IN VITRO AND IN VIVO CYTOKINE-ASSOCIATED IMMUNE RESPONSE TO BIOMATERIALS

by

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Duke University

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George Truskey

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

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ABSTRACT

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Abstract

The success of implanted medical devices, such as biosensors, is dependent on the immune reaction to the surface of the implanted material. This immune reaction, termed the foreign body reaction, is potentially affected by the physical and chemical properties of the implanted material. Macrophages interact with the surface of the implanted material and secrete intercellular signals, including cytokines and growth factors, which direct the actions of immune cells in the surrounding tissue. The type and quantity of cytokines and growth factors produced by macrophages at an implant surface could be an indicator of the outcome of the foreign body reaction.

This study investigated the effect of the surface chemistry of an implanted device on the production of cytokines and growth factors. First, microdialysis sampling was characterized as a technique for collecting cytokines and growth factors from the tissue surrounding an implant. Based on this characterization, it was determined that a direct sampling method would be more suitable than microdialysis sampling for determining accurate tissue concentrations of cytokines and growth factors. Second, an in vitro model was developed and utilized to assess cytokine and growth factor production from monocyte/macrophage cultures seeded onto commonly implanted polymeric biomaterials with varying surface chemistries. The materials included in this study were polyethylene (PE), polyurethane (PU), polymethyl methacrylate (PMMA), expanded polytetrafluoroethylene (ePTFE), and a
cytotoxic organo-tin polyvinyl chloride (ot-PVC) as a positive control. From this in vitro model, it was determined that the varying surface chemistries of these non-toxic materials, excluding ot-PVC, did not significantly affect the types and quantities of cytokines and growth factors produced. Finally, an in vivo model for evaluating the cytokine and growth factor response to an implanted biomaterial was utilized for comparison with the in vitro findings. In this model, biomaterials were implanted subcutaneously within the lumen of a stainless steel mesh cage. The mesh cage served to create a “pocket” where wound exudate fluid collected within the cage, surrounding the implanted biomaterial. The materials included in this study were PE, PU, and ot-PVC. Cytokines and growth factors produced at the material surface were sampled directly from the exudate fluid. The results from this in vivo study indicate that cytokine and growth factor production were not significantly impacted by the varying surface chemistries of the implanted biomaterials. The in vivo data support the findings from the in vitro model, suggesting that the foreign body reaction proceeds in a similar fashion for each of these non-cytotoxic, polymeric biomaterials with varying surface chemistries.
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<tr>
<td>FBGC</td>
<td>Foreign body giant cell</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptors</td>
</tr>
<tr>
<td>SR</td>
<td>Scavenger receptors</td>
</tr>
<tr>
<td>MR</td>
<td>Mannose receptor</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate-13-acetate</td>
</tr>
<tr>
<td>PES</td>
<td>Polyethersulfone</td>
</tr>
<tr>
<td>BMD model</td>
<td>Microdialysis model developed by Bungay, Morrison, and Dedrick</td>
</tr>
<tr>
<td>FEP</td>
<td>Fluorinated ethylene polymer</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>MWCO</td>
<td>Molecular weight cut-off</td>
</tr>
<tr>
<td>$E_d$</td>
<td>Extraction fraction</td>
</tr>
<tr>
<td>$C_0$</td>
<td>Analyte concentration in dialysate</td>
</tr>
<tr>
<td>$C_i$</td>
<td>Analyte concentration in perfusate</td>
</tr>
<tr>
<td>$C_s$</td>
<td>Analyte concentration in external solution</td>
</tr>
<tr>
<td>$Q_d$</td>
<td>Perfusate flow rate</td>
</tr>
<tr>
<td>$R_d$</td>
<td>Mass transport resistance in the dialysate</td>
</tr>
<tr>
<td>$R_m$</td>
<td>Mass transport resistance in the membrane</td>
</tr>
<tr>
<td>Symbol</td>
<td>Definition</td>
</tr>
<tr>
<td>--------</td>
<td>------------</td>
</tr>
<tr>
<td>$R_e$</td>
<td>Mass transport resistance in the external solution</td>
</tr>
<tr>
<td>$r_{β}$</td>
<td>Internal radius of the microdialysis membrane</td>
</tr>
<tr>
<td>$r_a$</td>
<td>External radius of the microdialysis probe cannula</td>
</tr>
<tr>
<td>$r_0$</td>
<td>External radius of the microdialysis membrane</td>
</tr>
<tr>
<td>$L$</td>
<td>Effective length of the microdialysis membrane</td>
</tr>
<tr>
<td>$D_d$</td>
<td>Solute diffusion coefficient in the dialysate</td>
</tr>
<tr>
<td>$D_m$</td>
<td>Solute diffusion coefficient in the membrane</td>
</tr>
<tr>
<td>$Φ_m$</td>
<td>Solvent accessible volume fraction of the membrane</td>
</tr>
<tr>
<td>$D_{LS}$</td>
<td>Diffusion coefficient determined by light scattering</td>
</tr>
<tr>
<td>$D_{PL}$</td>
<td>Diffusion coefficient calculated by a power law equation</td>
</tr>
<tr>
<td>$j$</td>
<td>Solute flux through the microdialysis membrane</td>
</tr>
<tr>
<td>$ΔC$</td>
<td>Concentration gradient across the microdialysis membrane</td>
</tr>
<tr>
<td>$k_1$</td>
<td>Mass transport coefficient in the external solution</td>
</tr>
<tr>
<td>$k_3$</td>
<td>Mass transport coefficient in the dialysate</td>
</tr>
<tr>
<td>$Δr_m$</td>
<td>Microdialysis membrane thickness</td>
</tr>
<tr>
<td>$SA$</td>
<td>Surface area of the microdialysis membrane</td>
</tr>
<tr>
<td>$v_0$</td>
<td>Dialysate bulk velocity</td>
</tr>
<tr>
<td>$ν$</td>
<td>Dialysate kinematic viscosity</td>
</tr>
<tr>
<td>$μ$</td>
<td>Dialysate viscosity</td>
</tr>
<tr>
<td>$V_0$</td>
<td>Dialysate volume</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1 beta</td>
</tr>
<tr>
<td>IL-1α</td>
<td>Interleukin-1 alpha</td>
</tr>
<tr>
<td>IL-1ra</td>
<td>Interleukin-1 receptor antagonist</td>
</tr>
<tr>
<td>IL-2</td>
<td>Interleukin-2</td>
</tr>
<tr>
<td>IL-4</td>
<td>Interleukin-4</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IL-10</td>
<td>Interleukin-10</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
</tr>
<tr>
<td>MIP-1α</td>
<td>Macrophage inflammatory protein-1 alpha</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Macrophage inflammatory protein-2</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor- alpha</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>PE</td>
<td>Polyethylene</td>
</tr>
<tr>
<td>PU</td>
<td>Polyurethane</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethyl methacrylate</td>
</tr>
<tr>
<td>ePTFE</td>
<td>expanded Polytetrafluoroethylene</td>
</tr>
<tr>
<td>ot-PVC</td>
<td>organo-tin Polyvinyl chloride</td>
</tr>
<tr>
<td>TCPS</td>
<td>Tissue-culture polystyrene</td>
</tr>
<tr>
<td>HEMA-PEG</td>
<td>2-hydroxyethyl methacrylate, 1-vinyl pyrrolidinone, polyethylene glycol acrylate</td>
</tr>
</tbody>
</table>
Acknowledgements

Thanks to my committee members and, in particular, Monty Reichert for providing support and guidance throughout my research project. Thanks to my labmates in the Reichert lab, as well as the CIEMAS and former LSRC lab members for your helpful suggestions and encouragement. Thanks to the undergraduate researchers, Shadia Oshodi, Dean Wang, Andreina Parisi-Amon, and Lola Xie for your contributions to this research. Thanks to my family for their support and optimism. Thanks, especially, to Amily and Ethan for your inspiration and continuous encouragement.
Chapter 1. Research Objectives

1.1 SIGNIFICANCE OF RESEARCH

Implanted medical devices, such as hip and knee replacements, glucose sensors, pacemakers, vascular grafts, and intraocular lenses, have revolutionized healthcare in the past several decades. These technologies have improved the quality of life of the millions of Americans suffering from osteoarthritis, heart disease, diabetes, and vision impairment. The success or failure of these implanted devices is largely dependent on the actions of immune cells in the surrounding tissue.

The aspects of the immune reaction that cause implant failure are driven by intercellular signaling molecules, such as cytokines and growth factors, derived from macrophages at an implant surface. Approaches toward controlling the immune reaction to implants primarily include surface modification and drug delivery. However, the signaling cascade that determines the fate of an implanted device is poorly understood. Characterizing the array of cytokines and growth factors derived from macrophage interactions with material surfaces is an important step in mediating inflammation-associated implant failure.

Monocytes activated into macrophage-like cells will be used in an in vitro biomaterial evaluation assay utilizing cytokine and growth factor production as an indicator of material-induced inflammation and wound healing. Cytokine and growth factor concentrations will be quantified using an antibody-bound microsphere array.
system which functions similar to a sandwich format immunoassay. To compare these in vitro experiments with in vivo data, samples containing cytokines and growth factors produced by the immune cells surrounding subcutaneously implanted biomaterial surfaces will be collected using a cage implant system. Comparisons between the in vitro and in vivo experiments will verify the in vitro biomaterial evaluation assay as an indicator of the in vivo immune reaction to an implanted material.

1.2 SPECIFIC AIMS

Specific Aim 1: Characterization of microdialysis sampling of macromolecules

Microdialysis sampling will be evaluated as a potential tool for sampling cytokines and growth factors from cell cultures and tissue. The efficiency of microdialysis sampling of macromolecules ranging from 3 to 150kD, encompassing the molecular weights of most cytokines and growth factors, will be characterized using 100kD MWCO polyethersulfone (PES) microdialysis membranes. Samples will be collected from microdialysis probes submerged in well-mixed solutions of fluorescently labeled macromolecules. Macromolecule diffusion coefficients will be calculated or determined experimentally for inclusion in models of mass transport in membranes. Experimental data will be evaluated for correlation with a published model for mass transport in microdialysis probes. In addition, cytokines and growth
factors will be sampled in vitro and in vivo using 100kD MWCO microdialysis probes. This technique will be evaluated as a tool for accurately quantifying tissue concentrations of cytokines and growth factors.

**Specific Aim 2: In vitro cytokine-associated immune response to biomaterials**

Cytokine and growth factor production will be analyzed from monocyte/macrophage cultures of various states of activation and differentiation seeded onto a selection of commonly implanted polymeric biomaterials with various surface chemistries. Production of cytokines and growth factors from monocytes/macrophages will be characterized as pro- or anti-inflammatory and pro- or anti-wound healing. These signals driving inflammation and wound healing will be analyzed for potential trends based on material surface chemistry.

**Specific Aim 3: In vivo cytokine-associated immune response to biomaterials**

Common biomaterials with different surface chemistries will be inserted into stainless steel mesh cages implanted subcutaneously into Sprague-Dawley rats. Cytokine and growth factor production will be analyzed from the exudate fluid contained within these stainless steel cage implants. In addition, the cell contents of the exudate fluid will be assessed throughout the eight week implantation. These
data will be analyzed for potential trends based on biomaterial surface chemistry and discussed in comparison to the results from the prior in vitro study.
Chapter 2. Background

The eventual success or failure of an implanted medical device is believed to be determined by the interaction between immune cells and the surface of an implant\(^1\). Immediately after implantation, the surface of the implanted material is adsorbed with an array of native proteins, such as fibrinogen, albumin, fibronectin, complement and IgG\(^4\). The first cells to arrive are neutrophils, which are responsible for clearing pathogens and damaged tissue from the site of implantation. Following neutrophils, monocyte-derived macrophages migrate into the tissue and attach to the implant surface. Macrophages are believed to serve as the bridge between the material surface and the host tissue, responsible for directing the tissue reaction to the foreign material, termed the foreign body reaction.

During the foreign body reaction, macrophages produce a milieu of intercellular signals, including cytokines and growth factors, in response to the foreign material. Researchers have deciphered some of the specific roles of the cytokines and growth factors produced during the foreign body reaction, but the induction of these signals is still poorly understood\(^5\). An analysis of the cytokines and growth factors produced in response to a biomaterial surface could improve upon our knowledge of the signaling processes involved in the foreign body reaction and drive the improvements in the design of implantable medical devices.
2.1 FOREIGN BODY REACTION

The foreign body reaction can be described as two phases: (1) an inflammatory phase immediately following implantation that functions to neutralize pathogens and (2) a repair phase that includes the healing and reconstitution of the implant site through regeneration of the damaged tissue. Although the inflammation and wound healing phases are characterized by a variety of cell types including neutrophils, fibroblasts, endothelial cells, and platelets, the predominant cell type at the surface of a long-term implant is the monocyte-derived macrophage. Macrophages at an implant surface secrete cytokines and growth factors that signal the recruitment and activation of lymphocytes, endothelial cells, and smooth muscle cells. These signals in turn drive the remodeling of tissue, formation of blood vessels, and the fibrous encapsulation of implants. In addition, cytokine activation of lymphocytes leads to the fusion of macrophages into foreign body giant cells (FBGCs), which have been shown to persist throughout the lifetime of an implanted device and attack the surface with degradative enzymes. The cascade of intercellular signals that determines the eventual fate of an implanted material originates from monocyte-derived macrophages at the implant surface.

Macrophages

Macrophages are myeloid lineage cells derived from monocytes that migrate into tissue from the bloodstream. Macrophages have specific functions dependent
on their location in the body, but are generally scavenger cells which degrade pathogens and damaged tissue and signal new tissue formation. Small particles can be degraded by macrophages through phagocytosis, a process by which the macrophage internalizes a particle into a phagolysosome and digests it with hydrolytic enzymes. For implant surfaces that are too large to be engulfed by a single macrophage, macrophages can fuse into FBGCs and attack the surface with secreted digestive enzymes.

The macrophages involved in the foreign body reaction migrate towards the site of implantation and adhere to the protein layer adsorbed onto an implanted material’s surface. McNally et al. suggest that macrophage adhesion to this protein layer is initially dependent on β2 integrins, and becomes dependent on β1 integrins as macrophages fuse into FBGCs. Integrin binding can initiate the formation of podosomes, which function in adhesion, migration, and degradative enzyme secretion. In addition, macrophages express an array of pattern recognition receptors, such as toll-like receptors (TLR), scavenger receptors (SR), and the mannose receptor (MR). SR are involved in apoptotic cell clearance, MR is an endocytic and phagocytic receptor, and TLR are implicated in recognition of microbial and foreign molecular targets. The mechanism by which macrophages recognize biomaterial surfaces remains unclear; however, transmembrane signals initiated by events such as macrophage receptor or integrin binding to an adsorbed
protein layer are known to stimulate inflammatory processes such as cytokine production\textsuperscript{10,15-17}.

**Cytokines**

Generally, the cytokines and growth factors involved in the foreign body reaction can be categorized as pro- or anti-inflammatory and pro- or anti-wound healing. Brodbeck et al. published a Gantt chart categorizing foreign body reaction cytokines and growth factors\textsuperscript{18}. The characterizations in Figure 2.1 have been adapted from the original source to include vascular endothelial growth factor (VEGF), which is considered pro-inflammatory and pro-wound healing for its role in promoting both dilation of blood vessels to maintain inflammation as well as angiogenesis to re-vascularize damaged tissue\textsuperscript{19}. Macrophage inflammatory protein-1 alpha (MIP-1\textalpha) and monocyte chemoattractant protein-1 (MCP-1) were also included in this figure as pro-inflammatory and anti-wound healing chemokines for their roles in recruiting monocytes, macrophages, neutrophils, T-lymphocytes, and inducing the production of other pro-inflammatory cytokines\textsuperscript{20,21}. 
**Figure 2.1 Cytokine categorization.** A Gantt chart categorizing the roles of cytokines and growth factors in the foreign body reaction (adapted from Brodbeck et al 2003). Additions to the published version are italicized.

Pro-inflammatory cytokines are responsible for activation and chemoattraction of inflammatory cells such as neutrophils, monocytes and lymphocytes, while anti-inflammatory cytokines downregulate this activity and suppress further cytokine production. Similarly, pro-wound healing cytokines are responsible for wound healing cell recruitment, such as fibroblasts, while anti-wound healing cytokines downregulate this recruitment and suppress cytokine production. Table 2.1 further details the functions of these cytokines and growth factors \(^5,22\).
Table 2.1 Cytokines in the foreign body reaction. From top to bottom, cytokines are grouped into pro wound healing, anti inflammatory; pro wound healing, pro inflammatory; anti wound healing, anti inflammatory; and anti wound healing, pro inflammatory.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Function in the foreign body reaction</th>
</tr>
</thead>
</table>
| IL-1ra   | • Competitive inhibitor of IL-1 receptor binding  
          • Preserves homeostasis in inflammation by modulating activities of IL-1 |
| IL-4     | • Blocks LPS-induced production of IL-1, IL-6, IL-8, IL-10, and TNF-α by monocytes  
          • Promotes proliferation and activation of B-cells  
          • Differentiates monocytes into macrophage-like dendritic cells |
| TGF-β    | • Promotes or inhibits angiogenesis dependent on concentration  
          • Deactivates macrophages and inhibits lymphocyte proliferation  
          • Stimulates synthesis of collagen, fibronectin, and integrins |
| IL-1β    | • Promotes proliferation of lymphocytes and activates monocytes  
          • Strong chemoattractant for leukocytes  
          • Promotes adhesion of neutrophils, monocytes, T-cells, B-cells  
          • Inhibits the growth of endothelial cells |
| VEGF     | • Potent mitogen for vascular endothelial cells  
          • Induces vascular permeability  
          • Chemoattractant for monocytes |
| IL-10    | • Inhibits production of pro-inflammatory cytokines  
          • Inhibits monocyte proliferation  
          • Downregulates macrophage-derived angiogenic factors (VEGF, IL-1β, TNF-α, IL-6) |
| IL-2     | • Stimulates T-cell proliferation  
          • Stimulates activated B-cell proliferation  
          • Induces secretion of TNF-α, IL-1 |
| IL-6     | • Stimulates B-cell proliferation and antibody production  
          • Differentiates T-cells into cytotoxic T-cells  
          • Plays a role in macrophage differentiation |
| IL-8     | • Can induce migration of macrophages, T-cells, and endothelial cells  
          • Exerts a variety of actions on neutrophils: degranulation, increase in intracellular calcium, respiratory burst, and adherence to endothelium  
          • Elicits a weak intracellular calcium increase and a weak respiratory burst in monocytes |
| MCP-1    | • Chemoattractant for monocytes  
          • Regulates the expression of IL-1 and IL-6 |
| MIP-1α   | • Causes local inflammatory responses in vivo  
          • Induces superoxide production by neutrophils in vitro  
          • Induces synthesis of IL-1, IL-6, and TNF in macrophages |
| TNF-α    | • Induces monocyte/macrophage production of IL-1, IL-8, and TGF-β  
          • Promotes monocyte/macrophage and neutrophil chemotaxis  
          • In vivo promoter of angiogenesis |
The concentrations of these cytokines and growth factors in tissue dictate the actions of neighboring cells, leading to a cumulative response such as blood vessel formation or fibrous encapsulation. Although other cell types contribute to the foreign body reaction, macrophages are considered primarily responsible for directing this tissue reaction\textsuperscript{6}. Therefore, an understanding of cytokine signal functions in combination with their induction by biomaterials could inspire the design of medical devices with improved function and longevity in vivo.

### 2.2 IN VITRO MACROPHAGE INTERACTIONS WITH BIOMATERIALS

In vitro investigations of macrophage interactions with biomaterials commonly examine macrophage adhesion and cytokine production\textsuperscript{23-25}. Macrophage cultures are typically mixed populations of monocytes and macrophages, either primary isolates or immortalized cell lines. This mixed population of cells can be stimulated to induce activation or monocyte differentiation into macrophages, in order to more closely mimic the macrophages involved in the foreign body reaction\textsuperscript{26}. Previous studies have indicated that stimulation can enhance monocyte/macrophage responsiveness to biomaterial surfaces\textsuperscript{27-29}. 
Monocyte stimulation

Two common methods of activating monocytes are incubation with lipopolysaccharide (LPS, endotoxin) or with phorbol 12-myristate 13-acetate (PMA). LPS treatment simulates bacterial activation of macrophages, while PMA treatment of a monocyte cell line has been shown to differentiate these cells into mature macrophages.\textsuperscript{26,30-32} Research has indicated that LPS and PMA each have a unique pathway for inducing monocyte activation.\textsuperscript{33} LPS forms a complex with LPS-binding protein (LBP), which binds membrane-bound CD14. LPS:LBP bound CD14, in concert with a toll-like receptor (TLR4), recruits the intracellular adapter molecule MyD88 and the interleukin-1 receptor associated kinase (IRAK). IRAK then dissociates from the receptor and interacts with tumor necrosis factor receptor associated factor 6 (TRAF6), which results in cell activation through NF-κB or c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAP), both of which are involved in inflammatory gene activation.\textsuperscript{34} PMA, a mimic of the natural ligand diacylglycerol (DAG), is an activator of protein kinase C (PKC)\textsuperscript{35,36}. Although the pathway is not completely understood, studies have shown that a PKC-dependent signaling pathway exists for the activation of NF-κB, which activates inflammatory genes.\textsuperscript{37} Each of these activators stimulates monocyte activation or differentiation into macrophages, which could more closely approximate the macrophages involved in the foreign body reaction.
Macrophage adhesion to biomaterials

With the expectation that adhesion to a surface propagates a macrophage attack on the implant via degradative enzymes and phagocytosis, many groups investigate macrophage adhesion as a negative indication of implant compatibility. Jenney et al. and Young et al. found that macrophage adhesion decreases with increasing hydrophobicity $^{38,39}$. In contrast, Brodbeck et al. found that macrophage adhesion is decreased on hydrophilic and anionic surfaces $^{23}$. Jones et al. reported no significant differences in macrophage adhesion on fluorocarbon, poly(ethylene oxide), and silicone modified surfaces $^{24}$. These findings suggest that while adhesion is somewhat dependent on surface charge and hydrophilicity, macrophages are capable of adhering to a variety of surface chemistries. Characterization of macrophage adhesion to materials is confounded by the complexity of the array of proteins that adsorb to the surface of an implanted device $^4$.

Biomaterial-induced cytokine production

A common measure of the reaction of macrophages to a material surface is cytokine and growth factor production, using either gene expression or protein production. Researchers generally measure up to three protein concentrations, or up to six genes, in a single experiment using ELISA or RT-PCR$^{23,39-42}$. Inflammatory cytokines and growth factors commonly studied are IL-1β, IL-1ra, IL-6, IL-8, IL-10, TNF-α, VEGF, MIP-1α, and MCP-1.
A study from Brodbeck et al. shows that macrophages, isolated from human peripheral blood, cultured onto hydrophilic or anionic surfaces exhibit decreased gene expression of the pro-inflammatory cytokine IL-8, but increased expression of the anti-inflammatory cytokine IL-10. This study indicates that macrophages recognize hydrophilic and anionic surfaces and respond with a less severe inflammatory reaction. However, in a more recent study from Jones et al., cytokine and growth factor concentrations in material-adherent macrophage cultures were shown to be independent of surface chemistry. When cytokine production was viewed on a per cell basis, hydrophilic surfaces induced increased production of both pro and anti-inflammatory cytokines. This contradictory finding illuminates the complexity of cytokine production from macrophages induced by material surfaces.

A number of additional studies have reported that macrophage production of cytokines, including TNF-α, IL-1β, IL-6, IL-10, VEGF, and bFGF, varies with biomaterial. Furthermore, cell surface marker expression and intracellular signaling proteins confirm that macrophages, as well as closely related dendritic cells, can behave differently on biomaterials of varying surface chemistry. However, the mechanism of this interaction between macrophages and biomaterials has yet to be elucidated. Further investigation is necessary to determine if meaningful changes to the foreign body reaction signaling or only modest differences are occurring due to variation in biomaterial surface chemistry. The advent of cytokine array technology allows for a broader look at the intercellular
signaling proteins that drive the foreign body reaction, which could be more indicative of the overall immune reaction to an implanted material.

### 2.3 In Vivo Immune Reaction to Biomaterials

While in vitro systems are useful for controlled studies of cellular interactions with materials, in vivo experimentation is necessary to examine the overall tissue reaction. The in vivo tissue reaction can be assessed using a cage implant system, whereby a biomaterial of interest is implanted within a stainless steel mesh cage. This system has been used regularly in the biomaterials literature to investigate the in vivo immune response to hydrogels, degradable scaffolds, polyurethanes, and biological microelectromechanical systems (BioMEMS). The stainless steel mesh cage creates a pocket in the subcutaneous space where wound exudate fluid, containing immune cells and signaling proteins, collects and interacts with the implanted biomaterial. Analysis of exudate fluid removed with a syringe allows for characterization of the immune cells and extracellular signaling proteins, including cytokines and growth factors, present at the site of implantation.

In one early study using the cage implant system, polyethylene (PE) was shown to induce a lesser degree of inflammation than a known cytotoxic poly(vinyl chloride) (PVC). This assessment of inflammation was based on macrophage concentration in the exudate fluid as well as alkaline phosphatase and acid
phosphatase concentrations; however, extracellular protein concentrations did not vary significantly between the PE and cytotoxic PVC. While the increased inflammation is likely due to a cytotoxic agent in the PVC, other evidence exists supporting the theory that surface chemistry impacts macrophage adhesion, FBGC fusion, apoptosis, microvessel growth, and surface protein expression in vivo\textsuperscript{56-60}. Brodbeck et al recently reported that material-adherent macrophages express mRNA for cytokines and growth factors such as IL-6, IL-8, IL-10, TGF-\(\beta\), IL-1\(\beta\), and IL-13 in vivo in a surface chemistry dependent manner \textsuperscript{18}. A small number of these cytokine mRNA findings have been supported with analysis of their respective proteins \textsuperscript{61,62}, but an in vivo study analyzing a wide array of cytokines and growth factors produced at the surface of an implanted biomaterial does not yet exist.

### 2.4 Microdialysis Sampling

One potential method for continuously sampling the local chemistry, such as cytokine and growth factor concentrations, of the tissue surrounding an implanted material is microdialysis. Microdialysis is a molecular sampling procedure performed by inserting a cylindrical semipermeable single hollow fiber membrane into the medium of interest \textsuperscript{63}. Microdialysis probes can be inserted with minimal damage into virtually any externally accessible tissue for continuous molecular sampling. As the probe is perfused with buffer, molecules of the external medium
diffuse through the membrane into the probe lumen to be collected in the exiting fluid, termed the dialysate. The dialysate containing sampled molecules is typically collected in a fraction collector and analyzed externally. Microdialysis sampling is most commonly used with small molecules such as pharmaceuticals and neurochemicals; however, there is increasing interest in using microdialysis to collect cytokines and growth factors\textsuperscript{64,65}. Since microdialysis sampling is governed by analyte diffusion through the probe membrane, the efficiency of collecting these larger molecules is critical for this use of microdialysis.

2.5 Organization and Presentation of This Dissertation

The objective of this dissertation is to analyze the impact of biomaterial surface chemistry on the foreign body reaction, with a specific focus on the production of cytokines and growth factors. In Chapter 3, microdialysis sampling will be characterized for the collection of macromolecules and evaluated as a tool for collecting cytokines in vitro and in vivo. Chapter 4 details an in vitro assessment of the cytokine and growth factor response from monocytes/macrophages seeded onto biomaterial surfaces. Chapter 5 discusses the in vivo cytokine and growth factor response to implanted biomaterials. Finally, Chapter 6 summarizes the current findings and suggests future projects for continuing this research.
Chapter 3. Characterization of Microdialysis Sampling of Macromolecules

3.1 SYNOPSIS

Experiments were performed to characterize the in vitro collection of macromolecules and assess the in vitro and in vivo collection of cytokines using microdialysis. Fluorescently labeled proteins and dextrans ranging from 3 to 150 kD were sampled using a 10 mm, 100 kD molecular weight cut-off (MWCO), polyethersulfone (PES) microdialysis probe. Published models describing microdialysis mass transport of small molecules were examined to determine their appropriateness for sampling of macromolecules. Collection efficiencies, reported as relative recoveries, for macromolecules from 3 to 70kD ranged from 5 to 44%. Collection efficiencies determined for microdialysis sampling of macromolecules follow the functionality of a published microdialysis model. In vitro cytokine collection efficiencies are dependent on the type of cytokine; TNF-α and IL-6 fall within the range predicted by proteins and dextrans, while IL-1β and MIP-2 recoveries were lower than the predicted range. In vivo experiments confirm the capability of microdialysis to collect cytokines from tissue. Implications of these results on the quantification of cytokines using microdialysis sampling are discussed.
3.2 Introduction

The microdialysis sampling of a given analyte is governed by the transport of the molecule from an external medium, through the membrane, and into the perfusing buffer. The efficiency of microdialysis sampling is commonly reported as the relative recovery, the percentage of available analyte collected by the probe. Factors such as membrane physical dimensions, accessible volume, perfusate flow rate, and analyte diffusion coefficient (and thus molecular weight) strongly influence relative recovery \(^{66}\). A theoretical model developed by Bungay, Morrison, and Dedrick (BMD) uses these parameters to describe mass transport of analytes in microdialysis experiments \(^{67}\).

Commercially available microdialysis probes have molecular weight cut-offs (MWCO) that range from 6 to 100 kD. MWCOs are defined as the molecular weight at which approximately 80% of the analytes are prohibited from diffusion through the membrane \(^{68}\). However, membrane MWCO is typically determined from equilibrium mass transport, which is not an exact representation of the non-equilibrium setting of microdialysis. Microdialysis sampling is most often used to collect pharmaceuticals and neurochemicals with smaller molecular weights than proteins, yet recent studies demonstrate the capacity of microdialysis to collect proteins such as cytokines and growth factors both in vitro and in vivo \(^{69-72}\). In vivo cytokine sampling via microdialysis could provide a wealth of information such as the local concentration of signaling and mediating molecules involved in inflammation, wound healing, and
vascular formation. Although a handful of researchers have used microdialysis to recover large molecular weight molecules, analyte collection efficiencies have varied markedly (Table 3.1) 69-71,73-77.
**Table 3.1. Literature examples of in vitro microdialysis collection efficiencies of macromolecules.**

*Comments include membrane length, molecular weight cut-off (MWCO), and material.
**Relative recovery here was reported as a range rather than a mean value.*

<table>
<thead>
<tr>
<th>Citation</th>
<th>Analyte (MW)</th>
<th>Relative Recovery (%)</th>
<th>Flow Rate (μl/min)</th>
<th>Comments*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kalantarinia et al (2003)</td>
<td>TNF-α (17kD)</td>
<td>73</td>
<td>1</td>
<td>5mm, 40-45kD MWCO, Filtral 20 (acrylonitrile/sodium methallyl copolymer)</td>
</tr>
<tr>
<td>Trickler et al (2003)</td>
<td>IL-1β (17.3kD)</td>
<td>1.9</td>
<td>1</td>
<td>4mm, 100kD MWCO, CMA/12 (polyethersulfone)</td>
</tr>
<tr>
<td></td>
<td>TNF (53kD)</td>
<td>0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter et al (2002)</td>
<td>IL-1β (17kD)</td>
<td>27.8</td>
<td>1</td>
<td>12-15mm, 3000kD MWCO, Asahi Plasmaseparator (polyethylene)</td>
</tr>
<tr>
<td></td>
<td>IL-6 (26kD)</td>
<td>45.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NGF (13.6kD)</td>
<td>22.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dabrosin et al (2002)</td>
<td>VEGF (28-42kD)</td>
<td>4</td>
<td>2</td>
<td>4mm, 100kD MWCO, CMA/20 (polyethersulfone)</td>
</tr>
<tr>
<td>Sjogren et al (2001)</td>
<td>IL-6 (26kD)</td>
<td>3.0</td>
<td>1</td>
<td>10mm, 100kD MWCO (polyethylenesulphone)</td>
</tr>
<tr>
<td></td>
<td>Trypsin (24kD)</td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phillips (2000)</td>
<td>IL-1 (17kD) IL-6 (20.3kD)</td>
<td>92-98**</td>
<td>1</td>
<td>10mm, 100kD MWCO, CMA/20 (polyethersulfone)</td>
</tr>
<tr>
<td>Woodroofe et al (1991)</td>
<td>IL-6 (17.5kD) IL-1 (28kD)</td>
<td>0.5-1.0**</td>
<td>2</td>
<td>16mm, 100kD MWCO, Diaflow Hollow Fiber</td>
</tr>
<tr>
<td>Yamaguchi et al (1990)</td>
<td>IGF-1 (7.6kD)</td>
<td>2.5</td>
<td>2</td>
<td>4mm, 100kD MWCO, Evaflux 4A fiber</td>
</tr>
<tr>
<td>Ao et al (2006)</td>
<td>TNF-α (52kD) IFN-γ (32kD) MCP-1 (26kD) IL-6 (22kD)</td>
<td>9, 3.8 2.1, 1.3 17.9, 9.6 5.7, 3.4</td>
<td>1,2</td>
<td>10mm, 200kD MWCO, CMA/20 (polyethersulfone)</td>
</tr>
</tbody>
</table>
Recently, Ao et al. described a system for improving the relative recovery of cytokines by perfusing anti-cytokine microspheres through the microdialysis probe\(^7\). Cytokine-bound microspheres are trapped within the membrane, thereby driving cytokine flux into the microdialysis probe. Although promising, a challenge in using this technique is maintaining the suspended beads in the microsyringe. Another method for improving the relative recovery of analytes incorporates ultrafiltration with microdialysis sampling\(^7\). With ultrafiltration, conditions within the microdialysis probe encourage convection of the external fluid to the inside of the probe membrane. The result of this convection is increased mass transport of analytes towards the inside of the microdialysis probe, which leads to higher relative recoveries. While this technique could be useful in improving the collection of cytokines, ultrafiltration of the exudate fluid could disturb the normal immune response associated with an implanted material.

To our knowledge, the current study is the first systematic characterization of the collection of macromolecules by microdialysis. In this study, 100kD MWCO polyethersulfone (PES) microdialysis probes were employed to collect proteins and dextrans ranging from 3 to 150 kD. Relative recovery data were used to determine the appropriateness of the BMD microdialysis mass transport model for macromolecule sampling. An additional model of general membrane flux was employed for estimating the apparent solute diffusion coefficient in the microdialysis membrane, and for calculating mass transport resistances for comparison to the BMD
model. Results show that macromolecule collection efficiencies fit the theoretical BMD model. Furthermore, in vitro microdialysis sampling was performed on cytokine solutions to determine if cytokine relative recoveries fall within the range predicted by proteins and dextrans. Finally, cytokines were sampled using microdialysis probes implanted into the subcutaneous tissue of LPS-stimulated rats to assess the feasibility of collecting cytokines using in vivo microdialysis sampling.

3.3 METHODS AND MATERIALS

Microdialysis system

Figure 3.1 shows the experimental configuration for the microdialysis measurements.
Figure 3.1. Diagram of the microdialysis system. Phosphate buffered saline (PBS) is perfused through the FEP tubing into the microdialysis probe at 1 μL/min. The macromolecule solution is buffered with PBS and continuously stirred. Dialysate from the microdialysis probe outlet is collected in a microcentrifuge tube. The capture depicts an enlarged image of the microdialysis probe where mass transport across the membrane occurs for molecules permeable to the membrane.

A 1 mL glass syringe attached to a microsyringe pump (Bioanalytical Systems) was connected to the distal end of a 45 cm long, 0.12 mm ID FEP (fluorinated ethylene polymer) tubing (CMA/Microdialysis, North Chelmsford, MA). The proximal end of the tubing was connected to the 20 cm long inlet of a 10mm PES microdialysis probe (CMA/Microdialysis) with a 100 kD MWCO. Tubing connections were made via CMA tubing adapters. The 20 cm long probe outlet tubing was inserted into a dialysate collection vial.
The macromolecules sampled in this experiment were fluorescently labeled proteins or dextrans ranging in molecular weight from 3 to 150 kD. The microdialysis probe was submerged in a 5 mL vacutainer vial (Becton Dickinson) filled with a macromolecule solution (0.4 mg/mL) in phosphate buffered saline (PBS). The solution was continuously rocked on a rotisserie (Labquake). Access to the microdialysis probe inlet and outlet tubing was through perforations in the vacutainer stopper, as described elsewhere.

**In vitro microdialysis sampling of proteins and dextrans**

Microdialysis probes were soaked for at least one hour in PBS prior to experimentation. The microdialysis system (syringe, probe, and tubing) was perfused with PBS for 5 minutes at a flow rate of 10 μL/min to remove glycerol (from packaging) and air bubbles. Fluorescently labeled dextrans (20 kD, 150 kD – Sigma-Aldrich; all others – Molecular Probes) were used as purchased. Proteins (Sigma-Aldrich) were labeled in house with Cy5 bisfunctional reactive dye (Amersham Biosciences, Piscataway, NJ). Free fluorescent dye was removed by dialysis against deionized water using 3.5 kD MWCO dialysis cassettes (Pierce) or 1 kD MWCO dialysis membranes (Fisher, only 3kD dextran). Protein and dextran microdialysis sampling was performed at a perfusate flow rate of 1 μL/min for nine sequential samples, each collected for 20 minutes. Macromolecule concentrations in the external solution and dialysate were determined by fluorescence spectrometry (SLM
Aminco) against calibrated standards. Microdialysis collection efficiency was reported as the relative recovery

\[
\text{Relative Recovery} = E_d \times 100\% \quad \text{(Eqn 3.1)}
\]

where \( E_d \) is the extraction fraction given by

\[
E_d = \frac{C_o - C_i}{C_s - C_i} \quad \text{(Eqn 3.2)}
\]

and \( C_i, C_o, \) and \( C_s \) are the analyte concentrations in the perfusate, dialysate, and the external solution, respectively (Fig 3.1).

**Fluid convection**

In order to assess fluid convection through the microdialysis probe, dialysate samples were massed to determine the dialysate volume after a separate microdialysis sampling of BSA. Microdialysis sampling was performed at a flow rate of 1 \( \mu \text{L/min} \) for seven sequential samples for a total duration of 3 hours. Fluid filtration or absorption from the microdialysis probe was calculated using the final dialysate mass, expected dialysate mass (perfusate flow rate \( \times \) time), and the density of water.
In vitro microdialysis models

BMD Model

A theoretical microdialysis model describing the relationship between the extraction fraction of the solute \( E_d \), the dialysate flow rate \( Q_d \), and the resistances to mass transport was previously derived from a series of steady state mass balances\(^67\).

\[
E_d = \frac{C_o - C_i}{C_s - C_i} = 1 - \exp \left( -\frac{1}{Q_d (R_d + R_m + R_e)} \right)
\]

(Eqn 3.3)

Resistances to solute mass transport defined in this model are resistance in the dialysate \( R_d \), resistance in the membrane \( R_m \), and resistance in the external environment \( R_e \) (Fig 1). In a well-stirred solution, one obtains\(^67\):

\[
R_d = \frac{13(r_\beta - r_\alpha)}{35(2\pi Lr_\beta)D_d}
\]

(Eqn 3.4)

\[
R_m = \frac{\ln(r_o/r_\beta)}{2\pi LD_m\phi_m}
\]

(Eqn 3.5)

\[
R_e = 0
\]

(Eqn 3.6)

where \( r_\beta \) is the internal radius of the microdialysis membrane, \( r_\alpha \) is the external radius of the cannula, \( r_o \) is the external radius of the microdialysis membrane, \( L \) is the effective length of the microdialysis membrane, \( D_d \) is the solute diffusion coefficient.
in the dialysate, \(D_m\) is the solute diffusion coefficient in the membrane, and \(\phi_m\) is the solvent accessible volume fraction of the membrane. The product, \(D_m\phi_m\) is defined as the apparent membrane diffusion coefficient.

**Cussler Model**

In order to see if values of \(R_d\) and \(R_m\) calculated via the BMD model (Eqns 4,5) fall within an acceptable range, an alternative model was employed for estimating mass transport resistances. In Cussler’s model, the mass transport of a solute through a membrane subject to a transverse laminar flow is given by

\[
j = \frac{\Delta C}{\frac{1}{k_1} + \frac{\Delta r_m}{D_m\phi_m} + \frac{1}{k_3}}
\]

(Eqn 3.7)

where \(j\) is the solute flux through the membrane, \(\Delta C\) is the log mean concentration gradient across the membrane, \(k_1\) and \(k_3\) are mass transfer coefficients in the external solution and dialysate, respectively, \(\Delta r_m\) is the membrane thickness, \(D_m\) is the solute diffusion coefficient in the membrane, and \(\phi_m\) is the solvent accessible volume fraction of the membrane. The product \(D_m\phi_m\) is the apparent solute diffusion coefficient in the membrane. For a well-mixed external solution, \(k_1\) approaches infinity, yielding

\[
j = \frac{\Delta C}{\Delta r_m / D_m\phi_m + 1/k_3}
\]

(Eqn 3.8)
By analogy with equation 3.3, incorporating the surface area (SA) of the microdialysis probe, we obtain

\[ \text{Mass Transfer Resistance} = \frac{1}{k_3(SA)} \sim R_d \]  
(Eqn 3.9)

\[ \text{Membrane Diffusive Resistance} = \frac{\Delta r_m}{D_m \phi_m(SA)} \sim R_m \]  
(Eqn 3.10)

For laminar flow within a cylindrical tube, an approximation for dialysate flow within the microdialysis probe, \( k_3 \) is given by

\[ k_3 = 3.657 \left( \frac{D_d}{2(r_\beta-r_\alpha)} \right) \]  
(Eqn 3.11)

where \( D_d \) is the solute diffusion coefficient in the dialysate, \( r_\beta \) is the internal radius of the microdialysis membrane, and \( r_\alpha \) is the external radius of the microdialysis probe cannula.

**Determination of aqueous diffusion coefficients (D\( d \))**

Jain reported a power law equation relating the aqueous diffusion coefficient at 37°C to molecular weight for dextran in the range of 10-147 kD. Combining this power law equation with the Stokes-Einstein equation yields the room
temperature diffusion coefficient at 23°C, the temperature at which microdialysis sampling was performed

\[
D_{d,23^\circ C} = \left(1.26 \times 10^{-4} \text{ [MW]}^{-0.478} \right) \left(\frac{296^\circ K}{310^\circ K} \right) \left(\frac{\mu_{37^\circ C}}{\mu_{23^\circ C}}\right) \quad \text{(Eqn 3.12)}
\]

where \(D_d\) is the dextran diffusion coefficient in the dialysate, MW is the dextran molecular weight, and \(\mu\) is the viscosity of water at the respective temperatures.

Unlike a homologous series of dextrans, the diffusion coefficients of the heterogeneous proteins had to be determined experimentally by dynamic light scattering. Proteins were dissolved in PBS at a concentration of 10 mg/mL and analyzed at 23°C using a DynaPro light scattering instrument (Protein Solutions, Charlottesville, VA) to determine the translational diffusion coefficients. Each protein diffusion coefficient was determined from a multimodal set of data indexed by molecular weight.

**Determination of apparent membrane diffusion coefficients (\(D_{m\phi_m}\))**

The apparent membrane diffusion coefficients (\(D_{m\phi_m}\)) for dextrans and proteins were calculated from the definition of mass flux through the membrane

\[
j = \frac{C_0 V_o}{SA t} \quad \text{(Eqn 3.13)}
\]
where $C_o$ is the concentration of analyte in the dialysate, $V_o$ is the dialysate volume, $SA$ is the surface area of the membrane, and $t$ is the collection time. Setting equation 3.13 equal to equation 3.8 yields

$$D_{m\phi_m} = \frac{\Delta r_m C_o V_o k_3}{(\Delta C SA t k_3) - (C_o V_o)} \quad \text{(Eqn 3.14)}$$

from which it is possible to estimate the apparent membrane diffusion coefficient ($D_{m\phi_m}$).

**Microdialysis from well-mixed cytokine solutions**

In order to determine the relative recovery rates of cytokines sampled with 100 kD MWCO microdialysis probes, a cytokine solution containing TNF-α, IL-6, and MIP-2 was created at 10 ng/mL in PBS with blocking buffer (1%BSA). Microdialysis probes were soaked for at least one hour in PBS prior to experimentation. The microdialysis system (syringe, probe, and tubing) was perfused with PBS for 5 minutes at a flow rate of 10 μL/min to remove glycerol (from packaging) and air bubbles. PBS was perfused at 1 μL/min through microdialysis probes inserted into cytokine solutions and the outflow was collected in eppendorf tubes in hourly intervals for 3 hours. The cytokine solutions were continuously rocked on a rotisserie (LabQuake) to ensure mixing.
**Microdialysis of cytokines from cell culture**

The efficiency of cytokine collection from biological conditions was assessed by sampling from the culture media of lipopolysaccharide (LPS) stimulated monocytes with 100 kD microdialysis probes. Microdialysis probes were soaked for at least one hour in PBS prior to experimentation. The microdialysis system (syringe, probe, and tubing) was perfused with PBS for 5 minutes at a flow rate of 10 μL/min to remove glycerol (from packaging) and air bubbles.

Human monocytes/macrophages (THP-1, ATCC) were cultured in RPMI 1640 media supplemented with HEPES buffer (10 mM), sodium pyruvate (1 mM), glucose (4.5 g/L), fetal bovine serum (10%), penicillin (100 U/mL), streptomycin (100 μg/mL), and 2-mercaptoethanol (0.05 mM), (complete monocyte/macrophage media). LPS (Sigma-Aldrich) was added to bring monocyte/macrophage cultures to a final concentration of 10 μg/mL, 1 μg/mL, or 0.1 μg/mL. Alternatively, PBS was added to the cultures as a negative control. Complete monocyte/macrophage media was perfused at 1μL/min through microdialysis probes inserted into the cell culture media and the dialysate was collected in hourly intervals after 3, 6, 24, and 48 hours. Dialysate samples were compared with samples removed directly from the supernatant for an evaluation of the cytokine recovery rates.
Microdialysis of cytokines from subcutaneous tissue

Microdialysis sampling was performed in rat subcutaneous and adipose tissue in order to evaluate the collection of cytokines in vivo. Two 100 kD microdialysis probes were implanted into the dorsal subcutaneous tissue of male Sprague Dawley rats. LPS (1 µg/g rat body weight) was injected into tissue near the probe to induce cytokine production. Microdialysis probes were soaked for at least one hour in PBS prior to experimentation. The microdialysis system (syringe, probe, and tubing) was perfused with PBS for 5 minutes at a flow rate of 10 μL/min to remove glycerol (from packaging) and air bubbles. PBS was perfused through the implanted probes at 1 μL/min and dialysates were collected in hourly intervals for 4 hours.

Cytokine measurement

Cytokine concentrations in the cytokine cocktail microdialysis experiment were measured using a cytokine microarray. Briefly, monoclonal antibodies (R & D Systems, Minneapolis, MN) specific to the cytokines of interest were robotically printed in a 8 by 5 array onto the nitrocellulose pads of FAST slides (Schleicher & Schuell, Keene, NH) using a Microsys 5100 microarrayer (Cartesian Technologies, Irvine, CA). Samples (60 µl) were incubated for 2 hours on monoclonal antibody-adsorbed pads using an incubation chamber (Schleicher & Schuell) to prevent contamination between samples. After aspirating the samples and washing each pad with wash buffer, the slides were incubated with a biotinylated detection antibody
cocktail (500 ng/mL) for 1 hour. Following a second aspiration/wash, the slides were incubated in the dark for 30 minutes with streptavidin-Cy5. Finally, slides were aspirated, washed, and dried with a stream of nitrogen. Fluorescence of dried slides was measured using a GenePix 4000B microarray scanner and analyzed using GenePix 5.0 software (Axon instruments, Union City, CA). Sample concentrations were calculated against fluorescence intensities of standard curves generated from cytokine cocktails of known concentration.

Cytokine concentrations in the cell culture microdialysis experiment were measure using enzyme-linked immunosorbent assay (ELISA DuoSet, R&D Systems, Minneapolis, MN). ELISAs were performed according to the manufacturer’s instructions. Briefly, monoclonal capture antibody (4.0 µg/mL) was incubated overnight in the wells of an immunosorbent 96-well plate (NUNC, Rochester, NY). After blocking with 1% BSA buffer for 1 hour, wells were aspirated, rinsed with wash buffer, and samples were then incubated for 2 hours. Following another aspiration/wash step, biotinylated detection antibodies (75 ng/mL for TNF-α, 100 ng/mL for IL-1β) were incubated for 2 hours. After another aspiration/wash step, streptavidin-horseradish peroxidase was incubated in the wells for 20 minutes. Following a final aspiration/wash step, substrate solution was incubated in the wells for 30 minutes. Optical densities at 450 nm were measured from these plates using a BioTek µQuant plate reader (BioTek Instruments, Toronto, Ontario, Canada). Sample concentrations were calculated against standard curves.
Cytokine concentrations in the in vivo microdialysis experiment were measured using the Luminex bead array according to the manufacturer’s instructions (BioRad). Briefly, antibody coupled beads were incubated with monocyte/macrophage supernatants for 1 hour. After washing, secondary antibody was incubated with the cytokine-bound beads for 30 minutes. Finally, phycoerythrin dye was bound to the beads via biotin-streptavidin binding for 30 minutes. All incubations occurred at room temperature on a plate shaker according to the manufacturers instructions. Sample concentrations (pg/mL) were determined from mean fluorescence intensities (MFI) compared against a 4 or 5-parameter logistic standard curve generated from standards of known concentration provided by the bead array manufacturer. The Luminex bead array is calibrated prior to every sample analysis and validated regularly per the manufacturer’s instructions.

3.4 RESULTS

Relative recovery of macromolecules

Fluorescently labeled dextrans and proteins ranging from 3 kD to 150 kD were sampled from buffered solution under identical conditions to examine the relationship between relative recovery and molecular weight. Relative recovery of an analyte was determined using steady state macromolecule concentrations in the external medium and dialysate (Eqns 3.1, 3.2). The time to reach steady state was
estimated to be 40 minutes for all microdialysis measurements. Steady state relative recoveries reported in Table 3.2 are the mean values calculated between 40 and 180 minutes.

**Table 3.2. Steady state relative recoveries of macromolecules**

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Steady State Relative Recovery (%)</th>
<th>pI/Charge</th>
<th>Shape</th>
</tr>
</thead>
<tbody>
<tr>
<td>Insulin (5.7kD)</td>
<td>18.5 ± 0.8</td>
<td>5.2</td>
<td></td>
</tr>
<tr>
<td>Lysozyme (14.4kD)</td>
<td>16 ± 2</td>
<td>11.35</td>
<td></td>
</tr>
<tr>
<td>SBTI(^1) (20.1kD)</td>
<td>13.4 ± 0.7</td>
<td>4.5</td>
<td></td>
</tr>
<tr>
<td>Ovalbumin (45.0kD)</td>
<td>8.6 ± 0.7</td>
<td>4.4-4.6</td>
<td>Globular</td>
</tr>
<tr>
<td>BSA (66.2kD)</td>
<td>8.1 ± 0.8</td>
<td>4.7-4.9</td>
<td></td>
</tr>
<tr>
<td>IgG (150kD)</td>
<td>1.7 ± 0.4</td>
<td>5.8-7.3</td>
<td></td>
</tr>
<tr>
<td>3kD dextran</td>
<td>44 ± 3</td>
<td>Anionic</td>
<td>Expandable</td>
</tr>
<tr>
<td>10kD dextran</td>
<td>14 ± 1</td>
<td>Neutral</td>
<td>coil</td>
</tr>
<tr>
<td>20kD dextran</td>
<td>6.6 ± 0.6</td>
<td>---------(^3)</td>
<td></td>
</tr>
<tr>
<td>40kD dextran</td>
<td>7.6 ± 0.4</td>
<td>Neutral</td>
<td>Highly</td>
</tr>
<tr>
<td>70kD dextran</td>
<td>5 ± 1</td>
<td>Anionic</td>
<td>branched</td>
</tr>
<tr>
<td>150kD dextran</td>
<td>1.0 ± 0.7</td>
<td>---------(^3)</td>
<td></td>
</tr>
</tbody>
</table>

Steady state relative recoveries decreased with increasing molecular weights of macromolecules, from 44% to 1.0% for dextrans and 18.5% to 1.7% for proteins.

An assessment of fluid convection through the microdialysis probe yielded a mean fluid outflow of 0.0 ± 0.4 μL/min.

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Macromolecule diffusion coefficients

Mass transport resistances calculated via the BMD and Cussler models require knowledge of analyte diffusion coefficients. Aqueous diffusion coefficients were determined by dynamic light scattering for proteins and calculated from a published power law relationship for dextrans (Table 3.3).

Table 3.3. Aqueous and apparent membrane diffusion coefficients determined for macromolecules

* Calculated from the power law relationship of Jain (Eqn 12)
Data are shown ± SEM (n=3)

<table>
<thead>
<tr>
<th>Protein</th>
<th>( D_{LS} ) ( 10^{-7} \text{cm}^2/\text{s} )</th>
<th>( D_{m \phi m} ) ( 10^{-7} \text{cm}^2/\text{s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (14.4kD)</td>
<td>12.2 ± 0.6</td>
<td>0.82 ± 0.01</td>
</tr>
<tr>
<td>Trypsin Inh. (20.1kD)</td>
<td>10.3 ± 0.5</td>
<td>0.67 ± 0.01</td>
</tr>
<tr>
<td>Ovalbumin (45.0kD)</td>
<td>8.3 ± 0.4</td>
<td>0.42 ± 0.01</td>
</tr>
<tr>
<td>BSA (66.2kD)</td>
<td>6.5 ± 0.3</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>IgG (150kD)</td>
<td>5.3 ± 0.3</td>
<td>0.078 ± 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dextran</th>
<th>( D_{PL} ) ( 10^{-7} \text{cm}^2/\text{s} )*</th>
<th>( D_{m \phi m} ) ( 10^{-7} \text{cm}^2/\text{s} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10kD dextran</td>
<td>11.0</td>
<td>0.69 ± 0.01</td>
</tr>
<tr>
<td>20kD dextran</td>
<td>7.9</td>
<td>0.32 ± 0.01</td>
</tr>
<tr>
<td>40kD dextran</td>
<td>5.7</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>70kD dextran</td>
<td>4.4</td>
<td>0.23 ± 0.01</td>
</tr>
<tr>
<td>150kD dextran</td>
<td>3.0</td>
<td>0.048 ± 0.001</td>
</tr>
</tbody>
</table>

For both proteins and dextrans, diffusion coefficients decreased with increasing analyte molecular weight. An apparent membrane diffusion coefficient
(\(D_m\phi_m\)), calculated from relative recovery data via the Cussler model (Eqn 3.14), also decreased with increasing analyte molecular weight.

**In vitro microdialysis resistances**

The membrane-induced hindrance to diffusion in microdialysis was examined using the resistances to mass transfer and diffusion determined from the BMD model (Eqns 3.4, 3.5). Resistances due to the dialysate and the membrane increased with increasing molecular weight of the analyte (Table 3.4).

<table>
<thead>
<tr>
<th>Protein</th>
<th>(R_{d, DLS} (\text{s/cm}^3))</th>
<th>(R_m (10^3 \text{s/cm}^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme (14.4kD)</td>
<td>6900 ± 300</td>
<td>338 ± 3</td>
</tr>
<tr>
<td>Trypsin Inh. (20.1kD)</td>
<td>8200 ± 400</td>
<td>414 ± 2</td>
</tr>
<tr>
<td>Ovalbumin (45.0kD)</td>
<td>10200 ± 500</td>
<td>661 ± 2</td>
</tr>
<tr>
<td>BSA (66.2kD)</td>
<td>12900 ± 600</td>
<td>712 ± 3</td>
</tr>
<tr>
<td>IgG (150kD)</td>
<td>16000 ± 800</td>
<td>3560 ± 13</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dextran</th>
<th>(R_{d, pL} (\text{s/cm}^3))</th>
<th>(R_m (10^3 \text{s/cm}^3))</th>
</tr>
</thead>
<tbody>
<tr>
<td>10kD</td>
<td>7700</td>
<td>403 ± 3</td>
</tr>
<tr>
<td>20kD</td>
<td>11000</td>
<td>878 ± 3</td>
</tr>
<tr>
<td>40kD</td>
<td>15000</td>
<td>752 ± 2</td>
</tr>
<tr>
<td>70kD</td>
<td>19000</td>
<td>1210 ± 10</td>
</tr>
<tr>
<td>150kD</td>
<td>28000</td>
<td>5790 ± 20</td>
</tr>
</tbody>
</table>

* Calculated from DPL (Table 3.3)

Data are shown ± SEM (n=3)
The membrane resistances of proteins and dextrans smaller than the microdialysis probe MWCO were 49 to 80 times greater than their respective dialysate resistances. In contrast, the membrane resistances of IgG and 150 kD dextran were 222 and 207 times greater than their respective dialysate resistances.

**Comparison of BMD and Cussler models**

Aqueous protein mass transport resistances calculated from the BMD model (Eqn 3.4) were compared to mass transport resistances calculated from the Cussler model (Eqn 3.9). The mass transport resistances estimated from the two models differed by less than one order of magnitude for the proteins indicated (Fig 3.2).

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Resistance values are shown for Lysozyme (L), Trypsin Inhibitor (TI), Ovalbumin (Ov), BSA (BSA), and IgG (IgG). Error bars represent ± SEM (n=3).

Membrane diffusive resistances were also compared for proteins using the BMD and Cussler models (Eqns 5,10). In this case, the estimated resistances from the two models were nearly identical, less than 2% different for each protein (Fig 3.2).

**Fit of Macromolecule Recovery to BMD Model**

Algebraic rearrangement of the BMD theoretical model (Eqn 3.3) yields the following log-normal equation for our experimental conditions ($R_c=0$)

\[
\ln[1-E_d] = \frac{1}{Q_d} \left( \frac{-1}{R_d+R_m} \right)
\]  
(Eqn 3.15)
where $1/Q_d$ is the slope, $(-1/R_d+R_m)$ is the independent variable, and the y-intercept is zero. Figure 3.3 depicts macromolecule recovery data plotted in the form of equation 3.15.

![Graph showing macromolecule microdialysis fit to the BMD model.](image)

<table>
<thead>
<tr>
<th></th>
<th>$Q_d$ (μL/min)</th>
<th>y-intercept</th>
<th>$R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Theoretical</td>
<td>1</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Dextran</td>
<td>0.990 ± 0.002</td>
<td>0.0001 ± 0.0002</td>
<td>0.99</td>
</tr>
<tr>
<td>Protein</td>
<td>0.988 ± 0.006</td>
<td>0.0001 ± 0.0007</td>
<td>0.99</td>
</tr>
</tbody>
</table>

**Figure 3.3. Macromolecule microdialysis fit to the BMD model.** Linear regressions were calculated for both proteins (●) and dextrans (Δ). The ideal line was calculated by inputting the experimental flow rate into the linear BMD model. Error bars represent ± SEM (n=3).

The dashed lines are linear regression fits (IGOR Pro, Wavemetrics, Lake Oswego, OR) to the dextran and protein data. The ideal fit calculated from the experimental flow rate is shown as the solid line. Figure 3.3 also contains the values of flow rate and y-intercept determined by linear regression, as well as theoretical
values calculated based on the experimental flow rate. The variance in the data presented in Figure 3.3 is expressed at the 95% confidence limit (as calculated with IGOR Pro).

**Cytokine microdialysis**

Relative recoveries were measured for cytokines sampled with 100 kD MWCO microdialysis probes from solutions of TNF-α (17 kD), IL-6 (21.8 kD), and macrophage inflammatory protein-2 (MIP-2, 8 kD) (Figure 3.4).

![Cytokine Microdialysis](image)

**Figure 3.4. Cytokine relative recoveries in well-mixed solutions.** Relative recoveries measured for cytokines sampled with 100kD MWCO microdialysis probes. Cytokine concentrations were measured using a cytokine microarray. Error bars represent ± SEM (n=3).
The highest relative recovery from the well-mixed cytokine cocktail was measured for TNF-α (17 kD), which was between 15% and 20% for all three time points. IL-6 (21.8 kD) was recovered at the second highest rate, between 8% and 12% at all time points. The lowest measured recovery was for MIP-2 (8 kD), ranging from 2% to 5%. Relative recoveries for all three cytokines remained constant throughout the 3 hours of microdialysis sampling.

Cytokine sampling from stimulated cell cultures

Microdialysis probes were submersed in the cell culture media of LPS-stimulated human monocytes/macrophages (THP-1). Dialysate was collected over 1 hour intervals at 3, 6, 24, and 48 hours after the addition of LPS. Direct samples were also collected for comparison with the dialysates (Figure 3.5).
Figure 3.5: LPS-induced cytokines from monocytes/macrophages. In vitro concentrations of TNF-α and IL-1β induced by varying concentrations of LPS. Data are shown as mean ± SEM (n≥3).

Samples removed directly from the culture supernatant reveal that TNF-α and IL-1β were produced by the activated monocytes/macrophages in an LPS dose-dependent manner. The concentration of TNF-α in the culture supernatant peaked at
3500 pg/mL after 3 hours of 10 µg/mL LPS stimulation. The concentration of IL-1β in the culture supernatant peaked after 48 hours of stimulation with 10 µg/mL LPS. Relative recoveries calculated from the samples collected via microdialysis are shown in Table 3.5.

Table 3.5. Relative recoveries of cytokines sampled from cell culture supernatant.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Relative Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α (17kD)</td>
<td>13 ± 5</td>
</tr>
<tr>
<td>IL-1β (17kD)</td>
<td>0.9 ± 0.7</td>
</tr>
</tbody>
</table>

Data are shown ± SEM (n=6)

Relative recoveries calculated for TNF-α and IL-1β sampled from activated monocyte/macrophage supernatant were 13 ± 5% and 0.9 ± 0.7%, respectively. The molecular weight of both of these cytokines is 17 kD.

**Cytokine sampling from rat tissue**

Microdialysis probes were also implanted into the dorsal subcutaneous tissue of Sprague Dawley rats stimulated with LPS. Dialysate samples, collected every 30 minutes for 4 hours after LPS injection, were analyzed for IL-1α, IL-1β, IL-6, IL-10, and TNF-α (Figure 3.6).
Figure 3.6: Cytokine concentrations sampled via microdialysis in vivo. In vivo microdialysis sampling of IL-1α, IL-1β, IL-6, IL-10, and TNF-α induced by LPS. Data are shown as mean ± SEM (n=2).
IL-6 was detected at the highest concentration of any cytokine, reaching a maximum concentration in the dialysate fluid above 3000 pg/mL. Dialysate concentrations of IL-1β reached 750 pg/mL, while dialysate concentrations of IL-1α and IL-10 reached 300 pg/mL. Dialysate concentrations of TNF-α were never greater than 150 pg/mL.

3.5 DISCUSSION

An important goal of our research is to use microdialysis probes as “mock sensors” to simultaneously (1) examine the impact of wound healing on glucose transport through the probe membrane, and (2) sample the host of cytokines and growth factors that populate the surrounding wound healing tissue. It is generally accepted that macrophage derived cytokines and growth factors are critical molecular mediators of acute inflammation, and ultimately whether an implant remains mired in chronic inflammation or progresses through the stages of wound healing to fibrous encapsulation. These events have a broad range of impact regarding implant performance, from a negligible effect to rendering the implanted device useless. For example, while stable fibrous encapsulation is a desirable condition for many implants, surrounding an implanted glucose sensor with avascular and densely collagenous encapsulation tissue limits sensor performance by restricting diffusional and perfusional access to blood borne glucose.
Although microdialysis has been used to collect proteins in vivo, the reported collection efficiencies vary markedly (Table 3.1) and it is difficult to determine an acceptable range of macromolecule collection efficiencies for commonly used microdialysis experimental conditions. Before this technique can be used reliably to sample macromolecules in vivo, it must be shown to perform predictably and quantitatively with macromolecules in vitro under favorable conditions, providing an upper limit of collection efficiencies compared to what may be observed in vivo.

The current study systematically measured the collection efficiency of macromolecules in vitro, and determined if these values adhered to published transport models that describe microdialysis sampling. Fluorescently labeled dextrans and proteins ranging from 3 to 150kD were sampled from a well-stirred solution via 100kD MWCO (10mm) microdialysis probes perfused with PBS at a flow rate of 1μL/min. In all cases, diffusion through the probe reached steady state after 40 minutes. Overall, the steady state relative recoveries decreased with increasing molecular weight of the macromolecule (Table 3.2). The lone exception to this trend was 20kD dextran, which was supplied exclusively from a different manufacturer than the other dextrans.

Furthermore, the current study assessed the capability of microdialysis to collect a sample group of cytokines from media of increasing complexity: from a well-stirred cocktail, to an unstirred biological medium, to an intact tissue. Initially,
relative recoveries were measured from a well-stirred cytokine cocktail to determine if these cytokines fall within the range expected based on the protein and dextran recoveries. Cytokines were then sampled from activated cell cultures to examine the recovery rates from an unstirred biological solution. Finally, microdialysis probes were implanted into subcutaneous tissue to determine the capability of microdialysis to sample cytokines from intact tissue.

The choice of a 100kD MWCO probe made possible the collection of proteins and dextrans with molecular weights typical of cytokines. Of particular interest were the collection efficiencies of proteins from 5.7 to 66.2kD that ranged from 8.1 to 18.5%, respectively. This relatively narrow range of recoveries differed significantly from the broad spectrum of protein collection efficiencies reported elsewhere (Table 3.1). Relative recoveries for 150kD dextran and IgG, both of which exceeded the MWCO of the membrane, were very small but non-zero.

The observed decrease in relative recovery with increased protein and dextran molecular weight (Table 3.2) arose from greater resistance to mass transport as molecular size increased. Mass transport resistances were estimated using a model derived by Bungay et al. Modifications to this model have been suggested for in vivo microdialysis sampling, but it remains acceptable for our in vitro microdialysis sampling from well-stirred solution. Although the BMD model is frequently applied to quantitative microdialysis, it has not, to our knowledge,
been applied to macromolecules. Calculation of mass transport resistances via the BMD model required accurate determination of aqueous diffusion coefficients (Table 3.3). Dynamic light scattering revealed similar aqueous protein diffusion coefficients to values in literature\(^{87}\). Aqueous dextran diffusion coefficients calculated via a published power law expression (Eqn 3.12) were comparable to similarly sized protein diffusion coefficients. Apparent membrane diffusion coefficients were calculated via Equation 3.14.

Apparent membrane diffusion coefficients for macromolecules smaller than the 100kD MWCO were 15-25 fold smaller than their respective aqueous diffusion coefficients, a relationship similar to previous studies\(^{85}\). Calculated membrane resistances of macromolecules with molecular weight smaller than the membrane MWCO were 49 to 80 fold larger than their respective dialysate resistances. Macromolecules larger than the microdialysis membrane MWCO had apparent membrane diffusion coefficients that were 63-68 fold smaller than aqueous diffusion coefficients, and membrane resistances that were 207-222 fold larger than dialysate resistances. In all cases, analyte collection was limited by passage through the membrane, and this effect was significantly more pronounced for molecules larger than the membrane MWCO.

Membrane transport can also be influenced by convection. Pressure gradients, including hydrostatic, hydrodynamic, and osmotic, across the
microdialysis membrane can cause fluid convection through the membrane. Depending on whether the direction of convection is into (absorption) or out of (filtration) the probe, relative recovery values can increase or decrease. For ideal microdialysis, all pressure gradients across the membrane are zero. For our microdialysis experiment, fluid convection through the membrane was measured as 0.0 ± 0.4μL/min. Although these data were variable, the net fluid convection is not expected to have a significant effect on the mass transport resistances.

The appropriateness of the BMD model for characterizing microdialysis sampling of macromolecules was examined in two ways. First, the Cussler model (Eqns 3.8-3.10) was employed to determine if values of $R_d$ and $R_m$ calculated via the Cussler model and the BMD model (Eqns 3.3-3.5) were of the same order, and were reasonable for macromolecules passing through a porous membrane. Calculations from the Cussler model produced membrane resistances nearly identical to those calculated by the BMD model (Fig 3.2). Additionally, the Cussler model estimates mass transfer resistances on the same order as the BMD model.

Second, the calculated resistances to mass transport and observed collection efficiencies were fit to a plot of $\ln(1-E_d)$ v. $[-1/(R_d+R_m)]$ (Fig 3.3; Eqn 3.15). Ideally, this treatment would have produced a straight line with a y-intercept of zero and a slope equal to the inverse of flow rate, which in our experiments was 1.0 μL/min (solid line in Figure 3.2). The dextran regression had a y-intercept 0.0001 ± 0.0002
and flow rate from the slope of 0.990 ± 0.002 μL/min. Similarly, the protein regression had a y-intercept of 0.0001 ± 0.0007 and flow rate from the slope of 0.988 ± 0.006 μL/min. The dextran and protein data were both linear and had near zero y-intercepts, suggesting that the behavior of these macromolecules follows the functionality of the BMD model. In addition, the calculated flow rates for the dextran and protein data were within two hundredths of the experimental flow rate of 1.0 μL/min. In sum, the mass transport of these macromolecules through 100 kD microdialysis probe membranes was in agreement with the theoretical BMD model.

Cytokine sampling from a well-stirred solution spiked with TNF-α, IL-6, and MIP-2 indicated that, with the exception of MIP-2, relative recovery rates for these cytokines were within the range predicted by the macromolecular characterization (8-20%). When microdialysis was performed from a biological solution, the recovery of TNF-α did not change significantly, although the recovery rate of IL-1β was smaller than for any of the cytokines from the well-stirred cocktail. This could be due to a characteristic of the IL-1β protein; however, molecular weight alone does not suggest such a difference. These in vitro data suggest that microdialysis recovery of cytokines is not predictable based on molecular weight. Thus, individual experiments would be required to determine relative recoveries for each cytokine examined in vivo. The expense of these additional experiments makes microdialysis sampling of cytokines impractical.
Cytokines were also sampled from the subcutaneous tissue of Sprague-Dawley rats. Microdialysis probes implanted in the subcutaneous tissue of LPS-stimulated rats sampled five cytokines at concentrations above 100 pg/mL. In particular, IL-6 was recovered as high as 3000 pg/mL. However, while this study verifies that cytokines can be collected from tissue using microdialysis sampling, this technique introduced significant error into the cytokine concentrations. In some instances for IL-1β and IL-10, the error was greater than 50% of the measured value (a signal-to-noise ratio less than 2). The additional error introduced by microdialysis could negatively affect statistical comparisons between cytokine samples. Furthermore, because microdialysis recovery rates for cytokines were never greater than 20%, the detection limit and analytical sensitivity of the cytokine measurement method decreases at least 5-fold when using microdialysis sampling. After considering the significant cost, error, and decreased detection limits and analytical sensitivity introduced by microdialysis sampling of cytokines, it was determined that a direct method of sampling cytokines from tissue would be advantageous.

3.6 Conclusions

Determining the level of relative recoveries expected for macromolecules under favorable in vitro conditions was the first step prior to performing quantitative in vivo microdialysis sampling of proteins. In this study, collection efficiencies of 3 to 70kD
macromolecules from well stirred solution ranged from 5 to 44% for a 100kD MWCO PES probe. Macromolecule sampling fit the functionality of the BMD model, yielding transport resistances similar in magnitude to values calculated via the Cussler model. While yielding results generally predictable according to established models, the in vitro relative recovery of proteins in the molecular weight range of cytokines did not exceed 20%. When cytokines were sampled under the same well-stirred conditions, TNF-α and IL-6 recoveries fell within the range predicted by the macromolecular characterization. Further, the feasibility of cytokine sampling from cell culture medium and subcutaneous tissue was confirmed. However, the impracticality of experimentally determining cytokine recovery rates in vivo and the decreased accuracy and sensitivity associated with microdialysis sampling indicate that a direct means of sampling tissue cytokine concentrations would be favorable.
Chapter 4. In Vitro Cytokine-Associated Immune Response to Biomaterials

4.1 SYNOPSIS

Cytokines, chemokines, and growth factors were assayed from the supernatants of monocytes/macrophages cultured on common biomaterials with a range of surface chemistries. TNF-α, MCP-1, MIP-1α, IL-8, IL-6, IL-1β, VEGF, IL-1ra, and IL-10 were measured from monocyte/macrophage cultures at different stages of activation and differentiation seeded onto polyethylene (PE), polyurethane (PU), expanded polytetrafluoroethylene (ePTFE), polymethyl methacrylate (PMMA), and a hydrogel copolymer of 2-hydroxyethyl methacrylate, 1-vinyl-2-pyrrolidinone, and polyethylene glycol acrylate (HEMA-PEG) in tissue culture polystyrene (TCPS) plates. Empty TCPS wells and organo-tin polyvinyl chloride (ot-PVC) served as “blanks” and positive controls, respectively. Results showed an overall increase in cytokine, chemokine, and growth factor production as monocytes are activated or differentiated into macrophages and that pro-inflammatory and anti-wound healing cytokines and chemokines dominate this profile. However, cytokine production was only modestly affected by the surface chemistry of these four stable and non-cytotoxic biomaterials.
4.2 Introduction

It is commonly held that the performance of an implanted device is dependent on the cascade of intercellular signals that originates from monocyte-derived macrophages at the implant surface. Upon implantation, proteins adsorb immediately to the implant surface. Monocytes migrate from blood vessels toward the site of implantation, encountering signals for differentiation into macrophages along the way. Once at the implant surface, macrophages attach to the adsorbed protein layer and secrete cytokines, chemokines, and growth factors that signal the recruitment and activation of lymphocytes, fibroblasts, endothelial cells, and smooth muscle cells and drive the formation of new tissue around implanted devices. Cytokine activation of lymphocytes also leads to the fusion of macrophages into foreign body giant cells (FBGCs) that persist throughout the lifetime of an implanted device and attack implant surfaces with degradative enzymes. A number of these cytokines, chemokines, and growth factors have been categorized as either pro or anti in terms of their inflammatory or wound healing character in the foreign body reaction (Figure 2.1).

One goal of the current study was to investigate the effect of monocyte activation and differentiation on cytokine production. In vitro investigations of the immune response rely on monocyte/macrophage cultures, either from primary sources or established cell lines. For our studies, an immortalized human monocyte cell line (THP-1) was utilized to assess cell-biomaterial interactions.
Although the untreated THP-1 monocyte cell line exhibits different surface marker expression and cytokine production from primary monocytes/macrophages isolated from peripheral blood, the addition of activation or differentiation factors promotes THP-1 monocytes to more closely resemble primary monocytes/macrophages \(^{91-94}\). Phorbol 12-myristate-13-acetate (PMA) is known to differentiate THP-1 cells into macrophage-like cells, which resemble native monocyte-derived macrophages with regard to morphology, membrane receptor and antigen expression, and production of secretory products \(^{92}\). In contrast, lipopolysaccharide (LPS) is generally considered an initiator of classical activation in monocytes/macrophages, rather than a differentiator \(^{32}\). Classically activated monocytes/macrophages migrate to sites of inflammation where they encounter and degrade pathogens, although they are not necessarily more phagocytic than resting cells \(^{26}\). PMA and LPS treated cells, in addition to untreated cells, were analyzed in this study for their impact on cytokine production in response to biomaterials.

Another goal of this study was to investigate the effect of surface chemistry, using commonly implanted materials, on the production of a range of cytokines known to be involved in inflammation and wound healing. Currently, there are two seemingly paradoxical views of surface mediated effects on the tissue surrounding implanted materials. One view, i.e. the “Anderson Hypothesis”, purports that certain biomaterial surface chemistries may dictate patterns of cytokine production, presumably affecting the outcome of the foreign body reaction \(^{18,23}\). Another
argument, i.e. the “Ratner Hypothesis”, suggests that healing surrounding an implant is essentially invariant one month post-implantation for many common biomaterials, regardless of surface chemistries, as long as the material is smooth, chemically inert, and non-degradable. Recent studies provide evidence to support either a surface chemistry-dependent immune reaction or an immune reaction independent of surface chemistry. To date, there remains no comprehensive correlation between biomaterial surface chemistry, cytokine production, and the outcome of the foreign body reaction.

The current study determined the initial cytokine profiles produced by human monocytes/macrophages (THP-1) with varying degrees of activation or differentiation seeded onto common biomaterials that cover a range of surface chemistries. Untreated, LPS-treated, and PMA-treated human monocytes/macrophages were seeded onto polyethylene (PE), polyurethane (PU), expanded polytetrafluoroethylene (ePTFE), polymethyl methacrylate (PMMA), and a hydrogel copolymer of 2-hydroxyethyl methacrylate, 1-vinyl-2-pyrrolidinone, and polyethylene glycol acrylate (HEMA-PEG). Organo-tin stabilized polyvinyl chloride (ot-PVC), which is recommended by the International Organization for Standardization as a positive control for cytotoxicity, was included as the positive control. In addition, since all measurements were performed in 24-well tissue culture polystyrene (TCPS) plates, empty wells of TCPS were employed as the “blank”. The production of cytokines, chemokines, and growth factors, including
TNF-α, MCP-1, MIP-1α, IL-8, IL-6, IL-1β, VEGF, IL-1ra, and IL-10, by monocytes/macrophages were examined over 48 hours for correlation with the different biomaterials. Results indicate that cytokine production was significantly affected by monocyte activation and differentiation, but only modestly affected by the surface chemistry of these stable and non-cytotoxic biomaterials.

4.3 MATERIALS AND METHODS

Biomaterials

High-density polyethylene (PE, US Pharmacopeia, Rockville, MD), polyurethane/pellethane 2363-80AE-050824, a soft segment polyurethane based on polyether and aromatic isocyanate (PU, Polyzen, Inc., Apex, NC), expanded polytetrafluoroethylene (ePTFE, Bard, Tempe AZ), polymethyl methacrylate (PMMA, Modern Plastics, Bridgeport CT), and organo-tin stabilized polyvinyl chloride (ot-PVC, Smiths Medical, Hythe, UK) were cut into 1 cm² squares and sonicated for 20 minutes in 70% ethanol. The materials were then rinsed with pyrogen-free water, dried with lint-free wipes, and sonicated in pyrogen-free water for 20 minutes. These cleaned materials were then gas-sterilized with ethylene oxide and outgassed for 8 hours at 135°F. Sterilized materials were tested for the presence of endotoxin using the QCL-1000 endotoxin assay. Endotoxin levels of all of the
sterilized materials were less than the FDA-approved limit for medical devices (0.5 EU/mL).

HEMA-PEG hydrogels were prepared using a modification of a previously described technique. The HEMA-PEG hydrogel solution was allowed to polymerize for 2 hours at room temperature in 24-well tissue culture plates. After polymerization, hydrogels were swelled in phosphate buffered saline (PBS, Sigma-Aldrich), pH 7.4, overnight at room temperature. To elute cytotoxic components of the hydrogels, PBS soaking was continued for 72 hours at room temperature.

Cell Culture

Human monocytes/macrophages (THP-1, ATCC) were cultured in RPMI 1640 media supplemented with HEPES buffer (10 mM), sodium pyruvate (1 mM), glucose (4.5 g/L), fetal bovine serum (10%), penicillin (100 U/mL), streptomycin (100 µg/mL), and 2-mercaptoethanol (0.05 mM). Monocytes/macrophages were treated with 1 µg/mL LPS (Sigma-Aldrich) for 24 hours, 50 nM PMA (Sigma-Aldrich) for 72 hours, or left untreated.

Untreated and LPS-treated cells were resuspended in 2 mL fresh culture medium at a cell concentration of 500,000 cells/mL and seeded onto PE, PU, PMMA, ePTFE, ot-PVC, HEMA-PEG, or empty wells in a 24-well TCPS plate. The plates were incubated for 24 and 48 hours. After 24 and 48 hours, the cells were loosened from the materials by mechanical dissociation and the cells were collected. Cells
were counted on a hemocytometer using a Nikon Diaphot light microscope (Nikon, Melville, NY). Live cells were determined via trypan blue dye exclusion. Supernatants collected at 24 and 48 hours were stored at -20°C until analyzed for cytokine concentrations.

The PMA treated cells were rinsed with PBS and detached with 4 mL of trypsin/EDTA (Clonetics, Walkersville, MD) per T-75 flask. The flask was incubated for 4 minutes before adding 8 mL of trypsin neutralizing solution (TNS, Clonetics). The cells were then re-suspended and seeded onto materials in the same manner as the untreated and LPS–treated cells. At 24 and 48 hours post-seeding the supernatants were collected and stored at -20°C. The cells were then loosened from the materials using 260 μL of trypsin/well, incubating for 4 minutes, and then adding 520 μL of TNS/well. Viable cells, determined by trypan blue dye exclusion, were counted on a hemocytometer via light microscopy.

**Flow Cytometry**

In order to assess levels of monocyte differentiation, the expression of the cell surface marker CD11b, a marker of macrophage differentiation, was measured by flow cytometry. Untreated, LPS treated, and PMA treated cells were detached as described above and fixed for 15 minutes with 3.7% paraformaldehyde. The cells were then rinsed in PBS, incubated with 10% goat serum (blocking buffer, Sigma), and incubated with 10 μg/mL mouse anti-human CD11b (Becton Dickinson). Cells
were again rinsed, then incubated with Alexa fluor 488 goat anti-mouse secondary antibody (Invitrogen), rinsed, and post-fixed in 10% formalin. Fluorescence intensity of the labeled cells was measured using a FACSCalibur flow cytometer (Becton Dickinson). At least 10,000 events were acquired per sample to determine the geometric mean fluorescence intensity (GMFI). Data analysis was performed using CellQuest software (Becton Dickinson).

**Fluorescent Imaging**

Untreated monocytes/macrophages were seeded onto PE, PU, PMMA, ePTFE, ot-PVC, and TCPS surfaces in 24-well tissue culture plates at a seeding density of 500,000 cells/mL. The materials were rinsed with PBS to remove loosely adherent cells then incubated with 1 µM Calcein-AM (Sigma-Aldrich) for 15 minutes. Images of fluorescent cells were obtained via fluorescence microscopy using an Olympus BX41 (Olympus, Center Valley, PA) microscope.

**Cytokine Measurement**

Supernatants collected from monocytes/macrophages seeded onto biomaterial surfaces were assayed for cytokines using a Luminex bead array (BioRad). Initially, a 27-plex human cytokine kit, which included TNF-α, MCP-1, MIP-1α, IL-8, IL-1β, IL-6, VEGF, IL-1ra, IL-10, IL-2, IL-4, IL-5, IL-7, IL-9, IL-12 (p70), IL-13, IL-15, IL-17, IFN-γ, IP-10, G-CSF, GM-CSF, RANTES, FGF-basic, Eotaxin, PDGF-bb,
and MIP-1β, was used to screen for potential cytokine targets (BioRad). Of this list of 27 cytokines, only the first nine were selected for continued analysis based on their measured concentrations (data not shown) and their roles in the foreign body reaction. Luminex bead array assays were performed according to the manufacturer’s instructions (Linco/Millipore). Briefly, antibody coupled beads were incubated with monocyte/macrophage supernatants for 1 hour. After washing, secondary antibody was incubated with the cytokine-bound beads for 30 minutes. Finally, phycoerythrin dye was bound to the beads via biotin-streptavidin binding for 30 minutes. All incubations occurred at room temperature on a plate shaker according to the manufacturers instructions. Sample concentrations (pg/mL) were determined from mean fluorescence intensities (MFI) compared against a 4 or 5-parameter logistic standard curve generated from standards of known concentration provided by the bead array manufacturer. The Luminex bead array was calibrated prior to every sample analysis and validated regularly per the manufacturer’s instructions.

**Statistics**

One-way ANOVA plus Bonferroni’s multiple comparison test *post hoc* analysis was performed to determine significance in the flow cytometry data (GraphPad Prism 4). A multivariate analysis of variance (MANOVA) was determined from nine four way ANOVAs (one per cytokine) to determine
significance in the cytokine per cell and normalized cytokine data (StatView 5.0.1). P-values of <0.1, as determined by a multivariate test using Roy’s Greatest Root, were considered significant for the material by treatment interaction term. Subsequently, p-values of <0.05 were considered significant in post-hoc tests (Fisher’s protected least square difference) used to compare between material effects.

4.4 RESULTS

Monocyte Activation and Differentiation

Human monocytes (THP-1) were treated with LPS, PMA, or left untreated. Phase contrast microscope images of these cells indicated that PMA-treated cells underwent a morphological change from untreated monocytes (Figure 4.1).
Figure 4.1. Characterization of monocyte/macrophage activation and differentiation. Phase contrast images (20x magnification) of untreated, LPS-treated, and PMA-treated monocytes/macrohages. The geometric mean fluorescence intensities (GMFI) from FACS results of CD11b induction were graphed as geometric means + standard errors. * Geometric mean fluorescence intensity is statistically greater (p < 0.05) for PMA-treated cells than for untreated or LPS-treated cells.

While untreated cells were generally round and grew in clusters, PMA-treated cells exhibited larger, irregular shapes with extended filopodia and decreased cell clustering. LPS-treated monocytes were similar in morphology to untreated monocytes. Immunostaining, in combination with fluorescence activated cell sorting
(FACS), was utilized to quantify the amount of expressed CD11b. FACS results showed a significant increase in CD11b expression for PMA-treated monocytes in comparison to untreated and LPS-treated monocytes.

**Cytokine production by monocytes/macrophages seeded onto biomaterial surfaces**

From an initial screen of 27 human cytokines, 9 were selected for inclusion in this study based on concentration (>20 pg/mL) and their relevance to the foreign body reaction: TNF-α, MCP-1, MIP-1α, IL-8, IL-1β, IL-6, VEGF, IL-1ra, and IL-10 (data not shown). The raw data depicting cytokine concentrations for each combination of material, cell treatment, and time is ideal for precise concentrations; however, patterns in cytokine production are difficult to ascertain from a standard graph of this data (Figure 4.2).
Figure 4.2. Raw cytokine concentrations from monocyte/macrophages in vitro. Raw cytokine concentrations detected from monocytes/macrophages on biomaterial surfaces are presented quantitatively. Data are shown as means ± SEM (n = 3).
In order to present the data in a manner more suitable for analyzing cytokine production patterns, these data were converted to a color map, similar to the familiar red-green gene expression grids. In addition, cytokine production was calculated on a per cell basis in order to account for variation in cell concentrations among the selected cell treatments and biomaterials. Cytokine production per cell was calculated by normalizing the cytokine concentrations measured from the supernatants of monocytes/macrophages seeded onto a material to their respective cell concentration. Cytokine production per cell, graphed on a color map using MATLAB (MathWorks, Natick, MA), is shown in Figure 4.3.
Figure 4.3. **In vitro biomaterial-induced cytokine production colormap.** Cytokine production per cell (ag/cell) from monocytes/macrophages seeded onto biomaterial surfaces after a) 24 h and b) 48 h. A scale bar is included at the top of each color grid. Data are shown as means (n = 3). *Biomaterial induced cytokine production is greater (p < 0.05) than TCPS-induced cytokine production.
Cytokine production per cell generally increased from 24 to 48 hours for all cytokines and all cell treatments. At both 24 and 48 hours, cytokine production per cell was greater for PMA-treated cells than either untreated or LPS-treated cells for all cytokines, reaching as high as 100,000 ag/cell for three cytokines: MIP-1α, IL-8, and IL-1ra. Patterns in material induced cytokine production were difficult to distinguish; however, cytokine production induced in untreated cells by each of the four non-cytotoxic biomaterials at 48 hours was greater than that induced by TCPS.

**Classification of total cytokine production**

Because individual cytokine production can be difficult to interpret, we also looked at the integrated response of classes of cytokines. Total cytokine production is presented for all cytokines grouped by their pro and anti roles in inflammation and wound healing (Figure 4.4).
Figure 4.4. Integrated biomaterial-induced cytokines in vitro. Production of cytokines per cell (fg/cell) grouped into pro- or anti-inflammatory and pro- or anti-wound healing based on each cytokine’s role in the foreign body reaction. Data are shown as means ± SEM (n = 3).
Untreated monocytes produced predominantly anti-inflammatory and pro-wound healing cytokines on all surfaces, with the exception of ot-PVC. In contrast, for LPS and PMA-treated monocytes, pro-inflammatory and anti-wound healing cytokines were produced in the greatest quantity. For all cell treatments, monocytes/macrophages seeded onto ot-PVC produced predominantly pro-inflammatory and anti-wound healing cytokines at both 24 and 48 hours. It should also be noted that there was no apparent materials-dependent effect on class of cytokine produced among non-cytotoxic materials.

**Evaluation of TCPS “blank”**

In order to examine whether TCPS was suitably benign to comprise a “blank”, we also examined cytokine production in response to a non-fouling HEMA-PEG hydrogel. Cytokine production per cell for monocytes/macrophages seeded onto HEMA-PEG was normalized to cytokine production per cell for monocytes/macrophages seeded onto TCPS (Figure 4.5).
Figure 4.5. **TCPS blank validation.** Cytokine production from monocytes/macrophages induced by HEMA-PEG hydrogels normalized to TCPS-induced cytokines. A scale bar is included above the color grid (normalized units). Data are shown as means (n = 3). Statistical significance is denoted as greater than (↑) or less than (↓) the TCPS blank (p < 0.05).

Of the 54 combinations in Figure 4.5, only in 11 cases was cytokine production induced by TCPS statistically different than HEMA-PEG. In eight of these cases, TCPS induced less cytokine production than HEMA-PEG. TCPS
induced more cytokine production than HEMA-PEG only for VEGF, a pro-wound healing cytokine, and IL-10, an anti-inflammatory cytokine.

**Cell viability on material surfaces and material contact angles**

After 24 and 48 hours, viability of monocytes/macrophages seeded onto material surfaces was measured via trypan blue dye exclusion. Viability was always greater than 92% for LPS-treated and untreated cells seeded onto PE, PU, ePTFE, PMMA, or TCPS (Table 4.1).

**Table 4.1. Monocyte/macrophage viability.** Viability for untreated, LPS treated, and PMA treated monocytes/macrophages at 24 and 48 hours post seeding onto material surfaces. Water contact angles are included for each material. Data are shown as mean ± SEM (n ≥ 3).

<table>
<thead>
<tr>
<th>Material (Contact angle)</th>
<th>Untreated Viability (%)</th>
<th>LPS treated Viability (%)</th>
<th>PMA treated Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24 hrs</td>
<td>48 hrs</td>
<td>24 hrs</td>
</tr>
<tr>
<td>PE (89 ± 2°)</td>
<td>99 ± 1</td>
<td>97 ± 1</td>
<td>98 ± 1</td>
</tr>
<tr>
<td>PU (89 ± 1°)</td>
<td>99 ± 1</td>
<td>97 ± 1</td>
<td>99 ± 1</td>
</tr>
<tr>
<td>ePTFE (117 ± 2°)</td>
<td>99 ± 1</td>
<td>98 ± 1</td>
<td>96 ± 1</td>
</tr>
<tr>
<td>PMMA (73 ± 1°)</td>
<td>96 ± 1</td>
<td>97 ± 1</td>
<td>94 ± 1</td>
</tr>
<tr>
<td>ot-PVC (69 ± 1°)</td>
<td>12 ± 1</td>
<td>11 ± 3</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>TCPS (68 ± 2°)</td>
<td>99 ± 1</td>
<td>98 ± 1</td>
<td>95 ± 1</td>
</tr>
</tbody>
</table>

74
Viability was much lower for PMA-treated cells, reaching as low as 29% for ot-PVC at 48 hours. Viability for PMA-treated cells increased from 24 to 48 hours for all materials except ot-PVC. Monocyte/macrophage viabilities were always the lowest for cells seeded onto ot-PVC, where monocytes/macrophages were 11-12% and 13-16% viable with no treatment and LPS-treatment, respectively. Water contact angles measured on the selected biomaterials ranged from mildly hydrophilic 68º for TCPS to very hydrophobic 117º ePTFE (Table 4.1).

**Cell adhesion to material surfaces**

At 24 hours post-seeding, untreated human monocytes/macrophages fluorescently labeled with 1 µM calcein AM were imaged. Calcein AM fluorescence revealed that adherent cells were prevalent on PE, PMMA, ePTFE, PU, and TCPS (Figure 4.6).
Figure 4.6. Biomaterial-adherent monocytes/macrophages. Fluorescent images (10x magnification) of untreated monocytes/macrophages adherent to biomaterial surfaces. Calcein AM was used for fluorescent staining.

Fluorescent images of ot-PVC surfaces showed substantially fewer viable, attached monocytes/macrophages than the other materials.

Cytokine production normalized to TCPS “blank”

In order to make comparisons between materials, cytokine production per cell induced by each material was normalized to cytokine production per cell induced by the TCPS blanks. Normalized cytokine production data was graphed on a color map using MATLAB (Figure 4.7).
Figure 4.7. In vitro biomaterial induced cytokines normalized. Material-induced cytokine production normalized to TCPS-induced cytokine production from monocytes/macrophages seeded onto material surfaces for (a) 24 h and (b) 48 h. A scale bar is included above each color grid (normalized units). Data are shown as means (n = 3). * Normalized cytokine production for ot-PVC is statistically greater (p < 0.05) than normalized cytokine production induced by all other materials. Bars indicate statistical differences in normalized cytokine production between the connected biomaterials (p < 0.05).
Normalized cytokine production was most frequently increased above the control (>1) for untreated monocytes. For untreated monocytes, few differences were noticed at 24 hours; however, at 48 hours, ot-PVC positive control induced significantly increased normalized production of TNF-α, MIP-1α, and IL-8, which were all greater than 10-fold higher than TCPS. Normalized IL-1ra and IL-10 production were the lowest for ot-PVC, although these differences were not statistically significant. The only statistically significant differences between non-cytotoxic biomaterials for untreated monocytes were increased normalized production of IL-10 induced by ePTFE as compared to PE or PU as well as increased VEGF induced by PMMA as compared to PU.

At 24 hours, normalized cytokine production was not significantly increased for LPS-treated cells seeded onto ot-PVC in comparison to the non-cytotoxic materials. At 48 hours, normalized production values for TNF-α, MCP-1, and MIP-1α were significantly higher for LPS-treated cells seeded onto ot-PVC as compared to other materials. No significant differences were noted between non-cytotoxic biomaterials.

For PMA treated cells, differences in normalized cytokine production with respect to material were not significant at 24 hours. At 48 hours, normalized cytokine production for PMA-treated cultures seeded onto PMMA and PE was increased for all cytokines, although these increases were only statistically significant.
with MCP-1, IL-6, and IL-10 as compared to ot-PVC. Again, no statistically significant differences were noted between non-cytotoxic biomaterials.

4.5 DISCUSSION

Macrophages at an implant surface are derived from monocytes that encounter molecular signals as they migrate into tissue from the vasculature. The arriving macrophages interrogate the implanted material and secrete an array of cytokines, chemokines, and growth factors to direct the foreign body reaction. Pro-inflammatory cytokines, such as TNF-α, IL-8, MIP-1α, and MCP-1, recruit immune cells such as lymphocytes and monocytes to the implant site to promote inflammation. Anti-inflammatory cytokines, such as IL-10 and IL-1ra, suppress pro-inflammatory signals in order to downregulate inflammation. Pro-wound healing cytokines such as VEGF play a reparative role by promoting neovascularization in the tissue surrounding the implant. While these classifications are useful in establishing general categories of cytokines, the specific functions of each individual cytokine are unique; thus, individual cytokine production levels are expected to vary for each cytokine within these classifications.

It is currently thought that the types and concentrations of cytokines produced by macrophages determine the outcome of the foreign body reaction to a given implanted material\(^1\). The goals of this study were to examine the roles of (1)
monocyte activation and differentiation into macrophages and (2) biomaterial surface chemistry on cytokine production.

An immortalized human monocyte line (THP-1) was selected for use in this study in order to minimize variability in cell populations. For our study, LPS and PMA mimicked the intrinsic activation and differentiation signals that monocytes encounter during the foreign body reaction as they migrate from the vasculature into tissues and become macrophages. LPS simulates bacterial activation of macrophages, while PMA mimics the intracellular signaling functions of diacylglycerol. Previous research indicates that LPS and PMA each have a unique signaling pathway in monocytes, though both induce cytokine production through activation of the transcription factor NF-κB. Because of these unique pathways, cells treated with LPS and PMA exhibited different phenotypes. For instance, PMA induced a noticeable change in monocyte/macrophage morphology, differentiating these loosely adherent cells into an adherent state. LPS-treated cells appeared morphologically similar to untreated cells. Fluorescence activated cell sorting (FACS) indicated upregulation of the macrophage differentiation marker CD11b, a subunit of the CD11b/CD18 integrin involved in cell surface adhesion, after stimulation with PMA. PMA induced the greatest increase in CD11b positive monocytes/macrophages, as evidenced by FACS and the increased spreading of monocytes/macrophages in Figure 2. Upregulation of CD11b and increased cell spreading suggested that PMA-treatment of
monocytes/macrophages led to a greater number of cell-surface contacts and a phenotype more typical of macrophages, as compared to LPS treatment or no treatment.

The change in cell behavior as monocytes were activated or differentiated into macrophages was also apparent in the variation in cytokine production (Figure 4.3). After just 24 hours, PMA-treated macrophages produced an increased amount of cytokines when compared to LPS-treated and untreated cells (Figure 4.3). This effect was even more apparent after 48 hours. To a lesser degree, LPS-treatment increased pro-inflammatory cytokine production as compared to untreated monocytes. These results were not surprising since PMA and LPS alone are known to induce cytokine production from cell cultures\textsuperscript{33}.

Interestingly, when the cytokines were grouped for their roles in the foreign body reaction, it became apparent that monocyte activation or differentiation with LPS or PMA, respectively, preferentially induced pro-inflammatory and anti-wound healing cytokines (Figure 4.4). These results suggest that uncommitted monocytes exhibit anti-inflammatory and pro-wound healing profiles until they are activated or differentiated into macrophages, at which point they adopt a decidedly pro-inflammatory and anti-wound healing profile. Perhaps activated monocytes and macrophages, which are known to have a role in chronic inflammation\textsuperscript{6}, are inherently more apt to promoting inflammation than unstimulated monocytes.
The interaction between macrophages and an implanted material is facilitated by a protein layer rapidly adsorbed to surfaces upon exposure to serum. Since the conformation of proteins in this layer is dependent upon the surface chemistry of a biomaterial, it is postulated that the surface chemistry of a biomaterial could impact cellular functions including cytokine secretion. The primary goal of the current study was to investigate this possibility by analyzing the initial cytokine production, up to 48 hours post-seeding, from monocytes/macrophages interrogating biomaterials with varying surface chemistries. A 27-cytokine kit was used to identify cytokines and growth factors induced by monocyte/macrophage interaction with material surfaces. Seven cytokines found in greater than 20 pg/mL concentrations, TNF-α, MCP-1, MIP-1α, IL-8, VEGF, IL-1ra, and IL-10, as well as the common pro-inflammatory cytokines IL-1β and IL-6 were selected from the original 27 cytokines for examination in this experiment.

Although TCPS is commonly used for culturing cells, it is a surface foreign to monocytes/macrophages much like the other biomaterials. For this reason, TCPS was evaluated as a biomaterial “blank” in comparison to a non-fouling HEMA-PEG (HEMA-VP-PEG copolymer) hydrogel. The HEMA-PEG surface induced similar cytokine production to TCPS for all cell treatments (Figure 4.5). Of the instances where TCPS and HEMA-PEG induced statistically significant differences in cytokine production, HEMA-PEG more often induced greater cytokine production than TCPS. The only instances where TCPS induced greater cytokine production
than HEMA-PEG involved the anti-inflammatory cytokine IL-10 and the pro-wound healing cytokine VEGF. In sum, as evaluated in comparison to a non-fouling hydrogel, TCPS is suitable as a “blank” for inducing cytokine production from monocytes/macrophages.

ot-PVC manufactured with an organo-tin stabilizer, a typical positive control in cytotoxicity testing \(^9^7\), was selected in this study as a positive control for inflammation. The cytotoxicity of ot-PVC was apparent in the first 24 hours after cell seeding (Table 4.1). The lack of viable cells adherent to ot-PVC could also be seen in the fluorescent images taken after calcein AM staining (Figure 4.6). In contrast, monocytes/macrophages were viable and adherent to the other 5 surfaces, including the background material, TCPS. Since significantly fewer cells survived in culture with ot-PVC, the amount of each cytokine produced would be expected to be lower as compared to other materials. To most accurately compare ot-PVC with the other materials, cytokine concentrations were calculated per concentration of viable cells present in culture with each material.

The positive control ot-PVC induced the greatest pro-inflammatory cytokine production by untreated and LPS-treated cells at 48 hours (Figure 3). The inflammatory effect of ot-PVC was even more apparent in the TCPS-normalized cytokine production, which was greater than 10 for the pro-inflammatory cytokines TNF-\(\alpha\), MCP-1, MIP-1\(\alpha\), and IL-8 (Figure 4.7). In addition, ot-PVC induced very little anti-inflammatory or pro-wound healing cytokine production. ot-PVC was the
only material to induce a pro-inflammatory and anti-wound healing cytokine profile for all cell treatments (Figure 4.4). For this material, the cells were likely responding to the cytotoxic effect of organo-tin leached from the ot-PVC surface, rather than the ot-PVC surface itself. Nevertheless, ot-PVC depicted a cytokine profile representative of a material that induced high signaling for promoting inflammation and low signaling for promoting wound healing.

The common biomaterials PE, PU, ePTFE, and PMMA were selected because they are stable, chemically inert, and they possess a range of surface chemistries, as evidenced by the water contact angles in Table 4.1. Differences in cytokine production among these materials were most easily interpreted from the TCPS-normalized cytokine production data (Figure 4.7). Normalized cytokine production values were greater than unity for all of these biomaterials. This suggests that the presence of these materials in TCPS culture plates increased cytokine production within the time frame of this study. In comparing materials, significant differences were rare among the non-cytotoxic biomaterials. Differences in cytokine production due to the effects of the biomaterials were indistinguishable from the increased cytokine production induced by LPS and PMA. The only significant differences in normalized cytokine production between non-cytotoxic biomaterials occurred with untreated cells (Figure 4.7). Most notably, ePTFE induced significantly higher IL-10 production than PU or PE from untreated monocytes at 48 hours. Since IL-10 is an anti-inflammatory cytokine, this finding suggests that untreated monocytes cultured
on ePTFE downregulate inflammation in comparison to those cultured on PE or PU. However, this result was not repeated for LPS and PMA-treated cells. The only other significant difference between non-cytotoxic biomaterials was increased VEGF production from untreated monocytes on PMMA as compared to PU. Again, this trend was not repeated for LPS or PMA-treated macrophages.

When cytokines were categorized based on their role in the foreign body reaction, cytokine production from the non-cytotoxic materials followed similar trends (Figure 4.4). Generally, more total cytokine was produced at 48 hours than at 24 hours. In addition, while the total amount of cytokine varied, each non-cytotoxic material induced similar patterns of pro- or anti-inflammatory and pro- or anti-wound healing for all cell treatments. This suggests that these materials with variable surface chemistries might not promote substantially different immune responses in vivo, although these highly controlled in vitro conditions are not completely representative of an in vivo environment.

Although this study and previous research have shown cytokine production from monocytes/macrophages can differ with material surface, caution should be used in correlating individual cytokine production with material properties. With the exception of cytotoxic ot-PVC, differences in cytokine concentrations among biomaterials in this study were only moderate and not reflective of a shift in the promotion of inflammation or wound healing. Additionally, when monocytes were activated or differentiated into macrophages, no differences were apparent between
these non-toxic biomaterials. Results from this investigation are consistent with the argument that, given time, the foreign body reaction resolves with the similar endpoint of fibrous encapsulation for nearly any commonly implanted material. While PE, PU, PMMA, and ePTFE are variable in surface chemistry, they are similar in being non-cytotoxic polymeric biomaterials; thus, macrophage interactions may be similar for these surfaces. Although surface chemistry undoubtedly impacts protein adsorption, a non-specific array of proteins adsorbs to material surfaces independent of surface chemistry. This adsorption of a non-specific protein layer may provide the signal that determines macrophage functions and, consequently, the foreign body reaction to implanted materials. Investigations of the foreign body reaction using animal models could provide evidence to determine whether or not material surface chemistry significantly affects cytokine and growth factor production in vivo.

4.6 CONCLUSIONS

In this study, cytokine production was examined from resting monocytes, activated monocytes and differentiated macrophages seeded onto biomaterial surfaces. A new method of presentation was developed to illuminate patterns within cytokine production data. Cytokine production increased as monocytes were activated or differentiated into macrophages, with specific increases in pro-inflammatory and anti-wound healing cytokines, suggesting activated monocytes and
differentiated macrophages were more prone to inducing inflammation than unstimulated monocytes. All biomaterials, including PE, PU, PMMA, and ePTFE, induced increased cytokine production in comparison with TCPS alone. With the exception of a known cytotoxic material, ot-PVC, the changes in cytokine responses with respect to material were not indicative of a significant shift in the balance of inflammation and wound healing. The minor differences in induced cytokine production in vitro between these polymeric biomaterials, PE, PU, PMMA, and ePTFE, could potentially indicate similar foreign body reactions and fibrous encapsulations in vivo.
Chapter 5. In Vivo Cytokine-Associated Immune Response to Biomaterials

5.1 SYNOPSIS

Cytokines, chemokines, and growth factors were analyzed periodically over eight weeks from the wound exudate fluid surrounding biomaterials implanted subcutaneously within stainless steel mesh cages. TNF-α, MCP-1, MIP-1α, IL-2, IL-6, IL-1β, VEGF, IL-4, and IL-10 were measured from exudate samples collected from cages containing polyethylene (PE), polyurethane (PU), and organo-tin polyvinyl chloride (ot-PVC). Empty cages served as negative controls. Cytokine, chemokine, and growth factor concentrations decreased from the initial implantation to eight weeks post-implantation. Additionally, results showed an overall increase in cytokine, chemokine, and growth factor production for material-containing cages. However, cytokine production was only modestly affected by the varying surface chemistries of these three implanted polymeric materials.

5.2 INTRODUCTION

The functionality of implanted devices, particularly tissue-interactive devices, is dependent on the immune reaction of the surrounding tissue, termed the foreign body reaction. In vivo, a number of cell types contribute to the foreign body reaction, including neutrophils, monocyte-derived macrophages, and lymphocytes.
Intercellular signals derived from these immune cells promote inflammatory events, such as macrophage recruitment, as well as wound healing events, such as collagen synthesis and neovascularization \(^6,^{10}\). The specific signaling molecules produced by cells at the surface of an implanted device may be indicative of the tissue environment surrounding the device. For example, high concentrations of monocyte chemoattractant protein-1 (MCP-1), which recruits monocytes to the site of implantation, would be indicative of prolonged inflammation \(^{104}\). Likewise, high levels of vascular endothelial growth factor (VEGF), a strong promoter of neovascularization, would be indicative of a vascularized tissue \(^{105}\).

Varying the surface chemistry of stable, non-toxic, polymeric devices has previously been shown to have only a modest impact on cytokine production from adherent monocyte/macrophage cultures. The goal of the current study is to investigate the in vivo production of cytokines in response to commonly implanted polymeric biomaterials with different surface chemistries using the cage implant system. In this study, a small stainless steel cage implanted in rat dorsal subcutis is utilized to create a “pocket” in the subcutaneous tissue where wound exudate fluid pools \(^{38,51-53,56-59,106-108}\). Biomaterials inserted within the implanted cage are bathed with exudate, but are not in contact with the surrounding tissue. The exudate is sampled periodically for an analysis of its cellular and cytokine content.

Prior investigations using the cage implant system have indicated that the type and number of immune cells present in the exudate fluid can be dependent on the
properties of the implanted material. In addition, Brodbeck et al showed that the production of cytokine mRNA is affected by material surface properties, such as surface charge and hydrophobicity. Other in vivo studies have indicated that the production of IL-6 is significantly increased for copper implants as compared to titanium implants; however, IL-10 concentrations were not affected by the material differences. The current study investigated the impact of two chemically dissimilar polymeric biomaterials on the infiltration of immune cells and the cytokine content in the exudate fluid sampled from the cage.

Specimens of polyethylene (PE), polyurethane (PU), and organo-tin stabilized polyvinyl chloride (ot-PVC), which is recommended by the International Organization for Standardization as a positive control for cytotoxicity, were placed within stainless steel mesh cages implanted subcutaneously in Sprague Dawley rats. The production of cytokines, chemokines, and growth factors, including TNF-α, MCP-1, MIP-1α, IL-2, IL-6, IL-1β, VEGF, IL-4, and IL-10, in the exudate surrounding the implanted materials was examined periodically up to 8 weeks post-implantation. Results indicate that cytokine production was impacted by the presence or absences of the implanted materials, but was only modestly affected by the different chemistries of these three materials.
5.3 MATERIALS AND METHODS

Biomaterials

High-density polyethylene (PE, US Pharmacopeia, Rockville, MD), polyurethane/pellethane 2363-80AE-050824, a soft segment polyurethane based on polyether and aromatic isocyanate (PU, Polyzen, Inc., Apex, NC), and organo-tin stabilized polyvinyl chloride (ot-PVC, Smiths Medical, Hythe, UK) were cut into 1 cm$^2$ squares and sonicated for 20 minutes in 70% ethanol. The materials were then rinsed with pyrogen-free water, dried with lint-free wipes, and sonicated in pyrogen-free water for 20 minutes. These cleaned materials were then gas-sterilized with ethylene oxide and outgassed for 8 hours at 135ºF. Sterilized materials were tested for the presence of endotoxin using the QCL-1000 endotoxin assay. Endotoxin levels of all of the sterilized materials were less than the FDA-approved limit for medical devices (0.5 EU/mL).

X-ray Photoelectron Spectroscopy (XPS)

XPS data were obtained using a Kratos Analytical Axis Ultra DLD electron spectrometer (Kratos Analytical Instruments, Manchester, UK). Monochromatic AlKα X-rays (1486.7 eV) were employed. Survey scans were generated using a 300 µm by 700 µm measurement area operating at 15 kV, 10 mA, and 150 W. Data are shown from 0 to 1200 eV in binding energy with 160 eV pass energy, a 1.0 eV step size, and a dwell time of 200ms per point. The data acquisition and processing were
done using CasaXPS software. The operation pressure of the spectrometer was typically in the \(2 \times 10^{-8}\) torr range with a system base pressure of \(6 \times 10^{-9}\) torr.

**Cage Implant System**

Stainless steel mesh (stainless steel type 316, 24 x 24 mesh per square inch, 0.014 inch wire diameter, MSC Industrial Supply Co., Melville, NY) was formed into cylindrical cages 1 cm in diameter and 4 cm in length. Protruding wire ends of the cages were turned inward. The cages were sterilized with ethylene oxide.

Male retired breeder rats (Sprague-Dawley) were anesthetized with isofluorane and their backs were shaved and skin prepped with chlorhexidine. Two 2-cm incisions were made exposing the dorsal subcutis. Subcutaneous space was cleared by blunt dissection. Two bilateral cages were implanted in the cleared space and the incisions were sutured. In order to effectively isolate the tissue reaction to an implanted experimental biomaterial from the tissue reaction to the implanted cage, two weeks were allowed for progression of healing in the tissue surrounding the implanted cages. After two weeks, access to the cage lumen was made by incision and PE, PU, and ot-PVC specimens were inserted within one of the stainless steel cages, while the other cage remained empty to serve as a control. Rats showing external signs of inflammation were immediately removed from the study.

As a positive control for inflammation, LPS (1 \(\mu g/g\) rat body weight, Sigma) was injected into empty cages implanted into male retired breeder rats (Sprague-
Dawley) for 24 hours. Exudate samples were collected as described below after 3 hours and 24 hours.

**Exudate collection**

Exudate samples were collected from empty and material-implanted cages on days 1, 2, 8, 28, and 56 after biomaterial implantation. Briefly, the rat was elevated to promote exudate fluid pooling at the posterior end of the cage. A 25 gauge, 1 inch needle inserted through the skin into the posterior end of the cage lumen, and exudate was extracted slowly to prevent contamination with blood. Collected exudate was divided into two eppendorf containers on ice, one for cellular analysis and one for cytokine analysis.

**Cell analysis**

Cellular composition of the exudate fluid was analyzed by the Duke Veterinary Diagnostic Laboratory. Briefly, exudate fluid samples were smeared onto glass slides and stained using a Diff-Quick staining kit. Neutrophils, lymphocytes, monocytes, basophils, and eosinophils were counted on each slide using light microscopy (100x magnification).
Cytokine measurement

Exudate fluid extracted from cages was assayed for cytokines with a Bioplex bead array (BioRad) using Lincoplex assay kits performed according to the manufacturer’s instructions (Millipore). Briefly, biotinylated, antibody coupled beads were incubated with exudate fluid at 8°C for 18-24 hours. After washing, secondary antibody was incubated with the cytokine-bound beads at room temperature for 1 hour. The biotinylated beads were developed by incubation of phycoerythrin-streptavidin to at room temperature for 30 minutes. All incubations occurred on a plate shaker according to the manufacturers instructions. Sample concentrations (pg/mL) were determined from mean fluorescence intensities (MFI) compared against a 4 or 5-parameter logistic standard curve generated from standards of known concentration provided by the bead array manufacturer. The Luminex bead array was calibrated prior to every sample analysis and validated regularly per the manufacturer’s instructions.

Statistics

A multivariate analysis of variance (MANOVA) was determined from nine three way ANOVAs (one per cytokine) to determine significance in the cytokine concentration data (StatView 5.0.1). P-values of <0.1, as determined by a multivariate test using Roy’s Greatest Root, were considered significant for the material by treatment interaction term. Subsequently, p-values of <0.05 were
considered significant in post-hoc tests (Fisher’s protected least square difference) used to compare between material effects. Significance in the normalized cytokine data was determined by p-values of <0.05 measured using a two-way ANOVA. Subsequently, p-values of <0.05 were considered significant in post-hoc tests (Fisher’s protected least square difference) used to compare between material effects.

5.4 RESULTS

Biomaterial surface characterization

The atomic composition of the biomaterial surfaces (PE, PU, and ot-PVC) was characterized by X-ray photoelectron spectroscopy (XPS). A survey scan of PE indicates an atomic percentage of 91% carbon, with the remainder composed of oxygen and nitrogen (Figure 5.1).
A survey scan of PU similarly indicates an atomic composition of primarily carbon (83%), with oxygen and nitrogen as the remaining elements (Figure 5.2).
Figure 5.2. XPS scan of polyurethane (PU). An XPS scan of PU depicts an elemental composition of carbon, oxygen, and nitrogen.

A survey scan of ot-PVC also indicates a large composition of carbon (78%), but chlorine (2.3%) and tin (0.3%) are present in addition to oxygen and nitrogen (Figure 5.3).
Survey

Exudate fluid extracted from stainless steel mesh cages with and without an implanted biomaterial was analyzed for the composition of immune cells, including neutrophils, lymphocytes, monocytes, basophils, and eosinophils (Figure 5.4).

**Cellular composition in exudate fluid**

Exudate fluid extracted from stainless steel mesh cages with and without an implanted biomaterial was analyzed for the composition of immune cells, including neutrophils, lymphocytes, monocytes, basophils, and eosinophils (Figure 5.4).

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**Table 5.3.** XPS scan of organo-tin polyvinylchloride (ot-PVC). An XPS scan of ot-PVC depicts an elemental composition of carbon, oxygen, nitrogen, chlorine, and tin.
Figure 5.4. Exudate cellular composition. a) Neutrophils, b) lymphocytes, and c) monocytes extracted from PE, PU, and ot-PVC filled cages, as well as empty cages. Data are shown as means ± SEM (n = 3).
At early time points, the exudate fluid collected from biomaterial-filled cages contained primarily neutrophils. As neutrophils decreased between day 2 and day 8, lymphocytes increased. From day 8 to day 56, lymphocytes remained elevated in the extracted exudate fluid. Monocytes never accounted for greater than 20% of the cell population in the exudates. Furthermore, basophils and eosinophils combined for less than 1% of the total cell population. There was no significant effect of material surface properties on the types of infiltrating cells.

**Cytokines in exudate surrounding implanted biomaterials**

Cytokine content was measured from the extracted exudate fluid using a Luminex® bead array system. The nine cytokines measured from this exudate included TNF-α, MCP-1, MIP-1α, IL-2, IL-6, IL-1β, VEGF, IL-4, and IL-10. The concentrations of these cytokines in the extracted exudate fluid are presented below in a colormap format, where magnitudes are represented by a color scale (Figures 5.5, 5.6, and 5.7).
On day 1 post implantation, there were twelve instances where cytokine concentrations were significantly different between cages containing PE, PU, ot-PVC, and empty cages (Figure 5.5). Nine of twelve instances were due to increased
cytokine production from material-containing cages in comparison to empty cages. The other three instances involved increased production of VEGF and IL-10, and decreased production of MCP-1 induced by PU as compared to PE. After the second day of implantation, significant differences were only detected for MCP-1, where PE induced a greater MCP-1 concentration than PU, ot-PVC, and the empty cage (Figure 5.5).

Cytokine concentrations on day 8 post implantation are presented in Figure 5.6.
Figure 5.6. Acute in vivo cytokine concentrations (day 8). Cytokine concentrations (pg/mL) in the exudate extracted from the tissue inside an implanted cage on day 8. A scale bar is included at the top of each color grid. Data are shown as means (n = 6).
Cytokine concentrations generally decreased from days 1 and 2 to day 8. Whereas IL-6, VEGF, and IL-10 were all present at concentrations above 10,000 pg/mL during the initial 2 days, only IL-10 reached this concentration after 8 days. No statistically significant differences were observed on day 8.

Cytokine concentrations in the exudate extracted after 8 weeks of material implantation are shown in Figure 5.7.
**Figure 5.7. Chronic in vivo cytokine concentrations (day 28, day 56).**
Cytokine production (pg/mL) from exudate extracted from the tissue inside an implanted cage on a) day 28 and b) day 56. A scale bar is included at the top of each color grid. Data are shown as means (n = 6). *Bars indicate statistically significant differences in cytokine concentrations (p<0.05) between materials.

Cytokine concentrations after 28 and 56 days were lower than after days 1, 2, and 8. After 28 days, MCP-1, IL-6, and VEGF remained present at concentrations of 1,000 pg/mL or higher. Only MCP-1 and IL-6 were present above 900 pg/mL after
56 days. The only significant difference after 28 days involved IL-6 production, which was significantly increased for the PU implants as compared to the PE, ot-PVC and empty cages. No significant differences were detected between materials after 56 days.

**LPS-induced cytokine production**

Cytokine concentrations were measured from exudate fluid extracted from an empty cage injected with LPS, which is known to induce an inflammatory reaction (Figure 5.8).
Figure 5.8. In vivo LPS-induced cytokines. Cytokine concentrations (pg/mL) from exudate extracted from the tissue inside an implanted cage treated with LPS after 3 hours and 24 hours. A scale bar is included at the top of each color grid. Data are shown as means (n = 3).

By 3 hours, and continuing to 24 hours, LPS induced the production of TNF-\(\alpha\), MCP-1, MIP-1\(\alpha\), IL-2, IL-6, IL-1\(\beta\), VEGF, IL-4, and IL-10. TNF-\(\alpha\), MCP-1, IL-6,
IL-1β, VEGF, and IL-10 were the cytokines produced at the highest concentrations, all of which were greater than 200 pg/mL at both 3 and 24 hours. IL-4 was produced at the lowest concentration, with a maximum of 20 pg/mL after 24 hours.

**Normalized cytokines from cage exudate**

In order to examine the relative differences between materials, all cytokine concentrations were normalized to their respective concentration in the empty cages (Figures 5.9, 5.10, and 5.11).
Figure 5.9. Acute in vivo normalized cytokines (day 1, day 2). Normalized cytokine production (normalized units) from exudate extracted from the tissue inside an implanted cage on a) day 1 and b) day 2. A scale bar is included at the top of each color grid. Data are shown as means (n = 6).

The normalized data presented in Figure 5.9 show a general increase in cytokine production induced by the implanted materials in comparison to the empty cage. For day one, 21 out of a possible 27 material and cytokine combinations were
upregulated in comparison to the empty cage (greater than unity), although these differences were not statistically significant. In particular, TNF-α, MCP-1, MIP-1α, IL-6, IL-1β, VEGF, and IL-10 were greater than unity for at least two of the three materials tested. After 2 days, the implanted materials continued to induce upregulation of TNF-α, MCP-1, MIP-1α, IL-6, IL-1β, VEGF, and IL-10; however, the magnitude of this upregulation was decreased from day 1.

Normalized cytokine production on day 8 post implantation is shown in Figure 5.10.
Day 8

**Figure 5.10. Acute in vivo normalized cytokines (day 8).** Normalized cytokine production (normalized units) from exudate extracted from the tissue inside an implanted cage on day 8. A scale bar is included at the top of each color grid. Data are shown as means (n = 6).

After 8 days, the magnitude of material-induced cytokine upregulation decreased from day 2, although MIP-1α, IL-6, IL-1β, and IL-10 continued to be upregulated. However, after 28 days, the frequency and magnitude of upregulation
increased and extended to VEGF and IL-4 (Figure 5.11). Normalized cytokine production after 56 days was similar to 28 days, with MIP-1α, IL-6, IL-1β, IL-4, and IL-10 upregulated by implanted materials as compared to the empty cage, though this upregulation was not statistically significant (Figure 5.11). In particular, MIP-1α and IL-6 were upregulated more than 10-fold for PE, PU, and ot-PVC as compared to the empty cages.
Figure 5.11. Chronic in vivo normalized cytokines (day 28, day 56). Normalized cytokine production (normalized units) from exudate extracted from the tissue inside an implanted cage on a) day 28 and b) day 56. A scale bar is included at the top of each color grid. Data are shown as means (n = 6).
**Classification of total cytokine production**

The integrated response of classes of cytokines was examined by combining the cytokines into categories based on their respective roles in the foreign body reaction. Total cytokine production is presented for all cytokines grouped by their role in promoting or suppressing inflammation and wound healing (Figure 5.12). For the material-implanted cages as well as the empty cages, the total amount of cytokine produced decreased after the first two days. In all cases, the majority of cytokines produced were pro-inflammatory and anti-wound healing. While the materials induced increased overall cytokine production during the first two days as compared to the empty cage, there was no apparent materials-dependent effect on the class of cytokine produced.
Figure 5.12. In vivo integrated cytokine production. Cytokine concentrations (pg/mL) grouped into pro- or anti-inflammatory and pro- or anti-wound healing based on each cytokine’s role in the foreign body reaction. Data are shown as means ± SEM (n = 3).
5.5 DISCUSSION

Immune cells such as macrophages, lymphocytes, and neutrophils recruited to the site of implantation react to the presence of a foreign material. The outcome of this foreign body reaction is critical in determining the eventual fate of an implanted device. It is widely accepted that extracellular signals direct the foreign body reaction; however, these signaling processes are poorly understood. The goal of this study was to analyze the infiltration of immune cells and the production of an array of signaling proteins, including cytokines and growth factors, from the tissue surrounding implant materials with varying surface chemistries.

In order to do so, a cage implant system was employed, whereby a material of interest was implanted subcutaneously within a stainless steel mesh cage, creating a pocket in the tissue for the collection of wound exudate fluid. Samples were removed from this exudate fluid, which was in direct contact with the surface of the implanted material. These samples were analyzed for the types of infiltrating cells and the concentrations of cytokines produced around the implanted material.

An analysis of the material surfaces using x-ray photoelectron spectroscopy (XPS) confirmed the presence of carbon, oxygen, and nitrogen in PE, PU, and ot-PVC (Figures 5.1, 5.2, 5.3). Additionally, ot-PVC was shown to contain chlorine, a component of PVC, and tin, a component of the cytotoxic organotin stabilizer used in the manufacture of ot-PVC. This organotin compound has been...
previously shown to have a cytotoxic effect on cell cultures and an inflammatory effect when implanted $^{55,112}$.

Exudate fluid extracted from the cages was analyzed for neutrophils, lymphocytes, monocytes, basophils, and eosinophils (Figure 5.4). As described in previous reports $^6$, neutrophils populated the site of implantation during the first two days, after which lymphocytes and to a lesser extent monocytes migrated into this tissue. Basophils and eosinophils were a minimal portion of the total cell population. There was no apparent affect of material surface property on the types of infiltrating cells.

The cytokine concentrations measured from the extracted exudates were as high as 10,000 pg/mL, specifically for IL-6, VEGF, and IL-10 (Figures 5.5, 5.6, and 5.7). In contrast, TNF-$\alpha$, MIP-1$\alpha$, and IL-4 were only detected in concentrations below 100pg/mL. Cytokines tended to decrease in concentration with time, although MCP-1 and IL-6 persisted at more than 900 pg/mL after 8 weeks of PE and PU implantation. The high concentration of IL-10 initially after implantation may signal the decrease in cytokine production observed at later time points, since IL-10 has been shown to downregulate cytokine production $^{113}$. As expected with tissue undergoing trauma, IL-6 is present in the exudate, likely promoting inflammation and pathogen clearance $^{114}$. VEGF in the exudate fluid presumably promotes the formation of vasculature to support the ingrowth of new tissue into the hollow cage $^{105}$. MCP-1 production remains steadily near 1,000 pg/mL during the first 8
days, and decreases slightly after 28 and 56 days post-implantation. This relatively constant production of MCP-1 through 8 weeks of implantation is indicative of continuous monocyte recruitment to the site of material implantation. IL-2 concentrations also reach 1,000 pg/mL during the first 8 days of implantation, potentially inducing T-cells and B-cells to remove antigens\textsuperscript{115,116}. The presence of IL-1β at the site of implantation also presumably serves to fight against infection\textsuperscript{117}.

Of the twelve total significant differences detected on day 1, nine involved increases in cytokine production induced by material-containing cages as compared to empty cages. These data suggest that the presence of these polymer materials within the stainless steel cages has an impact on the intercellular signaling that occurs in the healing tissue. The remaining significant differences on day 1 were increased production of MCP-1, but decreased production of VEGF and IL-10 by PE as compared to PU. Based on the individual roles of these cytokines, this would indicate that PE induces more pro-inflammatory and less pro-wound healing signals than PU at this early time point. After two days, MCP-1 production was significantly increased in cages implanted with PE as compared to PU, ot-PVC, or empty cages. Again, this suggests that PE induces greater pro-inflammatory, anti-wound healing signaling than PU, ot-PVC, or empty cages. After 4 weeks, IL-6 production by cages containing PU was significantly increased as compared to cages containing PE, ot-PVC, or empty cages. No significant differences were found in the exudates collected after 8 weeks. The finding at 4 weeks suggests that PU induces a greater
pro-inflammatory, anti-wound healing cytokine response than PE, ot-PVC, or empty cages, although the findings on days 1, 2, 8, and week 8 do not support this trend.

Exudates from LPS-injected cages were examined as a positive control for inflammatory cytokine production (Figure 5.8). As soon as 3 hours and up to 24 hours after LPS injection, cytokines were prevalent in the extracted exudate fluid. The cytokines induced by LPS were generally similar in magnitude to those induced by the implanted materials. However, LPS induced increased production of TNF-\(\alpha\) in comparison to the implanted polymer materials, but decreased production of IL-2. This finding was not surprising, since LPS has been shown previously to strongly induce TNF-\(\alpha\), but only weakly induce IL-2 \(^{118}\). Despite these differences, the impact of LPS-injected cages on cytokine and growth factor production was largely similar to the impact of biomaterial-implanted cages.

All cytokine concentrations were normalized to the empty cages in order to isolate the effect of the material implanted within the cage (Figures 5.9, 5.10, and 5.11). On days 1 and 2 post-implantation, the majority of material-cytokine combinations were upregulated as compared to the empty cage control, although this upregulation was not statistically significant. The upregulated cytokines include TNF-\(\alpha\), MIP-1\(\alpha\), IL-6, IL-1\(\beta\), VEGF, and IL-10. The frequency and magnitude of this upregulation decreased from day 1 to day 8, but then increased from day 8 to day 28 and day 56. The increase in normalized cytokine production after 28 and 56 days
could be representative of a continued immune response to the implanted polymer materials, while the immune response to the empty cages is more resolved.

In order view the integrated response of cytokines with similar functions, cytokines were grouped into categories based on their roles in the foreign body reaction (Figure 5.12). From this figure, it is clear that the largest amount of cytokines, particularly for cages containing materials, was produced during the first two days after implantation. This integrated depiction of cytokine concentrations also indicates that the majority of cytokines produced for all implanted cages were pro-inflammatory and anti-wound healing. However, it must be taken into consideration that, due to the limited availability of cytokines for measurement with the bead array, the cytokines selected for this experiment are primarily pro-inflammatory and anti-wound healing. Considering this, there were still no significant shifts in the relative production of inflammatory and wound healing cytokines among the implant materials, including cytotoxic ot-PVC.

Although the presence of a cytotoxic organo-tin component was confirmed by XPS analysis of ot-PVC, the impact of this cytotoxicity was not clearly exhibited in the corresponding cytokine production. One possible explanation for this phenomenon is the inability to precisely control the exudate fluid sampled from within the implanted cage. Fluid aspirated from a subcutaneous pocket follows the path of least resistance, which is dependent on the architecture of the subcutaneous tissue\textsuperscript{119}. Previous research indicates that this type of fluid sampling could extract
fluid from tissue channels distant to the sampling point. Since this fluid could be far from the biomaterial surface, its cytokine content is likely independent of the implant material. Another possible explanation is that the immune response directed at the stainless steel mesh cage overwhelms the more subtle effect of the differences in material, masking any material-dependent effects on cell infiltration and cytokine production. One potential design improvement in the cage implant system would involve implanting cages composed of the material of interest, thereby eliminating the confounding effect of the large surface area of stainless steel mesh.

Similar to the in vitro results, a small number of statistically significant differences were observed in cytokine production among the polymeric materials within the implanted cages, although these differences were not preserved over time. When cytokine production was viewed in an integrated fashion based on the roles of individual cytokines in inflammation and wound healing, there were no significant shifts among the implanted materials. In light of this, interpretation of the differences in individual cytokine production has been cautious.

In this in vivo study, differences in cytokine and growth factor concentrations among biomaterials were only moderate and not reflective of a shift in the promotion of inflammation or wound healing. These results are consistent with the prior in vitro results, further strengthening the argument that, given time, the foreign body reaction resolves with the similar endpoint of fibrous encapsulation for nearly any commonly implanted material. Alternatives to manipulating implant surface chemistry
include attaching cell or cytokine coatings to the surface of an implanted device, which could potentially direct the foreign body reaction through native signaling pathways. Ongoing research aimed at investigating the potential of these coatings to favorably affect the immune response is promising\textsuperscript{121,122}.

### 5.6 Conclusions

In this study, the cage implant system was utilized in rat subcutaneous tissue to assess the impact of material surface chemistry on cytokine production and cell infiltration around the implant. Cytokine production was increased for cages containing PE, PU, and ot-PVC in comparison to empty cages, although there was no apparent affect on the types of infiltrating cell. The production of individual cytokines was, in a few instances, significantly different among these materials. However, when cytokines were grouped based on their roles in inflammation and wound healing, the cumulative production of these cytokine groups was not significantly affected by these materials. As stated in previous in vitro studies, the differences between these polymeric materials are not expected to result in significant changes in the foreign body reaction in vivo, including tissue vascularization and fibrous tissue formation.
Chapter 6. Overview and Future Studies

6.1 OVERVIEW

In the initial portion of this study, microdialysis sampling was characterized for the collection of macromolecules. It was determined that the relationship between collection efficiency and the mass transport resistances of microdialysis sampling of macromolecules fit the functionality of an established theoretical model developed by Bungay et al.\(^{67}\) Additionally, while microdialysis sampling is feasible for collecting cytokines and growth factors, their collection efficiencies were never above 20% for the largest commercially available microdialysis probes (10 mm, 100 kD MWCO). The variation in collection efficiencies among cytokines necessitates the impractical characterization of collection efficiency for each individual cytokine of interest. Furthermore, microdialysis sampling created additional error in cytokine and growth factor measurements, as well as decreased the analytical sensitivity and increased the detection limit. For these reasons, a direct means of sampling cytokines and growth factors was determined to be preferable to microdialysis sampling.

An in vitro model was developed for assessing the cytokines and growth factors produced by monocyte/macrophage cultures with varying levels of activation and differentiation seeded onto biomaterial surfaces. The validity of this model was confirmed by the preferential production of pro-inflammatory and anti-wound healing cytokines induced by the cytotoxic positive control, organo-tin PVC. Additionally,
TCPS alone induced the lowest cytokine and growth factor production. While the varying levels of activation and differentiation impacted cytokine production, the four non-toxic biomaterials selected for this study (PE, PU, PMMA, and ePTFE) did not induce significantly different cytokine production profiles.

An in vivo model for analyzing the foreign body reaction to biomaterials was utilized for comparison with the in vitro model. The cage implant system was adopted in order to directly measure cell infiltration and cytokine concentrations produced in the tissue surrounding implanted biomaterials. The type and quantity of infiltrating cells was not significantly different among any of the implanted cages. PE, PU, and ot-PVC induced increased cytokine and growth factor concentrations in the surrounding exudate fluid as compared to the empty cages. When cytokine production induced by the materials was normalized to the empty cages, it was apparent that there was a prolonged affect on cytokine concentration induced by the materials. However, there were no significant differences in cytokine production among the different materials. These results support the in vitro model that cytokine and growth factor production do not vary significantly with the surface chemistry of the implanted material.

Regarding the impact of surface chemistry on the immune reaction to an implant, the findings of this study indicate that the variations in surface chemistry of commonly implanted, non-cytotoxic biomaterials did not have a significant effect on the production of cytokines and growth factors that drive the foreign body reaction.
These results support the argument that the foreign body reaction resolves similarly with nearly any implanted material. While material surface chemistry may impact the type and conformation of proteins adsorbed, all materials are adsorbed with a non-specific layer of proteins. Immune cells, particularly macrophages, may recognize and respond to any of these surfaces adsorbed with a non-specific layer of proteins in a similar fashion. Thus, the foreign body reaction induced by each of these commonly implanted, non-cytotoxic biomaterials is not expected to vary significantly.

6.2 Future Studies

The conclusions reported in this study provide direction for future research examining the immune reaction to implanted devices. While the relative impact of the surface chemistry of commonly implanted polymers appears to be modest, further research and optimization of techniques could reveal approaches for novel improvements in the design and analysis of biomaterials.

Improving cytokine collection efficiencies using microdialysis

Recently, attempts at improving the collection efficiencies of cytokines sampled with microdialysis probes have been suggested. A substantial improvement in the collection of cytokines and growth factors would allow for
continuous sampling from the tissue surrounding an implanted device and eliminate
the need for the cage implant system for in vivo cytokine and growth factor studies.
In vitro optimization of microdialysis sampling, including cytokine capturing
techniques and ultrafiltration to enhance mass transport through the probe membrane,
could substantially improve collection efficiencies and improve the capability for
cytokine collection in vivo.

**Effect of protein adsorption on the foreign body reaction**

Additional testing to determine the role of protein adsorption on cytokine and
growth factor production could further our understanding of the mechanism guiding
the behavior of adherent macrophages. An in vitro experiment could be developed to
measure cytokine and growth factor production from monocytes/macrophages
adhered to various protein layers. Further, in vivo experiments could be performed to
periodically determine the types of proteins adsorbed to an implanted material’s
surface over several months of implantation. Results from this study could address
whether or not any non-specific array of adsorbed proteins induces a similar foreign
body reaction.

**Cage fabrication from biomaterial**

One improvement to the cage implant system would be to fabricate the mesh
cages from the biomaterial of interest. This modification eliminates the large
stainless steel surface area, isolating the immune response to the test biomaterial. One challenge in this project would be maintaining consistent design specifications between cages fabricated from various materials. However, this design improvement would enable a more direct comparison between the in vivo immune reactions of various biomaterials.

**Cytokine production induced by surface-modified materials**

Because the results in this study indicated that variations in surface chemistry between commonly implanted, polymeric biomaterials did not have a significant impact on cytokine production, novel biomaterials should be engineered to direct the foreign body reaction. For instance, biomaterials that release IL-10 or VEGF could reduce inflammation or improve neovascularization in the surrounding tissue. Using a cage implant system, cytokine and growth factor profiles induced by these materials could be measured as indicators of the foreign body reaction. Additionally, the tissue surrounding these implanted biomaterials could be evaluated histologically for an analysis of the overall tissue response. This combination of cytokine and histological evaluation would be useful in determining the mechanism by which these engineered biomaterials direct the foreign body reaction.
Appendices
Appendix I. MATLAB Code

Author: R. Schutte

Colormap fabrication
(import data as logarithm of cytokine concentrations)

In Vitro Code

close all
A = data;
Z = data2;
figure(1)
imagesc(A)
caxis([0 5])
colormap(jet(256))

colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold',
'xticklabel',...%
{1','10','100','1000','10000','>10000'}

%sets and labels y ticks
set(gca,'ytick',1:1:18)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'yticklabel',[
'PE';' PU';' PMMA';'ePTFE';' PVC';
' TCPS';'TCPS2';'pHEMA';' PE';' PU';' PMMA';'ePTFE';' PVC';
' TCPS';'TCPS2';'pHEMA';' PE';' PU';' PMMA';'ePTFE';' PVC';
' TCPS';'TCPS2';'pHEMA']

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set(gca,'xticklabel', ([\' TNF-a\';\' MCP-1\';\'MIP-1a\'; \' IL-8 \';\' IL-6 \';\' IL-1b\';\' VEGF \';\'IL-1ra\';\' IL-10\'])

figure(9)
imagesc(Z)
caxis([0 5])
colormap(jet(256))

colorbar('northoutside','position', [0.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel',...,
{\'1\',\'10\',\'100\',\'1000\',\'10000\',\'>100000\'})

%sets and labels y ticks
set(gca,'ytick',1:1:18)
%note -- all strings must be same length -- put spaces in front to keep %everything 5 letters long
set(gca, 'yticklabel',['   PE';'   PU' ;' PMMA';'ePTFE';'  PVC';' TCPS';'TCPS2';'pHEMA';' PE';' PU';' PMMA';'ePTFE';'  PVC';' TCPS';'TCPS2';'pHEMA';' PE';' PU';' PMMA';'ePTFE';'  PVC';' TCPS';'TCPS2';'pHEMA'])
set(gca,'xticklabel', ([\' TNF-a\';\' MCP-1\';\'MIP-1a\'; \' IL-8 \';\' IL-6 \';\' IL-1b\';\' VEGF \';\'IL-1ra\';\' IL-10\'])

%%%% do 90 degree CC rotation and vertical flip -- changes axis orientation

figure(2)
B = rot90(A);
C = flipud(B);
imagesc(C)
caxis([0 5])
colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel', ... 
{'1','10','100','1000','10000','>100000'})

% sets and labels y ticks
set(gca,'xtick',1:1:18)
% note -- all strings must be same length -- put spaces in front to keep
% everything 5 letters long
set(gca, 'xticklabel',['   PE';'   PU';' PMMA';' ePTFE';' PVC';'
TCPS';'TCPS2';' pHEMA';' PE';' PU';' PMMA';' ePTFE';' PVC';'
TCPS';'TCPS2';' pHEMA';' PE';' PU';' PMMA';' ePTFE';' PVC';'
TCPS';'TCPS2';' pHEMA'])
set(gca,'fontsize',6)
set(gca,'yticklabel', [' TNF-a';' MCP-1';' MIP-1a'; ' IL-8';' IL-6';' IL-1b';' VEGF';' IL-1ra';' IL-10'])

figure(8)
Y = rot90(Z);
X = flipud(Y);
imagesc(X)
caxis([0 5])

colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel', ... 
{'1','10','100','1000','10000','>100000'})

% sets and labels y ticks
set(gca,'xtick',1:1:18)
% note -- all strings must be same length -- put spaces in front to keep
% everything 5 letters long
```matlab
set(gca, 'xticklabel', [' PE'; ' PU'; ' PMMA'; ' ePTFE'; ' PVC';
    ' TCPS'; ' TCPS2'; ' pHEMA'; ' PE'; ' PU'; ' PMMA'; ' ePTFE';
    ' PVC'; ' TCPS'; ' TCPS2'; ' pHEMA'])
set(gca, 'fontsize', 6)
set(gca, 'yticklabel', [' TNF-a'; ' MCP-1'; ' MIP-1a'; ' IL-8 '; ' IL-6 '; ' IL-1b'; ' VEGF '; ' IL-
    1ra'; ' IL-10'])

%% Put spacers in between treatments
% change colorbar so that 0 is white

figure(3)
% create a colormap where 0 is white
robcolor = [[0 0 0]; jet(255)];

% find size of original matrix
[r,c] = size(C);
C = C+.04; % bump original matrix by 0.1 so original zero values are not white
D = zeros(r,c+2); % make new matrix with spacer zeros
D(:,1:6) = C(:,1:6);
D(:,8:13) = C(:,7:12);
D(:,15:20) = C(:,13:18);

imagesc(D)
colormap(robcolor)
caxis([0 5])
axis equal tight
colorbar('northoutside', 'position', [.13 .77 .775 .02], 'fontsize', 10, 'fontweight','bold', 'xticklabel',... {'1','10','100','1000','10000','>100000 ag/cell'})
```

131
%sets and labels y ticks
set(gca,'xtick',1:1:20)
%note -- all strings must be same length -- put spaces in front to keep %everything 5 letters long
% set(gca, 'xticklabel',
\[ PE;\ PU ;\ ePTFE;\ PMMA;\ PVC;\ TCPS;\ \ PE;\ PU ;\ ePTFE;\ PMMA;\ PVC;\ TCPS;\ \ PE;\ PU ;\ ePTFE;\ PMMA;\ PVC;\ TCPS\])

%xticks
set(gca, 'xtick', []) %turns xticks off
text(1,10,'PE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(2,10,'PU', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(3,10,'PMMA', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(4,10,'ePTFE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(5,10,'TCPS', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(6,10,'ot-PVC', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(8,10,'PE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(9,10,'PU', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(10,10,'PMMA', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(11,10,'ePTFE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(12,10,'TCPS', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(13,10,'ot-PVC', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(15,10,'PE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(16,10,'PU', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(17,10,'PMMA', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(18,10,'ePTFE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(19,10,'TCPS', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(20,10,'ot-PVC', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
% yticks
set(gca,'ytick',[]) %turns yticks off

text(0,1, 'TNF\alpha', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,2,'MCP-1', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,3,'MIP-1\alpha', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,4,'IL-8', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,5,'IL-6', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,6,'IL-1\beta', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,7,'VEGF', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,8,'IL-1ra', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,9,'IL-10', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')

%print( '-dtiff', '-r600', 'conc-cell24.tif')

% set(gca,'yticklabel', [ ' IL-1b';' MCP-1';' TNF-a'; 'MIP-1a';' IL-6 ';' IL-8 ';' VEGF 
'],'IL-1ra'; ' IL-10'])

%set(gca,'fontsize',12)

%change colorbar so that 0 is white

figure(7)
%create a colormap where 0 is white
robcolor1 = [[0 0 0];jet(255)];

%find size of original matrix
[r,c] = size(X);
X = X+.04; %bump original matrix by 0.1 so original zero values are not white
W = zeros(r,c+2);  %make new matrix with spacer zeros
W(:,1:6) = X(:,1:6);
W(:,8:13) = X(:,7:12);
W(:,15:20)=X(:,13:18);

imagesc(W)
colormap(robcolor1)
caxis([0 5])
axis equal tight
colorbar('northoutside','position', [.13 .77 .775 .02], 'fontsize', 10, 'fontweight', 'bold', 'xticklabel', '...
{"1",","10","100","1000","10000",">,100000 ag/cell")

%sets and labels y ticks
set(gca,'ytick',1:1:20)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
%set(gca, 'xticklabel', [" PE ";' PU ';PMMA ';ePTFE'; PVC ';
TCPS';'TCPS2';'pHEMA';' PE ';PU ';PMMA ';ePTFE'; PVC ';
TCPS';'TCPS2';'pHEMA';' PE ';PU ';PMMA ';ePTFE'; PVC ';
TCPS';'TCPS2';'pHEMA']
%set(gca,'fontsize',6)

%xticks
set(gca,'xtick', []) %turns xticks off

text(1,10,'PE', 'horizontalalignment', 'center', 'FontSize', 7, 'fontweight', 'bold')
text(2,10,'PU', 'horizontalalignment', 'center', 'FontSize', 7, 'fontweight', 'bold')
text(3,10,'PMMA', 'horizontalalignment', 'center', 'FontSize', 7, 'fontweight', 'bold')
text(4,10,'ePTFE', 'horizontalalignment', 'center', 'FontSize', 7, 'fontweight', 'bold')
text(5,10,'TCPS', 'horizontalalignment', 'center', 'FontSize', 7, 'fontweight', 'bold')
text(6,10,'pHEMA', 'horizontalalignment', 'center', 'FontSize', 7, 'fontweight', 'bold')
text(8,10 ,'PE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(9,10 ,'PU', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(10,10 , 'PMMA', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(11,10 , 'ePTFE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(12,10 , 'TCPS', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(13,10 , 'ot-PVC', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(15,10 , 'PE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(16,10 , 'PU', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(17,10 , 'PMMA', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(18,10 , 'ePTFE', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(19,10 , 'TCPS', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')
text(20,10 , 'ot-PVC', 'horizontalalignment', 'center', 'FontSize',7, 'fontweight', 'bold')

% yticks
set(gca,'ytick',[]) %turns yticks off

%set(gca,'yticklabel', [' TNF-a';' MCP-1';'MIP-1a'; ' IL-8 ';' IL-6 ';' IL-1b';' VEGF ';'IL-1ra';' IL-10'])
%set(gca,'fontsize',8)
In Vivo Code
(import data as logarithm of cytokine concentrations)
close all

A1 = data1;
A2 = data2;
A3 = data3;
A4 = data4;
A5 = data5;

figure(1)
imagesc(A1)
caxis([0 4])
colormap(jet(256))
colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold',
'xticklabel',... 
{'1','','10','' ,'100','', '1000','','>10000',''})
%sets and labels y ticks
set(gca,'ytick',1:1:4)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'yticklabel',
[' PE'; ' PU' ; ' PVC';'Empty'])
set(gca, 'xticklabel', (' TNF-a';' MCP-1';'MIP-1a'; ' IL-2 ';' IL-6 ';' IL-1b';' VEGF ';' IL-4 ';' IL-10'])

figure(5)
imagesc(A2)
caxis([0 4])
colormap(jet(256))
colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel',...  
  {'10', '10', '100', '1000', '>10000', ''})
%sets and labels y ticks
set(gca,'ytick',1:1:18)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'yticklabel', ['   PE';'   PU' ;' PVC';'Empty'])
set(gca,'xticklabel', ([' TNF-a';' MCP-1';'MIP-1a'; ' IL-2 ';' IL-6 ';' IL-1b';' VEGF '; ' IL-4 '; ' IL-10'])

figure(10)
imagesc(A3)
caxis([0 4])
colormap(jet(256))
colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel',...  
  {'10', '10', '100', '1000', '>10000', ''})
%sets and labels y ticks
set(gca,'ytick',1:1:18)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'yticklabel', ['   PE';'   PU' ;' PVC';'Empty'])
set(gca,'xticklabel', ([' TNF-a';' MCP-1';'MIP-1a'; ' IL-2 ';' IL-6 ';' IL-1b';' VEGF '; ' IL-4 '; ' IL-10'])

figure(15)
imagesc(A4)
caxis([0 4])
colormap(jet(256))

colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel', ...{''1'',''10'',''100'',''1000'','''>10000''})
% sets and labels y ticks
set(gca,'ytick',1:1:18)
% note -- all strings must be same length -- put spaces in front to keep
% everything 5 letters long
set(gca, 'yticklabel', ['   PE';'   PU' ;'  PVC';'Empty'])
set(gca,'xticklabel',([' TNF-a';' MCP-1';' MIP-1a'; ' IL-2 ' ;' IL-6 ';' IL-1b';' VEGF ' ;' IL-4 ' ;' IL-10'])

figure(20)
imagesc(A5)
caxis([0 4])
colormap(jet(256))

colorbar('northoutside','position', [.13 .94 .775 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel', ... {''1'',''10'',''100'',''1000'','''>10000''})
% sets and labels y ticks
set(gca,'ytick',1:1:18)
% note -- all strings must be same length -- put spaces in front to keep
% everything 5 letters long
set(gca, 'yticklabel', ['   PE';'   PU' ;'  PVC';'Empty'])
set(gca,'xticklabel',([' TNF-a';' MCP-1';'MIP-1a'; ' IL-2 ' ;' IL-6 ';' IL-1b';' VEGF ' ;' IL-4 ' ;' IL-10'])

%% do 90 degree CC rotation and vertical flip -- changes axis orientation

figure(2)
B1 = rot90(A1);
C1 = flipud(B1);
imagesc(C1)
caxis([0 4])
colorbar('northoutside','position', [.38 .94 .275 .02],'fontsize', 8, 'fontweight', 'bold','xticklabel',...{
'1','10','100','1000','>10000pg/mL'})
%sets and labels y ticks
set(gca,'xtick',1:1:4)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'xticklabel', [' PE';' PU'; ' PVC';'Empty'])
set(gca, 'fontsize',6)
set(gca, 'yticklabel', [' TNF-a'; ' MCP-1';'MIP-1a'; ' IL-2 ';' IL-6 ';' IL-1b';' VEGF '; ' IL-4 '; ' IL-10'])
axis equal tight
%xticks
set(gca, 'xtick', []) %turns xticks off

text(1,10 , 'PE', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
text(2,10 , 'PU', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
text(3,10 , 'ot-PVC', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
text(4,10 , '  Empty', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
% yticks
set(gca,'ytick',[]) %turns yticks off

text(0,1, 'TNF\alpha', 'horizontalalignment', 'center', 'FontSize', 8, 'fontweight', 'bold')
text(0,2,'MCP-1', 'horizontalalignment', 'center', 'FontSize', 8, 'fontweight', 'bold')
text(0,3,'MIP-1\alpha', 'horizontalalignment', 'center', 'FontSize', 8, 'fontweight', 'bold')
figure(6)
B2 = rot90(A2);
C2 = flipud(B2);
imagesc(C2)
caxis([0 4])
colorbar('northoutside','position', [.38 .94 .275 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel',
{
'1','10','100','1000','>10000pg/mL'}
)

%sets and labels y ticks
set(gca,'xtick',1:1:4)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'xticklabel',[
' PE'; ' PU'; ' PVC'; ' Empty'])
set(gca,'fontsize',6)
set(gca,'yticklabel', [' TNF-a'; ' MCP-1'; ' MIP-1a'; ' IL-2 '; ' IL-6 '; ' IL-1b'; ' VEGF '; ' IL-4 '; ' IL-10'])
axis equal tight
%xticks
set(gca, 'xtick', []) %turns xticks off

140
text(2,10,'PU', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')

text(3,10,'ot-PVC', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')

text(4,10,' Empty', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')

% yticks
set(gca,'ytick',[]) %turns yticks off

text(0,1, 'TNF\alpha', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,2,'MCP-1', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,3,'MIP-1\alpha', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,4,'IL-2', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,5,'IL-6', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,6,'IL-1\beta', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,7,'VEGF', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,8,'IL-4', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,9,'IL-10', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')

print( '-dtiff', '-r600', 'cytokineday2.tif')

figure(11)
B3 = rot90(A3);
C3 = flipud(B3);
imagesc(C3)
caxis([0 4])

colorbar('northoutside','position', [.38 .94 .275 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel',...)

{%'1','10','100','1000','>10000pg/mL'}
%sets and labels y ticks
set(gca,'xtick',1:1:4)

%note -- all strings must be same length -- put spaces in front to keep
set(gca,'fontsize',6)
set(gca,'yticklabel', ['TNF-a'; 'MCP-1'; 'MIP-1a'; 'IL-2'; 'IL-6'; 'IL-1b'; 'VEGF'; 'IL-4'; 'IL-10'])
axis equal tight

set(gca, 'xtick', []) %turns xticks off

%yticks
set(gca,'ytick',[]) %turns yticks off

print( '-dtiff', '-r600', 'cytokineday8.tif')
B4 = rot90(A4);
C4 = flipud(B4);
imagesc(C4)
caxis([0 4])
colorbar('northoutside','position', [.38 .94 .275 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel', {
    '{1','10','100','1000','>10000pg/mL'})
%ssets and labels y ticks
set(gca,'xtick',1:1:4)

% note -- all strings must be same length -- put spaces in front to keep
% everything 5 letters long
set(gca, 'xticklabel', ['   PE'; '   PU' ; ' PVC'; 'Empty'])
set(gca,'fontsize',6)
set(gca,'yticklabel', [' TNF-a'; ' MCP-1'; 'MIP-1a'; ' IL-2'; ' IL-6'; ' IL-1b'; ' VEGF'; ' IL-4'; ' IL-10'])
axis equal tight
%xticks
set(gca, 'xtick', []) %turns xticks off

text(1,10, 'PE', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
text(2,10, 'PU', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
text(3,10, 'PVC', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')
text(4,10, 'Empty', 'horizontalalignment', 'center', 'verticalalignment', 'bottom', 'FontSize', 8, 'fontweight', 'bold')

% yticks
set(gca, 'ytick', []) %turns yticks off

text(0,1, 'TNF\alpha', 'horizontalalignment', 'center', 'FontSize', 8, 'fontweight', 'bold')
text(0,2, 'MCP-1', 'horizontalalignment', 'center', 'FontSize', 8, 'fontweight', 'bold')
text(0,3, 'MIP-1\alpha', 'horizontalalignment', 'center', 'FontSize', 8, 'fontweight', 'bold')
text(0,4,'IL-2', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,5,'IL-6', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,6,'IL-1\betaa', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,7,'VEGF', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,8,'IL-4', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,9,'IL-10', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
print( '-dtiff', '-r600', 'cytokineweek4.tif')

figure(21)
B5 = rot90(A5);
C5 = flipud(B5);
imagesc(C5)
caxis([0 4])
colorbar('northoutside','position', [.38 .94 .275 .02], 'fontsize', 8, 'fontweight', 'bold', 'xticklabel',...)
{'1','10','100','1000','>10000pg/mL'})
%sets and labels y ticks
set(gca,'xtick',1:1:4)
%note -- all strings must be same length -- put spaces in front to keep
%everything 5 letters long
set(gca, 'xticklabel',[' PE';' PU';' PVC';'Empty'])
set(gca,'fontsize',6)
set(gca,'yticklabel', [' TNF-a';' MCP-1';'MIP-1a'; ' IL-2 ';' IL-6 ';' IL-1b';' VEGF '; ' IL-
4 '; ' IL-10'])
axis equal tight
%xticks
set(gca, 'xtick', []) %turns xticks off
text(1,10 ,'PE', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')
text(2,10 , 'PU', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')
text(3,10 , 'ot-PVC', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')
text(4,10 , ' Empty', 'horizontalalignment', 'center', 'verticalalignment','bottom','FontSize',8, 'fontweight', 'bold')

% yticks
set(gca,'ytick',[]) %turns yticks off

text(0,1, 'TNF\alpha', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,2,'MCP-1', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,3,'MIP-1\alpha', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,4,'IL-2', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,5,'IL-6', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,6,'IL-1\beta', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,7,'VEGF', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,8,'IL-4', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
text(0,9,'IL-10', 'horizontalalignment','center', 'FontSize', 8, 'fontweight', 'bold')
print( '-dtiff', '-r600', 'cytokineweek8.tif')
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Biography

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Publications

