MODULATING THE INFLAMMATORY RESPONSE OF MACROPHAGES ON BIOMATERIAL SURFACES

by

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Abstract

The foreign body response is an inflammatory response to implanted materials and poses significant challenges for biomaterials-based medical applications, often leading to the degradation or fibrous encapsulation of an implanted device. The host response is largely dictated by the proteins that adsorb to the biomaterial surface, including damage-associated molecular patterns (DAMPs) that are released by cells upon necrosis or when under considerable amounts of stress. The immune response to implanted materials is carried out primarily by macrophages, which recognize DAMPs and other surface-adsorbed proteins with toll-like receptors (TLRs), initiating an inflammatory response. Macrophages play key roles in inflammatory and wound healing responses due to their ability to transition from a pro-inflammatory M1-like phenotype to a pro-resolution M2-like phenotype. This thesis explores the use of a model of biomaterial-induced macrophage inflammatory response, and the ability of two molecules to modulate this response.

A human macrophage-like cell line was treated with T6167923, a MyD88 signal transduction inhibitor. MyD88 is a critical component in the TLR pathway, which plays a significant role in inflammation and has been implicated in biomaterial-induced macrophage activation. Unfortunately, the inhibitor consistently precipitated in cell culture media, at all concentrations and with all dilution methods attempted. The presence of the particles may have encouraged pro-inflammatory activation of the cells via phagocytosis. However, some conditions did result in reduced activation with inhibitor treatment, suggesting further work with this molecule is warranted.

The second investigated molecule was Maresin 1 (MaR1), an endogenous pro-resolving mediator that contributes to the active transition from the inflammatory phase of wound healing
towards resolution. The effect of MaR1 on macrophage activation was studied in two macrophage-like cell lines and primary macrophages, and produced conflicting results. However, the inconsistency was attributed to the sensitivity of MaR1 to degradation. This could not only produce some confounding results, but also raised significant technical challenges in handling MaR1. Nonetheless, fresh, undegraded MaR1 did reduce pro-inflammatory cytokine expression in TLR2-activated dTHP1-XBlue macrophages.

Although both molecules posed significant technical challenges, their effects should be investigated further. Drug delivery systems should be explored to overcome these challenges and allow for direct delivery to cells.
Co-Authorship

I declare that this thesis incorporates some material that is the result of joint research. Flow cytometry analysis was performed by Laura McKiel (PhD candidate) under the supervision of the author. NIH/3T3 cell culture and lysate preparation was performed by Amanda Mills (undergraduate volunteer) under the supervision of supervisor Dr. Lindsay Fitzpatrick, Laura McKiel, and the author.

I certify that all other content contained in this thesis is my original work, completed under the supervision of Dr. Lindsay Fitzpatrick. All ideas and techniques of others included here are properly referenced in accordance with standard procedures.

Rosa Comas
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I would like to give special thanks to my friends and family, who always took an interest in my work and provided endless support. Your encouragement and unwavering confidence in me helped me more than you know. In particular to the Fitzpatrick and Wells labs, you made my experience in Kingston and in the lab more enjoyable than I could have imagined; I gained good friends and an excellent travel buddy, always had someone to share my love of food with, and looked forward to coming into the lab every day.

I would also like to thank Dr. Brian Amsden and Dr. Michael Blennerhassett for allowing me to use their laboratory equipment.

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<tr>
<td>15-epi LXA₄</td>
<td>15-epimer of lipoxin A₄</td>
</tr>
<tr>
<td>4PL</td>
<td>four-parameter logistics</td>
</tr>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
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<td>ALOX12</td>
<td>12-lipoxygenase</td>
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<td>AP-1</td>
<td>activator protein 1</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>BCA</td>
<td>Bicinchoninic Acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>E. coli</td>
<td>Escherichia coli</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>electrospray ionization mass spectrometry</td>
</tr>
<tr>
<td>FBGC</td>
<td>foreign body giant cell</td>
</tr>
<tr>
<td>FBR</td>
<td>foreign body reaction</td>
</tr>
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</table>
FBS  fetal bovine serum
FMO  fluorescence minus one
GM-CSF  granulocyte-macrophage colony-stimulating factor
H&E  hematoxylin and eosin
HI-FBS  heat-inactivated fetal bovine serum
hMDM  human monocyte-derived macrophages
HMGB1  high-mobility group box 1
HSP  heat shock protein
HSREB  Health Sciences Research Ethics Board
IFN-γ  interferon gamma
IL-1β  interleukin 1 beta
IL-  interleukin
IRF  interferon regulatory factor
LPS  lipopolysaccharide
Mac-1  macrophage-1 antigen
MaR1  Maresin 1
MCP-1  monocyte chemoattractant protein 1
M-CSF  macrophage colony-stimulating factor
MFI  mean fluorescence intensity
MyD88  Myeloid differentiation factor 88
m/z  mass/ion ratio
n-3 PUFA  omega-3 polyunsaturated fatty acid
NF-κB  nuclear factor κ-light-chain-enhancer of activated B cells
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PMN</td>
<td>polymorphonuclear neutrophil</td>
</tr>
<tr>
<td>Poly(I:C)</td>
<td>polyinosinic:polycytidylic acid</td>
</tr>
<tr>
<td>P/S</td>
<td>penicillin/streptomycin</td>
</tr>
<tr>
<td>PTFE</td>
<td>polytetrafluoroethylene</td>
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<tr>
<td>PRR</td>
<td>pattern recognition receptor</td>
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<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rosewell Park Memorial Institute</td>
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<tr>
<td>rSD</td>
<td>robust standard deviation</td>
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<tr>
<td>SEAP</td>
<td>secreted embryonic alkaline phosphatase</td>
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<tr>
<td>SPM</td>
<td>specialized pro-resolving mediator</td>
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<tr>
<td>TCPS</td>
<td>tissue culture polystyrene</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TIR</td>
<td>Toll/interleukin 1-R</td>
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<tr>
<td>TLR</td>
<td>toll-like receptor</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
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<tr>
<td>TRIF</td>
<td>TIR-domain-containing adaptor protein inducing interferon-β</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential cation channel subfamily V member 1ccl</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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Chapter 1

Introduction

1.1 Wound healing and inflammation

Normal wound healing is a critical physiological process that follows distinct, well-coordinated phases that can be roughly divided into two categories: inflammation and resolution. The wound healing cascade starts with homeostasis and inflammation, where a blood clot is formed to close the wound and stop bleeding, and neutrophils and macrophages infiltrate the wound site to clean up cellular debris and eliminate microbes\(^1-3\). This phase is when typical inflammation symptoms appear, such as pain, swelling, redness, and heat\(^2\). The immune cells present release a myriad of pro-inflammatory cytokines and nitrous oxide products, but also growth factors to encourage infiltration of other cells such as fibroblasts and more leukocytes\(^3\). Once the inflammatory stimuli have been removed, the wound healing response transitions to a resolution phase, and macrophages switch from an M1-like phenotype (pro-inflammatory) to an M2-like phenotype (pro-resolution). This transition was historically considered to be a passive process, where the inflammatory activity of the cells would dwindle and allow for tissue remodeling as a result\(^3\). It is now recognized that this is not true; resolution of a wound is in fact an active process. One mechanism through which inflammation is actively resolved is the synthesis of specialized pro-resolution mediators (SPMs) by macrophages and other effector cells of the inflammatory response\(^4\). These mediators act as stop-sIGNALS by inhibiting the release of pro-inflammatory cytokines, encouraging the release of pro-resolution/anti-inflammatory cytokines, and actively working to alter the phenotype of macrophages from an M1- to an M2-like state\(^5\). This leads to the proliferation of cells, such as fibroblasts, which work to rebuild the
epithelial layer, blood vessels, and other structures that may have been damaged. For surface wounds, the newly formed granulation tissue will eventually be remodeled into a relatively acellular collagen matrix (i.e. scar tissue) as one of the final steps in the resolution phase.

If the inflammatory phase fails to resolve, the wound can enter into chronic inflammation. There are several conditions that can contribute to the development of chronic inflammation, including autoimmune diseases, diabetes, infections, and biomaterial implants. These conditions interrupt the resolution process by perturbing the balance of pro-inflammatory and pro-resolution signals within the wound bed, promoting the continuous infiltration of immune cells and release of inflammatory signals. This in turn results in extended damage to surrounding tissues and, in cases such as diabetic wounds, an increased risk of infection.

1.2 Biomaterial-induced inflammation

In particular, medical implants activate macrophages to a pro-inflammatory state via interactions with the protein layer that adsorbs to the material surface immediately upon implant placement. Chronic inflammation, in this case, is characterized by the persistent presence of macrophages and macrophage-derived foreign body giant cells at the implant surface, and the eventual encapsulation of the implant by fibrous tissue that can potentially limit the implant’s clinical utility. In the United States alone, more than 13 million medical devices and biomaterials are implanted yearly, with variable success rates that are dependent on the type of device. For example, tooth implants are generally well accepted, with a 95% success rate. In contrast, many glucose sensing devices require replacement after one week due to fibrous encapsulation and subsequent blocking of the sensor. The inflammatory response to biomaterial implants, termed the foreign body reaction (FBR), can also cause cracking of pacemaker leads.
and failure of artificial ligaments\textsuperscript{14,15}. These issues often result in patient discomfort and the requirement for revision surgeries that incur significant medical costs\textsuperscript{16}.

1.3 Modulating the inflammatory response

Several methods of modulating the inflammatory response to biomaterials have been investigated. One such method has been to delivery anti-inflammatory drugs, such as corticosteroids, to the implant site through incorporation in biomaterial coatings\textsuperscript{12,17–19}. While these effectively prevent fibrosis from occurring around the implant, many pro-inflammatory M1-like macrophage processes are inhibited as well\textsuperscript{20,21}. The pro-inflammatory phase is recognized as being a critical component in clearing debris and initiating formation of granulation tissue\textsuperscript{6,21,22} so, consequently, inhibition of this phase would impair proper wound healing\textsuperscript{21,22}.

An alternative approach to modulating inflammation is to promote the natural transition from inflammation to resolution by regulating macrophage phenotype\textsuperscript{23–25}. The ability to modulate macrophage response by phenotype manipulation would be highly beneficial in promoting tissue healing and reducing chronic inflammation\textsuperscript{23,24,26} which could have significant implications for acceptance of biomedical devices and implants, as well as for the treatment of chronic wounds and inflammatory/autoimmune diseases. This thesis examined the inflammatory response of macrophages to polymeric biomaterial surfaces, using an \textit{in vitro} model of macrophage-material interaction adapted from McKiel, L. \textit{et al.}\textsuperscript{27}. Using this model, the ability of two molecules to modulate the macrophage response was explored. The identification of a suitable modulatory agent could lead to the development of a drug delivery system to be used for improved integration of polymeric biomaterial implants, which would reduce the frequency of revision surgeries and implant replacements, significantly decreasing associated medical costs.
This thesis examined two strategies for modulating macrophage activation on polymer biomaterial surfaces. Chapter 2 will provide a literature review of relevant background information for this thesis. Chapters 3 and 4 will provide the detailed experimental methods and the results and discussion, respectively. Finally, the conclusions and recommendations for future approaches to this project can be found in Chapter 5.
Chapter 2

Literature Review

2.1 Biomaterials

Biomaterials are an integral part of today’s medical system; there are upwards of 13 million biomaterials and medical devices implanted annually in the United States alone. They are used for a wide variety of applications, such as joint replacements, drug delivery systems, and biosensors. A critical aspect of their performance is the host response to the material, which is classically described as a FBR. The FBR is a series of events that describes the immune system’s recognition and response to a foreign material and generally results in attempts to degrade the material through the action of immune cells, or to isolate the implant from the rest of the body in a fibrous capsule. Depending on the biomaterial and its application, the FBR can be well-tolerated. However, in many situations the FBR can cause premature material failure or interfere with the performance of medical implant to perform its function by being encapsulated by thick, fibrous tissue. Previous studies have tried to modulate the FBR by delivering corticosteroids, such as dexamethasone, in drug delivery systems and biomaterial coatings to inhibit the pro-inflammatory response. Some studies have also incorporated the concurrent release of growth factors, for example vascular endothelial growth factor (VEGF), which can be included to counter the inhibition of angiogenesis caused by dexamethasone. However, the pro-inflammatory M1-like macrophage response is critical for clearing debris and dead cells from the wound area. They also release a variety of growth factors that are important for vascularization, epithelialization, and formation of granulation tissue. With advances in our understanding of the role of inflammation in tissue repair and regeneration, the traditional approach of broadly inhibiting the inflammatory response to an implanted material may impede our ability to
improve material integration with the body. Instead, I propose that targeted modulation of specific elements of the inflammatory response represents a strategy for achieving desired host response outcomes. This thesis focused on strategically targeting molecular pathways involved in macrophage-polymer interactions to reduce chronic inflammation and the development of the FBR, and promote a pro-wound healing immune response to implanted polymeric biomaterials.

2.2 Foreign body reaction

When a material is implanted, the body recognizes it as a foreign material. Its subsequent reaction, the FBR, is an attempt to remove or break down the material. Implantation of the material initiates an acute inflammatory response at the implant site, and the immediate adsorption of water molecules and ions to the implant surface. Proteins then adsorb from the bulk fluid phase onto the material surface; a process driven by the increase in entropy of the system due to the displacement of the structured water molecular layer into the bulk solution. This adsorbed protein layer mediates subsequent cell-material interactions.

Neutrophils are the first cells to arrive at the implant site, and attempt to clear the wound bed of foreign material and debris through phagocytosis. Chemoattractants, such as transforming growth factor β (TGF-β, from platelets and lymphocytes) and monocyte chemoattractant protein 1 (MCP-1, from keratinocytes), recruit monocytes towards the implant site, where they differentiate into pro-inflammatory or “M1-like” macrophages. During this early stage of inflammation, endogenous omega-3 polyunsaturated fatty acids (n-3 PUFAs) are metabolized by leukocytes into lipid mediators such as leukotrienes and prostaglandins, which further promote inflammation and the M1-like macrophage phenotype and act as chemoattractants to recruit more leukocytes. This macrophage population further clears the area of debris, bacteria, and necrotic or apoptotic cells, while releasing high levels of pro-inflammatory cytokines such as
tumor necrosis factor α (TNF-α), interleukin 8 (IL-8), and interleukin 1 beta (IL-1β), as well as reactive oxygen species (ROS)\textsuperscript{2,5,37}. Via the adsorbed protein layer, macrophages adhere to, and attempt to degrade and phagocytose the foreign implanted material. However, macrophages are unable to phagocytose particles larger than approximately 10 μm in diameter\textsuperscript{9}, and consequently enter into a frustrated phagocytosis, which can lead to macrophage fusion to form large, multinucleated foreign body giant cells (FBGC)\textsuperscript{9}. FBGCs release ROS, acid, and enzymes meant to degrade the material to allow for phagocytosis\textsuperscript{9}. FBGCs and macrophages also release soluble factors to recruit fibroblasts to the implant site and form a fibrous capsule around the implanted material to wall it off from the rest of the body\textsuperscript{9}. While the formation of a fibrous capsule is well-tolerated for certain applications\textsuperscript{31}, it can inhibit potentially important interactions between the material and its target cell population or tissue for many biomaterial applications such as glucose sensors\textsuperscript{12}, neural interfacing devices\textsuperscript{38}, and pacemakers leads\textsuperscript{39,40}. 
Figure 2-1. Diagram depicting the progression of the classical foreign body reaction to an implanted biomaterial: initially, proteins are adsorbed to the biomaterial surface, followed by neutrophil and monocyte/macrophage infiltration. Frustrated phagocytosis causes the macrophages to fuse into FBGCs, eventually leading to fibrous encapsulation. Reprinted with permission from RightsLink: Springer Nature, *Nature Biotechnology*, “All charged up about implanted biomaterials,” Grainger, D. (2013)41

2.2.1 Macrophages

Although macrophages are not the first cell type to migrate towards a wound site, they are arguably the most important in directing the inflammatory and wound healing response42. While neutrophils are the most abundant immune cell of the early inflammatory response for the first 2 to 5 days6, macrophages quickly take over and are subsequently present at all stages of the wound healing response42. Macrophages perform multiple important functions within the host response to biomaterials, including the phagocytosis of cellular debris and apoptotic neutrophils.
at the wound site, fusion to form FGBCs, and their critical role in tissue repair and remodelling through growth factor and cytokine secretion\textsuperscript{37}. As a result, they are commonly used in studies that aim to control the inflammatory host response to biomaterials.

2.2.1.1 Macrophage phenotypes and \textit{in vitro} polarization strategies

Macrophages are a heterogeneous cell population that can be activated towards various phenotypes in response to environmental cues, which enables macrophages to dictate their actions in a dynamic wound environment\textsuperscript{37,43}. Macrophage phenotype is easily defined by the expression levels of specific markers, which have been shown to vary widely based on the phenotype\textsuperscript{44}. Macrophage phenotypes can range from M0 (neutral), to M1 (pro-inflammatory) and M2 (pro-resolution).

Macrophages with an M0 phenotype are considered to have a neutral activation state and are neither pro-inflammatory nor pro-resolution\textsuperscript{44,45}. M0 macrophages generally have low cytokine and chemokine expression levels\textsuperscript{44}. However, they have been shown to highly express some of the same markers as M1 macrophages (CD14\textsuperscript{46}) and M2 macrophages (CD163\textsuperscript{47}), while having significantly lower expression levels of other markers, compared to M1 and M2 phenotypes\textsuperscript{44,45}.

The M1 phenotype is pro-inflammatory, and is the main phenotype present in the initial phase of inflammation\textsuperscript{6,25}. This phenotype is characterized by high expression of CD40\textsuperscript{45}, CD80\textsuperscript{44,47}, and CD86\textsuperscript{45,46}, for example. M1-like macrophages secrete cytokines such as TNF-\textalpha, IL-1\beta, and IL-8, which contribute to the inflammatory response by inducing vasodilation, activating other immune cells such as T-cells, and encouraging the further production of pro-inflammatory cytokines\textsuperscript{48}.
The M2 or “alternatively activated” phenotype possesses anti-inflammatory and pro-resolution properties\textsuperscript{6,25}. This macrophage phenotype is characterized by high expression of Arginase 1 (Arg1)\textsuperscript{45,46}, CD11b\textsuperscript{44}, and CD163\textsuperscript{46,47}, among others. M2-like macrophages secrete anti-inflammatory cytokines such as interleukin 10 (IL-10) and TGF-β, which suppress the release of pro-inflammatory cytokines and promote cell proliferation and tissue regeneration\textsuperscript{48}.

\textit{In vitro}, macrophages can be polarized towards an M1 or M2 phenotype using well-established protocols that generate strongly polarized populations. For example, M0 cells are commonly polarized to an M1 phenotype by culturing them with interferon gamma (IFN-γ)\textsuperscript{37,44,49,50}, TNF-α\textsuperscript{25,37}, lipopolysaccharide (LPS)\textsuperscript{25,43,44}, or a combination thereof\textsuperscript{43,44}. An M2 phenotype can be induced \textit{in vitro} by culturing M0 cells with interleukin 4 (IL-4)\textsuperscript{37,43,44,49}, interleukin 13 (IL-13)\textsuperscript{37,44}, IL-10\textsuperscript{25,43} alone, or in combination\textsuperscript{44,50}. From a materials approach, scaffold pore size has also been shown to influence macrophage polarization, with decreasing pore sizes inducing a stronger M1-like response, and M2-like phenotypes being promoted by increasing pore size\textsuperscript{50}. Similarly, the topography of a material can influence macrophage phenotype. McWhorter \textit{et al.} demonstrated that patterning the surface of a material to contain thin channels (20 μm) caused macrophages to elongate, which drove them towards an M2-like phenotype\textsuperscript{51}.

A significant focus in macrophage polarization research revolves around the ability of these polarizing factors, such as IL-4 or IFN-γ, to switch macrophages from an M1 phenotype to an M2 phenotype, and vice versa\textsuperscript{25}. However, examination of macrophages \textit{in vivo} often yields more complex phenotype populations that are frequently described as a spectrum or sliding-scale, as opposed to precise phenotypes\textsuperscript{37}. The ability to control the polarization of macrophages could have significant implications for modulating biomaterial host responses, as well as the
treatment of wounds, infections, auto-immune diseases, and many more applications. Kumar et al. loaded a multidomain peptide hydrogel with MCP-1 (macrophage chemoattractant) and IL-4 (anti-inflammatory cytokine) and analyzed macrophage polarization and infiltration using *in vitro* and *in vivo* studies. When these scaffolds were injected subcutaneously in mice, the MCP-1/IL-4 containing scaffold, compared to the unmodified scaffolds, had increased cellular infiltration as determined by hematoxylin and eosin (H&E) staining (p < 0.01) and achieved a two-fold increase in polarization of macrophages towards an M2-like phenotype (p < 0.01) by day 3, and by day 7 had also formed a greater number of distinct blood vessels. All unloaded and loaded scaffolds had degraded by the 14-day time point. This study suggests that manipulation of macrophage phenotype through the delivery of appropriate immunomodulatory factors could reduce chronic inflammation and promote tissue healing in various applications, including biomaterial host responses and biomaterial-based strategies for treating inflammatory diseases.

2.2.1.2 Cell sources for *in vitro* macrophage research

Many different primary cell sources and cell lines are available for studying macrophages *in vitro*. Cell lines are convenient for *in vitro* work due to their ability to grow indefinitely and their homogeneity. However, they are unable to represent the true and complicated nature of *in vivo* responses, and have been shown to have reduced cytokine expressions when compared to primary cells. Primary cells, while also unable to represent the full complexity of the *in vivo* environment, are more representative of cells in native tissue. However, they tend to be more difficult to work with as they have a limited lifespan, greater heterogeneity, and can be difficult to isolate in large numbers.
Two human cell lines commonly used for macrophage studies are the cultured monocyte-like THP-1 and U937 cell lines\textsuperscript{55}. Both of these cell lines are in grown in suspension and require activation to differentiate into adherent, macrophage-like cells\textsuperscript{52,55}. THP-1 cells are commonly differentiated into adherent macrophages-like cells (referred to here as dTHP-1) using phorbol 12-myristate 13-acetate (PMA)\textsuperscript{55,56}. Similarly, U937 cells differentiate into either mature monocytes or macrophages from the same treatment\textsuperscript{55}. Common examples of mouse macrophage-like cell lines are RAW264.7, IC-21, and J774A.1\textsuperscript{52}. Unlike the THP-1 and U937 cell lines, the mouse cell lines already possess an adherent, macrophage-like morphology and can be used for macrophage experiments without further manipulation\textsuperscript{52}. The RAW264.7 and THP-1 cells were chosen as the cell lines used for this thesis on the grounds that they both have modified reporter strains, RAW-Blues and THP1-XBlues, that express secreted embryonic alkaline phosphatase (SEAP) in response to nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) and activator protein 1 (AP-1) transcription factor activation. The enables the use of a convenient and simple alkaline phosphatase assay to indirectly measure NF-κB/AP-1 activity. The NF-κB and AP-1 transcription factors are key factors in the transcriptional regulation of pro-inflammatory cytokines\textsuperscript{57}, and consequently, NF-κB/AP-1 activity provides a useful insight into the inflammatory state of macrophages.

Common sources of primary macrophages are mouse bone marrow or human peripheral blood\textsuperscript{52,58}. Once bone marrow is retrieved from mice, the cells are differentiated into macrophages using either macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), with an incubation time of 6 to 7 days\textsuperscript{59}. The choice of growth factor used for macrophage differentiation determines the resulting macrophage phenotype, where GM-CSF differentiates monocytes towards a more M1-like phenotype\textsuperscript{60} and
M-CSF differentiates monocytes towards a more M2-like phenotype\textsuperscript{60}. Successful differentiation can be characterized by positive expression of both F4/80 and macrophage-1 antigen (Mac-1) surface markers, as determined by flow cytometry\textsuperscript{59}. Human peripheral blood mononuclear cells (PBMCs) are isolated from whole blood, then the monocyte and macrophage populations can be isolated with either negative or positive selection techniques, or by adherence\textsuperscript{61}. The isolated monocytes differentiate into macrophages on their own over a 4 to 7 day incubation period, although growth factors (M-CSF or GM-CSF) can be added to culture media as well to further promote differentiation\textsuperscript{62,63}. The expression of CD45, CD206, and CD11b are frequently used to confirm successful differentiation into human monocyte-derived macrophages\textsuperscript{62}.

2.2.2 The role of damage associated molecular patterns (DAMPs) and toll-like receptors (TLRs) in acute inflammation and the FBR

The host response to an implanted material largely depends on the proteins that adsorb to the material’s surface\textsuperscript{9}. The implantation of a biomaterial inherently causes some degree of tissue damage at the implant site, even when minimally invasive techniques are used\textsuperscript{64}. When cells and tissues are damaged and/or put under significant levels of stress, they release DAMPs which contribute to the inflammatory response\textsuperscript{65–67}. DAMPs can be released by cells passively (upon necrosis) or actively (when cells are under stress)\textsuperscript{65}. Some well-characterized DAMPs include high-mobility group box 1 (HMGB1), adenosine triphosphate (ATP), uric acid, heat shock proteins (HSPs), and deoxyribonucleic acid (DNA)\textsuperscript{65–67}. There are also extracellular DAMPs that are derived from extracellular matrix (ECM) components and released through proteolysis upon tissue damage and stress\textsuperscript{67}.

Both DAMPs and their pathogen-derived counterparts, pathogen-associated molecular patterns (PAMPs), are recognized as “danger signals” by pattern-recognition receptors (PRRs),
such as TLRs, which initiate an inflammatory response to address the source of danger by signalling through various adaptor proteins\textsuperscript{66,68}. In a sterile environment, DAMPs are the principal initiators of the inflammatory response\textsuperscript{67}, whereas PAMPs contribute significantly to inflammation in response to the presence of pathogens\textsuperscript{67}.

TLRs are one of the four families within PRRs, and are expressed by immune cells (macrophages, neutrophils, B cells, and dendritic cells), as well as tissue cells (e.g. endothelial cells)\textsuperscript{69}. TLR receptors are located either on the surface of the cell (TLR1, TLR2, TLR4, TLR5, TLR6, TLR11) or intracellularly, in the endosome (TLR3, TLR7, TLR9)\textsuperscript{57}. The variety of cell surface TLRs makes this particular PRR a family of interest in FBR research, as these receptors would enable direct interaction with the adsorbed protein layer on the material surface\textsuperscript{70}. Upon ligand binding, TLRs homo- or hetero-dimerize, and initiate an intracellular signalling cascade via their Toll/interleukin 1-R (TIR) domain\textsuperscript{68,69}, which activates downstream signalling by interacting with adaptor molecules\textsuperscript{57}.

Myeloid differentiation factor 88 (MyD88) is a critical TLR adaptor protein and is used by all TLRs except for TLR3, making it the most common TLR adaptor molecule\textsuperscript{57,69}. MyD88-dependent TLR signaling activates NF-κB and AP-1 transcription factors, which leads to the production of pro-inflammatory cytokines such as TNF-α, IL-1β, and interleukin 6 (IL-6)\textsuperscript{57,69}. TLR3 and TLR4 also use the adaptor molecule TIR-domain-containing adaptor protein inducing interferon-β (TRIF), which activates the NF-κB transcription factor and interferon regulatory factors 3 and 7 (IRF3, IRF7), leading to the production of pro-inflammatory cytokines as well as type 1 interferons such as interferon β (IFN-β)\textsuperscript{68,71}. TLR3 has been shown to act solely through the TRIF pathway, while TLR4 signals through both the MyD88 and TRIF pathways\textsuperscript{68}.
Studies have demonstrated that DAMPs frequently signal through TLR2 and TLR4\textsuperscript{27,67} leading to the activation of NF-κB/AP-1 transcription factors and pro-inflammatory cytokine release. Recently, the Fitzpatrick lab developed a protein adsorption model that uses cell lysate, alone or in combination with plasma or serum, to recapitulate the contributions of cell-derived DAMPs and blood-derived proteins within the adsorbed protein layer\textsuperscript{27}. The DAMP-containing adsorbates potently increased NF-κB activity and cytokine secretion in a mouse macrophage-like cell line in a TLR2-dependent mechanism\textsuperscript{27}. These data suggest that TLR receptors and/or specific adaptor molecules, such as MyD88, represent strategic targets for modulation of macrophage-material interactions within the inflammatory microenvironment of implanted polymeric biomaterials.

2.3 Resolution of inflammation

While many strategies for minimizing the FBR or improving host response outcomes focus on reducing the inflammatory response to the material, an alternative strategy for improving tissue-material interactions targets the active process of inflammation resolution\textsuperscript{25}. In normal wound healing, the acute inflammatory phase typically lasts 4 to 6 days, depending on the severity of the wound\textsuperscript{3}. As M1-like macrophages of the acute inflammation phase perform efferocytosis (clearing of apoptotic neutrophils)\textsuperscript{6}, they begin to generate greater levels of pro-wound healing IL-10 and TGF-β, and reduce their expression of pro-inflammatory interleukin 12 (IL-12)\textsuperscript{5}. This shift in the balance of pro- and anti-inflammatory signals induces the conversion of macrophages over to an M2-like phenotype\textsuperscript{72}. As the macrophage population in the wound shifts from a pro-inflammatory M1-like phenotype to an M2-like phenotype, the proliferation phase of wound healing is promoted by the increased secretion of IL-10, TGF-β, VEGF, and other pro-resolution cytokines\textsuperscript{3,5,72}. In addition to this shift in protein-based signalling, the
metabolism of n-3 PUFAs undergoes lipid-mediator class switching\textsuperscript{4} induced by the production and accumulation of key enzymes by prostaglandins\textsuperscript{73}. This shifts lipid metabolism from producing pro-inflammatory prostaglandins and leukotrienes\textsuperscript{4,73} to the production of SPMs\textsuperscript{3,4,73}, further promoting resolution and healing.

\textbf{2.3.1 Specialized pro-resolving mediators}

SPMs are endogenously derived molecules that originate from n-3 PUFAs, such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), and arachidonic acid (AA)\textsuperscript{73–75}. There are 4 classes of SPMs: lipoxins (from AA), resolvins (from EPA and DHA), protectins (from DHA), and maresins (from DHA)\textsuperscript{73–75}. Immune cells such as neutrophils, macrophages, and glial cells aid in the conversion of these n-3 PUFAs into SPMs, which have acute anti-inflammatory effects\textsuperscript{73–75}.
Figure 2-2. SPMs are derived from n-3 PUFAs; the metabolism of eicosapentaenoic acid created E-series resolvins, arachidonic acid creates lipoxins, and docosahexaenoic acid creates D-series resolvins, protectins, and maresins. Adapted with permission from RightsLink: Springer Nature, *Nature Reviews Immunology*, “Specialized pro-resolving mediators: endogenous regulators of infection and inflammation,” Basil, M. and Levy, B. (2015) \(^7^6\)

*In vitro* studies have demonstrated that treatment with SPMs prior to or concurrently with macrophage activation by TLR agonists (e.g. LPS, Pam3CSK4, or
polyinosinic:polycytidylic acid (poly(I:C)), resulted in decreased production of pro-inflammatory cytokines, specifically TLR-related cytokines\textsuperscript{77}, while increasing the release of anti-inflammatory cytokines\textsuperscript{77,78}. SPMs were also shown to reduce superoxide production and TNF-α-related NF-κB activation\textsuperscript{79}. In a study using an Alzheimer’s disease cell model, treatment of the cells with SPMs was able to reduce neuronal cell death and decrease the production of pro-inflammatory cytokines induced by β-amyloid 42 (a hallmark of Alzheimer’s disease)\textsuperscript{80}.

\textit{In vivo} mouse studies have also demonstrated that exogenous SPM administration decreased the production of pro-inflammatory cytokines in response to implanted chitosan scaffolds\textsuperscript{81,82}, as well as TNF-α-related NF-κB activation and superoxide production in a murine model of intimal hyperplasia\textsuperscript{79}. Exogenous SPM treatment was also shown to reduce the influx of pro-inflammatory cells, such as polymorphonuclear neutrophils (PMNs) and M1-activated macrophages, in models of dermatitis and intimal hyperplasia\textsuperscript{79,83}.

This family of molecules possesses distinctive polyunsaturated structures, with numerous unsaturated double bonds\textsuperscript{84}. These structures are susceptible to autoxidation/lipid peroxidation\textsuperscript{85}, making them very unstable in physiological environments\textsuperscript{83,84}. They are known to degrade when exposed to oxygen, heat, and light\textsuperscript{86} which limits their clinical utility. A study by Homann, J. \textit{et al.}\textsuperscript{83} evaluated the degradation of SPMs after being injected into mice, and observed that there was a significant decrease in SPM bioavailability after just 30 minutes, and by 2 hours the availability of some of the SPMs was reduced to 2%\textsuperscript{83}. DHA-derived SPMs were found to be the most stable, their availability having been reduced to 26-38% two hours after injection\textsuperscript{83}. Despite the short half-life of SPMs, lipoxin (15-epi-LXA\textsubscript{4}, the only SPM used for treatment in this study) was still able to significantly reduce PMN infiltration to the wound site by almost 50\%\textsuperscript{83}. 
2.3.2 Maresins

Maresins are the most recently discovered of the four SPM families, with maresin 1 (MaR1) being the first identified molecule in this group and the most potent\textsuperscript{74,87}. MaR1 is produced by macrophages through the metabolism of DHA by action of 12-lipoxygenase (ALOX12), followed by two enzymatic conversion steps to result in the final structure of MaR1\textsuperscript{88}. As maresins are the most recently discovered SPM, their active site and mechanism of action are not currently known\textsuperscript{89}. However, MaR1 has been shown to be more effective than other SPMs at an equal or lower dose for reducing PMN infiltration and pro-inflammatory cytokine release\textsuperscript{79,80}. In addition to the beneficial \textit{in vitro} effects of SPMs, MaR1 has individually been shown to increase the release of anti-inflammatory cytokines while decreasing the release of pro-inflammatory cytokines and ROS\textsuperscript{78,90}, reduce PMN infiltration\textsuperscript{79,90}, and alter the phenotype of macrophage from an M1-like state to an M2-like state\textsuperscript{91}. 
Figure 2-3. Depiction of stages and by-products from the conversion of DHA into MaR1. Macrophages use 12/15-lipoxygenase (12/15-LOX) to begin the process, followed by two enzymatic conversions. Reprinted with permission from RightLinks: Rockefeller University Press, *Journal of Experimental Medicine*, “Maresins: novel macrophage mediators with potent antiinflammatory and proresolving actions,” Serhan, C. *et al.* (2009)\textsuperscript{88}

Many murine *in vivo* studies have been performed with MaR1, demonstrating its ability to improve insulin sensitivity in diet-induced and genetically obese mice\textsuperscript{92}, protect mice against the effects of colitis in both acute and chronic conditions\textsuperscript{89}, improve the survival rate of mice with sepsis\textsuperscript{93}, reduce pain by inhibiting transient receptor potential cation channel subfamily V

...
member 1 (TRPV1) currents (involved in the reception of pain)\textsuperscript{94}, and to protect against carbon tetrachloride-induced liver damage\textsuperscript{95}. Exogenous MaR1 also protects against such a variety of inflammatory diseases by inhibiting PMN infiltration\textsuperscript{79,89,96} and reducing the expression of pro-inflammatory cytokines\textsuperscript{79,89,92,95,96} and ROS\textsuperscript{95}. MaR1, therefore, represents a promising mediator for the resolution of biomaterial-induced M1-macrophage activation, with the potential to alter acute and chronic inflammatory responses towards an M2-like, resolution and healing environment.

2.4 Research Objectives

The aim of this thesis was to examine two strategies for modulating the inflammatory macrophage response to DAMP-absorbed surfaces, as an \textit{in vitro} FBR model. Specifically, I hypothesized that MyD88 inhibition of TLR signaling and/or exogenous MaR1 delivery would reduce the NF-κB/AP-1 activity and/or cytokine secretion of human macrophages to DAMP-adsorbed surfaces.

\textit{Objective 1: Validate the ability of DAMPs to elicit an inflammatory response in human macrophages.} Previous research in the Fitzpatrick lab has demonstrated that lysate-adsorbed surfaces activate mouse macrophage-like cells in a TLR2-dependent manner\textsuperscript{27}. This objective validated this model in a human model using a macrophage-like cell line. Lysate was generated from 3T3 (murine fibroblast) cells and adsorbed onto tissue culture polystyrene (TCPS) surfaces. PMA-differentiated dTHP1-XBlue cells (a macrophage-like NF-κB reporter cell line) were seeded onto the lysate-adsorbed surfaces, and their inflammatory response was evaluated by NF-κB activity (dTHP1-XBlue reporter assay) relative the response generated by bare TCPS or TCPS pre-adsorbed with serum or plasma proteins.
**Objective 2: Evaluate the effectiveness of a MyD88 small molecule inhibitor in reducing the inflammatory macrophage response on DAMP-adsorbed surfaces.** Human macrophage-like cells (dTHP1-XBlue) were treated with a small molecule MyD88 inhibitor\(^{97}\) prior to seeding on lysate-adsorbed TCPS surfaces. The effect of the MyD88 inhibitor was examined through its ability to reduce NF-κB/AP-1 activity in DAMP-activated macrophages, relative to DAMP-activated macrophages that were not treated with the inhibitor.

**Objective 3: Evaluate the effectiveness of MaR1 in reducing the pro-inflammatory response of macrophages on DAMP-adsorbed surfaces.** This objective examined the ability of MaR1 to reduce macrophage activation in response to TLR stimulus and explored different MaR1 treatment schemes. Within the literature, the majority of *in vitro* studies treat cells with MaR1 prior to activation with LPS. While effective, this treatment scheme is not feasible for most clinical applications. Instead, I examined treatment schemes using pre- and post-LPS activation. Human macrophages (dTHP1-XBlues and human monocyte derived macrophages (hMDMs)) were treated with concentrations of MaR1 before or after activation with TLR agonists (LPS, Pam3CSK4, DAMP-adsorbed TCPS) and the macrophage activation was evaluated by cytokine expression.
Chapter 3

Materials and Methods

3.1 Materials and reagents

NIH/3T3 mouse fibroblast cells were purchased from the American Type Culture Collection (ATCC®, Manassas, VA, USA). Dulbecco’s Modified Eagle Media (DMEM; D6429), 1% penicillin/streptomycin (P/S), dimethyl sulfoxide (DMSO), 7000 series blunt 24-gauge Hamilton® syringe, poly(I:C), and PMA were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Wisent (St. Bruno, QC, Canada). RAW-Blue™ murine macrophage cells, THP1-XBlue™ human monocyte cells, 5 μg/mL plasmocin, 200 μg/mL zeocin, Escherichia coli (E. coli) K12 lipopolysaccharide (LPS), Pam3CSK4, and the QUANTI-Blue™ reagents were purchased from Invivogen (San Diego, CA, USA). Rosewell Park Memorial Institute (RPMI) 1640 (Gibco, A10491-01), RPMI 1640 (Gibco, 22400-089), TrypLE Express (Gibco), phosphate buffered saline (PBS; Gibco), Trypan Blue solution (Gibco), Micro Bicinchoninic Acid (BCA) protein assay kit, amber screw cap vial kit (amber vials and caps with polytetrafluoroethylene (PTFE)/Silicone/PTFE septa), and glass autosampler inserts were purchased from ThermoFisher Scientific (Ottawa, ON, Canada). Ficoll-Paque™ PREMIUM (GE Healthcare), bovine serum albumin (BSA), alamarBlue™ reagent, ethylenediaminetetraacetic acid (EDTA; Corning), red-capped vacutainers containing heparin, and green-capped vacutainers were purchased from Fisher Scientific (Ottawa, ON, Canada). ELISA MAX™ Deluxe (hIL-8, hTNF-α) and LEGEND MAX™ (pre-coated plates; mTNF-α, hIL-1β, hIL-8) enzyme-linked immunosorbent assay (ELISA) kits, Human TruStain FcX™ (422301), Zombie NIR™ Fixable Viability Kit, Brilliant Violet 421™ anti-mouse/human CD11b (101235), and Fixation Buffer were purchased from Biolegend (San Diego, CA, USA).
Lymphoprep was purchased from Alere (Waltham, MA, USA) and StemCell Technologies (Vancouver, BC, Canada). T6167923 (MyD88 inhibitor) was purchased from Enamine (Monmouth Junction, NJ, USA). Maresin 1 was purchased from Cayman Chemical (Ann Arbor, MI, USA).

3.2 Cell culture

3.2.1 Reporter cell lines

RAW-Blue™ (raw-sp, Invivogen) and THP1-XBlue™ (thpx-sp, Invivogen) cells express an NF-κB and AP-1 inducible SEAP reporter gene, which enables the rapid, indirect quantification of NF-κB/AP-1 activity using a colourimetric assay that measures the supernatant alkaline phosphatase activity, referred to as the QUANTI-Blue (Invivogen) or SEAP assay. As serum frequently contains alkaline phosphatases, reporter cell lines were cultured in heat-inactivated FBS (HI-FBS) in the culture media to prevent false positive results in the QUANTI-Blue assay, as recommended by the manufacturer. Briefly, FBS was heat-inactivated by placing a thawed bottle of FBS into a water bath set to 56°C for 30 minutes, then aliquoted and stored at -20°C until use.

3.2.1.1 THP1-XBlue™

THP1-XBlue cells are a human monocyte-like reporter cell line derived from the THP-1 acute monocytic leukemia cell line. THP1-XBlue cells were cultured in complete media, consisting of RPMI 1640 (A10491-01) media containing 10% HI-FBS, 1% P/S, 5 μg/mL plasmocin, and 200 μg/mL of zeocin. THP1-XBlues were maintained between 4x10⁵ and 2x10⁶ cells/mL, according to manufacturer’s instructions.
THP1-XBlue cells were differentiated into macrophage-like cells using 100 nM of PMA, then incubated at 37°C. After 48 hours, the activation media was replaced with complete media (without PMA) for an additional 24 hours. The macrophage-like phenotype was determined by their adherent morphology and expression of surface marker CD11b, measured by flow cytometry (Section 3.3). Differentiated THP1-XBlue cells are hereby referred to as dTHP1-XBlues.

3.2.1.2 RAW-Blue™

RAW-Blue cells are a mouse macrophage-like reporter cell line derived from RAW 264.7 macrophage-like cells. RAW-Blue cells were cultured in DMEM containing 10% FBS, 200 μg/mL zeocin, and 5 μg/mL plasmocin. Cells were passaged once they reached 80% confluency.

3.2.1.3 NIH/3T3 fibroblasts

NIH/3T3 cells were cultured in DMEM supplemented with 10% FBS and 1% P/S in T150 TCPS flasks. Fibroblasts were passaged once the flask reached 70% confluency using TrypLE Express.

3.2.2 Human peripheral blood-derived macrophages

3.2.2.1 Venipuncture

Blood was collected by a trained phlebotomist at Queen’s University from healthy volunteers to isolate serum, plasma, and primary monocytes/macrophages (approved by the Health Sciences and Affiliated Hospitals Research Ethics Board (HSREB); Protocol 6018530 “CHEM-005-16 Investigation of innate immune response to biomaterials;” informed consent was received from each volunteer). Throughout the study, peripheral blood was collected from 13
different volunteers. 40 to 100 mL of blood was collected at one time, at a maximum frequency of once per week.

3.2.2.2 Serum collection

Peripheral blood was collected from healthy volunteers into red-capped vacutainers. The blood was left to clot for at least 30 minutes, and then was centrifuged at 800 g for 15 minutes. The serum layer was pipetted off carefully, ensuring that no blood was being drawn up, and then was separated into 10 mL aliquots and either used immediately or stored at -20°C.

3.2.2.3 Plasma collection

Peripheral blood was collected from healthy volunteers into green-capped vacutainers containing 158 USP sodium heparin, which prevents the blood from clotting. The blood was distributed into 50 mL centrifuge tubes and centrifuged at 800 g for 20 minutes (brake off). The top layer of plasma was carefully removed to approximately 1 cm above the buffy layer, separated into 10 mL aliquots, and then stored at -20°C.

3.2.2.4 Human primary macrophage culture

Peripheral blood was collected from healthy volunteers into green-capped vacutainers containing 158 USP sodium heparin to prevent clotting. The blood was distributed into 50 mL centrifuge tubes and centrifuged at 800 g for 20 minutes, with the brake turned off to ensure the separated layers were not disrupted. The plasma layer was removed carefully to approximately 1 cm above the blood layer, and then the buffy coat was collected, transferred into new 50 mL centrifuge tubes, and diluted to 35 mL with the prepared wash buffer (PBS with 10 mM EDTA and 2% FBS). The 35 mL of diluted buffy coat was carefully layered on top of 15 mL of Ficoll-Paque™ PREMIUM (1.078 g/mL) or Lymphoprep (1.077 g/mL) and centrifuged (brake off) at
800 g for 20 minutes. The resulting mononuclear cell layer (located between the plasma layer and the Ficoll-Paque or Lymphoprep layer) was carefully collected using sterile filter pasteur pipettes with a dropper bulb, and transferred into new centrifuge tubes containing 5 mL of wash buffer. The mononuclear cells were washed twice, by centrifuging at 800 g for 5 minutes and then resuspending the pelleted cells in 10 mL fresh wash buffer to remove any of the density gradient that was collected with the cell layer. Two additional washes in 10 mL wash buffer were performed by centrifuging at 150 g for 5 minutes to remove platelets. Following the final wash, the concentration of viable cells was determined by Trypan Blue exclusion using a hemocytometer. Cells were resuspended and plated in TCPS 6-well plates at 1.25x10^6 cells/cm² in culture media (RPMI 1640 (22400-089) containing 25% autologous serum and 1% P/S). Full changes of culture media were performed at 2 hours and 48 hours, taking care not to disturb the adherent cells.

After 4 days, the media was removed and cold dissociation buffer (10 mM EDTA in PBS) was added to the wells. The cells were incubated on ice for 10 minutes and then detached by forceful pipetting. The collected cells were counted by hemocytometer with Trypan Blue exclusion, then centrifuged at 250 g for 5 minutes, resuspended in fresh media to 1x10^5 cells/cm² and plated in TCPS 6-well plates. The cells were incubated for an additional 72 to 96 hours to allow for their differentiation into macrophages, with full media changes every 48 hours.

### 3.3 Flow cytometry

Flow cytometry was used to analyze the expression of CD11b on monocytic-like THP1-XBlue and macrophage-like dTHP1-XBlue cells to confirm the differentiation into the macrophage-like phenotype. dTHP1-XBlue cells were washed with PBS and incubated in 10 mM EDTA on ice for 10 minutes, followed by forceful pipetting to detach them from the
surface. All wells for each differentiation state were combined into a single flow cytometry tube for staining to increase the total number of acquired events per condition. THP1-XBlue cells were centrifuged to remove culture media and resuspended in PBS. The Fc receptors of the cells were blocked with Human TruStain FcX™ for 10 minutes at room temperature. Cells were stained in 50 µL on ice for 20 minutes with the Zombie NIR™ Fixable Viability Kit and Brilliant Violet 421™ anti-mouse/human CD11b. The concentrations of ZombieNIR and CD11b were previously optimized in mouse bone marrow derived macrophages (work by L. McKiel). Cells were washed twice in cell staining buffer (5% FBS in PBS), fixed in 100 µL of Fixation Buffer, then washed two more times in cell staining buffer. Cells were suspended in PBS prior to flow cytometry analysis. Cells were gated using the histogram for the Zombie NIR dye to exclude dead cells (i.e. highly fluorescent, >10⁶) and the forward vs side scatter dot plot to remove cellular debris.

3.4 Preparation of adsorbed protein layers

3.4.1 Protein solution preparation

3.4.1.1 Plasma and serum preparation

Pooled human serum and plasma samples from 4 donors were collected as described in Sections 3.2.2.2 and 3.2.2.3 respectively. The total protein concentration of serum and plasma were measured with a micro BCA protein assay, using dilutions of 1/10, 1/100, 1/200, and 1/400 in PBS. Briefly, the micro BCA was performed according to the manufacturer’s instructions, using a standard curve of BSA ranging from 0.5-200 µg/mL. 150 µL of standards and samples were combined with 150 µL of working reagent in the wells of a TCPS 96-well plate, in triplicate. After shaking for 30 seconds on a plate shaker, the plate was sealed and incubated at
37°C for 2 hours, then read on a plate reader at 562 nm. The total protein concentrations found in the pooled serum and plasma were 59.6 mg/mL and 55.3 mg/mL, respectively.

3.4.1.2 3T3 lysate preparation

NIH/3T3 fibroblasts were grown to 80% confluency in T150 flasks, washed with PBS and then detached by incubating with TrypLE Express for 3 minutes. The cells were counted and washed 3 times with 10 mL PBS, centrifuging at 200 g for 5 minutes each time. The cells were then resuspended at 1x10^6 cells/mL in PBS. The suspended cells went through 3 freeze/thaw cycles at -80°C and 37°C, respectively, to create lysate. The lysate was then centrifuged at 1000 g for 15 minutes to remove cell debris, and the total protein concentration was measured using a micro BCA assay, as described in Section 3.4.1.1. The lysate was diluted to a standardized protein concentration of 100 μg/cm^2 and stored in 1 mL aliquots at -80°C.

3.4.2 Pre-adsorption of protein layers on TCPS surfaces

TCPS surfaces were pre-adsorbed with the following protein solutions: undiluted pooled human plasma, 20% (v/v) pooled human plasma in PBS, undiluted pooled human serum, 20% (v/v) pooled human serum diluted in PBS, 3T3 lysate diluted in PBS (final total protein concentration of 50 μg/cm^2), and 3T3 lysate diluted in pooled human plasma or pooled human serum (final total protein concentration of 50 μg/cm^2). The lysate-spiked serum and lysate-spiked plasma solutions were prepared by first diluting the lysate in pooled serum or pooled plasma (10% vol/vol lysate), then the entire solution was further diluted in PBS to obtain a total protein concentration of 50 μg/cm^2, unless otherwise noted. The proteins solutions were added to wells of a TCPS 48-well plate and incubated at room temperature for 30 minutes to allow protein adsorption. The wells were then washed with 250 μL PBS 3 times, for 5 minutes each and the plates were used immediately in macrophage activation assays.
3.5 THP1-XBlue activation on DAMP-adsorbed surfaces

3.5.1 Activation on pre-adsorbed protein layers

dTHP1-XBlues were plated at $2 \times 10^5$ cells/cm$^2$ in protein-adsorbed wells, bare TCPS wells containing cell culture media (negative control) or bare TCPS wells containing cell culture media supplemented with either 100 ng/mL LPS (TLR4 agonist) or 200 ng/mL Pam3CSK4 (TLR2 agonist) ($n = 3$ per condition, per experiment). The plate was incubated for 20 hours at 37°C, then supernatants collected and analyzed for SEAP activity (QUANTI-Blue assay, Section 3.6).

3.5.2 MyD88 Inhibitor

Reagent T6167923, which has been shown to inhibit the MyD88 pathway$^{97}$, was received in a powder form and resuspended to 100 mM in DMSO (stock solution). Prior to plating dTHP1-XBlue macrophages on test surfaces, cells were suspended at $2 \times 10^5$ cells/cm$^2$ in culture media and T6167923 was added to cell suspensions to achieve final inhibitor concentrations of 0 μM (untreated), 50 μM, 100 μM, 200 μM, 250 μM, or 500 μM. The cells with and without T6167923 were incubated at room temperature for 1 hour, then plated on bare TCPS (negative control) or TCPS pre-adsorbed with 20% pooled human plasma, 50 μg/cm$^2$ 3T3 lysate, or 1% (w/w) 3T3 lysate in pooled human plasma at a final concentration of 50 μg/cm$^2$ ($n = 3$ per condition, per experiment). A positive control of 200 ng/mL Pam3CSK4 (TLR2 agonist, MyD88-dependent) and a negative inhibition control of 10 μg/mL Poly(I:C) (TLR3 agonist, MyD88-independent) were included ($n = 3$ per condition, per experiment). The plates incubated for 20 hours at 37°C, then the supernatants were analyzed using a QUANTI-Blue assay (Section 3.6) according to manufacturer’s instructions.
3.5.2.1 DMSO toxicity assay

A DMSO toxicity assay was performed to verify that the concentration of DMSO in the culture conditions containing T6167923 did not affect dTHP1-XBlue viability or metabolic activity. Briefly, dTHP1-XBlue cells were cultured in 48-well plates in complete culture media. After 20 hours, the media was removed and fresh media containing 0.01%, 0.1%, 0.5%, 1%, and 2% vol/vol DMSO (n = 6), with a negative control of untreated cells (i.e. no DMSO). The plate was incubated for 20 hours at 37°C, at which point an alamarBlue assay (Section 3.7) was performed.

3.6 QUANTI-Blue assay

The QUANTI-Blue assay is an enzymatic assay that detects SEAP in cell supernatants, which is an indirect measure of the NF-κB/AP-1 activity in reporter cell lines (RAW-Blue and THP1-XBlue). HI-FBS was used in place of FBS in test media to ensure that any alkaline phosphatase present in the serum did not interfere with the QUANTI-Blue assay, which measures the activity of all alkaline phosphatases. RAW-Blue or dTHP1-XBlue supernatants were transferred to a 96-well plate in triplicate at a volume of 20 μL/well, followed by 180 μL of QUANTI-Blue reagent. The plate was sealed and incubated at 37°C for 2.5 hours, then read on a plate reader at 635 nm. All steps were performed per manufacturer’s instructions.

3.7 alamarBlue assay

alarmarBlue detects cell viability through the reduction of resazurin (dark blue) to resofurin (fuschia/pink) by living cells. alamarBlue (10% final well volume) was added to the supernatant of cells within a well plate. The plate was incubated at 37°C and visually inspected every 30-60 min for significant colour change. Each alamarBlue assay included PBS and media controls (n = 6), which consisted of 10% (vol/vol) alamarBlue in PBS or fresh media,
respectively. After 20 minutes to 2 hours, the supernatants were transferred to a 96-well plate (in duplicate) and the absorbance was measured at 570 nm and 600 nm using a plate reader. All steps were performed according to manufacturer’s instructions.

3.8 Macrophage treatment with aliquoted MaR1

3.8.1 Aliquoting MaR1

MaR1 was received at 100 μg/mL in ethanol. Due to MaR1’s sensitivity to light, oxygen, and water, received MaR1 was immediately aliquoted into small volumes (15 μL) in amber vials with glass autosampler inserts and caps with PTFE/Silicone/PTFE septa that were purged with nitrogen. Briefly, aliquoting was performed in a fume hood with the MaR1 on dry ice. All vials were assembled and then purged with nitrogen for approximately 30 seconds per vial. A 7000 series Hamilton syringe with a blunt 24-gauge needle was washed 38 times with 70% absolute ethanol and then 4 times with 100% absolute ethanol. The MaR1 was then diluted with 100% absolute ethanol to 100 μM. Without opening the vials, the Hamilton syringe was used to transfer 15 μL of diluted MaR1 to each vial by initially piercing through the septa with an 18-gauge beveled syringe followed by insertion of the Hamilton syringe. The original vial of MaR1 was continuously being purged with nitrogen to ensure no oxygen could enter during the multiple volume transfers. Aliquots were stored at -80°C.

3.8.2 Ethanol toxicity assay

As MaR1 was suspended in ethanol, a toxicity assay was performed using the relevant cell lines and primary macrophages to determine if the ethanol concentration in the cell culture media had any effect on cell viability or metabolic activity, measured by alamarBlue reduction.
3.8.2.1 RAW-Blue cell line

RAW-Blue cells were suspended at $1 \times 10^5$ cells/cm$^2$ in test media and plated into 48-well plate. The treatment groups were exposed to 100 nM, 500 nM, 1 μM, and 2 μM MaR1 ($n = 3$), with untreated cells as the negative control. The cells incubated for 20 hours at 37°C, then cell viability/metabolic activity was measured using an alamarBlue assay, as described in Section 3.7.

3.8.2.2 dTHP1-XBlues cell line

Immediately following the dTHP1-XBlue differentiation protocol, media was removed from the wells of dTHP1-XBlues in a 48-well plate and replaced with fresh media containing 0.01%, 0.1%, 0.5%, 1%, and 2% (vol/vol) absolute ethanol ($n = 6$), with a negative control of untreated cells (0% ethanol). The cells were incubated for 20 hours at 37°C, then cell viability/metabolic activity was measured using an alamarBlue assay, as described in Section 3.7.

3.8.2.3 hMDMs

Freshly isolated hMDMs cultured for 7 days in a 48-well plate, then their media removed and fresh media containing with 0.1%, 0.5%, and 1% (vol/vol) absolute ethanol ($n = 6$). Untreated cells were used as a negative control. The plate was incubated for 24 hours at 37°C, followed by analysis using an alamarBlue assay, as described in Section 3.7.

3.8.3 Preliminary MaR1 studies with RAW-Blue cells

Preliminary studies were performed with RAW-Blue cells by suspending and plating them at $1 \times 10^5$ cells/cm$^2$ into 48-well plates in test media, which contained 10% HI-FBS instead of FBS. MaR1 was prepared by adding test media to vials to achieve a desired concentration, followed by sonicating in a waterbath for 10 seconds and vortexing for 30 seconds$^{86}$. For these
preliminary experiments, cells that received neither LPS nor MaR1 were used as the negative control, the positive control were cells activated with 1 μg/mL LPS, and the treatment group was both activated with 1 μg/mL LPS and treated with 100 nM MaR1 (n = 3 per condition, per experiment). All experiments incubated for 20 hours at 37°C, at which point a QUANTI-Blue assay was performed on the supernatant, as per the manufacturer’s instructions. The experiment was performed with multiple variations, described below.

3.8.3.1 Concurrent activation and treatment

Once RAW-Blue cells were plated, 1 μg/mL LPS was added to the positive control and treatment wells, along with the simultaneous addition of 100 nM MaR1 to the treatment group.

3.8.3.2 Treatment with MaR1 pre-activation

100 nM MaR1 was added to the treatment wells and allowed to incubate for 30 minutes, at which point 1 μg/mL LPS was added to the treatment and positive control groups.

3.8.3.3 Treatment with MaR1 post-activation

The positive control and treatment groups were activated with 1 μg/mL LPS and allowed to incubate for 30 minutes, after which 100 nM of MaR1 was added to the treatment group.

3.8.3.4 Treatment with MaR1 post-activation with extended LPS activation

The positive control and treatment groups were activated with 1 μg/mL LPS and incubated for 2 hours. The media was subsequently removed from all cells and replaced with fresh media, and the treatment group was supplemented with 100 nM or 300 nM MaR1.

3.8.4 MaR1 treatment scheme 1

Treatment scheme 1 follows the pre-activation treatment scheme described in Section 3.8.3.2, which reproduces the methods reported in literature78,79,89,90. This treatment scheme was
applied to RAW-Blues, THP1-XBlues, and hMDMs, and cell-specific details are provided in the following sections. In general, cells were plated in TCPS 48-well plates, and treatment groups were exposed to a range of MaR1 concentrations (1 nM – 5 μM; n = 3 per condition) for 30 minutes at 37°C. Following incubation, the positive control and treatment groups were activated with 1 μg/mL LPS. As with the preliminary experiments, cells that received neither LPS nor MaR1 were used as the negative control (n = 3), while cells activated with 1 μg/mL LPS only were the positive control (n = 3). All experiments incubated for 20 hours at 37°C, at which point QUANTI-Blue assays or ELISAs were performed on the supernatant, per the manufacturer’s instructions.

3.8.4.1 RAW-Blue cells with treatment scheme 1

RAW-Blue cells were plated at 1x10^5 cells/cm^2 into 48-well plates in test media. MaR1 was added to the treatment wells at 100 nM, 500 nM, 1 μM, 2 μM, or 5 μM. After incubating for 30 minutes, 1 μg/mL LPS was added to the appropriate wells. After incubation for 20 hours, the supernatants were analyzed with a QUANTI-Blue assay (Section 3.6). These experiments were performed with the first batch of MaR1 acquired.

3.8.4.2 dTHP1-XBlue cells with treatment scheme 1

THP1-XBlues were counted using Trypan Blue, resuspended and plated into 48-well plates at 2x10^5 cells/cm^2 in media containing 100 nM PMA. After incubating at 37°C for 48 hours, the differentiation media was removed and replaced with fresh media for an additional 24 hours to obtain dTHP1-XBlue macrophage-like cell populations. Following differentiation, the culture media was removed and replaced with either fresh media containing 1 nM, 100 nM, 500 nM, 1 μM, or 2 μM MaR1 (treatment group) or fresh media with no MaR1 (untreated and positive control groups). Following a 30-minute incubation, the appropriate cells were activated
with 1 μg/mL LPS and then incubated for an additional 20 hours at 37°C. The experiment was analyzed with a QUANTI-Blue assay (Section 3.6) after incubating. This experiment was performed twice using the second batch of MaR1 6 months (first experiment) and 8 months (second experiment) after aliquoting.

3.8.4.3 hMDMs with treatment scheme 1

Once the hMDMs in 48-well plates had incubated for a total of 7 to 10 days, the treatment groups were exposed to 100 nM, 1 μM, or 2 μM MaR1 for 30 minutes, followed by activation with 1 μg/mL LPS. The cells incubated for 20 hours at 37°C, then the supernatants were collected and analyzed with a LEGEND MAX human IL-1β and IL-8 ELISA kits, according to the manufacturer’s instructions. The supernatants were not diluted for the IL-1β ELISA. The media control, maresin treated cells, and LPS control supernatants for the IL-8 ELISA were diluted by 1/10, 1/50, and 1/100, respectively. The MaR1 used in this experiment was from the second MaR1 batch, 5 months after aliquoting.

3.8.5 MaR1 treatment scheme 2

Treatment scheme 2 follows the post-activation treatment scheme described in Section 3.8.3.4. This treatment scheme was applied to RAW-Blue and THP1-XBlue macrophage cell lines, and cell-specific details are provided in the following sections. Generally, cells were plated into TCPS 48-well plates and activated with 1 μg/mL LPS for 2 hours. After incubation, media was removed from all wells and replaced with fresh media with or without MaR1 (50 nM – 2 μM) (n = 3 per condition), with the intent of examining the effect MaR1 had on LPS-activated macrophage-like cells without the continuous presence of LPS. Cells that received neither MaR1 nor LPS were used as the negative control, and the positive control was cells activated with 1 μg/mL LPS (n = 3). All experiments incubated for 20 hours at 37°C, at which point QUANTI-
Blue assays or ELISAs were performed on the supernatant, as per the manufacturer’s instructions.

3.8.5.1 RAW-Blue cells with treatment scheme 2

RAW-Blue cells were suspended in test media and plated into 48-well plates at $1 \times 10^5$ cells/cm$^2$. Following activation with 1 μg/mL LPS for 2 hours, the treatment groups were treated with 100 nM, 500 nM, 1 μM, or 2 μM MaR1 and then incubated at 37°C for 20 hours. This experiment was repeated 4 times; the first 3 used the first batch of MaR1, and the final experiment used MaR1 from the second batch one month after aliquoting. The supernatants from all repetitions were analyzed using QUANTI-Blue assays, and the final experiment was also analyzed with a LEGEND MAX mouse TNF-α ELISA kit according to the manufacturer’s instructions. The supernatants were diluted 1/10 for the TNF-α ELISA.

3.8.5.2 dTHP1-XBlue cells with treatment scheme 2

dTHP1-XBlues were prepared by seeding THP1-XBlues at $2 \times 10^5$ cells/cm$^2$ in 48-well plates. Following the 48-hour PMA differentiation and 24-hour resting period, the media was replaced with fresh media containing 1 μg/mL LPS for the treatment group and positive control, or no LPS for the negative control. After the 2-hour activation with LPS, media from all wells was replaced with fresh media containing 50 nM, 100 nM, 500 nM, 1 μM, or 2 μM MaR1, or no MaR1. After incubating for 20 hours, the supernatants were analyzed using a QUANTI-Blue assay. The MaR1 used in this experiment was from the second batch, 10 months after aliquoting.

3.8.6 Electrospray ionization mass spectrometry (ESI-MS) of MaR1

Three samples of MaR1 were prepared for analysis by ESI-MS. The first sample contained a fresh batch of MaR1 (batch 3), that was unopened and used as received. This sample
was expected to represent undegraded MaR1. The second sample was from the second batch of MaR1 and was analyzed 10 months after aliquoting, but was otherwise unmanipulated. This sample was expected to provide insight into the extent of MaR1 degradation throughout the aliquoting process and storage at -80°C. The final sample was an aliquot from the second batch of MaR1 (10 months after aliquoting) that was diluted to 10 μM with ethanol, placed in a sonicating bath for 10 seconds, and then vortexed for 30 seconds, according to the protocol used immediately before addition to the cell culture media. All samples were kept on dry ice prior to being analyzed. The samples were brought to the Mass Spectrometry Laboratory at Queen’s University, where it was analyzed by Dr. Jiaxi Wang using a ThermoFisher Orbitrap Velos Pro.

3.9 Treatment of macrophages with fresh MaR1

These experiments were performed with a new, previously unopened vial of MaR1 (i.e. batch 3).

3.9.1 hMDMs on pre-adsorbed protein layers with MaR1 treatment

PBMCs were isolated and plated at 1.25x10^6 cells/cm² for 4 days, with full media changes every 48 hours. Following this incubation, the cells were detached using 10 mM EDTA in PBS and resuspended in culture media with or without 100 nM MaR1 for 30 minutes. Cells were then seeded into TCPS 48-well plates at 1x10^5 cells/cm², with the following conditions: bare TCPS (negative control; n = 2 for donor 1, n = 1 for donor 2), lysate-adsorbed TCPS (donor 1, n = 3) (as described in Section 3.4.2), bare TCPS stimulated with 1 μg/mL LPS (TLR4 positive control; donor 1, n = 3), bare TCPS stimulated with 200 ng/mL Pam3CSK4 (TLR2 positive control; donor 2, n = 2), and 1% (w/w) 3T3 lysate in pooled human plasma (donor 2, n = 2) (as described in Section 3.5.2). Following a 24-hour incubation period at 37°C, the supernatants were analyzed with ELISA MAX Deluxe human IL-8 and TNF-α kits.
3.9.2 dTHP1-XBlues on pre-adsorbed protein layers with MaR1 treatment

TCPS surfaces were left bare or pre-adsorbed with following conditions: bare TCPS (negative control), 20% (v/v) pooled human plasma adsorbed to TCPS, lysate-adsorbed TCPS (as described in Section 3.4.2), 1% (w/w) lysate diluted in pooled human plasma (as described in Section 3.5.2), bare TCPS stimulated with 1 μg/mL LPS (TLR4 positive control), and bare TCPS stimulated with 200 ng/mL Pam3CSK4 (TLR2 positive control).

Following differentiation, dTHP1-XBlue cells were suspended in culture media with either 100 nM or 500 nM MaR1, or without MaR1 and incubated at 37°C for 30 minutes, prior to seeding into wells at 2x10^5 cells/cm^2. The plates were incubated for 24 hours at 37°C, at which point the supernatants were analyzed using ELISA MAX Deluxe human IL-8 and TNF-α kits.

3.10 Statistics

Data is presented as the mean +/- standard deviation, unless otherwise stated. One-way ANOVA with Tukey’s post-hoc tests were used to analyze data with GraphPad Prism 5, with a 95% confidence interval and p < 0.05 being considered significant (* p < 0.05, ** p < 0.01, *** p < 0.001).
Chapter 4

Results and Discussion

4.1 Differentiation of THP1-XBlues confirmed with flow cytometry analysis

THP1-XBlue differentiation into macrophage-like cells was induced using 100 nM PMA for 48 hours, after which the original non-adherent monocyte-like cell population became adherent and started to spread. THP1-XBlue differentiation was further evaluated by comparing the expression of surface marker CD11b using flow cytometry before and after PMA treatment (Figure 4-1). CD11b expression was increased in the differentiated macrophage-like THP1-XBlue population (dTHP1-XBlue) compared to the undifferentiated monocyte-like THP1-XBlue population (indicated as a greater shift in the stained population to the right, relative to the unstained population, for the dTHP1-XBlue compared to the THP1-XBlue cells, in Figure 4-1). Human macrophages are reported to have higher CD11b expression compared to monocytes\textsuperscript{98,99}, indicating that the treatment of THP1-XBlue cells with PMA results in successful differentiation from monocyte-like cells to macrophage-like cells.

The flow cytometry data presented in Figure 4-1 represents a sample of approximately 50000 events (1 cell per event) per treatment within a single experiment. Mean fluorescence intensity (MFI) for THP1-XBlue and dTHP1-XBlue sample populations was calculated and compared between the populations (Figure 4-1C). Robust standard deviation (rSD) was used as a measure of sample population variance, as flow cytometry data has a substantial number of outliers which would result in disproportionate standard deviation values. rSD is less influenced by the outliers present within the data, and therefore provides a more accurate representation of the deviation.
Figure 4-1. Flow cytometry analysis of CD11b expression levels for differentiated THP-1XBlues. Flow cytometry histograms of (A) non-differentiated THP1-XBlues (THP-1; pink) and (B) differentiated THP1-XBlues (dTHP-1; blue) stained for CD11b. The unstained controls for the THP-1 (black) and dTHP1-XBlue (grey) are also shown. (C) Mean fluorescence intensities of THP1-XBlues and dTHP1-XBlues. Data shown is mean fluorescence intensity +/- rSD.

4.2 Activation of dTHP1-XBlues in response to adsorbed DAMPs

McKiel et al.\textsuperscript{27} have previously shown that polymer surfaces with adsorbed protein layers from mouse 3T3 fibroblast lysate activated NF-κB signalling a murine macrophage-like reporter cell line, RAW-Blue. Furthermore, the NF-κB activity was attenuated by TLR-2 inhibition via neutralizing antibodies and the authors concluded that the TLR2-dependent NF-κB activity was due to the presence of cell-derived DAMPs within the lysate. Western blotting confirmed the presence of well-characterized DAMPs, HMGB1 and HSP60, further supporting this conclusion. The present study aimed to reproduce these results in a human model using dTHP1-XBlue cells.
in a procedure adapted from McKiel et al.\textsuperscript{27} to determine if human macrophages would be similarly activated by surface adsorbed DAMPs.

The 3T3 lysate was generated using freeze-thaw cycling, then diluted to a stock concentration of 100 μg/cm\textsuperscript{2}. The concentrations of the pooled plasma and serum solutions, obtained from BCA assays, were 11,640 μg/cm\textsuperscript{2} and 12,556 μg/cm\textsuperscript{2} respectively.

dTHP1-XBlue macrophages were seeded onto TCPS surfaces pre-conditioned with a range of protein conditions (Figure 4-2) to measure the NF-κB activation induced by adsorbed protein layers generated from plasma, serum and/or DAMPs. Plasma and serum were adsorbed at two concentrations to have a clinically relevant analysis (20% plasma/serum), as well as to determine if a greater concentration of those proteins would activate cells (100% plasma/serum). Lysate was tested at 100% (50 μg/cm\textsuperscript{2}) to observe the full response of cells to these proteins, but lysate-spiked conditions (10% vol/vol in 20% plasma/serum) were also examined due to clinical relevancy. These conditions allowed an overview of which components, and therefore proteins, can activate macrophages as well as the strength of this activation. After 20 hours, surfaces pre-adsorbed with only pooled human plasma or serum had no effect on the dTHP1-XBlues, relative to the negative control (p > 0.05). In contrast, all surfaces with adsorbates containing lysate-derived proteins (lysate, lysate-spiked plasma, and lysate-spiked serum) had significantly higher NF-κB-dependent SEAP activity of the dTHP1-XBlues relative to plasma (p < 0.001) and serum (p < 0.001) conditions, as well as the negative control (p < 0.001). Within the lysate-containing conditions, the undiluted lysate resulted in the similar level of activation as the Pam3CSK4 positive control (p > 0.05). Notably, the lysate-spiked serum and plasma still induced a significant increase in SEAP activity (compared to negative control, plasma and serum conditions), despite the relatively small amount of protein from lysate present (0.23% w/w of
total protein). Interestingly, there was a significant difference in NF-κB-related activation levels between the lysate-spiked serum and lysate-spiked plasma (p < 0.001). Serum and plasma have the same proteins aside from a few that are present exclusively in plasma (clotting factors and fibrinogen)\(^{100}\), so these results indicate that these plasma-specific proteins may be competing more strongly with the lysate’s proteins for space on the TCPS surface.

**Figure 4-2.** NF-κB/AP-1-dependent SEAP activity in dTHP1-XBlue cells on protein-adsorbed TCPS surfaces. dTHP1-XBlues were cultured on TCPS pre-adsorbed with serum (100% and 20%), plasma (100% and 20%), lysate (100%) or lysate-spiked serum and plasma solutions (10% v/v in 20% plasma or 20% serum) for 20 hours prior to measuring the SEAP activity in the culture supernatant. Untreated dTHP1-XBlue seeded on bare TCPS were used as the negative control (media), while dTHP1-XBlues stimulated by 100 ng/mL LPS (TLR4 agonist) and 200 ng/mL Pam3CSK4 (TLR2 agonist) were the positive controls. Concentrations of 100% pooled human plasma, pooled human serum, and fibroblast lysate were 11,640 μg/cm\(^2\), 12,556 μg/cm\(^2\), and 50 μg/cm\(^2\), respectively. Data shown as the mean +/- SD (n = 3, *** p < 0.001).

### 4.3 Small molecule inhibition of lysate-dependent NF-κB activity

MyD88 is a critical adaptor molecule for signal transduction for all TLRs except for TLR3\(^{57,69}\). Therefore, I hypothesized that inhibiting MyD88 would result in an effective
reduction in NF-κB/AP-1 activation levels, and represent a potential strategy for modulating the macrophage activation on implanted polymeric biomaterial surfaces. A literature review for MyD88 inhibitors yielded a novel small molecule MyD88 inhibitor discovered by Olson et al.\(^9^7\). Using computation methods, Olson et al.\(^9^7\) identified a novel small molecule MyD88 inhibitor, compound T6167923, which demonstrated dose-dependent reduction in NF-κB activity in an LPS-activation model in a human cell line and protected against lethal Staphylococcal enterotoxin B (SEB)-induced toxic shock in mice\(^9^7\). The T6167923 mechanism of action is the disruption of the homodimerization of MyD88, which is a critical step in MyD88-dependent signal transduction\(^1^0^1\). The following experiments were conducted to evaluate MyD88 inhibition by T6167923 using the model of TLR2-dependent macrophage activation on DAMP-adsorbed surfaces.

![Chemical structure of the T6167923 MyD88 small molecule inhibitor. Created in MoleculeSketch, Version 2.1.4 (56).](image)

**Figure 4-3.** Chemical structure of the T6167923 MyD88 small molecule inhibitor. Created in MoleculeSketch, Version 2.1.4 (56).

### 4.3.1 DMSO cytotoxicity

The small molecule MyD88 inhibitor active concentration range was reported as 50-500 μM. As the inhibitor was solubilized in DMSO, it was necessary to verify that the final DMSO concentration in the culture media was not cytotoxic to the dTHP1-XBlue cells. dTHP1-XBlue incubation in 0.01% to 2% DMSO (corresponding to 10 μM – 2 mM T6167923) for 20 hours
had no effect on alamarBlue reduction (Figure 4-4), suggesting this range of DMSO concentration did not induce cell death or alter metabolic activity of the cells.

![Graph showing DMSO cytotoxicity for dTHP-1XBlue macrophages. alamarBlue analysis of dTHP1-XBlue cells treated with 0.01%, 0.1%, 0.5%, 1%, and 2% DMSO. Untreated dTHP1-XBlue cells were the negative control. Data presented as the mean +/- SD (n = 6).]

**Figure 4-4.** DMSO cytotoxicity for dTHP-1XBlue macrophages. alamarBlue analysis of dTHP1-XBlue cells treated with 0.01%, 0.1%, 0.5%, 1%, and 2% DMSO. Untreated dTHP1-XBlue cells were the negative control. Data presented as the mean +/- SD (n = 6).

### 4.3.2 Effect of MyD88 inhibitor on lysate-dependent NF-κB activation of dTHP1-XBlues

dTHP1-XBlue cells were either left untreated or were treated with a range of concentrations of the T6167923 MyD88 inhibitor (50-500 μM) for one hour. Based on the results obtained in Figure 4-2, TCPS wells were coated with the following protein solutions: 20% pooled human plasma, 100% lysate, and 1% w/w lysate-spiked plasma. A negative inhibition control of poly(I:C) was included, as this is a TLR3-agonist and is MyD88-independent. The NF-κB/AP-1-dependent SEAP activity of the supernatants was then analyzed using a colourimetric alkaline phosphatase activity assay.

The initial experiment tested a broad range of T6167923 concentrations (100 μM, 250 μM, and 500 μM) to identify concentration-dependent effects of the inhibitor (Figure 4-5).
Despite Olson et al. showing promising results for the T6167923 compound as a MyD88 inhibitor, the results obtained (Figure 4-5) did not show a consistent reduction in NF-κB activation with increasing compound concentration. The compound only reduced activation levels on lysate-spiked plasma at 250 μM (p < 0.01) and with poly(I:C) at 250 and 500 μM (p < 0.001). For other conditions (100% lysate, Pam3CSK4), treatments with the compound instead increased activation levels. However, it was observed that the T6167923 compound came out of solution when being added to the cell suspension (Appendix B), which likely affected the results of the experiment.

The experiment was then repeated with lower concentrations of the compound (50 μM, 100 μM, and 200 μM), as an attempt to prevent the compound from precipitating (Figure 4-6). Interestingly, none of the inhibitor treatments resulted in a reduction of SEAP activity; instead cells became more activated on lysate-spiked plasma with the 50 μM and 100 μM treatments (p < 0.001). The TLR2 agonist control also demonstrated an increased activation level with the 50 μM treatment (p < 0.001). Despite the lower concentrations, the compound still precipitated when added to the cell suspensions.

Studies have demonstrated that nano and microparticles have the ability to activate macrophages towards an M1-like phenotype. Therefore, the presence of the compound particulate in the wells may have activated the cells to become more pro-inflammatory as an attempt to phagocytose the foreign material, thereby increasing NF-κB activation levels. This effect could have been dampened by a portion of the compound being processed by the cells and inhibiting the MyD88 pathway, as many conditions were not significantly affected by treatment with the compound.
**Figure 4-5.** Effect of MyD88 inhibitor T6167923 on the NF-κB/AP-1-dependent SEAP activity of dTHP1-XBlue cells on protein-adsorbed surfaces. dTHP1-XBlues were cultured on TCPS pre-adsorbed with 20% plasma, 100% lysate or lysate-spiked plasma (1% w/w in 20% plasma) for 20 hours prior to measuring the SEAP activity in the culture supernatant. Untreated dTHP1-XBlue seeded on bare TCPS were used as the negative control (media), while dTHP1-XBlues stimulated by 200 ng/mL Pam3CSK4 (TLR2 agonist) were used as the positive control. dTHP1-XBlues stimulated by 10 μg/mL poly(I:C) (TLR3 agonist, MyD88-independent) were used as a negative inhibition control. Concentrations of 100% pooled human plasma and fibroblast lysate were 11,640 μg/cm² and 50 μg/cm², respectively. Data presented as mean +/- SD (n = 3, ** p < 0.01, *** p < 0.001).
Figure 4-6. Effect of MyD88 inhibitor T6167923 on the NF-κB/AP-1-dependent SEAP activity of dTHP1-XBlues cells on protein-adsorbed. dTHP1-XBlues were cultured on TCPS pre-adsorbed with 20% plasma, 100% lysate or lysate-spiked plasma (1% w/w in 20% plasma) for 20 hours prior to measuring the SEAP activity in the culture supernatant. Untreated dTHP1-XBlue seeded on bare TCPS were used as the negative control (media), while dTHP1-XBlues stimulated by 200 ng/mL Pam3CSK4 (TLR2 agonist) were used as the positive control. dTHP1-XBlues stimulated by 10 μg/mL poly(I:C) (TLR3 agonist, MyD88-independent) were used as a negative inhibition control. Concentrations of 100% pooled human plasma and fibroblast lysate were 11,640 μg/cm² and 50 μg/cm², respectively. Data presented as mean +/- SD (n = 3, *** p < 0.001).

Many attempts were made to maintain the inhibitor in solution by using various methods of combining the inhibitor and cell culture media. Each method resulted in precipitation of the molecule, so experiments with it were discontinued. Furthermore, it is relevant to note that it is also possible the inhibitor was not simply precipitating, but potentially forming a more complex biphasic emulsion in combination with amphiphilic or lipophilic molecules within the culture media. Consequently, it would be beneficial to explore the possibility of developing a drug delivery system for this molecule, which could deliver it to cells without precipitating.
4.4 Pro-resolution activity of MaR1 in *in vitro* models of macrophage activation

The difficulties encountered with the T6167923 small molecule inhibitor suggest that further research is required to develop an appropriate delivery method to prevent precipitation. An alternative strategy for modulating macrophage activation on polymeric biomaterial surfaces aims to promote the resolution of pro-inflammatory macrophage activation towards a pro-wound healing phenotype. MaR1, of the SPM class of lipid molecules, has demonstrated a promising ability to reduce inflammation and pro-inflammatory cytokine release, as well as encourage a pro-resolution phase of healing\(^78,90,91\). Literature has shown that a range of MaR1 concentrations are effective at altering the pro-inflammatory response of various cell lineages, with the most effective *in vitro* concentration being 100 nM\(^79,90,94,95\). Three cell lineages were used with the intent of replicating the positive results seen in literature to evaluate MaR1 delivery as a potential strategy for altering the pro-inflammatory response of macrophages towards a pro-resolution phenotype. A major limitation of MaR1, however, is its highly unstable molecular structure, which is susceptible to degradation by hydrolysis and oxidation. While significant care and effort was made to maintain MaR1 activity through the following experiments, degradation of the molecule was a persistent issue. Consequently, three vials of MaR1 were purchased over a 16-month time span, although each vial was from the same lot. The following section reports the temporal progression of experiments using MaR1 and reflects upon the troubleshooting approaches taken in an effort to reproduce published results on this molecule.
4.4.1 Lot 1 of MaR1: Preliminary studies with RAW-Blues

RAW-Blue cells were chosen for preliminary studies with MaR1 due to the fact that they are easy to grow, do not require differentiation, and have been modified to allow for quick and easy analysis of NF-κB activation levels with the QUANTI-Blue assay. Furthermore, MaR1 has previously been shown to reduce LPS-induced activation of mouse macrophages\textsuperscript{89}. Three different chronologies of LPS activation and MaR1 treatment were tested on RAW-Blue cells; concurrent activation and treatment, treatment before activation (i.e. protective treatment), and activation before treatment (i.e. reactive treatment) (Appendix C). A treatment concentration of 100 nM MaR1 was chosen, as this is the most common dosage used within literature and has been found to be effective in several studies\textsuperscript{79,90,94,95}. These preliminary experiments did not demonstrate any reduction in NF-κB activity in maresin-treated conditions, relative to untreated conditions regardless of the treatment chronology used. Figure 4-7 shows the results from the experiment of concurrent activation with LPS and treatment with MaR1. The remainder of these results can be found in Appendix C (Figure C-1).
Figure 4-7. Initial evaluation of MaR1 effects on NF-κB/AP-1-related SEAP activity in RAW-Blue cells. RAW-Blue cells were concurrently activated with 1 μg/mL LPS and treated with 100 nM MaR1 for 20 hours prior to measuring SEAP activity in the culture supernatant. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. Data presented as mean+/− SD (n = 3).

In particular, the absence of reduction in NF-κB activity for the positive control treated with 100 nM MaR1 was unexpected, given the reported literature. Consequently, another experiment scheme was used to determine if the constant exposure to LPS in the culture media was masking or overpowering the effect of the MaR1. Therefore, cells were activated with LPS for 2 hours, then the LPS-containing culture media was replaced with fresh media supplemented with MaR1 (Figure 4-8). Under these conditions, MaR1 treatment resulted in a decreased NF-κB activation level for both concentrations tested (100 nM and 300 nM).
Figure 4-8. Effect of MaR1 on NF-κB/AP-1-related SEAP activity of RAW-Blue cells with constant LPS exposure removed. RAW-Blues were pretreated with 1 μg/mL LPS, then had media replaced with fresh media supplemented with (A) 100 nM and (B) 300 nM MaR1 for 20 hours prior to measuring SEAP activity in the culture supernatant. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. Data presented as mean +/- SD (n = 3, ** p < 0.01, *** p < 0.001).

Based on these results, two MaR1 treatment schemes were chosen to be used for future experiments. Treatment Scheme 1 treated cells with MaR1 for 30 minutes prior to LPS activation, as this is the most common chronology implemented in studies with MaR1. Treatment scheme 2 activated cells with LPS for 2 hours, then treated the cells with fresh media supplemented with MaR1.

4.4.1.1 MaR1 and ethanol cytotoxicity

As MaR1 is suspended in 100% ethanol to prevent degradation, it was necessary to verify the cytotoxicity of ethanol on the cells. RAW-Blues were treated with a range of MaR1 concentrations (100 nM – 2 μM) and analyzed with an alamarBlue assay to determine if the ethanol present had an effect on cell metabolism (Figure 4-9). There was no significant
difference seen between any of the conditions and the media control, indicating that neither MaR1 nor ethanol has any effect on cell metabolism or cell viability.

![Bar chart showing % difference in reduction compared to control across different MaR1 concentrations.](image)

**Figure 4-9.** Effect of MaR1 on RAW-Blue cell viability. RAW-Blue cells were treated with 0 nM (media), 100 nM, 500 nM, 1 μM, and 2 μM MaR1 for 20 hours prior to evaluation with an alamarBlue assay. Data presented as mean +/- SD (n = 3).

4.4.1.2 Treatment scheme 1 on RAW-Blues

Treatment scheme 1 (i.e. treat with MaR1 then stimulate with LPS) was applied to RAW-Blue cells with a range of MaR1 concentrations (100 nM, 500 nM, 1 μM, 2 μM and 5 μM) over two experiments. The NF-κB-related SEAP activation of the cells was analyzed with a QUANTI-Blue assay (Figure 4-10). Overall, all LPS stimulated cells had increased the NF-κB activity relative to the media control (p < 0.001), regardless of MaR1 treatment, and MaR1 did not significantly reduce the NF-κB activity within the LPS-stimulated populations at any concentration. However, it is important to note that there was variability within the data from two experiments. In the first experiment (highlighted using red dots), the SEAP activity was
increased relative to the LPS control for 100 nM and 500 nM MaR1 treatments and decreased for the 5 μM treatment (p < 0.05 vs LPS). However, when repeated (green dots) the NF-κB activity was moderately reduced with the 500 nM MaR1 treatment, and slightly increased with 2 μM MaR1 treatment (p < 0.05 vs LPS).

**Figure 4-10.** MaR1 treatment scheme 1 with RAW-Blue cells. NF-κB/AP-1-related SEAP activity was analyzed for RAW-Blue cells that were activated with 1 μg/mL LPS for 20 hours following pre-treatment with 100 nM, 500 nM, 1 μM, 2 μM and 5 μM MaR1. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. Data presented as mean +/- SD of two experiments; data from experiment 1 is shown as red points and experiment 2 as green points. n = 3 per experiment, *** p < 0.001 compared to untreated negative control).

The process of handling MaR1 during these experiments was still moderately new, so the variability seen could indicate that it may have been prepared somewhat differently between experiments which could have affected the results. Additionally, not only has the RAW264.7 cell line not previously been used with MaR1, but MaR1’s mechanism of action is also not known\(^8^9\), hence the method of analyzing the NF-κB activity in these cells may not be looking at the
appropriate inflammatory pathway. Most experiments performed with MaR1 use primary cells and evaluate its effect by looking at cytokine expression levels\textsuperscript{78,89}, so it is challenging to know the best method of analysis for cell lines.

Treatment scheme 2 was applied to the RAW-Blue cells to troubleshoot the issues encountered. The LPS-containing media was removed and replaced with media supplemented with MaR1 as a way to see if the effect of MaR1 was being overpowered by the continuous supply of LPS.

4.4.1.3 Treatment scheme 2 on RAW-Blues

RAW-Blue macrophage-like cells were subjected the treatment scheme 2, in which cells were first stimulated with LPS, then the media was replaced with fresh media containing MaR1. This experiment was repeated 4 times, where the first 3 replicates were performed with the first vial of MaR1, and the last replicate was performed with the second vial of MaR1 (used one month after aliquoting). The NF-κB-related SEAP data from all experiments were combined into Figure 4-11, which shows a decrease in NF-κB activity in a dose-dependent manner with increasing MaR1 concentration (100 nM to 2 μM).

In addition to performing the NF-κB reporter assay in the last replicate experiment, supernatant was also collected and the TNF-α cytokine release was analyzed by ELISA. Contrary to the SEAP activity results, the TNF-α ELISA (Figure 4-12) showed no significant difference in cytokine release from the cells compared to the LPS control, apart from the increase in expression shown in the 2 μM treatment (p < 0.01).
Figure 4-11. MaR1 treatment scheme 2 with RAW-Blue cells. NF-κB/AP-1-related SEAP activity of 4 combined experiments was analyzed, showing the effect of a 20-hour treatment with 100 nM, 500 nM, 1 μM, and 2 μM MaR1 on RAW-Blue cells that were pre-activated with 1 μg/mL LPS. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. MaR1 used was from the first batch, except for the final repetition which was taken from the second batch, 1 month post-aliquoting. Data presented as mean +/- SEM (n = 4, * p < 0.05, ** p < 0.01).
Figure 4-12. Effect of MaR1 treatment scheme 2 on RAW-Blue TNF-α secretion. RAW-Blue cells were pre-activated with 1 μg/mL LPS and treated with 100 nM, 500 nM, 1 μM, and 2 μM MaR1 for 20 hours prior to measuring TNF-α expression levels in culture supernatant using an ELISA. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. MaR1 used was from the second batch, 1 month-post aliquoting. Data presented as mean +/- SD (n = 3, ** p < 0.01). The curve fit was R² = 0.9999 (Figure E-1).

The conflicting NF-κB and TNF-α results further suggest that the RAW-Blue cell line may not be an appropriate model for analyzing MaR1’s effects on macrophages. However, it is important to note that although NF-κB activation and TNF-α release occur within a positive feedback loop, they are not mutually exclusive. It is possible that in this cell line, MaR1 is decreasing the activation of the NF-κB transcription factor but does not affect the release of TNF-α. Interestingly, these data show that the higher concentrations of MaR1 were reducing NF-κB-related SEAP activation, which contradicts the results found in literature. This result could indicate that some of the MaR1 had degraded, so there was a lower concentration being added than intended.
4.4.2 Lot 2 of MaR1: Troubleshooting using dTHP1-XBlues and primary human macrophages

4.4.2.1 Ethanol cytotoxicity on human cells

Since MaR1 is stored in 100% ethanol, the cytotoxicity of relevant ethanol concentrations was verified on the two human cell lineages used in this thesis. dTHP1-XBlues were treated with a range of ethanol concentrations from 0.01-2% in cell culture media, which corresponds to a MaR1 concentration of 10 nM - 2 μM (Figure 4-13A). Similarly, PBMCs were isolated and differentiated into hMDMs over 7 days, then treated with a range of ethanol concentrations (0.1-1%, Figure 4-13B). Fewer ethanol concentrations were tested on hMDMs due to a limitation in the amount of cells that could be harvested at once, and it was anticipated, based on literature, that lower concentrations of MaR1 would have a greater effect on primary cells, so the higher concentration would be tested less frequently. alamarBlue assays were performed to evaluate the effect of ethanol on cell viability and metabolism. No effect on alamarBlue reduction was observed, relative to the control, for any conditions except a slight decrease in alamarBlue reduction for the 0.1% ethanol concentration on hMDMs (p < 0.05). As higher concentrations did not exhibit a similar effect on alamarBlue reduction, it was assumed that this was due to experimental error and that ethanol at this concentration does not have an effect on hMDM viability.
Figure 4-13. The effect of ethanol of dTHP1-XBlue and hMDM viability. (A) dTHP1-XBlue cells treated with 0%, 0.01%, 0.1%, 0.5%, 1%, and 2% ethanol and (B) hMDMs treated with 0%, 0.1%, 0.5%, and 1% ethanol for 20 hours prior to evaluation with alamarBlue assays. Data presented as mean +/- SD (n = 6, * p < 0.05).

4.4.2.2 Treatment scheme 1 on hMDMs

While RAW-Blue cells were advantageous for preliminary data with MaR1, this molecule has mostly been used with primary cells. hMDMs were chosen as the next cell type to use because of the fact that they are primary cells, but also because it was desirable to translate the results obtained into human cells as this is a more clinically-relevant model.

PBMCs were collected and incubated for 7 days to differentiate into macrophages. With the intent to follow methods from literature, treatment scheme 1 was applied to the cells with varying concentrations of MaR1 (100 nM, 1 μM, and 2 μM), the supernatants subsequently collected and analyzed for cytokine release profiles with IL-1β and IL-8 ELISAs, both pro-inflammatory cytokines. This experiment was performed 5 months after aliquoting the second lot of MaR1. No conclusions could be drawn from the IL-1β results due to significant variability between replicates (Figure 4-14). The high variability was likely due to the low concentration of IL-1β, which fell within the low end of the assay range (0.5 pg/mL – 50 ng/mL). Consequently,
IL-1β concentrations were interpolated from the non-linear region of the standard curve, which could account for the variability between replicates that may have had slight differences in absorbance measured. Although there are no statistical differences among treatments due to the data variance, there does appear to be a decreased cytokine release with the 100 nM MaR1 treatment. However, the experiment would need to be repeated several times for any true trend to emerge, likely with an ELISA kit with greater sensitivity to low concentrations.

The IL-8 ELISA (Figure 4-15) results demonstrated a slightly decreased activation level with the 100 nM concentration of MaR1 but did not have a statistically significant effect. Again, it is likely that this experiment would need to be repeated for a trend to emerge, however the process of obtaining a sufficient amount hMDM cells limited the ability to repeat experiments.

![Figure 4-14](image)

**Figure 4-14.** The effect of MaR1 treatment scheme 1 on hMDM IL-1β secretion. hMDMs were treated with 100 nM, 1 μM, and 2 μM of MaR1 for 30 minutes, then treated with 1 μg/mL LPS for 20 hours prior to measuring IL-1β expression levels in culture supernatant using an ELISA. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. MaR1 used was from the second batch, 5 months post- aliquoting. Data presented as the mean +/- SD (n = 3). The curve fit was $R^2 = 0.9977$ (Figure E-2).
Figure 4-15. The effect of MaR1 treatment scheme 1 on hMDM IL-8 secretion. hMDMs were treated with 100 nM, 1 μM, and 2 μM of MaR1 for 30 minutes, then treated with 1 μg/mL LPS for 20 hours prior to measuring IL-8 expression levels in culture supernatant using an ELISA. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. MaR1 used was from the second batch, 5 months post- aliquoting. Data presented as the mean +/- SD (n = 3). The curve fit was R² = 1 (Figure E-3).

While treatment with MaR1 did yield slight differences at 100 nM, the results do not reflect the magnitude of effect typically reported in the literature. Commonly, MaR1 and other SPMs are able to reduce pro-inflammatory cytokine expression by half (compared to the positive control, frequently LPS) when treating human primary monocytes and macrophages. However, obtaining sufficient amounts of PBMCs was a barrier to troubleshooting with this cell model. Consequently, the THP1-XBlue cell line was used instead.

4.4.2.3 Treatment scheme 1 on dTHP1-XBlues

hMDMs presented difficulties with achieving an appropriate cell count, as well as the time and resources required to isolate them. dTHP1-XBlue cells were used to troubleshoot MaR1
treatment protocols as a human cell line that was easier to work with and would also benefit from the use of QUANTI-Blue reporter assays for NF-κB activity.

dTHP1-XBlue cells were treated with varying concentrations of MaR1 (1 nM, 100 nM, 500 nM, 1 μM, and 2 μM) according to treatment scheme 1, then analyzed for NF-κB-related SEAP activity (Figure 4-16). This experiment was first performed 6 months after aliquoting the second vial of MaR1 (Figure 4-16A), and resulted in a significant reduction in SEAP activation levels with the 100 nM concentration (p < 0.05) and a slight increase in activation with 2 μM of MaR1 (p < 0.05). There were no significant differences found among the other concentrations of MaR1 and the LPS control. These results were more consistent with the results reported in the literature, including the bell-shaped response curve96, as well as the 100 nM concentration of MaR1 being the most effective79,90,94,95. However, this data conflicted with the MaR1 response observed in the RAW-Blue cells (reported in Section 4.4.1.3). As there is very little published data on MaR1 response in the THP-1 or RAW264.7 cell lineages, it is difficult to say with certainty what response is expected.

This THP1-XBlue experiment was repeated with the same vial of MaR1, 8 months after aliquoting (Figure 4-16B). Interestingly, no reduction in NF-κB-related SEAP levels was seen at any MaR1 concentration. Instead, the 100 nM, 500 nM, 1 μM, and 2 μM concentrations increased NF-κB activity compared to the LPS control (p < 0.05), and the NF-κB activity increased with increasing MaR1 concentration.
Figure 4-16. Effect of MaR1 treatment scheme 1 on dTHP1-XBlues, performed 6 and 8 months after MaR1 aliquoting. NF-κB/AP-1-related SEAP activity was analyzed for dTHP1-XBlue cells activated with 1 μg/mL LPS following pre-treatment with 1 nM, 100 nM, 500 nM, 1 μM, and 2 μM MaR1. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. Maresin used for these experiments was from the second batch, (A) 6 months post-aliquoting and (B) 8 months post-aliquoting. Data presented as the mean +/- SD (n = 3, * p < 0.05, *** p < 0.001).
To investigate the possible cause for this response, treatment scheme 2 was applied to dTHP1-XBlues with the same MaR1 concentrations 2 months later (10 months after aliquoting lot 2). The results once again showed a slight but steady increase in activation levels with increasing MaR1 concentration (Appendix Figure D-1).

MaR1 and the other SPMs are known to degrade easily\(^8^6\), which can make them difficult to handle and limits their clinical utility. Following re-examination of the inconsistent results obtained with MaR1, I hypothesized that the MaR1 samples were degrading over time post-aliquoting.

Degradation of the MaR1 molecule would explain why it was no longer reducing the activation levels of the cells. Furthermore, I hypothesized that the degraded MaR1 molecule acted as an NF-\(\kappa\)B pathway stimulus due to the increased activation levels seen with higher concentrations of MaR1. In order to examine the potential degradation of the MaR1 molecule over the workflow used to aliquot, store and use MaR1 within the experimental protocol, MaR1 samples were prepared and analyzed with ESI-MS.

**4.5 ESI-MS of MaR1**

ESI-MS works by spraying a sample through a capillary tube containing high voltage potential, which disperses fine droplets of the charged solution into a gas ionization chamber. The charged droplets pass through this chamber while the solvent evaporates, leading to the ions eventually ejecting out of the droplets and being read by a mass analyzer\(^1^0^6,1^0^7\). A plot is generated based on the relative abundance of each ion detected, with the most abundant mass/ion (m/z) ratio represented as 100% relative abundance. This method was employed for analyzing MaR1 samples due to its ability to detect samples from a very low volume, its ease of use, and low cost.
Three samples were prepared for analysis: control (new, previously unopened vial of MaR1; the third lot purchased), aliquot (100 μM aliquoted sample from the second lot of MaR1, otherwise unopened for 10 months), and working aliquot (sample from the second lot of MaR1 diluted to 10 μM, sonicated for 10 seconds, and vortexed for 30 seconds to replicate standard experimental preparations). The samples were analyzed with ESI-MS to identify the peaks and ion composition present, which was used to determine the amount of original MaR1 that had degraded. The ESI-MS spectra of each sample can be seen in Figure 4-17 (larger versions can be found in Appendix F).

**Figure 4-17.** ESI-MS spectra of three samples of MaR1 to evaluate degradation levels. The three samples consisted of a control (fresh, unopened vial of MaR1), an aliquot (100 μM aliquoted sample from second lot of MaR1, otherwise unopened for 10 months), and a working aliquot (sample from second lot of MaR1 diluted to 10 μM, sonicated for 10 seconds, and vortexed for 30 seconds to replicate standard experimental preparations). The m/z peak at 359.22 represents intact MaR1, while all other peaks represent degraded forms of MaR1.

Table 4-1 displays the highest peak for each spectrum and the respective relative intensity of each peak. The largest peaks are easily identified in the spectra and always have a relative
intensity of 100. The molecular weight of a non-degraded MaR1 molecule is 360.5 g/mol; the highest peaks in both the Control and Aliquot samples were found to be 359.22 (Table 4-1 and Table 4-2), which represents a non-degraded molecule of MaR1 with a single ion removed (which allows for detection). In contrast, the largest peak found in the working aliquot sample had an m/z value of 253.81, representing a degraded form of MaR1.

Table 4-1. Comparison of largest m/z peaks found in each spectrum.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Control</th>
<th>Aliquot</th>
<th>Working Aliquot</th>
</tr>
</thead>
<tbody>
<tr>
<td>253.81</td>
<td>11.75</td>
<td>6.48</td>
<td>100.00</td>
</tr>
<tr>
<td>359.22</td>
<td>100.00</td>
<td>100.00</td>
<td>15.06</td>
</tr>
</tbody>
</table>

† Relative to the largest peaks found in the respective spectrum of each sample.

A summary of the three largest peaks found in the control sample is shown in Table 4-2. The largest m/z peaks from the control sample coincide with those in the aliquot spectrum, so this table represents the three largest peaks in both samples. The largest peak of the control and aliquot samples, an m/z of 359.22, has a relatively negligible intensity in the working aliquot sample with a relative intensity of 15.06. The second largest peak (m/z 255.23) is a degraded form of MaR1, however the relative intensity is similar between the aliquot and working aliquot samples, but these peaks are approximately twice the intensity of that found in the control sample, indicating that the control sample had the lowest amount of degradation. The final peak, the m/z ratio of 360.23, reported the exact same peak intensity for the control and aliquot samples, but was not found in the working aliquot sample of MaR1. This third peak (m/z 360.23)
represents a non-degraded molecule of MaR1 with a single ion removed, but with either one of
the carbons being a $^{13}$C instead of a $^{12}$C, or a hydrogen being a $^{2}$H instead of a $^{1}$H.$^{108}$

Table 4-2. Relative intensities of the largest m/z peaks identified in Control and Aliquot spectra.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Control</th>
<th>Aliquot</th>
<th>Working Aliquot</th>
</tr>
</thead>
<tbody>
<tr>
<td>255.23</td>
<td>27.00</td>
<td>51.53</td>
<td>52.80</td>
</tr>
<tr>
<td>359.22</td>
<td>100.00</td>
<td>100.00</td>
<td>15.06</td>
</tr>
<tr>
<td>360.23</td>
<td>24.16</td>
<td>24.16</td>
<td></td>
</tr>
</tbody>
</table>

† Relative to the largest peaks found in the respective spectrum of each sample.

Table 4-3 shows a summary of the three largest m/z peaks found in the working aliquot sample and the respective relative intensities between the 3 spectra. Only the m/z peaks of 359.22 and 360.23 represent fully intact MaR1 molecules, therefore all peaks identified in this table are degraded forms of MaR1.

Table 4-3. Relative intensities of the largest m/z peaks identified in Working Aliquot spectrum.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Control</th>
<th>Aliquot</th>
<th>Working Aliquot</th>
</tr>
</thead>
<tbody>
<tr>
<td>253.81</td>
<td>11.75</td>
<td>6.48</td>
<td>100.00</td>
</tr>
<tr>
<td>255.23</td>
<td>27.00</td>
<td>51.53</td>
<td>52.80</td>
</tr>
<tr>
<td>283.26</td>
<td>9.34</td>
<td>16.77</td>
<td>15.39</td>
</tr>
</tbody>
</table>

† Relative to the largest peaks found in the respective spectrum of each sample.

With this data, it appears that the Control sample must have degraded slightly to account for the presence of the peak at an m/z of 255.23, which may have happened when the vial was
opened during preparation to be analyzed. The Aliquot sample contained the same 3 highest peaks as the Control sample, indicating that the MaR1 was still mostly intact, but that it was degraded more due to the higher intensity of the 255.23 peak. Whereas the Working Aliquot sample only had a peak at 359.22 m/z of non-degraded sample equal to 15.06%, and significantly larger peaks at 253.81 and 255.23 m/z, indicating that it was mostly degraded when analyzed. It is reasonable to assume that the amount of degradation of the Working Aliquot sample is the reason the experiments with dTHP1-XBlue cells did not result in any decrease in activation.

4.6 Lot 3: MaR1 on DAMP-activated cells

There were 2 vials of MaR1 obtained with the final purchase, one used for ESI-MS analysis, and the other left unopened until the following experiments were performed. Due to the preliminary results seen as well as the ESI-MS results, which demonstrated that the previously aliquoted MaR1 was mostly degraded but the new lot was intact, MaR1 was used for one final experiment to determine if it can effectively reduce pro-inflammatory effects of macrophages activated on DAMPs. This experiment could only be repeated once, immediately after the final vial of MaR1 was opened.

4.6.1 dTHP1-XBlues on DAMPs with MaR1 treatment

dTHP1-XBlues were prepared and either left untreated or were treated with 100 nM or 500 nM MaR1 for 30 minutes. The following protein-adsorbed surfaces were prepared: 100% lysate, 20% pooled human plasma, and lysate-spiked plasma. TLR agonists were used as positive controls: 1 μg/mL LPS for TLR4 and 200 μg/mL Pam3CSK4 for TLR2. Following a 24-hour incubation, the supernatants were collected to be analyzed with TNF-α (Figure 4-18) and IL-8 ELISAs (Figure 4-19).
Figure 4-18 demonstrated a significant reduction in TNF-α production for the lysate (p < 0.001), LPS (p < 0.05 for 100 nM, p < 0.01 for 500 nM), and Pam3CSK4 (p < 0.001) conditions with MaR1 treatment. TNF-α production values were below the detection limit for the 20% plasma and lysate-spiked plasma conditions. Figure 4-19 also showed a decrease in IL-8 production with MaR1 treatment for the lysate and Pam3CSK4 conditions (p < 0.01 for 100 nM, p < 0.001 for 500 nM for both). Interestingly, no significant difference was found when LPS-activated cells were treated with MaR1, with the same results being seen for the 20% plasma and lysate-spiked plasma.

Previous research in the Fitzpatrick lab demonstrated that lysate activation of macrophages-like cells was TLR2 dependent and Pam3CSK4 is a TL2 agonist. Consequently, the present study in THP1-XBlues suggests that MaR1 inhibits the TLR2 pathway, which signals via the MyD88-dependent pathway to activate NF-κB/AP-1. It is unclear why MaR1 reduced LPS-induced TNF-α secretion but did not have an effect on IL-8 secretion, as these cytokines often follow similar expression patterns. However, contrasting release patterns have been previously documented in LPS-stimulated whole blood. LeForge et al. demonstrated a decrease in TNF-α expression by 30% between 4- and 24-hour time points, while IL-8 expression increased almost 4 times over the same period. Additionally, TLR4 has the ability to signal through both MyD88-dependent and TRIF-dependent signaling cascades, while TLR2 is MyD88-dependent. The consistent reduction in cytokine expression for lysate and Pam3CSK4 conditions (both signal through TLR2) and partial reduction for LPS activation (TLR4 agonist) could indicate the possibility of MyD88 inhibition by MaR1, with possible transcriptional or post-transcriptional effects through TRIF accounting for the unchanged IL-8 expression profile. Furthermore, MaR1’s mechanism of action has not yet been identified and
may have additional effects on transcriptional and post-transcriptional regulation of cytokine expression that are currently unknown.

Figure 4-18. Effect of fresh MaR1 on dTHP1-XBlue TNF-α secretion on DAMP-adsorbed surfaces. dTHP1-XBlues were cultured on TCPS pre-adsorbed with 20% plasma, 100% lysate or lysate-spiked plasma (1% w/w in 20% plasma) for 24 hours prior to measuring TNF-α expression levels in the culture supernatant using an ELISA. Untreated dTHP1-XBlue seeded on bare TCPS were used as the negative control (media), while dTHP1-XBlues stimulated by 1 μg/mL LPS (TLR4 agonist) and 200 ng/mL Pam3CSK4 (TLR2 agonist) were used as positive controls. Concentrations of 100% pooled human plasma and fibroblast lysate were 11,640 μg/cm² and 50 μg/cm², respectively. Data presented as the mean +/- SD (n = 3, * p < 0.05, ** p < 0.01, *** p < 0.001). The curve fit was R² = 0.9998 (Figure E-4).
**Figure 4-19.** Effect of fresh MaR1 on dTHP1-XBlue IL-8 secretion on DAMP-adsorbed surfaces. dTHP1-XBlues were cultured on TCPS pre-adsorbed with 20% plasma, 100% lysate or lysate-spiked plasma (1% w/w in 20% plasma) for 24 hours prior to measuring IL-8 expression levels in the culture supernatant using an ELISA. Untreated dTHP1-XBlue seeded on bare TCPS were used as the negative control (media), while dTHP1-XBlues stimulated by 1 μg/mL LPS (TLR4 agonist) and 200 ng/mL Pam3CSK4 (TLR2 agonist) were used as positive controls. Concentrations of 100% pooled human plasma and fibroblast lysate were 11,640 μg/cm² and 50 μg/cm², respectively. Data presented as the mean +/- SD (n = 3, ** p < 0.01, *** p < 0.001). The curve fit was R² = 0.9999 (Figure E-5).

### 4.6.2 hMDMs on DAMPs with MaR1 treatment

PBMCs were isolated and incubated for 4 days to differentiate into naïve macrophages, then treated with 100 nM MaR1 and activated with DAMPs and TLR agonists. Cells from two donors were used to obtain enough cells to run the experiment; the first donor’s cells had the untreated control, 100% lysate, and LPS conditions, while the second donor’s cells had untreated control, Pam3CSK4, and lysate-spiked plasma conditions. Following a 24-hour incubation, the supernatants were analyzed for TNF-α (Figure 4-20) and IL-8 (Figure 4-21) cytokine production.

The data obtained from the TNF-α ELISA (Figure 4-20) showed a significant increase in expression levels with MaR1 treatment for the LPS condition (p < 0.001), but no change in
cytokine expression levels for any other conditions. In contrast, Figure 4-21 demonstrated that
the 100 nM MaR1 treatment caused a decrease (p < 0.001) in IL-8 production with LPS-
activated cells but an increase (p < 0.001) in IL-8 production in cells that were activated by
lysate. No other conditions displayed a significant change in IL-8 expression levels.

These results suggest that 100 nM MaR1 enhances TLR2 activation in hMDMs, but more
experiments would be required to understand how it may be affecting the TLR4 pathway. It is
possible that differentiating the cells for 4 days instead of 7 influenced the cytokine expression
levels, as there may have been a more varied population of cells present. If there were more
monocytes present at 4 days than there would have been at 7, this could explain the
inconsistency seen in the data, as MaR1 and the agonists would affect those cells differently than
macrophages.
Figure 4-20. Effect of fresh MaR1 on hMDM TNF-α secretion on DAMP-adsorbed surfaces. hMDMs were cultured on TCPS pre-adsorbed with 20% plasma, 100% lysate or lysate-spiked plasma (1% w/w in 20% plasma) for 24 hours prior to measuring TNF-α expression levels in the culture supernatant using an ELISA. Untreated hMDMs seeded on bare TCPS were used as the negative control (media), while hMDMs stimulated by 1 μg/mL LPS (TLR4 agonist) and 200 ng/mL Pam3CSK4 (TLR2 agonist) were used as positive controls. Concentrations of 100% pooled human plasma and fibroblast lysate were 11,640 μg/cm² and 50 μg/cm², respectively. The 1% (w/w) lysate and 200 ng/mL Pam3CSK4 conditions were obtained from a second donor. Data presented as the mean +/- SD (media donor 2: n = 1; media donor 1, Pam3CSK4, 1% lysate: n = 2; lysate, LPS: n = 3, *** p < 0.001). The curve fit was R² = 0.9998 (Figure E-4).
Figure 4-21. Effect of fresh MaR1 on hMDM IL-8 secretion on DAMP-adsorbed surfaces. hMDMs were cultured on TCPS pre-adsorbed with 20% plasma, 100% lysate or lysate-spiked plasma (1% w/w in 20% plasma) for 24 hours prior to measuring IL-8 expression levels in the culture supernatant using an ELISA. Untreated hMDMs seeded on bare TCPS were used as the negative control (media), while hMDMs stimulated by 1 μg/mL LPS (TLR4 agonist) and 200 ng/mL Pam3CSK4 (TLR2 agonist) were used as positive controls. Concentrations of 100% pooled human plasma and fibroblast lysate were 11,640 μg/cm² and 50 μg/cm², respectively. The 1% (w/w) lysate and 200 ng/mL Pam3CSK4 conditions were obtained from a second donor. Data presented as the mean +/- SD (media donor 2: n = 1; media donor 1, Pam, 1% lysate: n = 2; lysate, LPS: n = 3, *** p < 0.001). The curve fit was R² = 0.9999 (Figure E-5).
Chapter 5
Conclusions and Future Work

The FBR poses significant challenges for successful biomaterial and medical device implantation\(^9\). While many methods to inhibit the FBR and fibrous encapsulation have been investigated through the use of anti-inflammatory drugs\(^{17-20}\), the benefits of an initial inflammatory phase have been recognized for clearing the wound of debris and initiating angiogenesis and the formation of granulation tissue\(^{6,21,22}\). Modulating the host response by targeting macrophages is a promising approach to promote proper wound healing and improve integration of biomaterial implants\(^{23,24,26}\). This thesis examined a model of the inflammatory response of macrophages to polymeric biomaterial surfaces\(^{27}\) and applied this model to assess the immunomodulatory properties of two molecules. The identification of an agent that is capable of modulating the inflammatory response could have significant implications for the acceptance of biomedical implants, leading to a decreased need for revision surgeries and a reduction in associated medical costs.

5.1 DAMP-adsorbed TCPS activates the NF-κB/AP-1 transcription factors in human dTHP1-XBlues

Previous research in the Fitzpatrick lab demonstrated the ability of lysate-adsorbed surfaces to activate mouse macrophage-like cells through the TLR2 pathway\(^{27}\). This thesis evaluated the ability of lysate-adsorbed TCPS to activate human dTHP1-XBlue macrophage-like cells, compared to human serum- and human plasma-adsorbed TCPS. As expected, serum and plasma had no effect on NF-κB activation, relative to the untreated control. Conversely, adsorbates derived from lysate or lysate-spiked serum or plasma significantly increased NF-κB-
dependent SEAP activity. While the spike-in conditions yielded modest increases in NF-κB activity, the lysate-only condition increased the SEAP activity of the cells to a similar level as 200 μg/mL Pam3CSK4, a TLR2 agonist, which was approximately 7 times greater than the negative control. Interestingly, the lysate-spiked plasma activated macrophages had lower NF-κB activity than the lysate-spiked serum, indicating that, compared to the proteins common between plasma and serum, plasma-specific proteins are competing more strongly with the lysate proteins for adsorption space. These results validate the ability of lysate-adsorbed surfaces to activate macrophages in a human model, even when lysate was present in only minute concentrations relative to blood-derived proteins.

5.2 The T6167923 small molecule MyD88 inhibitor’s effects on dTHP1-XBlues could not be properly evaluated due to precipitation

T6167923, a small molecule inhibitor, has previously been shown to disrupt the homodimerization of MyD88\textsuperscript{97}, which is a critical step for signal transduction\textsuperscript{101}, effectively blocking any activation of cells through the MyD88 pathway. dTHP1-XBlues were seeded onto protein-adsorbed TCPS and treated with a range of concentrations of the T6167923 inhibitor (50 μM – 500 μM) to evaluate its effects on the NF-κB/AP-1-dependent SEAP activity. Interestingly, no consistent results were seen with these experiments; SEAP levels decreased only in lysate-spiked plasma with 250 μM, and in poly(I:C) with 250 and 500 μM. The decrease seen in the poly(I:C) condition was unexpected, as this is a TLR3 ligand and is MyD88-independent\textsuperscript{69}. Furthermore, the inhibitor induced an increase in NF-κB activation for some conditions: all treatments except for the 200 μM concentration increased activation levels on lysate, and levels increased on Pam3CSK4 with 50, 100 (in the first of two experiments), and 500 μM of the
molecule. All other conditions and inhibitor concentrations resulted in no significant differences with inhibitor treatment, compared to the untreated controls.

A major challenge in this series of experiments was the solubility of the inhibitor in cell culture media. The small molecule inhibitor was suspended and stored in 100% DMSO and, upon addition to cell culture media, it formed a precipitate at all investigated concentrations. Multiple attempts to troubleshoot the inhibitor solubility failed to resolve the precipitate formation. In the experiments, the particles settled at the bottom of the well, which could have caused macrophage activation via phagocytosis, accounting for the increased NF-κB-dependent SEAP activity with certain conditions. The inconsistent results were likely a combination of the compound being processed by the cells, as well as being activated by the particles accumulated at the bottom of the wells. The unavoidable precipitation of the T6167923 compound suggests that further research would have to be performed to identify an appropriate method of evaluating its effect on the MyD88 pathway in cells.

5.3 MaR1 appears to have an effect on TLR2-dependent macrophage activation but is highly sensitive to degradation

Literature on MaR1 has demonstrated that this molecule was capable of reducing inflammation by limiting PMN infiltration and pro-inflammatory cytokine release, as well as encouraging macrophages to switch towards a pro-resolution phenotype. The initial goal of this thesis was to investigate MaR1 as a potential candidate for modulating the inflammatory response to polymeric biomaterials. However, there was significant difficulty in reproducing the positive effects reported in literature.

The results seen with RAW-Blues were inconsistent; the first treatment scheme (pre-treatment with MaR1 for 30 minutes, then activation with LPS) produced opposing results
between the two times it was tested, and the second treatment scheme (stimulated with LPS, then media was replaced and freshly supplemented with MaR1) demonstrated a dose-dependent decrease in NF-κB-dependent SEAP activity, but an increase in TNF-α release at the highest dose (2 μM). However, it was unclear if the variable results were due to the cell source and experimental measure (i.e. NF-κB activity) selected or due to MaR1 itself, as its mechanism of action is not yet known\textsuperscript{89}, the instability of the molecule\textsuperscript{86} may have led to degradation, and mouse cell lines have not commonly been used for testing MaR1 \textit{in vitro}.

Preliminary experiments were performed with dTHP1-XBlues as well, which produced results similar to what has been reported in literature\textsuperscript{79,90,94,95}; the 100 nM concentration of MaR1 was the most effective in reducing NF-κB-dependent SEAP activity. However, subsequent experiments with these cells were performed using the second vial of MaR1 purchased, which appeared to degrade unexpectedly. Two experiments performed using this second vial of MaR1 showed a slight but dose-dependent increase in NF-κB-dependent SEAP activity. The highly variable results obtained with MaR1 suggested the molecule was degrading over time. To test this hypothesis, a new vial of MaR1 was purchased and was compared to an aliquot (second vial, aliquoted but unopened for 10 months) and a working aliquot (second vial, opened, diluted, and prepared as usual for an experiment) using ESI-MS. The ESI-MS spectra indicated that while the fresh MaR1 and aliquoted MaR1 samples were mostly undegraded, the working aliquot sample was almost completely degraded.

A final experiment was performed using a fresh, unopened vial of MaR1 with dTHP1-XBlues and hMDMs. The cells were treated with MaR1 and then activated on protein-adsorbed TCPS. As expected, the dTHP1-XBlues demonstrated a decrease in both TNF-α and IL-8 cytokine release for most conditions. The exception was that there was no change in IL-8
cytokine expression for cells activated with LPS, which is a TLR4 agonist. This indicates that MaR1 may affect TLR2-dependent macrophage activation, as cells activated with Pam3CSK4 and lysate-adsorbed proteins both significantly decreased pro-inflammatory cytokine release with MaR1 treatment.

The results obtained with hMDMs demonstrated increased release in TNF-α but a decrease in IL-8 for LPS-activated cells when treated with MaR1, and increased IL-8 expression for cells activated by lysate-adsorbed proteins. These results suggest that MaR1 may increase TLR2 activation in hMDMs. However, achieving an appropriate cell count presented difficulties, and for this reason cells were used after a 4-day incubation as opposed to the usual 7 days, which could have resulted in a mixed population of macrophages and monocytes.

5.4 Future work

Despite the difficulties encountered with using the T6167923 small molecule inhibitor, the results seen in literature suggest this compound can effectively inhibit MyD88 signal transduction if it can stay in solution. The fact that many conditions remained unchanged or showed a decrease in NF-κB-dependent SEAP activity when treated with this molecule is also encouraging, as it suggests that although precipitated, the compound was able to decrease NF-κB activity in some conditions. The effects of this compound should be evaluated by looking at an earlier point in the signalling pathway, which would provide confirmation about the ability of the T6167923 compound to inhibit MyD88 signalling. In addition, it would be beneficial to further investigate a treatment model using this compound that prevents precipitation. The use of a drug delivery system could be explored to deliver the compound directly to cells, possibly avoiding precipitation of the compound.
MaR1 also yielded promising results, however its susceptibility to degradation makes this molecule technically challenging to work with and raises significant questions regarding the feasibility of this molecule as an exogenous therapeutic. Future research should consider only using fresh, unopened vials for experiments. Otherwise, preparing samples in a hood or chamber that has a continuous flow of nitrogen could prevent the molecule from degrading when the vial is open. Although the possibility of the molecule undergoing hydrolysis is difficult to avoid due to the composition of cell culture media, preventing oxygen from interacting with the molecule until it is added to cells could avoid significant degradation. Different preparation methods could also be explored and compared. The methods in this thesis followed the Serhan research group’s suggested process of sonicating for 10 seconds followed by vortexing for 30 seconds. However, adjustments to these times may produce reliable results with a potential for less degradation. Future studies should also investigate the use of a drug delivery system that would encapsulate the molecule and protect it from degradation.
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Appendix A - Queen’s University HSREB approvals

QUEEN’S UNIVERSITY HEALTH SCIENCES & AFFILIATED TEACHING HOSPITALS RESEARCH ETHICS BOARD (HSREB)

HSREB Renewal of Ethics Clearance

June 14, 2017

Dr. Lindsay Fitzpatrick
Department of Chemical Engineering
Queen’s University

ROME/O/TRAQ #: 6018531
Department Code: CHEM-006-16
Study Title: Characterizing transitional and turbulent blood flow conditions
Review Type: Delegated
Date Ethics Clearance Effective: June 24, 2017
Ethics Clearance Expiry Date: June 24, 2018

Dear Dr. Fitzpatrick,

The Queen’s University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application. This study, including all currently approved documentation, has been granted ethical clearance until the expiry date noted above.

Prior to the expiration of your ethics clearance, you will be reminded to submit your renewal report through ROMEO. Any lapses in ethical clearance will be documented below.

Yours sincerely,

[Signature]

Chair, Health Sciences Research Ethics Board

The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2), the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations, Canadian General Standards Board, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP). Federalwide Assurance Number: FWA#: 00004184, IRB#: 00001173

HSREB members involved in the research project do not participate in the review, discussion, or decision.
HSREB Renewal of Ethics Clearance

June 18, 2018

Dr. Lindsay Fitzpatrick
Department of Chemical Engineering
Dupuis Hall, Room 302
Queen's University

ROMEO/TRAQ: #6018536
Department Code: CHEM-005-16
Study Title: Investigation of innate immune responses to biomaterials
Review Type: Delegated
Date Ethics Clearance Effective: June 20, 2018
Ethics Clearance Expiry Date: June 20, 2019

Dear Dr. Fitzpatrick,

The Queen's University Health Sciences & Affiliated Teaching Hospitals Research Ethics Board (HSREB) has reviewed the application. This study, including all currently approved documentation has been granted ethical clearance until the expiry date noted above.

Prior to the expiration of your ethics clearance, you will be reminded to submit your renewal report through ROMEO. Any lapses in ethical clearance will be documented below.

Yours sincerely,

[Signature]
Chair, Health Sciences Research Ethics Board

The HSREB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP), Part C, Division 5 of the Food and Drug Regulations, Part 4 of the Natural Health Products Regulations, Part 3 of the Medical Devices Regulations, Canadian General Standards Board, and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The HSREB is qualified through the CTO REB Qualification Program and is registered with the U.S. Department of Health and Human Services (DHHS) Office for Human Research Protection (OHRP). Federallywide Assurance Number: FWA#00004184, IRB#: 00001173

HSREB members involved in the research project do not participate in the review, discussion or decision
Appendix B - T6167923 compound came out of solution when added to cell culture media containing dTHP1-XBlues

dTHP1-XBlues were treated with the T6167923 MyD88 small molecule inhibitor, which was suspended in DMSO and came out of solution when added to the cell culture media. Figure B-1A shows the negative control containing no precipitate. The cells in Figure B-1B were treated with 100 μM of the inhibitor and shows very little precipitate but is slightly darkened compared to the negative control. Figure B-1C shows cells treated with 250 μM of the inhibitor with a clear accumulation of precipitate, and even more showing in Figure B-1D when cells were treated with 500 μM.

Figure B-1. dTHP1-XBlue cells treated with the T6167923 small molecule MyD88 inhibitor at (A) 0 μM, (B) 100 μM, (C) 250 μM, and (D) 500 μM for 20 hours. Scale represents 400 μm.
Appendix C - Preliminary treatments of RAW-Blues with MaR1

RAW-Blue cells were activated with 1 μg/mL LPS and treated with 100 nM MaR1 in three treatment schemes: (1) LPS and MaR1 were added to cells simultaneously (Figure C-1A), (2) cells were pretreated with mar1 for 30 minutes before adding LPS (protective treatment, Figure C-1B), and (3) cells were activated with LPS for 30 minutes before treating with mar1 (reactive treatment, Figure C-1C). No significant difference was found between the conditions for any of the treatment schemes.

Figure C-1. Preliminary evaluation of MaR1 effects on NF-κB/AP-1-related SEAP activity of RAW-Blue cells. RAW-Blue cells were (A) activated with 1 μg/mL LPS and treated with 100 nM MaR1 simultaneously, (B) treated with 100 nM MaR1 for 30 minutes before being activated with 1 μg/mL LPS, and (C) activated with 1 μg/mL LPS for 30 minutes before being treated with 100 nM MaR1. Experiments incubated for 20 hours prior to measuring SEAP activity in the culture supernatant. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control for all experiments. MaR1 used was from the first batch purchased. (n = 3 per condition, per experiment)
Appendix D - MaR1 treatment scheme 2 applied to dTHP1-XBlues, after degradation

dTHP1-XBlues were treated according to treatment scheme 2, then analyzed using an NF-κB/AP-1-related SEAP assay (Figure D-1). As seen with treatment scheme 1 (Figure 4-16B), the results showed no decrease in cell activation for any of the MaR1 concentrations, and instead demonstrated an increase in activity (p<0.001) with 1 μM and 2 μM concentrations of MaR1.

**Figure D-1.** MaR1 treatment scheme 2 with dTHP1-XBlue cells. NF-κB/AP-1-related SEAP activity was analyzed for dTHP1-XBlues that were activated with 1 μg/mL LPS for 20 hours, following pre-treatment with 100 nM, 500 nM, 1 μM, and 2 μM. Untreated cells were used as the negative control and cells treated with only 1 μg/mL LPS were used as the positive control. The MaR1 used was from the second batch, 10 months post- aliquoting. (n = 3, *** p < 0.001)
Appendix E - ELISA standard curves with 4 parameter logistics (4PL) curves

Figure E-1. LEGEND MAX mouse TNF-α ELISA standard curve. 4PL curve fit was used to analyze cytokine release in RAW-Blue cells treated with MaR1.

\[ y = 5.0835915 + \frac{0.045564242 - 5.0835915}{1 + \left(\frac{0}{0.0305882}\right)^{1.1456494}} \]
\[ R^2 = 0.9999 \]

Figure E-2. LEGEND MAX human IL-1β ELISA standard curve. 4PL curve fit was used to analyze cytokine release in hMDM cells treated with MaR1.

\[ y = 551624 + \frac{0.079713185 - 551624}{1 + \left(\frac{0}{8.0796237}\right)^{1.0279995}} \]
\[ R^2 = 0.9977 \]
**Figure E-3.** LEGEND MAX human IL-8 ELISA standard curve. 4PL curve fit was used to analyze cytokine release in hMDM cells treated with MaR1.

\[
y = 59.066104 + \frac{0.02399973 - 59.066104}{1 + \left(\frac{x}{17363.232}\right)^{0.10147005}} \\
R^2 = 1
\]

**Figure E-4.** ELISA MAX Deluxe human TNF-α ELISA standard curve. 4PL curve fit was used to analyze cytokine release in dTHP1-XBlue and hMDM cells activated on DAMPs and treated with MaR1.

\[
y = 3.5145883 + \frac{0.016453372 - 3.5145883}{1 + \left(\frac{x}{5073562}\right)^{1.157288}} \\
R^2 = 0.9998
\]
Figure E-5. ELISA MAX Deluxe human IL-8 ELISA standard curve. 4PL curve fit was used to analyze cytokine release in dTHP1-XBlue and hMDM cells activated on DAMPs and treated with MaR1.
Appendix F - ESI spectra of MaR1 aliquots

Figure F-1. ESI-MS spectrum of Control sample of MaR1 (third lot purchased, previously unopened). The m/z peak at 359.22 represents intact MaR1, while all other peaks represent degraded forms of MaR1.
Figure F-2. ESI-MS spectrum of Aliquot sample of MaR1 (second lot purchased, previously aliquoted at 100 μM and unopened for 10 months). The m/z peak at 359.22 represents intact MaR1, while all other peaks represent degraded forms of MaR1.
Figure F-3. ESI-MS spectrum of Working Aliquot sample of MaR1 (second lot purchased, previously aliquoted and then diluted to 10 μM, sonicated for 10 seconds, and vortexed for 30 seconds to replicate standard experimental preparations). The m/z peak at 359.22 represents intact MaR1, while all other peaks represent degraded forms of MaR1.