Enhanced Vascularization of Modular Engineered Tissues Using Activated Macrophages

by

Michael West

A thesis submitted in conformity with the requirements for the degree of Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto

© Copyright by Michael West 2016
Enhanced Vascularization of Modular Engineered Tissues Using Activated Macrophages

Michael West
Master of Applied Science
Institute of Biomaterials and Biomedical Engineering
University of Toronto
2016

Abstract

Enhancing the vascularization of modular engineered tissues is critical for the survival of implanted therapeutic cells and the development of large, in vivo tissue constructs. However, modular implants elicit strong inflammatory responses, impairing this process. Mesenchymal stromal cells (MSC) are effective promoters of construct vascularization in vivo, and their immunomodulatory effect on inflammation and macrophage activation was assessed by flow cytometric analysis of enzymatically digested tissues. MSC limited macrophage infiltration into tissues at day 3 and caused increased vessel densities at day 7, but did not affect macrophage activation. Modular tissues containing bone marrow-derived macrophages (BMDM) activated to have pro- or anti-inflammatory phenotypes did not alter the course of inflammation or enhance vessel growth, but did stabilize existing vessels. Significant vessel regression occurred by day 14 in implants lacking BMDM, activated or otherwise. No effect due to BMDM activation was seen.
Acknowledgments

Thank you to my supervisor, Dr. Michael Sefton, for the opportunity to be a part of his lab and to learn from him. I am fortunate to have had his guidance and mentorship these last three years. Thank you also to my committee, Dr. Clinton Robbins, Dr. Myron Cybulsky, and Dr. Jonathan Rocheleau, for the advice, guidance, and technical expertise they have shared which made this work possible.

Many thanks to all the Sefton Lab members, but in particular to Dean Chamberlain, who was always available to help or to teach, to Alexandra Lisovsky, for all her support and guidance, and to Chuen Lo, for his surgical expertise. Thank you to Nicholas Cober, Yarden Gratch, Gabrielle Lam, Redouan Mahou, Ilana Talior-Volodarsky, Alex Vahos, Laura Wells, and David Zhang for all their assistance and insights.

Thanks also go to Dionne White for sharing her flow cytometry expertise, and to Sherine Ensan, for sharing important lab techniques.

Above all, thank you to Erin West, without whom none of this ever would have happened.
Table of Contents

Acknowledgments........................................................................................................ iii

Table of Contents........................................................................................................ iv

List of Tables ................................................................................................................ vi

List of Figures ................................................................................................................ vii

List of Appendices ........................................................................................................ ix

1 Introduction................................................................................................................. 1

1.1 Engineering vascularized tissues ........................................................................ 1

1.2 Modular tissue engineering .................................................................................. 1

1.3 Angiogenesis ......................................................................................................... 2

1.4 Inflammation ........................................................................................................ 4

1.4.1 Overview ........................................................................................................... 4

1.4.2 Mesenchymal stromal cells ............................................................................. 5

1.5 Macrophages ........................................................................................................ 6

1.5.1 Overview ........................................................................................................... 6

1.5.2 Activation .......................................................................................................... 6

1.5.3 Angiogenesis .................................................................................................... 9

1.5.4 Response to biomaterials ............................................................................... 10

1.5.5 In engineered tissues ...................................................................................... 10

1.6 Objectives ............................................................................................................. 11

2 Methods.................................................................................................................... 12

2.1 Bone marrow isolation ....................................................................................... 12

2.2 Cell culture .......................................................................................................... 12

2.3 Module fabrication .............................................................................................. 13

2.4 Module implantation ........................................................................................... 14
2.5 Macrophage depletion

2.6 Histology and immunohistochemistry

2.7 Histological analysis

2.8 Enzymatic digestion

2.9 Flow cytometry

2.10 Analysis of flow cytometry data

2.11 Statistical analysis

3 Results

3.1 Characterization of the inflammatory response to modular implants

3.1.1 Effect of macrophage depletion on implant vascularization

3.1.2 Standard inflammatory response to modular implants

3.2 Immunomodulatory and angiogenic effects of adMSC in modular implants

3.2.1 Effects of adMSC on implant vascularization

3.2.2 Effects of adMSC on inflammation

3.3 Effects of activated macrophages in modular implants

3.3.1 In vitro activation conditions

3.3.2 Effect of activated BMDM on vascularization

3.3.3 Effect of activated BMDM on inflammation

4 Discussion

4.1 The inflammatory response to modular implants

4.2 Impact of adMSC on vascularization and inflammation in modular implants

4.3 Impact of macrophage activation on vascularization and inflammation in modular implants

5 Conclusions

6 References

7 Appendices
List of Tables

Table 1. Standard components and treatments of half- \textit{(in vitro)} and full implants \textit{(in vivo)}...... 14

Table 2. Fluorescent antibodies used for flow cytometric analysis of explanted tissues. .......... 17

Table 3. Cell populations present in HUVEC/adMSC implants as a percentage of live cells ..... 24

Table 4. Percentage of macrophages from HUVEC/adMSC implants in MHCII vs. CD206 subpopulations ..............................................................................................................\textit{Error! Bookmark not defined.}
List of Figures

Figure 1. Macrophage activation. ................................................................. 8
Figure 2. Macrophage depletion reduces CD31 staining. ................................. 19
Figure 3. Macrophage depletion impairs vessel formation. ............................ 20
Figure 4. Vessel formation is partially rescued by incomplete macrophage depletion .......... 21
Figure 5. Implant mass is reduced at day 7. .................................................... 22
Figure 6. Representative gating of a day 1 HUVEC/adMSC implant. .................. 23
Figure 7. Representative gating of a day 3 HUVEC/adMSC implant. ................... 24
Figure 8. Distinct macrophage sub-populations at early time points..................... 25
Figure 9. Representative gating of a day 7 HUVEC/adMSC implant. ................... 26
Figure 10. Representative gating of a day 14 HUVEC/adMSC implant ................ 27
Figure 11. Cell populations present in HUVEC/adMSC implants by flow cytometry 28
Figure 12. Inflammatory cell recruitment by histology. ....................................... 28
Figure 13. Macrophage activation markers increase over time in HUVEC/adMSC implants. 29
Figure 14. MHCII and CD206 co-expression in response to HUVEC/adMSC implants .... 29
Figure 15. Identification of unknown populations by flow cytometry ................. 30
Figure 16. HUVEC/adMSC implants increase capillary-sized vessel density .......... 32
Figure 17. adMSC reduce macrophage infiltration into modular implants ............. 33
Figure 18. Histological analysis of inflammatory cell recruitment in HUVEC/adMSC vs. HUVEC-only implants ................................................................. 34
Figure 19. Macrophage activation in implants is unaffected by adMSC ................. 34
Figure 20. Macrophages express increasingly mixed activation over time ......................... 35

Figure 21. Module culture conditions alter macrophage activation ................................. 37

Figure 22. MHCII is a more reliable marker of classical activation than CD86 .................. 39

Figure 23. Macrophage modules promote endothelial cell presence in implants............. 40

Figure 24. Macrophages stabilize implant vasculature and prevent vessel regression ....... 41

Figure 25. Vessel size is unaffected by macrophage activation .................................... 42

Figure 26. Smooth muscle actin staining is increased at days 7 and 14 in all implants containing adMSC. ................................................................. 42

Figure 27. Representative histological staining of vessels at days 7 and 14. ...................... 43

Figure 28. Activated macrophages in implants do not alter host inflammatory cell recruitment. 44

Figure 29. Delivery of activated BMDM does not affect recruited macrophage activation .... 45

Figure 30. MHCII vs. CD206 activation subsets are unaffected by BMDM ....................... 46
List of Appendices

Appendix A. Function of important cytokines, surface markers, and cell products................. 62
1 Introduction

1.1 Engineering vascularized tissues

A critical limitation of engineered tissues is the requirement for a perfused vascular network throughout the tissue to provide nutrients and prevent hypoxia-induced apoptosis of their constituent cells. As oxygen diffusion is limited to a distance of approximately 100 – 200 µm[1,2], blood vessels must be present at least every 400 µm throughout a tissue to maximize cell density and survival. This necessarily limits the potential size of engineered tissues, and consequently their usefulness, until methods for their efficient vascularization can be developed.

1.2 Modular tissue engineering

Modular tissue engineering aims to create highly vascularized tissues through the random aggregation of individual microtissue building blocks, which are capable of simulating diverse tissue types[3]. The modules described herein are submillimeter-sized, cylindrical microtissues made of collagen or other extracellular matrix mimics which contain tissue-appropriate functional and supporting cells and are coated with endothelial cells (EC) on their surfaces[4]. Large numbers of modules can be implanted in an animal host by subcutaneous injection, where they form a tissue construct which undergoes remodelling over a period of several weeks to produce a mature tissue, fully engrafted with the host[5,6].

The primary advantage of modular tissue engineering is that it eliminates the requirement to equip tissues with a vasculature prior to implantation, or to even attempt to guide vessel formation. Instead, the components of the vasculature – EC which will form vessels, and mesenchymal stromal cells (MSC) which will function as pericytes – are implanted such that EC on the modules’ surfaces will form vessels in a bottom-up fashion at the inter-module junctions throughout the entire tissue, according to the conditions of the local cellular niche. Implanted modules are approximately 400 µm in diameter which permits effective diffusion of oxygen and nutrients into the center of modules from adjacent blood vessels. Modules larger in diameter have been shown to develop necrotic cores due to mass transfer limitations[7]. Seeding modules with EC is critical not only for vessel formation, but to maintain the integrity of the tissue construct. In a rat omental pouch implant model, collagen-only modules deformed rapidly in vivo and degraded by day 7, whereas EC-coated modules maintained their structures[8]. The EC...
migrated from the modules’ surfaces by day 7 and formed vessels that matured into distinct capillaries, arterioles, and venules which remained perfusable through day 60. Modular tissues also have the advantage of scalability and uniform cell distribution. If large tissues are to be formed, it is relatively straightforward to produce greater numbers of modules containing the standard complement of cells and aggregating them, or to implant sequential batches to a target location. This avoids the challenges associated with cellularizing large scaffolds in vitro.

Modular tissues are highly flexible in conformation, and can be modified to mimic a variety of tissues. Diverse cell types, such as cardiomyocytes[9], pancreatic islets[10], HepG2 cells[7], MSC[11], and a variety of endothelial cells have been used. Although generally type I bovine collagen is the sole ECM component, other conformations can be used to mimic tissues more specifically. Previously, collagen supplemented with Matrigel or alginate was used for the in vitro culture of cardiomyocyte-containing modules[9]. As well, collagen modules coated with HUVEC and treated with fibronectin showed significantly reduced HUVEC apoptosis in a mouse implant model, and produced twice as many HUVEC-lined vessels as modules without fibronectin[12].

The main limitation of modular tissue engineering is the slow rate of vessel maturation. Although vessels are routinely observed by day 3 in various implant models, indications of perfusion are generally not seen until day 7, and mature smooth muscle actin- (SMA) lined vessels are usually not observed until day 14. For optimal vasculature maturity, including robust perfusion and tight, non-leaking junctions, 21 days are normally required. This has negative implications for the survival and proper function of implanted therapeutic cells between the time of implantation and their connection with the host vasculature.

1.3 Angiogenesis

Two main routes of new vessel formation exist in vivo: vasculogenesis, in which vessels are formed de novo from precursor cells in the absence of an existing vasculature, and angiogenesis, in which vessels are formed by sprouting from pre-existing vessels. Generally, vasculogenesis is restricted to embryonic development, and for all subsequent vessels the main route of formation is angiogenesis. In embryonic development, vasculogenesis begins when mesodermal pre-cursors expressing vascular endothelial growth factor receptor 2 (VEGFR2) differentiate into hemangioblasts in response to fibroblast growth factor (FGF) and bone morphogenetic protein 4
(BMP4) signaling. These produce blood islands comprised of angioblasts and hematopoietic progenitor cells, which subsequently give rise to inter-connected networks of primitive vessels called capillary plexi[13]. Extension of these vessels through angiogenesis is ultimately the source of all other vasculature.

Angiogenesis generally occurs in one of two forms: non-sprouting (intussusceptive), and sprouting, of which sprouting is the more common[14]. Proliferation of EC is driven largely by VEGF-VEGFR2 signaling, and this also drives vessel sprouting. In response to VEGF, the walls of existing vessels become more permeable and individual cells become more mobile. The release of matrix metalloproteinases (MMP) which degrade the ECM allows migration along the VEGF gradient, which guides sprouting. The leading cell of the nascent sprout is called the tip cell, and VEGF upregulates its expression of DLL4, which in turn binds to Notch receptors on neighbouring EC. Notch downregulates VEGFR2, making those EC less responsive to the VEGF gradient although they continue to proliferate. The growing vessel thus forms a stalk which elongates behind the tip cell.

Vessel maturation involves the recruitment of mural cells, which includes smooth muscle cells and pericytes. The latter are smooth muscle cell-like, expressing α-SMA and residing within the basement membrane in direct contact with EC in capillaries, arterioles, and venules, where they facilitate cell coordination and reduce EC proliferation while enhancing EC survival[15]. A number of factors produced by EC, such as platelet-derived growth factor B (PDGF-B), sphingosine-1 phosphate, angiopoietin-1 (Ang1), transforming growth factor β (TGFβ), semaphorin-3A (Sema3a), and MMP, are largely responsible for the recruitment and proliferation of pericytes[16]. TGFβ can induce mesenchymal progenitors to differentiate into pericytes, and also increases deposition of ECM.[13] Pericytes themselves can produce PDGF-B which enhances this effect in an autocrine matter.

An important modulator of vessel growth and stability is the Ang1/2-Tie2 pathway. Both of these vascular growth factors compete for the Tie2 receptor and exert opposite effects, with Ang1 promoting vessel stability, and Ang2 promoting vessel growth. Secreted by pericytes, Ang1 promotes vascular quiescence and stability through increased cell-cell contact, pericyte coverage, and deposition of basement membrane. It also upregulates EC expression of PDGF-B and TGFβ, enhancing pericyte recruitment. Ang2, secreted by sprouting EC, promotes the
detachment of pericytes, reduction of cell-cell contact, and increased cell dissociation and vessel permeability. It also directly increases EC sprouting in an autocrine manner. However, in the absence of VEGF the effects of Ang2 are reversed, leading to EC death and vessel regression[17].

1.4 Inflammation

1.4.1 Overview

The inflammatory response protects the tissues of the body from infection and injury by clearing foreign bodies and pathogens, and is intimately involved with tissue development, remodelling, and repair. In general, inflammation-inducing signals from a variety of sources activate tissue-resident macrophages and mast cells. Microbes induce an inflammatory response when pathogen associated molecular patterns (PAMPs), are recognized by membrane-bound toll-like receptors on immune cells, or when PAMPs are phagocytosed and recognized by cytoplasmic NOD-like receptors. Inflammation can also be initiated by damage-associated molecular patterns (DAMPS), such as ATP, and various cytoplasmic proteins released from damaged and necrotic cells[18].

In response, macrophages and mast cells produce a variety of inflammatory mediators, such as vasoactive amines, cytokines, and chemokines which recruit neutrophils to the affected site. These signals activate EC to upregulate expression of P- and E-selectin, which bind P-selectin glycoprotein ligand 1 (PSGL1) on the surface of passing neutrophils in the blood. Tethered by selectins, neutrophils roll along the endothelium until they become more firmly attached to integrins, and become activated by endothelium-bound cytokines. The cytokine gradient guides them to extravasation sites where they enter the tissue and destroy the inflammatory agent by phagocytosis or by secreting reactive oxygen species (ROS) or antibacterial proteins[19].

Subsequently, neutrophils recruit monocytes by a variety of mechanisms. Granule proteins released from neutrophils bind to EC causing them to become more permeable and to induce adhesion of circulating monocytes. Activated, and apoptotic, neutrophils produce soluble interleukin-6 receptor (IL-6R) which forms complexes with IL-6 produced by local cells. These complexes induce EC upregulation of CCL2 and vascular cell-adhesion molecule 1 (VCAM1)
which cause recruitment and adhesion of monocytes, respectively, which differentiate into macrophages in the tissues[20].

Neutrophils have a short lifespan in tissues, and as they become apoptotic they inhibit further neutrophil recruitment. As apoptotic neutrophils are phagocytosed by macrophages they switch production of lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins, inhibiting neutrophil recruitment. Remaining apoptotic neutrophils secrete a number of factors that increase monocyte recruitment, while also upregulating CCR5 expression, which sequesters CCL3 and CCL5, and prevents further neutrophil recruitment. After efferocytosis of neutrophils, macrophages adopt an anti-inflammatory, pro-resolution phenotype and express anti-inflammatory cytokines TGFβ and IL-10[21].

1.4.2 Mesenchymal stromal cells

Mesenchymal stromal cells are multipotent, mesoderm-derived cells present in many mature tissues that are capable of self-renewal and differentiation into various cell lineages, including chondrocytes, osteoblasts, adipocytes, myocytes, and neurons[22]. MSC have been shown to have diverse immunomodulatory effects, secrete many cytokines and growth factors, and have been used in the treatment of chronic inflammatory diseases[23]. A number of tissue engineering strategies have made use of MSC to dampen host immune response[24], support cells implanted in vivo and to enhance construct vascularization[11,25–27], or generate specific tissues[5,28]. In constructs lacking MSC, modular implant studies have shown poor EC survival[29].

In response to hypoxia, several important angiogenic signals are upregulated in MSC including VEGF, Ang1, and PDGF-B[30], PIGF, and MMP-9[31], making them promising candidates to support initially avascular engineered tissues in vivo. In the SCID/Bg mouse model, inclusion of MSC in modular tissues produced perfused vessels as early as day 3 which appeared highly mature by day 14 and persisted beyond 90 days[5]. In a rat omental pouch modular implant model, MSC were seen migrating out of modules and assuming a pericyte-like position by day 7. By day 21 all MSC were fully incorporated into vessel structures with high SMA expression and reduced vessel leakiness compared to non-MSC implants[11]. The presence of MSC also caused changes in activation markers expressed by macrophages recruited to the implant site. Pro-angiogenic CD163+ macrophages migrated into the implant region, whereas in the absence of MSC CD163+ staining was only seen in the surrounding omental tissue, indicating MSC may
mediate some of their pro-angiogenic effects in this system by altering the recruitment and activation of immune cells. Cross-talk between MSC and macrophages has been shown to promote vascularization[24].

1.5 Macrophages

1.5.1 Overview

As described above, macrophages are responsible for initiation, and ultimately resolution, of the inflammatory response, and this is their primary function. However, macrophages have a variety of other functions relating to tissue homeostasis, angiogenesis, wound healing, and tissue remodelling[32]. In most tissues, a population of self-renewing tissue-resident macrophages is responsible for homeostatic functions and initiating the immune response. These are distinct from inflammatory macrophages of the mononuclear phagocyte system (MPS), which are derived from bone marrow progenitors and recruited to the tissues through the vasculature in response to inflammatory signals. Hematopoietic stem cells (HSC) in the bone marrow differentiate in response to macrophage colony stimulating factor 1 (CSF1), via transcription factor PU.1, through a series of myeloid progenitor states before ultimately becoming monocytes. These exist in two main subsets distinguished by high and low expression of Ly6C. Ly6C<sup>high</sup> monocytes express CCR2, the receptor for CCL2, which is responsible for monocyte chemotaxis to the site of inflammation. Ly6C<sup>low</sup> monocytes patrol the vascular endothelium and respond to initial inflammatory signals by enhancing neutrophil recruitment. As monocytes enter the tissue they differentiate into macrophages and express elevated levels of F4/80, an adhesion class g-coupled protein receptor[33] expressed by macrophages involved in the generation of antigen-specific regulatory T cells[34]. Tissue-resident macrophages are derived from erythro-myeloid progenitors in the fetal yolk-sac, whence they colonize the fetal liver by embryonic day 10.5 and give rise to tissue-specific populations throughout the body, including microglia, Langerhans cells, and Kupffer cells, which persist into adulthood[35,36]. Some replacement by HSC-derived macrophages was seen, and this increased with age but was generally limited.

1.5.2 Activation

Macrophage behavior is function of activation. Macrophages are highly plastic cells expressing diverse phenotypes or polarization states which can be influenced by many factors. The first
description of macrophage polarization drew a distinction between M1 and M2 macrophages based on differences in their metabolism of arginine. In response to bacterial challenge, Th1-derived IFNγ induced M1 macrophages to produce nitric oxide (NO) through inducible nitric oxide synthase (iNOS), whereas Th2-derived IL-4 caused M2 macrophages to metabolize arginine into ornithine. The M1/M2 labels referred to the ability to express different phenotypes regardless of T cell behavior, as the phenomenon had been seen in mice lacking T cells altogether. Nevertheless, the nomenclature mirrored and became associated with the Th1/Th2 dichotomy[37,38]. This was subsequently expanded to include many different phenotypes, such as M1, M2a, M2b, M2c, and others, expressing a variety of different markers of activation[39].

Classically-activated (M1) macrophages are distinguished from alternatively-activated (M2) macrophages by their source of activation, inflammatory products, and surface markers expressed. They arise in response to bacterial lipopolysaccharide (LPS) via toll-like receptors (TLR), or IFNγ from Th1 and natural killer cells. Production of inflammatory factors through Stat1 and NF-κB signaling induces expression of iNOS, tumour necrosis factor α (TNFα), IL-1β, IL-6, IL-12, and chemokines which recruit other inflammatory cells. Their role is microbial destruction and clearance of apoptotic cells. Alternatively-activated (M2) macrophages arise in response to cytokines such as IL-4 and IL-13 from Th2 cells and granulocytes, and IL-10 from regulatory T cells[40,41]. They encompass a number of sub-phenotypes which are collectively termed “alternative” not necessarily due to their similarities, but because they are anti-inflammatory and thus distinguishable from macrophages of the classical M1 activation pathway. In response to IL-4, Arg1, CD206, and a variety of scavenger receptors are upregulated via Stat6 signalling. TGFβ, PDGF, and IL-10 are produced, as well as chemokines. IL-10 activation promotes an anti-inflammatory phenotype which produces more IL-10 and TGFβ, MMP to degrade ECM for remodeling, and deposition of fresh ECM proteins.

Due to the large number of activation markers identified, and the increasingly complex and overlapping phenotypes now described, especially in vivo, it is widely recognized that this paradigm has become outdated[38,42,43]. While it is useful in in vitro contexts, it has little correlation with macrophage behavior and activation in vivo, such as that related to engineered tissues.
Figure 1. Macrophage activation. Macrophages are derived from circulating monocytes in response to inflammatory signals or by local proliferation in tissues. They can be activated by a variety of cellular and environmental signals to perform pro- or anti-inflammatory functions. Three activation states are pictured here, M(IFNγ), M(IL-4), and M(IL-10), along with a selection of their associated products, upregulated genes, and transcription factors.
1.5.3 Angiogenesis

The manner in which macrophages regulate angiogenesis has been thoroughly reviewed[44]. They are recruited to target tissues by chemotactic factors such as hypoxia-inducible factor (HIF)-mediated CCL2, VEGF, and SDF-1 signalling, and secrete pro-angiogenic mediators VEGF, FGF2, IL-1b, IL-8, and MMP critical for ECM degradation and vascular remodelling. In embryonic development, wound-healing, and tumour vascularization, macrophages share similar “M2”-like phenotypes[44,45], and play important roles in the regulation of angiogenesis [46,47]; their depletion generally leads to severely impaired vessel formation[48,49].

There are mixed reports as to which phenotypes are most conducive to angiogenesis. Wound healing studies have shown that macrophage phenotype and role in the healing process changes over time. Lucas et al. showed that depletion of macrophages at different stages of the healing process had different outcomes, with early stage depletion being the most detrimental to vessel formation [50]. Early depletion also prevented the typical M1 to M2 shift over the course of healing: this shift is normally characterized by increased IL-10, CD206, and CD163 expression. Subsequently, Willenborg et al. showed that recruitment of circulating Ly6C⁺CCR2⁺ monocytes is the source of a population of macrophages which produces high levels of VEGF and is critical to early-stage vascularization[51]. When myeloid-specific CCR2 was deleted, monocyte recruitment was reduced, and lower VEGF levels were seen in the wound tissue. Deletion of myeloid-specific VEGF did not prevent recruitment of this population, but was sufficient to cause a similar reduction in vascularization, indicating the small population of inflammatory, VEGF-producing early macrophages is critical for the induction of angiogenesis. In this case, the shift toward “M2”-associated gene expression still occurred, although the initial inflammatory population expressed a mix of M1 and M2 markers, so while this “M1-to-M2 shift” is surely an oversimplification, it is clear that distinct macrophage subsets play important, non-redundant roles in vascularization, especially at early time points.

Not surprisingly, macrophages have been shown to be equally critical in engineered tissues. For example, Melero-Martin et al. showed that CD11b⁺ cells were recruited to Matrigel plugs seeded with endothelial progenitor cells and MSC implanted in immunodeficient mice, and were necessary to achieve the formation of blood vessels from the implanted cells[52]. Depletion of circulating myeloid cells strongly impaired vessel formation. In an adipose tissue engineering
system, also Matrigel-based, a four-fold decrease in CD31+ endothelial cell staining was seen in response to depletion of macrophages with clodronate liposomes[53]. Evidence for the importance of direct EC-macrophage contact comes from studies of the developing zebrafish hindbrain, in which tissue-resident macrophages were shown to promote anastomosis of vessel sprouts by interacting directly with endothelial tip cells and guiding their fusion to form vascular networks[54].

1.5.4 Response to biomaterials

A topic of considerable interest in the biomaterials and tissue engineering community is the relationship between biomaterials and macrophage activation. All biomaterials generate an inflammatory response to some extent, and controlling it is critical to maximize implant function. When the inflammatory response is not well controlled it can persist for long periods, generating a prolonged foreign body response and impairing implant function[55].

Several groups have also shown that the structural and chemical properties of implanted tissue scaffolds affect the number and polarization of macrophages recruited to the implant site, as well as the degree to which the implants can be effectively remodeled by the host. In a comparison of 14 decellularized matrix-based biologic meshes, it was observed that the most efficient remodelling occurred in meshes into which large macrophage populations were recruited early, and had high proportions of CD206+ (“M2”-like) macrophages. The presence of CCR7+ (“M1”-like) macrophages in those populations did not impair remodelling, provided that the ratio of CD206+:CCR7+ cells remained high. High M2:M1 ratios at early time points (day 14) correlated strongly with improved vascularization and remodelling at later time points (day 35)[56].

Another study found that scaffolds designed to elicit an “M2” response were less effective at promoting vascularization that those eliciting a mixed “M1”-“M2” response. They proposed that mixed populations, each with specific phenotypes and specific, non-redundant roles in remodelling (e.g., M1 sprouting, M2a anastomosis, and M2c remodeling), will achieve optimal results[57].

1.5.5 In engineered tissues

Their ability to produce anti-inflammatory signals, promote angiogenesis, and actively remodel tissues makes manipulating macrophages to enhance engraftment of engineered tissues an
attractive approach. Their well-defined *in vitro* activation states make them readily adaptable for use as supporting cells in modular tissues to drive implant vascularization.

One of the first demonstrations that polarized macrophages can be effectively used to promote angiogenesis in vivo examined subcutaneously injected Matrigel plugs in a mouse model, supplemented with polarized macrophages. At day 14, plugs containing pre-polarized M(IL-4) macrophages showed increased EC counts (CD31+ staining) compared with M(IFNγ), M(0), and macrophage-free controls, indicating improved vascularization of the implants[58]. This was supported by similar results using in vitro tube formation assays, which suggested that direct cell-cell contact between EC and either M(IFNγ) or M(0) macrophages may inhibit tube formation.

### 1.6 Objectives

The purpose of this research was to investigate the impact of inflammation in general, and macrophages in particular, on the vascularization of modular implants *in vivo*. Previous work without MSC had identified large numbers of inflammatory cells, most likely macrophages, recruited to implants which were believed to be responsible for poor engraftment with host tissues. This limitation was overcome by incorporating MSC into implants, which secrete a number of immunomodulatory factors, suggesting the malleability of the immune response may be an important and useful tool to enhance vascularization.

**Objective 1:** Characterize the specific cell populations that constitute the inflammatory response during the normal course of modular implant vascularization.

**Hypothesis:** The initial intense inflammatory response to modular implants consists primarily of neutrophils and classically-activated macrophages which are detrimental to implanted cells and vascularization. This will be followed by a shift over time toward an alternatively-activated macrophage phenotype coinciding with vessel maturation and implant remodelling.

**Objective 2:** Determine the impact of adMSC on the recruitment and activation of inflammatory cells and their corresponding link with vascularization.

**Hypothesis:** The inclusion of adMSC in modular implants dampens the inflammatory response in one or more ways: by reducing the number of inflammatory cells recruited into implants, by
recruiting different cellular subsets, or by modulating the phenotype of macrophages which are recruited to a pro-angiogenic, alternatively-activated state. These effects will be clarified.

Objective 3: Enhance implant vascularization by direct delivery of activated exogenous macrophages in modules.

Hypothesis: Promoting the alternative activation of macrophages and increasing the ratio of anti-inflammatory to pro-inflammatory cells early in the vascularization process will mitigate the detrimental impacts of inflammation, and the rate and extent of vascularization can be enhanced.

2 Methods

2.1 Bone marrow isolation

Bone marrow was harvested from 6-10 week old Fox Chase SCID Beige (CB17.Cg-Prkdc<sup>scid</sup>Lyst<sup>Bg-1</sup>/Crl) mice (SCID/Bg; Charles River Laboratories). The hind legs were separated from the hip with the skin removed and placed in Hank’s buffered saline solution (HBSS; Gibco) on ice. After transfer to sterile conditions, muscle and connective tissue were removed from the bones while submerged in HBSS, and the surface of the bones was scraped clean with a scalpel. Femur and tibia were separated, wiped with sterile gauze to remove residual tissue, and rinsed first with 70% ethanol then with fresh HBSS. Scissors were used to remove the head of the femur at the lesser trochanter, and the malleolus of the tibia was cut at the junction with the fibula. A 20G needle was used to puncture the supracondylar region of the femur and the tibial plateau, respectively. Marrow was flushed from the bones by inserting a 25G needle into the open ends and rinsing thoroughly with HBSS. Clumped cells were disaggregated by repeated flushing through a 20G needle and then passed through a 40 µm nylon filter. Homogenized cells were resuspended in a 9:1 mixture of HI-FBS (Gibco) and dimethyl sulfoxide (DMSO; Sigma), frozen overnight at -80°C and transferred to liquid nitrogen for long-term storage.

2.2 Cell culture

HUVEC (Lonza) were cultured in EGM-2 medium (Lonza) in 150 cm<sup>2</sup> or 175 cm<sup>2</sup> tissue culture-treated polystyrene (TCPS) flasks (Sarstedt) at 37°C, 5% CO<sub>2</sub>. Medium was changed every 2-3 days and cells were passaged at 80% confluency (~5 days) at a ratio of 1:9. HUVEC were used between passages 2-6.
Human adipose-derived mesenchymal stromal cells (adMSC; Lonza) were cultured in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% (v/v) fetal bovine serum (FBS; Gibco) and 1% penicillin-streptomycin (P/S; Gibco) in 150 cm² or 175 cm² TCPS flasks at 37°C, 5% CO₂. Medium was changed every 3-4 days and cells were passaged at 80% confluency (~7-10 days) at a ratio of 1:4. adMSC were used between passages 2-5.

Bone marrow-derived macrophages (BMDM) were generated by culturing bone marrow from SCID/Bg mice in RPMI 1640 (Gibco) supplemented with 10% (v/v) HI-FBS, 1% P/S, and 20 ng/mL macrophage colony-stimulating factor 1 (CSF1; eBioscience) on 10 cm non-tissue culture-treated polystyrene dishes (Sarstedt) at a concentration of 10⁷ cells per dish at 37°C, 5% CO₂. Medium was changed after 4 days; after 7 days CSF1-treated cells were incubated for 10 minutes in PBS supplemented with 2mM EDTA at 4°C and harvested by gentle rinsing.

2.3 Module fabrication

Standard collagen modules are coated with EC and usually contain MSC. As both of these cell types have been shown to affect macrophage activation[22,59] modules containing BMDM were cultured in the absence of HUVEC or adMSC to preserve their M(IFNγ), M(IL-4), or M(0) activation states. Before implantation in vivo, BMDM-containing modules were combined in a 1:1 ratio with adMSC modules to produce standard-sized M(0), M(IFNγ), or M(IL-4) implants. HUVEC/adMSC implants (no BMDM) were similarly produced by combining a 1:1 ratio of HUVEC/adMSC modules with empty, collagen-only modules, and HUVEC implants were produced by combing a 1:1 ratio of HUVEC-coated modules (no BMDM or adMSC) with empty, collagen-only modules (Table 1). Thus, modules were fabricated as “half-implants” so they could be cultured separately in vitro and combined to produce “full implants” at the time of surgery.

Modules were produced under sterile conditions using PureCol type 1 bovine collagen solution (Advanced BioMatrix) supplemented with 128 µL/mL 10x concentrate Minimum Essential Medium-alpha medium (αMEM; Gibco) and neutralized with 14 µL/mL 0.8M NaOH, as described previously[4]. adMSC or BMDM were suspended separately in the collagen solution as appropriate, depending on module type (Table 1). Approximately 1 mL of collagen solution was drawn into two meters of PE-60 polyethylene tubing (Instech) with a syringe and allowed to gel for 1 hour at 37°C, 5% CO₂, producing one half-implant. Tubing was cut into 2 mm lengths
with an automatic tube cutter and collected in DMEM, and modules were removed from the tubing after 24 hours by vortexing. HUVEC/adMSC modules were seeded with HUVEC in a 1:1 mixture of EGM-2 and DMEM supplemented with 10% FBS and 1% P/S for 1 hour at 37°C with gentle rocking. In this pilot study, BMDM-containing modules were not coated with HUVEC, as described above, and were cultured in RPMI 1640 supplemented with 10% HI-FBS and 1% P/S and activated with IFNγ (20 ng/mL, Peprotech), IL-4 (20 ng/mL, Peprotech) or were left unactivated. All modules were cultured for 72 hours at 37°C, 5% CO₂ prior to implantation.

All modules were triple-rinsed with sterile PBS before implantation in mice. Immediately prior to implantation, one HUVEC-only and one collagen “half-implant;” or one HUVEC/adMSC and one collagen “half-implant;” or one HUVEC/adMSC and one BMDM-containing “half-implant” were combined to make “full implants.”

Table 1. Standard components and treatments of half- (in vitro) and full implants (in vivo).

<table>
<thead>
<tr>
<th>Modular components</th>
<th>Collagen (mL)</th>
<th>adMSC</th>
<th>BMDM</th>
<th>HUVEC</th>
<th>Cytokine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>HUVEC-only</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>3.0 x 10⁶</td>
<td>None</td>
</tr>
<tr>
<td>HUVEC/adMSC</td>
<td>1</td>
<td>2.0 x 10⁶</td>
<td>0</td>
<td>3.0 x 10⁶</td>
<td>None</td>
</tr>
<tr>
<td>M(0)</td>
<td>1</td>
<td>0</td>
<td>1.0 x 10⁶</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td>M(IFNγ)</td>
<td>1</td>
<td>0</td>
<td>1.0 x 10⁶</td>
<td>0</td>
<td>20 ng/mL IFNγ</td>
</tr>
<tr>
<td>M(IL-4)</td>
<td>1</td>
<td>0</td>
<td>1.0 x 10⁶</td>
<td>0</td>
<td>20 ng/mL IL-4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Modular components</th>
<th>Collagen (mL)</th>
<th>adMSC</th>
<th>BMDM</th>
<th>HUVEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>HUVEC-only</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>3.0 x 10⁶</td>
</tr>
<tr>
<td>HUVEC/adMSC</td>
<td>2</td>
<td>2.0 x 10⁶</td>
<td>0</td>
<td>3.0 x 10⁶</td>
</tr>
<tr>
<td>M(0)</td>
<td>2</td>
<td>2.0 x 10⁶</td>
<td>1.0 x 10⁶</td>
<td>3.0 x 10⁶</td>
</tr>
<tr>
<td>M(IFNγ)</td>
<td>2</td>
<td>2.0 x 10⁶</td>
<td>1.0 x 10⁶</td>
<td>3.0 x 10⁶</td>
</tr>
<tr>
<td>M(IL-4)</td>
<td>2</td>
<td>2.0 x 10⁶</td>
<td>1.0 x 10⁶</td>
<td>3.0 x 10⁶</td>
</tr>
</tbody>
</table>

2.4 Module implantation

Adult male (6-8 weeks old) SCID/Bg mice (Charles River) were individually housed in sterile conditions following surgery and fed ad libitum. Each mouse received 2 subcutaneous injections of ~2000 modules, one each to the left and right dorsum, in 1 mL PBS using an 18G needle.
Mice were euthanized at days 1, 3, 7, and 14 and modular tissues were explanted and fixed for histological analysis or subjected to enzymatic digestion for flow cytometric analysis. Studies were performed under the approval of the University of Toronto animal care committee.

2.5 Macrophage depletion

Clodronate liposomes or PBS liposomes (clodronateliposomes.com) were administered to SCID/Bg mice intravenously (200 µL) via the tail vein and subcutaneously (50 µL per site) per the manufacturer’s instructions at planned module implant sites 48 hours prior to module implantation. Some mice were sacrificed 3 days after implantation and tissues were explanted for histological analysis. Mice not sacrificed on day 3 received a second subcutaneous dose of liposomes (50 µL per site) on day 4 and were sacrificed on day 7.

2.6 Histology and immunohistochemistry

Explanted tissue was fixed in 10% buffered formalin (Sigma) for 48 hours, then processed for staining. Paraffin-embedded, 4 µm thick sections were stained using: Hematoxylin (Leica) & Eosin (Fisher), Masson’s trichrome (Fisher), anti-CD31 (Santa Cruz, SC1506, Tris-EDTA, 1/2000, 1 hr), anti-UEA-1 (Vector, B1065, 1/400, overnight), anti-SMA (Dako, MO851, 1/500, overnight), ant-F4/80 (Serotec, MCA4976A, trypsin, 1/2000, 1 hr), anti-MHCII (BD Bioscience, 556999, Tris-EDTA, 1/1000, overnight), anti-CD206 (Abcam, ab64693, citrate, 1/800, overnight), and anti-Ly6G (Abcam, ab25377, Tris-EDTA, 1/1000, overnight). Histological preparation was performed by the Pathology Research Program Laboratory at Toronto General Hospital. Slides were digitized using an Aperio ScanScope XT scanner at 20x magnification at the Advanced Optical Microscopy Facility (AOMF, Toronto).

2.7 Histological analysis

Digitized slides were analyzed using ImageScope v12 (Aperio). All areas containing modules were defined including a ~200 µm region directly adjacent to the implant region. The positively stained percentage of this area (positivity) was determined using the Positive Pixel Count v9 algorithm for CD31, CD206, F4/80, Ly6G, MHCII, and UEA-1, stains. Individual CD31\(^+\) vessels with clearly defined lumens were counted in three circular hotspots 1 mm in diameter per implant. Vessel density (# of vessels / mm\(^2\)) is the average of these hotspot counts.
2.8 Enzymatic digestion

The digestion protocol was adapted from Robbins et al. [60]. Whole implants were removed from the surrounding skin and connective tissue by carefully cutting away loose connective tissue with scissors. Explanted tissues were placed in 500 µL digestion buffer in a gentleMACS C Tube (Miltenyi). Digestion buffer consisted of 450 U/mL collagenase I (C0130, Sigma), 125 U/mL collagenase XI (C7657, Sigma), 60 U/mL DNase I (D5025, Sigma), 60 U/mL hyaluronidase (H3506, Sigma) in HBSS, with 50 µL of 1.0M HEPES buffer (H4034, Sigma). Samples were finely minced with scissors, then further dissociated using a gentleMACS Octo Dissociator (Miltenyi), and incubated for 60 minutes at 37°C and 250 rpm rotation. Under sterile conditions, samples were passed through a 40 µm filter and rinsed with flow cytometry (FC) buffer consisting of PBS supplemented with 2 mM EDTA (Sigma) and 0.5 % bovine serum albumin (BSA; Sigma). Red blood cells were lysed with 1X Pharm Lyse RBC lysis buffer (BD). Cells were counted using a hemocytometer, and samples were resuspended in FC buffer for staining.

2.9 Flow cytometry

Prior to cell preparation, a cocktail of standard antibodies was prepared, consisting of CD11b:PE, CD45:BV711, CD86:BUV395, CD206:PE-Cy7, F4/80:APC-eFluor780, Ly6G:V450, and MHCII:PerCP-eFluor710 in FC buffer supplemented with Brilliant Stain Buffer (BD). Final concentrations of each antibody are listed in Table 2. In some cases, other stains listed in Table 2 were added. Cocktail volume provided 50 µL per sample in which to be stained. Individual cocktails were prepared for each fluorescence minus one control (FMO), each containing all of the antibodies in the cocktail, less one. Cells were incubated for 5 minutes on ice in 100 µL FC buffer with 1 μg anti-CD16/32 (2.4G2, BD) per 10^6 cells to block FC receptors. Portions of each sample were removed and pooled for use as FMO controls. Samples were resuspended in 1 mL PBS + 1% BSA / 10^6 cells and stained (1 µL/mL) with LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies) for 30 minutes at 4°C in the dark to label dead cells. Samples were washed and resuspended in 50 µL FC buffer. 50 µL of staining cocktail or FMO cocktail was added to each sample. Final staining volume = 100 µL. Samples were incubated for 30 minutes at 4°C for 30 minutes in the dark. Samples were then washed with FC buffer and resuspended in 2% paraformaldehyde, and incubated for 15 minutes in the dark. Samples were then washed with
FC buffer and resuspended in 200 µL FC buffer and transferred to a 96-well plate. Samples were acquired on a 5 laser (355/405/488/561/640 nm) LSRFortessa X-20 flow cytometer (BD) equipped with a High Throughput Sampler (BD).

**Table 2. Fluorescent antibodies used for flow cytometry.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone</th>
<th>Fluorophore</th>
<th>Source (Product #)</th>
<th>Concentration (µg/100µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD11b</td>
<td>M1/70</td>
<td>PE-CF594</td>
<td>BD (562287)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD31</td>
<td>WM-59</td>
<td>FITC</td>
<td>eBioscience (11-0319)</td>
<td>0.1</td>
</tr>
<tr>
<td>CD45</td>
<td>30-F11</td>
<td>BV711</td>
<td>BD (563709)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD86</td>
<td>B7-2</td>
<td>BUV395</td>
<td>BD (564199)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD117</td>
<td>2B8</td>
<td>BUV395</td>
<td>BD (564011)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD206</td>
<td>C068C2</td>
<td>PE-Cy7</td>
<td>Biolegend (141719)</td>
<td>0.5</td>
</tr>
<tr>
<td>CD335</td>
<td>29A1.4</td>
<td>AlexaFluor700</td>
<td>BD (561169)</td>
<td>0.2</td>
</tr>
<tr>
<td>F4/80</td>
<td>BM8</td>
<td>APC-eFluor780</td>
<td>eBioscience (47-4801)</td>
<td>0.2</td>
</tr>
<tr>
<td>Ly6G</td>
<td>1A8</td>
<td>V450</td>
<td>BD (560603)</td>
<td>0.2</td>
</tr>
<tr>
<td>MHCII</td>
<td>M5/114.15.2</td>
<td>PerCP-eFluor710</td>
<td>eBioscience (46-5321)</td>
<td>0.2</td>
</tr>
</tbody>
</table>

2.10 Analysis of flow cytometry data

Total cell counts from digested implants were determined by acquiring samples on the cytometer from a known volume and dividing by the proportion of the sample acquired, as well as the proportion of the sample previously removed to create FMO controls. For example: 50% of a 200 µL sample was acquired, and 20% of that sample had previously been removed to create FMO controls. A total of 100,000 total cells were seen on the cytometer. The corrected cell count would be 100,000 / 0.5 / 0.8 = 250,000 cells. This correction factor allows comparison of cell numbers across samples collected at different times where technical requirements (e.g. instrument failure, high cell yields requiring substantial dilution, or larger proportions of each sample being required to create FMO controls when few samples were processed at once). Data were analyzed using FlowJo v10 (Tree Star), and minimum boundaries of gates were set according to maximum FMO boundaries for each given fluorophore subject to the same prior gating.
2.11 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics 20.0. (IBM). One-way or two-way analysis of variance (ANOVA) or independent t-tests were used to compare treatments across time points as indicated ($\alpha = 0.05$). Two-way ANOVA was preferred provided Levene’s test for equality of variances returned a non-significant result. When results were significant, data were natural log-transformed or one-way ANOVA was used. Tukey’s honestly significant difference (HSD) post-hoc test was used when sample sizes and variances were equal. When sample sizes were unequal, Gabriel’s pairwise comparisons post-hoc test was used. If variances were unequal and could not be transformed, the Games-Howell post-hoc test was used.

3 Results

3.1 Characterization of the inflammatory response to modular implants

The inflammatory response to standard modular tissue implants (HUVEC/adMSC) was examined by enzymatically digesting whole explants and characterizing recruited inflammatory cell populations by flow cytometry. The host inflammatory response was also inhibited by depleting macrophages with clodronate liposomes, and the extent of implant vascularization in the absence of an inflammatory response was assessed by histology. See appendix A for a summary of the function of important antigens, cytokines, and cell products.

3.1.1 Effect of macrophage depletion on implant vascularization

Although high levels of inflammation can be detrimental to engineered tissues, the presence of some amount of macrophages is critical for their efficient vascularization. This was demonstrated by depleting host macrophages in SCID/Bg mice with clodronate liposomes 48 hours prior to implantation of modules. Clodronate treatment showed significantly reduced F4/80$^+$ staining in HUVEC/adMSC implants at day 3 as well as correspondingly reduced CD31$^+$ staining at days 3 and 7 (Fig. 2). Representative histology of day 3 implants can be seen in Figure 3. Low-magnification images show that with both PBS and clodronate liposome treatments, most macrophages present are found immediately outside the implant region.
However, large numbers of macrophages are found within implants treated with PBS liposomes, whereas implants treated with clodronate are largely devoid of macrophages at day 3. High-magnification images show representative macrophage infiltration and vessel formation. When macrophages are present, CD31+ endothelial cells form vessels with clearly defined lumens. When macrophages are absent, vessel formation is severely impaired.

The amount of F4/80+ staining in PBS control implants was lower at day 7 than at day 3, reducing the magnitude of the difference between controls and clodronate treatment, which were not significantly different (Fig. 2A). CD31 staining was still significantly impaired by macrophage depletion at day 7. However, the depletion was less uniform. Some patches of implant tissue retained substantial macrophage populations. In these regions, vessel formation was less impaired (Fig. 4).

**Figure 2. Macrophage depletion reduces CD31 staining.** (A) Treatment of HUVEC/adMSC implants with clodronate liposomes (grey) significantly reduced F4/80+ staining at day 3 compared with PBS liposome controls (white). F4/80+ staining remained lower at day 7 but the difference was not significant despite a second clodronate dose on day 4. (B) CD31+ staining was significantly reduced in HUVEC/adMSC implants at days 3 and 7 when treated with clodronate liposomes (grey) compared with PBS liposome (white) controls (mean ± s.e.m., * p < 0.05, ** p < 0.01, n = 3 per group (independent t-test)).
Figure 3. Macrophage depletion impairs vessel formation. Representative histology from HUVEC/adMSC implants treated with clodronate liposomes shows reduced numbers of F4/80⁺ macrophages at day 3, as well as reduced macrophage infiltration into the implant region (top right, middle right), compared to control implants treated with PBS liposomes (top left, middle left). Dashed lines indicate boundary between implant and host tissue; black squares indicate high magnification regions. CD31⁺ endothelial cell staining is reduced following macrophage depletion and vessel formation is impaired. Many vessels with distinct lumens are visible in control implants (bottom left), whereas clodronate treatment (bottom right) limits vessel formation (n = 3, scale bar = 100 µm).
Figure 4. Vessel formation is partially rescued by incomplete macrophage depletion. At day 7, adMSC implants treated with clodronate show reduced F4/80 staining, although the difference between clodronate and PBS treatments is less pronounced than at day 3. Within implants, some regions are fully depleted (top center, middle center) whereas some retain macrophages (top right, middle right). Where depletion is relatively high (center) fewer vessels are seen (bottom center). Where depletion is relative low (right) more vessels are seen (bottom right) although less and smaller vessels than in controls (bottom left; n = 3 per group, scale bar = 100 µm).

3.1.2 Standard inflammatory response to modular implants

HUVEC/adMSC implants were enzymatically digested, then analyzed by flow cytometry to identify populations of inflammatory cells within the tissues. These were quantified and macrophages in particular were examined for signs of activation.

Day 7 implants were significantly smaller than those at days 1 and 3 (Fig. 5A). Similar decreases were seen across all implants and no significant effect was seen due to implant type (Fig. 5B). Accordingly, raw cell counts were normalized to the mass of the implant from which they were collected, and discussion of cell counts refers to these normalized values.
The typical gating strategy for a day 1 implant is shown in Figure 6. Debris, doublets, and non-viable cells were excluded, and remaining viable cells were divided into four target populations: (A) CD45<sup>-</sup> cells, (B) neutrophils (CD45<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>+</sup>), (C) macrophages (CD45<sup>+</sup>CD11b<sup>+</sup>F4/80<sup>+</sup>), and (D) other CD45<sup>+</sup> cells (CD45<sup>+</sup>Ly6G<sup>-</sup>F4/80<sup>-</sup>). Frequencies indicate the percentage of total live cells for the sample depicted; average population percentages for all HUVEC/adMSC implants are given in Table 3. Macrophages, the main population of interest, were sub-gated based on their expression of activation markers MHCII, CD86, and CD206. Frequencies in these sub-gates (in parentheses) indicate the percentage of total macrophages. Macrophages (C) are further sub-divided into two distinct sub-populations in Figure 6: CD11b<sup>low</sup>F4/80<sup>high</sup> (outlined in blue) and CD11b<sup>high</sup>F4/80<sup>med</sup> (outlined in red). The CD11b<sup>high</sup>F4/80<sup>med</sup> population showed signs of elevated – though still relatively low – levels of CD86, MHCII, and CD206 expression (Fig. 6, histograms).

Day 3 gating (Fig. 7) still identified two macrophage sub-populations, although they were generally less distinct and appeared to be merging (Fig. 7C). By this point the CD11b<sup>high</sup>F4/80<sup>med</sup> population had become CD11b<sup>high</sup>F4/80<sup>high</sup>, and in the example shown was expressing higher levels of CD206. Distinct CD11b<sup>high</sup> and CD11b<sup>low</sup> sub-populations were observed in 2/4 day 1 and 3/3 day 3 HUVEC/adMSC implants. Representative examples showing single and dual populations are shown in Figure 8A. Although several samples showed signs of increased activation in the CD11b<sup>high</sup> sub-population (e.g., Figs. 6 & 7, histograms) only CD86 expression

**Figure 5. Implant mass is reduced at day 7.** (A) The average mass of implants recovered at day 7 was significantly reduced compared with days 1 and 3 when implants of all types were pooled (mean ± s.e.m., n = 15-17 per day, * p < 0.05 *** p < 0.001 (one-way ANOVA)). (B) When each type of implant was considered individually, no significant difference in mass due to type of implant was seen (mean ± s.e.m., n = 3-6 per group).
was significantly increased in this group at day 1. At day 3, no significant differences were seen in CD86, MHCII, or CD206 expression (Fig. 8B).

By day 7, only one population of macrophages was distinguishable based on CD11b and F4/80

**Figure 6. Representative gating of a day 1 HUVEC/adMSC implant.** Debris, doublets, and dead cells are excluded and four populations of interest are identified: (A) CD45− cells, (B) CD11b+Ly6G+ neutrophils, (C) CD11b+F4/80+ macrophages, and (D) other CD45+ cells. Macrophages are divided into CD11b−F4/80− (blue) and CD11b+F4/80+ (red) sub-populations. Corresponding red and blue histograms show increased CD86+ and CD206+ cells in the CD11b-F4/80− (red) population. Activation of the entire C gate is assessed by both MHCII vs. CD206 and CD86 vs. MHCII expression. Frequencies indicate percentages of live cells; frequencies in parentheses indicate percentages of gate C (total macrophages).
Table 3. Cell populations present in HUVEC/adMSC implants as a percentage of live cells.

<table>
<thead>
<tr>
<th>Day</th>
<th>(A) CD45-</th>
<th>(B) Neutrophils</th>
<th>(C) Macrophages</th>
<th>(D) Other CD45+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.1 ± 4.8</td>
<td>75.1 ± 13.1</td>
<td>16.8 ± 14.7</td>
<td>4.2 ± 1.2</td>
</tr>
<tr>
<td>3</td>
<td>28.5 ± 39.1</td>
<td>25.7 ± 48.5</td>
<td>31.9 ± 23.4</td>
<td>14.0 ± 13.0</td>
</tr>
<tr>
<td>7</td>
<td>38.4 ± 14.8</td>
<td>1.1 ± 0.7</td>
<td>38.5 ± 19.2</td>
<td>22.1 ± 32.2</td>
</tr>
<tr>
<td>14</td>
<td>29.9 ± 46.3</td>
<td>17.9 ± 36.6</td>
<td>35.6 ± 4.8</td>
<td>16.6 ± 7.1</td>
</tr>
</tbody>
</table>

expression (Fig. 9C), and it expressed substantially higher levels of MHCII and CD206 than those seen at day 3. Expression was bimodal, indicating a sizeable unactivated sub-population (Fig. 9, histograms). This was reflected in MHCII and CD206 co-expression, where unactivated cells (MHCII/CD206) accounted for 26.1 ± 12.2% of total macrophages, approximately the

![Figure 7. Representative gating of a day 3 HUVEC/adMSC implant.](image)

Debris, doublets, and dead cells were excluded (not shown) and four populations of interest are identified: (A) CD45- cells, (B) CD11b+Ly6G+ neutrophils, (C) CD11b+F4/80+ macrophages, and (D) other CD45+ cells. Macrophages are sub-divided into CD11b+low F4/80+high (blue) and one CD11b+high F4/80+high (red) populations. Corresponding red and blue histograms show increased CD86+ and CD206+ cells in the CD11b+high F4/80+high (red) population, although the two groups are less distinct than at day 1. Frequencies indicate percentages of live cells; frequencies in parentheses indicate percentages of gate C (total macrophages).
same amount as the MHCII\(^+\)CD206\(^+\) cells account for \((27.0 \pm 5.6\%; \text{Table 4})\). The proportion of neutrophils was also substantially reduced down to \(1.1 \pm 0.7\%\) of all cells. Surprisingly, at day 14 neutrophil counts were increased in multiple samples, bringing the proportion to \(17.9 \pm 36.6\), although variance was high and in some cases counts remained at lower day 7 levels. Figure 10 depicts a sample with a small neutrophil population at day 14. Gating at day 14 is otherwise similar to that seen at day 7, with increased expression of activation markers for the macrophage population as a whole, in particular MHCII (Fig. 10, histograms).

Considering the absolute numbers of infiltrating cells shows that a large reduction in neutrophil

Figure 8. Distinct macrophage sub-populations at early time points. (A) At day 1 distinct CD11b\(^{\text{low}}\) (blue) and CD11b\(^{\text{high}}\) (red) macrophage populations were seen in 2 of 4 HUVEC/adMSC samples. Representative single population (left) and dual populations (right) are shown. (B) At day 3 dual populations are still present but less distinct, and were seen in 3 of 3 samples. (C) CD11b\(^{\text{high}}\) macrophages show increased expression of CD86 at day 1 (mean ± s.e.m., n = 3-4, ** p < 0.01, (independent t-test)).
numbers between days 1 and 3 was the main cellular event (Fig. 11C). Although decreases in total cell and macrophage numbers (Fig. 11A,B) were seen for the same period, neither was statistically significant, likely due to the small sample size (n = 3). Changes in other CD45+ and CD45- cell counts were negligible. Somewhat contradictory results were seen when analyzing neutrophil and macrophage recruitment by histology. Figure 12 shows the results of Ly6G+ (neutrophil) and F4/80+ (macrophage) staining. The percentage of the implant area positively stained (positivity) for Ly6G decreased over time, in agreement with the flow cytometry results, although the decrease was much more gradual, only becoming significant by day 7. F4/80 positivity was much lower at day 1 than flow cytometry results suggested, and reached a maximum at day 14, which is consistent with earlier analysis of macrophage infiltration in a rat omental pouch implant model[11].

Although gating examples show instances of macrophage activation, Figure 13 shows the overall

Figure 9. Representative gating of a day 7 HUVEC/adMSC implant. Debris, doublets, and dead cells were excluded (not shown) and four populations of interest are identified: (A) CD45- cells, (B) CD11b+Ly6G+ neutrophils, (C) CD11b+F4/80+ macrophages, and (D) other CD45+ cells. Only one population of macrophages was visible with bimodal expression of activation markers MHCII and CD206. Frequencies indicate percentages of live cells; frequencies in parentheses indicate percentages of gate C (total macrophages).
Table 4. Percentage of macrophages from HUVEC/adMSC implants in MHCII vs. CD206 subpopulations

<table>
<thead>
<tr>
<th>Population</th>
<th>Day</th>
<th>MHCII-CD206-</th>
<th>MHCII+CD206-</th>
<th>MHCII-CD206+</th>
<th>MHCII+CD206+</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>87.9 ± 11.9</td>
<td>2.3 ± 2.8</td>
<td>8.8 ± 9.6</td>
<td>1.2 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>61.1 ± 39.1</td>
<td>10.0 ± 7.1</td>
<td>22.9 ± 30.9</td>
<td>6.0 ± 6.2</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>26.1 ± 12.2</td>
<td>29.2 ± 43.1</td>
<td>17.6 ± 26.1</td>
<td>27.0 ± 5.6</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>15.9 ± 13.5</td>
<td>27.2 ± 16.5</td>
<td>6.9 ± 12.1</td>
<td>50.1 ± 30.0</td>
<td></td>
</tr>
</tbody>
</table>

trend is toward significantly increased expression of MHCII and CD206 over time. CD86 was expressed by greater than 40% of cells at day 1 and this proportion did not change significantly by day 14. In contrast, both MHCII and CD206 were expressed by relatively small numbers of cells at day 1 and expression of both increased steadily, significantly so for CD206 by day 7 and highly significantly for both markers by day 14. Looking at MHCII vs. CD206 co-expression (Fig. 14 & Table 4) shows a significant increase in the percentage of double positive cells from

Figure 10. Representative gating of a day 14 HUVEC/adMSC implant. Debris, doublets, and dead cells were excluded (not shown) and four populations of interest are identified: (A) CD45− cells, (B) CD11b+Ly6G+ neutrophils, (C) CD11b+F4/80+ macrophages, and (D) other CD45+ cells. Greater than 60% of macrophages express all three activation markers. Frequencies in the first row indicate percentage of the preceding gate. Frequencies indicate percentages of live cells; frequencies in parentheses indicate percentages of gate C (total macrophages).
Figure 11. Cell populations present in HUVEC/adMSC implants by flow cytometry. (A) Populations as a percentage of all cells. (B) Total cell, (C) neutrophil, (D) macrophage, (E) other CD45+, and (F) CD45- cell counts in HUVEC/adMSC implants normalized to implant mass. Neutrophil counts differed significantly and were reduced from day 3 onward (mean ± s.e.m., n = 3-4 per day, * p < 0.05 (one-way ANOVA)).

Figure 12. Inflammatory cell recruitment by histology. (A) Ly6G+ staining decreases slowly and is only significantly reduced from day 1 levels at day 14. (B) F4/80+ staining increases over time is significantly increased by day 7, and reaches a maximum at day 14 (mean ± s.e.m., n = 3-4, * p < 0.05 (one-way ANOVA)).
Figure 13. Macrophage activation markers increase over time in HUVEC/adMSC implants. The percentage of macrophages expressing CD206 was significantly increased by day 7 and for MHCII expression was significantly increased by day 14. No change in CD86 expression was observed (mean ± s.e.m., n = 3-4, * p < 0.05 *** p < 0.001 (one-way ANOVA)).

Figure 14. MHCII and CD206 co-expression in response to HUVEC/adMSC implants. The majority of macrophages recruited to the implant site at day 1 were unactivated (MHCII/CD206-). This proportion decreased significantly beyond day 3. A significant increase in MHCII+CD206- cells was seen at day 3, and a steady increase in double positive macrophages (MHCII+CD206+) was seen through day 14 (mean ± s.e.m., n = 3-4, * p < 0.05 ** p < 0.01 *** p < 0.001 (one-way ANOVA)).
1.2 ± 1.4\% at day 1 up to 50.1 ± 30.0\% at day 14, and a corresponding decrease in the number of double negative cells from 87.9 ± 11.9\% down to 15.9 ± 13.5\% by day 14. An increase in MHCII\(^+\)CD206\(^-\) cells is also seen, which accounted for 27.2 ± 16.5\% of macrophages by day 14.

Attempts to identify other CD45\(^+\) cells were inconclusive. Screening with anti-CD117 (mast cells) and anti-CD335 (natural killer cells) antibodies was conducted as these were considered plausible candidates. Natural killer cells are present although non-functional in SCID/Bg mice.

Figure 15. Identification of unknown populations by flow cytometry. (A) Other CD45\(^+\) cells do not express mast cell marker CD117 or natural killer cell marker CD335. (B) A portion of CD45\(^-\) cells stain positively for anti-human CD31, and are likely HUVEC. This population diminishes over time and is negligible by day 14 (n = 1 per time point). Dashed grey histograms indicate fluorescence minus one controls.
The unidentified population did not stain positively for either antibody (Fig. 15A). Anti-CD31 screening (Fig. 15B) was also conducted to identify endothelial cells, and a substantial portion of the CD45– population was identified as CD31+ at days 1 and 3, but was no longer detected by day 7 (n = 1). The CD31 antibody used was specific to human cells in order to estimate the number of implant-derived HUVEC rather than host-derived endothelial cells, and should therefore be considered an estimate of HUVEC presence within implants as opposed to an estimate of implant vascularization.

3.2 Immunomodulatory and angiogenic effects of adMSC in modular implants

HUVEC/adMSC implants were compared with HUVEC-only implants to determine whether or not the pro-angiogenic effects of adMSC in modules seen previously were a result of their immunomodulatory effects. Inflammatory cell recruitment and activation was assessed by flow cytometry, and implant vascularization was assessed histologically.

3.2.1 Effects of adMSC on implant vascularization

Contrary to expectations, the presence of adMSC in modular tissues did not significantly affect CD31 positivity compared with HUVEC-only implants at any time point (Fig. 16A). An increase in the total density of CD31+ vessels (vessels / mm²) with defined lumens in representative hotspots was seen at days 1, 7, and 14 when adMSC were present (Fig. 16B). This increase was in capillary-sized vessels (< 9 µm diameter), and when these are considered separately from other vessels a significant increase was seen at all time points (Fig. 16C). No significant differences were observed in the densities of larger vessels, sub-divided into small vessels 9-15 µm in diameter and large vessels greater than 15 µm in diameter (Fig. 16D,E).
Figure 16. HUVEC/adMSC implants increase capillary-sized vessel density. (A) CD31 positivity in histological sections of HUVEC-only (black) and HUVEC/adMSC implants (white). No differences were seen in CD31 positivity due to the presence of adMSC. (B) HUVEC/adMSC implants increased overall CD31+ vessel density at days 1, 7, and 14 compared with HUVEC-only implants. (C) This increase was predominantly due to increased capillary density at all time points. No significant changes in (D) small or (E) large vessels were observed. Densities are the average of three hotspots per implant (mean ± s.e.m., n = 3 per group, * p < 0.05 ** p < 0.01 *** p < 0.001 (two-way ANOVA)).

3.2.2 Effects of adMSC on inflammation

The total cell counts normalized to implant mass recovered from HUVEC-only (black) and HUVEC/adMSC implants (white) are shown in Figure 17A. HUVEC-only implants contained significantly more cells than implants containing adMSC at days 3 and 14. The same data are shown in Figure 17B, sub-divided into cell populations to show relative population sizes, where it is clear that the difference is largely due to a change in macrophages. Inflammatory sub-groups are shown in greater detail in subsequent panels. Neutrophil cell counts were unaffected by adMSC (Fig. 17C), and this was confirmed histologically (Fig. 18A). Significant decreases in both other CD45+ cells (Fig. 17E) and macrophages were seen at day 3, with macrophage counts being significantly reduced at day 14 as well (Fig. 17D). Confirmation with histological analysis
Figure 17. adMSC reduce macrophage infiltration into modular implants. (A) Total cell counts per implant by flow cytometry (normalized to implant mass) from HUVEC-only (black) and HUVEC/adMSC implants (white). HUVEC/adMSC implants contained less cells than HUVEC-only implants at days 3 and 14. (B) The same data are presented as in (A) but subdivided to show the relative proportions of each cell population: F4/80+ macrophages (bottom, solid), Ly6G+ neutrophils (second from bottom, hatched), Other CD45+ cells (second from top, solid), and CD45- cells (top, hatched). (C) Neutrophil counts steadily decreased over time with no differences based on implant type, whereas (D) macrophage counts were reduced in HUVEC/adMSC implants at days 3 and 14. (E) Other CD45+ cells were significantly decreased
in HUVEC/adMSC implants at day 3 (mean ± s.e.m., * p < 0.05 ** p < 0.01 *** p < 0.001, n = 3-6 per group (one-way ANOVA)).

![Graph](image)

**Figure 18.** Histological analysis of inflammatory cell recruitment in HUVEC/adMSC vs. HUVEC-only implants. **(A)** Ly6G⁺ staining was not affected by adMSC presence in modules. **(B)** F4/80⁺ staining was significantly reduced at day 3 in HUVEC/adMSC implants (white) compared with HUVEC-only implants (black) (mean ± s.e.m., n = 3-6 per group, ** p < 0.01 (one-way ANOVA)).

![Graph](image)

**Figure 19.** Macrophage activation in implants is unaffected by adMSC. No significant differences were seen in the percentage of macrophages expressing CD86 (left), MHCII (center), or CD206 (right) when comparing HUVEC/adMSC implants (white) with HUVEC-only (black) controls. For both implant types, MHCII and CD206 expression both increased significantly over time (p < 0.001) (mean ± s.e.m., n = 3-4 per group).

also showed decreased macrophage numbers in HUVEC/adMSC implants at day 3, although the decrease in day 14 numbers was not replicated (Fig. 18B). Although the numbers of macrophages recruited were lower in the presence of adMSC, no impact on their activation states was observed at any time point relative to HUVEC-only macrophages. The percentage of
macrophages expressing CD86 was relatively high even at day 1, as seen previously, and did not change significantly over time (Fig. 19). The expression of both MHCII and CD206 started low and increased significantly over time, as previously (Fig. 13), but no difference was seen between implant types. Considering MHCII and CD206 expression together gives a clearer picture of activation by dividing total macrophages into MHCII−CD206− (black), MHCII single-positive (dark grey), CD206 single-positive (light grey), and MHCII+ CD206+ (white) populations (Fig. 20). The proportion of unactivated macrophages decreased steadily over time in HUVEC-only implants, with a corresponding increase in the proportion of double-positive cells. Both categories of single-positive cells remained relatively stable over the course of the experiment. The same pattern of activation is clear in both HUVEC (Fig. 20A) and HUVEC/adMSC (Fig. 20B) implants.

Figure 20. Macrophages express increasingly mixed activation over time. Similar patterns of macrophage activation were observed in (A) HUVEC-only and (B) HUVEC/adMSC implants. In both cases the percentage of unactivated macrophages (MHCII−CD206−) decreased steadily over the course of the experiment (p < 0.001) while the percentage of double-positive macrophages (MHCII+CD206+) steadily increased (p < 0.001). MHCII single-positive percentages (MHCII+CD206−) increased significantly between days 1 and 3 (p < 0.05) then remained at moderate levels. No change was observed in the proportion of CD206 single-positive (MHCII−CD206+) macrophages (mean ± s.e.m., n = 3-6 per group, (one-way ANOVA)).
3.3 Effects of activated macrophages in modular implants

Bone marrow-derived macrophages were activated \textit{in vitro} to produce distinct phenotypes with pro- or anti-inflammatory characteristics, and these were incorporated into modular tissues in order to drive construct vascularization by promoting an anti-inflammatory, pro-angiogenic cellular niche.

3.3.1 In vitro activation conditions

To confirm the activation treatments for modules containing BMDM were stable during culture and not altered by the implant digestion procedure, activated modules were digested and analyzed by flow cytometry for CD86, MHCII, and CD206 expression. BMDM modules with and without HUVEC were also compared to assess the impact of HUVEC co-culture on BMDM activation after 72 hours of \textit{in vitro} culture. This timeframe was selected to match the HUVEC/adMSC culture protocol, as modules seeded with HUVEC were cultured for 72 hours to allow EC proliferation and the formation of a confluent monolayer. Digested modules were compared with similarly activated BMDM (not in modules) cultured on non-tissue culture polystyrene plates (non-TCPS). For culture on non-TCPS and in modules without HUVEC, standard conditions described in section 2.3 were used. For HUVEC co-culture, BMDM medium and HUVEC medium were mixed in a 1:1 ratio, although the total concentrations of cytokines used for activation were kept constant. BMDM were activated with IFNγ, IL-4, or left unactivated (M(IFNγ), M(IL-4), and M(0), respectively).

Figure 21 illustrates the differences in BMDM activation when cultured on non-TCPS, in collagen modules alone (modules), and in collagen modules coated with HUVEC (HUVEC). Under standard conditions (M[0]) on non-TCPS, typical \textit{in vitro} activation was seen\cite{39}. CD86 expression was moderate when unactivated, high in response to IFNγ, and low in response to IL-4 (Fig. 21A). MHCII expression was low when unactivated, high in response to IFNγ, and low in response to IL-4 (Fig. 21B). As expected, CD206 expression went in the opposite direction, and was moderate when unactivated, low in response to IFNγ, and high in response to IL-4 (Fig. 21C).

CD86 and MHCII are considered markers of classical activation; CD206 is considered a marker of alternative activation. Under standard conditions (M[0]), CD86 (Fig. 21A) and MHCII
Figure 21. Module culture conditions alter macrophage activation. BMDM were cultured on non-tissue culture polystyrene plates (non-TCPS), alone in collagen modules (Modules), or in HUVEC-coated collagen modules (HUVEC). BMDM were activated with IFNγ, IL-4, or left unactivated (M[0]). (A) The percentage of BMDM expressing CD86 was reduced by culture in modules when BMDM were activated with IFNγ or unactivated compared with non-TCPS culture. Co-culture with HUVEC reduced CD86 expression in IFNγ-treated BMDM but increased its expression in IL-4-treated BMDM. (B) MHCII expression was reduced in BMDM cultured in collagen modules when activated with IL-4 or left unactivated compared with non-TCPS culture. HUVEC co-culture significantly reduced expression of CD86 in IFNγ-treated BMDM compared with non-TCPS and collagen culture, but no differences were seen when BMDM were treated with IL-4 or untreated. (C) CD206 expression was unaffected by collagen
culture regardless of activation treatment. HUVEC co-culture significantly increased CD206 expression in unactivated and IFNγ-activated cells, but decreased its expression in IL-4-treated cells (mean ± s.e.m., n = 3, * p < 0.05 ** p < 0.01 *** p < 0.001 (one-way ANOVA)).

expression (Fig. 21B) were significantly reduced when BMDM were cultured in modules both with and without HUVEC. Even when activated with IFNγ, culture in modules significantly reduced CD86 expression, although it did not affect MHCII expression. When modules were seeded with HUVEC, large, significant decreases in both CD86 and MHCII expression were seen, suggesting an anti-inflammatory effect. When activated with IL-4, mixed effects were seen on inflammatory markers. Collagen modules did not affect CD86 expression (Fig. 21A), but significantly reduced MHCII expression (Fig. 21B). When HUVEC were added, no effect was seen on MHCII (Fig. 21B), but CD86 expression was significantly increased (Fig. 21A).

CD206 expression was not affected by culture in modules. Regardless of activation, no significant differences were seen compared with non-TCPS culture (Fig. 21C). Co-culture with HUVEC increased CD206 expression significantly in M(0) and M(IFNγ) treatments, although, somewhat surprisingly, it produced a small but significant decrease in CD206 expression in the presence of IL-4, which on its own produces high CD206 expression. This reduced expression, combined with the increased expression levels in M(0) modules co-cultured with HUVEC, resulted in no significant difference between CD206 expression levels of cells from M(0) and M(IL-4) treatments in the presence of HUVEC. HUVEC co-culture therefore produces an alternative activation in otherwise unactivated BMDM. CD206 expression levels in BMDM co-cultured with HUVEC and activated with IFNγ, although elevated, were significantly lower than those with either M(0) or M(IL-4) activation.

The decrease in CD86 expression due culture in collagen modules even when cells are exposed to IFNγ makes it less suitable as a marker of classical activation in this system. MHCII was not decreased in this manner: 46.6% ± 28.4% of macrophages expressed CD86, whereas MHCII was expressed by 76.1% ± 9.9%. In addition, 86.4% ± 6.5% of CD86+ cells also expressed MHCII, whereas only 52.7% ± 28.9% of MHCII+ cells also expressed CD86, indicating MHCII is the more reliable marker of IFNγ activation in this system (Fig. 22).
Figure 22. MHCII is a more reliable marker of classical activation than CD86. Representative BMDM modules activated with IFNγ express both (A) CD86 and (B) MHCII as assessed by flow cytometry following enzymatic digestion. MHCII provides better separation from negative cells and identifies a larger percentage of cells than does CD86. CD86⁺ cells are generally MHCII⁺, whereas gating on MHCII⁺ cells also identifies many cells that are CD86⁻ and would otherwise be missed. Dashed histogram is fluorescence minus one control.

3.3.2 Effect of activated BMDM on vascularization

Modules containing activated BMDM were incorporated into in vivo implants with the aim of promoting the rate and extent of vascularization, as well as the maturity and stability of the nascent vessels. As macrophages have been shown to be necessary for effective implant vascularization, but also a potentially rate-limiting factor when inflammation is uncontrolled, it was undertaken to directly control the initial macrophage inflammatory response by delivering exogenously activated macrophages. Control implants were the HUVEC/adMSC implants described previously, as this modular configuration had produced the most robust vascularization seen thus far in modular implants. Macrophage implants therefore contained the standard complement of 2.0 x 10⁶ adMSC but were supplemented with 1.0 x 10⁶ BMDM.

CD31 positivity was slightly increased in BMDM implants compared with HUVEC/adMSC implants (a finding which was not seen comparing HUVEC/adMSC with HUVEC-only
Figure 23. Macrophage modules promote endothelial cell presence in implants. (A) CD31+ staining is significantly increased at day 7 when implanted modules contain macrophages (grey) compared with HUVEC/adMSC (white) controls (mean ± s.e.m., n = 3-9 per group, * p < 0.05 (two-way ANOVA)). The increase in (B) CD31 positivity is not statistically significant when each macrophage implant type (HUVEC/adMSC, M(0), M(IFNγ), M(IL-4)) is considered individually, and (C) similar results were seen with UEA-1 positivity (mean ± s.e.m., n = 3-4 per group).
In terms of vessel formation, there were no differences observed between M(0), M(IFNγ), and M(IL-4) implants, but all three led to the formation of a more persistent, stable vasculature compared with HUVEC/adMSC implants (Fig. 24). Vessel densities in implants of all types were relatively low at days 1 and 3 but increased significantly in all cases by day 7. This increase did not persist for HUVEC/adMSC implants, however, as day 14 densities had returned to day 3 levels. In contrast, vessel densities in BMDM implants at day 14 persisted at day 7 levels, avoiding the vessel regression seen here in HUVEC/adMSC controls. This was true for overall vessels (Fig. 24A), capillary-sized vessels (Fig. 24B), and small vessels (Fig. 24C), but not for large vessels, where relatively low densities were observed in all cases. There was no impact on average vessel diameter, which was similar across all implants (Fig. 25). These results differed from an earlier study using similar implants which saw consistent decreases in the number of

![Figure 24](image_url)

**Figure 24.** Macrophages stabilize implant vasculature and prevent vessel regression. (A) Total vessel density remains higher in BMDM implants of all types compared with HUVEC/adMSC controls at day 14. (B-D) This difference was seen in capillaries (B) and small vessels (C) but not in large vessels (D). Densities are the average of three hotspots per implant (mean ± s.e.m., n = 3 per group, *** p < 0.001 (A,B two-way ANOVA; C one-way ANOVA)).
UEA-1⁺ vessels through day 14, and a consistent increase in the diameter of the remaining vessels over the same period[5]. SMA positivity was increased at day 7 in HUVEC/adMSC implants, as well as in all BMDB implants (Fig. 26). Interestingly, although vessels did not persist in HUVEC/adMSC implants at day 14, SMA⁺ staining did, and was not significantly different than that expressed in BMDB implants. Representative histological sections of day 7 and day 14 vessels can be seen in Figure 27.

Figure 25. Vessel size is unaffected by macrophage activation. No difference in vessel size was seen between implant types (HUVEC/adMSC white, M(0) dark grey, M(IFNγ) light grey, M(IL-4) hatched; mean ± s.e.m., n = 3 per group).

Figure 26. Smooth muscle actin staining is increased at days 7 and 14 in all implants containing adMSC. Although SMA expression is increased at later time points, no significant difference was seen between implant types (mean ± s.e.m., n = 3-4 per group).
Figure 27. Representative histological staining of vessels at days 7 and 14. At day 7 HUVEC/adMSC implants and M(0) implants are well vascularized. Most vessels are CD31+ and UEA-1+, and have substantial pericyte coverage (SMA+). At day 14, only the M(0) implant is well vascularized. SMA staining shows pericytes are present but not associated with the few remaining vessels. F4/80 staining shows macrophage throughout tissues, although not directly associated with vessel structures.

3.3.3 Effect of activated BMDM on inflammation

The expected impact of BMDM on cell recruitment into implanted tissues – either by promoting recruitment of pro-angiogenic cells or inhibiting recruitment of pro-inflammatory cells – was never seen. Contrary to expectations, the addition of BMDM of all activation states to modular implants failed to influence the recruitment of host cells into the implant tissue. Explanted tissues subjected to enzymatic digestion and analyzed by flow cytometry at days 1, 3, 7, and 14 showed no significant differences in the number of total cells recovered (Fig. 28A-D). Normalized macrophage (Fig. 28E), neutrophil (Fig. 28F), and other CD45+ cell counts (not shown) were similarly unaffected. A small decrease in the number of CD45− cells was seen at day 1 in M(0)
Figure 28. Activated macrophages in implants do not alter host inflammatory cell recruitment. (A-D) Total cells per implant normalized to implant mass measured by flow cytometry from HUVEC/adMSC, M(0), M(IFNγ), and M(IL-4) implants at (A) day 1, (B) day 3, (C) day 7, and (D) day 14. Within bars, total cells are sub-divided into macrophages (black), neutrophils (dark grey), other CD45+ cells (light grey), and CD45- cells (white). No differences in total cell recruitment were observed due to implant type (mean ± s.e.m., n = 3-4 per group). (E) Macrophage and (F) neutrophil counts normalized to implant mass from HUVEC/adMSC (white), M(0) (dark grey), M(IFNγ) (light grey), and M(IL-4) implants (hatched). No differences in macrophage or neutrophil numbers due to implant type were observed (mean ± s.e.m., n = 3-4
per group). (G) At day 1, M(0) implants had reduced numbers of CD45<sup>-</sup> cells compared with adMSC controls, and M(IFNγ) implants had reduced numbers of CD45<sup>-</sup> cells compared with both adMSC and M(IL-4) implants (mean ± s.e.m., * p < 0.05, ** p < 0.01, n = 3-4 per group (two-way ANOVA)).

and M(IFNy) implants, but this was an extremely small number of cells and is unlikely to be meaningful (Fig. 28G).

Similarly, no changes in the activation of recruited macrophages were seen due to implant type. The general increase in macrophage activation described previously when comparing HUVEC and HUVEC/adMSC implants (increased MHCII and CD206 expression and unchanged CD86 expression [Figs. 13,19]) was in evidence again (Fig. 29), and subdividing these cells into MHCII vs. CD206 expression again shows a large decrease in the percentage of unactivated cells over time, particularly between days 3 and day 7, and a steady increase in the percentage of double-positive cells which peaks at day 14 in all implant types (Fig. 30).

![Figure 29. Delivery of activated BMDM does not affect recruited macrophage activation.](image)

In implants of all types MHCII and CD206 expression increased significantly with time (p < 0.001, one-way ANOVA) whereas CD86 expression did not change. No effect of implant type on the expression of any activation marker was seen (mean ± s.e.m., n = 3-4 per group).
Figure 30. MHCII vs. CD206 activation subsets are unaffected by BMDM. For all implant types, most macrophages are activated by day 14, and the majority have a mixed phenotype. The percentage of MHCII-CD206- macrophages decreased steadily over time in all cases while the percentage of double-positive macrophages (MHCII+CD206+) steadily increased (p < 0.001). MHCII single-positive percentages (MHCII+CD206-) increased significantly between days 1 and 3 (p < 0.05; mean ± s.e.m., n = 3-4 per group, (one-way ANOVA)).

4 Discussion

4.1 The inflammatory response to modular implants

Inflammation is important to the vascularization process, as the large number of macrophages recruited mediate much of the process of angiogenesis and remodelling that follows it. Macrophage phenotype, shown to be a reliable predictor of constructive remodelling[56], can be directly affected by the composition of biomaterials[61], and attempts to design biomaterials to elicit macrophage phenotypes favourable to angiogenesis and remodeling have shown success[57,62,63].

Here, eliminating the inflammatory response through macrophage depletion strongly impaired vessel formation, and also limited EC survival (Figs. 2-4). Other tissue engineering studies have shown similar results[53]. More complex experiments in wound healing models showed that depletion at specific times over the course of healing, and therefore the depletion of different subsets of macrophages, had variable effects on wound closure[50]. In particular, the depletion of an early inflammatory subset high in VEGF production critically impaired vascular sprouting[51]. In a contrary example, HUVEC-only implants in the omental pouch of nude rats survived poorly beyond day 3, but when treated with clodronate survival was extended to day 7, suggesting macrophages inhibited HUVEC survival, although this was a xenogeneic implant.
model[29]. Ideal inflammatory conditions, therefore, fall somewhere between these two extremes, with macrophages being a necessary part of the engraftment process, provided their numbers and activation are appropriate.

The standard inflammatory response to HUVEC/adMSC implants, as measured by flow cytometry, was characterized by high neutrophil infiltration at day 1, followed by a sharp decrease at day 3 and subsequent negligible levels, which is typical of the normal inflammatory response[20]. At day 1 neutrophils outnumbered macrophages by about 4:1. By day 3 the ratio was approximately even, and by day 7 the virtually all CD45+ cells were macrophages or “other” CD45+ cells. Like neutrophils, macrophages were also at their highest levels on day 1 and were lower on all subsequent days, although not significantly so.

The discrepancy between flow cytometric and histological analysis of these numbers was unexpected. Ly6G+ staining (neutrophils) decreased steadily and ultimately reached very low levels by histology (Fig 12A), but took much longer to do so than when measured by flow cytometry (Fig. 11C). This may be explained by apoptotic neutrophils still present in the tissue at days 3 and 7 staining positively, whereas these cells would be excluded by viable cell gating in flow cytometry. The discrepancy in F4/80+ staining (macrophages) is more difficult to account for, although it could potentially be due to the use of different clones for anti-F4/80 staining (BM8 was used for flow cytometry and CI:A3-1 was used for histology). Some differences between the two have been reported in flow cytometry applications although not nearly to the degree seen here[64]. It was noted by flow cytometry that the intensity of F4/80+ expression in macrophages increased over time. It may be the case that the CI:A3-1 clone has been optimized for use on F4/80bright cells and fails to detect early F4/80low/med macrophages, causing staining to increase over time as a reflection of increased intensity of expression, as opposed to increases in cell numbers. As was the case for neutrophils, the accumulation of dead cells may play a role, and the flow cytometry results are therefore considered more representative.

The distinct sub-populations of CD11bhighF4/80med and CD11blowF4/80high macrophages observed at day 1 (Fig. 6) likely represent monocyte-derived and tissue-resident macrophages, respectively. Tissue resident macrophages proliferate locally in tissues and have been shown to be CD11blowF4/80high, whereas bone-marrow derived macrophages recruited from circulating monocytes have been shown to be CD11bhighF4/80low[35,36]. The recruitment of local tissue-
resident macrophages from areas adjacent to the implant, which account for a large percentage of cells in normal tissues, explains the presence of this population at early time points[65]. In response to module implants, both populations become activated and express increasing levels of CD11b and F4/80 over time, as well as MHCII and CD206 (Fig. 13), becoming indistinguishable at day 7 onward (Figs 9,10). Further, the CD11b\textsuperscript{high}F4/80\textsuperscript{med} population expressed significantly higher levels of CD86 – a marker of classical inflammation – at day 1, consistent with this interpretation (Fig. 8B).

By day 14, in all implant types examined, macrophages eventually displayed a mix of phenotypes with variable MHCII and CD206 expression. According to the “M1”/”M2” interpretation of macrophage activation, CD86 is a marker of “M1” and “M2b” activation, MHCII is a marker of “M2a” and “M2b” activation, and CD206 is a marker of “M2a” and “M2c” activation[39]. Because “M2b” activation is induced through immune complexes and TLR, it can be ignored in this model. Applied to these data, the average breakdown in HUVEC/adMSC modules at day 14 would indicate macrophages were 27.2% “M1” (MHCII$^+$CD206$^+$), 50.1% “M2a” (MHCII$^+$CD206$^+$), 15.9% “M2c” (MHCII$^-$CD206$^+$), and 15.9% unactivated (MHCII$^-$CD206$^-$) (Table 4). However, swapping MHCII for the equally valid “M1” activation marker CD86 gives the following, drastically different, result: 11.4 % “M1” (CD86$^+$CD206$^+$), 17.2% “M2a/c” (CD86 CD206$^+$), 44.1% “M2b” (CD86$^+$CD206$^+$), and “15.5%” unactivated (CD86 CD206$^-$). This illustrates the potential for misinterpretation when using this system, which is useful in vitro, but it should not necessarily be assumed to be applicable in vivo on the basis of selected surface marker expression – especially as a means of explaining cell function – without more conclusive gene expression analysis.

4.2 Impact of adMSC on vascularization and inflammation in modular implants

Vessel density was dramatically increased with HUVEC/adMSC implants compared with HUVEC-only implants at day 7, which was not seen in previous work. Previously in the SCID/Bg mouse, HUVEC/adMSC implants were shown to result in consistent vessel densities of approximately 30-50/mm$^2$ in hotspots at days 3, 7, and 14[6]. Here, densities were consistently in the range of 20-25/mm$^2$ at days 3 and 14, but increased to approximately 80/mm$^2$ at day 7. This may be related to slightly altered module parameters. Modules used previously all contained
adMSC and were coated with HUVEC. Here, only half the modules used were of this type. The other half were collagen-only modules to serve as a control for BMDM implants. Total EC presence, as measured by CD31 positivity based on pixel counts from digitized histological sections, was unchanged in HUVEC/adMSC implants compared with HUVEC-only implants, as shown in Figure 16A. Previous studies using HUVEC-only implants showed similar trends, although in those cases UEA-1+ as opposed to CD31+ cells were counted using different methodology from that described here[12,29]. However, UEA-1 and CD31 expression were shown here to be roughly comparable in HUVEC/adMSC implants (Fig. 23B,C).

The increase in vessel formation due to adMSC appears to be correlate with limited macrophage infiltration at day 3. In the absence of adMSC, the classic neutrophil response immediately following implantation is seen followed by a sharp decrease after 72 hours (Fig. 17C). The classic macrophage response follows, increasing sharply at day 3 as the neutrophils are resolved. However, this large influx of macrophages does not occur with adMSC. Presumably, this prevents HUVEC from being damaged by inflammatory macrophages and provides an environment more conducive to angiogenesis, the effects of which can be seen in increased vessel density at day 7.

Like macrophages, other CD45+ cells – so far unidentified – were decreased in HUVEC/adMSC implants at day 3 compared with day 1 (Fig. 17E). This decrease suggests these cells are monocytes. Circulating monocytes are CD11b+F4/80low/neg, and expression is only upregulated following extravasation into the tissues. The cell counts of macrophages (Fig. 11D) and other CD45+ cells (Fig. 11E) show remarkably similar trends at all time points in HUVEC/adMSC implants, suggestive of the fact that they are actually two samplings of the same population. Dendritic cells would likely express CD11c and share many surface markers with macrophages[66]. Previous experiments (not shown) found little to no CD11c+ staining. Negative staining for CD117 and CD335 excluded mast cells and natural killer cells (Fig. 15), and SCID/Bg mice lack B cells and T cells, which leaves few candidates besides monocytes.

The activation of infiltrating macrophages was unchanged between HUVEC/adMSC implants and HUVEC-only implants. This was unexpected, as there is much evidence that MSC affect macrophage activation[22,67], and this was hypothesized to be a major mechanism by which MSC promote vessel formation in this tissue engineering model. Earlier studies showed changes
in macrophage activation in module implants in response to MSC. In a rat omental pouch implant, MSC induced the expression of CD163 – a marker of alternative activation – in macrophages within the implant region. In the absence of MSC, no CD163+ staining was seen in the implant region, although CD163+ cells could be seen just outside the implant region. CD68+ staining (pan-macrophage) was seen in both cases. Whether CD163+ was being induced in macrophages already present in the implant or different subset was being recruited was unclear.

It may be the case in this system that the HUVECs coating the modules themselves are sufficient to control macrophage activation, and adMSC promote angiogenesis in this model by other means. As shown in Figure 21, BMDM modules co-cultured with HUVECs showed low expression levels of inflammatory markers CD86 and MHCII and high levels of CD206 expression, consistent with alternative activation. One study showed that co-culturing immortalized EC with myeloid progenitors could form stable EC-macrophage colonies, which led macrophages to adopt an “M2”-like phenotype, expressing CD206 and high levels of VEGF, but only when in direct contact with EC. Transplanting the EC-educated macrophages into a mouse tumour model promoted tumour vascularization[59]. This suggests EC alone may be able polarize macrophages in modular implants, where infiltrating macrophages and HUVEC are in close contact, even in the absence of MSC.

4.3 Impact of macrophage activation on vascularization and inflammation in modular implants

Implants containing BMDM did not increase vessel densities (Fig. 24) or produce larger vessels (Fig. 25) than HUVEC/adMSC implants. They did, however, prevent the vessel regression that was observed in HUVEC/adMSC implants between days 7 and 14. In that case, regression reduced vessel densities back to day 3 levels. Contrary to expectations, the activation state of BMDM did not influence this result; all three activations were equivalent. It had been anticipated that M(IL-4) implants would be the most angiogenic. Evidence for this had been seen by Jetten et al.[58], who showed increases in CD31+ staining in vivo at day 14 in response to M(IL-4) and M(IL-10) activation, but not in response to M(IFNγ) or M(0) activation. However, no effect on CD31+ staining was seen here due to the activation of BMDM implants. An increase in CD31+ staining was seen at day 7, but only when explicitly ignoring activation and considering all BMDM implants collectively. Individually, changes compared with HUVEC/adMSC implants
were not seen at any time point. One of their observations was confirmed here: in both studies, the number of inflammatory cells recruited to the implant was not affected by the presence, or the activation, of BMDM.

Notable differences between these studies include the source of macrophages and matrix employed. Their BMDM were derived from immunocompetent C57BL/6 mice as opposed to immunocompromised SCID/Bg, and their implants used FGF2-supplemented Matrigel rather than collagen.

Activation effects related to the encapsulation of BMDM in collagen modules were observed (Fig. 21). Modules containing unactivated BMDM without HUVEC showed reduced levels of CD86 and MHCII expression, and moderate levels of CD206 expression, suggestive of mild alternative activation. CD86 is a widely used inflammatory marker, but here its expression was significantly reduced when BMDM were in collagen modules – even in the presence of IFNγ – to levels equivalent with those of unactivated BMDM cultured on non-TCPS (Fig. 21A). Compared to CD86, MHCII expression remained high following IFNγ treatment and was considered to indicate classical, inflammatory activation. However, in some contexts MHCII has been associated with alternative activations[43]. There is also evidence that monocyte-to-macrophage differentiation mediated by CSF1 can itself upregulate many genes associated with alternative activation[68].

Macrophage-ECM interactions affecting cell shape may be the principal explanation for these observations. An important study showed that cell shape was an important regulator of macrophage activation independent of cytokine activation. Recognizing that BMDM activated with IL-4 in vitro adopt elongated shapes, unactivated BMDM were forced to adopt elongated shapes using culture substrates micropatterned with narrow channels. Elongation induced alternative activation in the absence of cytokines, as identified by increased CD206 and Arg1 expression, although not to the same extent as with cytokine activation[69]. It was also shown that this elongation-induced activation was mediated by cytoskeletal contraction, such as that which occurs when macrophages migrate through the ECM. Inhibiting actin polymerization prevented elongation from upregulating Arg1. This suggests interaction with the ECM itself – or an ECM mimic such as collagen modules – may promote alternative activation. Critically, elongation-induced alternative activation made macrophages resistant to the effects of
subsequent IFNγ treatment, suggesting collagen-encapsulated BMDM may be resistant as well. This implies that the M(0) and M(IFNγ) treatments used here may have in fact been closer to M(IL-4) treatments than they appeared, as BMDM were not activated with cytokines until 24 hours after they had been encapsulated in collagen. This would have allowed ample time for all cells to develop elongation-induced alternative activation and rendering them resistant to subsequent attempts to polarize them with cytokines, potentially resulting in one truly M(IL-4) group, and two expressing mild alternative activation (i.e., upregulated Arg1), despite lower CD206 expression.

This mechanism of activation may explain why all three BMDM implant types showed similar results and prevented vessel regression, in spite of constituting a relatively small portion of total macrophages and failing to alter the activation state of infiltrating macrophages. Alternatively activated macrophages produce high levels of TGFβ[68], which is essential for maintaining EC-pericyte contact[16]. This small, protected core of alternatively activated macrophages, producing TGFβ, may promote pericyte coverage in the BMDM implants. As expected, SMA staining, indicative of adMSC differentiation into pericytes, is high in HUVEC/adMSC implants as well as in BMDM implants at day 14, as all contain adMSC (Fig. 26). Corresponding vessel densities remain high in BMDM implants; however, significant vessel regression was seen in HUVEC/adMSC implants, suggesting pericytes are not effectively maintaining vessels, perhaps due to receiving less TGFβ than those in BMDM implants.

5 Conclusions

The presence of macrophages was shown to be critical for the vascularization of modular tissues, and they were recruited to implants in large numbers, particularly in the absence of adMSC. The standard pattern of inflammation was observed when adMSC were not present in modules, with high neutrophil infiltration at day one, which resolved by day three, alongside a large macrophage population which persisted through day fourteen and became increasingly CD206+.

When present, adMSC significantly reduced macrophage recruitment at day 3, and kept it low through day 14. This diminished inflammatory response had the effect of increasing vessel densities by day 7, despite having no effect on macrophage activation, which appeared to be dictated by other elements of the modular implant niche.
Bone marrow-derived macrophages embedded in modules and co-implanted with HUVEC/adMSC modules caused blood vessels formed in the presence of adMSC to persist to day 14; in their absence, vessel regression occurred by day 14. This was not a function of activation, as M(0), M(IFNγ), and M(IL-4) implants all produced similar results. Otherwise, no effect of BMDM was seen on vessel formation, inflammatory cell recruitment, or activation of host macrophages.

It is proposed that activated macrophages embedded in collagen modules promote vessel maturation and persistence in the following manner: embedding bone marrow derived-macrophages in collagen modules induces and preserves an alternative activation state characterized by high expression of Arg1 and CD206, and therefore the production of PDGF-B and TGFβ, which drive pericyte recruitment and adhesion to vessels. Initial implantation elicits the standard response of pro-inflammatory macrophages from the host; however, through co-implantation of HUVEC/adMSC modules, adMSC reduce the scope of the inflammatory response without eliminating it, so that early inflammatory macrophages are still recruited to implants in reduced numbers where they produce high levels of VEGF, and undergo the standard transition to alternative activation as inflammation is resolved and remodelling occurs. Increased vessel formation is mediated by adMSC-derived pro-angiogenic factors, and pericyte adhesion to vessels is enhanced by the production of TGFβ by embedded bone marrow-derived macrophages, preventing vessel regression.
6 References


## 7 Appendices

### 7.1 Appendix A - Function of important cytokines, surface markers, and cell products

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Full Name</th>
<th>Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arg1</td>
<td>arginase 1</td>
<td>Enzyme upregulated in alternatively activated macrophages which converts arginine to ornithine</td>
</tr>
<tr>
<td>CCR2</td>
<td>chemokine (C-C motif) receptor 2</td>
<td>Receptor for CCL2 (monocyte chemoattractant protein-1) responsible for monocyte chemotaxis</td>
</tr>
<tr>
<td>CCR7</td>
<td>chemokine (C-C motif) receptor 7</td>
<td>Receptor for CCL19 and CCL21, involved in monocyte/macrophage trafficking, commonly used as a marker of classical macrophage activation</td>
</tr>
<tr>
<td>CD11b</td>
<td>cluster of differentiation 11b</td>
<td>Integrin expressed by all cells of myeloid lineage</td>
</tr>
<tr>
<td>CD31</td>
<td>cluster of differentiation 31 (platelet/endothelial cell adhesion molecule 1)</td>
<td>Endothelial cell marker and component of EC intercellular junctions</td>
</tr>
<tr>
<td>CD45</td>
<td>cluster of differentiation 45 (protein tyrosine phosphatase receptor type c)</td>
<td>Surface marker expressed by all leukocytes</td>
</tr>
<tr>
<td>CD68</td>
<td>cluster of differentiation 68</td>
<td>Scavenger receptor expressed by all macrophages</td>
</tr>
<tr>
<td>CD86</td>
<td>cluster of differentiation 86</td>
<td>Regulates T cell activation through CD28 and cytotoxic T-lymphocyte-associated protein 4, marker of classically activated macrophages</td>
</tr>
<tr>
<td>CD117</td>
<td>cluster of differentiation 117 (mast/stem cell growth factor receptor)</td>
<td>Marker of mast cells, hematopoietic stem cells, melanocytes, and interstitial cells of Cajal</td>
</tr>
<tr>
<td>CD163</td>
<td>cluster of differentiation 163</td>
<td>Hemoglobin scavenger receptor, used as a marker of alternative macrophage activation</td>
</tr>
<tr>
<td>CD206</td>
<td>cluster of differentiation 206 (mannose receptor C type 1)</td>
<td>Scavenger receptor for mannose and other glycoproteins, used as a marker of alternative macrophage activation</td>
</tr>
<tr>
<td>CD335</td>
<td>cluster of differentiation 335</td>
<td>Activating receptor specific to natural killer cells</td>
</tr>
<tr>
<td>F4/80</td>
<td>adhesion G protein-coupled receptor E1</td>
<td>Surface marker specific to macrophages and eosinophils, involved in generation of antigen-specific regulatory T cells</td>
</tr>
<tr>
<td>IFNγ</td>
<td>interferon gamma</td>
<td>Primary effector cytokine of classical macrophage activation produced by T cells and NK cells</td>
</tr>
<tr>
<td>IL-4</td>
<td>interleukin-4</td>
<td>Primary effector cytokine of alternative macrophage activation. Upregulates CD206, Arg1, and inhibits Stat1 and inflammatory cytokines through Stat6 signalling</td>
</tr>
<tr>
<td>IL-10</td>
<td>interleukin-10</td>
<td>Anti-inflammatory cytokine, promotes alternative macrophage activation, downregulates Th1 cytokines, MHCII, and inhibits NF-κB and Stat1 signalling</td>
</tr>
<tr>
<td>IL-12</td>
<td>interleukin-12</td>
<td>Inflammatory cytokine, promotes T cell and NK cell activation, marker of classical activation</td>
</tr>
<tr>
<td>IL-13</td>
<td>interleukin-13</td>
<td>Anti-inflammatory cytokine, promotes alternative macrophage activation, downregulates Th1 cytokines and inhibits Stat1 signalling</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
<td>Function</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
<td>----------</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
<td>Enzyme upregulated in classically activated macrophages which converts arginine to nitric oxide</td>
</tr>
<tr>
<td>Ly6C</td>
<td>lymphocyte antigen 6 complex, locus C</td>
<td>Marker of classical inflammatory monocytes (epitope of Gr-1 with Ly6G) and functions in inflammatory cell migration</td>
</tr>
<tr>
<td>Ly6G</td>
<td>lymphocyte antigen 6 complex, locus G</td>
<td>Neutrophil-specific marker (epitope of Gr-1 with Ly6C)</td>
</tr>
<tr>
<td>MHCII</td>
<td>major histocompatibility complex 2</td>
<td>Surface molecule on antigen presenting cells used for presentation of phagocytosed antigens to T cells</td>
</tr>
<tr>
<td>MMP-9</td>
<td>matrix metalloproteinase-9</td>
<td>Enzyme important to degradation of extracellular matrix for angiogenesis and tissue remodelling</td>
</tr>
<tr>
<td>SMA</td>
<td>α-smooth muscle actin</td>
<td>Major constituent of the contractile apparatus of smooth muscle, indicative of vessel maturity and stability</td>
</tr>
<tr>
<td>UEA-1</td>
<td>Ulex europaeus agglutinin 1</td>
<td>Lectin specific to human endothelial cells</td>
</tr>
</tbody>
</table>