Inducing and Characterizing M2c Macrophages at a Non-Degradable Implant In Vivo

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Inducing and Characterizing M2c Macrophages at a Non-Degradable Implant In Vivo
Inducing and Characterizing M2c Macrophages at a Non-Degradable Implant In Vivo

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy in Cell and Molecular Biology

By

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Abstract

The implantation of biomaterials in the body leads to a reaction known as the foreign body response (FBR) which leads to the eventual encapsulation of the biomaterial. This encapsulation proves to be detrimental to certain biomaterials, such as implanted glucose sensors, which rely on interacting with the extracellular space for proper function. Altering the FBR has become of interest in an effort to increase the longevity and integration of biomaterials. One strategy for altering the FBR is by targeting the macrophage, shown to play an important role in the FBR. In this work, the microdialysis sampling technique was used to deliver modulators to an implant site in an effort to alter the predominant macrophage activation state present. Simultaneously, analytes were collected from the same implant site to further characterize the biochemical signals present during the FBR as well as in a predominantly M2c environment. With the use of 100kDa molecular weight cut-off microdialysis membranes, it was first necessary to find a suitable perfusion fluid to use for this work which did not cause additional inflammation. An acceptable perfusion fluid was found to consist of Ringer’s + 4% w/v Dextran-500 + 0.1% w/v BSA. Further, it was found that Dexamethasone-21-phosphate (Dex), when delivered immediately through the microdialysis probe, resulted in a significant increase in M2c macrophages found in the tissue surrounding the treatment. Immediate Dex administration also resulted in the significant down-regulation of CCL2 and IL-6 transcripts as well as significantly decreased CCL2 concentrations found in treatment dialysates. The delayed infusion of Dex was also investigated to determine if a different/more optimal effect was seen as compared to an immediate administration. The delayed administration of Dex resulted in a significant increase in the number of M2c macrophages in the tissue surrounding the treatment probe. Interestingly, at the transcription level, delayed Dex resulted in a significant up-regulation of IL-6. Further, CCL2 concentrations were reduced in treatment dialysates but in a pattern different from what was seen in response to immediate Dex administration. This work shows that modulators can be used to alter the macrophage activation state at a non-degradable implant site.
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Keeler GD, Durdik JM, Stenken JA, Effects of Delayed Delivery of Dexamethasone-21-Phosphate via Subcutaneous Microdialysis Implants on Macrophage Activation in Rats. Acta Biomater
Abbreviation List:

ANOVA – Analysis of Variance
Arg – Arginase
BSA – Bovine Serum Albumin
CCL – (C-C motif) Ligand
CCR – C-C Chemokine Receptor Type
CD – Cluster of Differentiation
cDNA – Complimentary Deoxyribonucleic Acid
Dex – Dexamethasone-21-Phosphate
ECF – Extracellular Fluid
ECM – Extracellular Matrix
ECS – Extracellular Space
ELISA – Enzyme-Linked Immunosorbent Assay
FBGC – Foreign Body Giant Cell
FBR – Foreign Body Response
H&E – Hematoxylin and Eosin
HPLC – High Performance Liquid Chromatography
IACUC – Institutional Animal Care and Usage Committee
IFN-γ – Interferon Gamma
IgG – Immunoglobulin G
IL – Interleukin
iNOS – Inducible Nitric Oxide
kDa – Kilodalton
Lipo-1 – Lipocortin-1
LPS – Lipopolysaccharide
MAPK – Mitogen-Activated Protein Kinase
M-CSF – Macrophage Colony Stimulating Factor
MCP-1 (aka CCL2) – Monocyte chemoattractant protein-1

MerTK – Mer Tyrosine Kinase

MHC II – Major Histocompatibility Complex II

MWCO – Molecular Weight Cut-off

NF-κB – Nuclear Factor Kappa-Light-Chain-Enhancer of activated B cells

NIH – National Institutes of Health

OCT – Optimal Cutting Solution

PAMP – Pathogen-associated Molecular Pattern

PBS – Phosphate Buffered Saline

pFN – Plasma Fibronectin

PRR – Pattern Recognition Receptor

qRT-PCR – Quantitative Real Time Polymerase Chain Reaction

REST – Relative Expression Software Tool

RNA – Ribonucleic Acid

RSA – Rat Serum Albumin

SOCS3 – Suppressor of Cytokine Signaling 3

TGF-β – Transforming Growth Factor Beta

TLR – Toll Like Receptor

TMB - 3,3',5,5'-Tetramethylbenzidine

TNF-α – Tumor Necrosis Factor alpha
Chapter 1

Introduction
Implanted biomaterials have become common place in today’s society with many people having prosthetic joints (artificial knees, hips), screws holding bones together, dental implants, and pacemakers. A common phenomenon with implanted biomaterials is the body's immune response to the foreign material known as the foreign body response (FBR) [1-4]. This response leads to the eventual encapsulation of the material. While this response does not affect the performance of many biomaterials, such as the ones named above, it can be detrimental to other biomaterials. Implanted glucose sensors rely on interacting with the extracellular fluid (ECF) to make measurements. In the case of implanted glucose sensors, the FBR leads to outputs which do not reflect the body's glucose level but rather the glucose level found within the microenvironment of the encapsulation surrounding the implant. Due to these detrimental effects, understanding the FBR is a field of interest with hopes to better integrate biomaterials into host tissue.

Macrophages are cells in the body which have been shown to exist in many forms and play an essential role in the integration of a biomaterial. The study of the different phenotypes of macrophages at the implant site, and the dominance of certain types of macrophages at the implant site is thought to result in improved outcomes in relation to biomaterials [5]. For this reason, inducing certain macrophage activation states at an implant site may be beneficial. While different materials have been used to elicit a desired macrophage activation state, it is unknown whether modulators can be used to shift the macrophage activation state at a non-degradable implant site. Further, the molecular properties which are associated with different activation states are incompletely understood, in vivo.

This work is the first to use modulators to attempt regulating the macrophage activation state at a non-degradable implant site. The primary hypothesis is that by producing a predominantly M2c activation state, improved healing will be seen at an implant site. It was the aim of this work to determine the efficacy of different modulators to shift macrophages to a predominantly M2c activation state at a microdialysis probe implant site, as well as characterize the molecular response. Through these studies a multitude of discoveries were made. First, when using microdialysis sampling, Dextran-70 should be avoided as it can lead to inflammation at the implant site as well as tissue necrosis. However, Dextran-500 can be used as a safe alternative to Dextran-70. Dexamethasone-21-phosphate (Dex) can be
delivered through the microdialysis probe to alter the activation state of macrophages. The delivery of Dex results in an increase in the percentage of M2c macrophages seen in the tissue surrounding the microdialysis probe. Interestingly, differences were seen when Dex was delivered immediately following probe implantation as compared to a delayed delivery. In tissue where Dex was immediately delivered, fewer macrophages were seen as compared to delayed delivery. The gene expression profiles also differed. While both CCL2 and IL-6 were significantly down-regulated in the tissue from immediate Dex delivery, IL-6 was seen to be significantly up-regulated in response to a delayed Dex infusion. Finally, CCL2 protein concentrations were decreased in the dialysate in response to both immediate and delayed Dex infusions. However, a decrease is seen much sooner in response to immediate Dex infusion as compared to delayed infusion with the response in the delayed infusion being more transient. These results show that it is possible to use modulators to shift macrophages in vivo to a desired activation state at a non-degradable implant site while also further characterizing a predominantly M2c environment.

Macrophage Activation

Monocytes are the largest of the leukocytes (white blood cells) with an ameoboid shape and kidney bean shaped nucleus. These cells are known to play a role in innate immunity. Innate immunity is the body's first line of defense to microbes or any foreign invading molecule. This is a non-specific response consisting of epithelial cells (physical barrier), phagocytic cells (e.g. macrophages which ingest and destroy invading material), natural killer cells (cells which will kill microbe infected cells), the complement system (which promotes the phagocytosis of microbes as well as attracts neutrophils, a white blood cell), and cytokines (signaling proteins which mediate inflammation and immune responses) [6]. One of the roles monocytes are known to play is to replenish resident macrophages. Inflammatory monocytes are known to be recruited to a wound site where they are differentiated to macrophages [7]. Macrophages are known to have multiple morphologies, from round, to pancake shaped, to elongated [8], and are characterized as having large nuclei with noticeable lysosomes. These cells are known to be much larger than monocytes with human macrophages measuring ~20 microns.
Macrophages have long been recognized as scavenger cells due to their ability to phagocytose, or engulf, cellular debris and dead cells. These cells also play a role in innate immune responses by phagocytosing pathogens. This process is facilitated by the expression of pattern recognition receptors (PRR) being present on the cell surface of macrophages. PRRs do not recognize any one specific molecule but rather recognize a pattern. These PRRs are able to identify an array of molecules known as pathogen-associated molecular patterns (PAMPs) such as bacterially-derived carbohydrates (lipopolysaccharide (LPS) or mannose) found on gram negative bacteria, peptidoglycans found on gram positive bacteria, nucleic acids such as bacterial or viral DNA or RNA, glucans from fungi, and lipoproteins [9-11]. The recognition of one or more PAMPs, by a PRR found on the cell surface of a macrophage, results in the phagocytosis of the pathogen. Once the pathogen has been phagocytosed, it is destroyed due to an oxidative burst within the macrophage. For this reason, resident macrophages are found throughout the body in areas likely to encounter pathogens, i.e., the skin, lungs, and liver.

Macrophages are also known to play a role in adaptive immunity, or acquired immunity because people are not born with it but develop it over time. Adaptive immunity is called into action in response to an innate immune response, the non-specific recognition of microbes due to conserved molecular patterns [11]. Adaptive immunity differs from innate immunity in that adaptive immunity is a highly specific response to a particular antigen and is characterized as producing long-lived protection against the antigen [12]. Adaptive immunity is carried out via antibody responses where B cells are activated to produce antibodies specific for the antigen which mounted the response or by cell mediated responses where T cells kill infected cells or release signaling molecules which activate macrophages to destroy microbes which have been phagocytosed [12].

While the ability of macrophages to phagocytose cellular debris, dead cells, and pathogens, as well as their role as antigen presenting cells has long been recognized, more recently different states of macrophages have been shown to exist. The different states of macrophages have been coined as polarization states. In 2000, Mills et al. coined these polarization states as being M1 and M2 [13]. The two prominent macrophage polarization states are M1, or classically-activated macrophages, and M2, or alternatively activated macrophages [14] with the polarization state present depending on the cues
present in the microenvironment [15-17] (Figure 1). At this point it should be noted that this classification of macrophages is somewhat simplified in that the classification represents the two extremes of a continuum in which macrophage populations will exist [15]. Further, it should be recognized that these cells have been shown to be plastic, switching from an M1 to M2 state and vice versa *in vitro* by altering the cytokines present in the culture media [18, 19] as well as reverting to their initial state after the removal of the stimulus [20]. More recently, it has been suggested that 'polarization states' should be avoided for the use of 'activation states' and that instead of using the M1/M2 nomenclature, the modulator which was used to create the macrophage should be stated, e.g. M (LPS) for a macrophage which was created in response to LPS treatment [21]. For this document, the M1/M2 nomenclature will be used as a generalization to describe cells which have been elicited in response to one of the many modulators which may result in an M1 or M2 macrophage while the M (modulator) will be used to describe specific macrophages or macrophages elicited in this work.
Fig. 1. Image showing the different polarization states of macrophages. The phenotype of macrophage present depends on the inducers which are present in the microenvironment. These different phenotypes are characterized by the cell surface markers they express as well as differential expression of cytokines and genes. Abbreviations: DTH=Delayed type hypersensitivity, IC=immune complex, MR=mannose receptor, PTX3=the long pentraxin, RNI=reactive nitrogen intermediates, SRs=scavenger receptors
Reprinted from Trends in Immunology. 25/12 Mantovani, Alberto et al. The chemokine system in diverse forms of macrophage activation and polarization. 2004 with permission from Elsevier.
M1 macrophages are considered to be pro-inflammatory macrophages. These cells are responsible for eliciting a Th1 type response due to a bacterial infection. These cells are induced by LPS, interferon gamma (IFN-γ), and tumor necrosis factor alpha (TNF-α). As discussed earlier, LPS is a bacterial carbohydrate found on the cell walls of gram negative bacteria. IFN-γ is a pro-inflammatory cytokine produced primarily by Th1 cells and is thought to ‘prime’ macrophages making them more responsive to LPS challenge [22, 23]. TNF-α is a pro-inflammatory cytokine produced primarily by macrophages in response to activation of toll like receptor (TLR) 4, a specific type of PRR found on the cell surface of macrophages, by LPS [24-27]. The M1 response to biomaterials is generally accepted as a low grade response where M1 macrophages are seen in the early stages of wound healing and then transition to M2 macrophages [28]. In fact, the low grade response has recently been shown in a rat model where collagen discs were implanted subcutaneously [29]. However, the severity of the M1 response has been shown to depend on a multitude of different factors. In a recent study, a mouse model was used to show that higher numbers of M1 macrophages were seen in response to increased acetylation of a chitosan scaffold [30]. Further, Badylak's group has shown that surgical meshes which are cross-linked, the chemical joining of two molecules via a covalent bond, result in a larger M1 response than those which are not [31, 32]. Also those which contain a cellular component result in a larger M1 response [33] in rats.

M1 macrophages are characterized as being highly phagocytic and bactericidal [14, 34]. These cells are characterized as producing high amounts of pro-inflammatory cytokines such as Interleukin (IL) 1β, IL-6, IL-12, IL-15, IL-18, IL-23, and TNF-α, [14, 35]. These cells are also known to secrete chemokines CCL15, CCL20, CXCL9, CXCL10, CXCL11, and CXCL13 [14] as well as reactive oxygen intermediates [36, 37]. M1 macrophages also show increased amounts of the receptor CCR7 (C-C Chemokine Receptor type 7) [35, 38] (which binds both CCL19 and CCL21 [39] and is responsible for the homing of immune cells to secondary lymphoid organs [39, 40]), MHC II (which plays a role in the presentation of extracellular proteins [41]) [18, 37, 42], CD80 (a molecule known to be a co-stimulator for T cell activation [6]), and CD64 (a cell surface receptor able to bind monomeric IgG with high affinity [43].
resulting in activated macrophages [6]) [44]. Further, M1 macrophages are capable of converting L-arginine to nitric oxide via inducible nitric oxide (iNOS). It should be noted at this point that while increased iNOS production has been seen in M1 macrophages from murine models [45], iNOS production from human macrophages has proven to be far less reliable with iNOS not being detected in human macrophages in vitro but detected in human tissue samples [45-48]. It has been argued, by Murray and Wynn, that this may be due to culture media missing components needed for the production of iNOS by human macrophages [49]. In this work, the gene expression levels of iNOS have been utilized as an indicator of macrophage activation state.

M2 macrophages are a broad category of macrophages which are considered to be activated in response to stimuli other than what has been stated for M1 macrophages, e.g. in response to apoptotic or necrotic cells, or material which is too large to be phagocytosed. For this reason, these macrophages have been dubbed as alternatively-activated macrophages. However, unlike the M1 class of macrophages which currently consists of no subclasses, the M2 class can be further subdivided into three subclasses: M2a, M2b, and M2c. Each of these subclasses have unique characteristics and are elicited by different stimuli. With this said, M2 macrophages as a whole have been characterized as being anti-inflammatory in nature and in fact have been shown to be an indicator of biologic scaffold outcome where predominantly M2 wound sites result in improved healing and integration as compared to wound sites which are predominantly M1 [31]. It is important to note at this point that wound sites typically express a combination of M1 and M2 macrophages.

M2 macrophages are shown to increase arginase activity, resulting in arginine being converted to ornithine and urea [50] resulting in reduced iNOS production [51]. M2 macrophages show an increased expression in the cell surface receptor, CD206 (Cluster of Differentiation 206), previously called the macrophage mannose receptor, mannose receptor C type 1. This receptor was one of the first to be used to identify M2 macrophages, primarily macrophages induced by IL-4/IL-13 [(M(IL-4/IL-13)]. It has recently been shown, in human monocytes collected from buffy coats and then differentiated to M2 macrophages using macrophage colony stimulating factor (M-CSF), that CD206 can be used as a non-specific M2 marker instead of an M2a marker [52] with expression levels of CD206 being shown to
significantly increase in response to M-CSF, dexamethasone (Dex), transforming growth factor beta (TGF-β), and IL-4 in human macrophages [52].

For many years, inducing macrophages with IL-4/IL-13 was said to produce an M2 macrophage. It now is recognized that the use of IL-4/IL-13 to induce macrophages elicits an M2a response [14, 35, 53]. M2a macrophages are described as eliciting a Th2 type response, type II inflammation, and playing a role in the encapsulation and killing of parasites and helminths [15, 35] as well as in allergic responses [35, 44] but do not present antigen to T cells in vitro [15]. These cells produce anti-inflammatory cytokines IL-1ra, IL-4, and IL-10 as well as suppressing the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12, and TNF-α [14, 35]. Further, it has been shown that IL-4 plays a role in the fusion of macrophages to form foreign body giant cells (FBGC), though other factors, such as cell density, cell source, time of exposure to IL-4, and type of adherent substrate, may play a role in the effectiveness of IL-4 to produce FBGCs [2, 54-58]. The ability of IL-4 to promote FBGC formation may help explain the role these macrophages play in response to parasites, helminths, and biomaterials.

At the cell surface, M2a macrophages are characterized as expressing increased amounts of MHC II along with CD206 [59]. The expression of CD206 is important in that it can be used to help distinguish these cells from M1 macrophages as CD206 is decreased in response to IFN-γ, a common M1 inducer [60]. Further, in human macrophages, CD209 (previously recognized as DC-SIGN) has been shown to be specifically up-regulated in response to IL-4 [38, 52] whereas Ambarus et al. found that CD200R was specifically up-regulated by IL-4 and not IFN-γ or IL-10 [44] making it a possible M2a marker. CD209 is a C-type lectin, a carbohydrate binding protein, which was originally identified as a specific marker for dendritic cells [61] but has been shown to be expressed by macrophages [62, 63]. CD209 is known to bind viruses, fungi, bacteria, and mycobacteria, and [61, 64, 65], parasites [63] as well as aid in immunosuppression by different pathogens, including HIV, to evade immune surveillance [63, 66].

M2b macrophages are quite possibly the least characterized M2 activation state of macrophages. M2b macrophages differ from M2a macrophages in many ways, primarily by the fact that these macrophages produce pro-inflammatory cytokines while M2a macrophages do not. M2b macrophages
are induced by the ligation of immune complexes (e.g. Fc or complement) [67], TLRs [35], and IL-1 receptor [35]. These cells produce pro-inflammatory cytokines such as IL-1, IL-6, TNF-α [35, 67], and CCL1 [68, 69] as well as producing nitric oxide more abundantly than arginase, similar to M1 macrophages [67, 70]. However, like M2 macrophages, these cells produce little to no IL-12 but high amounts of IL-10 (an anti-inflammatory cytokine) [35, 67, 70]. M2b macrophages are identified by their expression of LIGHT (Tumor Necrosis Factor superfamily 14) and SPHK1 (sphingosine kinase 1) genes [68, 70, 71] as well as MHC II on the cell surface [35]. These macrophages are known to play a role in the remission of lupus, promote Leishmania infection, suppress pro-inflammatory responses, and are induced by the pro-inflammatory clearance of apoptotic neutrophils [67].

The final subset of M2 macrophages are the M2c macrophages, currently recognized by their expression of CD163 (to be discussed later) at the cell surface. M2c macrophages are induced by IL-10, glucocorticoids, and TGF-β [14, 16, 35]. Like other M2 macrophages, M2c macrophages are characterized as being anti-inflammatory and suppressing the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, IL-12, and TNF-α [14]. The anti-inflammatory nature of M2c macrophages is further developed by the production of the IL-1 decoy receptor IL-RII, IL-10, and TGF-β [14, 35]. M2c macrophages are similar to M2a macrophages in that they metabolize L-arginine to ornithine resulting in an increase in arginase and a decrease in iNOS.

At the cell surface, M2c macrophages are known to have decreased expression of MHC II making them poor antigen presenting cells. However, M2c macrophages have an increased expression of scavenger receptors on their cell surface which alludes to their role as tissue remodeling cells. Immunohistochemically, these cells have been identified by their cell surface expression of CD163 which was recently shown to be specific to M2c cells [44, 52]. CD163 is a scavenger receptor which is a member of the scavenger receptor cysteine-rich group B family [72]. Kristiansen et al. originally identified CD163 as being a scavenger receptor responsible for the Ca^{2+} dependent endocytosis of hemoglobin-haptoglobin (Hb-Hp) complexes [73]. They showed that CD163 only binds the Hb-Hp complex, preferably the 2-2 Hb-Hp complex, and not free Hb [73]. As the implantation of a biomaterial results in rupturing of blood vessels, hemoglobin is released which will bind haptoglobin. CD163 is thought to play an anti-
inflammatory role by binding the Hb-Hp complex, endocytosing the complex, and converting the Hb to anti-inflammatory metabolites bilirubin and carbon monoxide [74, 75].

Recently, human macrophages, differentiated from monocytes collected from blood donors, which were treated with either IL-10 in conjunction with macrophage colony stimulating factor (M-CSF) [M(IL-10+M-CSF) macrophages] or dexamethasone alone [M(Dex) macrophages] have been shown to express Mer tyrosine kinase (MerTK) on their cell surface resulting in the clearance of early apoptotic cells [52]. MerTK is a protein which recognizes unmodified phosphotidylserine, an apoptotic marker, resulting in the engulfment of the apoptotic cell by macrophages [52]. The clearance of apoptotic cells is important to proper wound healing as late apoptotic cells become necrotic, resulting in a pro-inflammatory clearance of these cells [52, 76, 77]. Table 1 shows a breakdown of the different activation states of macrophages as well as their characteristics.
Table 1. Activation States of Macrophages as Well as Their Characteristics.

<table>
<thead>
<tr>
<th>Pro-/Anti-inflammatory</th>
<th>M1</th>
<th>M2a</th>
<th>M2b</th>
<th>M2c</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inducers</strong></td>
<td>Pro- Anti-</td>
<td>Ligation of immune complexes (Fc or complement), Toll-Like Receptors, IL-1 receptor</td>
<td>Dexamethasone, IL-10, TGF-β</td>
<td></td>
</tr>
<tr>
<td>IFN-γ, LPS, TNF-α</td>
<td>IL-4, IL-13</td>
<td>IL-1β, IL-6, IL-12, IL-15, IL-18, IL-23, TNF-α, CCL15, CCL20, CXCL9, CXCL10, CXCL11, CXCL13 [14, 35]</td>
<td>IL-1 receptor decoy IL-RII, IL-10, TGF-β. Suppress IL-1β, IL-6, IL-8, IL-12, TNF-α [14, 35]</td>
<td></td>
</tr>
<tr>
<td><strong>Cytokines/Chemokines secreted</strong></td>
<td>Pro- Anti-</td>
<td>Primarily iNOS</td>
<td>Produce arginase</td>
<td></td>
</tr>
<tr>
<td>IL-1β, IL-6, IL-12, IL-15, IL-18, IL-23, TNF-α, CCL15, CCL20, CXCL9, CXCL10, CXCL11, CXCL13 [14, 35]</td>
<td>IL-1ra, IL-4, IL-10. Suppress IL-1β, IL-6, IL-12, TNF-α [14, 35]</td>
<td>CCL1, IL-1, IL-6, IL-10, TNF-α [35, 66-69]</td>
<td>IL-1 receptor decoy IL-RII, IL-10, TGF-β. Suppress IL-1β, IL-6, IL-8, IL-12, TNF-α [14, 35]</td>
<td></td>
</tr>
<tr>
<td><strong>Arginine Metabolism</strong></td>
<td>Produce iNOS</td>
<td>Produce arginase</td>
<td>Primarily iNOS</td>
<td>Produce arginase</td>
</tr>
<tr>
<td><strong>Cell Surface Markers</strong></td>
<td>CCR7, CD64, CD68, CD80, MHC II [18, 35, 37, 38, 42, 44]</td>
<td>CD68, CD200R, CD206, CD209 (Humans), MHC II [38, 51, 53, 44]</td>
<td>CD68, MHC II [35]</td>
<td>CD68, CD163, MerTK (Human) [44, 53]</td>
</tr>
<tr>
<td><strong>Physiological role</strong></td>
<td>Produce Th1 response to bacterial infection.</td>
<td>Produce Th2 type response, type II inflammation, play a role in encapsulation and killing of parasites and helminths</td>
<td>Play a role in the remission of lupus, promote Leishmania infection, suppress pro-inflammatory response</td>
<td>Scavenge hemoglobin-haptoglobin complexes, clearance of apoptotic cells, matrix deposition, tissue remodeling</td>
</tr>
</tbody>
</table>
The M2c macrophage state has become of interest, especially in the field of biomaterials, due to its being an anti-inflammatory, pro-wound healing, pro-tissue remodeling cell [17, 35]. Due to these characteristics of the M2c macrophage, it is hopeful that eliciting a predominantly M2c environment around an implant will result in better integration of the implant with the tissue and improved outcomes. To this point, the field has focused on the use of different materials-based approaches to elicit a predominantly M2 environment around an implant. This approach has garnered a better understanding of the relationship that exists between biomaterial constituents and macrophage activation state. However, this work is the first to look at an M2c response around a non-degradable biomaterial.

In a study where mesh scaffolds were implanted in rats, it was shown that implants which had been chemically cross-linked elicited a predominantly M1 response whereas non cross-linked scaffolds elicited a predominantly M2c response [32]. The cellularity of scaffolds has also been shown to affect macrophage activation state with scaffolds having a cellular component eliciting a predominantly M1 state while de-cellularized scaffolds, whether allogeneic (from the same species) or xenogeneic (from different species), elicit a predominantly M2c state [33]. Further, it has been shown that the predominantly M2c environment surrounding these implants results in improved integration and outcome with less fibrosis and scarring and more vessel formation as compared to the M1 predominant environment [33]. While the previous work was done using degradable biomaterials, it does suggest a means for studying other biomaterials. The work shows that if the environment surrounding a biomaterial consists predominantly of M2c macrophages, improved integration and outcome are achieved.

Previous studies have been performed in which drug releasing biomaterials were used to modulate the FBR. Many modulators including basic fibroblast growth factor, bone morphogenetic protein, CCL2, dexamethasone, icaritin, IL-10 gene, mastinib, platelet derived growth factor, TGF-β1, TGF-β2, and vascular endothelial growth factor [78-93] have been used in drug eluting biomaterials. However, other than the recent work by Boehler et al., these studies have relied on histology to determine the effectiveness of their modulator and none have looked at immunohistochemistry to determine the predominant macrophage phenotype present in the tissue surrounding the implant. Further, these drug
releasing biomaterials suffer from initial burst of modulator followed by a continuous slow release which cannot be controlled. The work presented in this dissertation is the first to utilize a non-degradable implant to deliver modulators to a wound site in an attempt to shift the activation state of macrophages (outlined in Table 2). The non-degradable implant utilized is a microdialysis probe which prevents the initial burst of modulators seen in other drug releasing biomaterials and allows for the controlled release of the modulator over time. If a two pronged approach is used where modulators successful at shifting macrophages to the desired activation state are found, followed by investigation as to the optimal time point to administer those modulators, this could open the door for increased longevity of biomaterials such as implanted glucose sensors by allowing better integration of the biomaterial into the host tissue.
Ability of proven *in vitro* modulators to shift macrophage activation state *in vivo*. Per fused IL-10 and Dex through microdialysis probe and looked at activation state of macrophages in the tissue surrounding both control and treatment tissue via IHC. (CD68 and CD163 for both modulators, and CCR7 and CD206 for IL-10)  

**Result**: IL-10 had minimal effects on the amount of CD163⁺ macrophages. Dex resulted in a significant increase in the percentage of M2c (CD68⁺CD163⁺) macrophages.

**Effect of modulator on protein conc.** Measured CCL2 conc. in dialysate from both control and treatment probes.  

**Result**: IL-10 had no significant effect on CCL2 concentration. Dex significantly decreased the concentration of CCL2 collected in the dialysate.

**Effect of modulator on gene expression.** Studied regulation of multiple genes via qRT-PCR. (Arg₂, CCL2, CD163, CD206, IL-1ra, IL-6, IL-10, Lipo-1, iNOS₂, TGF-β1, TNF-α)  

**Result**: Dex significantly down-regulated CCL2 and IL-6 when administered immediately following probe implantation. Dex significantly up-regulated IL-6 when administered 3 days post implantation.

**Effect of an immediate delivery of modulator as compared to a delayed delivery on macrophage activation state.** Dex delivered immediately following microdialysis probe implantation as well as 3 days post implantation.  

**Result**: The percent of M2c macrophages present were similar in the immediate and delayed infusion Dex studies. However, more macrophages were seen in the tissue from the delayed Dex infusion study.

**Effect of modulator on tissue histology.** Tissue surrounding the microdialysis probe subjected to hematoxylin and eosin and Masson's Trichrome stains.  

**Result**: IL-10 resulted in undesirable effects (ring of blood, cellular debris) immediately surrounding the treatment probe but reduced cellularity and collagen further away. Immediate Dex infusion resulted in mostly unusable treatment tissue sections with those that were obtained showing decreased cellularity but no difference in collagen. Delayed Dex infusion resulted in increased cellularity in treatment tissue but no appreciable differences in collagen.
Foreign Body Response

Park and Lakes defined a biomaterial as “A synthetic material used to replace part of a living system or to function in intimate contact with living tissue” [94]. The use of biomaterials, e.g. prostheses, biosensors, breast implants, heart valves, pacemakers, in recent decades have increased to the point where it is not uncommon to know someone with a biomaterial implant. Given the extremely common nature of these implants, it has become of great interest to understand how the body reacts to these implants and reduce the amount of inflammation and rejection, with the highest rates seen in hip replacements at 40%, due to these implants. Materials which enter the body and are > 10 μm in size cannot be phagocytosed [2]. In the case that an object cannot be phagocytosed and is recognized as a foreign material, the body has a specific way of dealing with the object. The FBR is the body’s response to any recognized implanted material, helminth, or foreign material which is too large to be phagocytosed (Figure 2). For this work we will focus on biomaterials which are > 10 μm in size.
Fig. 2: Image showing the steps involved in the foreign body response. The foreign body response is the body’s response to a biomaterial or material which enters the body and is too large to be phagocytosed. Reprinted from Surface Science.,500, Castner, D.G., Ratner, B. D. Biomedical surface science: Foundations to frontiers 2002, 28-60 with permission from Elsevier.
The response is initiated by the implantation of the biomaterial. During the implantation process, mechanical injuries occur to the tissue which include the destruction of cells and blood vessel severing, resulting in the initiation of the inflammatory response, thrombus activation, the activation of the complement system, and platelets [2]. Immediately following the implantation of the biomaterial, a layer of proteins, including fibrinogen, fibronectin, vitronectin, IgG, von Willebrand factor, and others, spontaneously adsorbs to the surface of the biomaterial [95]. This deposition of proteins on the surface of the biomaterial is known as the formation of a provisional matrix [1]. The provisional matrix is a biodegradable substance which contains an array of biological substances such as chemoattractants, cytokines, and growth factors.

The perturbation of the tissue sets into effect acute inflammation. Acute inflammation is inflammation which lasts minutes to days depending on the amount of injury with resolution generally occurring within one week. Following the adsorption of proteins to the biomaterial surface, cells arrive at the implant to investigate the biomaterial. This response happens within hours of the biomaterial being implanted. Initially, acute inflammation is characterized by the wound site being predominated by neutrophils [1, 2]. These neutrophils migrate, via capillaries, to the implant site where they extravasate to the tissue surrounding the implant. The primary role of these neutrophils is to phagocytose any foreign material including microorganisms. Due to its size, the neutrophil is unable to phagocytose the biomaterial. While phagocytosing does not occur, the neutrophils release enzymes to degrade the biomaterial through extrusion or exocytosis [96, 97]. Neutrophils immigrate to the wound site for a short amount of time and are short lived cells which die after about 24-48 hrs. It is generally accepted that the wound site will be predominated by neutrophils for ~ 3 days [1]. Three to five days post-implantation, a shift in the cell type which predominates the wound site occurs with macrophages becoming predominant.

The leukocytes, first the neutrophils then the macrophages/monocytes, are drawn to the wound site in response to the chemokine gradient, a chemical ligand gradient, which exists causing cells to move to an area of high chemokine concentration [98]. The chemokine gradient is established when chemokines are secreted in response to different cytokines and bind to glycosaminoglycans which
preserve higher concentrations of chemokines at the site of inflammation [99]. Chemokines are responsible for the recruitment of leukocytes, e.g. monocytes, neutrophils, lymphocytes, to a wound site. Chemokines are a subset of cytokines, which can be subdivided into four families: C, CC, CXC, CX3C with the X representing the number of amino acids separating the conserved cysteine residues at the N-terminal [100, 101].

Different members of the chemokine families are responsible for the recruitment of specific cell types to a wound site. The neutrophils are recruited to the wound site in response to CXCL8 [IL-8 (KC/GRO in rats and mice)] and to some extent TNF-α [102] while CCL2 (MCP-1) has been shown to be the primary chemokine responsible for the migration of monocytes to a wound site [103]. Further, CCL2 has been shown to be present in the exudate macrophages collected from high molecular weight polyethylene implants placed in the subcutaneous space [104]. However, it has been argued that CCL2 is not imperative for the recruitment of monocytes to subcutaneous biomaterial implants but rather plays a role in FBGC formation [105]. It should be noted though that these experiments were carried out using different biomaterials as well as different species. In the case where CCL2 was seen to not be imperative for monocyte recruitment, the experiments were carried out in CCL2 null mice and it is not unreasonable to think that more than one chemokine is capable of recruiting monocytes to a wound site in the case where one chemokine is unavailable.

Once the macrophages arrive at the wound site, they are attracted to the provisional matrix which has formed around the biomaterial. This 'attraction' is a result of integrin binding motifs which are found on the surface of biomaterials. Integrins are adhesion molecules which allow the movement of cells through the ECF allowing the cell to respond to its environment [106]. Macrophages are known to express three different classes of integrins: β1, β2, β3. These integrins recognize integrin binding motifs found in proteins which compose the provisional matrix thereby allowing macrophages to bind. While the primary binding event of macrophages to the provisional matrix is yet to be elucidated, it has been suggested that multiple proteins may play a role. Early in vivo studies, by Tang and Eaton using a mouse model, showed that animals with low levels of fibrinogen were unable to mount a response to the biomaterial unless the material was coated with fibrinogen or fibrinogen was injected into the animal, thus
concluding that fibrinogen was the primary protein needed for adhesion [107]. In vitro studies by Anderson's group have shown that macrophages/monocytes will initially adhere via β2 integrins which bind to various proteins, e.g. fibrinogen, complement, fibronectin, and IgG [108, 109]. These β2 integrins have also been associated with podosome formation in macrophages [110, 111]. A podosome is a protrusion of the plasma membrane which represents attachment sites for cells to the extracellular matrix [112] and has been identified as the major adhesive structure in macrophages in vitro [1]. The formation of these podosomes allows for the spreading of the macrophages over the surface of the biomaterial.

Once macrophages have adhered and spread over the surface of the biomaterial, a process which can be described as interrogating the biomaterial, the macrophages will fuse together to form foreign body giant cells. The formation of these FBGCs occurs in response to the size disparity that exists between the biomaterial and the macrophage and the inability of the biomaterial to be phagocytosed. The formation of these FBGCs around implants have been seen in many different models including but not limited to, a rat model where polyether-polyurethane sponge discs were implanted subcutaneously [113], a rat model where multiple different scaffolds were implanted subcutaneously [114], where nitrocellulose is injected in the foot pads of mice [115], around both polyetherurethane and polyethylene terephthalate implanted subcutaneously in mice [116], as well as in my own work where microdialysis probes were implanted subcutaneously for 14 days in rats.

Many different factors have been identified with the extent to which FBGCs form around a biomaterial. Anderson's group has done much work in this area and has shown that IL-4 can be used to induce FBGC formation in vitro with IL-13 being shown to be an equally potent inducer of FBGCs [55, 117]. Further, their group was able to show that IL-4 is an important factor in the formation of FBGCs in vivo [54]. While the mechanism behind this FBGC induction is unclear, it may be in part due to the fact that both IL-4 and IL-13 significantly up-regulate CD206 which is thought to play a role in the fusion process as this receptor is localized at areas of the cell where fusion is occurring [2]. Further, in a mouse model where polyethylene terephthalate disks were implanted in the subcutaneous space, it was found that plasma fibronectin (pFN) played a role in the formation of FBGCs with results showing three times as many FBGCs in pFN knockout mice as compared to controls [118].
The formation of these FBGCs results in a microenvironment which exists between the FBGCs and the biomaterial. While the biomaterial is unable to be phagocytosed, a process known as 'frustrated phagocytosis' occurs. Frustrated phagocytosis is a term which was coined by Henson in which macrophages and FBGCs are capable of releasing degradative substances, e.g. reactive oxygen intermediates, phagosomes, oxygen free radicals, and enzymes, into the area between these cells and the biomaterial [96]. This puts the surface of the biomaterial in direct contact with high concentrations of these substances, and low pH (phagosome can have a pH as low as 4 [119]) which may destroy some materials, such as polyethylene and polypropylene, resulting in failure of the device.

FBGCs not only release degradative substances at the implant site but also release cytokines, in fact it has been shown that FBGCs release IL-1α and TNF-α at four weeks post nitrocellulose injections in mice [115]. This suggests that FBGCs may play a role in prolonging the inflammatory response. In a mouse model, it was shown that the release of TGF-β1 coincided with the predominance of FBGCs in response to nitrocellulose injections at 45 and 60 days post injection [115]. This might suggest that FBGCs play a role in the formation of a fibrotic capsule. Also it is known that IL-4/IL-13 play a role in the formation of FBGCs. The presence of IL-4/IL-13 as well as the release of TGF-β by FBGCs may play a role in the activation and recruitment of fibroblasts to the implant site [120]. The activated fibroblasts will become myofibroblasts as they migrate toward the implant [120]. The myofibroblasts are responsible for the production of collagen. The myofibroblasts and collagen production initially promote the closing of a wound. The correct balance of collagen production and degradation result in the wound healing without the formation of scar tissue. However, in the case of biomaterials, an acellular fibrotic capsule is created around the biomaterial resulting in the biomaterial being in its own microenvironment separate from the body. The formation of this fibrotic encapsulation is due in part to the rate of collagen being produced exceeding the rate at which the ECM (Extracellular Matrix) is degraded [120]. The formation of this encapsulation proves to be detrimental to some biomaterials, e.g. biosensors, which require interaction with the body for proper function. For this reason, it would be of great interest to alter the formation of the fibrotic encapsulation around biomaterials.
In an effort to further understand and reduce the formation of the fibrotic encapsulation as well as improve integration, many researchers have investigated different materials as well as different chemistries. Through these works many aspects that play a role in the size of the fibrotic encapsulation have been discovered. Biomaterials that are constructed with porous materials show a decreased FBR and fibrotic capsule size as well as an increase in the amount of vascularity [121-124]. Brauker et al. was the first to show this effect on fibrosis and vascularity [125]. However, there is debate as to the optimal pore size. In a series of experiments, Sharkawy et al. showed that a 60 µm pore size was superior to no pores, 5 µm, and 700 µm pores resulting in increased diffusivity, increased vascularization, and optimal response times to changes seen in the plasma [121-123]. The Ratner group showed that a pore size of 34 µm was superior to no pores and pores of 160 µm [124, 126]. While the 34 and 60 µm pores have never been compared directly, it might be assumed that the response would be similar as they are of the same relative size. These studies support the use of a microdialysis probe as a model due to the porosity of the membrane. While the pores of the membrane are much smaller than those in the above studies, the pores should still result in improved healing, as compared to a non-porous material, as well as allow the delivery of modulators. Unfortunately, no studies have been performed to determine the activation state of macrophages surrounding a non-degradable implant. Instead, conclusions on the predominant macrophage activation state surrounding an implant must be drawn from degradable implants.

Badylak's group has investigated the cross-linking and cellular component of implanted scaffolds. They have found that non-cross-linked scaffolds degrade in less time than do cross-linked scaffolds, produce an M2 vs M1 response, and avoid the chronic inflammation associated with cross-linked scaffolds [32, 127]. In another study, Badylak's group found that scaffolds with a cellular component, whether autologous (from the same animal) or xenogeneic, resulted in a predominantly M1 macrophage response and dense collagen deposition and scarring [33]. Further, acellular scaffolds were shown to produce a predominantly M2 macrophage response and resulted in improved healing containing more vascularization, no scarring, and islands of skeletal myoblasts [33]. These findings are important in that they show the macrophage phenotype can be altered in vivo, and show improved healing is seen when a predominantly M2 response is established. However, it must be considered that a degradable biomaterial was utilized. In the case of non-degradable implants, cellularity, cross-linking, and porosity may be fixed.
aspects of the biomaterial, as is the case for the microdialysis probe. For this reason, alternative methods to changing the material itself must be investigated. The use of modulators at an implant site might allow for the shift of macrophage activation state to a predominantly M2c state resulting in improved tissue integration leading to increased longevity of some biomaterials.

**Microdialysis Sampling Technique**

Microdialysis sampling technique is a minimally-invasive, widely used, *in vivo* sampling technique for monitoring bioactive molecules. Any technique is designated as being invasive if the skin is broken or a body cavity is entered. Non-invasive techniques are those which do not break the skin such as X-rays while invasive techniques, such as open heart surgery, allow the doctor to fully see the surgical site. Minimally-invasive techniques, are any techniques which are less invasive than open surgery with sub-dermal implants being included in this category.

Microdialysis sampling technique was originally designed on the premise that the microdialysis probe works by mimicking a blood vessel *in vivo* [128]. The microdialysis probe consists of inlet tubing, inner cannula, semi-permeable membrane (with a defined molecular weight cut-off (MWCO)), and outlet tubing. This device is capable of being implanted in almost any tissue type and has been used in multiple different types. During sampling procedures an isotonic fluid with physiological pH is passed through the inlet, down the inner cannula, between the outside of the inner cannula and the semi-permeable membrane, and out the outlet tubing (Fig 3). This allows microdialysis sampling technique to be used to collect molecules found in the extracellular fluid (ECF) which can then be quantified.

Microdialysis sampling works based on diffusion wherein the concentration gradient that exists between molecules found in the ECF and the perfusion fluid inside the probe, results in molecules smaller than the MWCO crossing the probe membrane and being collected in the dialysate [129]. Likewise, this
concentration gradient allows for molecules at a higher concentration inside the microdialysis probe to cross the membrane and be delivered to the ECF.
Fig. 3: Image showing a microdialysis probe and schematic of how it works. Adapted from Chapter 3 Figure 1. Reprinted from European Journal of Pharmaceutical Sciences, 57, Geoffrey D. Keeler, Jeannine M. Durdik, Julie A. Stenken, Comparison of microdialysis sampling perfusion fluid components on the foreign body reaction in rat subcutaneous tissue, Pages 60-67, Copyright (2013), with permission from Elsevier.
Given that the perfusate is continuously moving through the microdialysis probe, the length of the membrane is defined, and the flow rate is sufficiently high to collect enough dialysate for analysis while maintaining temporal resolution (~1 µL/min), collection of analytes does not happen at an equilibrium state. For this work, equilibrium state is defined as the concentration of a given analyte being the same inside the probe as it is outside the probe, meaning the amount of analyte entering the probe is equal to the amount of analyte concentration in the tissue surrounding the probe. Instead, the amount of analyte recovered via microdialysis sampling technique is a fraction of the analyte present in the ECF [130].

The efficiency at which an analyte crosses the microdialysis probe membrane and is collected is known as the extraction efficiency of that molecule. The extraction efficiency (EE) of an analyte is generally given as a percentage and is defined as shown below in Equation 1, where $C_{\text{inlet}}$ is the concentration of the analyte in the probe, $C_{\text{outlet}}$ is the concentration of the analyte leaving the probe, and $C_\infty$ is the concentration of the analyte far away from the probe.

$$EE = \frac{C_{\text{outlet}} - C_{\text{inlet}}}{C_\infty - C_{\text{inlet}}}$$

One common way to increase the EE is to lower the flow rate. Lower flow rates have been shown to increase the EE [131, 132]. However, while a lower flow rate has been shown to increase EE, 60 µL of sample are needed to perform an ELISA, greatly restricting the lowest flow rate which can be used while maintaining temporal resolution. Thus, a compromise must be struck between the flow rate and the amount of sample needed. For much of the work presented in this document, a flow rate of 1 µL/min was used as this was seen to meet this compromise.

Another characteristic of microdialysis sampling which must be considered is the difference in concentration of a modulator in the probe as compared to far away from the probe. That is, only a percentage of the modulator which is perfused through the probe will actually cross the membrane and enter the ECF. Further, as the modulator enters the ECF it will diffuse, resulting in higher concentrations close to the probe with concentrations dropping further away from the probe.
Since its inception, microdialysis sampling technique has gained popularity for a multitude of reasons. The technique is considered to be a minimally-invasive technique which allows for the collection/monitoring of molecules at a given wound/trauma site in awake and freely-moving animals as well as humans. This is an important advantage over many other techniques such as biopsies, which are capable of sampling tissue at a wound site but are considered to be highly invasive, or blood draws which are considered to be minimally invasive but are not indicative of what is happening at the wound site, rather in the periphery.

The microdialysis sampling technique is also capable of taking continuous samples post implantation at a given wound site via one implantation procedure. This characteristic has three main advantages, 1) increased temporal resolution per wound is gained as compared to other techniques such as suction blisters or biopsies, 2) reduced numbers of wounds, 3) reduced animal numbers for a study. At this point it should be mentioned that while multiple samples can be collected from one microdialysis probe, increasing temporal resolution per wound, these samples are an average of the molecular concentrations collected over a sampling period. Microdialysis sampling technique is also unique in that the same probe can be used to simultaneously collect molecules from the ECF while delivering modulators to the ECF. This allows for the near real-time view of the effects different modulators may have on a wound site. Furthermore, given that multiple probes can be implanted into a single animal, one can be used to deliver a modulator while one does not, allowing each animal to serve as its own control thereby greatly reducing the number of animals needed for a given study. Finally, the samples collected from microdialysis sampling technique are considered to be analytically-clean. This means that samples require little to no sample preparation before being analyzed allowing for a multitude of chemical analyses to be used [133].

Initially, this technique was used to collect small, hydrophilic compounds such as glucose, pyruvate, and catecholamines. Probes used for this type of work consisted of small MWCO membranes, usually 20 kDa or less. For this type of collection, balanced salt solutions, such as Ringer's (an isotonic, physiologically pH matched solution), were often used as these contained the same ions at the same strength as were found in the ECF [134] and resulted in no loss of fluid across the membrane. With the
The advent of high MWCO (100 kDa) membranes it became possible to collect larger molecules such as cytokines as well as other proteins [135]. Further, with the advent of commercially available high MWCO membranes this type of sampling gained popularity as it gave a means to investigate a multitude of disease states where cytokine levels may be dysregulated [136-144]. However, when a salt solution alone, such as Ringer's, is used as a perfusion fluid in high MWCO membranes, an undesirable side effect is seen, fluid loss or ultrafiltration.

Ultrafiltration is problematic in that it results in decreased fluid recoveries making quantification techniques with volume requirements, i.e. ELISA, difficult to perform. Furthermore, the physiological effects of the fluid leaving the probe and collecting in the ECF are poorly understood. While poorly understood, it could be speculated that the fluid loss would lead to edema at the probe implant site which could lead to the dilution of molecules resulting in concentrations which are not indicative of a typical trauma site. In an effort to combat this problem different osmotic reagents were added to the perfusion fluid shifting the osmotic balance in a manner which prevents ultrafiltration. While different osmotic agents have been used, the two most commonly used are Dextrans and bovine serum albumin (BSA) or a combination of the two [145-147]. While dextran-60 and dextran-70 were osmotic agents of choice for decades, it has recently been shown that these dextrans are able to diffuse across the microdialysis membrane and enter the ECF resulting in inflammation [144]. The diffusion of dextrans across a 100 kDa MWCO membrane has further been shown by Chu et al. who showed that both Dextran-40 and Dextran-250 are capable of diffusing across a 100 kDa membrane, in vitro [148]. While BSA was shown to cause no increased inflammation at the wound site, it was shown to be needed at high concentrations, >10% w/v, to appreciably reduce ultrafiltration [144]. However, Dextran-500 was shown to result in no increased inflammation at the wound site while giving 100% fluid recovery in vitro [149] and in this work acceptable fluid recovery in vivo have been observed when Dextran-500 was used at 4-6% w/v.

While the use of Dextran-500 successfully eliminates the problem of ultrafiltration, it does nothing to solve the second issue which plagues the collection of proteins, non-specific binding of proteins to the inner walls of the tubing during collections. To circumvent this problem, BSA is commonly included in perfusion fluids at low concentrations, i.e. 0.1% w/v, to reduce non-specific binding of collected proteins.
BSA is an acceptable additive to perfusion fluids, at least in a rat model, as it shows no appreciable increase in inflammation surrounding a microdialysis probe as compared to rat serum albumin. Given the non-inflammatory effects of BSA as well as being inexpensive and commercially available, it is an appropriate choice as a blocking agent for microdialysis tubing.

Given the characteristics of microdialysis sampling technique, it is a valuable tool for the investigation of the molecular mechanisms occurring at a wound site and shows great promise for the investigation of the FBR. It can be used to deliver modulators to a wound site, while monitoring the biochemical processes which are occurring. For these reasons, it was used in this work as a model system for studying macrophage activation at a non-degradable implant site. More specifically, the technique was used to determine if modulators could be used to shift the activation state of macrophages at a non-degradable implant site to a predominantly M2c state while monitoring the effects on protein levels, gene transcription, and tissue histology.

The work in this dissertation focused on whether proven in vitro modulators can be used to shift macrophages to an M2c state in vivo. To determine if this was possible, modulators (IL-10 and Dex) were delivered to an implant site and immunohistochemistry was used to determine the predominant macrophage activation state present. Many different strategies were utilized to fully elucidate the effects of IL-10 on macrophage activation state, in vivo with minimal success (Appendix A). What was found was that IL-10 had a minimal effect on the presence of M2c macrophages in the tissue surrounding a microdialysis probe. However, Dex (when administered immediately following implantation) significantly increased the number of M2c cells in the tissue surrounding the probe while also altering gene transcription and protein levels. Further, this work looked at whether a delayed Dex infusion might be more beneficial than an immediate infusion. To determine this, Dex was not infused until three days post implantation of the microdialysis probe. This type of infusion resulted in significantly more M2c macrophages in the tissue surrounding the treatment probe as compared to the control probe while altering gene transcription as well as protein levels in a manner different than was seen in response to immediate Dex infusion. This work has shown that a modulator can be delivered via a non-degradable biomaterial to successfully shift macrophages in the tissue to an M2c activation state. While this work is
exciting and the first of its kind, more is needed to fully understand how this shift in macrophage activation will affect the integration of the biomaterial into the surrounding tissue.
References


Chapter 2

Materials and Methods
Chemicals

The following chemicals were used in this study: Anti-CD68 Antibody (Santa Cruz Biotechnology, Inc., Dallas, TX); Anti-CD163 Antibody (Santa Cruz Biotechnology, Inc., Dallas, TX); Apex™ Antibody Labeling Kits (Alexa Fluor 488 and Alexa Fluor 647) (Life Technologies, Carlsbad, CA); BDOptEIA™ Rat IL-10 ELISA Set (BD Biosciences, San Jose, CA); BD OptEIA™ Rat MCP-1 ELISA Set (BD Biosciences, San Jose, CA); bovine serum albumin (BSA) (Rockland Immunochemicals, Gilbertsville, PA); Chloroform (MP Biomedicals LLC, Solon, OH); Dexamethasone-21-phosphate disodium salt (Dex) (Sigma Aldrich, St Louis, MO); Dextran-70 and Dextran-500 (Sigma Aldrich, St Louis, MO); Ethylene oxide (Anderson Sterilizers, Inc, Haw River, NC); formalin, 10% and neutral buffered (BDH, VWR, West Chester, PA); Halt Protease Inhibitor (Pierce, Rockford, IL); HPLC grade water (Fisher Scientific, Waltham, MA); isoflurane (Abbott Laboratories, North Chicago, IL); Optimal Cutting Temperature solution (Sakura® Finetek, Torrance, CA); povidone-iodine (Professional Disposables International Inc, Orangeburge, NY); Proteinase K (Qiagen, Venlo, Limburg); Rat Serum Albumin (RSA) (Sigma Aldrich, St Louis, MO); Recombinant Interleukin-10 (R&D Systems, Minneapolis, MN); RINalater (Life Technologies, Carlsbad, CA); Trizol (Life Technologies, Carlsbad, CA); Taqman® Gene Expression Assays (Arg2, CCL2, CD163, CD206, IL-1ra, IL-6, IL-10, iNOS2, Lipo-1, TGF-β1, Taf9b, and TNF-α) (Life Technologies, Carlsbad, CA); Ringer’s solution contained 150 mM NaCl, 5.4 mM KCl, 2.3 mM CaCl₂ (For all experiments up to Perfusion Fluid Comparison, Chapter 3), and 147mM NaCl, 4.6mM KCl, 2.3 mM CaCl₂ (For all experiments after Perfusion Fluid Comparison, after Chapter 3) pH 7.4 and was prepared in HPLC-grade water. All other chemicals were reagent-grade or higher.

Microdialysis Sampling

All microdialysis sampling procedures were performed using CMA 20 microdialysis probes with polyethersulfone (PES) membranes, 100 kDa MWCO and 10 mm length (Harvard Apparatus, Holliston, MA). Prior to implantation, microdialysis probes were sterilized using ethylene oxide (Anderson Sterilizers, Inc, Haw River, NC). The probes are perfused using a BAS Bee pump with appropriate
syringes (Bioanalytical Systems Inc., West Lafayette, IN). After completion of the surgical procedures to implant the microdialysis probe, the animal was placed into a CMA\(^1\) 120 freely moving animal collection system (CMA Microdialysis, Solna, Sweden\(^1\)), except where otherwise stated.

**Surgical Procedures**

Male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) in the weight range of 230-400 grams were used and were housed in a climate controlled room at 72°F. Prior to surgical procedures, rats were allowed access to both food and water *ad libitum*. All animal experiments were approved by the University of Arkansas Institutional Care and Use Committee (IACUC) and were in compliance with the NIH standards for the ethical treatment of animals.

Rats were anesthetized in an induction chamber with 5% isoflurane in 0.8 L/min oxygen. The rat was then maintained on a nose cone via 2.5% isoflurane in 0.4 L/min oxygen during probe implantation. During the surgical procedure the body temperature was maintained using a CMA 150 temperature controller (CMA Microdialysis, Solna, Sweden). Surgical procedures were performed using aseptic technique. All surgical tools were autoclaved prior to use. The surgical site was shaved and then swabbed with povidone-iodine prior to any incisions.

To implant the microdialysis probe, a ‘┴’ shaped incision was made into the posterior dorsal subcutaneous tissue followed by a ‘------’ shaped incision made near the base of the neck. Both incisions were about 0.5 cm in length. An autoclaved straw was then passed through the subcutaneous tissue from the posterior to anterior incisions. The tubing lines of the microdialysis probe were then run from the posterior to anterior end of the straw such that outlet lines were located on the anterior side of the rat. The straw was then removed from the animal. A plastic introducer that has split tubing (Harvard Apparatus, Holliston, MA) was then placed subcutaneously at the posterior incision site. The microdialysis probe was then placed in the introducer and the introducer was removed by pulling the tubing to the side and the fluid lines were pulled tight. The posterior incision was closed using Vetbond\(^\text{TM}\) (3M, St Paul, MN). A second microdialysis probe was then implanted on the opposite side of the spine using the same procedure. The two probes were separated by at least 2.5 cm (Fig 1). Post collection, \(^1\)CMA now owned by Harvard Apparatus
the animal was placed under light anesthesia and the lines were placed under the skin at the anterior incision in a subcutaneous pocket. The anterior incision was then closed using surgical staples and the animal was returned to housing.
Fig. 1. Image showing the placement of the microdialysis probes. Image taken by author.
Comparison of microdialysis sampling perfusion fluid components on the foreign body reaction in rat subcutaneous tissue

Perfusion Fluid Comparisons.

For all perfusion fluid comparisons, two probes were implanted into the rats. Three animals were used in each set. Unless otherwise specified, perfusions through the probes were performed every day for four days. At this point, the anterior incision with the pocket for tubing lines was closed using Vetbond™. On the seventh day post implantation, the animals were euthanized via CO₂ asphyxiation and the probes, along with the tissue immediately surrounding the probe, were explanted by using scissors to remove a ~1 inch by ~2 inch piece of tissue which contained the probe. A sterile scalpel was then used to cut around the probe removing any excess tissue not in immediate contact with the probe. The tissue containing the probe was then held in a fashion which allowed for a scalpel blade to be used to cut the probe and its encapsulation tissue, which can be seen surrounding the probe (~ 1-2 mm), away from the remaining tissue. The probe and tissue were then stored in 10% neutral buffered formalin.

Probe Only vs. Ringer’s Solution Filled Probe.

One probe had no fluid passed through it while the other had Ringer’s solution perfused through at a flow rate of 3 µL/min until fluid was observed exiting the outlet tubing at which point the flow was stopped. Fresh Ringer’s solution was infused through the implanted dialysis probe as described above each day for four days, including the day of implantation. On the fourth day, the anterior incision was permanently closed using Vetbond™ and the animals were returned to housing.
**Ringer’s vs. Ringer’s + Dextran-70**

One probe was perfused using Ringer’s solution while the other was perfused using Ringer’s solution with 6% (w/v) Dextran-70. After implantation of the probes, the animals were moved to a CMA 120 freely moving bowl which allows for microdialysis sampling to be performed in awake and freely-moving animals. Upon placement of the animal in the freely-moving animal system, a flush (3 µL/min) through the implanted probe was performed and reduced to 0.5 µL/min, in increments of 0.5 µL/min over 25 minutes. Infusions were then performed at 0.5 µL/min for 1 hour. At the end of the 1 hour infusion, a flush (3 µL/min) was performed using filter sterilized HPLC grade water.

**RSA vs. BSA.**

The same procedure was followed as the Ringer’s vs Ringer’s with Dextran-70 except for the following differences. One probe was perfused with Ringer’s solution with 3% (w/v) BSA and the other with 3% (w/v) Rat Serum Albumin (RSA). The 3% albumin solutions were necessary to prevent ultrafiltration.

**Dextran-500 vs. Dextran-500 + BSA.**

The same procedure was followed as the Ringer’s vs. Ringer’s with Dextran-70 except for the following differences. One probe was perfused with Ringer’s solution containing 6% (w/v) Dextran-500 (Sigma Aldrich St Louis, MO) and the other was perfused with Ringer’s solution plus 6% (w/v) Dextran-500 and 0.1% (w/v) Bovine Serum Albumin (BSA).

**Histological Analyses.**

Tissue was fixed in 10% neutral buffered formalin. It was subsequently embedded in paraffin blocks and cut using a microtome (~5 µm). The tissue sections were de-paraffinized, and then stained using standard protocol for both hematoxylin and eosin (H&E) and Masson’s Trichrome by Mr. David
Cross with the USDA Histology Lab, University of Arkansas. Tissue sections were then analyzed using a Zeiss Axioskop II plus microscope (Carl Zeiss Inc., Thornwood, NY) with Canon EOS Digital Software for Rebel T2i camera.

Localized Delivery of Dexamethasone-21-Phosphate via Microdialysis Implants in Rat Induces M2c Macrophage Polarization and Alters CCL2 Concentrations

Collection Procedure

Two probes were implanted with one serving as the control and the other as the treatment. The control perfusion fluid consisted of Ringer's + 0.1% BSA + 4% Dextran-500 while the treatment perfusion fluid contained an added 20 µg/mL Dex. Dextran-500 was used as an osmotic agent [1] and BSA was used to reduce non-specific binding [2, 3]. Prior to implantation, control and treatment probes were flushed with Ringer's or Ringer's + 20 µg/mL Dex, respectively. Post probe implantation, animals were moved to a CMA 120 freely moving animal system. An initial flush was then performed using the perfusion fluids which started at 3 µL/min and was reduced to 1.0 µL/min in 0.5 µL/min increments over a 20 min period. Infusions were then performed in 1 hour increments for 6 hours. All collection vials contained Halt Protease Inhibitor and were immediately placed in ice once collection was complete. A final flush was performed following the 6th hour of collection for 30 mins at 3 µL/min using Ringer's or Ringer's + 20 µg/mL Dex for the control and treatment, respectively. Following the flush, the animal was anesthetized and the lines were placed in a subcutaneous pocket at the anterior (Fig 1) incision. The animal was then returned to housing. This procedure was performed every day for 3 days. At the end of the 3rd day the animal was euthanized and the probes and tissue surrounding the probes were harvested as previously stated [4]. The tissue was either removed from the probe and stored in RNAlater for PCR studies or the probe and the tissue surrounding it were placed in optimal cutting temperature (OCT) solution and flash frozen using liquid nitrogen for immunohistochemical studies.
**qRT-PCR Procedure**

Tissue was harvested from around the membrane portion of the microdialysis probe and placed in RNAlater and stored on ice. Once all tissue was harvested, it was moved to 4°C where it was stored until RNA was extracted. RNA was extracted using the Trizol method and was purified using an RNeasy Minikit (Qiagen, Venlo, Limburg) per the manufacturer's instructions. Prior to conversion of RNA to cDNA, the integrity of the RNA was checked by running samples on a gel and looking at the 18s and 28s bands (Fig 2). RNA was then converted to cDNA using a high capacity cDNA reverse transcription kit (Life Technologies, Carlsbad, CA), per the manufacturer's instructions. Taqman gene expression assays (Life Technologies, Carlsbad, CA) were then used to determine the relative gene expression ratios via a 7500 Real Time PCR Instrument (Life Technologies, Carlsbad, CA). Data were analyzed using REST Gene Quantification Software [5] (http://rest.gene-quantification.info/).
Fig. 2. Image showing non-degraded 28S and 18S bands from RNA integrity check. Image taken by author.
**Immunohistochemical Staining Procedure**

Primary antibodies specific for Cluster of Differentiation 68 (CD68) and Cluster of Differentiation 163 (CD163) (Santa Cruz Biotechnology, Dallas, TX) were used to stain macrophages and M2c macrophages, respectively. CD68 was conjugated with Alexa Fluor 488 and CD163 was conjugated with Alexa Fluor 647 using APEX Antibody Labeling Kits (Life Technologies, Carlsbad, CA). Tissue sections were cut at a thickness of ~5µm via a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on microscope slides. Tissue was then fixed in methanol at -20°C for 20 mins. Tissue sections were then encircled with a hydrophobic pen and incubated in blocking solution for 30 mins at room temperature in a humidified chamber. Once blocked, slides were washed 4 times for 5 mins per wash in phosphate buffered saline (136.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4). Wash solution was then wicked away from the tissue and tissue was incubated with CD68 (1:125 dilution) and CD163 (1:50 dilution) (both diluted in blocking solution) antibodies overnight at 4°C in a humidified chamber. Slides were then washed 4 times for 5 mins per wash and wash solution was wicked away from the tissue sections using a Kimwipe. VECTASHIELD® (Vector Laboratories, Burlingame, CA) was then applied and cover slips were sealed. Images were then visualized using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany).

**CCL2 Quantification in Microdialysis Dialysates**

Dialysates were collected from both the control and treatment probes over a 6 hour period plus the initial 25 min flush. CCL2 concentrations in the dialysates were determined using a BD OptEIA™ rat MCP-1 ELISA set (BD Biosciences, San Jose, CA) per the manufacturer's protocol with the exception that only 60 µL of sample were loaded and the remaining reagents were adjusted accordingly. Briefly, a 96 well plate was blocked for 1 hour, standards of known concentration along with samples were then loaded and incubated for 2 hours, samples were incubated with enzyme working solution for 1 hour, TMB substrate solution for 30 mins in the dark, and then the reaction was stopped by adding Stop Solution (1M
phosphoric acid). The absorbance was then read at 450 nm with a 570 nm reference on a Tecan Infinite M200 (Tecan, Maennendorf, Switzerland).

Statistical Analysis

A Shapiro-Wilk test was performed to determine normality using Origin 8.6 software. If normality was not met, a Kruskal-Wallis ANOVA was performed. A Bonferroni post-hoc test was performed to determine significance, with $p \leq 0.05$ being considered statistically significant.

Effects of Delayed Delivery of Dexamethasone-21-Phosphate via Subcutaneous Microdialysis Implants on Macrophage Activation in Rats

Microdialysis Procedure

CMA 20 microdialysis probes with a 10 mm 100 kDa molecular weight cut-off (MWCO) polyethersulfone membrane (Harvard Apparatus, Holliston, MA) were used for all microdialysis sampling procedures. All microdialysis probes were ethylene oxide sterilized (Anderson Sterilizers Inc, Haw River, NC) prior to use. A BAS Bee microdialysis pump (Bioanalytical Systems Inc, West Lafayette, IN) with 1 mL BAS syringes (Bioanalytical Systems Inc, West Lafayette, IN) were used to perform infusions. All perfusion fluids were autoclaved and filter sterilized prior to use. Post microdialysis probe implantation, the inlet and outlet lines were placed in a subcutaneous pocket at the anterior incision. Animals were then returned to the vivarium until three days post implantation. On days 3-6 post implantation, the animals were anesthetized, lines removed from the subcutaneous pocket, and placed in a CMA 120 freely moving collection system (CMA Microdialysis, Solna, Sweden).
Surgical Procedure

All surgeries were performed on male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) in a weight range of 280-315 g. Prior to surgery and post collection, animals were kept in a temperature controlled vivarium at 72°F. Animals were allowed access to food and water *ad libitum*. All surgical procedures and experiments were approved by the University of Arkansas Institutional Animal Care and Use Committee (IACUC) and conformed to the NIH standards for the ethical treatment of animals.

Animals were initially anesthetized using an induction chamber and 5% isoflurane in 1 L min⁻¹ O₂. Animals were then moved to a nose cone where they were maintained using 3% isoflurane in 1 L min⁻¹ O₂ during the surgical procedure. Body temperatures were maintained using a CMA 150 temperature controller (CMA Microdialysis, Solna, Sweden) during surgical procedures. All surgical procedures were performed using aseptic technique. All tools were autoclaved prior to use.

The techniques for implanting microdialysis probes into the rats have been previously described [6, 7]. Two probes were implanted on opposite sides of the spine with approximately 2.5 cm separation between them. After collection techniques have commenced each day, the inlet and outlet tubing is connected with a tubing connector and placed in the subcutaneous pocket. The anterior incision was then closed using sterile Reflex wound clips (Fine Science Tools, Foster City, CA). On collection days, animals were anesthetized, the incision was swabbed with alcohol and the lines were removed. All lines were cleaned with alcohol prior to being placed in the subcutaneous pocket.

Collection Procedure

With two microdialysis probes implanted, one probe serves as a control and the other as the treatment. The control probe is perfused with Ringer’s + 4% Dextran-500 + 0.1% BSA while the treatment probe is perfused with 20 µg/mL dexamethasone-21-phosphate added. Dexamethasone-21-phosphate was used as a derivative which is more water soluble than dexamethasone and is converted
to dexamethasone by esterases, *in vivo* [8]. Dextran-500 is an osmotic agent used to reduce fluid loss in high MWCO probes which has been shown to cause no additional inflammation at the implant site [6]. Bovine serum albumin (BSA) is used in the perfusion fluid to prevent non-specific binding to microdialysis materials [2]. Prior to implantation, microdialysis probes were flushed with sterile Ringer’s. On the third day post implantation, animals were placed in a CMA 120 freely moving animal system. An initial flush was performed starting at 3 µL/min and the flow rate was reduced by 0.5 µL/min every 5 mins until a flow rate of 1 µL/min was reached. Collections were then performed in 1 hr increments for 6 hours. All collection vials contained Halt Protease Inhibitor, were immediately placed on ice following collection, and stored at -80ºC following the entire collection period. Following the final collection hour, a final flush was performed at 3 µL/min for 30 mins. For the final flush, Ringer’s or Ringer’s + 20 µg/mL Dex were used as perfusion fluids for the control and treatment, respectively. On the seventh day post implantation, the animal was euthanized and the probe as well as the tissue surrounding the probe were harvested as previously described [6]. The probe and tissue surrounding it were then stored in optimal cutting temperature (OCT) solution and flash frozen using liquid nitrogen for histological and immunohistochemical analyses or the tissue surrounding the membrane was removed and stored in RNAlater for qRT-PCR analyses.

**qRT-PCR Procedure**

Tissue immediately surrounding (~1-2 mm) was harvested from the membrane portion of the probe and placed in RNAlater and stored on ice. Harvested tissue was stored at 4ºC until RNA was extracted. RNA was then extracted from the tissue and purified using the Trizol method and an RNeasy Minikit (Qiagen, Venlo, Limburg). The integrity of the RNA was confirmed by comparing the 18s and 28s band ratios via gel electrophoresis. RNA was converted to cDNA using a high capacity reverse transcription kit (Life Technologies, Carlsbad, CA). Gene expression ratios were determined using Taqman gene expression assays (Life Technologies, Carlsbad, CA) using a 7500 Real Time PCR instrument (Life Technologies, Carlsbad, CA). Gene expression data were analyzed using REST Gene
Quantification Software with Taf9b (Transcription initiation factor TFIIID subunit 9B) being used as a control gene.

CCL2 Quantification

CCL2 was quantified in dialysates collected from both the control and treatment probes for each of the 6 collection hours as well as the initial flush period using a BD OptEIA™ rat MCP-1 ELISA set (BD Biosciences, San Jose, CA). The ELISA was performed per the manufacturer's protocol with the exception that 50 µL of sample were used and the remaining reagents were adjusted accordingly. Briefly, a 96 well plate was incubated overnight with detection antibody, the plate was then blocked using assay diluent for 1 hour, standards and samples were then loaded and incubated for two hours, substrate solution was then incubated for 30 mins, and the reaction was stopped using stop solution (1M phosphoric acid). All the appropriate wash steps were performed per the manufacturer's protocol. Absorbance was then read at 450 nm and a reference of 570 nm was used via a Tecan Infinite M200 (Tecan, Maennedorf, Switzerland).

Immunohistochemistry Procedure

Monoclonal antibodies specific for Cluster of Differentiation (CD) 68 and CD163 (Santa Cruz Biotechnology, Dallas, TX) were used as a pan macrophage marker and M(Dex) marker, respectively. Fluorophore conjugation to the antibodies was performed using APEX Antibody Labeling Kits (Life Technologies, Carlsbad, CA). Alexa Fluor 488 was conjugated to CD68 and Alexa Fluor 647 was conjugated to CD163. Tissue sections were cut to a thickness of ~5 µm using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany). Sections were then mounted on microscope slides. Sections were fixed by placing them in cold methanol for 20 mins at -20ºC. The methanol was allowed to dry from the slide and the tissue sections were encircled with a hydrophobic pen. Blocking solution (PBS + 2% v/v horse serum + 0.05%(w/v) Tween 20 + 0.0001% (w/v) BSA) was then applied and slides were incubated
in a humidity chamber for 30 mins at room temperature. Once blocking was completed, slides were washed 4 times for 10 mins each wash in phosphate buffered saline. Wash solution was then wicked away from the tissue sections using Kimwipes. Antibodies diluted in blocking solution were then applied to tissue sections, CD68 (1:125) and CD163 (1:50), and sections were incubated overnight at 4ºC in a humidity chamber. Slides were then washed 4 times for 10 min per wash and excess wash solution was wicked away using a Kimwipe. A nuclear counterstain (Hoechst 34580) was then applied to the tissue sections and allowed to incubate at room temperature for 12 mins in the dark. Slides were then washed 3 times for 5 mins/wash and excess solution was wicked away using a Kimwipe. VECTASHIELD® (Vector Laboratories, Burlingame, CA) mounting media was then applied to tissue sections; cover slips were placed on slides and sealed. All images were obtained using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were then subjected to manual counts of cells staining positive for CD68 as well as CD68 and CD163. Using the data from the manual counts, the percentage of M(Dex) macrophages was determined.

Histological Analyses

Tissue sections (~5 µm) were obtained using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on microscope slides. Tissue sections were fixed in 10% neutral buffered formalin. Tissue sections were subjected to hematoxylin and eosin (H&E) as well as Masson's Trichrome stains. Images were obtained using a Zeiss Axioskop II plus microscope (Carl Zeiss Inc., Thornwood, NY) with Cannon EOS Digital Software for Rebel T2i camera.

Statistical Analysis

A Shapiro-Wilk test was performed to determine normal distribution of the data. For CCL2 concentrations, a Kruskal-Wallis ANOVA was performed with a Bonferroni post-hoc test to determine
significance. For the percent M(Dex) cells present, a two sample t-test was performed to determine significance. Origin 2015 statistical software was used for all statistical tests.

Effects of IL-10 Delivery at a Non-Degradable Implant Site on Macrophage Activation State via Microdialysis Sampling in Rats

IL-10 (10 ng/mL) Perfusion

Two microdialysis probes were implanted in the subcutaneous space of male Sprague Dawley rats. One probe served as a control utilizing Ringer’s + 6% Dextran-70 + 0.1% BSA as a perfusion fluid while the other served as a treatment utilizing the same perfusion fluid with 10 ng/mL IL-10 added. After probe implantation, animals were moved to a CMA 120 freely moving animal collection system. An initial flush was performed starting at a flow rate of 3 µL/min and was reduced by 0.5 µL/min every 5 minutes until a flow rate of 1 µL/min was reached. A one hour perfusion was performed to deliver IL-10 to the treatment side. After the first hour of perfusion, the treatment perfusion fluid was changed to Ringer’s + 6% Dextran-70 + 0.1% BSA, same as the control. Perfusions were performed in 1 hour intervals for the next 6 hours. Following collections, a final flush was performed with HPLC water (filter sterilized through 0.2 µm PES filter) at a flow rate of 3µL/min for 30 minutes. Dialysates were stored on ice initially, then at -80°C until an ELISA was performed to determine the concentration of CCL2. Animals were then returned to housing where they remained until 7 days post-implantation. On day 7 post-implantation, the animals were euthanized and the probe as well as the tissue immediately surrounding the probe was harvested for histological and immunohistochemical analyses, as previously stated [4] (Fig. 3).
Two microdialysis probes were implanted in the subcutaneous space of male Sprague Dawley rats. One probe served as a control utilizing Ringer's + 1% Dextran-70 + 0.1% BSA as a perfusion fluid while the other served as a treatment utilizing the same perfusion fluid with 10 ng/mL IL-10 added. After probe implantation, animals were moved to a CMA 120 freely moving animal collection system. An initial flush was performed starting at a flow rate of 3 µL/min and was reduced by 0.5 µL/min every 5 minutes until a flow rate of 1 µL/min was reached. A one hour perfusion was performed using the 1% Dextran solution. After the first hour of perfusion, both the treatment and control perfusion fluids were changed to Ringer's + 6% Dextran-70 + 0.1% BSA. Perfusions were performed in one hour intervals for seven hours. Following collections, a final flush was performed with HPLC water (filter sterilized through 0.2 µm PES filter) at a flow rate of 3µL/min for 30 minutes. Animals were then returned to housing where they remained until 7 days post-implantation. On day 7 post-implantation, the animals were euthanized and the probe as well as the tissue immediately surrounding the probe (Fig 3) was harvested for histological and immunohistochemical analyses. Dialysates were stored on ice initially, then at -80°C until an ELISA was performed to determine the concentration of IL-10.
Fig. 3. Image showing tissue removed from microdialysis probes. Image taken by author.
Four Day IL-10 Infusion

Two microdialysis probes were implanted in the subcutaneous space of male Sprague Dawley rats. One probe served as a control utilizing Ringer's + 6% Dextran-70 + 0.1% BSA as a perfusion fluid while the other served as a treatment utilizing the same perfusion fluid with 10 ng/mL IL-10 added. After probe implantation, animals were moved to a CMA 120 freely moving animal collection system. An initial flush was performed starting at a flow rate of 3 µL/min and was reduced by 0.5 µL/min every 5 minutes until a flow rate of 0.5 µL/min was reached. A one hour perfusion was performed to deliver IL-10 to the treatment side. After the first hour of perfusion, the treatment perfusion fluid was changed to Ringer's + 6% Dextran-70 + 0.1% BSA, same as the control. Perfusion was performed in 1 hour intervals for the next 7 hours at a flow rate of 1 µL/min. Following collections, a final flush was performed with HPLC water (filter sterilized through 0.2 µm PES filter) at a flow rate of 3 µL/min for 30 minutes. Dialysates were stored on ice initially, then at -80°C until an ELISA was performed to determine the concentration of CCL2. Animals were then returned to housing. The one hour IL-10 infusion was performed every day until the fourth day post-implantation while the seven hour collections were performed on the day of implantation and 4 days post-implantation. On day 4 post-implantation, the animals were euthanized and the probe as well as the tissue immediately surrounding the probe was harvested for histological and immunohistochemical analyses.

IL-10: 200 and 1200 µg/mL Infusions

Two microdialysis probes were implanted in the subcutaneous space of male Sprague Dawley rats. One probe served as a control utilizing Ringer's + 6% Dextran-500 + 0.1% BSA as a perfusion fluid while the other served as a treatment utilizing the same perfusion fluid with either 200 ng/mL or 1200 ng/mL IL-10 added. After probe implantation, animals were moved to a CMA 120 freely moving animal collection system. An initial flush was performed starting at a flow rate of 3 µL/min and was reduced by 0.5 µL/min every 5 minutes until a flow rate of 1 µL/min was reached. On the day of implantation and four
days post-implantation, collections were performed for five hours in one hour intervals with the IL-10 being delivered for the entire time in the treatment probe. Following collections, a 30 minute flush at 3 µL/min on the day of implantation was performed but not on day 4 post-implantation. On days 1, 2, 3 post-implantation, probes were infused for one hour at 1 µL/min followed by a 30 minute flush at 3 µL/min. The final flush was performed with HPLC water (filter sterilized through 0.2 µm PES filter). Animals were then returned to housing where they remained until 7 days post-implantation. On day 7 post-implantation, the animals were euthanized and the probe as well as the tissue immediately surrounding the probe was harvested for histological and immunohistochemical analyses.
References


Chapter 3

Comparison of microdialysis sampling perfusion fluid components on the foreign body reaction in rat subcutaneous tissue

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1. Introduction

Microdialysis sampling is a widely used in vivo collection technique that has been used for more than 35 years for numerous life science applications [1-3]. This diffusion-based separation method uses an isotonic perfusion fluid that flows through inlet tubing into an inner cannula, the inner fiber lumen of a semi-permeable membrane, an outer cannula, and exits via an outlet tube where the dialysate is collected (Fig. 1). These devices are then implanted into tissue space to allow for collection of solutes from the extracellular fluid (ECF). The solute concentration gradient that exists between the perfusion fluid inside the probe and the surrounding extracellular fluid allows analytes smaller than the membrane molecular weight cutoff (MWCO) to diffuse into the membrane lumen to be collected and then quantified [4]. The primary reasons for the success and variety of biomedical applications of microdialysis sampling include 1) it is minimally invasive allowing collections to be performed from targeted tissue sites in awake and freely-moving animals; 2) analytically-clean samples that require either no or minimal sample preparation are collected allowing a wide variety of chemical analysis schemes to be applied [5]; 3) animal numbers can be reduced since the animal in which the probe is placed serves as its own control significantly reducing the number of animals needed in different studies.
Fig. 1. Microdialysis probe schematic.
Microdialysis sampling was originally developed to collect small hydrophilic molecules such as the catecholamine and amino acid neurotransmitters. With the advent of commercially-available high MWCO membranes incorporated into microdialysis probes, it is now possible to collect peptides and proteins of biological significance including cytokines [6-8]. This has opened a wide range of possibilities for researchers investigating multiple disease states in different tissues that are believed to incur dysregulated cytokine function [8-15].

Prior to the use of high MWCO membranes during microdialysis sampling, it was common practice to use a saline solution, such as Ringer’s or Ringer’s-Krebs, as a perfusion fluid since these solutions contain a balance of different ions (Na⁺, K⁺, Ca²⁺ and Cl⁻) believed to exist in the extracellular fluid space [16]. When Ringer’s solution is the perfusion fluid through a high MWCO membrane, a significant reduction in expected fluid volumes can be observed due to a difference in hydrostatic pressure causing the perfusion fluid to pass through the membrane pores. This phenomenon is called ultrafiltration and would be expected for high MWCO ultrafiltration membranes, which is defined as any membrane with a MWCO of greater than 50 kDa.

Microdialysis sampling of large bioactive proteins including cytokines can be fraught with two major difficulties – ultrafiltration and non-specific adsorption to the device materials. Ultrafiltration is problematic because fluid is lost across the membrane into the tissue resulting in lower than expected sample volumes. This causes difficulties with chemical analysis techniques such as ELISA that have defined volume specifications. Additionally, the loss of fluid into the tissue space and its effect on tissue physiology is poorly understood. Non-specific adsorption is problematic especially with large bioactive proteins since their concentrations are often in the pg/mL range. If non-specific adsorption is not reduced by inclusion of albumin as a blocking protein, proteins in such low concentrations may adsorb to the probe materials precluding their quantitation in dialysates.

When using microdialysis membranes with 100 kDa MWCO or greater, colloids (high molecular weight dextrans, albumin, or a combination of the two) are added to the perfusion fluid to reduce ultrafiltration via an increase solution osmotic pressure within the membrane lumen [17, 18]. Bovine
serum albumin (BSA) and human serum albumin have also been used to reduce ultrafiltration and non-specific adsorption [19, 20].

An unexplored area of research is whether these added colloids to the microdialysis perfusion fluid affect the surrounding tissue in deleterious ways. Fluid loss across the membrane may cause edema or other tissue damage. Albumin (~ 66 kDa), dextran-60, and dextran-70 can diffuse through the 100 kDa MWCO into the tissue. Bovine serum albumin is commonly used in rat studies since it can be procured at a significantly lower cost than rat serum albumin. However, there are considerable homology differences between these two albumins that could lead to the potential for immune response.

To date no studies have been performed to determine the effects of commonly used perfusion fluid reagents, i.e. Ringer's solution, Dextran-70, or BSA, on tissue surrounding implanted microdialysis probes. Likewise, it has not been determined if there are differences in the tissue reactions to perfused microdialysis probes vs. non-perfused (simply implanted) probes. These issues related to microdialysis sampling are critical to elucidate since microdialysis sampling is being widely used to collect cytokines and other bioactive proteins for various disease states. In this work, we demonstrate that a significant portion of the trauma caused at the site of a microdialysis probe appears to be due to the act of perfusion as compared to the implantation process.

2. Results

2.1 Probe vs. Ringer's filled probe

Fig. 2 shows the (H&E) and Masson's Trichrome images for the probes that were either just implanted (no solution in the membrane fiber lumen) or allowed to have Ringer's solution reside in the inner fiber lumen. Cells are seen interrogating both the probe (Fig. 2a) and the Ringer's fluid-filled probe (Fig. 2b). No significant difference in the density of cells was observed surrounding either probe. The Masson's Trichrome stained sections show more collagen surrounding the probe only (Fig. 2c) as compared to the Ringer's fluid-filled probe (Fig. 2d). There appears to be some vascularity surrounding the probe only (top right of Fig. 2c) whereas no vascularity is seen surrounding the fluid-filled probe (Fig.
2d). When these images are compared to those for the probes that were continuously perfused with
Ringer's solution, the similarities in cell density, cellular organization, and collagen formation are striking.
For this reason, all further treatment comparisons are made in relation to probes through which Ringer's
solution was perfused.
Fig. 2. Probe vs. Ringer’s Filled Probe. 10x Magnification. White size bar represents 100 µm. Top: H&E stain (a) Microdialysis probe implanted in the subcutaneous tissue (no perfusion fluid). (b) Microdialysis probe with Ringer’s solution residing in the fiber lumen. Bottom: Masson’s Trichrome stain. (c) Microdialysis probe implanted in the subcutaneous tissue (no perfusion fluid). (d) Microdialysis probe with Ringer’s solution residing in the fiber lumen.
**Fig. 3.** Ringer’s vs. Ringer’s + Dextran-70. 10x Magnification. White size bar represents 100 µm. Top: H&E stain (a) Microdialysis probe with Ringer’s solution perfused. (b) Microdialysis probe with Ringer’s + 6% (w/v) Dextran-70 solution perfused. Bottom: Masson’s Trichrome stain. (c) Microdialysis probe with Ringer’s solution perfused. (d) Microdialysis probe with Ringer’s + 6% (w/v) Dextran-70 solution perfused.
2.2 Ringer’s vs. Ringer’s with 6% Dextran-70

With the H&E stain, the probe which had Ringer’s solution perfused through shows cells near the membrane pores of the probe (Fig. 3a). However, these cells, while well organized, are less densely packed around the probe as compared to the probe which had Ringer’s with 6% Dextran-70 perfused (Fig. 3b). The Masson’s Trichrome staining shows far less collagen found around the Ringer’s-perfused probe (Fig. 3c) as compared to the Ringer’s with 6% Dextran-perfused probe (Fig. 3d), and the collagen seen around the Ringer’s + Dextran probe consists of thicker strands and is more densely packed than is seen around the Ringer’s probe. Furthermore, there is a ~25–50 µm thick layer of densely packed cells immediately surrounding the probe membrane of the Ringer’s with 6% Dextran probe which is lacking in the Ringer’s-perfused probe site.

Occasionally, probes will fail and fluid flow will no longer be observed exiting the fluid line. Suspecting that the more pronounced inflammatory response observed for the Dextran-70 infused probes may be due to Dextran-70 diffusing out of the probe, we decided to let probes that failed remain in the tissue followed by histological analysis. Fig. 4 shows both the H&E and the Masson’s Trichrome stain for a microdialysis probe implant that had failed. A significant amount of cellular material is located at the probe site with the failed probes indicating a nearly necrotic response (Fig. 4a). The space is significantly inflamed with little collagen remaining as shown in Fig. 4b.
Fig. 4. Probe Failure with Dextran-70. 10x Magnification. White size bar represents 100 µm.
2.3 RSA vs. BSA

Fig. 5a and b show the H&E staining for the RSA- vs. BSA-infused probes. Cells are seen near the pores of the membrane (Fig. 5a and b). While these cells are well organized, they are not as densely packed as is seen with Dextran. The density of cells surrounding both probes closely resembles that for tissue surrounding the probes through which only Ringer’s was perfused. Additionally, the amount of collagen seen around each probe is also similar to that with only Ringer’s perfused (Fig. 5c and d).
Fig. 5. RSA vs. BSA. 10x Magnification. White size bar represents 100 µm. Top: H&E stain (a) Microdialysis probe perfused with Ringer’s + 3% (w/v) BSA solution. (b) Microdialysis probe perfused with Ringer’s + 3% (w/v) RSA. Bottom: Masson’s Trichrome stain. (c) Microdialysis probe perfused with Ringer’s + 3% (w/v) BSA solution. (d) Microdialysis probe perfused with Ringer’s + 3% (w/v) RSA.
2.4 Ringer’s + Dextran-500 vs. Ringer’s + Dextran-500 + BSA

The H&E stains for probe explants through which Ringer’s and Dextran-500 vs. Ringer’s, Dextran-500, and BSA are shown in Fig. 6a and b. Cells appear to be embedded within the membrane pores in the H&E stained tissue (Fig. 6a and b). There appears to be no difference in the cellular density around the two probes. The density of cells seen around both probes seems to be the same as is seen in response to Ringer’s only perfused probes and probes perfused with Ringer’s and either BSA or RSA. Likewise, cells seen around these probes seem to be less densely packed than what is seen around the probes which had Dextran-70 infused. Also, there is no layer of extremely densely packed cells immediately surrounding the probe as is seen in the Dextran-70 infused probes. The collagen content observed around the two probes is also similar, suggesting there is no difference with respect to the tissue reaction to this infusion fluid between the two treatments (Fig. 6c and d). The prevalence of collagen seen around these probes is similar to the collagen seen around the Ringer’s only probe as well as the Ringer’s with either BSA or RSA.
Fig. 6. Ringer’s + Dextran-500 vs. Ringer’s + Dextran-500 + BSA. 10x Magnification. White size bar represents 100 µm. Top: H&E stain (a) Microdialysis probe perfused with Ringer’s + 6% (w/v) Dextran-500. (b) Microdialysis probe perfused with Ringer’s + 6% (w/v) Dextran-500 + 0.1% (w/v) BSA solution. Bottom: Masson’s Trichrome stain. (c) Microdialysis probe perfused with Ringer’s + 6% (w/v) Dextran-500. (d) Microdialysis probe perfused with Ringer’s + 6% (w/v) Dextran-500 + 0.1% (w/v) BSA solution
3. Discussion

The inception of microdialysis sampling was based on an analogy that an implanted microdialysis sampling probe behaves like a benign capillary in the tissue allowing free diffusion of solutes across its porous semi-permeable membrane [21]. Despite over 14,000 publications that have used microdialysis sampling, relatively few have investigated how the implant procedure or the perfusion fluids influence the tissue surrounding the implant. The majority of the research literature that has described tissue reactions to microdialysis sampling has been focused on neural tissue [22, 23]. Ultrastructural studies have shown tissue disruptions to be seen as far as 1.4 mm away from the implant site in the brain [24]. However, histological analyses of microdialysis probes implanted into other tissues have also revealed the expected presence of inflammatory cells surrounding the probe implant.

Our group has previously reported, microdialysis probe implants in the subcutaneous space that only had phosphate buffered saline solution passed through the probes showed a dense, avascular, cellular layer surrounded by a collagenous, vascular layer at 14 days post implantation [25]. A long-term investigation of implanted dialysis fibers (not microdialysis probes and not infused) showed that at 3 weeks the probes were encapsulated by a highly cellular disorganized capsule, at 6 weeks a highly cellular yet highly organized capsule, and at 12 weeks a mature fibrotic capsule was seen characterized by low cellularity and dense bands of collagen on the membrane surface [26]. These results are consistent with Sanders' study which showed that a height threshold of 5.9 µm exists and is the most important factor in determining the formation of a fibrotic capsule around an implant [27]. The microdialysis probes used in this study had an outer diameter of 500 µm which exceeds the 5.9 µm reported as the threshold indicating that a fibrotic capsule would be expected. However, while the effects of implanting different types of polymeric membranes have been investigated, no investigations have looked at the effects of perfusion fluid components i.e. Dextran, BSA, and RSA with respect to their effect on tissue responses to the microdialysis probe implant.
Our work is the first to demonstrate that perfusion fluid additives used for microdialysis sampling lead to different inflammatory responses. While the insertion of a microdialysis probe is considered to be minimally invasive, it must be recognized that there is a certain amount of trauma associated with the implantation procedure. This can be seen by the densely packed cells surrounding an implanted microdialysis probe. These cells are responsible for the initial clearance of any cellular debris, the formation of the fibrotic capsule, and the eventual wound healing process. While the trauma due to probe implantation cannot be avoided, it is important to minimize the trauma due to the sampling procedure to gain a better understanding of the biochemical processes occurring at the implant site. While we have shown that there are differences in the cellular response seen surrounding a Ringer's perfused probe as compared to an implanted microdialysis probe where no perfusion occurs, they can be considered negligible. Given the nature of microdialysis sampling experiments in that the technique is used to sample from tissue, it is unavoidable to prevent a perfusion and thus the low-level inflammatory response to the perfusion fluid will be present.

The inclusion of high MWCO membranes into microdialysis probes has allowed for the collection of larger molecular weight solutes such as bioactive proteins. The tradeoff is that ultrafiltration, or loss of fluid across the membrane pores, can become a significant problem. To circumvent this problem, osmotic agents such as Dextran-60 (~60 kDa) or -70 (~70 kDa) were being added at high concentrations (1 to 4% w/v). These agents have since been widely used in microdialysis sampling with 100 kDa MWCO membranes [11, 15, 28-42]. Our results show that Dextran-70, when allowed to enter the extracellular space, causes an intense inflammatory response. While theoretically the use of Dextran-70 in perfusion fluids could result in the dextran leaving the probe, it has never been determined if its use results in an increased inflammatory response at the implant site. We found that Dextran-70 diffuses out of the probe resulting in increased inflammation at the probe site. This response cannot be seen as negligible and must be considered when collecting bioactive proteins such as the cytokines.

Due to the fact that Dextran-70 was shown to invoke an inflammatory response when perfused through the probe, alternative osmotic agents were tested. The use of BSA is commonly used in perfusion fluids at high concentrations (3.5-10% w/v) to reduce fluid loss [19]. Simultaneously, low concentrations of BSA (~0.1% w/v) have been used in perfusion fluids to prevent non-specific binding of...
proteins to the microdialysis outlet tubing. Theoretically, BSA (~66,000 Da) could exit the probe yet its effects on the tissue surrounding the implanted microdialysis probe have never been studied. Our results show that the tissue surrounding a BSA treated probe shows no appreciable difference to tissue seen surrounding a RSA treated probe or a Ringer's only perfused probe. This suggests that there is no significant inflammatory reaction seen in response to the BSA.

While the use of BSA as an osmotic reagent was shown to have no inflammatory effect on the tissue, it was shown to give lower fluid recoveries (data not shown) as compared to Dextran-70. Dextran-500 has been shown to give fluid recoveries of 100% when used at lower flow rates [43]. In fact, we have found that Dextran-500, when used at 6% w/v, yields 100% fluid recovery in vitro (data not shown). Dextran-500, with or without 0.1% BSA, has also been shown to result in no additional inflammatory response as compared to Ringer's only perfused probes in vivo. While the data presented here shows Dextran-70 causes an inflammatory response in vivo, Dextran-500 appears to be a viable alternative as it retained the fluid recovery without causing an excessive inflammatory response.

Conclusion

While microdialysis sampling has gained popularity, there have been no studies reporting the effects of perfusion fluid components on inflammation surrounding the probe. In this study we investigated the effects of the actual perfusion of fluid through the probe, Ringer's solution, Dextran-70, BSA, RSA, and Dextran-500 on the tissue surrounding the probe. What we found shows that the use of Dextran-70 in the perfusion fluid results in some of the Dextran diffusing into the ECM. The result of this Dextran diffusing out is highly inflamed tissue seen surrounding the probe. For this reason, we would suggest that Dextran-70, or any lower molecular weight Dextran, not be used in perfusion fluids during microdialysis sampling. Instead, we would suggest the use of Dextran-500 as this does not diffuse out of the probe and appears to not cause additional inflammation on top of the inflammation that is caused by the surgical implantation process.
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References


Localized Delivery of Dexamethasone-21-Phosphate via Microdialysis Implants in Rat Induces M2c Macrophage Polarization and Alters CCL2 Concentrations

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1. Introduction

Macrophages play key roles in both innate and adaptive immunity. Their importance to the field of biomaterials has been recognized for decades [1, 2]. Macrophages are known to play opposing roles such as inflammatory vs anti-inflammatory and tissue destruction vs tissue remodeling. The macrophage role is driven by micro-environmental chemical signals present within the extracellular matrix. These cues result in different macrophage polarization states [2-4], which comprise a continuum of macrophage phenotypes. In the case of bacterial infections, pro-inflammatory cytokines, such as Interferon-gamma (IFN-γ) and Tumor Necrosis Factor-alpha (TNF-α), are released, inducing an M1-type macrophage. This macrophage phenotype is responsible for a Th1 type response resulting in a pro-inflammatory, phagocytic response that effectively clears pathogens. M1 macrophages are further characterized as producing high levels of pro-inflammatory cytokines including interleukin (IL)-1β, IL-6, IL-12 and TNF-α [5-7].

At the other end of the polarization continuum are the M2 macrophages, which are subdivided into three classifications: M2a,b,c [6]. The M2a macrophages are induced by IL-4 and/or IL-13. This polarization state is thought to promote a Th2 type response and the formation of foreign body giant cells (FBGC), as well as clearance of parasites [6]. M2a macrophages are characterized as producing collagen type VI, fibronectin, Transforming Growth Factor Beta (TGF-β) and Transforming Growth Factor Beta Induced (TGFβI) [8]. M2b macrophages are induced by FC gamma receptors and toll-like receptor activation. M2b macrophages are characterized as producing high levels of the anti-inflammatory cytokine, IL-10, but also high levels of inflammatory cytokines such as IL-1β, IL-6 and TNF-α [2]. M2c macrophages are induced by IL-10 and glucocorticoid steroids and are characterized as producing high levels of IL-10 and low levels of inflammatory cytokines such as IL-1β, IL-6 and IL-12 [5]. M2c macrophages are thought to be anti-inflammatory, pro-wound healing, and pro-tissue remodeling cells.

A recent publication by Murray et al. provides suggestions for a nomenclature change for macrophage polarization states [9]. These authors suggest that instead of using the M1, M2 nomenclature that the polarizing agent be used to identify the macrophage such as M(LPS) for LPS induced macrophages as a replacement for M1 or M(GC) for macrophages induced by glucocorticoids.
instead of M2c [9]. In accord with these newly suggested guidelines, this paper will use the new nomenclature for descriptions of specific subtypes of macrophages elicited in this study.

Chemokine (C-C motif) ligand 2 (CCL2), also known as monocyte chemoattractant protein-1, is a 13kDa monomeric chemokine [10]. Chemokines are responsible for the recruitment of leukocytes such as monocytes, neutrophils, and lymphocytes, to a wound via chemokine gradients. CCL2 is known to be one of the primary chemokines responsible for the migration and infiltration of monocytes to a wound site [11]. CCL2 has been implicated in biomaterials contexts for attracting monocytes to the site of an implant [12]. Once at the wound site, monocytes differentiate to macrophages. Depending on the cytokine signals present at the wound site, these macrophages will then polarize to either a predominantly M1 or M2 state.

Glucocorticoid steroids are a class of steroids that bind to the glucocorticoid receptor which regulates inflammation and have been used extensively to treat inflammatory conditions [13, 14]. Dexamethasone, a synthetic glucocorticoid steroid, has been shown to regulate pro-inflammatory cytokines in two ways: (1) the glucocorticoid receptor interacts directly with glucocorticoid response elements in the promoter region of genes, thereby inhibiting gene expression [15, 16], (2) by interfering with transcription factors nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and Activator Protein 1 (AP-1) [17-19]. Glucocorticoids have been shown to reduce the transcription of several genes including CCL2, IL-6, and TNF-α and inducible Nitric Oxide Synthase (iNOS) [20] as well as reducing protein levels of CCL2 in plasma and granuloma tissue [13, 21, 22], IL-6 in plasma and sponge exudate [23, 24], and TNF-α in sponge exudate [24]. In human monocytes, dexamethasone has been shown to induce a specific anti-inflammatory subtype marked by the up-regulation of many anti-inflammatory genes and the down regulation of pro-inflammatory genes [25].

Dexamethasone has been widely used in the field of biomaterials to reduce inflammatory responses to implanted materials [26, 27]. There are numerous examples of the use of dexamethasone to reduce inflammation in a biomaterials context [14, 28-31]. However, the endpoint analyses in these studies are frequently histological analysis of H&E and Masson’s Trichrome stains.
Microdialysis sampling is a widely-used, minimally-invasive, diffusion-based *in vivo* collection technique in which a probe, with a defined molecular weight cut-off (MWCO) semi-permeable membrane, is surgically implanted allowing in situ collection of many different solutes [32]. An isotonic fluid with a pH and ionic strength matching the extracellular space (ECS) is perfused through the membrane lumen in contact with the extracellular fluid allowing free solute exchange and collection of solutes. Since a solute concentration gradient exits between the perfusion fluid and the ECS, solutes diffuse into the membrane lumen and the resulting dialysate is collected and quantified [33]. This allows microdialysis to be used to simultaneously collect analytes from the ECS as well as deliver modulators to the ECS provided they are smaller than the MWCO of the membrane [34]. Dexamethasone-21-phosphate is a water-soluble derivative of the hydrophobic dexamethasone that is converted to dexamethasone *in vivo* by esterases [35].

Macrophage polarization in the context of biomaterials has been identified as an important process to consider when developing materials that can integrate with the host and provide improved outcomes [36]. Much effort has been focused on reducing the FBR to implanted sensors. Thus, having means to control this outcome via macrophage polarization that provides acceptable outcomes would be highly beneficial to many biomaterials applications. While there has been significant work on macrophage polarization *in vitro*, there are relatively few *in vivo* reports. Knowing that M2c macrophages are pro-wound healing and pro-tissue remodeling, it has been postulated that if modulators can be used to shift macrophages to a predominately M2c state, the longevity of the implant would be expected to increase.

In this work, microdialysis sampling probes have been used to locally deliver dexamethasone-21-phosphate at an implant site to alter the cytokine environment toward polarizing macrophages to their M (GC) state. To assess if cytokine responses were altered from the dexamethasone treatment, we quantified CCL2 protein levels in dialysates and performed gene expression studies combined with immunohistochemistry of the tissue surrounding the microdialysis probe implant.
2. Results

2.1 qRT-PCR

Transcription levels of seven different genes (Arginase (Arg2), CCL2, CD163, IL-6, iNOS2, TGF-β, and TNF-α) were analyzed using qRT-PCR from the extracted tissue in which the microdialysis probe was implanted (Figure 1). CCL2, IL-6, TGF-β, and TNF-α were chosen as markers to determine if Dex had any effect on the cytokine environment which would be expected in the FBR. Both IL-6 and CCL2 transcript levels were found to be significantly reduced, 2.7 fold (p≤0.01) and 3.4 fold (p≤0.001) respectively, in response to the Dex treatment as compared to controls. The transcription levels between TNF-α and TGF-β showed no significant difference between Dex treatments and controls. CD163, Arg2, and iNOS2 were chosen to investigate the effects of Dex on known differential markers for macrophage polarization state at the implant site. Transcript levels of CD163, Arg2, or iNOS2 were unchanged in response to Dex treatment as compared to controls.
Fig. 1. Relative gene expression ratios from excised tissue in response to Dex (20 µg/ml) infused as compared to controls (n = 3). Error bars represent the SEM with *p ≤ 0.01, **p ≤ 0.001.
2.2 CCL2 quantification

Figure 2 shows the CCL2 concentrations in dialysates from control and treatment probes. On the day of implantation (Fig 2a), CCL2 concentrations from the control probes steadily rose over the 6 hour collection period ranging from ~100 pg/mL in the first hour of collection to ~1300 pg/mL by the 6th hour. In dialysates collected from the Dex-treatment probes, CCL2 concentrations were found to be ~100 pg/mL during the 1st hour, and peaked during hours 2 through 4 to a level of approximately 400 pg/mL. During hours 5 and 6, CCL2 concentrations decreased to ~200 pg/mL. There was no significant difference in CCL2 levels found in control and treatment dialysates in the flush or hours 1 through 4. In hours 5 and 6, CCL2 levels in the treatment were found to be significantly lower (~200 pg/mL) than in control (~1200 pg/mL).

One day post implantation (Fig 2b), CCL2 concentrations collected from control probes ranged from 1100 pg/mL to 600 pg/mL during the collection period (1 to 6 hrs), decreasing from 1-3 and stabilizing over hours 4-6 but showing no statistical difference over the collection period. CCL2 concentrations collected from treatment probes decreased from ~750 to ~300 pg/mL during hours 1 through 3, remained constant in hour 4 and decreased in both hours 5 and 6 to a level similar to the first hour of collection on the day of implantation. No significant difference was seen between CCL2 concentrations collected from control and treatment probes during the flush or hours 1, 2, and 4, but CCL2 concentrations were found to be significantly lower in the treatment probes as compared to the control probes in hours 3, 5, and 6.

Two days post implantation (Fig 2c), CCL2 concentrations from control probes ranged from ~750 pg/mL to ~400 pg/mL and seemed to remain stable over the collection period showing no statistically significant difference. CCL2 concentrations from treatment probes were ~450 pg/mL during the 1st hour of collection and steadily decreased during the 6 hour collection period. During hours 3 through 6, CCL2 concentrations in dialysates collected from treatment probes were significantly lower than CCL2 concentrations in dialysates collected from control probes. Furthermore, by the 6th hour of collection, two
days post implantation, Dex reduced CCL2 concentrations from the treatment probes to the level that was seen during the initial flush on the day of implantation.
Fig. 2. Bar graphs showing the concentration of CCL2 collected from the control and treatment probes perfused at 1 µl min⁻¹ for each hour on the day of implantation (top, 2a), 1 day post implantation (middle, 2b), and 2 days post implantation (bottom, 2c). Error bars represent the standard error of the mean with *p ≤ 0.05 for n = 8 (top) and n = 6 (middle, bottom) animals. F represents the initial flush period.
2.3 Immunohistochemistry

Immunohistochemistry was used to identify macrophages in the tissue surrounding an explanted microdialysis probe using a CD68 antibody. To determine if Dex caused macrophages to convert to the M(GC) polarization state and formerly called M2c state, a CD163 antibody was used to immunohistochemically stain these types of macrophages. At lower magnifications (20x) there appeared to be no CD163+ cells immediately surrounding the microdialysis probe (Figure 3A). At the lower magnification, pockets of CD163+ cells were seen more distal (350-500 µm) to the microdialysis probe in both control and treatment tissue, with these cells appearing to be more distal to the control probe (Figure 3A). However, at higher magnification (40x) it was found that CD163+ cells are present in both control and treatment tissue immediately surrounding the microdialysis probe (Figure 3B). Further, in the higher magnification images, it can be seen that there is a greater amount of CD163+ cells surrounding the treatment probe than the control (Figure 3B). The percentage CD163+ cells found in the 40x images were calculated for both treatment and control. The percentage of CD163+ cells found was significantly greater in the treatment tissue than in the control tissue (Figure 4).
Fig. 3. Immunohistochemical staining for CD68 (green), CD163 (red) and nuclei (blue) in tissue surrounding a microdialysis probe. Overlapping colors represent M(GC) macrophages. (A) Images of both control and treatment tissue surrounding a microdialysis probe membrane, 20x magnification. (B) Images of both control and treatment tissue surrounding a microdialysis probe membrane, 40x magnification. (C) Images of isotype controls, 20x magnification. (D) Images of isotype controls at 40x magnification. M indicates the microdialysis probe membrane. Scale bars in lower right of each image represent 100 µm.
Effect of Dex-21-P on the Percentage of M2c Macrophages Surrounding a Microdialysis Membrane

Fig. 4. Graphical representation of the percentage of CD163⁺ (M(GC)) cells found in both control and treatment tissue. Error bars represent the standard error of the mean. n = 5 where n is equal to the number of measurements *p ≤ 0.05.
2.4 Histological analyses

Histology (H&E and Masson’s Trichrome) was performed to determine the effects of Dex on the cellular density and collagen amounts surrounding the probe (Figure 5). The Dex-treated tissue was more fragile than control tissue resulting in unusable treatment sections. The Dex-treated tissue fragility may be a result of reduced cellular density. Tissues with Dex treatment are often difficult to process since the tissue surrounding the implant is not well integrated. In the cases where sections were obtained, it is clear the Dex treatment has reduced the number of infiltrating cells.
Fig. 5. H&E and Masson’s Trichrome staining of the tissue surrounding the microdialysis probe implanted into the subcutaneous space. Top: (H&E) stained tissue (nuclei: blue; eosinophilic structures: red; basophilic structures: purple; erythrocytes: bright red). (A) Control microdialysis probe. (B) Treatment microdialysis probe (20 µg ml⁻¹ Dex). Bottom: Masson’s Trichrome stained tissue (nuclei: dark brown/black; cytoplasm: light red/pink; collagen: blue). (C) Control microdialysis probe. (D) Treatment microdialysis probe (20 µg ml⁻¹ Dex). Images are 10x magnification with 100 µm scale bar and are representative of tissue where tissue sections were obtainable.
3. Discussion

The implantation of biomaterials is known to lead to the FBR and eventual fibrotic encapsulation. While the encapsulation of some biomaterials is not problematic, other biomaterials suffer from loss of reliable function due to encapsulation [37]. In the case of degradable biomaterials, the formation of scar tissue at the implant site as opposed to wound resolution is considered problematic. Until recently, these tissue outcomes of biomaterial implantation were viewed as detrimental, yet unavoidable.

Macrophage polarization has been described as playing a potential key role in the outcome of biomaterial implantation [36]. Controlling macrophage polarization states at an implant site may lead to improved tissue remodeling and reduced fibrosis and has been the topic of several recent review articles [38-40]. However, the available literature describing macrophage polarization as an observed outcome in biomaterials studies are limited. Most of the macrophage polarization literature is derived from the field of tumor biology. One recent work has used genetic engineering of IL-10 to different types of macrophages in cell culture to promote macrophage polarization [41]. The same group has used genetic engineering techniques in tissue to alter leukocyte infiltration in vivo [42].

Use of microdialysis sampling procedures allows both the localized delivery of modulators to the tissue space and a concomitant collection of bioactive solutes that may be affected by the modulator. This characteristic makes microdialysis sampling a useful model technique in biomaterials science for investigating responses to locally-delivered modulators and their effects on the local tissue biochemistry. Solutes collected by the microdialysis probe reflect the localized chemical milieu surrounding the implanted microdialysis probe. Combining bioactive chemical content analysis with tissue analysis that includes judicious choices regarding gene transcription coupled with immunohistochemical staining of defined macrophage surface markers provides a comprehensive approach for elucidating the molecular mechanisms of macrophage polarization in vivo.

Dexamethasone was used in this study for several reasons. First, glucocorticoids are known to dampen inflammation and Dex has been shown previously to reduce fibrosis at an implant site and has been widely applied in biomaterials studies [26, 43]. Additionally, Dex is known to induce macrophages
to an M(GC) state which was our desired goal in these experimental studies. Dex likely reduces fibrosis at implants due in part to Dex's ability to decrease pro-inflammatory cytokines at both the gene and protein level [44, 45]. It is interesting to note that most of the work with biomaterials and Dex for in vivo studies have relied on histological outcomes and have not elucidated alterations in molecular mechanisms in vivo. To our knowledge, this work is the first that aims to gain a more complete molecular snapshot of how modulators affect the FBR in vivo.

Dexamethasone delivery from the microdialysis probe caused significant decreases in both IL-6 and CCL2 at the gene transcript level. Since both of these cytokines are considered to be inflammatory, it is expected that Dex treatment would cause significant decreases in their overall transcript levels. While PCR analysis has been used to elucidate gene transcript changes in the presence of implanted biomaterials in an in vivo setting [46], it has not been used in vivo in the context of using modulators to affect macrophage polarization. The changes in transcription levels with Dex are in accordance with previous studies which have shown Dex treatment to be able to reduce transcript levels of CCL2 in rat pancreatitis models [17] as well as in an inflammatory state induced by potassium permanganate [21]. Dex has been shown to reduce IL-6 transcript levels in a rat arthritis model [47].

While Dex significantly decreased CCL2 and IL-6 gene transcripts, it did not significantly change the gene transcripts for another inflammatory cytokine, tumor necrosis factor-α (TNF-α). While this was unexpected, it is not novel in that TNF-α levels have been shown to be constitutively expressed in the spleen, liver, and small bowel of rats even when treated with Dex [48]. Further, TNF-α transcript levels have been shown to rise immediately following Dex treatment in a rat arthritis model [47]. The remaining genes showed no differential expression due to Dex treatment.

In the case of TGF-β1, reports of transcript regulation have been controversial. Dex has been shown to have different effects including no changes in TGF-β1 transcript levels in human T cells collected from asthma patients [49], increases in TGF-β1 transcript levels in human T cell cultures [50] as well as decreases in TGF-β1 transcript levels in rats [51]. The effect Dex has on TGF-β1 may be transient and dose dependent and perhaps the time course and/or dosage was unable to produce a difference in TGF-β1 transcript levels.
The genes for CD163, Arg2, and iNOS2 were chosen for analysis based on reports of differential expression of their protein products in an M(GC) state. While transcript levels of CD163, Arg2, and iNOS2 were unchanged, this does not mean that protein levels also remained unchanged as changes in transcript and protein levels do not always share a correlated response [52, 53]. Since quantitative analysis was not performed on Arg2 or iNOS2 protein concentrations, it is not possible to determine if these protein levels changed in response to the Dex-treatment. However, evidence of increased expression of CD163 was found using immunohistochemical staining. This increase may be due to tissue being harvested and RNA processed after the transcript levels peaked. Alternatively, this may be due to the relative amount of total tissue obtained relative to that which would be nearest to the microdialysis probe releasing Dex.

CCL2 is a chemokine known to recruit monocytes to a site of inflammation. Dex reduces CCL2 concentrations in rats during different inflammatory states in both plasma and whole tissue [13, 17, 21]. On the day of implantation, a steady increase in CCL2 concentration was quantified in the dialysate of the control probe during the collection period. This was expected as CCL2 is needed at the wound site to recruit macrophages. On one and two days post implantation, no statistically significant difference was seen in CCL2 concentrations over the 6 hour collection period, possibly in result of CCL2 reaching homeostasis. Interestingly, in the dialysate from the treatment probe, an increase in CCL2 concentration is seen between the first and second hour followed by a decrease in the fifth and sixth hours of collection on the day of probe implantation. This suggests that while the Dex may begin exerting anti-inflammatory effects as early as two hours post administration, as indicated by the leveling off of CCL2 concentrations in the treatment dialysates, the anti-inflammatory effects do not peak until later time points as indicated by suppression of CCL2 amounts quantified. On the following two collection days a general trend of suppression of CCL2 in treatment dialysates was seen in response to Dex, with the CCL2 concentration by hour 6 two days post implantation being similar to the concentrations initially detected after microdialysis probe implantation. Interestingly, CCL2 concentrations were found to be much higher in the initial hours of collection as compared to the final hours of collection the previous day. In fact, CCL2 concentrations in the initial flush each day showed no difference between control and treatment dialysates even though Dex was allowed to remain in the probe overnight and freely diffuse out into the
ECS from the treatment probe. It is not entirely clear why this has been observed. The lack of difference in the initial flush may suggest that a certain continuous dose has to be applied. Alternatively, from an area-under-the-curve approach, it is clear the overall amount of CCL2 is significantly lower surrounding the probe. This could be a combination of diffusion/mass transport changes combined with CCL2 generation that lead to these differences. If CCL2 is being produced or generated locally by macrophages in the control implant, then its concentration will not be drained from the tissue space through the continuous sampling (removal) process with the microdialysis sampling probe.

Glucocorticoids, such as Dex, have been reported to shift macrophages to a predominantly M2c (M(GC)) state [5, 6]. In a recent study it was shown that dexamethasone was able to shift human macrophages to an M2c (M(GC)) state resulting in increased CD163 expression and increased clearance of early apoptotic cells, in vitro [54]. The desire to shift macrophages to an M2c (M(GC)) state at an implant site is based on the hypothesis that by doing so the implant will better integrate into the surrounding tissue resulting in reduced fibrosis, reduced failure rates, and improved healing. Badylak’s group has shown that a predominantly M2c macrophage response to a scaffold implantation results in improved healing and integration of the scaffold [55, 56]. It is important to note at this point that while the Badylak group’s findings are important, they differ from this work in that (1) they investigated degradable biomaterials whereas our study utilizes a non-degradable biomaterial, (2) the Badylak group looked at the polarization state of macrophages as a predictor of outcome in response to different biomaterials whereas we focus on using a modulator to shift the polarization state of macrophages at an implant site. However, the same factors can be used as a predictor of outcome. What we found was that two distinct populations of macrophages were found surrounding the implant at lower magnification (20x). This gave the appearance that tissue proximal to the implant was predominantly CD68⁺ CD163⁻ macrophages, while tissue more distal from the probe was predominantly CD68⁻CD163⁺ macrophages, representing the expected conversion for M(GC) macrophages. This finding is in concert with a previous report that showed areas of predominantly M2 macrophages at the periphery of an implant, though these cells were stained with the pan M2 marker (CD206) and not CD163, which is an M2c specific marker [57]. However, upon further investigation, we found that at higher magnification CD163⁺ macrophages were seen in areas proximal to the probe. Further, we found that there were more CD163⁺ macrophages surrounding
the treatment probe as compared to the control probe. We also found that the tissue surrounding the Dex probes contained a significantly higher percentage of CD163⁺ macrophages. This suggests that Dex is able to shift macrophages to a pro-wound-healing state in vivo.

Previous studies have used different biomaterials to release Dex in an effort to reduce fibrosis using histological analyses as an endpoint [14, 28, 29, 31, 58]. Our own histological findings are similar to this previous work since Dex is able to reduce the cellular density surrounding the biomaterial. However, it should be noted that these studies have a wide range (5.3 µg-7 mg) of Dex being delivered to the biomaterial implant site. These are much higher doses than used in this study, with the amount of delivered Dex being ~3.6 µg per 6h collection period, giving a total of ~10.8 µg of Dex being delivered over the three days. It is important to note that these numbers do not take into account the Dex which was left in the probes at the conclusion of each sampling period and allowed to freely diffuse out. Interestingly, even though less Dex was delivered in this study, our findings are consistent with previous findings which showed Dex to result in a decrease in the amount of infiltrating cells [14, 28, 29, 31, 58]. This may be due in part to the fact that our technique does not suffer from the initial high bursts of drug that is commonly associated with controlled release biomaterials.
4. Conclusions

Implanted microdialysis sampling probes were used to locally deliver Dex to the subcutaneous space of rats. This treatment altered the localized concentration of the chemokine, CCL2, and damped gene transcription for CCL2 and IL-6. While no changes in gene transcription were observed for the macrophage CD163 protein, this marker was identified in immunohistochemical analyses. While different materials have been used successfully in vivo to polarize macrophages to a pro-wound healing M2c (CD163⁺) state, the use of modulators to shift macrophage polarization states has been focused primarily on in vitro studies. The ability to bridge the gap between successful in vitro modulators and in vivo implants could result in improved tissue remodeling outcomes to a host of biomaterials. This study is the first to show that Dex can be used to shift macrophages to a CD163⁺ state at a non-degradable implant site. While this study has some limitations with respect to a limited number of markers and investigation as to the long-term effects on tissue remodeling, it shows great promise for the ability to use modulators to shift macrophage polarization states in vivo resulting in improved tissue remodeling.

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References


Chapter 5

Effects of Delayed Delivery of Dexamethasone-21-Phosphate via Subcutaneous Microdialysis Implants on Macrophage Activation in Rats

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1. Introduction

Millions of different types of biomaterials (artificial heart valves, breast implants, implanted biosensors, pacemakers, and prosthetic joints) are implanted worldwide every year. Each of these different biomaterials elicits a foreign body reaction (FBR) which is an immune response to the implanted biomaterial [1]. While normal wound healing consists of hemostasis, inflammation, proliferation and remodeling, the FBR consists of acute inflammation, chronic inflammation, and the eventual fibrotic encapsulation of the biomaterial. This encapsulation results in the biomaterial being 'walled off' from the rest of the body, residing in its own microenvironment. For many biomaterials, this encapsulation poses no significant clinical concerns, but for other materials such as sensors, this encapsulation proves to be detrimental resulting in loss of function [2]. For this reason, much effort has been put into controlling or altering the FBR to eliminate or reduce the formation of the fibrotic encapsulation.

Macrophages play a role in both innate and adaptive immunity and can phagocytose foreign materials including microbes and cellular debris [3]. Beyond being phagocytic cells, macrophages play dueling roles in both driving and resolving inflammation, antigen presentation vs. scavenging, and tissue destruction vs tissue remodeling. Macrophages have been historically-identified as playing a critical role in the outcome of implanted biomaterials and their FBR [4].

Macrophages are highly plastic cells exhibiting a wide range of phenotypes [5]. Macrophage activation is a term used to describe the ability of macrophages to change phenotypes in response to biochemical signals [6, 7]. Mills identified this phenotypic change as macrophage polarization and identified the extremes of these phenotypes along this plastic continuum as either M1 or M2 [8]. M1 macrophages are classically activated by lipopolysaccharide (LPS), interferon gamma (IFN-γ), or tumor necrosis factor alpha (TNF-α). M1 macrophages secrete pro-inflammatory cytokines (IL-1β, IL-6, IL-12, and TNF-α) and high concentrations of nitric oxide [7, 9]. M1 macrophages are characterized as being pro-inflammatory, highly microbicidal, and efficient antigen presenting cells expressing high amounts of major histocompatibility complex II (MHC II) [10]. M2 macrophages consist of three subclasses, M2a,b,c, and are considered to be anti-inflammatory. M2 macrophages are induced by a variety of different modulators, IL-4 and/or IL-13 (M2a), immune complex, toll-like receptor, or IL-1 receptor ligation (M2b).
and IL-10, glucocorticoids, and secosteroids (M2c) [10, 11]. M2c macrophages are considered to be anti-inflammatory, pro-tissue remodeling, and pro-wound healing. Macrophage activation has become of wide interest in the fields of biomaterials and regenerative medicine [12-15]. It has been postulated that by switching macrophages to a predominantly M2c activation state at an implant site, improved wound healing and improved implant integration into the host tissue will be achieved. Recently, a nomenclature change was requested to denote macrophage polarization as macrophage activation with the macrophage phenotype being termed according to the modulator which was used to elicit the macrophage, i.e., M(LPS) rather than M1 [16]. In this paper, the M1/M2 nomenclature will be used as a broad categorization for comparisons to past literature while the modulator nomenclature, M(Dex), will be used to describe macrophages that were altered via the delivery of Dex through the implanted microdialysis probe.

Microdialysis sampling is a minimally-invasive, diffusion-based sampling technique [17]. The microdialysis probe consists of an inlet and outlet tubing, inner cannula, and a semi-permeable membrane with a defined molecular weight cut-off (MWCO). Microdialysis sampling has been used in vivo to sample low molecular weight soluble analytes from the extracellular space (ECS) which are smaller than the MWCO of the probe membrane. This technique works by passing a fluid (perfusate), which is of physiological ionic strength and pH, through the inlet and down the inner cannula. Once the perfusate leaves the inner cannula, it passes by the membrane and exits through the outlet tubing and is collected as a dialysate. Any concentration gradient which exists between the perfusate and the ECS allows the diffusion of molecules into or out of the microdialysis probe. This feature allows microdialysis sampling to be used for the simultaneous collection of analytes from and delivery of modulators to the ECS [18]. Once collected, analytes in the dialysate can be quantified using a wide variety of chemical analysis methods.

Dexamethasone (Dex) is a synthetic glucocorticoid that is widely used as an anti-inflammatory and immunosuppressant drug [19, 20]. Once inside the cell, dexamethasone binds to glucocorticoid receptors which then translocate to the nucleus [21]. In the nucleus, Dex carries out its anti-inflammatory effects via two means: transactivation and transrepression [22]. Transactivation is the increase in the
expression of certain anti-inflammatory genes including lipocortin-1 and the type II IL-1 receptor [23, 24]. Transrepression is the down regulation of pro-inflammatory genes through the direct binding of the glucocorticoid receptor to negative glucocorticoid receptor elements [25, 26] and by interfering with activator protein-1 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [27, 28]. Through these actions Dex has been shown to down-regulate pro-inflammatory cytokines such as CCL2, IL-6, and TNF-α at the transcriptional level [29] as well as reducing CCL2, IL-6, and TNF-α protein concentrations in different animal models [30].

Chemokine (C-C motif) ligand 2 (CCL2) is a 13 kDa chemokine which exists as a monomer and homodimer at physiological concentrations [31]. CCL2 was formerly known as monocyte chemoattractant protein-1 (MCP-1) due to its ability to result in monocyte migration. CCL2 has been shown to be one of the primary factors for attracting monocytes to a wound site [32]. Once at the wound site the monocytes differentiate into macrophages. The activation state of these macrophages depends on the biochemical milieu present at the wound site.

Dexamethasone has been widely used as a release agent from biomaterials studies in an effort to improve integration of the biomaterial with the host tissue [33]. Dex has also been identified as a modulator which produces a phenotype that has characteristics of the former M2c macrophage [7, 10]. While much work has been done with incorporating Dex-release into implanted biomaterials, quantifying the molecular response, particularly in vivo attempts to shift macrophages to an M(Dex) phenotype has not been reported. Most work with Dex-releasing biomaterials uses standard histological, rather than molecular assessments, of implant/tissue outcomes. Our previous work has shown that Dex delivered immediately after microdialysis probe insertion is capable of shifting macrophages to a more CD68^+CD163^+ state, the former M2c designation [34]. However, there is a body of literature suggesting that an initial inflammatory response is critical for proper wound healing [35]. Therefore, we sought to determine if a different or even more optimal response in terms of converting macrophages to an M(Dex) state can be gained if the start of Dex infusion is delayed to allow the initial inflammatory response to commence. The resulting foreign body reaction to the implanted dialysis probes was characterized not only with standard histological means, but also immunohistochemical and molecular means at the gene
and protein level. Thus, this work aimed to gain a better molecular understanding of macrophage-activation modulators effectiveness at controlling the foreign body reaction.

2. Results

3.1 qRT-PCR

Figure 1 shows the gene expression ratios of eleven different genes: Arginase (Arg2), CCL2, CD163, CD206, Interleukin-1 receptor antagonist (IL-1ra), IL-6, IL-10, iNOS2, Lipocortin-1 (Lipo-1), TGF-β1, and TNF-α. Gene expression ratios were determined from tissue excised from around the microdialysis probe using qRT-PCR. IL-6, CCL2, IL-1ra, IL-10, Lipo-1, TGF-β1, and TNF-α were chosen to determine if delayed Dex was able to alter the cytokines involved in the FBR at the gene level. IL-6 was found to be significantly up-regulated in response to delayed Dex treatment. CCL2, IL-1ra, IL-10, Lipo-1, TGF-β1, and TNF-α were not affected by delayed Dex treatment. Arg2, CD163, CD206, and iNOS2 were chosen to determine if Dex had any effect on indicators of macrophage activation state at the gene level and none of these were affected by delayed Dex treatment.
Fig. 1. Relative gene expression ratios between treatment and control tissue. Tissue samples from around the microdialysis probe were used to determine relative gene expression ratios of eleven different genes in response to delayed dexamethasone-21-phosphate (20 µg/mL) treatment. (n=3) **p≤0.001 Error bars represent SEM.
2.2 CCL2 Quantification

CCL2 concentrations were quantified in the dialysates collected from both control and treatment probes for each hour of the six hour collection period as well as the initial flush (Figures 2-5). Three days post implantation (Figure 2), CCL2 concentrations were found to be highest in both the control and treatment dialysates during the initial flush with concentrations of ~1500 pg/mL and ~1700 pg/mL, respectively. After the initial flush, CCL2 concentrations in the control dialysate dropped to ~900 pg/mL during the first hour of collection and remained within ~200 pg/mL of that concentration for the entirety of the collection period. In the treatment dialysate, CCL2 concentrations remained high, ~2000 pg/mL, during the first hour of collection and then began to decrease over the remainder of the collection period with concentrations being ~450 pg/mL during the last hour of collection. No significant difference in CCL2 concentrations was seen in the treatment as compared to the control in any of the time points tested at three days post implantation.
Three Days Post Implantation

![Bar graph showing concentration of CCL2 pg/mL over hours of collection.]

**Fig. 2.** Concentration of CCL2 3 days post implantation. Bar graphs showing the concentration of CCL2 collected in control and treatment dialysates. n=8 where n represents the number of animals, *p≤0.05 with the error bars representing the SEM, F represents the initial flush.
Four days post implantation (Figure 3), CCL2 concentrations were found to be the highest during the initial flush at ~2750 pg/mL and ~2000 pg/mL for control and treatment, respectively. After the initial flush, CCL2 concentrations in the control dialysate dropped and remained relatively constant, fluctuating from a high of ~1500 pg/mL to a low of ~1000 pg/mL. In the treatment dialysate, CCL2 concentrations dropped during the first hour of collection to ~1500 pg/mL. Interestingly, a spike in CCL2 concentration was seen during the second hour of collection in the treatment dialysate with values being the same as was seen in the initial flush, ~2000 pg/mL. In hour 3, the concentration of CCL2 in the treatment dialysate fell to ~450 pg/mL and was found to be significantly lower than the concentration of CCL2 in the control dialysate. The concentration of CCL2 remained constant during hour 4 and showed a slight decrease in hours 5 and 6 with CCL2 concentrations being significantly lower in the treatment dialysates as compared to the control in hours 5 and 6.
Fig. 3. Concentration of CCL2 4 days post implantation. Bar graphs showing the concentration of CCL2 collected in control and treatment dialysates. n=8 where n represents the number of animals, *p≤0.05 with the error bars representing the SEM, F represents the initial flush.
Five days post implantation (Figure 4), CCL2 concentrations were again found to be highest in
the initial flush. After the initial flush, CCL2 concentrations reduced and were found to be relatively stable
over hours 1-6 ranging from ~1900 pg/mL to ~1200 pg/mL. In the treatment dialysate, CCL2
concentrations were found to steadily decrease from hours 1-6, ranging from ~1300 pg/mL to ~200
pg/mL. In hours 3-6, the concentration of CCL2 was found to be significantly lower in the treatment
dialysate as compared to the control dialysate.
Fig. 4. Concentration of CCL2 5 days post implantation. Bar graphs showing the concentration of CCL2 collected in control and treatment dialysates. n=8 where n represents the number of animals, *p≤0.05 with the error bars representing the SEM, F represents the initial flush.
Six days post implantation (Figure 5), CCL2 concentrations were found to be ~2400 pg/mL and ~2100 pg/mL in the control and treatment dialysates during the initial flush, respectively. During hours 1-6, CCL2 concentrations remained fairly constant at ~1500 pg/mL with a range of ~1750 pg/mL - ~1300 pg/mL in the control dialysate. In the treatment dialysate, CCL2 concentrations were found to steadily decrease over the collection period from ~1350 pg/mL during hour 1 to ~500 pg/mL during hour six. Though CCL2 concentrations were seen to decrease over time in the treatment dialysate, significance was not reached in any of the collection periods tested.
Fig. 5. Concentration of CCL2 5 days post implantation. Bar graphs showing the concentration of CCL2 collected in control and treatment dialysates. n=6 where n represents the number of animals, *p≤0.05 with the error bars representing the SEM, F represents the initial flush.
2.3 Immunohistochemistry

Immunohistochemistry was used to investigate the density and number of total macrophages (CD68$^+$) compared with M(Dex) macrophages (CD68$^+$CD163$^+$) present in the tissue surrounding both control and treatment microdialysis probes. At lower magnifications (Figure 6A) few macrophages are seen surrounding the microdialysis probe in the control tissue. Further, there are minimal M(Dex), CD68$^+$CD163$^+$, macrophages seen with none seen less than 100 µm away from the microdialysis probe. In the tissue surrounding the treatment probe, at lower magnification, many macrophages are seen surrounding the probe with a high number of M(Dex) macrophages being present. At higher magnification (Figure 6B), few M(Dex) macrophages are seen in the tissue immediately surrounding the control probe. In the tissue surrounding the treatment probe, M(Dex) macrophages are seen immediately surrounding the probe with M(Dex) macrophages seen as close as 30 µm away from the membrane. The percent of M(Dex) macrophages found in the tissue surrounding both the control and treatment probes was determined via manual counts of macrophages staining CD68$^+$ as well as macrophages staining CD68$^+$CD163$^+$ in the high magnification (40x) images (Figure 6C). The percentage of M(Dex) macrophages found in the tissue surrounding the treatment probe was statistically higher than the percentage of M(Dex) macrophages found in the tissue surrounding the control probe (Figure 7).
Fig. 6. Immunohistochemical staining of tissue surrounding a microdialysis probe. Immunohistochemistry was used to identify CD68⁺ (green) cells, CD163⁺ (red) cells, and nuclei (blue). Cells which show an overlap of colors (stain both red and green or yellow/orange) represent a M(Dex) macrophage. A) Tissue surrounding both control and treatment microdialysis probes at 20X magnification. B) Tissue surrounding both control and treatment microdialysis probes at 40X magnification. M indicates the microdialysis probe membrane. Scale bars in lower right of each image represent 100 µm (20X) and 50 µm (40X). Images representative of three animals.
Fig. 7. Percent of CD68⁺ Macrophages which Stain CD68⁺CD163⁺ [M (Dex)] macrophages surrounding a microdialysis probe. Bar graph representing the percentage of M(Dex) macrophages found in the tissue surrounding both the control and treatment probe. Error bars represent the SEM where *p≤0.01, n=12 where n represents the number of measurements taken.
2.4 Histology

Figure 8 shows the hematoxylin and eosin (H&E) and Masson’s Trichrome analyses performed to determine the effects of delayed Dex infusion on the cellular density and collagen in the tissue surrounding the microdialysis probe seven days post-implantation. The tissue surrounding the treatment probes was characterized as having a higher cellular density as well as more macrophages as compared to the control probe. Further, the tissue is better integrated surrounding the treatment probe as compared to the control as evidenced by the lack of acellular cytoplasmic material surrounding the treatment probe which is seen in the control. Of the cells seen surrounding the control probe, there was a higher number of foreign body giant cells as compared to the treatment probe. The Masson’s Trichrome showed there to be no appreciable difference in the amount of collagen seen in the tissue surrounding the treatment probe as compared to the control probe. Additionally, for both the control and treatment probes, few aggregated cells or foreign body giant cells were observed.
Fig. 8. Histological staining of tissue surrounding a microdialysis probe. **Top:** H&E Stained tissue (Six panes) (nuclei-blue, eosinophilic structures-red, basophilic structures-purple, erythrocytes-bright red). **Bottom:** Masson’s Trichrome stained tissue (Six panes) (nuclei-dark brown/black, cytoplasm-pink/light red, collagen-blue)  

**A)** Control and treatment tissue immediately surrounding a microdialysis probe, 10X magnification, 100 µm scale.  

**B)** Control and treatment tissue distal to the microdialysis probe, 10X magnification, 100µm scale.  

**C)** Control and treatment tissue immediately surrounding a microdialysis probe, 40X magnification, 20µm scale bar. Images representative of three animals. **M** represents the microdialysis probe membrane or tissue at the membrane interface.
3. Discussion

Macrophages have long been recognized as pivotal cells for directing the foreign body reaction to implanted biomaterials [4]. Within the biomaterials community, there is a tremendous interest in modulating macrophage phenotype from what has been termed the M1, inflammatory phenotype, to the anti-inflammatory, M2, phenotype [12]. In order for wounds to heal properly, there are multiple stages through which a healing wound must pass [35]. In these stages, macrophages play many significant roles and removal of macrophages is detrimental to wound healing [39]. Early in the wound stage, macrophages release cytokines that attract additional leukocytes. Macrophages also clear neutrophils from the site allowing for the resolution of inflammation. It is believed that during this stage, macrophages can begin to alter their phenotype to a reparative state stimulating tissue regeneration [5, 40]. Within this macrophage phenotype continuum at the host/biomaterial implant interface, there is an approximate three- to five-day window before the arrival of fibroblasts that begin to deposit collagen [41]. In this study, we chose to target altering macrophage phenotype within this approximate five-day window. While much work has been done to understand the different activation states of macrophages in vitro, understanding how shifts in macrophage activation states occurs in vivo is still lacking. Indeed, a review of many different studies aimed to affect or knockout different cells involved in wound repair concludes that no one cell type appears to be absolutely essential for repair, but inflammation is known to inhibit repair processes by either slowing down the process or causing excessive fibrosis [42].

Dexamethasone is a synthetic glucocorticoid known to have powerful anti-inflammatory and immunosuppressive effects. Dex has been widely used as a controlled-release agent in many different biomaterials studies as a means to dampen the fibrotic response [33, 43-46]. Macrophages possessing the M2c characteristic phenotype (CD163⁺) have been implicated as being positive for appropriate tissue remodeling at an implant site [47, 48]. Shifting macrophages to the M(Dex) (M2c) activation state has become of interest in the field of biomaterials as it is thought that by doing so, the biomaterial will better integrate into the surrounding tissue leading to reduced fibrosis, reduced scarring, and increased longevity of some biomaterials. Dexamethasone is known to be able to shift macrophages to an M2c
activation state [7, 10]. This M2c [M(Dex)] characteristic phenotype has been shown to result in the increased expression of CD163 at the cell surface [49]. Additionally, since little is known about the time-course or optimum timing necessary for altering macrophage activation, we chose to investigate a 3-day post-implantation time for initiating Dex infusion. Others have suggested that immediate intervention to the wound healing response may not be the most effective way to control the FBR as interfering with normal wound healing processes leads to improper healing [50]. Following this rationale, allowing macrophages to enter the wound site prior to attempting to modulate their phenotype may prove to be more effective in long-term reduced fibrosis, reduction in fibrotic capsule, and better integration of the biomaterial.

Delayed delivery of dexamethasone from the microdialysis probe resulted in the significant increase of IL-6 at the gene transcript level. Similarly, we showed in a previous study that immediate and daily infusion of Dex to a subcutaneous wound site in rats, resulted in the significant decrease in IL-6 gene transcription [34]. It should also be noted that IL-6 is known to exhibit either pro- and anti-inflammatory properties [51]. While this finding was unexpected, it may be due to the delayed delivery of Dex to the wound site. Additionally, while Dex has been widely used in biomaterials studies in the rat, direct measurements of cytokine gene or protein expression are lacking in the literature.

The delayed delivery of Dex to the wound site had no effect on the transcription levels of the remaining genes tested. In the cases of TNF-α and TGF-β1, these results are not surprising as TNF-α levels have been shown to immediately rise following Dex administration [52] and the effects of Dex on TGF-β1 have been shown to be highly variable [53-55]. These results were unexpected in the case of CCL2 which has been shown to be decreased in response to Dex in a rat pancreatitis model [56], and in response to locally delivered Dex to the subcutaneous space [34]. The inability of Dex to down-regulate CCL2 gene transcription may be due to the delayed delivery of the Dex, the concentration of the Dex delivered being insufficient to reduce CCL2, or a combination of the two. Further, this may be due to Dex being able to decrease CCL2 mRNA stability as opposed to transcription rates as has been shown in vitro [57].
In the case of IL-1ra, Dex has been shown to decrease transcript levels in vitro in human monocytes [58, 59]. Lipocortin-1 transcript levels have been shown to increase in response to dexamethasone treatment in vitro in rat astrocytoma cells [60]. In this study, no difference in the expression levels of IL-1ra or lipocortin-1 transcripts was observed. This may be due to the time course of the study in which tissue was harvested after four days of Dex infusion. In the case of lipocortin-1, previous up-regulation in gene transcription was seen four hours after Dex administration [60]. This might imply that the effects of Dex on transcript levels had already peaked and returned to basal levels at the time of tissue harvesting in this work. However, since direct protein concentration measurements were not performed, it is not possible to say if Dex had any effect on their concentrations.

The transcription levels of Arg2, CD163, CD206, and iNOS2 were chosen as markers of macrophage activation state. In M2c macrophages, protein levels of Arg2, CD163, and CD206 are increased while iNOS2 is decreased. Although there was no differential expression of these transcripts, this does not mean that there is no differential expression of the protein as it has been shown that transcript levels and protein levels are poorly correlated [61]. The immunohistochemical analyses show there to be an increase in the amount of CD163 protein present in response to Dex treatment. Since protein levels for Arg2, CD206, or iNOS2 were not measured in this study, it is inconclusive whether delayed delivery of Dex had an effect on their expression. Again, these differences may be due in part to the timing where CD163 transcript levels may have peaked prior to tissue harvest.

CCL2 is a chemokine responsible for the recruitment of monocytes to a wound site. This cytokine has been implicated as playing a significant role in wound repair and has recently been implicated in promoting healing in diabetic wounds by restoring macrophage response [62]. However, in other work, CCL2 knock-out mice have demonstrated an inability to form a fibrous capsule around implants [63]. In a previous study, when Dex was immediately infused through the dialysis probe, the concentration of CCL2 was decreased roughly three hours after infusion initiation [34]. However, this effect was not immediately apparent with the delayed Dex infusions. Here, the reduction in CCL2 concentrations was not altered until the second day (four days post implant) of Dex infusion. Additionally, the reduction in CCL2 concentration was also not as great as with immediate Dex infusion. Moreover, while some reductions in
CCL2 concentrations were observed on the third day of infusion (5th day post-implantation), no significant alterations in CCL2 concentrations are observed on the fourth day (6th day post-implantation) of Dex infusion. While in all cases, the CCL2 concentrations decrease after initiation of the dialysis collection, what is notable is the initial collected concentrations in the flush collection. While many microdialysis sampling practitioners would generally not even analyze initial flush solutions through the probe, this is actually fluid that may be more representative of the approximate external concentrations residing in the extracellular fluid space (ECS) in the tissue in contact with the dialysis probe. The dead volume within the dialysis probe shaft is roughly 1 µL. However, if solutes diffuse into the shaft, they can continue to diffuse through the tubing lines. While these initial flush concentrations were variable from day to day, the average is roughly 2000 to 2500 pg/mL. The decreasing concentrations of CCL2 after initiation of dialysis indicate that CCL2 production in the ECS is not sufficient to compete with the dialysis removal process. However, the significant decrease in CCL2 with Dex-delivery in some days suggests bioactivity of the Dex at the tissue site. Why this decrease in CCL2 is not as significant as with the daily infusions of Dex that we have previously reported is not clear. The lack of difference in CCL2 concentrations between control and treatment six days post implantation may suggest that after three days of Dex infusion, higher concentrations of Dex are needed to illicit the same effect. This may also be due to the arrival of more CCL2-secreting cells, such as macrophages, to the tissue surrounding the treatment probe, as is seen in the histological analyses. These macrophages may be receiving competing endogenous signals from within the extracellular space such that addition of Dex does not effectively modulate the CCL2 levels.

Immunohistochemical analyses were performed to determine the effects of delayed Dex infusion on the amount of macrophages (CD68⁺) present in the tissue surrounding the microdialysis probe as well as the amount of M(Dex) (CD68⁺CD163⁻) macrophages present. The interesting finding here is that CD68⁺CD163⁺ macrophages are not found adjacent to the dialysis probe, but rather further away (~100 µm in control tissue and ~30 µm in treatment tissue). This is consistent with a recent scaffold implant study in rats that measured CD68 and CD206 (a pan M2 macrophage marker), but not CD163, where they observed CD68⁺ cells near the scaffold/tissue interface and CD68⁺CD206⁺ cells farther away [64]. While these findings are consistent, we note that CD206 was not quantified in this study. While all M2 macrophages would be expected to express CD206, we have not stained for this marker in this work and
thus cannot say if this would hold true in this work. More importantly, CD68^+CD163^+ macrophages are observed in both control and treatment tissue, but with more M(Dex) macrophages at the treatment site. This suggests that while the biomaterial itself may result in the previously described M2c activation state (CD68^+CD163^+), it is clear that adding Dex appears to promote a significantly higher wound healing macrophage phenotype. Additionally, few foreign body giant cells were observed in this study which is consistent with a recent non-degradable implant study in the rat [65].

Dexamethasone has been previously used extensively in biomaterials studies in an effort to reduce fibrosis where histological analyses were used as an endpoint [44-46, 66]. These studies have shown that tissue surrounding dexamethasone eluting materials are characterized as having reduced cellular density. We have previously reported that immediate infusion of Dex results in a loose capsule surrounding the dialysis probe resulting in difficulties in obtaining the tissue for histological analysis [34, 67]. However, with delayed Dex infusion, the results demonstrated an increased cellular density surrounding the treatment probe as compared to the control probe. Further, we found no difference in the amount of collagen present in the tissue surrounding the treatment probe as compared to the control probe. These differences may be attributed to three factors: 1) initial burst associated with other techniques, 2) the time course of Dex infusion, 3) the concentration of Dex used. While many controlled release biomaterials suffer from initial bursts of drug (dexamethasone), the microdialysis sampling approach allows a constant (zero-order) delivery of Dex. Further, controlled release biomaterials begin eluting drug immediately following implantation (the initial burst) and continuously deliver the drug over time. Our technique allows for the delivery of Dex for defined amounts of time with the ability to begin delivery at any given time post implantation. This is important due to increasing knowledge that an initial wounding response is necessary to elicit appropriate wound healing [35, 50]. Finally, the concentrations of Dex used in this study differ from the previously mentioned studies. In the previously mentioned studies, a concentration range from 5.3 µg-7 mg of Dex was used. In this work, ~3.6 µg of Dex was delivered per six hour collection period resulting in a total of ~14.4 µg of Dex being delivered over the entire collection period. It should be noted at this point that the ~14.4 µg of Dex does not take into account the Dex which was allowed to remain in the probe and freely diffuse out between collection periods. While our previous study showed ~10.6 µg of Dex to be sufficient to reduce cellular density
around the treatment probe, this was in response to immediate Dex delivery following implantation [34] while in this study Dex was not delivered until three days post implantation. To our knowledge, this is the first study to look at the effects of delayed Dex infusion on a subcutaneous implant site. An additional concern is that we do not know the range of Dex actually needed to induce macrophages to the CD68\(^+\)CD163\(^+\) state.

4. Conclusions

In this study, microdialysis sampling probes were implanted in the subcutaneous space of rats and used to locally deliver Dex over a four day period starting three days post probe implantation. The delayed delivery of Dex resulted in an up-regulation of IL-6 gene transcripts as well as a moderate decrease in collected CCL2 concentrations. Histologically, the delayed Dex treatment resulted in an increase in cellular density in the tissue surrounding the microdialysis probe. Further, the delayed delivery of Dex was able to shift macrophages to an M(Dex) activation state (CD68\(^+\)CD163\(^+\) macrophages (M2c) in the tissue). The use of modulators to shift the activation state of macrophages has focused primarily on \textit{in vitro} studies. This is the first study to show that a delayed delivery of Dex can be used to shift macrophages to an M(Dex) activation state \textit{in vivo} at a non-degradable implant site.

Acknowledgements

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References


Conclusion

The work presented in this dissertation focused on switching the activation state of macrophages to a predominantly M2c state at a non-degradable implant site via microdialysis sampling technique. While microdialysis sampling has many characteristics which make it an ideal technique for this work, colloids must be used to prevent ultrafiltration, the loss of fluid across the microdialysis membrane, when using high MWCO membranes. Initially perfusion fluids contained Dextran-70, a commonly used colloid. However, increased inflammation was seen at the implant site. After a series of experiments, it was determined that this increase in inflammation was due to the diffusion of Dextran-70 across the microdialysis membrane, allowing the Dextran-70 to enter the extracellular space. Upon further investigation, a suitable perfusion fluid which resulted in no additional inflammation at the implant site was discovered. The replacement of Dextran-70 with Dextran-500 proved to be acceptable as Dextran-500 successfully limited ultrafiltration while resulting in no increased inflammation at the implant site.

Once a suitable perfusion fluid was found, Dex was delivered to the implant site immediately following implantation via the microdialysis probe in an attempt to shift the activation state of macrophages to a predominantly M(Dex) state. At the gene level, the immediate administration of Dex resulted in the significant down-regulation of both CCL2 and IL-6 in the tissue surrounding the treatment probe as compared to the control probe. Immediate Dex infusion also resulted in the significant decrease of CCL2 concentration collected in the treatment dialysate as compared to the control dialysate. Histologically, Dex resulted in mostly unusable treatment sections which may have been due to the decrease in cellular density seen in the tissue surrounding the treatment probe as compared to the control probe, where sections were obtained. Most importantly, as it was the main aim, Dex resulted in a significant increase in the percentage of M(Dex) macrophages in the tissue surrounding the treatment probe as compared to the control probe. This showed that it is possible to use modulators to alter the activation state of macrophages at a non-degradable implant site.

Once it was determined that Dex could be used to alter macrophage activation states, in vivo I set out to determine if a different/more optimal response could be garnered from the delayed delivery of Dex to the implant site. For this work, Dex was not administered until three days post implantation.
point allows for the initial inflammatory response to occur. The delayed delivery of Dex gave results which were similar yet very different from what was seen in response to immediate Dex infusion. At the gene level, it was found that IL-6 was significantly increased in the tissue surrounding the treatment probe as compared to the control. This result differs from what was seen in response to the immediate delivery of Dex. At the protein level, delayed Dex administration also resulted in the significant decrease in CCL2 concentrations collected from treatment dialysates as compared to control dialysates. However, the response seen in the delayed delivery varied greatly from what was seen in response to the immediate infusion and was seen to be much more transient. Histologically, an increase in cellular density was seen in the tissue surrounding the treatment probe as compared to the control which is in stark contrast to what was seen in response to immediate Dex delivery. Immunohistochemically, Dex proved to be able to significantly increase the percentage of M(Dex) macrophages in the tissue surrounding the treatment probe as compared to the control. Interestingly, roughly the same percentage of CD68⁺CD163⁺ macrophages were seen in the tissue surrounding the treatment probes in both the delayed and immediate Dex delivery experiments (~65%). However, a greater number of macrophages (both CD68⁺CD163⁻ and CD68⁺CD163⁺) were seen in the delayed Dex experiment as compared to the immediate Dex experiment, suggesting that this time point allows for a more predominantly macrophage response to be targeted as compared to the immediate Dex delivery.

This work shows that it is possible to use modulators to shift macrophages to a predominantly M(Dex) activation state at a non-degradable implant site while giving a better understanding of what is happening at the molecular level. Further, it shows that differential effects are seen in response to the immediate and delayed delivery of Dex to the implant site. While much work has been done on understanding macrophage activation, most of this work was done in vitro. Understanding how in vitro modulators may affect macrophages in vivo as well as characterizing this response may lead to increased longevity and improved integration/healing of biomaterials and their wound sites in the future. At this point, experiments where Dex is delivered both immediately and at 3 days post implantation with 14+ day end points are needed. These experiments will determine if the initial effects of the Dex are maintained and if there are any differences in integration and healing. Further, it is important to study other modulators which may be used to shift macrophages to an M2c activation state as well as characterize
the molecular response associated with these modulators. Through this work it may be possible to improve the integration of biomaterials into host tissue as well as increase longevity while reducing failure rates.
Appendix A

Effects of IL-10 Delivery at a Non-Degradable Implant Site on Macrophage Activation State via Microdialysis Sampling in Rats
1. Introduction

In today's society, implanted biomaterials have become common place with the use of such things as prostheses, heart valves, artificial breasts, and biosensors. The foreign body response (FBR) is the body's response to the implantation of most biomaterials. This response is characterized initially by acute inflammation at the wound site and eventual encapsulation of the biomaterial causing the biomaterial to reside in its own microenvironment. While this encapsulation poses no threat to many biomaterials, e.g. artificial breasts, artificial knees, screws, the encapsulation is detrimental to other biomaterials which rely on proper communication with the extracellular space (ECS), e.g. biosensors. In fact, the encapsulation of these biomaterials results in their inability to be used after seven days in vivo. Controlling or altering the FBR would lead to better integration of all biomaterials into the host tissue as well as possibly increasing the longevity of many biomaterials such as biosensors.

Interleukin-10 is a homodimeric cytokine with each dimer ranging in size from 17 - 18 kDa [1, 2]. IL-10 is known to be a potent anti-inflammatory cytokine which reduces levels of pro-inflammatory cytokines such as IL-1, IL-6, IL-12, and TNF-α as well as reducing the expression of major histocompatibility complex II (MHC II) [3]. IL-10 has also been shown to affect chemokine (C-C motif) ligand 2 (CCL2) but reports are conflicting as to whether CCL2 is increased or decreased in response to IL-10 [4-6]. Interleukin-1 receptor antagonist has been shown to be increased in response to IL-10 adding to the anti-inflammatory effects [3]. The exact mechanisms through which IL-10 exerts its' anti-inflammatory effects are unknown but it is thought to alter nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) [3], and mitogen-activated protein kinase (MAPK) signaling pathways [7], as well as activating suppressor of cytokine signaling 3 (SOCS3) which has regulatory effects on many cytokine genes [3, 8, 9]. Recently, a viral vector was used to deliver IL-10 at a scaffold implant in mice resulting in reduced leukocyte infiltration of the scaffold, reduced IFN-γ production, and increased IL-10
production [10]. Interleukin-10 shows promise as a modulator in the field of biomaterials due to the anti-inflammatory effects as well as IL-10's ability to induce M2c macrophages.

The focus of this work is to use microdialysis sampling probes to locally deliver IL-10 to a wound site. IL-10 was chosen due to its ability to shift macrophages to an anti-inflammatory, pro-wound healing, pro-tissue remodeling activation state. To determine the effects of IL-10 on the wound site, cytokine responses (primarily CCL2) were investigated as well as histological and immunohistochemical analyses.

2 Results

2.1 IL-10 (10 ng/mL) Perfusion (Dextran-70)

Figure 1 shows a box plot of the concentration of CCL2 in dialysates collected from a control probe and a treatment probe which had 10 ng/mL IL-10 added to the perfusion fluid. CCL2 concentrations rose steadily over the collection period in the dialysates of both the control and treatment probes. In the first hour of collection, the average CCL2 concentration was slightly higher in the control dialysate as compared to the treatment dialysate at ~800 pg/mL as compared to ~500 pg/mL respectively. Over the following 6 hours, CCL2 concentrations were found to be higher in the treatment dialysate as compared to the control dialysate. At the end of the collection period, hour 7, the average CCL2 in the control dialysate was ~6200 pg/mL while the average CCL2 concentration in the treatment dialysate was ~9500 pg/mL. While a trend was seen where CCL2 concentrations were higher in the treatment dialysate as compared to control dialysates, there was no statistical difference between these values at a 95% confidence interval.
Quantification of CCL2 in Response to IL-10 Treatment

Fig. 1. Box plot showing concentration of CCL2 collected in control and treatment (10 ng/mL IL-10) dialysates over a 7 hour period on the day of implantation.
Figure 2 shows images of hematoxylin and eosin (H&E) and Masson's Trichrome stained tissue surrounding a control probe and a 10 ng/mL IL-10 treatment probe. These two stains are used to identify cells (H&E) and collagen (Masson's Trichrome). The H&E stain shows that the cells surrounding the treatment probe are slightly more densely packed than the cells surrounding the control probe. The Masson's Trichrome stained sections, show no appreciable difference in the amount of collagen surrounding the control and treatment probes. Further, there is no appreciable difference in the organization of the collagen or in the collagen strand thickness surrounding the control and treatment probes.

Immunohistochemistry was used to determine the effect IL-10 had on the phenotype of macrophages found in the tissue surrounding the microdialysis probe. To identify macrophages, an antibody specific for CD68 was used. To determine if more M (IL-10) macrophages (formerly known as M2c macrophages) were found surrounding the treatment probe as compared to the control probe, an antibody specific for CD163 was used. Further, antibodies specific for CCR7 and CD206 were used to determine the effects of IL-10 infusion on the amount of M1 and M2 macrophages found in the tissue surrounding the microdialysis probe, respectively. There was no appreciable difference in the amount of macrophages surrounding the treatment probe as compared to the control probe (Fig 3 A&B). Further, there was no appreciable difference in the phenotype of macrophages surrounding the treatment probe as compared to the control probe, that is, there was no difference in the number of cells staining CCR7⁺ (Fig 3 C&D), CD206⁺ (Fig 4 A&B), or CD163⁺ (Fig 4 C&D). Interestingly though, there appeared to be more cells which stained both CD206⁺ and CD163⁺ in both tissues than there were cells that stained CD 68⁺.
Fig. 2. Control vs. 10 ng/mL IL-10 treated probe. Top: H&E stain (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (10 ng/mL IL-10) perfusion fluid. Bottom: Masson's Trichrome stain. (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (10 ng/mL IL-10) perfusion fluid. 10x magnification, white bar represents 100 µm.
Fig. 3. Immunohistochemistry for CD68⁺ and CCR7⁺ macrophages. **Left:** CD68⁺ macrophages present around the microdialysis probe. **Right:** CCR7⁺ macrophages present around the microdialysis probe. **Top:** Tissue surrounding a microdialysis probe implanted subcutaneously which utilizes a control perfusion fluid. **Bottom:** Tissue surrounding a microdialysis probe implanted subcutaneously which utilizes a treatment (10 ng/mL IL-10) perfusion fluid. 20x magnification, paraffin embedded sections.
Fig. 4. Immunohistochemistry for CD206+ and CD163+ macrophages. **Left:** CD206+ macrophages present around the microdialysis probe. **Right:** CD163+ macrophages present around the microdialysis probe. **Top:** Tissue surrounding a microdialysis probe implanted subcutaneously which utilizes a control perfusion fluid. **Bottom:** Tissue surrounding a microdialysis probe implanted subcutaneously which utilizes a treatment (10 ng/mL IL-10) perfusion fluid. 20x magnification, paraffin embedded sections.
CCL2 concentrations were measured in the dialysates of control and treatment (10 ng/mL IL-10) probes. Figure 5 shows a box plot of the concentrations of CCL2 collected in both the control and treatment dialysates over the 7 hour collection period. In the first hour of collection, concentrations of CCL2 were approximately the same in the control and treatment dialysates. Over the 7 hour collection period, the concentration of CCL2 steadily rose in the dialysates of the control probes. In the dialysates from the control probes, average CCL2 concentrations ranged from ~500 pg/mL during the first hour of collection to ~5500 pg/mL during the final hour of collection. In the dialysates collected from the treatment probes, average CCL2 concentrations ranged from ~500 pg/mL in the first hour of collection to ~5800 pg/mL during the final hour of collection. While the average concentration of CCL2 collected from the treatment probe increased over the collection period, there was no steady increase seen in CCL2 concentration as was seen in the dialysates collected from the control probes. In fact, the concentration range of CCL2 collected in the dialysates of treatment probes peaked during the second hour of collection with no difference seen between the second hour and the following hours.
Quantification of CCL2 in Response to IL-10 Ultrafiltration

Fig. 5. Box plot showing concentration of CCL2 collected in control and treatment (10 ng/mL IL-10) dialysates over a 7 hour period on the day of implantation in response to ultrafiltration.
Histological studies were performed to determine the effects of IL-10 ultrafiltration on the morphology of the tissue surrounding the microdialysis probe. H&E stains were performed to determine the cellular density of the tissue surrounding the probes as well as the organization of cells in the tissue surrounding the probes. In the IL-10 ultrafiltration tissue, the cells were less densely packed in the tissue surrounding the probe as compared to the tissue surrounding the control probe (Fig 6 A&B). There also appears to be more cells interrogating the control probe membrane as compared to the treatment probe membrane. Further, the tissue around the control probe is more organized than that surrounding the treatment probe. Masson's Trichrome stain was performed to investigate the amount of collagen present in the tissue surrounding the probes as well as the strand thickness and organization of the collagen. The Masson's Trichrome stained tissues show there to be more collagen in the tissue surrounding the treatment probe as compared to the control probe (Fig 6 C&D). Further, the collagen is well organized and seen much closer, to the treatment probe, immediately surrounding, as compared to the control probe, ~150 µm before significant amounts of collagen are seen. Interestingly though, the tissue surrounding the treatment probe is more vascular than the tissue seen surrounding the control probe where practically no blood vessels are seen.

Immunohistochemistry was also performed to determine the amount of macrophages in the tissue surrounding both the control and treatment probes as well as the predominant phenotype of these macrophages. No appreciable difference was seen in the amount of macrophages (CD68+) in the tissue surrounding the probe. Further, there was no appreciable difference in the phenotype of the macrophages. That is, there was no difference in the amount of CCR7+, CD206+, or CD163+ macrophages.
Fig. 6. Ultrafiltration of control and 10 ng/mL IL-10 treated probe. **Top:** H&E stain (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid and ultrafiltration. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (10 ng/mL IL-10) perfusion fluid and ultrafiltration. **Bottom:** Masson's Trichrome stain. (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid and ultrafiltration. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (10 ng/mL IL-10) perfusion fluid and ultrafiltration. 10x magnification, white bar represents 100 µm.
2.3 Four Day IL-10 Infusion (Dextran-70)

Collections were performed on the day of implantation and 4 days post implantation. The concentration of seven different analytes were quantified each day: CCL2, IL-1β, IL-6, IL-10, KC/GRO, MIP-2, and RANTES. Concentrations of the seven analytes were quantified in both control and treatment (10 ng/mL IL-10) dialysates (Fig 7). On the day of implantation, CCL2 concentrations ranged from 15 pg/mL – 1795 pg/mL in control and from 20 pg/mL – 510 pg/mL in treatment, IL-1β concentrations ranged from 15 pg/mL – 450 pg/mL in control and from below 5 pg/mL – 680 pg/mL in treatment, IL-6 concentrations ranged from below 75 pg/mL – 515 pg/mL in control and from below 75 pg/mL – 595 pg/mL in treatment, IL-10 concentrations ranged from 15 pg/mL – 1035 pg/mL in control and from below 10 pg/mL - 805 pg/mL in treatment, KC/GRO concentrations ranged from 15 pg/mL - 640 pg/mL in control and from 5 pg/mL – 555 pg/mL in treatment, MIP-2 ranged from below 25 pg/mL - 1610 pg/mL in control and from below 25 pg/mL – 415 pg/mL in treatment, and RANTES ranged from 15 pg/mL – 1170 pg/mL in control and from 15 pg/mL – 510 pg/mL in treatment. Four days post implantation, CCL2 levels ranged from below 10 pg/mL-3930 pg/mL in the control and from below 10 pg/mL-145 pg/mL in the treatment, IL-1β concentrations ranged from 15 pg/mL -over 10,000 pg/mL in control and from 5 pg/mL- 155 pg/mL in treatment, IL-6 concentrations ranged from below 75 pg/mL - over 300,000 pg/mL in control and from below 75 pg/mL - 165 pg/mL in treatment, IL-10 concentrations ranged from 15 pg/mL - 3620 pg/mL in control and from 20 pg/mL-260 pg/mL in treatment, KC/GRO concentrations ranged from 15 pg/mL - 15835 pg/mL in control and from 5 pg/mL – 260 pg/mL in treatment, MIP-2 ranged from below 25 pg/mL – 5550 pg/mL in control and from below 25 pg/mL - 210 pg/mL in treatment, and RANTES ranged from 10 pg/mL – 7830 pg/mL in control and from 15 pg/mL – 300 pg/mL in treatment. A significant difference between control and treatment was found only in the concentration of IL-10 collected during the initial flush four days post implantation.
Fig. 7. Bar graphs showing the concentration of seven analytes in both control and treatment (four day infusion of 10 ng/mL IL-10) dialysates. Collections were performed on the day of implantation and four days post implantation. *p≤0.05
Figure 8 shows histological results from tissue surrounding a control probe and tissue surrounding a treatment probe where 10 ng/mL IL-10 was infused for four days. H&E stained tissue shows a thin, ~50 µm or less, area of densely packed cells immediately surrounding the control probe with the remaining tissue consisting of well-organized but more scattered cells. The tissue surrounding the treatment probe consists of a larger area of densely packed cells surrounding the probe, ~100 µm or more. Past the initial area of densely packed cells, cells are found to be scattered and not well organized. The Masson's Trichrome stained tissue shows there to be more collagen surrounding the treatment probe as compared to the control though this difference is negligible and may be due in part to the cells distal to the probe in the control tissue being more organized therefore making the collagen seem more dispersed. Interestingly, in the Masson's Trichrome stained tissue, a ring of tissue ~200 µm wide is present immediately surrounding the treatment probe which is absent in the control tissue. This ring of tissue consists of neutrophils, macrophages, foreign body giant cells, blood, and possibly cellular debris and dead cells.

Immunohistochemistry was also performed on the tissue surrounding both the control and treatment probes to determine if the treatment had any effect on the amount of macrophages present or on the predominant macrophage activation state present. No appreciable difference was seen in the amount of macrophages (CD68⁺) surrounding the treatment probe as compared to the control probe. Further, there was no difference in the amount of CCR7⁺, CD206⁺, or CD163⁺ macrophages surrounding the treatment probe as compared to the control probe.
Fig. 8. Control and four day infused 10 ng/mL IL-10 treated probe. Top: H&E stain (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (10 ng/mL IL-10) perfusion fluid infused for four days. Bottom: Masson’s Trichrome stain (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (10 ng/mL IL-10) perfusion fluid infused for four days. 10x magnification, white bar represents 100 µm.
2.4 IL-10: 200 and 1200 ng/mL Infusion (Dextran-500)

200 ng/mL Infusion

Histological analyses were performed to determine cellular density (H&E) and collagen presence in the tissue surrounding a control probe and a treatment probe (200 ng/mL IL-10 infused). Figure 9 shows the H&E stained tissue. The tissue surrounding the control probe is well organized and densely packed immediately surrounding the probe. This tissue remains well organized and densely packed in areas as far as ~500 µm away from the probe. The tissue surrounding the treatment probe has a dense ring of cells, ~100 µm, immediately surrounding the probe. Further, there is an area of tissue immediately surrounding the probe consisting of neutrophils, macrophages, foreign body giant cells, blood, and possibly cellular debris and dead cells, much like was seen in the tissue surrounding the four day IL-10 infused tissue only exacerbated. Interestingly, once past the initially dense ring of cells, the tissue is found to be much less organized with cells being far more scattered in the treatment tissue as compared to the control tissue. The Masson’s Trichrome stained tissue shows collagen immediately surrounding both the control and treatment probes (Fig 10). However, there appears to be more collagen immediately surrounding the control probe as well as consisting of much thicker strands as compared to the treatment probe. In areas more distal to the probe, the amount of collagen in the tissue increases in the tissue surrounding the control probe with thick strands persisting throughout (Fig 10B). In the tissue surrounding the treatment probe, very little collagen is found in areas distal to the probe (Fig 10D).
Fig. 9. H&E stained control and 200 ng/mL IL-10 treated tissue. **Left:** Tissue immediately surrounding the microdialysis probe. (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (200 ng/mL IL-10) perfusion fluid. **Right:** Tissue distal to the microdialysis probe. (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (200 ng/mL IL-10) perfusion fluid. 10x magnification, white bar represents 100 µm.
**Fig. 10.** Masson's Trichrome stained control and 200 ng/mL IL-10 treated tissue. **Left:** Tissue immediately surrounding the microdialysis probe. (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (200 ng/mL IL-10) perfusion fluid. **Right:** Tissue distal to the microdialysis probe. (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (200 ng/mL IL-10) perfusion fluid. 10x magnification, white bar represents 100 µm.
Immunohistochemistry was performed to determine if there was any difference in the amount of macrophages (CD68+) or the predominant phenotype (CD206+ and CD163+) in the tissue surrounding the IL-10 treated probe as compared to the control probe. Antibodies specific for rat CD68 were used to identify macrophages in the tissue surrounding the microdialysis probe. Antibodies specific for CD206 and CD163 were used to identify M2 and M(IL-10) (M2c) macrophages in tissue surrounding the microdialysis probe. No appreciable difference was seen in the number of macrophages (CD68+) in the tissue surrounding the treatment as compared to the control probe (Fig 11). Further, no difference was seen in the amount of M2 (CD206+) macrophages present in the tissue surrounding the treatment probe as compared to the control probe (Fig 12). There seemed to be a slightly more CD163+ [M(IL-10)] macrophages in the tissue surrounding the treatment probe as compared to the control but this difference is negligible (Fig 13).
Fig. 11. Macrophages surrounding the microdialysis probe. These cells are preferentially stained using antibodies specific for CD68. **Top:** Tissue surrounding a control microdialysis probe. **Bottom:** Tissue surrounding a treatment (200 ng/mL IL-10) microdialysis probe. Images are 20X magnification, paraffin embedded tissue.
Fig. 12. M2 macrophages surrounding the microdialysis probe. These cells are preferentially stained using antibodies specific for CD206. **Top:** Tissue surrounding a control microdialysis probe. **Bottom:** Tissue surrounding a treatment (200 ng/mL IL-10) microdialysis probe. Images are 20X magnification, paraffin embedded tissue.
Fig. 13. M(IL-10) (M2c) macrophages surrounding the microdialysis probe. These cells are preferentially stained using antibodies specific for CD163. **Top:** Tissue surrounding a control microdialysis probe. **Bottom:** Tissue surrounding a treatment (200 ng/mL IL-10) microdialysis probe. Images are 20X magnification, paraffin embedded tissue.
1200 ng/mL IL-10 Infusion

Tissue surrounding both the control and treatment (1200 ng/mL IL-10) were stained using H&E and Masson's Trichrome. Figure 14 shows H&E stained tissue surrounding both control and treatment probes. In the tissue surrounding the control probe, a dense cellular layer, ~200 µm or greater, is seen immediately surrounding the probe. Distal to this area, the tissue is well organized and cells are dense, though not as densely packed as is seen immediately surrounding the probe. Immediately surrounding the treatment probe, a highly dense area of cells is seen which stain dark purple to black representing neutrophils, macrophages, foreign body giant cells, blood, and possibly cellular debris and dead cells. This area is similar to what was seen in the 200 ng/mL treated IL-10 tissue. Immediately surrounding this highly dense area, an area of tissue is seen which seems to be necrotic. However, beyond the necrotic tissue, the cells are scattered and less well organized as compared to the control. Figure 15 shows Masson's Trichrome stained tissue surrounding both control and treatment probes. In the tissue surrounding the control probe, collagen is seen immediately surrounding the probe with increased amounts of collagen seen in areas distal to the probe. In the treatment tissue, very little collagen is seen in areas within the necrotic tissue with a ring of thick collagen seen surrounding the necrotic tissue. Interestingly, distal to the necrotic tissue, less collagen is seen as compared to the control tissue.
Fig. 14. H&E stained control and 1200 ng/mL IL-10 treated tissue. **Left:** Tissue immediately surrounding the microdialysis probe. (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (1200 ng/mL IL-10) perfusion fluid. **Right:** Tissue distal to the microdialysis probe. (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (1200 ng/mL IL-10) perfusion fluid. 10x magnification, white bar represents 100 µm.
Fig. 15. Masson's Trichrome stained control and 1200 ng/mL IL-10 treated tissue. **Left:** Tissue immediately surrounding the microdialysis probe. (A) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (B) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (1200 ng/mL IL-10) perfusion fluid. **Right:** Tissue distal to the microdialysis probe. (C) Microdialysis probe implanted in the subcutaneous space utilizing a control perfusion fluid. (D) Microdialysis probe implanted in the subcutaneous space utilizing a treatment (1200 ng/mL IL-10) perfusion fluid. 10x magnification, white bar represents 100 µm.
Immunohistochemistry showed there to be differences in the amount of macrophages and the predominant phenotype present in the tissue surrounding the treatment probe as compared to the control probe. The most significant differences were seen in macrophages staining CD68\(^+\) and CD163\(^+\) (Fig 16). In the tissue surrounding the treatment (1200 ng/mL IL-10), more CD68\(^+\) macrophages are seen as compared to the tissue surrounding the control probe. Further, more CD163\(^+\) [M(IL-10)] macrophages are seen in the tissue surrounding the treatment probe as compared to the tissue surrounding the control probes. Interestingly, when the tissue surrounding the 200 ng/mL treatment probes is compared to the tissue surrounding the 1200 ng/mL treatment probes, a difference is seen in the amount of CD68\(^-\), CD206\(^+\), and CD163\(^+\) macrophages. More macrophages (CD68\(^+\)) are seen in the 1200 ng/mL treatment tissue as well as more M2 (CD206\(^+\)) and M(IL-10) (CD163\(^+\)) as compared to the 200 ng/mL treatment tissue (Fig 17-19).
Fig. 16. Macrophages surrounding the microdialysis probe. **Left:** These cells are preferentially stained using antibodies specific for CD68. **Right:** These cells are preferentially stained using antibodies specific for CD163. **Top:** Tissue surrounding a control microdialysis probe. **Bottom:** Tissue surrounding a treatment (200 ng/mL IL-10) microdialysis probe. Images are 20X magnification, paraffin embedded tissue.
Fig. 17. Macrophages surrounding the microdialysis probe. These cells are preferentially stained using antibodies specific for CD68. **Top:** 200 ng/mL IL-10 treatment tissue. **Bottom:** 1200 ng/mL IL-10 treatment tissue. 20X magnification, paraffin embedded tissue.
Fig. 18. M2 Macrophages surrounding the microdialysis probe. These cells are preferentially stained using antibodies specific for CD206. **Top:** 200 ng/mL IL-10 treatment tissue. **Bottom:** 1200 ng/mL IL-10 treatment tissue. 20X magnification, paraffin embedded tissue.
Fig. 19. M(IL-10) (M2c) macrophages surrounding the microdialysis probe. These cells are preferentially stained using CD163. **Top:** 200 ng/mL IL-10 treatment tissue. **Bottom:** 1200 ng/mL IL-10 treatment tissue. 20X magnification, paraffin embedded tissue.
3. Discussion

Any material which enters the body and is too large to be phagocytosed, results in the foreign body response (FBR). The FBR results in the encapsulation of the material separating the material from the rest of the body causing it to reside in its own microenvironment. This phenomena holds true for the implantation of biomaterials as well. While the process of encapsulation and subsequent residing of the biomaterial in a microenvironment has long been known to occur, it has been seen as an unfortunate yet unavoidable consequence.

Macrophage activation (formerly macrophage polarization) is a process which plays a role in the outcome of implanted biomaterials [12] and in fact has been shown to be an indicator of biomaterial implantation outcome [13-15]. While much is known about how macrophages change activation state in vitro little is known about the ability of macrophages to do so in vivo. Further, much of the in vivo work has focused on using degradable biomaterials while there is very little to no information on the use of non-degradable biomaterials in conjunction with modulators to shift the activation state of macrophages. The ability to change and/or control the activation state of macrophages in vivo at an implant site might result in better integration of the biomaterial into the surrounding tissue.

Microdialysis sampling technique is unique in its ability to simultaneously collect analytes from and deliver modulators to the implant site. For this reason, microdialysis sampling technique is a useful tool for investigating the effects of locally-delivered modulators on the biochemical processes at an implant site. Coupling microdialysis sampling technique with histological analyses and immunohistochemistry allows for the determination of whether a modulator is having advantageous effects on fibrosis and shifting macrophages to a desired activation state, respectively.

Interleukin-10 was used in this study for a multitude of reasons. First, IL-10 is known to be an activator of macrophages. It is known that IL-10 is capable of inducing a switch of macrophages to an M2c [M(IL-10)] state which is characterized as being anti-inflammatory and pro-wound healing [16] and is
known to play a role in limiting inflammatory responses [2, 17]. However, no studies have been performed to determine the ability of IL-10 to induce M(IL-10) macrophages at a non-degradable implant site. Second, while IL-10 is said to result in a pro-wound healing environment, it is unknown how the subcutaneous space is affected by IL-10 during the FBR. Third, there are conflicting results on the effects IL-10 has on the regulation of different cytokines such as CCL2 [4-6]. Knowing this information will better illuminate the molecular mechanisms through which IL-10 is able to exert anti-inflammatory effects and shift macrophage activation state.

In the first experiment, IL-10 (10 ng/mL) perfusion, the release of IL-10 from the microdialysis probe resulted in a trend where CCL2 concentrations were higher in the dialysate collected from the treatment probes as compared to the control probes. While a trend was seen, this trend was not significant at the 95% confidence interval, only at a 90% confidence interval. While this is in disagreement with studies which show IL-10 to inhibit CCL2 [18] it is not novel as reports show that IL-10 can cause an increase in CCL2 mRNA expression levels in a mouse lung fibrosis model where IL-10 was over-expressed [19] and also in CCL2 protein concentrations [4]. Given that IL-10 seemed to cause an increase in CCL2 concentrations, it was postulated that by increasing the amount of IL-10 delivered to the implant site, a greater increase in IL-10 concentrations would be seen in treatment dialysates. This increase in IL-10 concentration would thereby lead to a larger difference in CCL2 concentrations found in treatment and control dialysates.

To deliver higher concentrations of IL-10 to the implant site, ultrafiltration was utilized where there was a ~50% fluid loss (~30 µL/hour). Ultrafiltration is simply the loss of fluid across the microdialysis membrane. While this phenomenon is generally seen as problematic in microdialysis sampling and therefore avoided, it can be used to deliver higher concentrations of a modulator to an implant site. In the IL-10 ultrafiltration experiment, the trend that was seen previously in the IL-10 (10 ng/mL) perfusion disappeared and there was found to be no difference nor trend in the concentration of CCL2 found in the dialysate from the treatment probe as compared to the control probe. To verify that this was due to differential IL-10 effects on CCL2 and not due to analytes in the ECS possibly being diluted by the fluid lost during ultrafiltration, IL-10 was delivered for four days (Four Day IL-10 Infusion). Again, no difference

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was seen in the concentration of CCL2 in the dialysate collected from the treatment probe as compared to the control probe. Further, the concentrations of six other analytes (IL-1β, IL-6, IL-10, KC/GRO, MIP-2, and RANTES) were tested and found to show no significant difference between treatment and control.

Since IL-10 is said to be anti-inflammatory and result in macrophages which produce a pro-tissue remodeling, pro-wound healing state, histological analyses were performed to determine the effects IL-10 had on tissue. In the IL-10 (10ng/mL) perfusion, there was no appreciable difference seen in the H&E or Masson's Trichrome stained tissue. While this may imply that IL-10 is not resulting in the anti-inflammatory, pro-tissue remodeling, pro-wound healing state as expected, it may also be a result of insufficient IL-10 being delivered to the wound site. It is important to note at this point that the relative recovery of IL-10 through a CMA 20 100 kDa probe is ~1% (data not shown). This means that if 10 ng/mL IL-10 are being perfused through the microdialysis probe that only ~0.1 ng/mL of IL-10 is being delivered to the wound site.

Firstly, ultrafiltration was used to increase the amount of IL-10 being delivered to the wound site. When ultrafiltration was utilized, differences in cellular density as well as collagen formation were seen in the treatment tissue as compared to the control tissue. The treatment tissue had less cellular density and increased collagen surrounding the microdialysis probe. The decrease in cellular density would suggest a decrease in inflammation at the implant site. On the contrary, the increase in collagen might suggest reduced healing and scar tissue formation, especially given its organization. Again, it is not apparent whether these findings are a result of the IL-10 being infused or from mechanical injury being sustained from the fluid leaving the probe due to ultrafiltration. In the four day IL-10 infusion experiment, we find a layer of densely packed cells immediately surrounding the treatment probe as well as more collagen surrounding the treatment probe as compared to the control. Further, for the first time we see a large extremely dense cellular area surrounding the treatment probe which is absent from the control tissue. These results suggests that the IL-10 is not resulting in an anti-inflammatory, pro-tissue remodeling, pro-wound healing environment but rather is resulting in increased inflammation.

Given the anomalies seen in the IL-10 work thus far, a new hypothesis was derived with two options: 1) IL-10 has very transient and dose dependent effects on tissue or 2) there is something
entering the tissue abrogating the effects of IL-10 and resulting in an increase in inflammation. To elucidate which issue was leading to the unexpected results, a series of experiments were performed to determine if any components of the perfusion fluid were causing increased inflammation (Chapter 3). What was found was that Dextran-70, a commonly used osmotic agent in microdialysis sampling technique, is able to exit the probe resulting in increased inflammatory responses and even necrosis at high concentrations.

Once Dextran-70 was identified as a pro-inflammatory agent and eliminated from the perfusion fluid, experiments were performed where either 200 ng/mL or 1200 ng/mL IL-10 were infused. Similar histological results were obtained from both 200 ng/mL and 1200 ng/mL IL-10 infusions. In the tissue immediately surrounding the treatment probes, the same extremely dense cellular area was seen as was seen in the four day IL-10 infusion only to a much greater extent. IL-10 has been shown to cause apoptosis in monocytes [20, 21] which may lead to reduced clearance of apoptotic neutrophils and an increase in apoptotic cells at the membrane/tissue interface resulting in the extremely dense cellular area surrounding the probe. Once beyond this initial area, there is a decrease in cellularity which is not seen in the tissue surrounding the control probe. In the 1200 ng/mL treated tissue, there is a tightly bound ring of collagen which is parallel to the wound indicative of scar tissue formation [22, 23] with less collagen seen beyond that as compared to the tissue surrounding the control probe. Interestingly, in the 200 ng/mL treated tissue, there is no sign of the scar tissue formation with very little collagen being seen beyond the 'apoptotic/necrotic' area. This suggests that at 200 ng/mL, IL-10 has some anti-inflammatory, pro-wound healing, pro-tissue remodeling effects. These effects might possibly be intensified if a lower dose of IL-10 were used or if IL-10 were used in conjunction with a second modulator that would help overcome the apoptotic effects.

The activation state of macrophages at an implant site has been shown to be a predictor of implant outcome with early M2 predominance at the wound site leading to improved outcome as compared to early M1 predominance at the wound site [15]. Further, Badylak’s group has shown that a scaffold wound site which is predominated by M2c macrophages results in improved integration and outcome of the implanted scaffold [13, 14]. IL-10 has been reported to shift macrophages to the M(IL-10)
It is hypothesized that by delivering IL-10 to a wound site, macrophages will shift to the M(IL-10) activation state resulting in improved integration of the biomaterial with the host tissue and therefore increased longevity of the biomaterial. In this work, IL-10 was found to have no effect on the predominant macrophage phenotype present at the wound site when delivered at 10 ng/mL regardless of whether the IL-10 was delivered for one day, ultrafiltrated, or delivered for four consecutive days. However, at higher concentrations, 200 ng/mL and 1200 ng/mL, IL-10 was seen to be able to cause a minimal shift in the activation state of macrophages surrounding the microdialysis probe to an M(IL-10) state. It should be clearly noted at this point that different dextrans were used for 10 mg/mL studies and 200 ng/mL and 1200 ng/mL studies and the difference in dextran cannot be excluded as a possible factor in the results. The shift in activation state was most evident in the 1200 ng/mL IL-10 infusion which would suggest that either this concentration is needed to deliver sufficient IL-10 to the wound area to cause any effect or that the effects of the IL-10 on the activation state of macrophages is dose dependant. Surprisingly, the histological results do not support this outcome but rather show increased inflammation at the implant site in response to the higher concentrations of IL-10. This anomaly may be due to the fact that IL-10 has been shown to cause apoptosis in macrophages [21]. Further, it can be expected that the predominance of M(IL-10) macrophages would be found close to the microdialysis membrane as this is where the IL-10 is delivered and at the highest concentration. While the microdialysis probe should cause a shift to a M2 phenotype on its own, if IL-10 is causing apoptosis in M(IL-10) macrophages this might result in the remaining M2 macrophages present not being M(IL-10) macrophages. Contrary to IL-10 and IL-10 macrophages which are considered pro-wound healing and pro-tissue remodeling, M2 macrophages as a whole have been implicated in increased fibrosis in some instances [25, 26]. This may in part explain the increased inflammation seen at the implant site in response to high concentrations of IL-10.

At this point it should be explicitly noted that all the immunohistochemistry slides prepared were formalin fixed paraffin embedded sections. Formalin fixed paraffin embedded sections are classically used for histological analyses due to the high retention of tissue morphology. While this would also be advantageous to immunohistochemical analyses, cross-linking becomes an issue as some epitopes become blocked during the process. This requires that an antigen retrieval step be performed. Also, the
paraffin must be removed from the tissue sections. Protocols have been established to perform immunohistochemistry on formalin fixed, paraffin embedded tissue sections where an antigen retrieval step and paraffin removal step are performed. Also, more antibodies are becoming available which are capable of being used on formalin fixed, paraffin embedded tissue sections. While many in the field use formalin fixed, paraffin embedded tissue sections for immunohistochemistry, we found that this technique resulted in high background noise. Even though antigen retrieval and paraffin removal techniques were used, we found there to be residual paraffin left on the tissue sections. When the tissue sections were analyzed, we found that this residual paraffin fluoresced. This fact may in part explain why no differences were seen in the immunohistochemistry in response to lower concentrations of IL-10 and why at high concentrations of IL-10 very minimal differences were seen in the immunohistochemistry. Formalin fixed, paraffin embedded tissue sections were abandoned for frozen sections in all remaining immunohistochemistry analyses. Switching to frozen sections had two main advantages: 1) the high amount of background noise seen with the paraffin was eliminated as there is no paraffin involved and the frozen sections showed little to no background noise or non-specific binding of antibodies 2) a more accurate profile of the macrophage activation state at the implant site could be obtained as monoclonal antibodies can be used with the frozen sections which cannot be done with paraffin sections as the antibodies are not available. It would be interesting and worth further investigation to use frozen sections and determine if IL-10 is capable of causing a shift in the macrophage activation state at an implant site.

4. Conclusion

In this study microdialysis sampling probes were implanted in the subcutaneous space of male Sprague Dawley rats. The microdialysis probe was used to locally deliver IL-10 to the wound site while simultaneously collecting analytes from the ECS. Further, tissue histology, and immunohistochemistry were performed to determine the effects of IL-10 on cellular density and collagen formation as well as macrophage activation state, respectively. While IL-10 caused no alterations in the cytokine levels tested, differences were seen in the tissue histology and macrophage activation state. The use of high
levels of IL-10 resulted in increased inflammation at the wound site. However, high levels of IL-10 also caused a minimal increase in the number of M(IL-10) macrophages at an implant site, though these effects as well as any possible effects at lower levels might become more obvious through the use of frozen tissue sections. While much work has been done to better understand macrophage activation, the majority of this work has been performed \textit{in vitro} with few \textit{in vivo} studies being performed. It has been postulated that through the use of modulators, which have been proven to shift macrophage activation states \textit{in vitro}, the activation state of macrophages can be altered or controlled resulting in improved integration of biomaterials into the host tissue and increased longevity of these biomaterials. It is important to understand how the use of these \textit{in vitro} modulators correlates to \textit{in vivo} responses. This is the first study to investigate the ability of locally delivered IL-10 to alter macrophage activation state at an implant site.

\section*{Acknowledgements}

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References


Appendix B

Research Compliance Protocol Letter
MEMORANDUM

TO: Julie Stenken

FROM: Craig N. Coon, Chairman
Institutional Animal Care and Use Committee

DATE: May 2, 2014

SUBJECT: IACUC APPROVAL
Expiration date: May 11, 2017

The Institutional Animal Care and Use Committee (IACUC) has APPROVED protocol 14041: "Inducing and Monitoring Macrophage Polarization at an Implant Site in a Rat Subcutaneous Model" for the period of May 12, 2014 thru May 11, 2017.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any further changes to the protocol during the research, please notify the IACUC in writing (via the Modification form) prior to initiating the changes. If the study period is expected to extend beyond May 11, 2017 you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at a time.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for involving animal subjects.

CNC/aem

cc: Animal Welfare Veterinarian