD post hoc test with an alpha-value of 0.05 to determine statistical significance. All values are reported as mean ± standard deviation with p-values.
4.2.7 VEGF ELISA

Conditioned media was collected following macrophage stimulation and at each media change for the 3D cell cultures. VEGF-A concentration was assessed via VEGF ELISA (R&D systems) according to the protocol in the kit. Data are shown as mean ± standard deviation at the indicated day in culture (n= 3 samples for 2D stimulation and 4 gels per condition). Statistical analysis utilized a two-tailed ANOVA followed by Tukey’s HSD post hoc test with an alpha-value of 0.05 to determine statistical significance (p-value reported in text).

4.2.8 Proliferation of primary endothelial cells

For studies involving proliferation, three media conditions were considered: endothelial cells in their aforementioned original media, endothelial cells in M1 conditioned media, and endothelial cells in their media supplemented with 10 ng/mL of IFNγ (ProSpec). In order to understand the effects of M1 conditioned media and IFNγ on endothelial cell proliferation, each group was compared to the endothelial cell media control. M1 conditioned media was assessed to understand the influence of soluble M1 factors on endothelial cell proliferation while IFNγ supplement was included to compare to the M1 conditioned media group. Each sample was cultured for 3 days, fixed and stained for CD31 and Ki67. To evaluate % of Ki67+ cells, Image J was utilized. First, a Z-projection of each stack was created (n=3 gels per conditioned media sample, 4 stacks per gel). Both the DAPI+ and the Ki67+ channels were separated from the stack and
converted into two masks. The analyze particle function was then utilized in order to count the number of DAPI+ structures and the number of Ki67+ cells. The mask of the Ki67+ cells was then overlaid onto the Z-projection of the DAPI channel in order to validate each Ki67+ structure was in fact a cell. Quantification utilized a t-test with an alpha-value of 0.05 to determine statistical significance as a direct comparison was made between the AEC only and AEC + M1 conditioned media groups. All values are reported as mean ± standard deviation with p-values.

4.3 Results

4.3.1 Macrophage phenotype profiles after stimulation

Macrophages were stimulated towards an M1 phenotype through exposure to IFNγ and LPS. M2 macrophages were stimulated in the presence of IL-4 and IL-13 [108], [116], [152]–[154]. By comparison, M0 macrophages are cultured in basal media alone. To confirm appropriate stimulation of macrophages, immunocytochemistry, Western blot and flow cytometry were conducted. M1 stimulated macrophages were positive for iNOS (a known M1 macrophage marker) as demonstrated via immunocytochemistry (Figure 4.1A) and Western blot (Figure 4.1B). M2 stimulated macrophages were positive for arginase-1 (a known M2 macrophage marker; Figure 4.1A and 4.1B). Additionally, flow cytometry was used to assess CD86 expression (a M1 associated surface marker) and CD206 expression (a M2 associated surface marker). M1 stimulated macrophages had a higher mean fluorescence intensity (MFI= 639 a.u.) for CD86 while M2 and M0
macrophages had MFIs of 140 a.u. and 42 a.u., respectively. M2 stimulated macrophages had a MFI of 837 a.u. for CD206 while M1 and M0 macrophages had MFIs of 111 a.u. and 167 a.u., respectively (Figure 4.1C). The presence of these markers in each respective stimulated group indicates the stimulation conditions were able to induce both the M1 and M2 phenotypes.

Figure 4.1 Demonstration of macrophage phenotypes upon stimulation. (A) Immunocytochemistry of macrophages stimulated with LPS/IFNγ or IL-4/IL-13. iNOS, Arg-1, DAPI. Scale Bar = 100 µm. (B) Western blot of M0 (unstimulated macrophages), LPS/IFNγ stimulated macrophages (M1) or IL-4/IL-13 stimulated macrophages (M2). (C) MFI (Mean Fluorescence Intensity) from Flow Cytometry for CD86 (an M1 associated marker) and CD206 (an M2 associated marker).
4.3.2 Macrophage phenotypes influence vessel assembly

To assess the influence of each macrophage phenotype on endothelial tubule formation, macrophages were first stimulated towards the M0, M1, or M2 phenotypes as described above. Following stimulation, each group of macrophages was co-encapsulated with AECs at a 1:1 ratio in hydrogels. After 7 days in culture within the 3D hydrogel matrix, samples were fixed and stained for CD31, iNOS and Arg-1 to identify vessel structures and macrophage phenotypes. Representative images (confocal slices from stacks) of each group are shown in Figure 4.2A. To calculate the volume of endothelial tubule structures within the 3D hydrogels, the total endothelial tubule volume of each stack was calculated and then divided by the volume of each stack, thus the numbers below are unit less as they are represented as a fraction of the total stack volume. AECs alone had an average tubule volume fraction of 0.15 ± 0.03 while AEC + M0 macrophage had an average tubule volume fraction of 0.27 ± 0.09, a nearly two-fold increase in tubule volume (Figure 4.2B). AEC + M1 macrophage had an average tubule volume fraction of 0.02 ± 0.02, a dramatic reduction to nearly one tenth the tubule volume seen in AECs alone. AEC + M2 macrophage, however, had an average tubule volume fraction of 0.44 ± 0.12 per stack volume, a nearly three-fold increase when compared to the tubule volume of AECs alone.
Figure 4.2 M2 macrophages and M0 macrophages enhance tubule volume when encapsulated with primary aortic endothelial cells (AECs). M1 macrophages inhibit tubule volume in co-culture with AECs. (A) Representative images are shown. Day 7. CD31, iNOS, Arg-1 and DAPI (nuclear stain). Scale bar= 100 µm. (B) Tubule volume of AECs alone compared to AEC + M0, AEC + M1, and AEC + M2. Bars that do not share a common letter are statistically significant, (A-B, p = 0.015, A-C p= 0.0023, B-D p=0.0002, A-D, B=C, C-D p= <0.0001).

4.3.3 Macrophage interactions with MS1 endothelial cells vary by macrophage phenotype

MS1 endothelial cells were used to verify the macrophage phenotype influence on tubule volume and to assess macrophage-endothelial cell interactions. Specifically, MS1-macrophage interactions were considered at day 3 as this was previously demonstrated as the time point for MS1 tubule formation (see Chapter 3.3.3, Figure 3.3). Representative images (confocal slices from stacks) are shown in Figure 4.3A. M0 and M2 macrophages enhanced MS1 tubule volume by nearly two-fold (Figure 4.3B). MS1s
alone had an average tubule volume fraction of 0.19 ± 0.03 while MS1 + M0 macrophage had an average tubule volume fraction of 0.33 ± 0.14 (Figure 4.3B). MS1 + M1 macrophage had an average tubule volume fraction of 0.01 ± 0.004. MS1 + M2 macrophage had an average tubule volume fraction of 0.36 ± 0.12, an increase compared to the tubule volume of MS1s alone. We also utilized the MS1s to investigate the cell-cell interactions between endothelial cells and macrophages (Figure 4.4A). The MS1 + M0 macrophage group and MS1 + M2 macrophage group demonstrate macrophages interacting with MS1s as the tubules form within the 3D hydrogel matrix. In the MS1 + M1 macrophage group, little to no interaction was seen between macrophages and endothelial cells. As demonstrated previously, cell interactions between macrophages and endothelial cells can be quantified within this hydrogel matrix [150]. Close associations between macrophages and endothelial cells are assessed by considering overlap between the vessel structures and the macrophages within the hydrogel. The MS1 + M0 macrophage had an average of 28.5 ± 10 macrophage-endothelial cell interactions per stack (where stack volume was 7.2 x 10^6 μm³), MS1s + M1 macrophage had an average of 1.17 ± 1.2 interactions per stack and MS1 + M2 macrophage had an average of 31.5 ± 11 interactions per stack (Figure 4.4A). The MS1 + M0 macrophage condition and MS1 + M2 macrophage condition demonstrated higher interactions than the MS1 + M1 macrophage group. However, no statistical difference was seen between
the interactions in the MS1 + M0 macrophage group and the MS1 + M2 macrophage group.

We next used the macrophage phenotype markers to quantify on the population of macrophage phenotypes present within each condition (Figure 4.4B). Specifically, iNOS and CD206 markers were considered. At day 3 in culture, we found the ratio of iNOS/CD206 cells was greatest in the MS1 + M1 macrophage group and lowest in the MS1 + M2 macrophage group. However, no statistically significant difference was seen.

Figure 4.3 M2 and M0 macrophages enhance tubule volume of MS1 endothelial cells in PEG-based hydrogels. (A) Representative images of MS1 + M0 are shown. Day 3. CD31, CD206, Arg-1 and DAPI (nuclear stain). Scale bar= 100 µm. (B) Tubule volume of MS1s alone compared to MS1 + M0, MS1 + M1, and MS1 + M2. Bars that do not share a common letter are statistically significant, (A-B, p = 0.05, A-C p= 0.007, B-C p <0.0001.
Figure 4.4 M0 and M2 macrophages readily interact with MS1s in PEG-based hydrogels. (A) Quantification of the cell-cell interactions seen between MS1+ M0, MS1+ M1, and MS1 + M2 co-cultures. Bars that do not share a common letter are statistically significant (A-B p< 0.0001). (B) iNOS/CD206 macrophage ratio within each group at day 3 in culture. A higher iNOS population is found in the MS1+M1 group.

4.3.4 VEGF Secretion in 3D culture

To elucidate potential mechanisms by which the M2 macrophages enhanced AEC tubule volume, we probed the influence of soluble cues in the ability of M2 macrophages to promote AEC tubule formation. Specifically, the presence of secreted VEGF was assessed. AEC + M2 macrophage encapsulated within the same hydrogel secreted more VEGF than AECs alone at day 1 and 3 than AECs alone, AECs + M0 macrophages and AECs + M1 macrophages (Figure 4.5A,B). At day 1, AECs alone in the hydrogels secreted an average of 0.37 ± 0.8 pg/mL while the AEC + M2 macrophage condition had an average VEGF concentration of 12.7 ± 3.9 pg/mL. No VEGF was detected in the AEC + M0 or AEC + M1 macrophage groups. There was also an increased amount of VEGF seen at day 3 (Figure 4.5B) in the AEC + M2 macrophage condition.
compared to the AECs alone. However, no statistical significant difference was seen between the groups.

Figure 4.5 AEC+ M2 macrophages secrete more VEGF in PEG-based hydrogels. (A) VEGF concentration of conditioned media of AECs alone, AEC + M0, AEC + M1 and AEC + M2 macrophages at day 1, (B) and day 3. (* statistical significant to all other groups, * p=0.0006).

4.3.5 Influence of M1 conditioned media on endothelial tubule formation

To probe the reasons for reduced tubule volume in the M1 macrophage co-cultures, we investigated the role of soluble factors in influencing AEC and MS1 tubule formation. In particular, we hypothesized that soluble factors from M1 macrophages inhibited endothelial cell migration and tubule formation. To test this, we cultured AECs (and MS1s) in M1 macrophage conditioned media. As seen in the representative images (confocal slices from stacks), AECs in M1 conditioned media displayed retarded cell spreading when compared to AECs cultured in their EC media (Figure 4.6A). Because
the M1 conditioned media reduced endothelial cell spreading, we next assessed the influence of these conditions on endothelial cell proliferation via Ki67 staining (Figure 4.6B). AECs encapsulated alone in the hydrogels demonstrated higher levels of proliferation than the AECs cultured in M1 conditioned media. On average, AECs alone had 15 ± 4.8% Ki67+ cells while no AECs cultured in M1 conditioned media were Ki67+. The same trend in reduced spreading of endothelial cells upon exposure to M1 conditioned media was seen for the MS1 endothelial cells as well (Figure 4.7).

Figure 4.6 Soluble Influence of M1 conditioned media on AECs. (A) Representative images of AECs and AECs in M1 conditioned media. Day 3. CD31 (red), Ki67(white) and DAPI (nuclear stain). Scale bar= 100 µm. (B) Ki67 comparison between AECs alone and AECs in M1 conditioned media. * p < 0.0001.
Figure 4.7 Soluble Influence of M1 conditioned media on MS1s. Representative images of MS1s and MS1s in M1 conditioned media. Day 3. CD31 and DAPI (nuclear stain). Scale bar= 100 µm.

4.4 Discussion

Within this work, we demonstrated the roles of macrophage phenotypes in influencing vessel formation within PEG-based hydrogels. A beneficial response was demonstrated between M2 macrophages while a detrimental response seen between M1 macrophages and endothelial cells (Figure 4.8). This dichotomy helps to elucidate the influences of various macrophage phenotypes within vessel development. Specifically, this 3D in vitro system allowed the study of paracrine and juxtacrine influences between macrophages and endothelial cells to be assessed within a controlled 3D environment.
Figure 4.8 M1 and M2 macrophages influence vessel development when encapsulated with endothelial cells in a PEG-based hydrogel. M2 macrophages enhance vessel formation (as evidence via cell-cell contact and increased VEGF). M1 macrophages retard vessel formation (as evidenced through reduced Ki67 of endothelial cells).

It is known that macrophage phenotypes exist along a broad spectrum of functions and states [70], [71]. While the restrictive classification of M1 or M2 macrophages is myopic [63], for this work, the use of these classical stimulation profiles was employed given the context in which these cells are studied. In particular, this work sought to understand the influence of these two extremum of macrophage phenotypes within the context of in vitro culture and, specifically, within the context of 3D in vitro blood vessel development. Thus, the adoption of the M1 and M2 macrophage phenotypes was justified; however, only considering M1 and M2 macrophage phenotypes is restrictive in a broader context [76]. For this work, M1 macrophages are defined by stimulation with LPS and IFNγ. They are positive for iNOS and CD86 (Figure 4.1). M2 macrophages are defined by stimulation with IL-4 and IL-13. They are
positive for Arginase-1 and CD206 (Figure 4.1). M0 macrophages are artificially defined by the absence of stimulants LPS/IFNγ or IL-4/IL-13.

With these stimulating conditions, each macrophage phenotype was encapsulated with AECs. As demonstrated in our prior work, macrophages possess the ability to enhance tubule volume of AECs when both cells are encapsulated together into a PEG-based hydrogel [150]. The encapsulation of macrophage phenotypes with endothelial cells yielded two novel results: M1 macrophages encapsulated with AECs retarded the formation of tubule networks and M2 macrophages encapsulated with AECs enhanced tubule volume to a greater degree than any of the other groups (Figure 4.2B). Other studies have demonstrated the role of M2 or M2-like macrophages in enhancing vessel formation and tissue formation within in vivo models [45], [96], [97]. In particular, fetal testis macrophages were studied within the gonads of mice from E10.5-E13.5 (embryo day) [96]; M2-like macrophages were shown to play a dominating role in vascular reorganization. Specifically, these macrophages were responsible for vascular pruning and reorganization within the gonads. Moreover, Kubato’s group found that M2-like macrophages in wound models closely associated with the endothelial cells at the wound site; removal of these cells resulted in decreased blood vessel density suggesting that M2-like macrophages contribute to vessel repair [97]. Additional in vitro studies have introduced conflicting results in which some M1 stimulated macrophages have been seen having a beneficial impact on vessel sprouts or other aspects of vessel
development [66] while others have demonstrated that M2 and M2-like macrophages enhance tubule formation [108], [110]. Specifically, Spiller’s group has also noted the beneficial roles of M2 macrophage secreted factors in enhancing tube length and fusion of vessel sprouts on a 2D surface of Matrigel utilizing human derived cells [108], [109]. Donner’s group also demonstrated that M2-like macrophages enhanced tube formation on the 2D surface of Matrigel [110]. Contrary to prior in vitro studies, within this work, only murine derived cell types were utilized. Moreover, primary endothelial cells were encapsulated with primary macrophages of the same mouse strain. These cells were then assessed in a highly controlled 3D environment in which the influence of macrophages on vessel structures can be studied from both a soluble and cell-cell context. Our results demonstrated both the beneficial role of M2 stimulated macrophages and the detrimental influence of M1 stimulated macrophages on tubule volume.

Based off of the enhanced tubule volume of AECs when encapsulated with M2 macrophages, we hypothesized the beneficial influence of M2 macrophages was a due to a combination of the soluble signals and cell-cell interactions between the macrophages and the endothelial cells within the PEG-based hydrogel. In particular, we hypothesized that M2 macrophages encapsulated with endothelial cell encourage VEGF secretion. We also hypothesized that these macrophages interacted with endothelial cells in a manner similar to pericytes or support cells [12], [40], [70], [72], [110]. To quantify the M2
macrophage influence, we analyzed VEGF levels in conditioned media and analyzed the interactions between macrophages and endothelial cells. While no statistically significant difference was seen between the number of interactions between M0 and M2 macrophages cultured in hydrogels with MS1 endothelial cells, M2 macrophages were more likely to interact with MS1 endothelial cells (Figure 4.4A). In particular, interactions between endothelial cells and macrophages have been shown to enhance vessel formation [12], [150]. We speculate M2 and M0 macrophages may be behaving as support cells as a result of their interactions with endothelial cells [14]. Additionally, the AEC + M2 macrophage condition secreted more VEGF than AECs alone within the hydrogel (Figure 4.5). It is known that, in the presence of VEGF, macrophages migrate to endothelial cells, facilitate endothelial tip cell anastomosis, and benefit vessel formation [12], [96]. Thus, we propose that M2 macrophages are enhancing vessel formation within our PEG-based hydrogel through cell-cell interaction with the endothelial cells and by influencing VEGF levels when encapsulated with the AECs (Figure 4.8).

In our system, M1 macrophages inhibited the formation of endothelial tubules. Additionally, we noticed the AECs encapsulated with M1 macrophages appeared less spread than AECs encapsulated alone (Figure 4.2A). It is known that M1 macrophages are capable of secreting IFNγ and other inhibitory factors [94], [155]. In particular, IFNγ is known to inhibit endothelial cell proliferation. The reduced spreading seen in the AEC + M1 macrophage group led us to probe the proliferation of AECs when exposed to M1
macrophages conditioned media (Figure 4.6A). The AECs cultured with M1 conditioned media had a significant reduction in Ki67+ cells when compared to AECs alone (Figure 4.6B). M1 macrophages, via soluble factors [94], [155], can thus influence endothelial cells in a manner that inhibits tubule formation by reducing endothelial cell proliferation within the PEG-based hydrogels.

While these studies have revealed dominant roles of M1 and M2 macrophage phenotypes in influencing vessel formation, there are certain limitations to this work. In particular, the macrophage markers used were primarily limited to iNOS and arginase-1 due to the lack of overlap anticipated between the markers based on macrophage metabolism [81]. It is appreciated that alternative markers should be used to identify macrophage phenotypes within a broader range of M1 and M2 markers [71], [72]. It is also important to note that CD206+/iNOS+ cells were found in all of the groups indicating that the macrophage phenotypes, upon co-culture with endothelial cells, were altered from their previously stimulated states.

4.5 Conclusion

This work aimed to utilize the PEG-based hydrogel platform in order to probe the roles of M1 and M2 macrophage phenotypes within vessel development. This research demonstrated the ability for M2 macrophages to enhance tubule volume through increased VEGF secretion and cell-cell contacts. M1 macrophages were shown to have a deleterious effect on vessel formation as evidenced through reduced
endothelial cell proliferation and endothelial tubule volume. Thus, this work has demonstrated that macrophage phenotypes can be exploited to influence vessel formation within a 3D PEG-based system.
5. Development of stimulating hydrogels to design an stimulating environment for macrophage phenotypes

In order to exploit the effects of the macrophage phenotype on vessel development, this chapter seeks to design stimulating hydrogels capable of directing the macrophage phenotype. In particular, we designed hydrogels that considered the covalent incorporation of PEGylated factors in order to direct the phenotype of any macrophages encapsulated within the hydrogel. The stimulating factors (IFN\(\gamma\), IL-4 and IL-13) were reacted with PEG-derivative (acryl-PEG-SVA) and verified via gel electrophoresis. The PEG-stimulants were then assessed for bioactivity in 2D culture. Next, stimulating hydrogels were created. The stimulating hydrogels include the PEG-stimulants (PEG-IFN\(\gamma\) or PEG-IL4/PEG-IL13) within the PEG-PQ-PEG hydrogel matrix. Within the stimulating hydrogels, vessel volume fraction of both endothelial cells alone and endothelial cells with macrophages were assessed.

5.1 Immobilization of stimulating factors

Our work has demonstrated that M2 macrophages can enhance vessel formation within PEG-based hydrogels, following transient stimulation of the macrophages with soluble IL-4 and IL-13 (chapter 4). To better translate these findings into potential regenerative medicine applications, the design of a hydrogel construct capable of stimulating macrophages was considered.
Because macrophage phenotypes are known to be highly dynamic, most of the work in the field has stimulated macrophages in a 2D environment for periods of less than 48 hr [101], [105], [116]. As detailed in chapter 1, transient stimulation has consisted of macrophage exposure to soluble stimulating factors with cells in standard 2D tissue culture over a time range of 24-48 hr. The stimulating factors are then removed from the macrophages and assays are thereafter conducted. Importantly, it is not known how long the macrophages persist in their stimulated phenotype once the stimulating factors are removed [108] (Figure 5.1). This has generally not been investigated within the literature. Moreover, evidence in our studies has indicated that macrophage phenotype is altered within 7 days of culture (see chapter 4.4) Thus, this traditional model of 2D macrophage stimulation for 24 hr results in limited control over macrophage phenotype. Moreover, it is known that macrophages are highly plastic and have the ability to undergo phenotype switching dependent on microenvironmental cues which are sensitive to time [53], [63], [68].

In particular, microenvironmental cues are often presented via the ECM. Within in vivo settings, bidirectional dynamic reciprocity occurs between the cells and sequestered factors in the ECM [149]. The ECM often sequesters growth factors thus providing continued presentation, concentration gradients, enzymatic protection and dynamic release of the factors [35]. The function of the ECM in sequestering growth factors is of critical importance in the wound environment and within angiogenesis.
[149], [156]; for example, VEGF binding to heparin plays a critical role in capillary formation [157]. Shima’s group found that the removal of the binding domain of VEGF-A resulted in a significant reduction in capillary sprouting and altered vessel formation in a spatially directed manner [157]. This is a powerful example of the role of ECM (in this case, heparin) binding a growth factor and informing cell function within a restricted manner. Within the context of macrophages stimulation, it is known that IFNγ, IL-4, IL-10 (as well as many other interleukins) are sequestered within heparin sulfate [158]–[161]. It is not known if IL-13 is also sequestered in heparin sulfate, however, it is known that IL-4 and IL-13 share a common receptor and often compete for similar signaling cascades (specifically, with regards to STAT6) [162]. Thus, the immobilized presentation of specific factors would replicate the interactions macrophages have in vivo. In addition, the immobilization of factors would address the role of temporal stimulation of the macrophages and better replicate the macrophage stimulation process that occurs in vivo via ECM presentation.
Figure 5.1 2D soluble stimulation versus 3D immobilized stimulation of macrophages. 2D soluble stimulation of macrophages can yield altered macrophage phenotype states over time as macrophage phenotypes vary dependent on microenvironmental cues.

While it was previously hypothesized that the immobilization of growth factors would retard or inhibit the cell response to the factors (as growth factor receptor dimerization and growth factor internalization are required for signaling), work in Dr. Linda Griffith’s group demonstrated that the immobilization of epidermal growth factor (EGF) via PEG linkers on glass did not yield any differential cell response when compared to soluble EGF [163]. It has been demonstrated that immobilized factors are capable of directing cell function, migration and vessel organization. Moreover, it is known that PEGylated factors show a decrease in bioactivity when delivered solubly
due both to steric hindrance of the PEG as well as the non-specific PEGylation which could alter the protein structure [164], [165]. When immobilized, however, the PEGylated factors have been shown an increase in bioactivity. In particular, prior work in the West lab has studied the influence of PEGylated growth factors on cell behavior: VEGF, to promote tubulogenesis in PEG hydrogels [136]; ephrin A1, to enhance growth, differentiation and patterning of microvascular formation [38]; PDGF-BB to recruit pericytes [166]; bFGF, to promote the migratory and proliferative behaviors of SMCs [167]; EGF, to promote fibroblast migration [168]; stem cell factor (SCF) and SDF-1α, for the maintenance and expansion of HSCs in vitro [169]. This prior work demonstrates the ability to successfully PEGylate factors and subsequently direct cell function.

This chapter investigates the temporal aspects of macrophage phenotype and subsequent macrophage influence on endothelial cell interactions by PEGylating stimulating factors and covalently grafting the factors into the PEG-based hydrogel. In particular IFNγ, IL-4 and IL-13 are the factors considered. The aim of this work is to better mimic the cell-matrix interactions within an engineered PEG-based platform.

5.2 Materials and methods

5.2.1 PEGylation of IFNγ, IL-4 and IL-13

Previous work in our lab has demonstrated the ability to PEGylate proteins while retaining their bioactivity [169], [170]. Briefly, acryl-PEG-SVA (Laysan) was reacted with carrier free IFNγ, IL-4 or IL-13 (ProSpec, East Brunswick, NJ) at a molar ratio of 100:1
(acryl-PEG-SVA to stimulant). This reaction was performed in protein conjugation buffer at pH 8.5, as described in chapter 2.2.1. To assess PEGylation, PEGylated proteins were compared to non-PEGylated proteins via gel electrophoresis using Any kD Mini-PROTEAN® TGX precast protein gels (Bio Rad). The gel was then incubated with BioSafe Coomassie Gel Stain (Bio Rad) and imaged under white light.

5.2.2 Bioactivity of PEGylated stimulants

After confirming successful conjugation of the PEG-stimulants (in this case, PEG-IFNγ, PEG-IL4 and PEG-IL-13) via PAGE, bioactivity of the PEG stimulants was assessed via soluble stimulation in 2D (as described in chapter 4.2.1 and 2D immunocytochemistry in chapter 4.2.3). Briefly, the PEGylated stimulants were introduced to the bone marrow macrophages via soluble delivery. Bone marrow macrophages were seeded at 20,000 cells/cm². 24 hr after thaw, the macrophages were exposed to stimulating conditions. Initially, a dose response range of PEG-IL4 and PEG-IL13 concentrations was assessed in comparison to soluble IL-4 and IL-13 in order to assess levels of bioactivity of the PEG-stimulants. Following the dose response, the stimulating conditions were used: 100 ng/mL of PEG-IFNγ or IFNγ, 50 ng/mL of PEG-IL4/PEG-IL13 or 50 ng/mL of IL-4/IL-13. The PEG-stimulants were compared to the non-PEGylated stimulants at the same concentration. At 24 hr following soluble stimulation, the samples were fixed and stained as described in chapter 4.2.3. Analysis of each cell for iNOS⁺ or CD206⁺ was conducted as described in chapter 4.2.5 utilizing ImageJ.
Comparisons were made between each PEGylated group (i.e. IFNγ vs. PEG-IFNγ) to assess number of cells positive for each indicated marker. Quantification utilized a t-test with an alpha-value of 0.05 to determine statistical significance (n > 130 macrophages per group).

**5.2.3 Generation of stimulating hydrogels**

To generate stimulating hydrogels, the procedures outlined in chapter 5.2.2 and 5.2.3 were combined. PEGylated stimulants were added to the polymer solution (2.5% PEG-PQ-PEG and 3.5 mM PEG-RGDS dissolved in a HBS with 1.5% TEAO, 10 µM eosin Y and 0.35% (v/v) NVP at pH 8.3, see chapter 2.2.2). In particular, the PEGylated stimulant solution was added at 10% (v/v) of the polymer solution (or in the case of the blank hydrogel without PEGylated stimulants, 10% (v/v) of HBS-TEOA was added). Three groups were considered in total: (1) PEG-PQ-PEG only (referred to as PEG); (2) PEG-PQ-PEG + 10 ng/mL of PEG-IFNγ (referred to as M1SH or M1stimulating hydrogel, normalized for protein concentration); and (3) PEG-PQ-PEG + 20 ng/mL PEG-IL4 and 20 ng/mL of PEG-IL13 (referred to as M2SH). The precursor solution was mixed as detailed in chapter 2.2.2. Upon exposure to white light, crosslinking occurred to form the hydrogel.

**5.2.4 Tubule volume analysis in stimulating hydrogels**

Tubule volume analysis is described in chapter 4.2.4. As described in section 5.2.5, AECs alone and AECs + M0 macrophages were assessed in the stimulating
hydrogels at 8 million cells/mL. AECs alone were introduced to assess the endothelial cell response to the stimulating hydrogels. AEC + macrophage tubule volume was used to quantify the response to the stimulating hydrogels upon co-encapsulation with macrophages. MS1s were also considered to assess an alternative endothelial cell response to the stimulating hydrogels. MS1s (with or without macrophages) were encapsulated at 15 million cells/mL. Statistical analysis was conducted as previously described. A two-tailed ANOVA followed by Tukey’s HSD post hoc test was utilized to determine statistical significance with an alpha-value of 0.05. All values are reported as mean ± standard deviation.

5.3 Results

5.3.1 PEGylation of PEG-stimulants

PEGylation of IFNγ, IL-4 and IL-13 was determined via PAGE. A smear is seen in the PEG-IFNγ lane demonstrating an increase in molecular weight of the sample PEG-IFNγ in comparison to the IFNγ alone (Figure 5.2A). Successfully PEGylated proteins are known to increase in size as a result of the PEGylation, specifically when compared to the size of the unmodified protein. The same smear is seen for PEG-IL4 and PEG-IL13 when compared to IL-4 and IL-13 (Figure 5.2B,C).
Figure 5.2 Verification of PEGylated stimulants. PAGE gel with proteins false-colored blue showing (A) IFNγ compared to PEG-IFNγ, (B) IL-14 compared to PEG-IL4 and (C) IL-13 compared to PEG-IL13. Brackets indicate smear respective lane.

5.3.2 Bioactivity of PEG-stimulants

To assess bioactivity of the PEG-stimulants, macrophages were stimulated using PEG-stimulants and compared to the soluble stimulants alone. Initially, a dose response was assessed in order to determine the range of concentrations that would allow the PEG-stimulants to stimulate the macrophages (Figure 5.3). The dose response ranged from 20 ng/mL to 100 ng/mL IL-4/IL-13 (Figure 5.3). The concentration of 50 ng/mL was selected as no discernable difference in stimulation of the macrophages towards M2 was detected at that concentration (via CD206+ assessment).
Figure 5.3 Dose response of PEG-IL4/PEG-IL13. CD206+ macrophages are shown in red in the PEG-IL4/PEG-IL13 group (top column) and IL-4/IL-13 (bottom column). CD206 and DAPI. Scale Bar = 100 µm. Following 24 hr stimulation.

PEG-IFNγ vs. IFNγ was used to assess M1 phenotype. As seen in Figure 5.4B, both groups expressed iNOS as determined via immunocytochemistry. For the M2 phenotype, both the PEG-IL4/PEG-IL13 and the soluble IL-4/IL-13 groups were positive for CD206 (Figure 5.4C).
Figure 5.4 2D Bioactivity of PEGylated stimulants. (A) Unstimulated macrophages are shown. (B) iNOS+ macrophages shown in both IFNγ and PEG-IFNγ groups. (C) CD206+ macrophages shown in IL-4/IL-13 and PEG-IL4/PEG-IL13 groups. iNOS, CD206, and DAPI. Scale Bar = 100 µm. Following 24 hr stimulation.

After 24 hr stimulation, there was no statistical difference in the number of cells positive for each marker when comparing soluble stimulants to PEG-stimulants.

Unstimulated macrophages were 38% iNOS+; IFNγ stimulated macrophages were 98% iNOS+ and PEG-IFNγ were 97% iNOS+. Unstimulated macrophages were 26% CD206+; IL-4/IL-13 stimulated macrophages were 95% CD206+ while PEG-IL-4/PEG-IL-13 stimulated macrophages were 92% CD206+. 
5.3.3 AEC tubule formation in stimulating hydrogels

To assess the influence of the stimulating hydrogels on tubule formation, M0 macrophages and AECs were encapsulated together in each hydrogel condition (PEG, M1SH, and M2SH) (Figure 5.5). In order to assess the influence of the stimulating hydrogels alone on vessel volume, control gels of AECs alone were compared (Figure 5.6). Representative images of AECs in the stimulating hydrogels are shown in Figure 5.6A. Tubule volume was assessed to probe the influence of the stimulating hydrogels on AECs tubule formation. AECs alone in PEG hydrogels had a tubule volume fraction of 0.04 ± 0.02. In M1 stimulating hydrogels, AECs alone had a tubule volume fraction of 0.03 ± 0.02 and AECs alone in M2 stimulating hydrogels had a volume of 0.05 ± 0.03 (Figure 5.6B). For the AECs alone in the stimulating hydrogel conditions, there was no statistically significant difference in AEC tubule volume fraction between the three groups (Figure 5.6B). We next considered the role of macrophage in AEC tubule formation within the stimulating hydrogels (Figure 5.7). AECs +macrophages had a tubule volume fraction of 0.08 ± 0.03 in PEG hydrogels (Figure 5.8A). As has been previously reported, macrophages enhance AEC tubule volume fraction by nearly two-fold when compared to AECs alone. In M1 stimulating hydrogels, AECs encapsulated with M0 macrophages had a tubule volume fraction of 0.05 ± 0.03. In M2 stimulating hydrogels, AECs encapsulated with M0 macrophages had a tubule volume fraction of 0.09 ± 0.04 (Figure 5.8B).
Figure 5.5 A schematic of endothelial cells encapsulated in PEG stimulating hydrogels.
Figure 5.6 Vessel volume of AECs alone in stimulating hydrogels. (A) Representative images are shown. Day 7. CD31 and DAPI (nuclear stain). Scale bar= 100 µm. (B) Tubule volume of AECs in PEG blank, M1SH and M2SH conditions. No statistical significance was observed between the groups.
Figure 5.7 A schematic of endothelial cells and macrophages encapsulated in PEG stimulating hydrogels.
Figure 5.8 M2 stimulating hydrogels and PEG hydrogels enhance vessel volume of AECs when compared to M1 stimulating hydrogels. (A) Representative images are shown. Day 7. CD31 and DAPI (nuclear stain). Scale bar= 100 µm. (B) Tubule volume of AECs alone, AECs + M0 in PEG compared to M1SH and M2SH conditions. Bars that do not share a common letter are statistically significantly different from one another, * p<0.05.

5.3.4 MS1 tubule formation in stimulating hydrogels

MS1s were also considered as a secondary endothelial cell source to verify the influence of the stimulating hydrogels on tubule formation. Importantly, as noted previously, MS1s form tubules independent of a secondary cell source (see chapter
Thus, MS1s are included as a comparison endothelial cell source and to verify the results seen in the AEC group. Initially, the influence of the stimulating hydrogels on MS1 tubule volume fraction was assessed (Figure 5.9). No statistical difference was seen between the MS1s in PEG hydrogels or the MS1s in M1SH or M2SH (Figure 5.9). Next, MS1s + M0 macrophages were encapsulated together in each hydrogel condition (PEG, M1SH, and M2SH). Representative images of MS1s + macrophages are shown in Figure 5.10A. Additionally, tubule volume was assessed to probe the influence of the stimulating hydrogels on MS1 tubule volume fraction when encapsulated with macrophages (Figure 5.10B). MS1s + macrophages in PEG hydrogel had a tubule volume fraction of 0.05 ± 0.01 and MS1s + macrophages in M1SH had a tubule volume fraction of 0.05 ± 0.01. MS1s + macrophages in M2SH had a tubule volume fraction of 0.11 ± 0.04.

![Total Tubule Volume](image)

**Figure 5.9** Vessel volume of MS1s alone in stimulating hydrogels. Tubule volume of MS1s in PEG blank, M1SH and M2SH conditions. No statistical significance was observed between the groups.
Figure 5.10 M2 stimulating hydrogels enhance vessel volume of MS1s when compared to M1 stimulating hydrogels and PEG hydrogels. (A) Representative images are shown. Day 7. CD31, iNOS, CD206 and DAPI (nuclear stain). Scale bar= 100 µm. (B) Tubule volume of MS1s + M0 in PEG compared to M1SH and M2SH conditions. Bars that do not share a common letter are statistically significantly different from one another, * p<0.05.

5.4 Discussion

In this chapter, we have explored the influence of PEGylated stimulants in governing macrophage mediated endothelial cell vessel formation within PEG-based hydrogels. As discussed in the introduction, the role of soluble influence of macrophage phenotype does not readily mirror the stimulation exposure that occurs within in vivo
situations [72], [78], [171]. This system provides an the ability to maintain a stimulating environment over time. Following verification of the PEG-stimulants via PAGE, the PEG-stimulants were compared to soluble stimulants via 2D stimulation. After assessing a dose response, no statistically significant difference was seen between the PEG-stimulant macrophages and the macrophages stimulated via unmodified soluble stimulants. However, it is important to note that the concentration of PEG-stimulant and soluble stimulant considered within this chapter was higher than the soluble factor conditions outlined in chapter 4. As expected, a higher concentration of PEG-stimulant was required to stimulate the macrophages in 2D, likely due to decreased bioactivity of the PEG-stimulants (Figure 5.3). This phenomena has been seen in prior work with PEGylated proteins [165].

Following verification of the PEG-stimulants ability to create the stimulation profiles of macrophages in 2D, the stimulating hydrogels were created. In particular, PEG-stimulants were incorporated into the PEG-PQ-PEG and PEG-RGDS system (Figure 5.5) [172]. We encapsulated the AECs within the stimulating hydrogel groups to ascertain the influence of each stimulating hydrogel on the AECs alone. As has been referenced previously, it is known that IFN\(\gamma\) can have anti-proliferative effects on endothelial cells [93]. Within this context, there was no statistical difference seen in AEC cell volume fraction when comparing PEG hydrogels, M1 stimulating hydrogels or M2 stimulating hydrogels. Macrophages and AECs were then encapsulated together within
each of the stimulating hydrogel conditions (Figure 5.7). We found that AECs and macrophages in PEG hydrogels and in M2 stimulating hydrogels significantly enhanced AEC vessel volume fraction when compared to AECs alone in PEG hydrogels (Figure 5.8). Of critical note is the difference in tubule volume within the M1 stimulating hydrogels. In chapter 4.3.2, we saw that the encapsulation of M1 stimulated macrophages with AECs resulted in retarded vessel volume (to nearly one tenth the tubule volume fraction seen in AECs alone). In the case of the macrophages and AECs in the M1 stimulating hydrogels, the tubule volume fraction was slightly greater than the AECs alone (AECs alone had a tubule volume fraction of 0.03 ± 0.02 while AECs + macrophages in M1SH had a tubule volume fraction of 0.05 ± 0.03) (Figure 5.8B). The difference in AEC and macrophage response to the M1SH environment when compared to the previously stimulated M1 macrophages could be explained through stimulation conditions used in each study. While the use of LPS and IFNγ has been explored extensively within the context of macrophage phenotypes, it has also been argued that the true stimulating factors of macrophage phenotypes should only consider the influence of IFNγ in the absence of LPS to adhere to the T\(H_1\) cell dichotomy of macrophage stimulation [70], [71]. Alternative cytokines (such as TNFα) have also been considered to explore the range of M1 stimulation profiles [41]. The absence of LPS no doubt alters the stimulation state of the M1-stimulated macrophages and could play a
role in the macrophage function and subsequent influences on endothelial cells during vessel formation.

The presence of PEG-IL4/PEG-IL13 enhanced the tubule volume fraction of the AECs and macrophages. This is a similar trend that was introduced in chapter 4. M2 macrophages (or in this case an M2 stimulating hydrogel environment) motivate the formation of vessel structures and enhance tubule volume beyond that of AECs alone and AECs + macrophages in an M1 stimulating hydrogel. Interestingly, no difference was seen between the AECs + macrophages in the PEG hydrogels and the AECs + macrophages in the M2 stimulating hydrogel although the trend indicates greater tubule volume fraction in the M2 stimulating hydrogel (Figure 5.8). This trend is more evident in the MS1 + macrophage groups (Figure 5.10). However, it is important to note that the mechanisms of macrophage stimulation are altered in the immobilized hydrogels and thus the stimulation state and subsequent influence of the stimulated macrophage on tubulogenesis is altered as well. In this work, we assessed the time periods of 3 days and 7 days in culture. This was significantly longer than the typical 24-48 hr studies conducted in this field [15], [116]. However, it is known that macrophages are present at wound sites for upwards of months [56], [173]. The interactions and the stimulation states of these macrophages can be further explored within these stimulating hydrogels in order to shed light on the influence of immobilized stimulation within the context of longer time periods.
Overall, IFNγ, IL-4 and IL-13 were able to be PEGylated, maintain macrophage stimulation in 2D when compared to their soluble counter parts and be incorporated into stimulating hydrogels. M2 stimulating hydrogels encouraged the greatest tubule volume fraction when AECs and macrophages were encapsulated into the hydrogel. The M1 stimulating hydrogels did not retard vessel formation in the AECs alone or in the AECs + macrophages condition.

5.5 Conclusions

In this chapter, we altered the PEG hydrogels to design a stimulating environment for the macrophages. We assessed the influence of the stimulating hydrogels on vessel formation (both with and without the macrophages). This work introduces the idea of designing a synthetic hydrogel capable of directing the macrophage phenotype and thus, capable of directing the vascular response within a specific environment. Specifically, the development of the stimulating hydrogels could be utilized for in vivo regenerative medicine applications in order to manipulate the macrophage microenvironment within the hydrogels upon implantation.
6. Conclusions and future directions

6.1 Concluding remarks

The work presented in this dissertation introduces macrophage-mediated vasculogenesis within a reductionist PEG-based microenvironment. This work supports the notion that macrophages can be considered as a support cell source in furthering vasculogenesis of biomimetic scaffolds. Additionally, depending on the phenotype of the macrophages, the degree of support or inhibition in vasculogenesis can be tuned. These findings further the fields of tissue engineering, specifically vascular tissue engineering, as the incorporation of macrophages can be considered to augment vascular development both within in vitro models and within in vivo implantations (with regards to anastomosis of vascular structures in and surrounding the implant). Moreover, the PEG-based system utilized in this work is a modifiable platform in which other matrix mechanics, growth factors and/or peptides, and cell types can be easily introduced. The amendment of this platform to incorporate peptides and growth factors along with cells promotes the ability to assess the roles of macrophages in synthetic ‘tissue’ environments under controlled conditioning. These types of studies will allow a greater understanding of both macrophage-material stimulation and macrophage-assisted tissue development. These studies may ultimately impact the way tissue scaffolds for implantation are developed and address current limitations within the context of tissue engineering.
6.2 Future research directions

There are many future applications for the work developed in this dissertation. This work has demonstrated macrophages can play a critically beneficial role in vessel development within PEG-based hydrogels. The next section will cover various aspects of the hydrogel model that can be enhanced in order to consider the broader context. Secondly, new applications and translations of these findings will be explored within the context of wider tissue engineering developments.

6.2.1 Enhancing the current understanding of macrophage phenotypes within the context of vasculogenesis

6.2.1.1 Alteration of hydrogel mechanics

In addition to creating a hydrogel capable of manipulating macrophage phenotype in vitro, the ability to stimulate vessel invasion in vivo is of interest, specifically as it relates to mechanical properties of the implanted hydrogel. As previously mentioned in chapter 1, prior work in the West lab has demonstrated that the recruitment of macrophages into an implant site led to greater vessel density and structural organization than conditions which did not recruit macrophages [14]. Thus, macrophage recruitment at sites of implant can enhance vessel formation. Moreover, studies have investigated the effects of substrate stiffness on macrophage function and phenotype [174]. Bryant’s group found that stiffer PEGDA substrates (of 840 kPa) only influenced macrophage secretions when stimulated with soluble LPS [175]. Without soluble stimulation, there was no difference in the murine bone marrow secretion when
comparing 130 kPa to 840 kPa [175]. However, it is important to note that the stiffness range of this work is beyond the realm of most soft tissues [139]. Additional studies have been conducted in which macrophage cell lines were assessed within the context of 2D migration (durotaxis) and cell adhesion [133], [176]. Macrophage response to stiffness in 3D has not widely been studied.

More recent work in our lab has introduced the ability to alter the mechanical properties of the PEG-based hydrogel by introducing an amended PQ peptide capable of competing with free radicals during polymerization [37]. This amended peptide, PQ(alloc), differs from the PQ peptide through the incorporation of an alloc group on the amino acid Lys(alloc) (Figure 6.1).

![Figure 6.1](image)

**Figure 6.1** (A) Allyloxycarbonyl protecting group is present on the (B) Lysine creating Lys(alloc) which is used in the PQ(alloc) sequence

The PQ(alloc) sequence was amended to GGGGPQGIWGQGGG-Lys(alloc)-GK; in order to compare peptides the new PQ* peptide sequence was amended to GGGGPQGIWGQGGGGK. The Lys(alloc) group contains a vinyl group that competes with acrylates in crosslinking while acrylate groups tend to propagate the free radicals
during the addition reaction, the alloc group tends to terminate the reaction[177], [178] (Figure 6.2). Thus, the crosslink density and the hydrogel network formation is altered when the hydrogels are composed of PEG-PQ(alloc)-PEG in comparison to PEG-PQ*-PEG hydrogels. As a result of the architecture of the crosslinked network, the mechanical properties of the PEG-PQ(alloc)-PEG hydrogel are transformed. PEG-PQ(alloc)-PEG hydrogels have been shown to be softer than PEG-PQ*-PEG hydrogels; in fact, 5% (w/v) PEG-PQ(alloc)-PEG have a compressive moduli of 0.66 ± 0.52 kPa while 5% (w/v) PEG-PQ*-PEG hydrogels have a compressive moduli of 15.63 ± 3.72 kPa [179]. This difference in compressive moduli resulted in PEG-PQ(alloc)-PEG supporting accelerated vessel formation in vitro and enhanced vessel invasion in vivo [180]. Thus, the PEG-PQ(alloc)-PEG can be used to assess the influence of matrix stiffness on macrophage phenotype in 3D within the realm of soft tissues < 15 kPa. This PEG-PQ(alloc)-PEG can also investigate the influence of matrix stiffness as macrophages influence vessel formation in vivo.
Figure 6.2 PEG-PQ(alloc)-PEG hydrogels alter crosslinking sites and hydrogel mechanics when compared to PEG-PQ*-PEG hydrogels due to the alloc group participation with the free radicals. Adapted from: [179].

6.2.1.2 Introduction of alternative factors in order to assess macrophage-material response

Given the in vivo context of macrophage stimulation, it is also important to consider the role of ECM in stimulating or directing the macrophage phenotype [35], [58], [119]. There is a limited understanding of how hydrogels (collagen, fibrin and Matrigel) within alternative in vitro models effect macrophage stimulation (Figure 6.3). In order to better understand how natural materials are capable of stimulating and or directing the macrophage phenotype, alternative ECM components can be considered within the PEG-based hydrogel system (Table 6.1). Specifically, prior work in the West
lab has investigated the roles of: YIGSR (a laminin derived mimetic peptide) [39], [181], IKVAV (laminin derived) [39], and DGEA (a collagen derived peptide) [181]. Additionally, peptide sequence KQAGDV, derived from fibrinogen, has been considered in alternative contexts such as smooth muscle cell growth on PEGDA hydrogels [182], [183]. Altering ECM components within the PEG-based hydrogel will allow selective control over ECM presentation to the macrophages. This work would complement the current literature in which natural systems are used as a platform to assess macrophage-endothelial cell types. Moreover, this work would elucidate the influence of ECM on macrophage stimulation alone and stand as a comparison to natural biomaterial platforms.

Figure 6.3 Stimulating factors embedded within hydrogels are capable of stimulating macrophages. In particular, various stimulating factors (green, yellow and pink) can be incorporated into the PEG hydrogel to alter the function of macrophages nearby.
6.2.1.3 Introduction of alternative cell types to assess macrophage-dependent vasculogenesis

Additionally, it is known that more relevant cell types can be of use in terms of translating the findings in this dissertation towards a clinical application in humans. To that end, the use of human blood plasma monocytes and/or blood plasma macrophages can be considered in future work. As covered in chapter 1, macrophages are derived from two main sources: EMPs (which develop into tissue resident macrophages) or the bone marrow [43], [44] [19]. For clinical relevance and due to differences in function between the resident vs. bone marrow macrophages, this work considered murine bone marrow derived macrophages [46]. However, the availability of human bone marrow sample is limited [190]. Thus, monocytes/macrophages derived from the blood plasma should also be considered in future studies.
In the same vein, human endothelial cells such as HUVECs can be considered when translating these findings into a human cell model. However, as noted in chapter 1, donor (and species) matching is critical for all co-cultures [111]. Thus, HUVECs do not provide an ideal endothelial cell source for the translation of this work into human cells. Endothelial progenitor cells (EPCs) have been identified as a clinically available cell source capable of developing vessel structures in natural material and within PEG-based hydrogels [191], [192]. Thus, utilizing EPCs as an endothelial cell source would allow macrophage donor matching and better recapitulate the in vivo interactions between the cell types at sites of injury.

Smooth muscle cells or pericytes are established support cells for stabilizing nascent vessel structures [9], [22], [147], [148]. The critical presence of these cells within the context of vasculogenesis and vascular tissue engineering is well established [7], [8], [21]. The incorporation of support cells such as pericytes would more closely mimic the cellular environment of the vasculogenesis [66], [78], [141]. Additionally, the incorporation of pericytes would elucidate whether the roles of roles of macrophages are distinct from the roles of pericytes in the context of vasculogenesis (Figure 6.4).
Figure 6.4 A more clinically relevant macrophage-mediate vasculogenesis model. The combination of human endothelial cells (in the form of EPCs), blood plasma macrophages, and pericytes (or a smooth muscle cells source) would generate a more relevant vascular tissue engineering model.

6.2.1.4 Introduction of alternative stimulating factors

Lastly, in terms of altering the macrophage phenotype, it is known that several cytokines are capable of stimulating (and thus altering the function of) macrophages [58], [75]. As covered in chapter 1, the classical definition of the M1 and M2 phenotype scheme recommends the consideration of a select number of cytokines (i.e.- M1- IFNγ and M2-IL-4) [71]. However, it is also important to consider the role of alternative cytokines and chemokines in the stimulation of macrophages. Importantly, based on prior work in the West lab, a noticeable difference was seen in the presence and vascular-supporting function of macrophages recruited in the presence of PGDFBB/bFGF compared to VEGF alone and VEGF/MCSF [14]. To exploit the benefit of
macrophages, it is important to consider how each of these factors (in combination and individually) stimulate macrophages. The potential to utilize alternative factors to stimulate macrophages supports a realm of macrophage phenotypes beyond that of limitations of the M1/M2 paradigm. Moreover, the application for in vivo implants, based on soluble secretion of specific factors, could dramatically enhance vascular tissue engineering efforts within the context of a myriad of scaffolds.

### 6.2.2 Assessing macrophages in tissue engineered models

Because of the ubiquitous nature of macrophages in many tissues, considerations should be given for the role of macrophages in the development of tissue engineered applications and models. In particular, the following sections will highlight potential use in liver tissue engineering and tumor angiogenesis progression.

### 6.2.3 Macrophages in liver tissue engineering

Currently, liver tissue engineering is limited by the difficulty in maintaining hepatocyte function outside of the body [193]. Hepatocytes, the main cell in the liver, require a diverse set of cues to maintain function outside of the body; specifically, work within the West lab (conducted by Asli Unal) has developed a liver tissue engineering model comprised of hepatocytes, endothelial cells and pericytes within PEG-based hydrogel scaffolds (Figure 6.5) [194]. Within this model, we have maintained hepatocyte function (via albumin output) for upwards of 56 days in culture. This work was recently
published and supports the notion that heterotypic interactions between the hepatocytes and the vascular cells enhance hepatocyte function in vitro.

Figure 6.5 Hepatocytes co-cultured with ECs and pericytes proliferate in PEG-based hydrogels (A) at day 7 and (B) at day 28. Both sets were stained for Ki-67 (green, a proliferation marker), albumin (pink, used as a hepatocyte marker here), CD31 (red), and DAPI (blue). Arrows point to some proliferating hepatocytes. Scale bar = 100 µm. Adapted from: Unal et al.[194].

Alternative cells, such as lymphocytes, stellate cells and macrophages, are also present in the liver. These cells play a critical role in maintaining liver function via heterotypic interactions between cells and structural organization. Specifically, macrophages known as Kupffer cells reside in the liver as a tissue resident macrophage [195]. These cells are derived from EMPs and play a key role in clearance of red blood cells (RBCs) and immunologically labeled cells [196], [197]. Given the context of liver tissue engineering, macrophages can be investigated to understand how the presence of these cells influences hepatocyte function. Within our PEG-based liver tissue engineering system, macrophages can be incorporated in order to assess their influence
on the vascularization and hepatocyte function. Additionally, critical outputs could be considered within the context of macrophage phenotypes and disease progression. Alcoholic liver disease is associated with an M1 like phenotype as the macrophages secrete TNFα [198]. An M2 phenotype has been associated with halting insulin insensitivity in obese patients and may play a role in type 2 diabetes [199].

6.2.4 Macrophages incorporated into a tumor angiogenesis models

Prior work within the West Lab has also included the development of a tumor angiogenesis model. This model included two stacked hydrogels in which vasculogenesis occurred in the top gel while cancer clusters developed in the bottom gel. The interaction between cancer clusters and vessels were studied specifically, at the plane in which the two hydrogels overlapped and compared to other regions of the hydrogel. Circularity of the cancer clusters was used as a measurement to indicate metastatic potential of the cancer clusters; more spread and invasive clusters were seen at the interface with the vessel hydrogel (Figure 6.6) [138], [200]. This model can be enhanced through the inclusion of macrophages to assess the role of macrophages in promoting metastatic potential.
Figure 6.6 Images of cancer cells interacting with vessels at the hydrogel interface in the tumor angiogenesis PEG-based model. (A) A parallel view to the interface in which clusters are seen interacting with the white vascular cells (*, ∧ and arrows). (B) An orthogonal view of the interface demonstrating cancer cells interacting with vessel structures. Day 12. Phalloidin (red), CD31 (cyan), PEG-RGDS (green), and DAPI (blue). Scale bar =100µm. Image adapted from: Roudsari et al.[138].

Tumor associated macrophages have gained recent interest due to their presence in many cancers [201]–[203]. In fact, in many cancers tumor associated macrophages (TAMs) compose a significant fraction of the total cells present within the tumor [204], [205]. It is also speculated that TAMs play a critical role in promoting EMT, vascularization of the tumor and metastasis [206]. In fact, the increasing population of the TAMs was correlated with increased aggressiveness of the cancer. In particular, these TAMs are associated with the M2 phenotype [69], [201], [207]. Thus, considering the role of TAMs in the tumor angiogenesis model is critically important. Moreover, the
clinical impact of assessing TAM-targeting therapeutics can be incorporated into therapeutic assessments from the tumor angiogenesis model [206].

6.3 Overall summary

In this thesis, we have demonstrated the influence of macrophages on vessel development within a 3D in vitro PEG-based model. In this model, we analyzed the macrophage response to the presence of endothelial cells, the macrophage interactions with endothelial cells and the macrophage influence on vessel formation. We also investigated the roles of macrophage phenotypes in enhancing vessel development and found that M2 macrophages are supportive of vessel formation in contrast to M1 macrophages. Findings in this work advance our understanding of the influence macrophages can have on vessel formation within 3D in vitro constructs. This thesis has also commented on macrophages in vascular tissue engineering as these cells can be used as an additional cell source to promote vessel formation within the context of vascularized tissue engineered constructs. As described in this chapter, macrophages can also be used to enhance alternative tissue engineering endeavors such as liver tissue engineering and the development of tumor angiogenesis models. Because macrophages are ubiquitous in most tissues and are known to play critical roles in tissue formation, the findings reported in this dissertation have widespread impact on vascular tissue engineering and the development of tissue engineered constructs now and in the future.
References


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Biography

Erika Michelle Moore was born in Menlo Park, California on June 11th, 1991.

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2013  National Science Foundation Graduate Research Fellow, National Science Foundation
2013  Pre-doctoral Ford Foundation Fellow (awarded but declined), Ford Foundation Fellowship Program
2013  James B. Duke Fellowship, The Graduate School of Duke University
2013  Dean’s Graduate Fellowship, The Graduate School of Duke University

She has authored the following publications during her graduate career: