Dissertation

AN EVALUATION OF BIOLOGICAL RESPONSES TO MODEL BIOMATERIALS *IN VIVO* AND *IN VITRO*

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LISA CHAMBERLAIN ENTITLED AN EVALUATION OF BIOLOGICAL RESPONSES TO MODEL BIOMATERIALS *IN VIVO* AND *IN VITRO* BE ACCEPTED AS FULLFILING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

AN EVALUATION OF BIOLOGICAL RESPONSES TO MODEL BIOMATERIALS *IN VIVO* AND *IN VITRO*

The use of *in vitro* and *in vivo* models to study inflammatory responses is extremely common in the pre-clinical evaluation of implantable materials and anti-inflammatory drugs. In this body of work we performed comparative studies of the inflammatory responses elicited by different biomaterials when interacting with cells in the implanted host. The results demonstrate first the non-equivalence between immortalized cell lines and primary-derived cell types in the inflammatory response and second temporal effects on differences in responses. Additionally, differences between *in vivo* and *in vitro* models are clearly demonstrated, and potential differences between our *in vivo* and other published models are seen. The results obtained from this comparative study will help to explain many discrepancies found between previous studies reported in the literature.

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CHAPTER 1: MACROPHAGE INTERACTIONS WITH IMPLANTED MATERIALS

This chapter was written by Lisa M. Chamberlain and edited by Mercedes Gonzalez-Juarrero.

1.1 Introduction

Biomaterials are widely used in medicine. All biomedical devices including pacemakers, stents, contact lenses, and total joint replacements are made from different types of biomaterials, and all interact with cellular and acellular components of the body.[1] The results of these interactions can range from completely benign, to adverse biological reactions, inflammation, and/or failure of the device. With the introduction of micro- and nano-materials into this realm, the cellular responses elicited to smaller and smaller sizes of biomaterials are being explored and a greater understanding of the underlying biological processes involved in these responses are needed.

Upon implantation (via surgery or injection), all materials immediately adsorb a layer of host proteins on their surface.[2] This layer of host proteins mediates all subsequent interactions between the material and the host. One of the major cellular players in the response to implanted materials is the macrophage cell type. Macrophage cells are highly differentiated cells of the mononuclear phagocyte system; these cells start as hematopoietic stem cells in the bone marrow, and mature into monocytes in the blood

prior to moving into the tissue as macrophages. At the site of an implanted material, recruited macrophages adhere to or ingest the foreign objects and typically this process triggers activation of these cells. A greater understanding of macrophage response to materials on the macro, micro, and nano scales is vital to the engineering of these materials for therapeutic uses.

In the medical field biomaterials are largely used on a macro scale, and the biological response to materials at this level is well documented with both *in vitro* and *in vivo* studies. *In vivo* biological responses to implanted materials results in the fusion of macrophages at the surface of the implant, and the eventual surrounding of the implant within a collagenous capsule, the thickness of which seems to depend on the type of material used. *In vitro* testing of novel biomaterials with macrophages has a history reaching back at least a decade in the literature,[3-9] and has similarly demonstrated cytokine expression by macrophages in response to surfaces at varying levels depending on the particular surface.[10-13]

1.2 The macrophage

Macrophage cells represent a highly differentiated cell phenotype of the mononuclear phagocyte system that orchestrate host inflammatory response and wound healing.[14] These cells originate in the bone marrow as monoblasts and promonocytes which mature into a peripheral blood monocyte and finally differentiate into a tissue-resident macrophage (Figure 1.1). Cells exist along this maturation pathway in a gradient of immature to mature phenotypes.[14] Macrophages are found in many different tissue

microenvironments where they perform multiple functions and differentiate into further subcategories of tissue and pathological specific phenotypes. These subcategories of macrophages serve the specific needs of their location. Alveolar macrophages for example are found in the lung and as one of the first lines of defense against inhaled pathogens are very active phagocytes. Populations of alveolar macrophages are enriched in CD14+, CD16+ cells and appear to be self replicating. A different population of macrophages found in the liver called Kupffer cells express Fc receptor, the mannosyl-fucosyl receptor, CD14, and CD33. Kupffer cells clear particulate and soluble substances from the liver and their activation triggers the recruitment of large numbers of monocytes.[14] Several subsets of macrophage are found in the central nervous system. These include microglia, perivascular macrophages, meningeal macrophages, and choroid-plexus macrophages.[15] These all serve tissue-specific roles of innate immunity in their respective locations. Splenic macrophages aid in the removal of blood-borne pathogens and express pattern-recognition receptors as well as scavenger receptors.[14,15] At sites of inflammation, yet another subset of macrophage exists. As monocytes are recruited to inflammatory lesions, they are differentiated into macrophages which in combination with lymphocytes direct the inflammation. These inflammatory monocyte derived macrophages can be activated by different stimuli to produce different activation states.[15]

1.3 Macrophage activation

Classically, immune and inflammatory responses have been divided into two distinct types on the basis of cytokine profiles secreted by immune cells. Initiation of both types of inflammation is thought to be highly regulated by macrophages.[14] 'Classical' activation occurs in macrophages in response to lipopolysaccharide, a bacterial cell wall

component, and IFNγ.[16] Cells of the T helper 1 (TH1) lineage are associated with type of activation, also termed TH1-type responses. Classical immune responses are characterized by production of potent activators of cell-mediated immunity including the cytokines TNF-α, IL-12, IFNγ, and IL-8. 'Alternative' activation of macrophages, associated with cells of the T helper 2 (TH2) lineage, also termed TH2-type responses occurs in response to TH2-associated cytokines, such as IL-4, IL-5, IL-10 and IL-13, all potent activators of B-cell immunoglobulin production.[17]

Immune pathogenesis that results from deregulated TH1 responses can promote tissue destruction and chronic inflammation whereas deregulated TH2 responses can cause allergy and asthma.[17] Recently, this nomenclature has been adapted to the responding macrophage populations which can polarize under these conditions into either M1 or M2 populations, each specialized to respond optimally to the type of inflammatory response occurring.[18] Classically activated macrophages (M1) are highly phagocytic and release many proinflammatory cytokines and substances. In a TH2 immune response, the cytokines present lead to "alternatively" activated macrophages (M2) and an immune response that is effective against parasitic organisms. Alternatively activated macrophages release large amounts of cytokines and molecules that induce cellular proliferation and extracellular matrix production.[19] Some cytokines released by macrophages (e.g., TNFα, IL-12, IL-8, and IL-1) are termed inflammatory cytokines because they actively participate in the inflammatory responses or recruitment of cells to sites of both inflammation types.[20] Other soluble immuno-modulators, the chemokines (e.g., monocyte chemotactic protein (MCP-1)) are chemoattractant substances that function to attract and regulate the traffic of immune cells to the foreign body site.

1.4 Macrophage adhesion to surfaces

Macrophages are highly efficient at phagocytosis, thus their latin name translation of "big eater." This ability to adhere to small particles and ingest them is reflected in their ability to bind to and proliferate on surfaces that cause other adhesion-dependant cells to ball up and die.[21] Multiple receptors are involved in macrophage adherence to surfaces, the particular repertoire of proteins, carbohydrates, and other molecules determining the exact receptors used. *In vitro* studies have demonstrated that macrophage attachment to surfaces can involve multiple integrins,[22,23] and sugar-binding receptors,[24] which serve biologically as ways for the cells to adhere to ECM, ingest bacteria, and to ingest debris from necrotic and apoptotic cells. When interacting with a biomaterial *in vivo,* the protein layer adsorbed to the material allows for interactions between the material and macrophage surface receptors. As adherence of macrophages at this interface is highly dependent on the density and type of proteins present, and the species and density of protein adsorption to a surface is highly material dependent, it can be reasoned that macrophage activation following adhesion is also highly material dependent. The physical characteristics of a surface can also impact macrophage activation. As an example, the commonly used J774A.1 macrophage-like cell line when grown on rough titanium surfaces significantly change their production of IL-1β, IL-6, and nitric oxide when compared to those cells grown on smooth surfaces of the same material.[25]

1.5 Macrophage uptake of particles

As clinically relevant uses of micro- and nano- materials increase, the importance of understanding the biological response to materials of those sizes increases as well. Phagocytosis of objects by macrophages is a highly regulated process, involved in the ingestion of invading microorganisms, and cellular debris.[26] It has been demonstrated

that blocking receptors involved in small particle uptake does not inhibit macrophage adhesion to surfaces, implying that different receptors are involved in the two proccesses.[27,28] However, recent studies have indicated that these processes may be linked to some degree. When a macrophage interacts with a particle that has a high local curvature the cell will try to ingest the particle, but if a macrophage interacts with a particle of the same material that has low local curvature the macrophage will not try to ingest the particle, but seems to try to adhere to it.[29] These data point out the possible involvement of cellular skeleton dynamics in the alternate pathways of ingestion versus adhesion.

Ingestion of particles can be activating to macrophages, and there are definite material and possibly size dependence to it. Human peripheral blood monocytes (PBMC) derived macrophages release significantly more inflammatory cytokines following incubation with CoCr or alumina, while submicron ultra high molecular weight polyethylene (UHMWPE) induces little expression.[30] Size dependence can be seen from experiments *in vitro* where UHMWPE particles are incubated with PBMC. Larger particles (1.7μM to 88μM) caused little PGE_2, TNF-α, IL-6, or GMCSF, secretion while smaller particles (0.24μM to 0.45μM) induced secretion of these compounds at levels similar to LPS stimulation.[31] A contrasting *in vitro* study demonstrated little activation following incubations of PBMC derived macrophages incubated with UHMWPE particles with mean diameters of 0.48 μM. Analysis of media using protein chips indicated no change in TNF-α, but increases in IL-6 and IL-8.[30] This either implies that the macrophages can discern between particles with very slight size differences, activating at sizes below 0.45μM while showing little response to sizes of 0.48μM. Yet other studies found no increase in inflammatory cytokines by biodegradable microspheres, but did see an associated lengthened time of antigen stimulation by macrophages.[32]

The effects of nanotopographies on macrophages have demonstrated few changes in the expression of inflammatory cytokines, although an increase in the expression of fibronectin has been observed.[33] When looking at nanoparticles however, carbon nanoparticles induced macrophages express to produce more TNF-alpha and ICAM-1, an effect that was exacerbated by the addition of iron to the particles.[34] Some of these effects may have *in vivo* implications as studies have demonstrated that while inhalation of multi-walled carbon nanotubes does not result in lung impairment, it can impair immune function within the lung.[35] There are many differences in the responses of macrophages to surfaces versus particles. Attachment of cells to surfaces can involve a multitude of different cell surface receptors depending on the material. However, the uptake of nanoparticles of differing chemistries has been demonstrated by a single surface protein. For example it is known that the macrophage scavenger receptor with collagenous structure (MARCO) is involved in the uptake of TiO_2, Fe_2O_3, and polystyrene beads from 20nm to 1μm.[28] As more materials are used at the micro- and nano-scales, the importance of understanding cellular interactions with them will continue to grow.

1.6 Protein adsorption to materials

Several factors are involved in protein adhesion from an aqueous milieu to a solid interface. The bulk protein concentration of the solution, the surface concentration, the on and off-rate are all involved in the initial kinetics of protein adhesion. The on-rate is related to the bulk concentration as well as the diffusion constant of the protein while the off-rate of a protein is related to the spreading pressure of the protein and it's denaturation on the surface. Spreading proteins will have larger contact points with the surface and will tend to have slower off rates than proteins with less spreading pressure. Proteins which begin a denaturizing process on the surface are also likely to have slower

off rates, particularly when globular proteins with hydrophobic cores come in contact with hydrophobic surfaces.[2]

As material sizes decrease and approach nanoscale dimensions, they vastly increase their surface area while attaining similar volumes to proteins that would normally adsorb to the material.[36] Adsorption of protein to a material surface occurs immediately upon its interaction with a protein solution.[2] This adsorbed layer rapidly changes *in vivo* and *in vitro* and can vary significantly depending on the material being used, with certain materials adsorbing particular proteins non-proportionally to the bulk solution.[37,38] The protein layer adsorbed to a surface has clear effects on cell adhesion, and the use of pre-adsorbed proteins to induce or inhibit cellular adhesion from mammalian and bacterial cell sources is well documented.[21,37,39] The type of surface involved in protein adhesion to a substrate greatly influences the surface residency of an adherent globular protein. The on and off-rates of a protein depend on the attraction of the protein to the surface and its diffusion rate in solution[40]. As an example, fibronectin adsorption to different surfaces differs greatly. Thus, when serum is incubated with polystyrene (PS) or "tissue culture" treated polystyrene (TCPS, PS that has been plasma treated making it more hydrophillic), fibronectin deposition from serum is much greater onto the more hydrophilic TCPS than onto PS.[41] This would indicate a faster off-rate for fibronectin on PS and a longer off-rate on TCPS. The higher off-rate of fibronectin from PS would indicate a very dynamic surface with many molecular-level changes in the protein coat occurring, this state is reflected in fibroblast adhesion and spreading to PS surfaces. Fibroblast adhesion and spreading is largely dependant on contacts with fibronectin, and with the faster off-rate of fibronectin, fibroblast spreading on PS of fibroblasts is much less than fibroblast spreading and adhesion on TCPS.[40]

This model also correlates in the case of TCPS and Teflon AF. We can describe the molecular events involved in protein adsorption to the surface and the resulting support or non-support of fibroblast growth. TCPS, as mentioned before has been plasma treated, making the surface relatively hyrophillic and increasing its wetability, whereas Teflon-AF is a highly hydrophobic polymer. Upon initial exposure to serum proteins, albumin will be the predominant protein adhered on both surfaces due to its high concentration.[2] However the hydrophobicity of Teflon-AF will allow for more spreading and denaturation of albumins hydrophobic core on its surface, thus reducing the albumin off-rate. TCPS on the other hand, as a more hydrophilic surface will have a greater albumin off-rate and will eventually gain a coating which includes other serum proteins as well.

Fibroblast adhesion to the resulting protein layers on TCPS and Teflon-AF is quite different between the two materials.[42] The albuminated surface of Teflon-AF is non-supportive to fibroblast growth and development. The cells cannot adhere to the surface, and due to their adherence dependant nature, they die. TCPS on the other hand, with it's more diverse protein coat provides many adherence points for the fibroblasts and they thrive on this surface.[42] It is important to note however, that when these principles are transferred to other cell types, different results occur as in the case of monocyte/macrophage cells which adhere well to the albuminated surfaces of Teflon-AF.[21,23,43-45]

1.7 The wound healing environment

The creation of a wound initiates with a primary acute inflammation step, a short-lived process which serves to start the process of wound healing. After initial vasoconstriction of the wound site and the formation of a scab, vasodilatation of the area is triggered by release of cytoplasmic granules by mast cells. This results in infiltration of fluids and immune cells to the wound site which helps to dilute toxic agents in the wound as well as to clear infective agents and necrotic tissue. Acute inflammation can last from minutes to days depending on the severity of the injury. Chronic inflammation follows the acute phase and is responsible for completing the wound healing process.[46]

The major cellular players in chronic inflammation are lymphocytes, macrophages, and plasma cells. Lymphocytes are recruited to the site by cytokines and chemokines produced during acute inflammation which serve to direct and recruit other cells involved in the chronic inflammatory response. Chemotactic factors attract macrophages to the site of inflammation, and IFN-γ (a macrophage activating factor) serves to increase the phagocytic activity of macrophages. Macrophages serve two primary roles in chronic inflammation, phagocytosis of foreign bodies and cell debris as well as production of several secreted products involved in wound healing and clearing of necrotic tissue. Plasma cells are cells which aid in the clearance of foreign bodies by the production of antibodies. Macrophages have surface receptors for Fc portion of antibodies and complement proteins. Binding of these surface receptors with their respective proteins will increase the rate of phagocytosis by the macrophage[46].

The large collection of soluble protein mediators of inflammation collectively referred to as cytokines serve multiple purposes during chronic inflammation. IL-1 is a cytokine produced by multiple cell types which strongly promotes proliferation of lymphocytes,

and is necessary for their activation. IL-2 is largely produced by lymphocytes, and like IL-1 also promotes lymphocyte proliferation. IFN-γ, a cytokine produced by activated lymphocytes functions as a strong signal to recruit macrophages and serves as a strong migratory signal. Migration inhibitory factor (MIF) is also produced by activated lymphocytes (primarily T-cells) and functions similarly to IFN- γ in its ability to recruit macrophages and keep them at the site of inflammation. MIF can also play a role in macrophage activation. TGF-β1 is a pleiotropic (multifunctional) cytokine, it functions as a chemoattractant for lymphocytes, an inducer of angiogenesis, a regulator for the production of other cytokines, and an inhibitor of extracellular matrix (ECM) degradation. Overproduction of TGF-β1 can allow for overproduction of ECM and result in scarring and fibrosis. Activated macrophages produce TNF-α which serves several functions. TNF-α binds to "death domains" on certain cell types resulting in apoptosis of the cell, and so functions in tissue remodeling. A protein related to TNF-α, TNF-β, is produced by several types of activated lymphocytes. In addition to induction of apoptosis, TNF-β also activates angiogenesis and can be involved in macrophage activation[46].

1.8 *In vivo* responses to implanted materials

The pathology of *in vivo* responses to implanted materials has been well documented however the molecular mechanisms that triggers the foreign body response (FBR) are not well understood. An important contributing factor to the FBR is its presence within a wound healing event, initiated by the implantation of the biomaterial. In these surroundings proteins from plasma and disrupted ECM are the first moieties that encounter the material. Adsorption of proteins to the surface of the implant occurs quickly, creating a material-specific layer that will serve as the bio-interface for the duration of the FBR.[2,47] Over the first 1-3 days following implantation, neutrophils,

granulocytes, and monocytes are recruited to the wound site.[1] These are the first cells to encounter the foreign body and their numbers are reduced greatly as the first stages of wound healing complete.[48] Macrophages at the site are sourced from differentiated monocytes and recruited from nearby tissue.[49] They are highly immunomodulatory, and are thought to become alternatively activated near the site of the implant, initiating new cytokine cascades that cause normal wound healing to go awry.[10] This alternate course of events is referred to as the FBR. During their residence at the surface of the implant, macrophage cells begin to fuse with each other to form large, multinucleated, foreign body giant cells (FBGC) which are the hallmark of the FBR. The molecular events that cause macrophages to fuse into FBGC are not well understood, but *in vitro* studies have implicated two TH2 cytokines named IL-4 and IL-13 in their development.[50,51] As the FBR continues, recruited fibroblasts encapsulate the implant in an avascular, acellular, collagenous shell through signaling of TGF-β.[52] This effectively walls off the implant from the normal tissue environment while maintaining a continued attack against the foreign body via activated macrophages and FBGC trapped within (Figure 1.2).

1.9 Experimental design

The main goal of this body of work was to explore macrophage responses to biomaterials. There were four main aims within this goal. The first aim was to characterize the expression of cell derived signals and membrane receptors of primary derived macrophages and macrophage cell lines common to *in vitro* testing of biomaterials under standard culture conditions. Additionally, the expression of these proteins following exposure to physical (surface) and chemical (LPS) stimuli was explored. The second aim, was to observe phenotypic stability of macrophages in culture by comparing cells from short-term and longer term cultures, observing changes

in morphology, cytokine production, and surface receptor expression. The third aim was, to quantify rates of fusion for murine macrophage cells as a function of culture conditions. Our final aim was to characterize initial *in vivo* signaling events in response to material implantation by macrophages, monocytes, and foreign body giant cells, and to evaluate similarities and differences between *in vitro* and *in vivo* models.

To complete these aims, we utilized a murine model. In choosing this model, comparisons of the resulting encapsulation of foreign bodies by the foreign body response (FBR) to lesions found in tuberculosis (TB) were made. TB lesions and foreign body capsules both exhibit walling off of a foreign substance which macrophages have had trouble eliminating (mycobacteria or a biomaterial implant), as well as the presence of large multi-nucleate cells (langerhans cells and FBGC, respectively) in and around the site.[51,53] Current *in vivo* and *in vitro* TB research is largely performed in a murine model,[54,55] and the similarities between immune responses to TB and those involved in the FBR indicate that a similar model will likely work for studies of the FBR. Within mouse strains, there is very little biological variability.[56] However, considerable genetic variability exists between mouse strains.[57] Comparisons of gene expression between mouse strains in brain and muscle have exhibited relatively low variation in gene expression between inbred mouse strains. Due to these findings, we postulate that similar variability between non-purpose bred animals and the cells compared in this study.[58,59]

Choice of materials is central to studies of the FBR. In studies utilizing *in vitro* cell culture, materials used must be conducive to working within a laboratory setting. This means that they must be relatively transparent for visualizing morphology of adherent cells, and that they be easily sourced or created. For our purposes we utilized common

cell culture surfaces, polystyrene and tissue culture treated polystyrene (polystyrene that has been plasma treated) as control materials, due to their availability and ease of use. Additionally, two model biomaterials were utilized. One being non-biodegradable Teflon-AF®, which is similar to normal Teflon® but has an inserted block copolymer which makes it transparent and soluble by certain solvents, allowing it to be used to coat surfaces easily in a lab setting. The second model biomaterial is poly-L-lactide, a bio-resorbable biomaterial commonly used in bio-resorbable sutures. This material is easily solvated and can be used to solvent-cast glass dishes to create a culture surface, and is transmits light sufficiently to visualize adherent cells. Poly-L-lactide is available in many molecular weights, the higher of which take longer to degrade. We chose a 100KDa polymer for our studies.

In choosing the number of replicates for these studies, we utilized the method described by W.E. Berndston in 1991.[60] CV values within cell populations using flow cytometric methods were typically between 5 and 7. Using these numbers and the charts provided in the method we determined that in order to design an experiment of 80% power, with the potential to differentiate changes of 25% from the control, that a minimum of three replicates would be necessary.

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Figure 1.1: *Overview of macrophage differentiation.* Macrophages differentiate from hematopoietic stem cells in bone marrow which move into the blood stream as they mature into a monocytic phenotype. Certain signals will encourage the movement of these monocytes into the tissues where they will further differentiate into a macrophage phenotype.

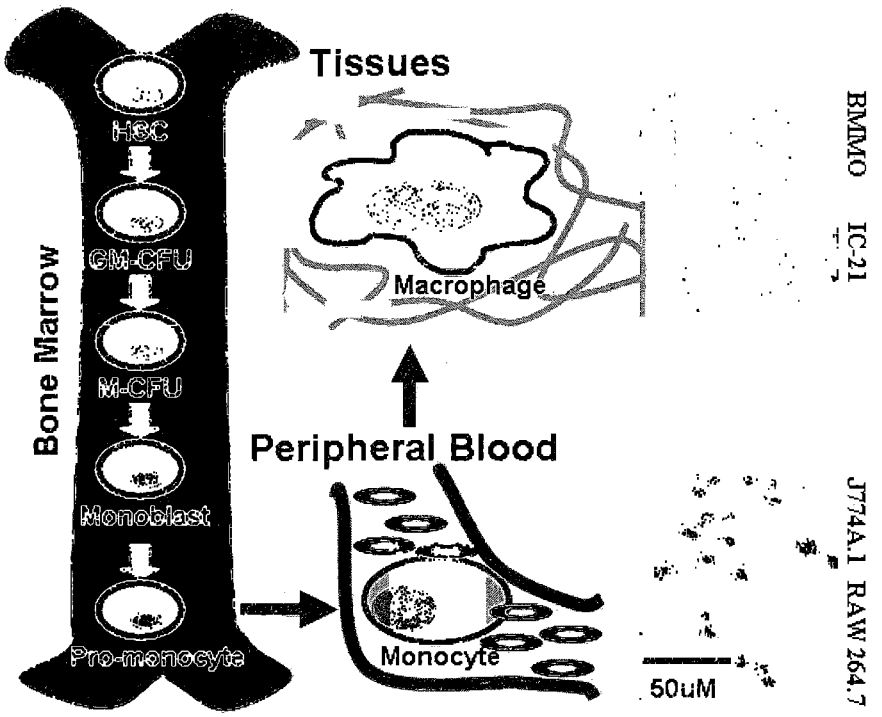
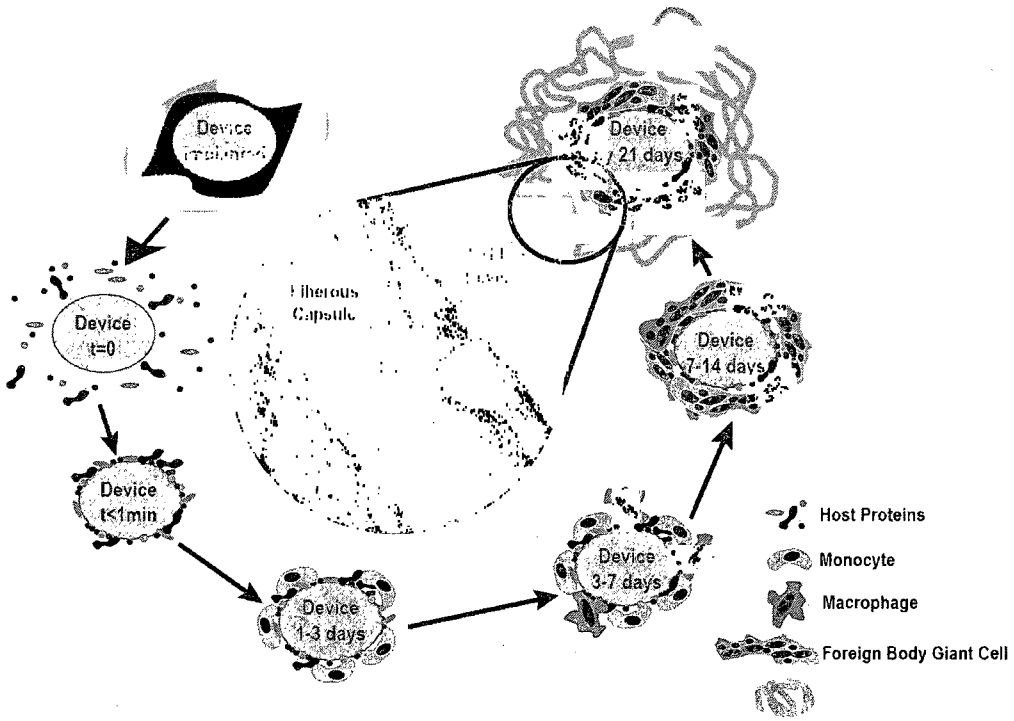


Figure 1.2: *Overview of the foreign body response.* Following device implantation, protein adsorption occurs at a very fast rate. As the wound healing process ensues, monocytes and eventually macrophages adhere to the protein coat and activate. Monocytes and macrophages fuse to form foreign body giant cells, and over time surrounding fibroblasts are activated. These fibroblasts replicate rapidly and produce a collagenous layer surrounding the implant.



CHAPTER 2: PHENOTYPIC NON-EQUIVALENCE OF MURINE (MONOCYTE-) MACROPHAGE CELLS IN BIOMATERIAL AND INFLAMMATORY MODELS

This chapter was written by Lisa M. Chamberlain, edited by David W. Grainger and Mercedes Gonzales-Juarrero, and contains contribtutions from Marisha L. Godek. It has been published in The Journal of Biomedical Materials Research Part A, 2009[1] (DOI: 10.1002/jbm.a.31930) and is reprinted here with permission of the journal.

2.1 Abstract

Cells of the mononuclear phagocytic system including monocytes and macrophages (e.g., pooled human monocytes, bone marrow derived macrophages, etc.) are often employed for *in vitro* assessment of novel biomaterials and to assay anti-inflammatory drug activity. In this context, numerous macrophage cells are treated interchangeably in the literature despite a lack of demonstrated equivalence among immortalized cell lines and further, between cell lines and primary-derived macrophages of different species. Three murine (monocyte-) macrophage cell lines (IC-21, J774A.1, and RAW 264.7), commonly utilized in biomaterial and pharmaceutical screening research, have been compared to primary-derived murine bone marrow macrophages. Significant differences were discovered in the expression of cell surface proteins requisite for cell adhesion and activation among cell lines and primary-derived cells as well as between the different cell lines. Results demonstrate activation but with reduced cytokine expression to chemical

stimulus (lipopolysaccharide) by cell lines compared to that of primary-derived macrophages. Limited correlation between cultured primary and immortalized cells in cytokine production, phenotype and intrinsic activation states has relevance to fidelity for *in vitro* testing. These differences warrant justification for selection of various cell lines for specific assay purposes, and merit caution if comparisons to primary cell types (i.e., for biocompatibility) are required.

2.2 Introduction

Animal models have been extensively utilized in pre-clinical studies of pharmaceuticals and biomaterials testing for nearly a century.[2] Inflammatory disease and related biomaterials/biocompatibility research have both exploited numerous *in vitro* and *in vivo* experimental systems required prior to human clinical trials. Due to an abundance of reagents and species-specific probes, cost effectiveness, and abundant gene knock-in and knock-out variants, murine models are often employed in survey and pre-clinical studies as the first step in the transition between *in vitro* models and pre-clinical trials. The prevalence of the murine model in pre-clinical *in vivo* studies has led to extensive use of murine cells as *in vitro* models.[3,4] Significantly, use of the same species for *in vivo* and *in vitro* studies allows identical assay reagents to be used, and permits facile comparisons of *in vitro* to *in vivo* data. However, equivalence of murine models to humans is frequently questioned; obvious differences in comparative disease pathology and healing, as well as physiology and anatomy exist (e.g., murine vs. human bone, skin, blood). Hence, human cells are often used for *in vitro* studies of inflammation, and many similarities between immortalized human monocytic cell lines (e.g., U937, THP-1) and primary human blood monocytes have been demonstrated.[5-7] Significantly, studies employing these immature phenotypes often require addition of a stimulant

phorbol ester (e.g., 12-O-tetradecanoylphorbol-13-acetate (TPA))[8,9] to promote differentiation to a mature, adherent phenotype. This further complicates the study of inflammatory outcomes related to these cell types given that these stimulant molecules are known to have specific effects on cellular behavior, including activation of protein kinase C, calcium release, and increases in proliferation.[9,10] Additionally, maturation protocols vary across the literature, with concentrations of TPA varying from 10 to 100 ng/mL, and incubation times ranging from hours to days.[7,8,11,12]

In contrast, murine cell lines offer the advantage of an immortalized, relatively stable, mature, adherent macrophage phenotype. The murine cell lines J774A.1, RAW 264.7 and IC-21 all exhibit the maturity markers F4/80 and Mac-1,[13,14] indicating their macrophage-like phenotype, and have been used for models of macrophage activation in numerous studies. [3,15-23] Thousands of published reports use these murine cells interchangeably as putative analogs to human macrophages without sufficient assessment of their functional changes according to their stage of differentiation.[3,15-22] To date, extensive characterization studies which examine basal expression levels of relevant biomarkers (extracellular matrix proteins, signaling proteins, cytokines, chemokines, etc.) in 2-D tissue culture systems employed for *in vitro* modeling have not been performed sufficiently on these models. Although several murine cell types have been repeatedly employed for *in vitro* studies,[16,22,24-26] limited validation by direct comparison to either primary murine macrophage cells or to each other has been reported. Thus, accurate comparisons between numerous studies using different immortalized murine cell lines are difficult, making precise relevance and fidelity to *in vivo* murine or human inflammatory responses indeterminable.

Macrophage cells represent a highly differentiated cell phenotype of the mononuclear phagocyte system that orchestrate host inflammatory response and wound healing.[27] These cells originate in the bone marrow as monoblasts and promonocytes which mature into a peripheral blood monocyte and finally differentiate into a tissue-resident macrophage. Cells exist along this maturation pathway in a gradient of immature to mature phenotypes.[27] *In vitro* studies commonly involve culture of commercially sourced macrophage cell lines on medical device surfaces or culture surfaces (e.g,, 96-well plastic plates).[19,28] Macrophage cells involved in inflammatory responses are activated by certain chemical (e.g., pathogenic) and physical (e.g., medical implant) stimuli, initiating the release of signaling molecules including cytokines and chemokines that activate other cell types and chemotactically recruit new cells to the site that play a role in healing and wound resolution.[29] As noted above, assessments of novel anti-inflammatory drugs and biomaterials regularly utilize the adherent (monocyte-) macrophage cell lines IC-21,[15-17] J774A.1,[3,18,19] and RAW 264.7,[20-23] to profile cytotoxicity, inflammatory response and biocompatibility. Current phenotyping of these cells indicates an intermediate monocyte-macrophage stage of development for J774A.1 cells and RAW 264.1 cells, and a mature macrophage phenotype for IC-21 cells.[30,31] Despite the abundance of inflammatory assays performed with numerous inflammatory cells, molecular phenotyping of these cells, as well as comparisons to assert relative equivalence to each other, to primary derived murine macrophages, or to human models has not been undertaken. As the desired endpoint for most anti-inflammatory pharmaceutical and biomaterials research and development is demonstrated efficacy and safety in humans, some confidence in reliability, fidelity and equivalence of *in vitro* testing is necessary. Developing a greater understanding

of the maturation, activation, and cytokine response of these cell lines will facilitate development of models for studies, and allow for greater equivalence between *in vitro* and *in vivo* studies.

Activation of macrophage cells can occur through multiple pathways, resulting in different types of inflammatory responses. Classical activation of macrophages is initiated by pathogens, (e.g., lipopolysaccharide (LPS), a gram-negative bacterial cell wall component), and the production of interferon gamma (IFN-γ) by T-helper 1 (TH1) cells.[29,32] This TH1 type response requires the interaction of LPS, the CD14 receptor and toll-like receptor 4 (TLR-4), and is propagated by macrophage production of the cytokines IL-12 and IL-18.[33,34] Alternative activation of macrophages involves T-helper 2 (TH2) cell production of IL-4 and IL-13, and primes macrophages to combat parasitic and extracellular pathogens. This type of macrophage activation results in the up-regulation of the macrophage mannose receptor (MMR), and induces the expression of cytokines and enzymes involved in granuloma formation.[29,32] Macrophage activation at or on the surface of biomaterial implants and other foreign bodies is ubiquitous and a major challenge in medical implant interfacing within the human body.[35] Inflammation derived from macrophages activated at biomaterial surfaces is similar to a TH2 type response: in a chronic phase, granulomatous tissue forms at the implant surface, often producing implant fibrous encapsulation within an avascular collagenous capsule.[35-37] Macrophages can also be activated innately *via* a humoral route through binding of antibody or opsonins to cell Fc or complement receptors.[32,38] Therefore, establishing a model system that reliably and accurately recapitulates essential cellular and molecular features of inflammatory signaling is essential for proper *in vitro* testing of

anti-inflammatory drugs and implanted biomaterials. Understanding the behavior and relevance of cellular components in these *in vitro* inflammatory models is critical.

The murine cell lines (IC-21, J774A.1, and RAW 264.7) and primary bone marrow derived macrophage (BMMΦ) cells were compared with respect to phenotype and cytokine production upon exposure to 1) a positive control stimulus (endotoxin, LPS) and 2) control and model biomaterial surfaces: tissue culture polystyrene (TCPS), polystyrene (PS), poly-L-lactide (PLLA) and Teflon®-AF, using *in vitro* assay techniques previously described (Scheme 2.1).[30,31] Results indicate that these cell types exhibit different phenotypes relevant to monitoring the foreign body response. Specifically these cells exhibit different activation states based on macrophage lineage (F4/80, CD14, and Fc receptor) and activation markers (CD40, TLR-4 and MMR). The cell lines tested, irrespective of maturity, do not reflect a primary-derived phenotype. Cytokine responses of cell lines to LPS and surface stimulation differ significantly from BMMΦ, with lower or no response by cell lines to LPS, and different cytokine profiles resulting from culture on model biomaterials.

2.3 Materials and Methods

2.3.1 Mice.

Specific-pathogen-free female C57BL/6 mice (6-8 weeks old, Jackson Laboratory, Bar Harbor, ME) were maintained in the biosafety level 2 facilities at Colorado State University, and given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments. The specific pathogen-free nature of the mouse colonies at these facilities is demonstrated by testing sentinel animals, shown to be negative

for 12 known mouse pathogens. Animal guidelines for the care and use of laboratory animals have been observed, all experimental protocols used in this study were approved by the Animal Care and Use Committee of Colorado State University.

2.3.2 Primary cell harvest.

Bone marrow cells were harvested from murine tibias and femurs and differentiated into macrophage cells using previously described methods.[31,39] Briefly, bone marrow cells were flushed from long bones, and then differentiated into a macrophage phenotype by incubating in complete DMEM (cDMEM, 10% heat inactivated fetal bovine serum (FBS, Hyclone®, Logan, UT), 10% L-929 fibroblast conditioned medium, 1% penicillin-streptomycin (Gibco, Carlsbad, CA), 0.01M Hepes buffer, 1 mM sodium pyruvate, and 1% of a 100X MEM non-essential amino acids solution (all Sigma) in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc, Herndon, VA). Cells were differentiated for 7 days with media changes every 2 days. All cell cultures were incubated under "standard conditions" (37℃, 5% CO_2, 98% humidity). Primary cells used in experiments are defined as mature bone marrow-derived macrophage cells from 7-day cultures (BMMΦ). Replicates were defined as cells sourced from different mice.

2.3.3 Immortalized murine cell culture.

Adherent murine (monocyte-)macrophage cell lines IC-21, J774A.1 and RAW 264.7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 and IC-21 cells were cultured in RPMI-1640 (Mediatech, Inc, Herndon, VA) and J774A.1 cells were cultured in DMEM (Mediatech, Inc, Herndon, VA) per vendor recommendations. All growth media used to culture cell lines were

supplemented with 10% FBS (Hyclone®, Logan, UT), 1% penicillin-streptomycin (Gibco, Carlsbad, CA), and 0.01 M Hepes buffer (Sigma). Cell cultures were maintained below 80% confluence in TCPS flasks and passaged by incubation with divalent cation free Dulbecco's Phosphate Buffered Saline (dPBS Hyclone®, Logan, UT) prior to scraping with a rubber policeman. All cells were used at or below passage number 30 as received from ATCC and incubated under standard conditions. Replicates were defined as cells harvested from different passages and/or flasks.

2.3.4 Biomaterials and surface preparation.

Model and control materials used in this study have been characterized previously for cell culture: standard tissue culture polystyrene (TCPS, 15 x 100 mm Petri dishes, Falcon®, BD Biosciences, San Diego, CA); polystyrene (PS, Corning Inc.); poly-L-lactide (PLA, Polysciences Inc., Warrington, PA) and Teflon-AF® (DuPont Fluoroproducts).[40] PS and TCPS were selected for their known differences in surface chemistries[39] that distinguish TCPS over PS as highly conducive to cell attachment and profileration, as well as for their ubiquitous availability and common use in various tissue culture protocols. Teflon-AF® surfaces were prepared as previously reported.[30,31] Briefly, 100-mm PS Petri dishes were coated with Teflon-AF® (3 mL of a 0.1% solution diluted from stock in 3M™ Fluorinert™ Electronic Liquid FC-40 solvent, 3M Corp. St. Paul, MN) prior to vacuum drying overnight at 65°C. PLA surfaces (50,000 MW) were prepared as described previously by solvent casting a 0.2% w/v solution of PLA in methylene chloride.[30,31] Glass Petri dishes (ø =100 mm) were coated with 10 mL of PLA solution, loosely covered, and allowed to dry in a fume hood for approximately one hour. Teflon-AF® and PLA-coated plates were misted with 70% ethanol in cell-grade water (Hyclone®, Logan, UT) in a

biosafety cabinet and sterilized by treatment with culture-hood UV light for 15 minutes (a process shown to have no detectable effect on surface chemistry).[41] All surfaces and cell culture materials were tested for the presence of contaminating endotoxin using a Pyrogene™ Assay kit (Cambrex, East Rutherford, NJ), and endotoxin levels were determined to be below the kit detection limit (0.02 EU/mL).

2.3.5 Cell culture on model surfaces.

All model and control surfaces were equilibrated with media containing 10% serum for approximately 4 hours prior to plating. BMMΦ, IC-21, J774A.1 and RAW 264.7 cells were removed from culture flasks by rinsing with divalent-cation free dPBS prior to scraping with a rubber policeman. Equilibration media were removed from surfaces, and cells were plated at sub-confluent levels (to avoid profiling of quiescent cells) on model and control surfaces and incubated for 36 hours under standard conditions, these conditions resulted in adherent cultures of approximately 80% confluence. Cells were lysed *in situ* for RNA collection.

2.3.6 Cell imaging.

Live adherent cells were photographed using phase contrast microscopy on a Nikon Eclipse TE 2000-U microscope (Nikon Inc., Torrance, CA), a Photometrics Coolsnap ES camera (Roper Scientific, Tucson, AZ), and Metamorph™ software (Molecular Devices, Downingtown, PA).

2.3.7 Flow cytometric analysis of surface markers.

Cells from adherent, sub-confluent cultures propagated on TCPS under standard conditions were assayed for expression of membrane proteins. Cells were scraped

from flasks after rinsing with divalent cation-free dPBS. After removal from culture surfaces, cell suspensions were incubated with purified monoclonal antibodies (MAbs) CD16/32 (clone 93, rat IgG2a anti-mouse, eBioscience, San Diego, CA) using at least 1 μg of MAbs per million cells in staining solution (dPBS with 1% FBS and 0.01% w/w NaN_3) at 4°C for 15 minutes to block Fc receptors.[42] After rinsing Fc-blocked cells twice with staining solution, cell suspensions were transferred to a 96-well plate for staining with 1 μg of antigen-specific MAbs at 4°C for 30 minutes in the dark. MAbs against CD11b (clone M1/70, rat anti-mouse IgG2b), CD18 (clone m18/2, rat anti-mouse IgG2a), CD11c (clone N418, armenian hamster anti-mouse IgG), CD54 (clone YN1/1.7.4, rat anti-mouse IgG2b), F4/80 (clone BM8, rat anti-mouse IgG2a), Fc (clone 93, rat anti-mouse IgG2a), CD14 (clone Sa2-8, rat anti-mouse IgG2a), CD40 (clone MR5D3, rat anti-mouse IgG2a), TLR-4 (clone UT41, mouse IgG1, shown to cross-react with mouse, rat, and human), and CD206 (MΦ mannose receptor, MMR, clone MR5D3, rat anti-mouse IgG2a) were used in this study. All MAbs were purchased from AbD Serotec Inc (Raleigh, NC) or eBioscience (San Diego, CA) as direct conjugates to FITC or Alexa Fluor 488. Data acquisition and analysis used a FACscalibur (BD Biosciences, Mountain View, CA), CellQuest software (BD Biosciences, San Jose, CA), and WinMDI 2.9 software (J. Trotter, The Scripps Research Institute, La Jolla, CA). A minimum of 3 replicates for each cell type were performed.

2.3.8 Intracytoplasmic cytokine staining.

Measurement of intracellular TNF-α, IL-6, IL-12, IL-10, and MCP-1 in all cell types from TCPS cultures was conducted by plating cells at sub-confluent levels in TCPS flasks. Cells were allowed to adhere to surfaces overnight prior to incubating cells with 1 μL/mL of GolgiPlug™ (monensin solution, BD Pharmingen, San Diego, CA)

and/or 5 µg/mL lipopolysaccharide (*E.coli* lipopolysaccharide, Sigma-Aldrich, St. Louis, MO) for 8 hours under normal culture conditions. This treatment stops export of cellular products, allowing for buildup of cytokines within the cell. Cells were removed from the culture surface, fixed and permeabilized in suspension using Perm Fix/Perm Wash (BD Pharmingen, San Diego, CA) and finally stained for intracellular TNF-α (clone MP6-XT22, rat IgG1), IL-6 (clone MP5-20F3, rat anti-mouse IgG1), IL-12 (clone C17.8, rat anti-mouse IgG2a), IL-10 (clone JES5-16E3, rat anti-mouse IgG2b) and monocyte chemotactic protein (MCP-1, also known as CCL-2, clone 2H5, armenian hamster anti-mouse IgG). All MAbs were purchased from eBioscience (San Diego, CA) as direct conjugates of phycoerythrin. Data acquisition and analysis for this study was done using a FACScalibur (BD Biosciences, Mountain View, CA), CellQuest™ software (BD Biosciences, San Jose, CA), and Summit™ software (DakoCytomation, Carpinteria, CA). Data presented represent at least 3 replicates per cell type.

2.3.9 Analysis of flow cytometric data.

Data from flow cytometry was used to determine both the percent positive cells as well as the mean fluorescent channel (MFC, correlated to marker expression intensity). Histograms of isotype control and antigen-specific stained cells were overlaid. A gate containing 5% of the negative control was set, and any cells in the antigen-specific stained sample above the gate were considered to be positive for the antigen. MFC values were calculated by subtracting the mean isotype control intensity from the mean antigen specific intensity.

2.3.10 Multiplex and single-plex RT-PCR.

RNA was extracted from BMMΦ, IC-21, J774A.1 and RAW 264.7 cells at sub-confluent levels on TCPS, PS, PLA and Teflon-AF® surfaces, as well as from all cell types cultured on TCPS treated with 5 μg/mL LPS for 6 hours, using an RNeasy kit (Qiagen Inc. Valencia, CA) per the manufacturer's instructions. Up to 4 μg of RNA was used to make cDNA with the SuperScript III 1st strand RT kit for PCR (Invitrogen, Carlsbad, CA), poly $d(T)_{15}$ primers were used for reverse transcription. The resulting cDNA stocks were used for multiple PCR reactions. Two MPCR kits were purchased from Maxim Biotech. The Inflammatory Cytokines MPCR kit was used to assay mRNA expression of TNF-α, IL-6, IL-1β, TGF-B, and GM-CSF from all cDNA samples. The TH1 and TH2 cytokine kit was used to assay mRNA expression of IL-10, IL-2, IL-4, IL-5, IFN-γ, IL-12, and IL-13 from non-LPS treated cDNA samples. Single-plex PCR primers (table 2.1) were designed for MMR and MCP-1 using Primerquest software from Integrated DNA Technologies (IDT, Coralville, IA). Primers were also purchased from IDT. The specificity of designed primers was confirmed by sequencing at least two positive results. MPCRs were performed per manufacturer's suggested protocols using 2 μL of the cDNA stock. Single-plex PCRs were performed with 1.25 units of BioRad iscript™ DNA polymerase, 1.5 mM magnesium chloride, 200 μM each of dNTP's, 500 nM of each primer, and 2 μL of the cDNA stock. MPCRs used manufacturer's recommended thermal cycling settings while single-plex PCRs were performed with 30 cycles of a 95℃ melt, 60℃ anneal and 72℃ extension. Analysis of PCR product s was performed on ethidium bromide-stained TBE based 2% agarose gels run at 100 volts for one hour and visualized with UV light. A minimum of three replicates were performed for each condition. 100% agreement among all replicates was required to determine a positive or negative result.

2.3.11 Statistical Analysis

Statistical significance among cell types was determined using ANOVA followed by post-hoc t-tests. Two tailed student's t-tests were used to determine significant increases in cytokine/chemokine production following LPS treatment for individual cell types. Significance is determined by P-values less than 0.05. Error bars represent the standard error of the mean.

2.4 Results

2.4.1 (Monocyte-) macrophage cells exhibit different morphologies in 2-D.

Characteristic morphological features of each cell type in 2-D culture are shown in Figure 2.1. Similar to previous observations, adherent BMMΦ and IC-21 cells show greater spreading and more cytoskeletal features than less mature RAW and J774A.1 cells.[30,31] Cells were able to adhere, grow and proliferate on all culture surfaces tested. BMMΦ and IC-21 cells are larger, and exhibit numerous filopodial extensions and membrane ruffling while J774A.1 and RAW 264.7 cells have a smaller, more rounded phenotype with fewer cytoplasmic extensions.[27,43]

2.4.2 Surface antigen expression indicates significant phenotypic differences between primary macrophage cultures and (monocyte-) macrophage cell lines.

The percent of cells in BMMΦ and IC-21 populations expressing the macrophage marker F4/80 was significantly higher than that from J774A.1 and RAW 264.7 cells Figure 2.2. However, the MFC values of F4/80 molecules were not significantly different among cell types (83.79, 275.24, 50.42, and 35.57, for BMMΦ, IC-21, J774A.1, and RAW 264.7, respectively). The percentage of cells expressing the CD14 marker was significantly higher in BMMΦ, J774A and RAW 264.7 cells than in

IC-21 cells, with RAW cells expressing CD14 in significantly more cells than BMMΦ (>80% in BMMΦ, J774A.1 and RAW 264.7 versus 62% in IC-21) MFC values of J774A.1 and RAW 264.7 were also significantly higher than IC-21 (MFC of 103.10 in BMMΦ, 35.73 in IC-21, 196.91 in J774A.1, and 306.64 in RAW 264.7). The Fc receptor included in this study as an additional differentiation marker,[44] was found to be expressed by more than 60% of all cells in each cell type. However, the highest MFC corresponded to the J774A.1 cell line (MFC of 263.22 for J774A.1 versus MFC less than 60 for all other cell types).

Cell adhesion molecules CD11b and CD18 were expressed by more than 75% of cells in all cell types. The MFC for the same molecules showed no statistical differences between cell lines. Other integrins do exhibit demonstrable differences (e.g., CD11c and CD54). CD11c is expressed at a higher level in IC-21 cells than all other cell types (MFC of 21.05 for BMMΦ, 50.61 for IC-21, 19.45 for J774A.1, and 20.77 for RAW 264.7) while CD54 shows higher percentages of positive cells in the cell lines than in BMMΦ (61.39 for BMMΦ, 81.44 for IC-21, 78.14 for J774A.1, and 86.28 for RAW 264.7). MFC for CD54 on IC-21cells was also significantly higher than that on BMMΦ (31.53 for BMMΦ, 121.52 for IC-21, 68.79 for J774A.1, and 66.46 for RAW 264.7).

The constitutive levels of expression of activation markers CD40 and TLR-4 were assessed in each cell type. The amount of CD40 per cell varied between cell types with BMMΦ expressing the least amount, and IC-21 expressing the most. TLR-4 was expressed by less than 35% of the population in each cell type. MFC and percent positive values for TLR-4 showed no significant differences. The expression of MMR was observed at a higher level in macrophages versus monocytes with levels of

26.26% of cells in BMMΦ cultures, 20.83% in IC-21 cells, 7.18% in J774A.1, and 5.08% in RAW 264.7. However, the MFC values for MMR were not significantly different across the cell types.

2.4.3 Primary macrophages and (monocyte-) macrophage cell lines exhibit different cytokine expression profiles upon exposure to LPS activation.

Cells assayed were cultured on TCPS and tested before and after stimulation with LPS. Expression of cytokines TNF-α, IL-6, IL-1β, TGF-β and GM-CSF was observed at the transcript level using non-quantitative multiplex RT-PCR. Flow cytometry was used to confirm protein expression of these cytokines, chemokines TNF-α, IL-6, IL-12, IL-10 and MCP-1 (related to inflammation and the foreign body reaction).

As shown in Table 2.2, all cell types expressed mRNA for all cytokines tested following stimulation with LPS. Notably, despite the similar expression profiles of all cells for TLR-4 (i.e., LPS-sensitive receptor), significant differences in cell activation were observed when cytokine expression was studied by intracellular staining for cytokines and chemokines of activated cells. Results of these experiments are shown in Figure 2.3 for percent of cells and the MFC in each population producing cytokines (TNF-α, IL-6, IL-10, and MCP-1) when cultured on TCPS. These results compare the un-stimulated cytokine expression among cell types, changes in cytokine expression prior to and after stimulation in each cell type, and changes in expression among cell types. Basal cytokine levels are represented by white bars in Figure 2.3. TNF-α expression varied significantly among all cell types when normalized to the percent of positive cells in populations ($p \leq 0.05$, ANOVA followed by post-hoc t-tests). However, the MCF was negligible for all cell types (MCF for TNF-α was 4.53, 14.19, 0, and 21.41 for BMMΦ, IC-21, J774A.1, and RAW 264.7,

respectively). IL-6 cytokine expression did not differ among cell types, and MCF data was similarly low (MFC of 2.23, 7.10, 0, and 0.93 in BMMΦ, IC-21, J774A.1, and RAW 264.7 cells, respectively). Constitutive levels of IL-12 were low for all cell types (MFC of 4.56 for IC-21, vs. 0.73 for J774A.1 and 0 for RAW 264.7 and BMMΦ). Percent positive cells for IL-10 were significantly lower than BMMΦ for all cell lines, with 14.43% of BMMΦ cells expressing IL-10 prior to LPS treatment, MCF values were again very low for all cell types. MCP-1 was expressed at very low levels if at all prior to activation with LPS (0.05% of BMMΦ, 7.40% of IC-21, and no J774A.1 or RAW 264.7 with MCF values of 0, 1.8, 0, and 0 respectively). Following LPS treatment, cytokine expression significantly increased for many cytokines. TNF-α data shows significant increases in the percent positive cells for BMMΦ, J774A.1, and RAW 264.7 cells but not for IC-21 cells, and significant increases for all cell types in the MCF. The percent positive cells for IL-6 increased significantly for only BMMO and J774A.1, but MCF increased for BMMΦ, IC-21, and J774A.1. RAW 264.7 cells were unable to induce IL-6 expression under our conditions. IL-12 data showed significant increases only in he percent positive cells for BMMΦ and J774A.1, and IL-10 showed a significant decrease for the percent positive cells only for BMMΦ. MCP-1 expression increased following LPS treatment for the percent positive cells and MCF for all cell types. The final observation we made was the change in expression from control to LPS treated cells. Changes in cytokine expression following LPS stimulation induced a significantly higher change of TNF-α and MCP-1 in BMMΦ cells than all other cell lines at the MCF level.

2.4.4 Cytokine mRNA expression differs in primary macrophages versus macrophage-like cell lines when cultured on different surfaces.

Different materials induce foreign body reactions of varied severity *in vivo.*[37] Cytokine expression at the mRNA level in each cell type was used to assess constitutive levels of mRNA expression for cells cultured on TCPS, PS, PLA or Teflon-AF® surfaces to correlate material composition to inflammatory response *in vitro*. Reverse transcriptase PCR (RT-PCR) produced qualitative expression data for TNF-α, IL-6, IL-12, MCP-1, IL-10, IL-1β, TGF-β, GM-CSF, IFN-γ, IL-2, IL-4, IL-5 and IL-13 from activation by surfaces, as well as expression of two surface molecules implicated in macrophage fusion (CCR-2 and MMR). Results are summarized in Table 2.3. mRNA for TNF-α, TGF-β, MCP-1, and the surface receptors CCR-2 and MMR was expressed by all cell types on all surfaces, whereas IL-1β, IL-2, IL-4, IL-5 and IL-13 mRNA were not detected in any of the cells. Interestingly, mRNA for IL-10 was differentially expressed by different cells. The immunosuppressive cytokine IL-10 was expressed at the mRNA level by J774A.1 cells cultured on all surfaces, but not detected in any other cell type. Expression of mRNA for other cytokines (IL-6, IL-12, GM-CSF and IFN-γ) appeared to be affected by the type of material used during culture conditions; (e.g., mRNA for IL-12 was only expressed in BMMΦ cultured on Teflon-AF® and IFN-γ was only expressed in J774A.1 on PLA). Similarly, mRNA expression of GM-CSF, was detected only for IC-21 cells on PS, PLA and Teflon-AF®, and RAW 264.7 cells on TCPS and PS surfaces. Finally, mRNA expression of IL-6 was the most variable of all cytokines studied, detected in BMMΦ on TCPS, J774A.1 on Teflon-AF®, and RAW 264.7 on Teflon-AF® and PLA.

2.5 Discussion

Morphology, macrophage cell surface markers, integrin expression, and cell surface proteins associated with cell activation state, as well as cytokine response to chemical (LPS) and physical (model surfaces) stimuli, demonstrate significant differences among murine macrophage cell types commonly used in inflammatory research. In this study, murine (monocyte-) macrophage cells routinely employed in published studies of wound healing, pathogenesis, implant reactions, the foreign body reaction, drug testing and inflammation mechanisms (BMMΦ, IC-21, J774A.1, and RAW 264.7) are shown to differ in their phenotypes and responses to stimuli. Significant *in vitro* differences observed for responses to stimuli among these cell types may reflect extended exposures to 2-D culture substrates, or intrinsic cell-cell differences rather than cell-based biocompatibility or anti-inflammatory analyses *in vitro* or *in vivo*.

Macrophages represent a diverse, dynamic family of phenotypes derived by cues received from individual environments. Physiologically, the macrophage differentiates from hematopoietic stem cells in bone marrow, exhibiting heterogeneity along differentiation pathways from monocytes to macrophages as well as between macrophage populations from different tissue microenvironments in the body.[45,46] Such heterogeneity stems from different functions required by macrophages in different tissues and can result in different morphologies, signaling, phenotypes and responses to stimuli. Visual morphological characterization of adherent macrophage lineage cells largely agrees with previously observed differentiation states, assigning BMMΦ and IC-21 cells a macrophage phenotype, and J774A.1 and RAW 264.7 as monocyte-macrophage phenotypes, respectively.[30,31] Molecular characterization supports these designations. F4/80 is a surface marker preferentially expressed on

highly differentiated macrophages;[47] less-differentiated monocytes preferentially express CD14 and the Fc receptor.[27,46] Our analysis of these monocyte/macrophage markers corroborates morphological characterizations, with relative differentiation states of monocyte to macrophage ranked as RAW 264.7<J774A.1<BMMΦ<IC-21. This is consistent with precedent phenotyping of these cells.[30,31,48,49]

Macrophage adhesion to biomaterial surfaces is an essential step in initiation of the foreign body reaction. Therefore, cell adhesion markers could reflect the incumbent cell inflammatory response relevant to inflammatory testing of biomaterials. Interestingly, our previous work showed very few differences in expression and activation of intracellular GTPase signaling molecules involved in macrophage adhesion (i.e., Rho, Rac1, Cdc42) despite gross morphological differences between these same adherent cell types.[31] Similarly, our current findings show only a few detectable differences in adhesion molecule expression (i.e., integrins) despite important phenotypic differences (Figure 2.1, 2.2). Data sets from current and precedent work support our contention that macrophage cells share certain common non-distinguishing features despite important phenotypic differences in culture. All cell adhesion proteins assayed were expressed in all cell populations, but at varying levels for some markers (CD11c, CD54, Figure 2.2). Similar repertoires of adhesion molecules in all cell types might therefore promote similar adherence properties to these cultures. Nonetheless, some quantitated phenotypic disparities in expression of adhesion molecules among primary and secondary cell types were also clearly exhibited.

In addition to phenotypic roles in adhesion, monocyte and macrophage surface receptors have diverse functions in activation and initiation of inflammation. CD40 is

involved in antigen presentation and its expression is elevated in activated macrophages,[45] while TLR-4 in conjunction with CD14 binds LPS as an initiating step in "classical" inflammatory responses. CD40 was expressed in greater than 50% of all cell populations tested (Figure 2.2). However, IC-21 and RAW 264.7 cells had significantly higher numbers of cells expressing this cell receptor than BMMΦ, which may indicate a higher classical activation state in these cells. TLR-4 was expressed at similar levels in all cell types (Figure 2.2). Combined with CD14 expression in all cell types (Figure 2.2), this indicates similar potentials for activation by LPS. Yet, BMMΦ cells show greater induction of cytokine expression following LPS stimulation compared to all other cell types (Figure 2.3). Immortalized cells exhibit reduced cytokine production in response to LPS stimulus. MMR supports macrophage maturity, activation, and potential for fusion into foreign body giant cells during a foreign body reaction.[37,50] Expression of MMR was observed in significantly fewer immortalized cells than primary derived cells, and also less in monocyte (J774A.1 and RAW 264.7) than macrophage (BMMΦ and IC-21) phenotypes, indicating that BMMΦ cells may have more potential for fusion than cell lines, and that these (monocyte-) macrophage cell lines are not equivalent in maturity or activation potential to primary derived macrophage cells.

Adhesion of monocytes and macrophages to surfaces engages cell surface receptors, inducing cell signaling cascades and thus affecting the production of immunomodulatory substances.[51] Therefore, activation of adherent monocyte/macrophage cells in culture is often correlated with the inflammatory potential of the surface.[19,52] Some concern remains that cell culture surfaces are intrinsically activating to monocyte and macrophage phenotypes, causing adherent populations to become maximally activated in culture under all conditions and

therefore indistinguishable. To assess the activation of monocyte/macrophages from adherent culture, multiplex RT-PCR was used to assess qualitative expression of common inflammatory cytokines under standard culture conditions on model biomaterials, and after 8 hours of LPS treatment on TCPS. Additionally, these same cytokines plus additional cytokines and cell surface receptors were assayed for cells propagated on all model biomaterials Results show that all cell types can be further activated by LPS stimulation and are not maximally activated simply by adhesion to culture surfaces. Multiplex and single-plex RT-PCR of these and additional cytokines, chemokines and surface receptors were used to assess cellular response to surfaces (Table 2.3). All cell types expressed cytokines TNF-α, MCP-1 and TGF-β as well as the surface receptors CCR-2 and MMR on all materials. However, IL-6, IL-10, GMCSF and IFNγ are only detected in certain cells in response to particular surfaces (Table 2.3). Clear differences in cytokine response to surfaces demonstrate non-equivalence of cell lines to each other and to primary-derived cells. Expression of IFNγ by J774A.1 cells on PLLA surfaces is interesting. Expression of IFNγ by mononuclear phagocytes is reported but often attributed to contaminating T-cells.[53] By contrast, mRNA expression of IFNγ here is from a mononuclear cell line where T-cell contamination is not an issue. This indicates that monocyte/macrophage cells can produce IFNγ at the mRNA level, but production of the protein product has yet to be shown.

RT-PCR assays deliver only qualitative data, and mRNA expression does not always reflect protein translation. Thus, two important cytokines probed at the mRNA level (TNF-α and IL-6) as well as a cytokine involved in TH-1 type immune responses (IL-12), an anti-inflammatory cytokine (IL-10), and a chemokine implicated in inflammation and macrophage fusion (MCP-1) were assayed using flow cytometry

techniques both before and after LPS treatment. Up-regulation of TNF-α protein after LPS treatment further defines the gap between BMMΦ and cell lines, as BMMΦ cells expressed significantly more TNF-α following LPS stimulation than all other cell types. This indicates a greater sensitivity to LPS induction of TNF-α compared to immortalized cell lines, despite similar TLR-4 and CD14 receptor expression profiles (Figure 2.2, 2.3). IL-6 was also expressed in significant numbers of cells prior to LPS stimulation (Figure 2.3), but expressed at significantly higher levels in BMMΦ cells than in all cell lines. Following LPS treatment, BMMΦ, IC-21 and J774A.1 cells increase IL-6 expression. By contrast, RAW 264.7 cells did not up-regulate this cytokine in response to LPS. Lack of IL-6 protein production by RAW 264.7 cells after stimulation with LPS could easily produce false negative assay results *in vitro* regarding inflammatory potential. Observed inability of IC-21 and RAW 264.7 cells to initiate significant IL-12 protein translation after LPS treatment groups these cells together despite their maturity differences. This demonstrates yet another discrepancy both among the cell lines and between primary cells and cell lines, and demonstrates that macrophage maturity is not always indicative of increased response to stimuli.

IL-10 is an anti-inflammatory cytokine with TH-1 inhibiting properties. The observed decrease in the number of BMMΦ cells expressing these molecules after LPS stimulation indicates increasing TH-1 activation. No IL-10 expression was observed for any cell lines, demonstrating one more distinction between primary derived and immortalized cell lines. Interestingly, IL-10 mRNA was only detected in the J774A.1 cell line (Table 2.3) yet only BMMΦ cells showed detectable expression of this cytokine at the protein level (Figure 2.3). Assays of mRNA production were conducted after 72 hours of culture on model surfaces to allow for cells to proliferate

to near-confluent numbers, while protein production assays occurred after 32 hours of culture on surfaces. This result indicates that cytokine expression may change over time following adhesion to a surface. MCP-1, while up-regulated by all cell types following LPS stimulation, was greatest in BMMΦ. Overall, cytokine expression at the protein level varies greatly between primary derived macrophages and immortalized (monocyte-)macrophage cell lines, with primary cells initiating stronger and more diverse responses than all cell lines tested.

2.6 Conclusions

BMMΦ, IC-21, J774A.1 and RAW 264.7 cells have all been routinely employed to assess biomaterials and pharmaceutics *in vitro* and to predict pre-clinical murine *in vivo* performance testing.[3,15-23] These cultured cell responses may or may not be relevant to *in vitro* models of material biocompatibility. Fidelity of these cell phenotypes on materials in culture has been *presumed* to represent *in vivo* states rather than actually proven. This study delineates specific phenotypic and molecular-level differences and similarities in these commonly used murine cells that should be useful to guide their utility in inflammatory cell-based assays. Significant differences in cell morphology, membrane protein, cytokine expression and LPS activation are observed between all murine macrophage cell types in this study. Not only are cultured murine primary macrophages distinct from immortalized (monocyte-) macrophage cell lines phenotypically, but markers used for inflammatory predictions are irregular across the cell types on different culture substrates. Substantial evidence for non-equivalence of these cells in common assay configurations exists for various *in vitro* tests. Correlations between cultured primary and immortalized cells in cytokine production, phenotypes, and intrinsic states of activation relevant to *in vitro* testing are inconsistent.

Such differences warrant justification for the selection of various cell lines for specific purposes, and merit caution if comparisons to primary cell types are required. Interchangeable use of these multiple cell types generically as macrophages in *in vitro* methodologies makes extrapolation of results published across different macrophage inflammatory activation studies difficult. Intensive comparisons at the molecular level have been reported for immortalized human monocyte cell lines (i.e. U937 and THP-1)[6,54] allowing some assessment of their potential relevance in experiments on inflammatory activation. Murine cells for *in vitro* use in such experimentation should be carefully characterized in culture for standard macrophage markers (F4/80, CD14, CD11b, Fc receptor) and reliable cytokine response to chemical stimuli. Validation of phenotypic fidelity for commonly used macrophage-like cell phenotypes against accepted benchmarks for a standard phenotype would improve comparison and correlation in cell-based responses reported in inflammatory biomaterials and drug assays. This could also instill confidence in comparing published outcomes as well as pre-clinical *in vivo* results on biomaterials and experimental therapeutics.

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Table 2.1. RT-PCR Primers. Primers used for amplicons not included in multiplex PCR kits.

Gene	Primer sequence	Amplicon length
MCP-1	TCACCTGCTGCTACTCATTCACCA AAAGGTGCTGAAGACCTTAGGGCA	250 bp
MMR	AGCTACCATGGCATGAAGCAGAGA ACCCATTCGAAGGCATTCCAGAGA	464 bp
CCR-2	TGTTACCTCAGTTCATCCACGGCA AGCCCTGTGCCTCTTCTTCTCATT	746 bp

Table 2.2. Inflammatory cytokine expression at the mRNA level with and without LPS treatment. Under standard culture conditions (see Materials and Methods) all cell types expressed mRNA for TNF-α and TGF-β; only BMMΦ cells expressed IL-6. Following LPS treatment, all cell types expressed all tested cytokines (TNF-α, IL-6, IL1-β, TGF-β, and GM-CSF).

Cell Type		BMMO	IC-21	J774A.1	RAW 264.7
TNF-α	- LPS	+	+	+	+
IL-6	- LPS	+	-	-	-
IL-1β	- LPS	-	-	-	-
TGF-β	- LPS	+	+	+	+
GM-CSF	- LPS	-	-	-	-
TNF-α	+ LPS	+	+	+	+
IL-6	+ LPS	+	+	+	+
IL-1β	+ LPS	+	+	+	+
TGF-β	+ LPS	+	+	+	+
GM-CSF	+ LPS	+	+	+	+

Table 2.3. Constitutive mRNA expression of select cytokines, chemokines and cell surface receptors as determined by RT-PCR. Primary macrophages (BMMΦ) and macrophage cell lines were cultured on control surfaces and model biomaterials to observe induction of cytokine expression by surface. Macrophages were cultured on model biomaterial surfaces ≥24 hours prior to RNA extraction and RT-PCR analysis.

Cell Type		BMMO				IC-21				J774A.1				RAW 264.7				Bone Marrow Cells
Culture Surface		TCPS	PS	PLA	Teflon-AF™	TCPS	PS	PLA	Teflon-AF™	TCPS	PS	PLA	Teflon-AF™	TCPS	PS	PLA	Teflon-AF™	no surface
Cytokines	TNF-α	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	IL-6	+	-	-	+	-	-	-	-	-	-	-	+	-	-	+	-	+
	IL12	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
	MCP-1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	IL10	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-
	IL-1β	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	TGF-β	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	GM-CSF	-	-	-	-	-	+	+	+	-	-	-	-	+	+	-	+	-
TH1 Cytokines	IFNγ	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
	IL2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
TH2 Cytokines	IL4	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IL5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	IL13	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Surface Receptors	CCR-2	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+
	MMR	+	+	+	-	+	+	+	+	+	+	-	+	+	-	+	+	+

Scheme 2.1. Flowchart of experimental procedures. Methods for comparison of cell types included culture with various stimuli (model surfaces, lipopolysaccharide), followed by data collection at both the mRNA transcript (PCR, MPCR) and the protein (Flow Cytometry) level.

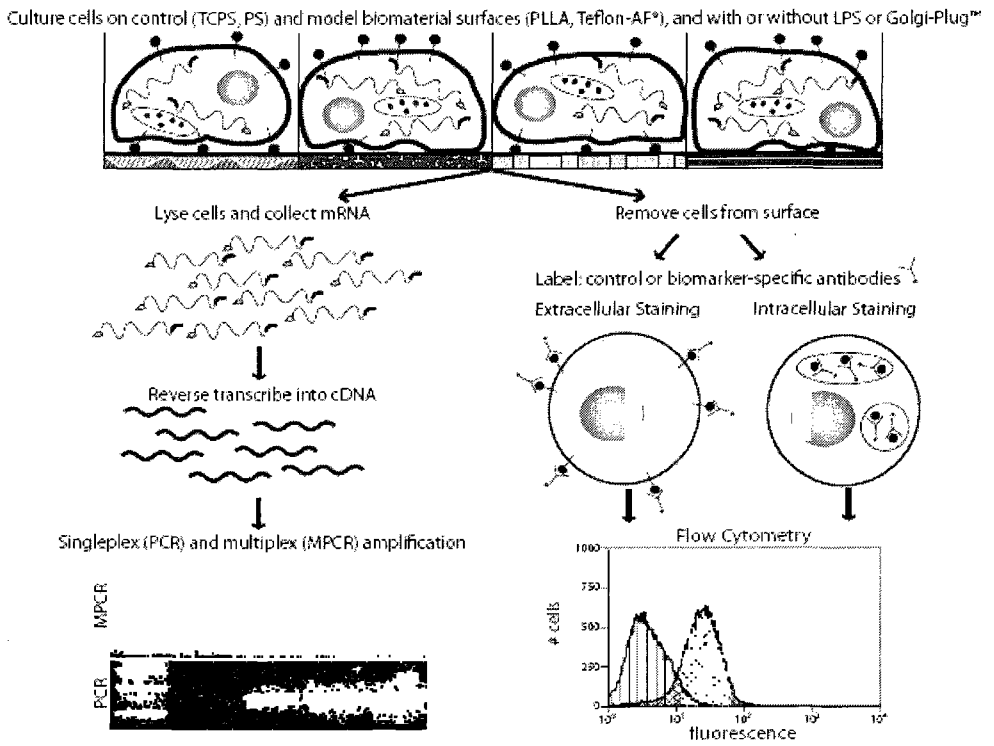


Figure 2.1. Morphology of macrophage-lineage cell types. Phase contrast microscopy images of RAW 264.7, J774A.1, IC-21 and BMMΦ cells on tissue culture treated polystyrene (TCPS).

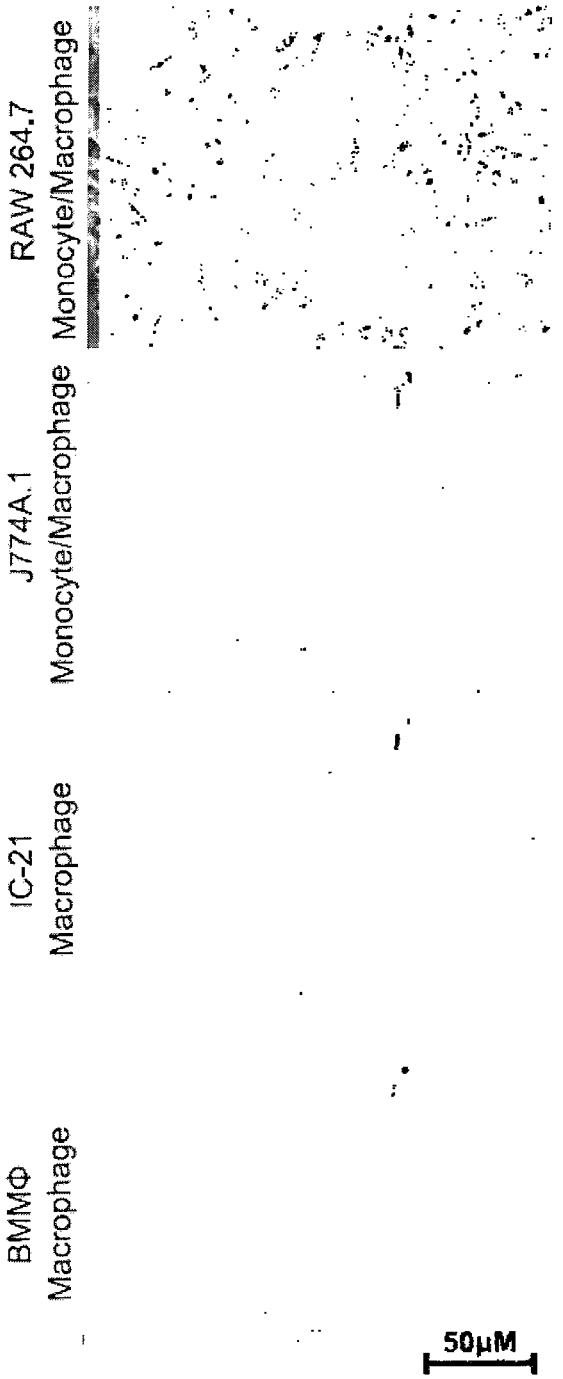


Figure 2.2. Phenotypic characteristics of primary-derived macrophages and macrophage cell lines. Flow cytometric mean percent positive and mean fluorescent channel (MFC) data for macrophage cell-surface markers F4/80, CD14, FcR, CD11b, CD18, CD11c, CD54, CD40, TLR-4, and MMR, +/- the standard error (data are representative of at least 3 experiments, statistical significance of $p \leq 0.05$ is indicated by an *). Cells were cultured on TCPS for 24 hours in serum containing media as described in Materials and Methods.

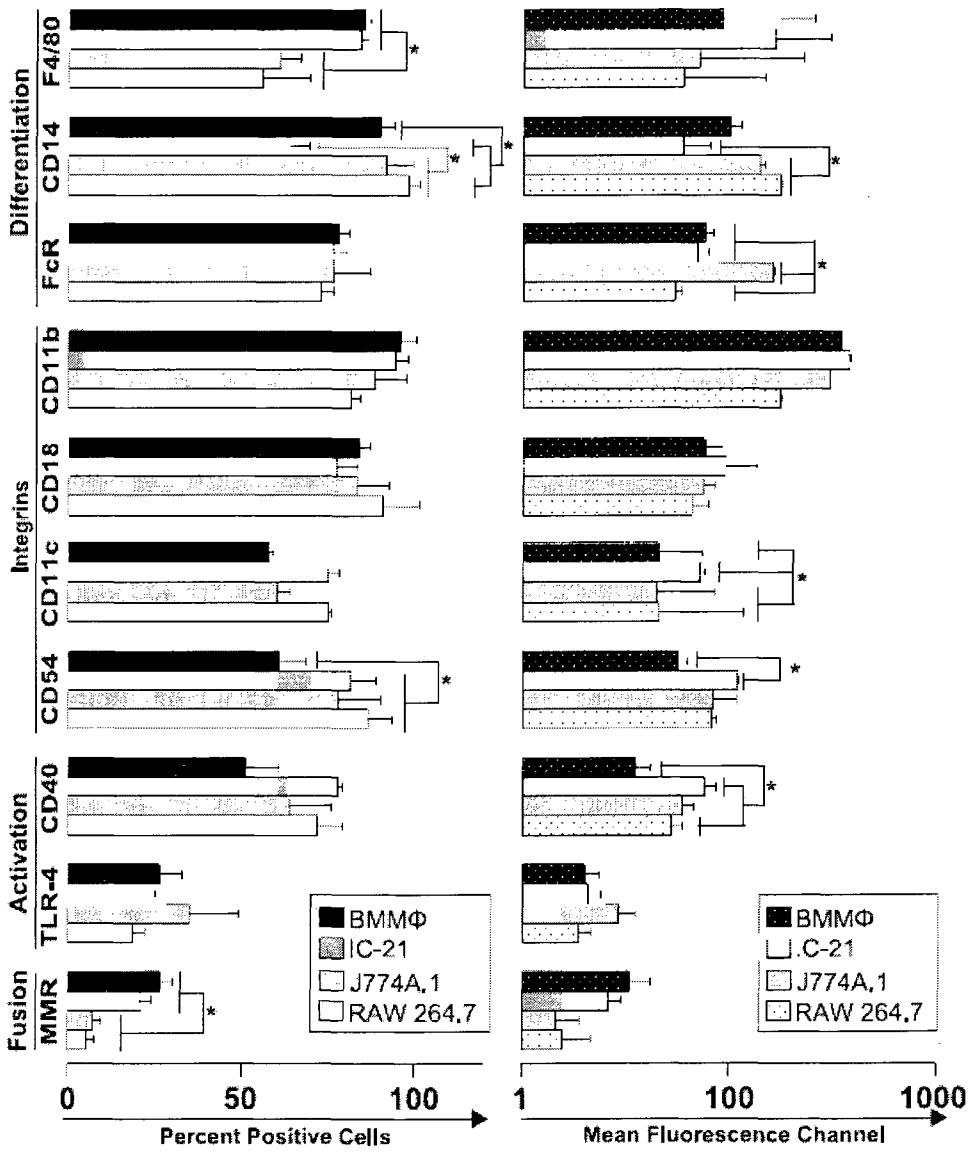
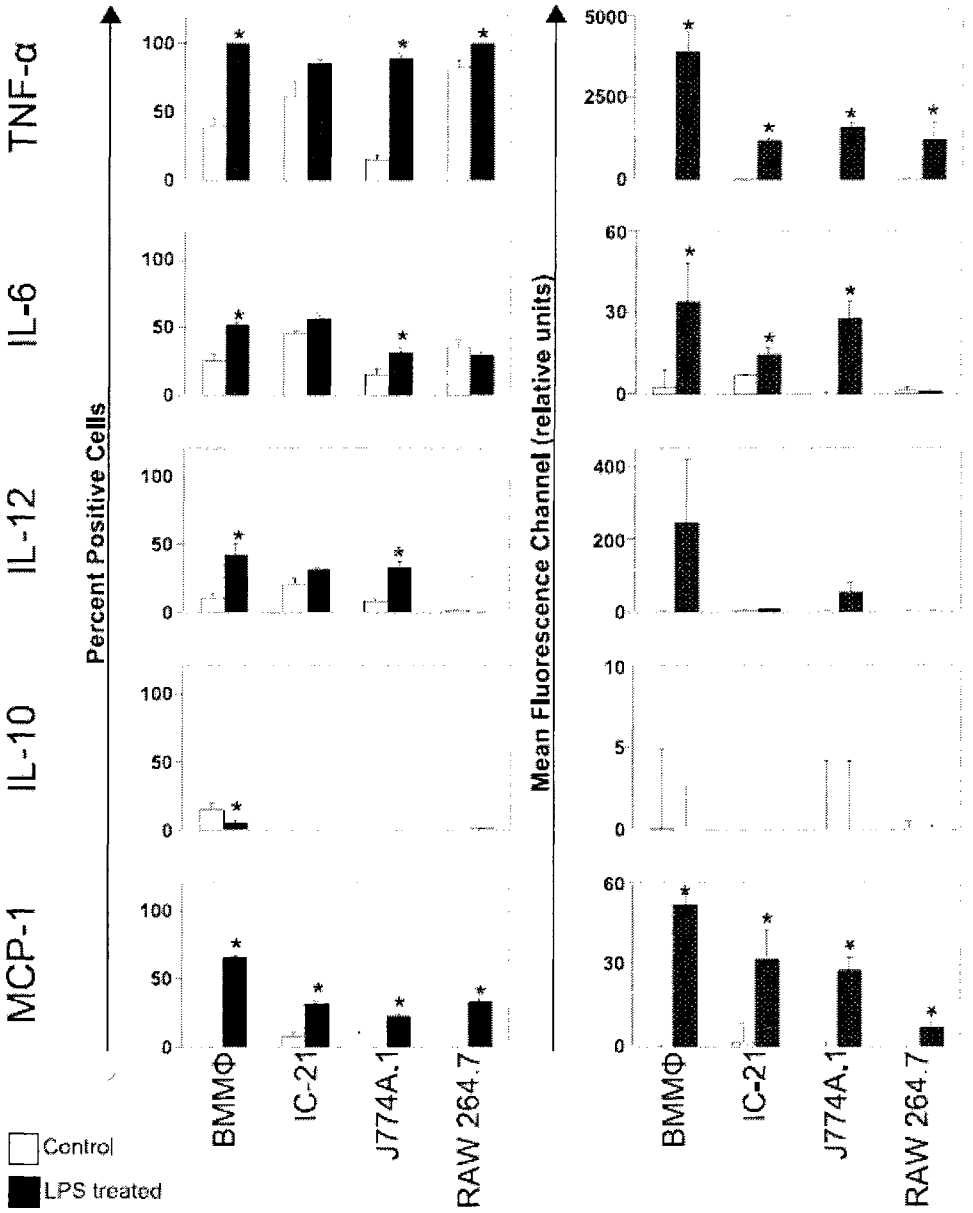


Figure 2.3. Flow cytometric analysis of inflammatory cytokines. Cells were incubated with LPS and/or a complex to stop Golgi complex export of cytokines for 8 hours prior to intracellular immunostaining and flow cytometry analysis. White bars indicate basal cytokine expression on TCPS and black bars indicate cytokine expression after treatment with LPS. Error bars represent standard error. The induction of cytokine expression varies greatly from cell type to cell type. (Data are representative of at least 3 experiments, statistical significance of $p \leq 0.05$ is indicated by an *).



CHAPTER 3: *IN VITRO* INFLAMMATORY RESPONSES BY MACROPHAGES OF DIFFERENT SOURCING AND MATURITY TO MODEL BIOMATERIALS PRODUCE AN M2 PHENOTYPE

This chapter was written by Lisa M. Chamberlain, edited by David W. Grainger and Mercedes Gonzalez-Juarrero, and submitted to *Biomaterials.*

3.1 Abstract

Macrophages are immuno-modulatory cells highly involved in both innate wound healing and the inflammatory response to implanted biomaterials. Macrophage involvement in the *in vivo* response to foreign bodies has prompted many to adopt short term *in vitro* assays of macrophage cells on biomaterials as an initial test of biocompatibility. Unfortunately, differences in duration, media and conditions of culture, cell types used, and activation markers assessed make comparisons of these assays across the literature difficult. Minimal knowledge of key molecular events that occur following macrophage attachment to surfaces further confounds both interpretation of these results and the design criteria for new biomaterials that might avoid the foreign body response. Additionally, despite longer term in vivo implant models that focus on macrophage involvement,[1-4] the effects of longer-term interactions between macrophages and surfaces has not been assayed *in vitro.* Recently, longer-term cultures have been used to study macrophage responses to biomaterial surfaces, intending to link in vitro and in vivo results querying the cell-material interface.

Specifically this timepoint links in vitro assays with the 21-day point in vivo where the foreign body response matures.[5]

Herein we report long-term (21-day) in vitro culture of macrophage cells at different stages of maturity, an extended period of culture not previously reported but correlated to in vivo foreign body maturation. Cell morphology and cytokine production were tracked throughout the experiment, whereas the expression of cell surface markers was only assayed prior to and after the extended culture. We report material- and time-dependent morphology and cytokine expression, as well as differences in cytokine expression and material-dependent morphology between primary-derived macrophages and immortalized macrophage cell lines. All cell types exhibited up-regulation of cell surface molecules characteristic of alternative macrophage activation, implying that extended culture of several different macrophages on different surfaces leads commonly to the development of an M2-characteristic phenotype defined by Mantovani in 2002.[6].

3.2 Introduction

The use of *in vitro* assays to assess cell responses to implantable biomaterials is ubiquitous in the literature.[7-18] These assays often involve the culture of immortalized cells on biomaterial surfaces for one to three days followed, in cases of inflammatory cell lines, by observations of cell inflammatory responses. The use of different cell lines, different time points, and different inflammatory markers have resulted in considerable conflicting datasets and conclusions in the literature. Recent efforts to understand some of these differences have involved assaying large arrays of cytokines following macrophage culture on model biomaterial surfaces.[19,20] These studies have monitored the production of numerous cytokines by human macrophages for up to ten days and have shown a peak of inflammatory cytokine expression at early time points, followed by

attenuations on further times to basal levels. This change in cytokine expression over time has prompted the hypothesis that changes in macrophage phenotype and maturation are also occurring in these cells over time in culture.[19]

Macrophages are phagocytic cells involved in inflammation, wound healing, infection, and the response to implanted materials. They represent not a single phenotype but a diverse collection of cell types exhibiting different phenotypes depending on their tissue location, environment and differentiation stage. While this heterogeneity produces a continuum of different macrophage phenotypes, additional categories exist based on surface markers and cytokine expression. Among these different types of macrophages, those activated by different stimuli can comprise a spectrum ranging between M1 and M2 categories: the "classically" and "alternatively" activated macrophages, respectively.[6,21] These are not generally considered to be truly distinct phenotypes as there is evidence for macrophages changing between M1 and M2 polarization in the literature.[21] However, there are several markers that signify a macrophage cell to be in one state or the other, such as Toll-like receptors (e.g. TLR-4) for M1, and the macrophage mannose receptor (MMR) for M2.[6] The complexities of macrophage maturity and activation state can make interpretation of *in vitro* data from different cell types difficult. Nonetheless, these culture data are often used to screen materials "biocompatibility" despite some lack of confidence or consensus in the predictive value of the assay for in vivo outcomes.

Here we report new studies of longer-term in vitro culture responses by secondary macrophage cell lines and primary derived macrophages at different stages of differentiation to understand effects of extended culture. Cell surface proteins assayed included molecules associated with cell adhesion (CD18, CD11b, CD11c, CD54), state

of maturation (F4/80, CD14, and Fc receptor), and several markers of associated with cell activation (CD40, TLR-4, and MMR). We observe similar cell cytokine responses to materials over time among all cell types, with an initial early-phase burst of cytokine expression with subsequent attenuation to basal levels over time. Additionally, evidence of alternative macrophage activation, or an M2 phenotype,[6] is demonstrated following extended culture of all cell types on all biomaterial surfaces.

3.3 Materials and Methods

3.3.1 Model biomaterials and surface preparation.

Model and control materials used in this study have been characterized previously for cell culture: standard tissue culture polystyrene (TCPS, 15x100mm petri dishes, Falcon®, BD Biosciences, San Diego, CA); poly-L-lactide (PLLA, Polysciences Inc., Warrington, PA) and Teflon-AF™ (DuPont Fluoroproducts).[22] Teflon-AF™ surfaces were prepared as previously reported.[23,24] Briefly, 100mm PS Petri dishes were coated with Teflon-AF™ (3 mL of a 0.1% solution diluted from stock in 3M™ Fluorinert™ Electronic Liquid FC-40 solvent, 3M Corp. St. Paul, MN) prior to overnight vacuum exposure at 65°C. PLA surfaces (50,000 MW) were prepared as described previously by solvent casting a 0.2% w/v solution of PLA in methylene chloride.[23,24] Glass petri dishes (ø=100mm) were coated with 10mL of PLA solution, loosely covered, and allowed to dry in a fume hood for approximately one hour. Teflon-AF® and PLA-coated plates were sterilized inside a laminar flow hood after misting with 70% ethanol in cell-grade water (Hyclone®, Logan, UT) by treatment with culture-hood UV light for 15 minutes (a process shown to have no detectable effect on surface chemistry).[25] All surfaces and cell culture materials were tested for the presence of contaminating endotoxin using a Pyrogene™ Assay kit (Cambrex, East Rutherford, NJ), and endotoxin levels were determined to be below the

kit detection limit (0.02 EU/mL).

3.3.2 Primary murine macrophage cell harvest.

Specific-pathogen-free female C57BL/6 mice, 6 to 8 weeks old, were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the University of Utah animal facilities, and were given sterile water, and mouse chow for the duration of the experiments. Animal guidelines for the care and use of laboratory animals have been observed; all experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the University of Utah.

Bone marrow cells were harvested from murine tibias and femurs and differentiated into macrophage cells using previously described methods.[24,26] Bone marrow cells were flushed from long bones, and then differentiated into bone-marrow derived macrophages (BMMΦ) by incubating in complete DMEM (cDMEM, DMEM supplemented with 10% heat inactivated FBS, 10% of supernatant from L-929 fibroblast cells (ATCC, Manassas, VA), 1% penicillin-streptomycin (Hyclone®, Logan, UT), 0.01M Hepes buffer (Hyclone®, Logan, UT), 1mM sodium pyruvate (Hyclone®, Logan, UT), and 1% of a 100X MEM non-essential amino acids solution (Hyclone®, Logan, UT)). Cells were cultured for 7 days on TCPS, with media changes every 2 days, selecting adherent cultured cells as mature macrophages (BMMΦ) for further studies. This protocol has been shown to produce a mature macrophage phenotype.[26] Replicates are defined as cells from different mice. A minimum of three replicates were completed for all experiments.

3.3.3 Immortalized murine cell (secondary cell line) culture.

Adherent murine (monocyte-) macrophage cell lines IC-21, J774A.1 and RAW 264.7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 and IC-21 cells were cultured in RPMI-1640 (Mediatech, Inc, Herndon, VA) and J774A.1 cells were cultured in DMEM (Mediatech, Inc, Herndon, VA) per vendor recommendations. All growth media used to culture cell lines were supplemented with 10% FBS (Hyclone®, Logan, UT), 1% penicillin-streptomycin (Gibco, Carlsbad, CA), and 0.01 M Hepes buffer (Sigma). Cell cultures were maintained below 80% confluence in TCPS flasks and passaged by incubation with divalent cation-free Dulbecco's Phosphate Buffered Saline (dPBS Hyclone®, Logan, UT) prior to scraping with a rubber policeman. All cells were used at or below passage number 20 as received from ATCC and incubated under standard conditions. Replicates are defined as cells harvested from different passages and/or flasks. A minimum of three replicates were performed for each experiment.

3.3.4 Extended Cell Culture.

Cell lines and mature primary macrophages were removed from passage or differentiation surfaces and seeded at approximately 80% confluence onto test surfaces for continuous culture. Media was changed every 24-hours for 21 days and cells were imaged prior to all media changes. Media was collected for analysis of cytokine production from days 1, 2, 3, 7, 14, and 21 and stored at -80°C. Cells were removed from surfaces on day 21 by incubation with non-cationic PBS (Gibco) followed by gentle scraping and analysis by flow cytometry for phenotypic surface markers. Three replicates were performed for each cell type on each surface.

3.3.5 Cell imaging.

Live cells were photographed using phase contrast microscopy on a Nikon Eclipse TE 2000-U microscope (Nikon Inc., Torrance, CA), a Photometrics Coolsnap ES camera (Roper Scientific, Tucson, AZ), and Metamorph™ software (Molecular Devices, Downingtown, PA). Cell images were analyzed for the number of cells per field. The mean counts of at least 5 random frames were used to estimate the number of cells on each plate.

3.3.6 Flow cytometric analysis of surface markers.

Control cells (freshly differentiated BMMΦ or secondary cell lines from stock flasks), and cells from 21-day cultures were removed from culture surfaces. Cell suspensions were incubated with purified monoclonal antibodies (MAbs) CD16/32 (clone 93, rat IgG2a anti-mouse, eBiosciences, San Diego, CA) using at least 1μg of MAbs per million cells in 100μL of staining solution (PBS with 1% FBS and 0.01% w/w NaN_3) at 4°C for 15 minutes to block Fc receptors.[27] After rinsing Fc-blocked cells twice with staining solution, cell suspensions were transferred to a 96-well plate for staining with 1μg of fluorescently conjugated MAbs diluted to 100μL with staining solution at 4°C for 30 minutes in the dark. MAbs against CD11b (clone M1/70, rat anti-mouse IgG2b), CD18 (clone m18/2, rat anti-mouse IgG2a), CD11c (clone N418, armenian hamster anti-mouse IgG), CD54 (clone YN1/1.7.4, rat anti-mouse IgG2b), F4/80 (clone BM8, rat anti-mouse IgG2a), Fc (clone 93, rat anti-mouse IgG2a), CD14 (clone Sa2-8, rat anti-mouse IgG2a), CD40 (clone MR5D3, rat anti-mouse IgG2a), TLR-4 (clone UT41, mouse IgG1, shown to cross-react with mouse, rat, and human), and CD206 (macrophage mannose receptor, MMR, clone MR5D3, rat anti-mouse IgG2a) were used in this study. All MAbs were purchased from AbD Serotec Inc (Raleigh, NC) or eBioscience (San Diego, CA) as direct conjugates to Alexa Fluor 488 (TLR-4) or FITC (all others). Cells were rinsed

twice with additional staining solution to reduce background fluorescence from unbound antibody prior to analysis. Data acquisition and analysis for this study used a FACScan (BD Biosciences, Mountain View, CA), CellQuest software (BD Biosciences, San Jose, CA), and WinMDI 2.9 software (J. Trotter, The Scripps Research Institute, La Jolla, CA).

3.3.7 Quantitation of cytokine expression over time.

Cytometric bead array (CBA) assays were purchased from BD Biosciences and used per manufacturer's instructions. Media samples from days 1, 2, 3, 7, 14, and 21 on all surfaces were surveyed. CBA assays were performed on a minimum of 3 replicates for all time points, for all surfaces, and for all cytokines/chemokines available (GMCSF, MIP-1β, TNF, RANTES, MCP-1, IFN-γ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 p70, and IL-13). Standard curves were used to generate quantitative data, and the estimated number of cells per plate (from cell density numbers) was used to transform the data to control for cell population fluctuations.

3.3.8 Statistical Analysis

Statistical significance was determined using Anova followed by post-hoc t-tests where appropriate. P-values less than 0.05 are considered significant.

3.4 Results

3.4.1 Cell morphology and density depend on culture surface.

Cell morphology was observed for all cell types on all surfaces at days 1 and 21. Unlike IC-21, J774A.1, and RAW 264.7 secondary cells, BMMΦ cells exhibited surface-dependent morphology at both time points. At day 1, BMMΦ cells cultured on TCPS displayed a spiny, oblong-like morphology not observed when these cells were cultured on Teflon-AF® or PLA surfaces. Over time, BMMΦ morphology continued to be

surface-dependent. BMMΦ cells cultured on Teflon-AF® surfaces developed large lammelipodia, whereas as on TCPS and PLA surfaces, these cells maintained the spiny, oblonged morphology as on day 1. This phenomenon was more pronounced on the PLA surfaces (Figure 3.1).

IC-21, J774A.1, and RAW 264.7 cells exhibited no surface-dependent morphology at day 1 or 21 (Figure 3.1). Unlike BMMΦ cells, they proliferated and reached culture confluence by day 21, producing a rounded morphology very different from general morphologies presented at day 1 (Figure 3.1).[23]

Cellular proliferation in analogous culture conditions is represented in Figure 3.2 as the number of cells per frame at 40X magnification over a 21-day period for each cell type on all surfaces. Overall, proliferation of BMMΦ cells was higher when these cells were cultured on TCPS surfaces than on Teflon-AF® and PLA surfaces. Similarly, IC-21 cells also demonstrated higher proliferation when cultured on TCPS than on Teflon-AF® or PLA surfaces. However, differences between TCPS and Teflon-AF® were not observed until after day 7 of culture, indicating that early-stage cell cultures would not distinguish macrophage differences on these two surfaces. J774A.1 proliferation on these surfaces was similar to that observed for BMMΦ cells. However, the differences in cell number between both cell types on each surface indicated that the proliferation rate was higher in J774A.1 cultures than in BMMΦ cells.

By contrast, RAW 264.7 cells proliferated only during the initial 3 days when cultured on TCPS, Teflon-AF®, or PLLA surfaces. Thereafter, the number of cells decreased (Figure 3.2). Additionally, similar numbers of cells per 40X field were observed between

TCPS and Teflon-AF® surfaces at all time points, indicating that these cells grow equally well on either of these surfaces.[23,28,29]

3.4.2 Cytokine expression by macrophages depends on culture surface.

CBA assays were used to analyze relative expression levels of cytokines and chemokines in supernatants obtained from the various cell cultures. Our results indicated positive expression in supernatants collected at any time point over the duration of the experiment from only five of the 14 analytes assayed by the CBA (Table 1). Supernatants collected from all cultures were found to contain two common chemokines (RANTES, MCP-1). However, while BMMΦ supernatants contained the inflammatory cytokine, IL-6, supernatants collected from IC-21, J774A.1, and RAW 264.7 cultures after 21 days of culture had positive expression for two inflammatory cytokines; TNFand MIP-1β. The data shown in the left column of figures 3.3-3.6 represent concentrations of chemokines or cytokines expressed in pg/mL to allow comparison with previous reports. In the right column from the same figures, the data are represented as pg cytokine per 10^{10} cells to normalize for changes in cell density on these surfaces over time.

Cultures of BMMΦ cells showed several significant differences in cytokine expression on different surfaces. Supernatants from these cells cultured on TCPS contained much more MCP-1 on TCPS at day 2 than from Teflon-AF® or PLLA cultures, while at day 7, the concentration of MCP-1 was much higher on Teflon-AF® than on TCPS or PLLA. On the other hand, RANTES and IL-6 expression was significantly higher on PLLA surfaces at early time points. However, when taking cell density into account, these data show that the supernatants obtained from BMMΦ cells when cultured on PLLA surfaces had higher levels of MCP-1, RANTES and IL-6 per cell than supernatants from cells

cultured on TCPS or Teflon-AF® surfaces (Figure 3.3). Considering cell density, this indicates that BMMΦ cell cytokine-chemokine response is initiated upon cell adhesion to surfaces and drops to basal levels by day 7 in culture (Figure 3.3, Tables 3.2 and 3.3).

MCP-1, RANTES, TNF and MIP-1β were positive in supernatants obtained from IC-21, J774A.1 and RAW 264.7 cell cultures over a 21-day period (Table 1). In contrast to supernatants obtained from similar cultures of BMMΦ cells, there were no significant differences in expression of these cytokines and chemokines for IC-21, J774A.1, or RAW 264.7 when cultured on different surfaces However, when the data was normalized to pg per 10^{10} cells, there was a similar trend for concentration of these cytokines and chemokines as that shown in supernatant obtained from BMMΦ cell cultures, with a burst of expression that drops to basal levels by day 7 (Figure 3.4-3.6, Tables 3.2 and 3.3).

3.4.3 Changes in expression of cell surface markers following extended culture of macrophages on model biomaterials depend on cell type.

The expression of cell surface markers associated with macrophage maturity, adhesion, and activation were compared. Specifically, changes of these markers in primary BMMO and cell lines included in this comparative study were compared. The markers chosen were associated with cell differentiation (F4/80, CD14, Fc receptors), intergrin expression (CD18,CD11b, and CD11c), intracellular adhesion (CD54) and cell activation (CD40, TLR-4 and MMR) in each cell culture. Furthermore, changes in the relative expression of these markers over the course of the study were monitored. Changes in the expression of each of these cell markers over time were determined using flow cytometry using a comparative analysis of the mean fluorescence channel (MFC) and the percent of positive cells for each cell population and for each cell marker. Data

obtained from this study are listed in Tables 3.2 and 3.3, and are presented in graphical format in figures 3.7-3.10. Furthermore, significant changes in expression of each surface marker in each cell type and culture are summarized in Table 3.4.

BMMΦ cells had few changes in cell surface marker expression following 21 days of culture on different model surfaces. Changes in markers associated with cell differentiation include significant increases in the MFC of the F4/80 for these cells cultured on PLLA surfaces. Additionally, BMMΦ cells cultured on TCPS surfaces showed significant changes in the MFC for CD14 and the Fc receptor. Assays for the integrins, CD18, CD11b, and CD11c, showed no significant differences in surface expression nor percentage of positive cells for BMMΦ on all surfaces between cells prior to plating and after extended culture. However, the cell-cell adhesion molecule CD54 exhibited significant decreases in MCF following extended culture on all surfaces, despite a lack of change in the percent of positive cells. Similar to the three integrins assayed, no significant changes were observed in the MCF or the percentage of cells positive for CD40. The percentage of BMMΦ cells positive for TLR-4 significantly decreased on all surfaces over time. In addition, there were significant decreases in the MFC for TLR-4 in these cells on both PLLA and Teflon-AF® surfaces. Finally, the percentage of BMMΦ cells positive for the MMR marker significantly increased when cultured on any of the surfaces, and the MFC of MMR was increased significantly when these cell were cultured on TCPS (Tables 3.1-3.3, Figure 3.7).

In contrast to the lack of changes observed in the percentage of positive cells for each marker in BMMO cell cultures, IC-21 cells cultured on all surfaces demonstrated significant increases in the percentage of positive cells for most cell markers under analysis (Figure 3.8, Tables 3.1-3.3). The few exceptions (i.e., little change) were for

CD14 of IC-21 cells cultured on TCPS and PLLA, as well as CD11b and TLR-4 for IC-21 cells cultured on TCPS and PLLA. Significant decreases in the MFC for F4/80 were seen on TCPS and Teflon-AF® surfaces, and for CD14 on TCPS surfaces.

Also in contrast, J774A.1 and RAW 264.7 cells exhibited few changes in expression of most cell surface markers following extended culture. J774A.1 cells cultured on TCPS exhibited decreases in percentages of cells positive for CD11c marker, These cells cultured on TCPS and PLLA also showed decreases in the percentage of cells positive for the CD54 marker. Similarly to BMMΦ and IC-21 cells, J774A.1 cells cultured on all surfaces exhibited increases in the percentage of cells positive for the MMR marker. The MFC for the MMR marker was also increased when these cells were cultured on TCPS and PLLA (Figure 3.9, Tables 3.1-3.3). RAW 264.7 cells cultured on TCPS and PLLA surfaces exhibited decreases in the percentage of cells positive for CD54, as well as an increase in the percentage of cells positive for MMR if cultured on any of the surfaces (Figure 3.10, Tables 3.1-3.3).

3.5 Discussion

Despite short culture assays, extended culture of macrophage cells is rarely reported in the study of their *in vitro* responses to biomaterials. Two recent studies of human macrophage cells cultured on a variety of model biomaterials have demonstrated differences in cell density and magnitude of cytokine production over time. These changes are characteristic of the type of biomaterial used in the culture system.[19,20] These two studies have demonstrated that there are temporal changes in cytokine production and imply that a change in cell phenotype is also occurring following extended culture.[19,20,30] We have previously shown that short-term cultures of the four macrophage cell types used maintain varying states of differentiation when cultured on

different model biomaterials.[31] Extending these early-phase studies to 21-day cultures yields a time period repeatedly referenced as a point of maturity for the foreign body response in vivo and allows for observations of changes in phenotype that require a longer time course.[32,33] Our results demonstrated that cell adhesion and their proliferation, as well as their morphology and cytokine expression, differed across macrophages of different differentiation states.[34] Additionally, increases in the expression of the macrophage mannose receptor by all cell types was observed when cultured on all material surfaces under study. Finally these long-term cell culture data showed that all cells cultured for extended periods of time produced a phenotype associated with the alternative activation pathway, or M2 macrophage phenotype, a result which has been recently hypothesized in a current opinions paper and is associated with the foreign body response.[6,30,35]

We have previously reported differences in cell morphology with minimal changes in expression of inflammatory proteins following short-term (<3 day) culture of monocyte/macrophage cells cultured on different surface materials.[23,24,34] Our 24 hour data confirmed that BMMΦ morphology following 24 hours of culture differed according to different materials surfaces. Over the next 21 days, this cell morphology continued to change over time and was distinct on different surface chemistries. In contrast, all cell lines (IC-21, J774A.1, and RAW 264.7) developed rounded morphologies by day 21 with no time or surface-dependent distinctions in morphology at days 1 or 21. This is in contrast to previous work from our group that showed differences in the morphology of IC-21 cells at a later time point (3 days of culture) on the same materials.[23,24] This discrepancy in behavior of primary-derived versus immortalized macrophages at late culture time points is likely due to a loss of contact inhibition, a common result of cellular immortalization specific to the secondary cell lines.[36] Lack of surface-dependent

changes in morphology by cell lines at earlier time points implies that immortalized cell lines and primary-derived cells are not equivalent in their responses to surfaces materials, a point previously emphasized and significant to their common use in biocompatibility assays that assert relative equivalence.[31]

TCPS, the gold-standard for cell culture, consistently yielded highest cell densities for most cell types (BMMΦ, IC-21, and J774A.1) compared to cultures on PLLA or Teflon-AF® surfaces, although at early time points macrophage culture densities and growth rates on Teflon-AF surfaces were very comparable. This has been noted in previous work on early cultures as well.[23,29] RAW 264.7 cells, as previously demonstrated,[23] displayed no difference in proliferation when cultured on TCPS or Teflon-AF® surfaces. Similarly to RAW 264.7, proliferation rates for IC-21 and J774A.1 cells were comparable when cultured on TCPS or Teflon-AF® surfaces at early time points. However, over longer times, these cells cultured on TCPS surfaces reached higher cell densities than when cultured on other material surfaces. These growth data on surfaces indicate inherent phenotypic differences between primary-derived and immortalized cell lines, and among cell lines themselves, likely reflecting intrinsic differences in their respective expression of surface proteins involved in adhesion as discussed below.[34]

Similar to short-term culture studies, BMMΦ, J774A.1, or RAW 264.7 cells cultured over a 21-day period produced very few noticeable changes in their levels of expression of cell surface markers. However, cell changes that do occur indicated cellular phenotypic transition towards the known alternative activation pathway for macrophages.[6] This pathway describes and distinguishes two general phenotypes for contrast – the M1 and M2 phenotypes characterized by Mantovani et.al.,[6] although it is more likely that macrophages represent a continuum of states between these M1 and M2 arbitrary

designations. Surface proteins assayed included molecules associated with cell adhesion (CD18, CD11b, CD11c, CD54), state of maturation (F4/80, CD14, and Fc receptor), and several markers of associated with cell activation (CD40, TLR-4, and MMR). BMMΦ cells did not show significant changes in the expression of integrin markers despite having demonstrated major changes in adherent cell morphology reflecting changes in cell adhesive properties. One noticeable change for BMMΦ cultures on any of these biomaterials was the decrease of their CD54 expression, a cell-cell adhesion marker. J774A.1 and RAW 264.7 cells on the other hand also showed reduced expression of the same marker when cultured either on TCPS or PLLA surfaces. This observation may indicate a cell-specific change towards cell adhesion to the surface rather than cell-cell relationships, with the cells changing their focus towards the implant.

The few distinct changes observed in markers of macrophage differentiation for BMMΦ, J774A.1, and RAW 264.7, as well as the many changes seen with IC-21 cells suggested that the macrophage phenotype of these cells was maintained. Changes in F4/80, CD14 and Fc receptor, markers associated with macrophage maturity of BMMΦ and IC-21 cells cultured for 21 days indicated an increase in cell maturity with long term culture.[27,37,38] This observation is strengthened by increases in MMR expression for all cell types.[39]

Our flow cytometry data demonstrated the decrease in a marker of macrophage "classical" activation (TLR-4) for BMMΦ cells following extended culture on all surfaces, and the increase in a marker of "alternative" activation (MMR)[21,40] for all cell types following extended culture on all surfaces under study. These data support the hypothesis that macrophages in all systems assayed in this study preferentially

differentiate towards an alternative activation state, or M2 phenotype with extended culture.[6,30] This phenotypic convergence for all cells at extended culture times is significant in several regards. First, it shows that initial short-term differences in macrophage cultures on different biomaterials may not accurately reflect longer-term endpoints that tend to converge on a common phenotype. Second, distinct macrophage morphology as an early phenotypic indicator in culture may not necessarily distinguish activation phenotypes at longer times. Third, as the foreign body response is often considered to correlate with an alternative type of inflammatory response,[21,41] and such response is regarded as relatively mature at the 21-day point,[5] extended macrophage culture appears to also induce this alternative pathway of activation, regardless of biomaterial chemistry (or cell lineage), consistent with many foreign body responses that occur despite differences in surface chemistry.

Common cytokines and chemokines present in supernatants from these macrophage cell cultures were MCP-1, RANTES, TNF, MIP-1β, and IL-6. Interestingly, while all cell types expressed MCP-1 and RANTES, only immortalized cell cultures expressed TNF or MIP-1β. In contrast, IL-6 was only present in supernatants obtained from BMMΦ cultures. Primary human macrophages in another study produced positive expression of TNF in culture on different material surfaces, demonstrating significant differences between species, and immortalized versus primary cells.[19] IL-10 was also detected previously in human macrophage cultures but at concentrations below the current limit of detection.[19] Detectable presence of chemokines MCP-1 and RANTES demonstrates the macrophage's role in recruiting cells to sites of inflammation, both mononuclear and T-cells.[42,43] Additionally, the production of inflammatory cytokines (IL-6 for BMMΦ, and TNF and MIP-1α for all cell lines) suggests a role in the initiation and potentially continuation of inflammation at the surface of an implant. Cytokine production is

material-dependent only for BMMΦ cells, again demonstrating differences between primary and secondary macrophage cell types.

Protein production data reported in raw assay units (pg/mL) show very little similarity from surface to surface for cytokine expression. However, when normalized for cell density per plate, chemokines and cytokines amounts produced by BMMΦ show an initial burst of expression followed by attenuation to a baseline level that remains steady to 21 days. While data from cell lines are far more erratic than BMMΦ in pg/mL form, cell number corrections cause these data to largely mimic the BMMΦ trend with initial spike and following attenuation over the long-term culture.

Correlating differences in macrophage expression of cytokines and chemokines, and cell morphology, in cultures on different surfaces *in vitro* to the severity of the observable foreign body response *in vivo* is frequently difficult. Few consistent in vitro – in vivo correlatons exist, although different materials have been demonstrated to produce collagen capsules of different thicknesses in vivo.[44] Additionally recent studies have demonstrated positive correlations between the number of CD11b-positive cells and fibrous capsule thickness, indicating a relationship between the number of macrophages present and the severity of the FBR.[32,45] Thus, the amount of cytokine and chemokine generated by macrophages *in vitro* could be relevant to the *in vivo* response as the amount of cytokine and chemokine generated at an implant site would affect macrophage recruitment, particularly as macrophage recruitment to a cage-implant model has been seen to be material dependent.[45,46]

3.6 Conclusions

Macrophages of several different immortalized lineages representing several distinct levels of macrophage maturity were compared to primary bone marrow macrophages in vitro for extended culture times not generally assayed. All macrophage cells cultured to 21-day extended time points on model biomaterials exhibit bursts of cytokine production immediately following adhesion while maintaining a characteristic macrophage phenotype based on the expression of surface markers. Additionally, primary cell morphology in these long-term cultures changes continuously, while key cytokines and chemokines thought to be critical to macrophage activation (e.g., TNF, MIP-1β, IL-6, MCP-1, RANTES) undergo a very early, transient expression burst but with no subsequent changes (i.e., in integrins among others) during cell morphological evolution. By contrast, secondary immortalized cells of several macrophage maturities do not alter morphology long term in culture but do exhibit significant changes in cytokine expression over time and between different lineages. However, changes in MMR, a marker of alternative activation in all cultures on all surfaces over 21 days indicate a consistent phenotypic shift towards an alternative activation M2 phenotype.

These data support the hypothesis that macrophages in all systems assayed in this study preferentially differentiate towards an alternative activation state, or M2 phenotype with extended culture.[6,30] This phenotypic convergence for all cells at extended culture times is significant in several regards. First, it shows that initial short-term differences in macrophage cultures on different biomaterials may not accurately reflect longer-term endpoints that tend to converge on a common phenotype. Second, distinct macrophage morphology as an early phenotypic indicator in culture may not necessarily distinguish activation phenotypes at longer times. Third, as the foreign body response is often considered to correlate with an alternative type of inflammatory response,[21,41] and such response is regarded as relatively mature at the 21-day point,[5] extended macrophage

culture appears to also induce this alternative pathway of activation, regardless of biomaterial chemistry (or cell lineage), consistent with many foreign body responses that occur despite differences in surface chemistry.

3.7 References

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Scheme 3.1. Overview of experimental setup. Methods used to track cell progression over time on model surfaces included culture over 21 days, microscopy, media collection, and flow cytometry.

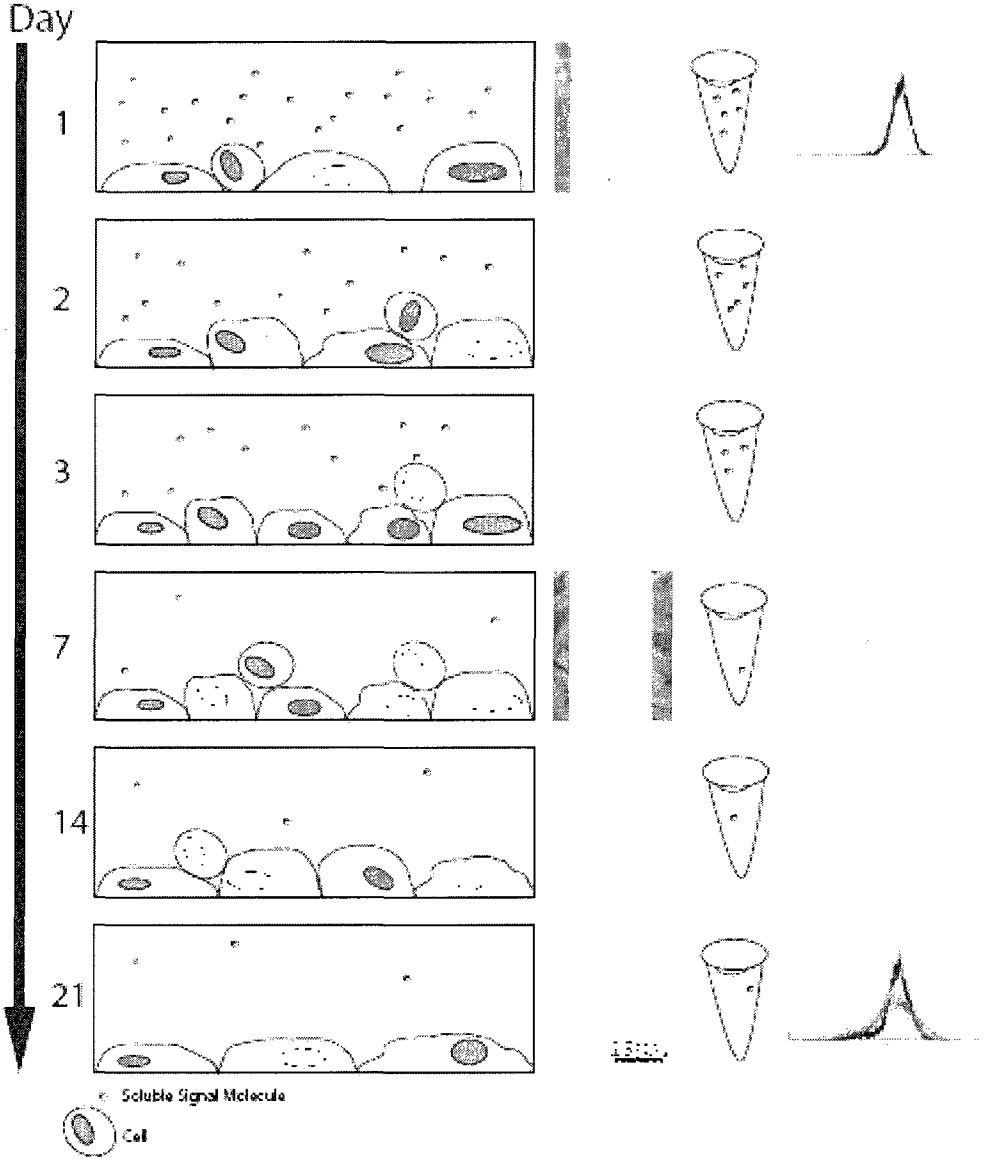


Table 3.1. Cytokine and chemokine detection is cell-type dependent. Of 14 cytokines (black box = detected) and chemokines (grey box = detected) assayed, only 3 cytokines and 2 chemokines were detected in these experiments.

	BMMΦ	IC-21	J774A.1	RAW 264.7
RANTES	+	+	+	+
MCP-1	+	+	+	+
IL-6	+			
TNF		+	+	+
MIP-1β		+	+	+
IL-10				
GM-CSF				
IL-12 P70				
IFN-γ				
IL-2				
IL-4				
IL-5				
IL-9				
IL-13				

Table 3.2. Percent of cells positive for extracellular markers using flow cytometry. The percent of positive cells and standard error of the data prior to and following 21 days of culture on model biomaterials are listed for all cell types and all surface proteins assayed. Significant changes from control are in bold.

		Control			21 day TCPS			21 day PLLA			21 day Teflon-AF®		
		mean		S.E.	mean		S.E.	mean		S.E.	mean		S.E.
BMMΦ	F4/80	88.58	+/-	2.76	89.28	+/-	3.99	95.95	+/-	1.48	84.63	+/-	4.22
	CD14	89.89	+/-	1.41	97.65	+/-	0.94	**98.63**	**+/-**	**0.19**	89.50	+/-	4.21
	Fc	85.76	+/-	4.52	85.95	+/-	4.79	63.88	+/-	6.81	94.03	+/-	1.45
	CD18	87.15	+/-	21.34	93.80	+/-	4.13	91.33	+/-	3.80	75.60	+/-	8.89
	CD11b	96.97	+/-	1.18	94.90	+/-	2.74	97.25	+/-	1.32	93.50	+/-	2.21
	CD11c	67.13	+/-	5.59	63.30	+/-	5.24	61.85	+/-	12.09	60.43	+/-	6.97
	CD54	59.02	+/-	4.62	67.88	+/-	7.31	62.20	+/-	7.00	41.45	+/-	8.86
	CD40	50.54	+/-	9.04	28.28	+/-	6.62	37.40	+/-	6.06	20.18	+/-	5.60
	TLR-4	27.59	+/-	3.60	**15.65**	**+/-**	**3.54**	**11.73**	**+/-**	**3.33**	**16.60**	**+/-**	**1.91**
	MMR	33.32	+/-	6.23	**66.55**	**+/-**	**4.83**	**48.73**	**+/-**	**6.88**	**77.43**	**+/-**	**3.42**
IC-21	F4/80	84.58	+/-	5.76	**99.35**	**+/-**	**0.19**	**97.20**	**+/-**	**1.23**	**99.40**	**+/-**	**0.34**
	CD14	64.55	+/-	3.14	69.30	+/-	2.88	70.00	+/-	3.60	**73.28**	**+/-**	**2.80**
	Fc	76.58	+/-	7.31	**98.48**	**+/-**	**0.42**	**96.93**	**+/-**	**1.00**	**98.98**	**+/-**	**0.31**
	CD18	77.75	+/-	5.23	**95.35**	**+/-**	**0.82**	**95.03**	**+/-**	**1.19**	**94.93**	**+/-**	**3.46**
	CD11b	94.28	+/-	1.74	96.25	+/-	1.60	96.80	+/-	0.89	97.18	+/-	0.47
	CD11c	75.21	+/-	3.75	**97.68**	**+/-**	**0.27**	**94.75**	**+/-**	**1.41**	**98.75**	**+/-**	**0.35**
	CD54	81.44	+/-	3.67	**96.68**	**+/-**	**1.19**	**95.08**	**+/-**	**1.48**	**94.23**	**+/-**	**0.53**
	CD40	78.06	+/-	1.30	**92.53**	**+/-**	**2.31**	**84.93**	**+/-**	**3.40**	**97.53**	**+/-**	**1.15**
	TLR-4	19.28	+/-	5.39	18.68	+/-	4.26	14.00	+/-	1.53	**37.53**	**+/-**	**2.65**
	MMR	20.83	+/-	3.02	**44.78**	**+/-**	**3.11**	**35.05**	**+/-**	**4.07**	**61.85**	**+/-**	**8.44**
J774A.1	F4/80	61.27	+/-	9.06	59.27	+/-	8.95	71.17	+/-	3.86	77.10	+/-	3.51
	CD14	91.68	+/-	3.91	99.03	+/-	0.23	99.53	+/-	0.09	99.50	+/-	0.15
	Fc	76.27	+/-	12.21	99.63	+/-	0.27	99.53	+/-	0.12	99.83	+/-	0.03
	CD18	83.15	+/-	7.51	99.40	+/-	0.10	98.97	+/-	0.07	99.58	+/-	0.10
	CD11b	88.59	+/-	5.39	98.20	+/-	0.32	93.63	+/-	0.60	98.03	+/-	1.15
	CD11c	60.30	+/-	11.13	**45.03**	**+/-**	**8.46**	67.60	+/-	5.82	71.73	+/-	8.48
	CD54	78.15	+/-	8.86	**65.77**	**+/-**	**5.52**	**69.83**	**+/-**	**7.32**	93.73	+/-	1.32
	CD40	63.92	+/-	11.90	70.37	+/-	8.43	74.03	+/-	1.46	72.20	+/-	4.87
	TLR-4	34.82	+/-	14.49	28.90	+/-	12.79	17.03	+/-	1.56	35.20	+/-	12.21
	CD206	7.18	+/-	2.39	**38.30**	**+/-**	**4.59**	**45.60**	**+/-**	**7.91**	**18.60**	**+/-**	**2.94**
RAW 264.7	F4/80	55.90	+/-	10.22	53.60	+/-	7.42	33.87	+/-	11.67	51.85	+/-	14.42
	CD14	98.17	+/-	0.62	99.65	+/-	0.15	98.23	+/-	1.52	99.88	+/-	0.07
	Fc	72.71	+/-	7.26	68.30	+/-	9.51	44.30	+/-	19.01	76.33	+/-	12.34
	CD18	90.81	+/-	3.12	97.38	+/-	0.81	74.40	+/-	21.41	97.63	+/-	1.17
	CD11b	95.13	+/-	1.52	94.63	+/-	2.24	88.63	+/-	4.64	96.93	+/-	0.83
	CD11c	75.23	+/-	3.57	62.75	+/-	11.07	43.27	+/-	16.90	73.93	+/-	5.73
	CD54	86.28	+/-	2.66	**67.45**	**+/-**	**7.11**	**50.57**	**+/-**	**12.80**	71.58	+/-	10.40
	CD40	72.02	+/-	7.22	78.68	+/-	2.37	62.07	+/-	18.10	74.20	+/-	7.12
	TLR-4	18.49	+/-	3.57	33.80	+/-	17.65	27.77	+/-	10.41	24.23	+/-	4.55
	CD206	5.08	+/-	2.55	**38.13**	**+/-**	**16.22**	**38.67**	**+/-**	**15.21**	**37.25**	**+/-**	**19.58**

Table 3.3. Mean flow cytometry fluorescence channel for extracellular markers. The mean fluorescence channel and standard error of the data prior to and following 21 days of culture on model materials are listed for all cell types and all surface proteins assayed. Significant changes from control are in bold.

		Control			21 day TCPS			21 day PLLA			21 day Teflon-AF®		
		mean		S.E.	mean		S.E.	mean		S.E.	mean		S.E.
BMMΦ	F4/80	23.53	+/-	5.92	20.43	+/-	2.62	**41.36**	**+/-**	**8.07**	16.20	+/-	1.70
	CD14	86.60	+/-	17.50	**24.61**	**+/-**	**2.59**	53.00	+/-	2.16	34.31	+/-	1.74
	Fc	31.69	+/-	8.70	**75.19**	**+/-**	**11.68**	12.69	+/-	3.72	31.89	+/-	9.91
	CD18	30.65	+/-	9.99	18.66	+/-	6.43	40.22	+/-	14.76	28.08	+/-	5.31
	CD11b	118.68	+/-	36.82	68.51	+/-	17.24	104.66	+/-	30.91	93.20	+/-	21.14
	CD11c	12.77	+/-	2.27	7.11	+/-	0.42	16.96	+/-	5.55	8.04	+/-	1.51
	CD54	20.61	+/-	5.21	**9.97**	**+/-**	**1.92**	**8.11**	**+/-**	**2.16**	**8.60**	**+/-**	**1.67**
	CD40	6.57	+/-	2.28	3.40	+/-	0.59	4.54	+/-	0.50	2.63	+/-	0.40
	TLR-4	2.67	+/-	0.42	2.95	+/-	0.17	**1.22**	**+/-**	**0.42**	**1.28**	**+/-**	**0.21**
	MMR	6.86	+/-	2.84	**19.58**	**+/-**	**1.10**	6.11	+/-	0.73	8.99	+/-	1.13
IC-21	F4/80	275.24	+/-	89.84	**107.89**	**+/-**	**17.66**	**59.09**	**+/-**	**14.09**	**120.37**	**+/-**	**33.40**
	CD14	35.73	+/-	7.41	**14.04**	**+/-**	**3.11**	19.21	+/-	6.48	18.38	+/-	2.67
	Fc	47.84	+/-	3.99	84.00	+/-	21.93	66.64	+/-	18.89	84.72	+/-	22.91
	CD18	93.73	+/-	28.45	51.37	+/-	13.84	66.56	+/-	23.26	84.75	+/-	25.23
	CD11b	449.48	+/-	74.82	**133.03**	**+/-**	**35.85**	**183.80**	**+/-**	**59.06**	280.73	+/-	63.09
	CD11c	50.61	+/-	13.51	48.03	+/-	9.80	38.33	+/-	9.00	67.61	+/-	15.70
	CD54	121.52	+/-	25.35	86.86	+/-	21.36	73.36	+/-	19.88	98.48	+/-	20.42
	CD40	58.38	+/-	19.25	30.86	+/-	9.67	25.83	+/-	10.53	54.34	+/-	15.26
	TLR-4	4.24	+/-	1.38	3.35	+/-	0.94	3.25	+/-	1.24	6.20	+/-	1.28
	MMR	6.87	+/-	2.19	7.55	+/-	2.23	7.25	+/-	3.01	13.57	+/-	3.97
J774A.1	F4/80	55.60	+/-	21.66	73.22	+/-	27.55	78.54	+/-	26.44	63.75	+/-	16.91
	CD14	2.11	+/-	1.51	9.18	+/-	3.51	9.01	+/-	0.97	2.00	+/-	0.59
	Fc	50.42	+/-	19.20	32.88	+/-	14.14	20.37	+/-	5.14	30.07	+/-	10.56
	CD18	196.91	+/-	49.66	251.63	+/-	85.29	269.45	+/-	81.26	376.38	+/-	105.71
	CD11b	263.22	+/-	51.17	357.58	+/-	142.71	305.64	+/-	69.76	225.17	+/-	49.37
	CD11c	35.23	+/-	10.26	21.01	+/-	8.53	17.67	+/-	4.70	14.95	+/-	3.22
	CD54	189.14	+/-	59.22	224.51	+/-	93.64	213.04	+/-	59.56	300.37	+/-	86.91
	CD40	68.79	+/-	28.17	47.30	+/-	22.76	45.21	+/-	11.33	62.89	+/-	15.83
	TLR-4	19.45	+/-	9.42	31.10	+/-	15.18	33.71	+/-	11.11	29.99	+/-	9.74
	CD206	8.55	+/-	3.87	**26.76**	**+/-**	**11.77**	**19.87**	**+/-**	**5.53**	7.30	+/-	3.41
RAW 264.7	F4/80	35.57	+/-	18.38	12.47	+/-	4.56	15.75	+/-	8.98	10.98	+/-	3.71
	CD14	306.64	+/-	118.01	213.43	+/-	135.53	297.59	+/-	208.98	196.29	+/-	100.85
	Fc	29.32	+/-	9.25	27.67	+/-	19.16	27.96	+/-	19.81	24.29	+/-	12.39
	CD18	43.09	+/-	13.30	75.69	+/-	46.40	69.76	+/-	45.19	48.02	+/-	22.78
	CD11b	95.02	+/-	17.72	228.92	+/-	152.89	200.65	+/-	150.63	157.85	+/-	86.62
	CD11c	20.77	+/-	4.78	23.95	+/-	14.93	21.44	+/-	14.22	18.31	+/-	7.52
	CD54	66.46	+/-	15.35	47.13	+/-	33.08	43.73	+/-	35.86	41.31	+/-	23.01
	CD40	27.26	+/-	8.06	23.81	+/-	10.45	35.87	+/-	17.47	20.34	+/-	8.16
	TLR-4	3.44	+/-	1.27	16.31	+/-	11.73	6.16	+/-	3.07	4.38	+/-	1.92
	CD206	2.43	+/-	2.12	5.83	+/-	3.41	14.06	+/-	11.69	14.43	+/-	12.92

Table 3.4. Summary of flow cytometry experiments. Changes in the expression of surface markers following extended culture of various macrophage cell lineages on model biomaterials point to few changes in phenotype. However, a universal increase in the macrophage mannose receptor (MMR) implies a move towards alternative activation or an M2 phenotype for all cells on all materials surfaces over longer-term culture.

		BMMΦ			IC-21			J774A.1			RAW 264.7		
		TCPS	PLLA	Teflon-AF®	TCPS	PLLA	Teflon-AF®	TCPS	PLLA	Teflon-AF®	TCPS	PLLA	Teflon-AF®
Markers of Macrophage Maturity	F4/80		↑		↑↓	↑	↑↓						
	CD14	↓	↑		↓		↑						
	Fc	↑			↑	↑	↑						
Macrophage adhesion molecules	CD18				↑	↑	↑						
	CD11b												
	CD11c				↑	↑	↑	↓					
	CD54	↓	↓	↓	↑	↑	↑	↓	↓				
Macrophage activation markers	CD40				↑	↑	↑						
	TLR-4	↓	↓	↓			↑						
	MMR	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑

Figure 3.1. Morphology of adherent macrophage-lineage cells before and after extended culture. BMMΦ cells exhibit surface-dependent morphology which changes over time in culture, while morphologies of all immortalized cell lines display no surface dependence, and the consistent development of a cobblestone morphology over time.

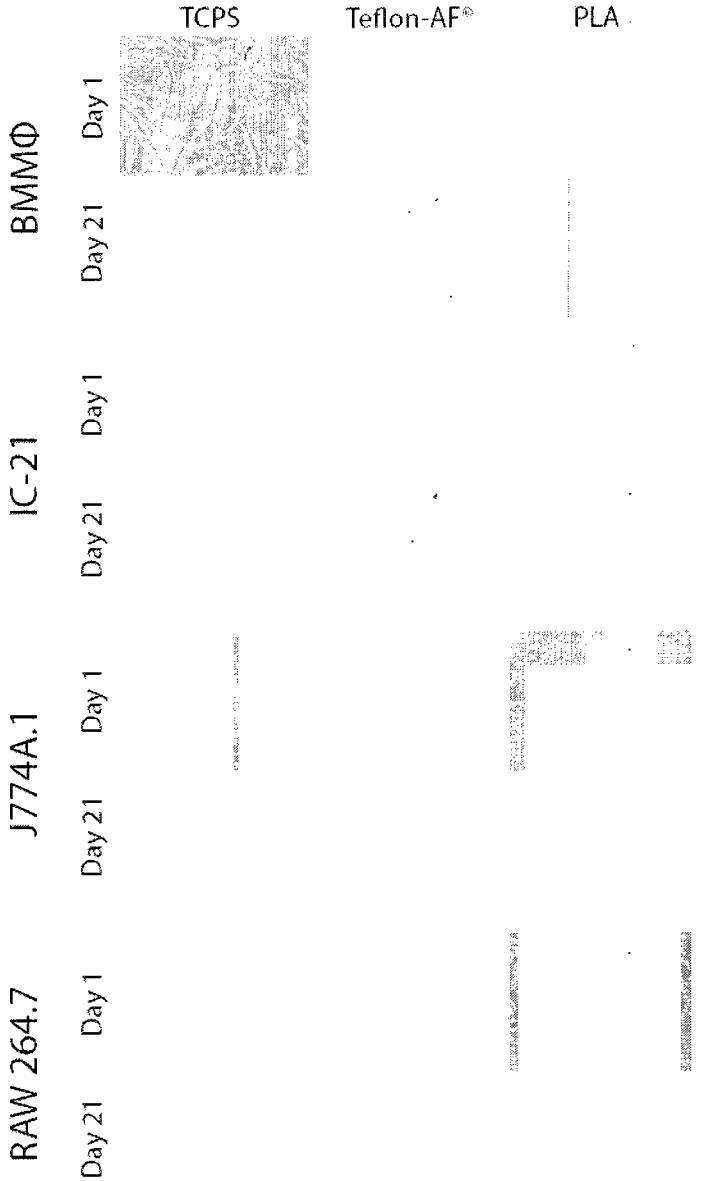


Figure 3.2. Cellular proliferation over time is surface-dependent. Cell proliferation over time is dependent on cell type and culture surface.

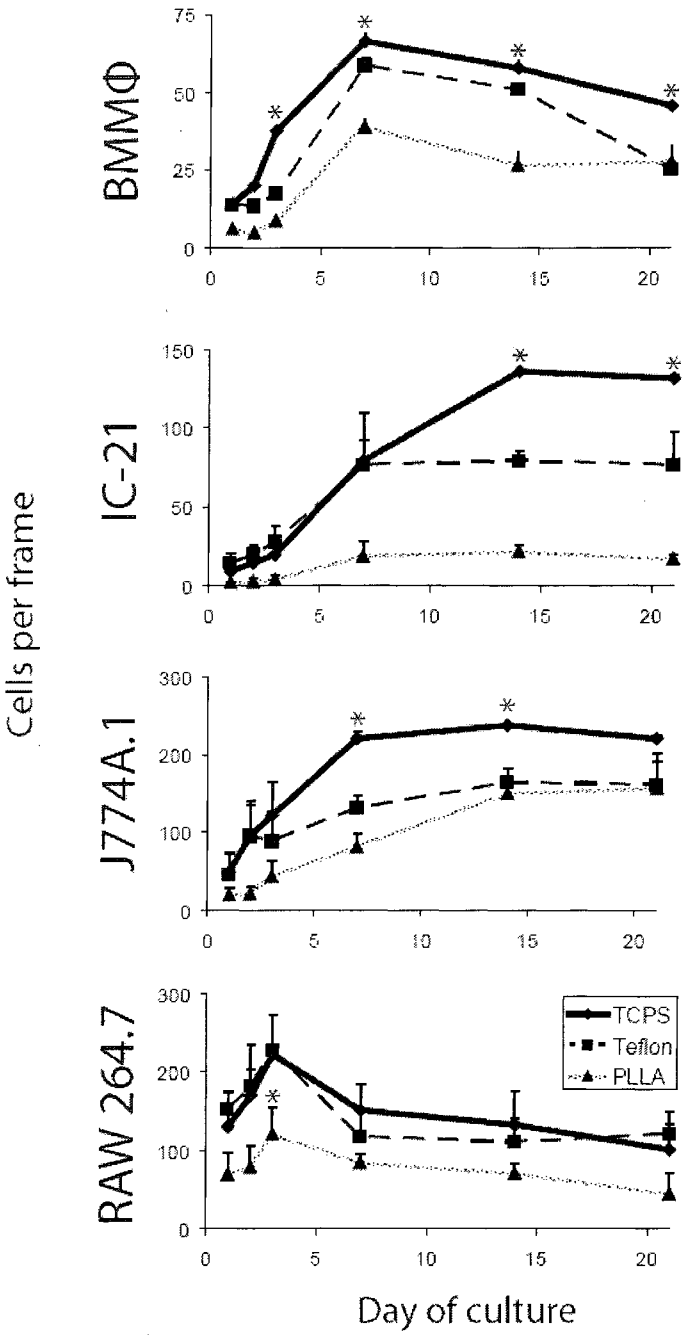


Figure 3.3. BMMΦ cytokine/chemokine expression is dependent on surface and time. After correcting cytokine amounts by the number of cells per plate, expression appears to occur in a large initial burst followed by attenuation by day 7.

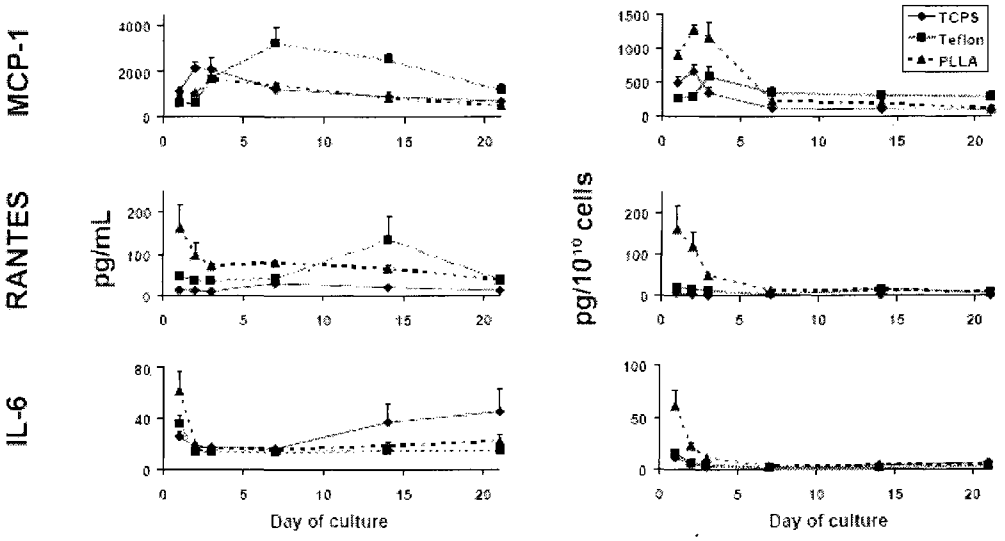


Figure 3.4. IC-21 cytokine/chemokine expression is dependent on surface and time. After correcting for cytokine amounts by the number of cells per plate, expression appears to occur in a large initial burst followed by attenuation by day 7.

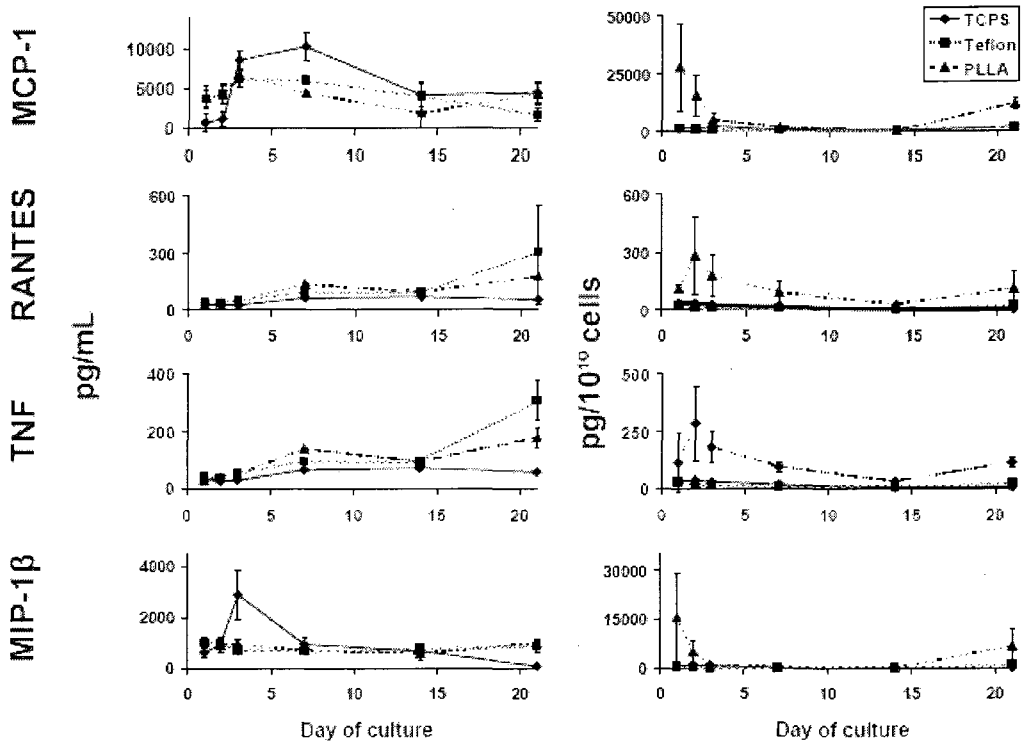


Figure 3.5. J774A.1 cytokine/chemokine expression is dependent on surface and time. After correcting for cytokine presence by the number of cells per plate, expression appears to occur in a large initial burst followed by attenuation by day 7.

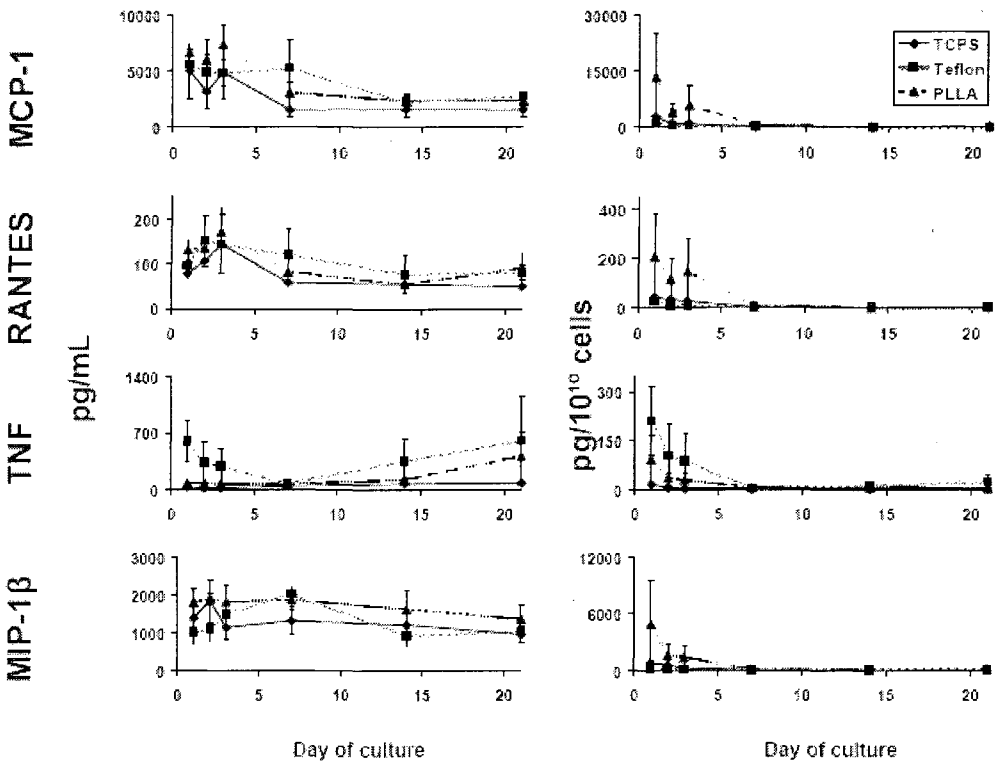


Figure 3.6. RAW 264.7 cytokine/chemokine expression is dependent on surface and time. After correcting for cytokine amounts by the number of cells per plate, expression appears to occur in a large initial burst followed by attenuation by day 7.

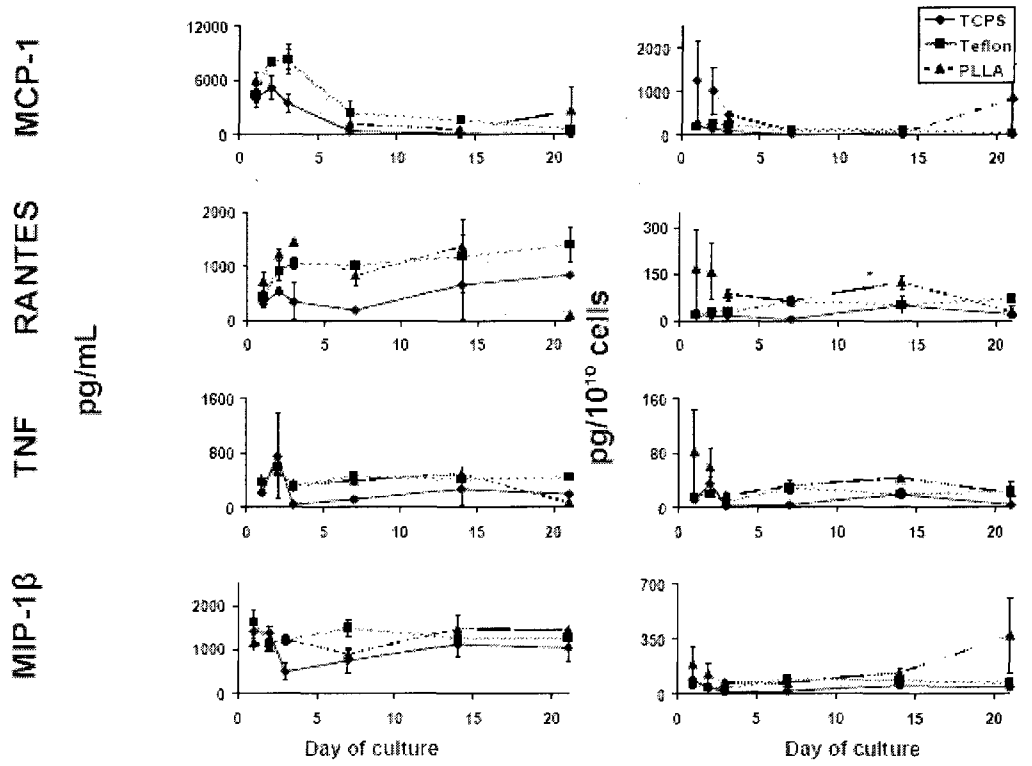


Figure 3.7. Cell surface marker expression in BMMΦ remains relatively stable following extended culture. Detection of BMMΦ surface markers by flow cytometry shows no changes in integrin expression (CD18, CD11b, and CD11c) following 21 days of culture on model surfaces. Changes observed occur in markers of differentiation (F4/80, CD14, Fc), of intracellular adhesion (CD54), and of activation (CD40, TLR-4, and MMR).

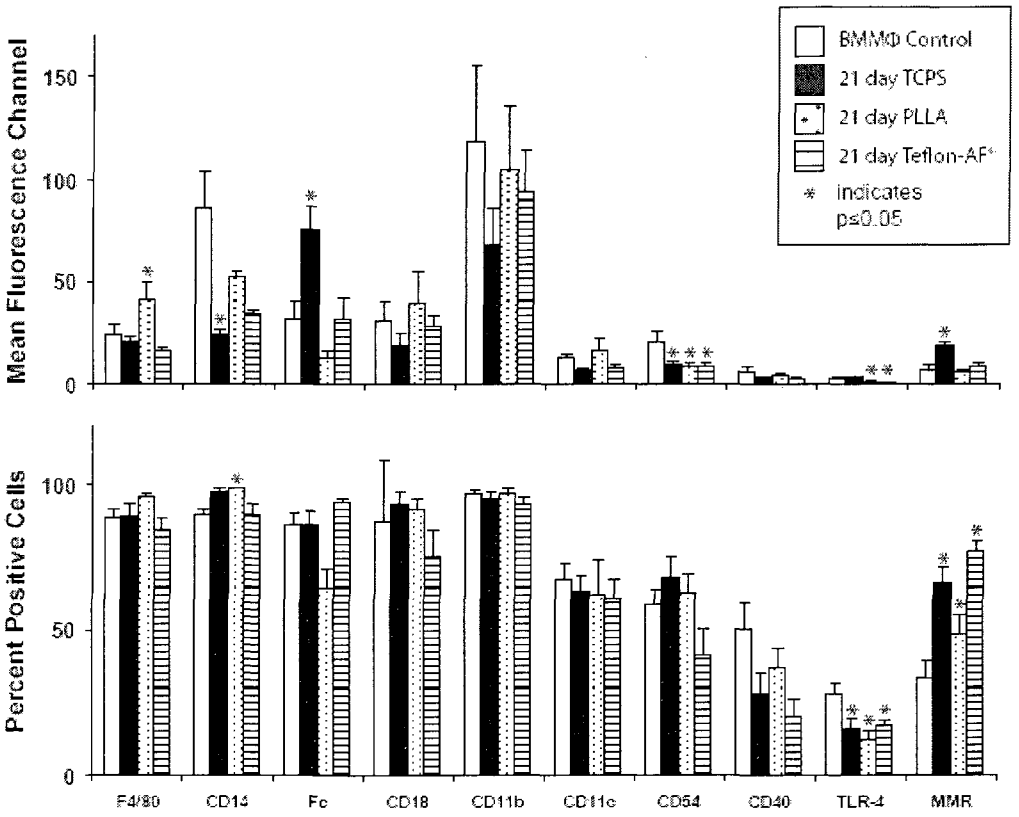


Figure 3.8. Cell surface marker expression in IC-21 exhibits many changes following extended culture. Detection of IC-21 surface markers by flow cytometry shows changes in integrin expression (CD18, CD11b, and CD11c), markers of differentiation (F4/80, CD14, Fc), of intracellular adhesion (CD54), and of activation (CD40, TLR-4, and MMR) following 21 days of culture on model surfaces.

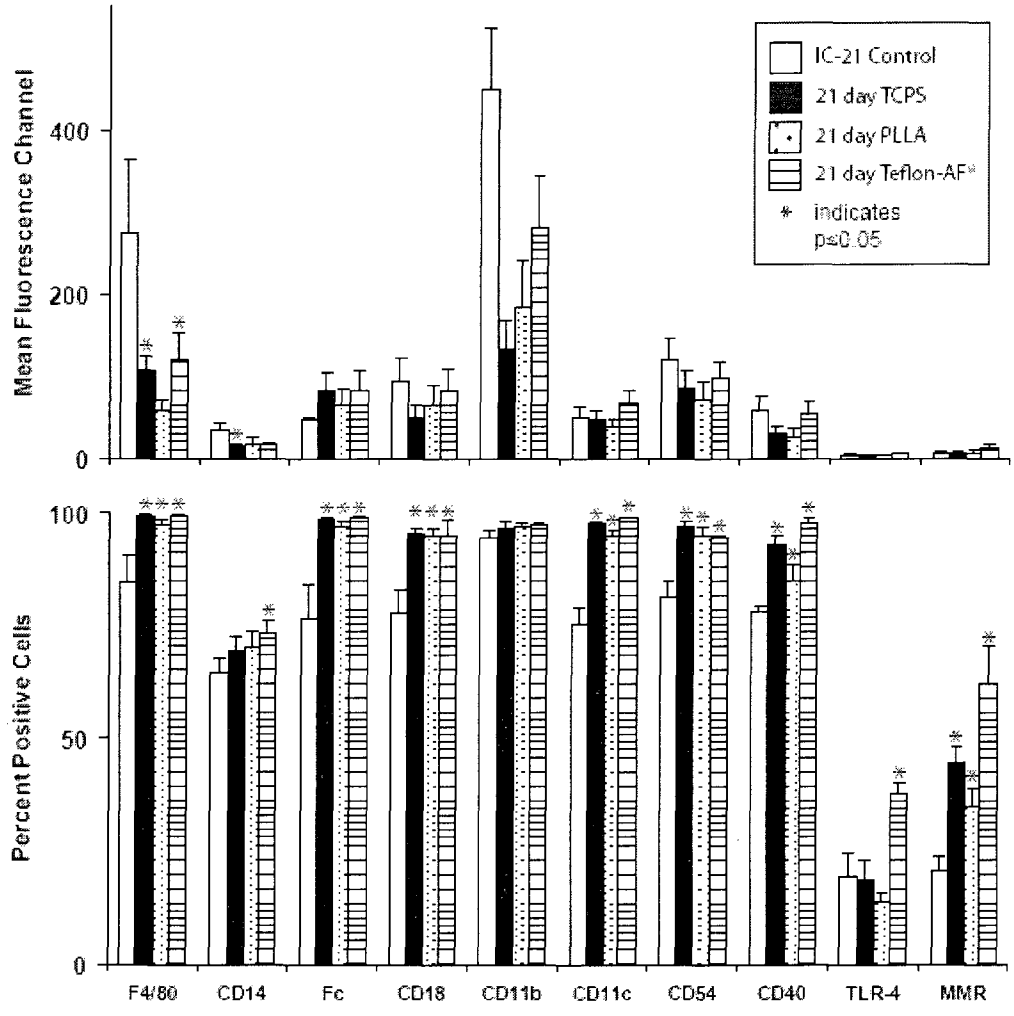


Figure 3.9. Cell surface marker expression in J774A.1 remains relatively stable following extended culture. Detection of J774A.1 surface markers by flow cytometry shows no changes in markers of differentiation (F4/80, CD14, Fc) following 21 days of culture on model surfaces. Changes observed occur in integrin expression (CD18, CD11b, and CD11c), markers of intracellular adhesion (CD54), and markers of activation (CD40, TLR-4, and MMR).

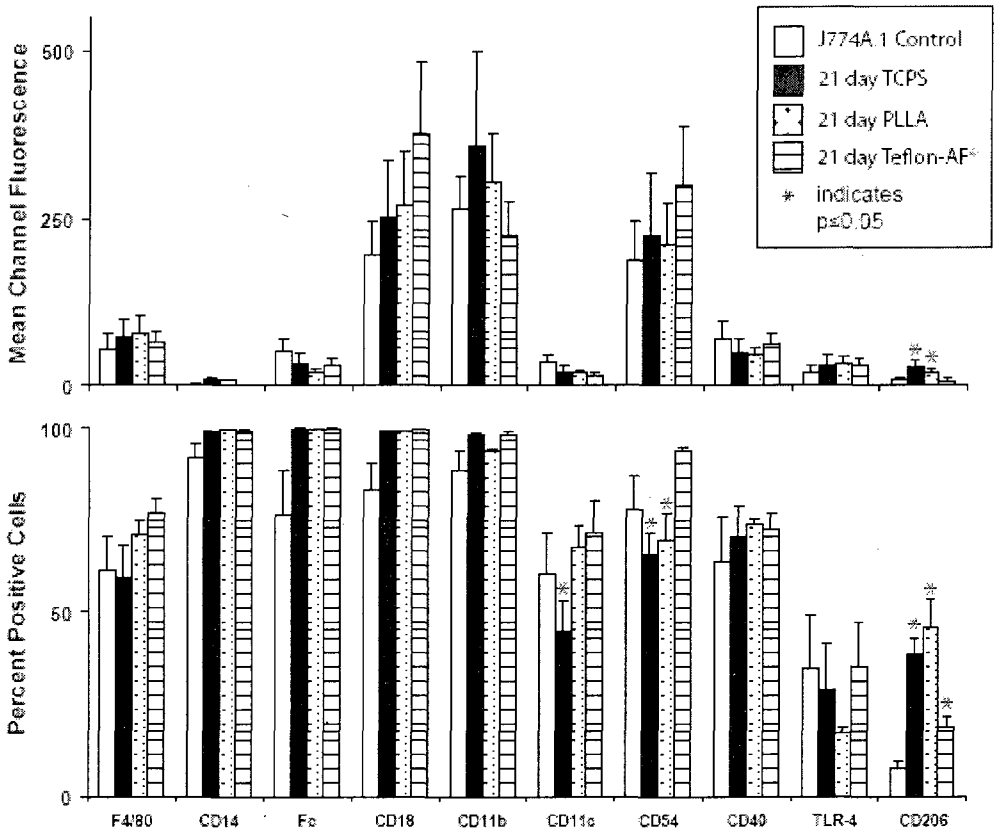
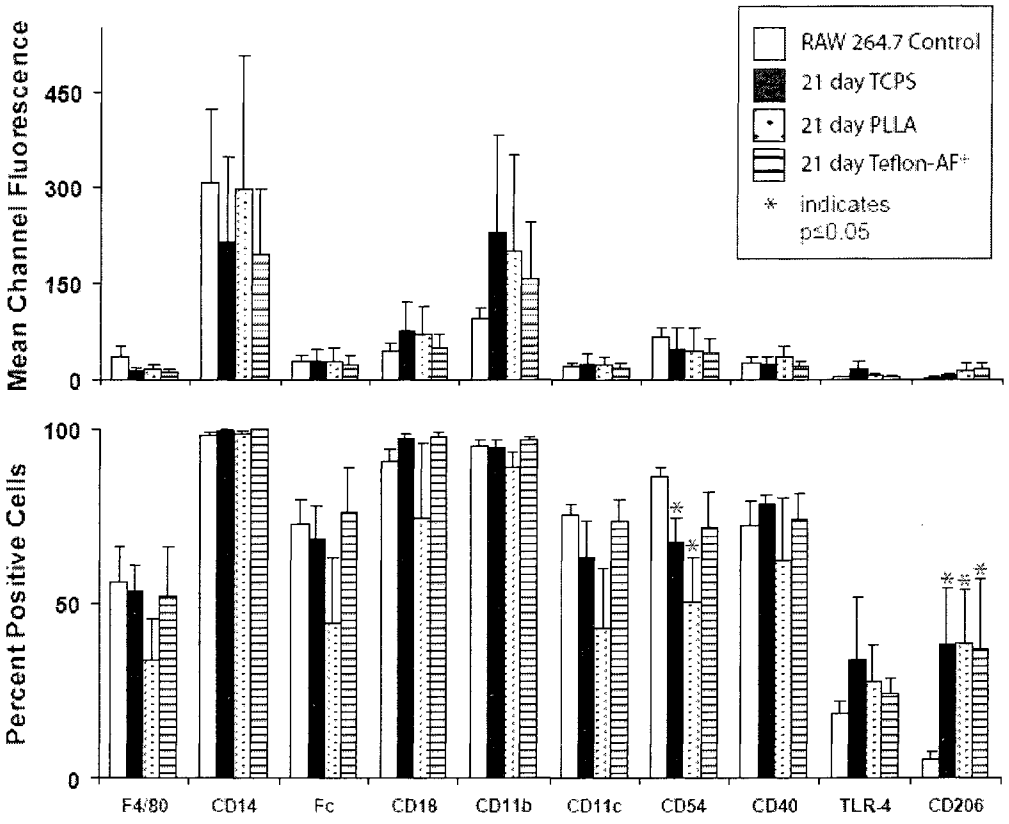


Figure 3.10. Cell surface marker expression in RAW 264.7 remains relatively stable following extended culture. Detection of RAW 264.7 surface markers by flow cytometry shows no changes in markers of differentiation (F4/80, CD14, Fc), or integrin expression (CD18, CD11b, and CD11c) following 21 days of culture on model surfaces. Changes observed occur in markers of intracellular adhesion (CD54), and markers of activation (CD40, TLR-4, and MMR).



CHAPTER 4: AN *IN VITRO* MURINE MACROPHAGE FUSION MODEL DEMONSTRATES SIGNIFICANT DECREASES IN SURFACE MARKERS FOLLOWING CELL-CELL FUSION.

This chapter was written by Lisa M. Chamberlain, and edited by Mercedes Gonzalez-Juarrero and is submitted to *Biochemical Biophysical Research Communications*.

4.1 Abstract

Fusion of macrophage-lineage cells into multi-nucleate foreign body giant cells (FBGCs) is a histological and phenomenological hallmark of the host foreign body response. Murine macrophage cells were tested in several known *in vitro* conditions of FBGC development as a working method of developing foreign body giant cells from murine bone marrow cells over a 10-day culture period. Foreign body giant cells formed using this method exhibit reliably high efficiency in producing FBGCs in culture, and significant decreases in several macrophage markers compared to non-fused cells, implying clear divergence from the original molecular macrophage phenotype. That these changes represent a FBGC phenotype or some significance to FBGC function at the implant site remains to be determined.

4.2 Introduction

Consequent to surgical biomaterial placement *in vivo*, a complex immune response is initiated that substantially changes the healing conditions and response at the implant site. This abnormal host-initiated and sustained inflammatory response, the foreign body response (FBR), involves the recruitment of mononuclear lymphocytes,

neutrophils, monocytes, and macrophages to the implant site at various times and with varying intensity and duration.[1] As the FBR ensues, recruited macrophages fuse near or at the surface of the implant to form foreign body giant cells (FBGC) that persist at the site for the duration of the implant.[2] FBR maturation/completion in soft tissue is characterized by the production of a dense, avascular collagenous capsule by recruited and activated fibroblasts. This capsule effectively "walls off" the implant and can ultimately result in implant failure through several adverse events.[3] This implant-initiated inflammatory response remains unresolved, with FBGC and the avascular collagenous capsule, and associated risk factors for failure, persisting throughout the life of the implant.[4]

Current protocols for prompting *in vitro* FBGC formation use harvested primary peripheral blood monocytes (PBMC) incubated with 10 ng/mL IL-4 and 10 ng/mL GMCSF.[5] PBMC are costly and require a cohort of healthy individuals to be included in the study on a regular basis. Another approach is to generate FBGCs from primary mouse cells as well as various cell lines in cultured systems. Several current protocols cited in the literature produce varying yields of these cells.[6,7] Variations include using multiple combinations and varying concentrations of mixtures of IL-4, IL-3, IL-13, and GM-CSF. Inconsistent fused cell yields, with highly variable numbers of FBGC cell nuclei, and poor cell fusion efficiencies (i.e., <50%) for these procedures then complicate further cellular and molecular characterization of these FBGC cultures since essentially it comprises a mixed culture where fused and unfused, but cytokine-treated, monocytes co-exist.

The presence of FBGC at the site of an implant has been correlated with increased material degradation, but knowledge of the FBGC purpose, whether these correlations are also causative, and why these cells persist for so long at the implant site is not well understood.[8] Few characterizations of FBGC phenotype and residual macrophage marker expression have been performed.[9] New information on FBGC surface markers in improved cultures where co-culture effects might be minimized may strengthen the field's understanding of FBGC function at implant sites.

4.3 Materials and methods

4.3.1 Mice.

Specific-pathogen-free female C57BL/6 mice (6-8 weeks old, Jackson Laboratory, Bar Harbor, ME) were maintained in the biosafety level 2 facilities at Colorado State University, and given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments. The specific pathogen-free nature of the mouse colonies at these facilities is demonstrated by testing sentinel animals, shown to be negative for 12 known mouse pathogens. Animal guidelines for the care and use of laboratory animals have been observed, all experimental protocols used in this study were approved by the Animal Care and Use Committee of Colorado State University.

4.3.2 Primary cell harvest.

Bone marrow cells were harvested from murine tibias and femurs and differentiated into macrophage cells using previously described methods.[10,11] Briefly, bone marrow cells were flushed from the femurs and tibias , and then cultured in media which causes the cell to differentiate towards a macrophage phenotype. All primary cultures were grown in BMMΦ media, which contained 10% heat inactivated fetal bovine serum (FBS,

Hyclone®, Logan, UT), 10% L-929 fibroblast conditioned medium, 1% penicillin-streptomycin (Gibco, Carlsbad, CA), 0.01M Hepes buffer (Gibco, Carlsbad, CA), 1 mM sodium pyruvate (Gibco, Carlsbad, CA, and 1% of a 100X MEM non-essential amino acids solution (Gibco, Carlsbad, CA) in Dulbecco's modified Eagle's medium (DMEM, Mediatech, Inc, Herndon, VA). Cell cultures were incubated under "standard conditions" (37℃, 5% CO_2, 98% humidity).

4.3.3 Immortalized murine cell culture.

Adherent murine (monocyte-) macrophage cell lines IC-21, J774A.1 and RAW 264.7 were purchased from the American Type Culture Collection (ATCC, Manassas, VA). RAW 264.7 and IC-21 cells were cultured in RPMI-1640 (Mediatech, Inc, Herndon, VA) and J774A.1 cells were cultured in DMEM (Mediatech, Inc, Herndon, VA) per vendor recommendations. All growth media used to culture cell lines were supplemented with 10% FBS (Hyclone®, Logan, UT), 1% penicillin-streptomycin (Gibco, Carlsbad, CA), and 0.01 M Hepes buffer (Sigma). Cell cultures were maintained below 80% confluence in TCPS flasks and passaged by incubation with divalent cation free Dulbecco's Phosphate Buffered Saline (dPBS Hyclone®, Logan, UT) prior to scraping with a rubber policeman. All cells were used at or below passage number 30 as received from ATCC and incubated under standard conditions. Replicates were defined as cells harvested from different passages and/or flasks.

4.3.4 Fusion using published protocols

Bone marrow cells were added to 12-well plates and allowed to differentiate in BMMΦ media for three days, allowing these cells to mature to a monocytic phenotype, after which media was changed to contain 10ng/mL IL-4 (R&D Systems), and 5ng/mL GM-

CSF (R&D Systems). Bone marrow cells were incubated in this cytokine cocktail for 7 days, with media and cytokine changes every 2 days. Cell lines were added to 12-well plates and incubated with 10ng/mL IL-4 and 5ng/mL GM-CSF for periods of 7 days. Media and cytokines were changed every 2 days. To facilitate the visualization of nuclei, a Hema-3 kit (Fisher Scientific) was used to fix and stain the cells.

4.3.5 Manipulation of fusion protocols

The effects of cytokine concentration on cell fusion were observed by significantly increasing the concentration of cytokines, and including another fusogenic cytokine in the culture system (IL-13). For primary-derived macrophages, we developed two protocols that result in significant fusion. Both included incubation of bone marrow cells in L-929 media for 3 days. Following this incubation, media was changed by adding a cocktail of cytokines to the media. 1) 5ng/mLGMCSF and 10mg/mL IL-4, 2) 50ng/mL GMCSF and 100ng/mL IL-4, 3) 5ng/mL GMCSF, 10ng/mL IL-4, and 10ng/mL IL-13, and 4) 50ng/mL GMCSF, 100ng/mL IL-4, and 100ng/mL IL-13. Cocktail 1 was basic BMMΦ media containing 10ng/mL IL-4, 10ng/mL IL-3, and 5ng/mL GM-CSF. Cocktail 2 was basic BMMΦ media containing 100ng/mL IL-4, 100ng/mL IL-3, and 50ng/mL GM-CSF. Cocktail 3 was media containing 10ng/mL IL-4, 10ng/mL IL-13, and 5ng/mL GM-CSF. Cocktail 4 was media containing 100ng/mL IL-4, 100ng/mL IL-13, and 50ng/mL GM-CSF. Primary cells matured to a monocytic phenotype over 3 days in BMMΦ media were incubated in one of the four cytokine cocktails for 7 days prior to analysis. Cell lines were incubated in cocktails 2 and 4, for periods up to 7 days. Media and cytokines were changed every 2 days.

4.3.6 Quantitation of percent cell fusion

Nuclei per field, FBGC per field, and the number of nuclei within each FBGC were tallied using a microscope under (40X magnification). For each sample, a minimum of 100 nuclei were counted. Fields were randomly selected by starting at the left side of a well and moving the stage a defined amount between fields in a straight transect. The percent of cell fusion per cell type was calculated by dividing the number of nuclei present in FBGC by the number of all nuclei present.

4.3.7 Immunofluorescent staining

BMMΦ and FBGC cultures were fixed in 4% paraformaldehyde for 30 minutes. Cells were rinsed twice with PBS, and blocked in PBS containing 3% bovine serum albumin (BSA) and 0.02% Tween-20 (blocking solution) for thirty minutes. Primary antibodies (CD11c clone N418 armenian hamster anti-mouse IgG, CD18 clone m18/2 rat anti-mouse IgG2a, F4/80 clone BM8 rat anti-mouse IgG2a, CD206 (macrophage mannose receptor, MMR) clone MR5D3 rat anti-mouse IgG2a, CD44 clone IM7 rat anti-mouse IgG2b, CD11b clone M1/70 rat anti-mouse IgG2b, all from eBiosciences, San Diego, CA) were diluted 1:100 with blocking solution and incubated with cells at 37℃ for 1 hour. Cultures were then rinsed 5 times with blocking solution prior to incubation with secondary antibodies (FITC-conjugated goat anti-armenian hamster IgG and FITC-conjugated goat anti-rat IgG, both from Santa Cruz Biotechnology, Santa Cruz, CA, items sc-2446 and sc-2011, respectively), which were diluted 1:1000 with blocking solution, at 37℃ for 1 hour. Cultures were rinsed 5 more times with blocking solution prior to adding DAPI solution to stain nuclei (1:1000 dilution in PBS, Invitrogen, Carlsbad, CA). Cells were imaged while immersed in DAPI solution.

4.4 Results

4.4.1 Primary-derived cells fuse at a higher rate than cell lines.

Fusion of bone marrow derived (BMMΦ) cells demonstrated higher rates of cell fusion than similar cultures from cell lines using 10ng/mL IL-4 and 10ng/mL GMCSF. Thus, in RAW 264.7, J774A.1, and IC-21 cultures, the cell fusion rate was below 10%, while primary-derived cultures exhibited fusion rates exceeding 20%.

4.4.2 Primary-derived macrophages and J774A.1 cells exhibit greater morphological changes following fusion than other cell lines.

While fusion was observed in all cell types, BMMΦ and J774A.1 cells exhibited large changes in morphology for their FBGCs compared to IC-21 and RAW 264.7 cell fusions. FBGCs from J774A.1 and primary-derived cell cultures exhibit a very large adherent area "footprint" with enhanced spreading of the cytoplasm whereas FBGC morphologies in cultures of RAW 264.7 and IC-21 remained similar to non-fused cells (Figure 4.1).

4.4.3 Increasing cytokine concentrations is toxic to cell lines, but fusogenic for primary cell cultures.

Protocols using media cocktail 4 significantly increases fusion rates in primary cultures compared to cocktails 1, 2, or 3 (Figure 2). Protocols using media cocktail 4 provide the highest fusion rates (42.34%). Cocktails containing high concentrations of cytokines (cocktails 2 and 4) were highly toxic to all secondary cell lines, with no visible adherent cells remaining at day 3 in any cultures.

4.4.4 Several macrophage markers are significantly decreased in fusogenic cultures.
The ratio of antibody fluorescence to DNA fluorescence among surface receptors tested using image analysis from fluorescence microscopy decreased for all assays but for CD206 markers in fusogenic cultures compared to BMMΦ cultures. The macrophage marker F4/80, integrins CD11b, CD11c, and CD18, and a surface protein thought to be involved in fusion, CD44, all exhibited significant decreases in FBGC cultures (Figure 4.4).

4.5 Discussion

This study sought to produce a reliable method to induce cell-cell fusion to form FBGCs from murine bone marrow cells differentiating towards a macrophage phenotype, and to compare these results to the same using secondary commercial cell lines. The cell fusion rate obtained using primary murine bone marrow cells and protocol "4" described in this study (42.34%) was similar to previous cell fusion rates published for primary human cell fusion models.[6,12] Recently, another murine FBGC model has been reported in the literature.[13] This newly reported protocol required less cytokine (10-fold less) over an extended period of time (17 versus 10 days). Both methods should now be evaluated to compare fusion efficiencies and their relevance to *in vivo* developed FBGCs.

Clear differences between primary derived cells and cell lines can be seen from the morphological differences observed in FBGCs formed from different cell types (Figure 4.1). Macrophage fusion has been shown to be dependent on the phenotype of the macrophage.[14] Thus, differences in cell fusion rates and resulting adherent FBGC morphology likely reflects differences in macrophage differentiation and activation states such as those previously reported for these same cell types.[15] The need for prolonged incubation times and increased cytokine concentrations to induce fusion in the murine

cells investigated here is likely indicative of a lack of sufficient cell receptors on the surface of these cells that prompt the FBGC fusion mechanism.

Decreases in macrophage markers (F4/80), adhesion proteins (CD11b, CD11c, CD18), and fusion-related proteins (CD44) were observed in BMMΦ cultures following fusion (Figure 4.3). These changes suggest a shift towards a new cellular phenotype distinctly different from the FBGC precursors. While gross morphological changes are easily observable between macrophages and FBGC, changes in surface protein expression have not yet been reported. CD44 is a marker that is up-regulated at the time of fusion, yet is clearly decreased in fused cells (Figure 4.3 B).[9] To understand the full implications of these changes from macrophage markers on FBGCs and, more importantly, on the foreign body response, further work is necessary. However, it is clear from these protein profiles before and after cell fusion in culture that cell phenotypic change is occurring as reflected in their protein production following fusion suggesting an associated change in function and behavior of FBGCs compared to macrophage precursors.

4.6 Conclusions

Both primary and secondary murine macrophages require substantially larger concentrations of cytokines to induce fusion into FBGCs than primary human macrophages, demonstrating distinct differences between the two cell sources and culture models. As most in vivo preclinical studies for materials biocompatibility testing are performed in mice, this comparison brings the relevance of these murine *in vivo* protocols into question with regard to their relevance to the human FBR condition. Additionally, macrophage fusion in vitro results in significant decreases in the expression of many cell surface markers, suggesting a change in the role of the cell following fusion.

4.7 References

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Figure 4.1 Morphology of cell types before and after fusion. Fusion of macrophages into FBGC results in a large increase in cell size for primary cells and J774A.1. IC-21 and RAW 264.7 however exhibit rare fusion events that result in little morphological change.

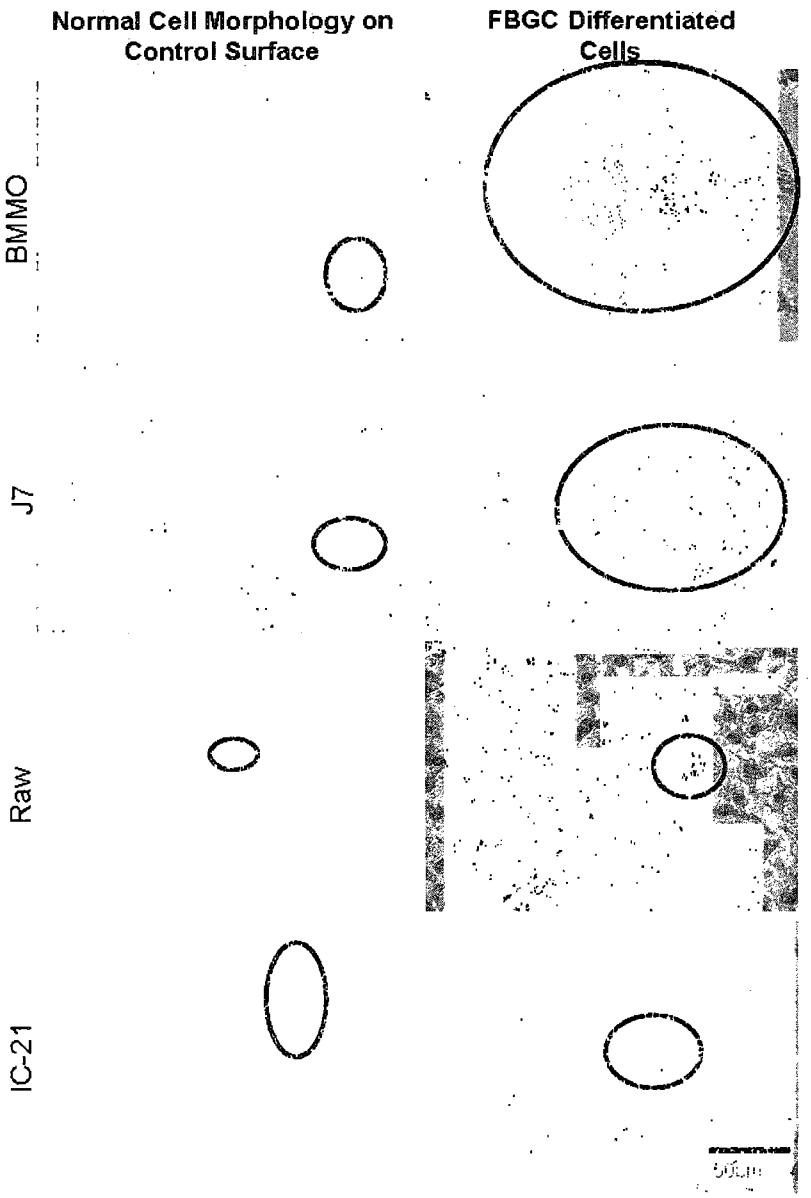


Figure 4.2 Cell fusion rates increase with optimal cytokine cocktails. Bone marrow cells differentiated 3 days in L-929 conditioned media, then 7 days in the following distinct media conditions (represented as corresponding numbers under the x-axis): 1) 5ng/mLGMCSF and 10mg/mL IL-4, 2) 50ng/mL GMCSF and 100ng/mL IL-4, 3) 5ng/mL GMCSF, 10ng/mL IL-4, and 10ng/mL IL-13, and 4) 50ng/mL GMCSF, 100ng/mL IL-4, and 100ng/mL IL-13. * represents $p \leq 0.05$ compared to all other cytokine cocktails. Error bars represent the standard error of the mean.

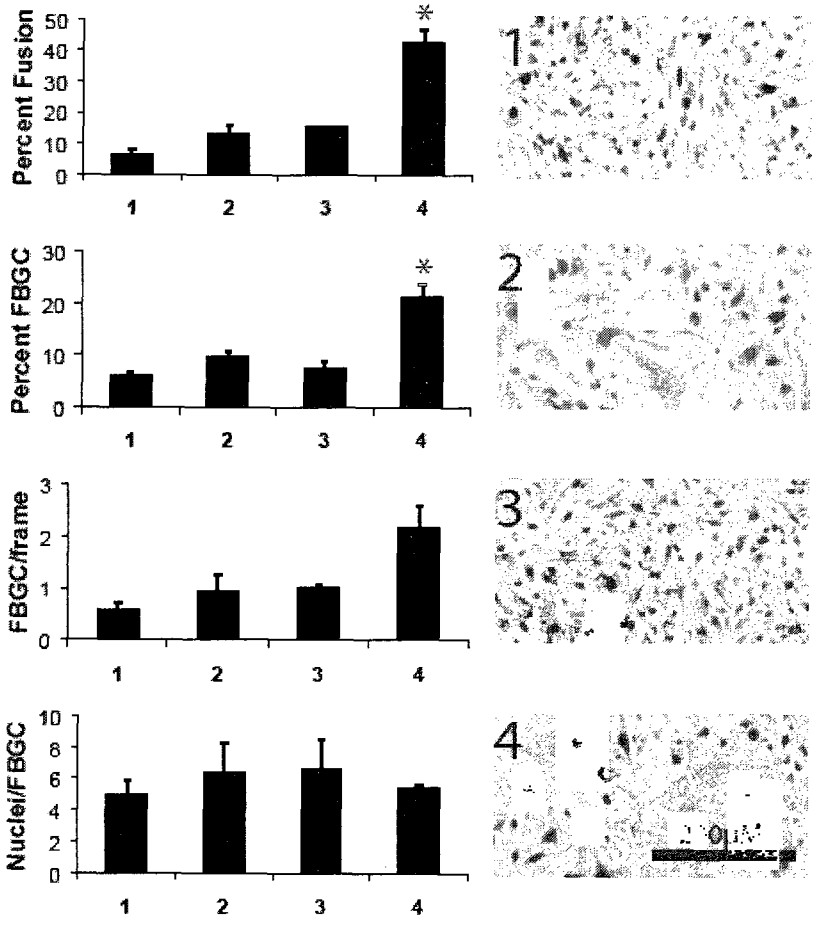
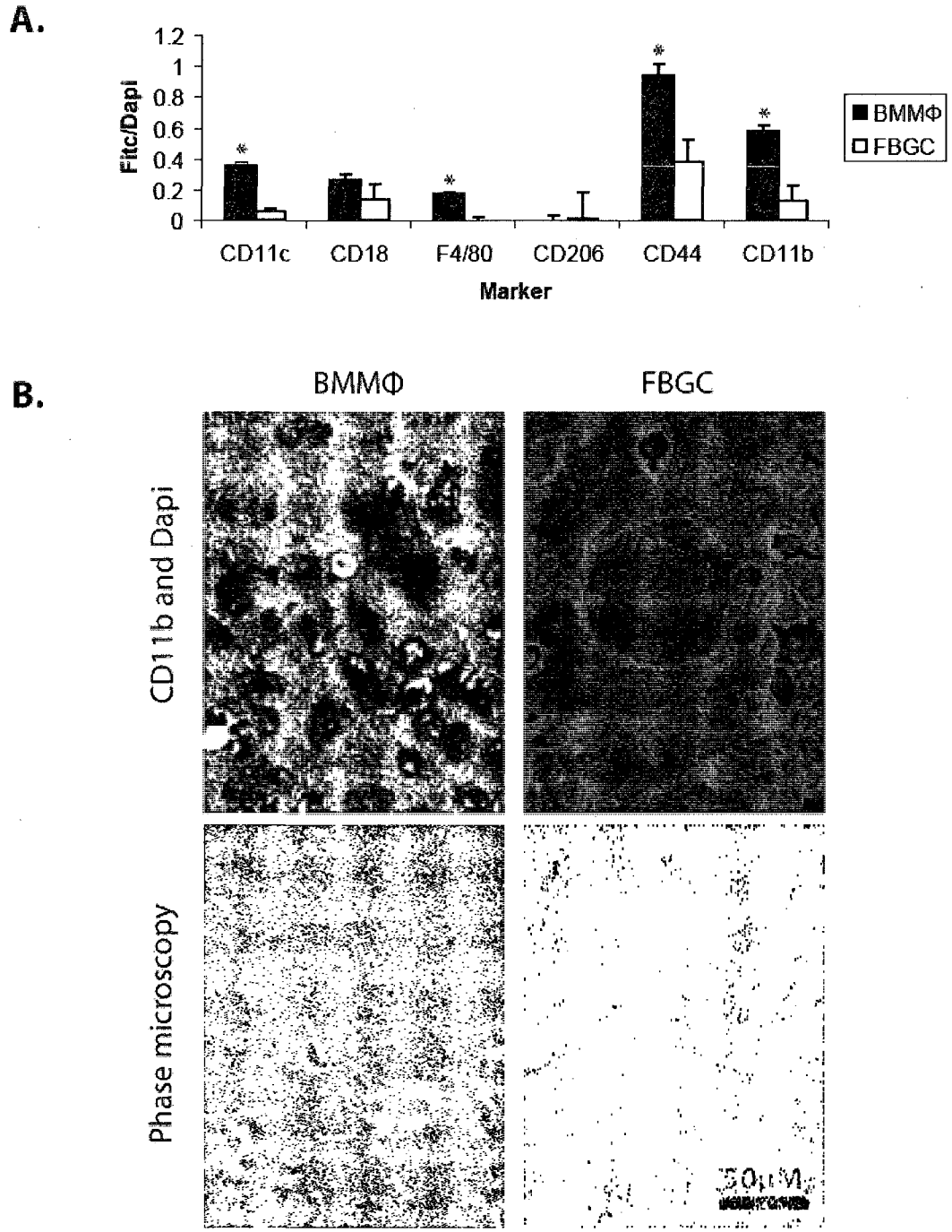


Figure 4.3. Macrophage cell surface antigens decrease in FBGC cultures. The ratio of FITC fluorescence from monocolonal antibodies to dapi fluorescence of nuclei significantly decreases for several macrophage markers when exposed to fusogenic stimuli in culture. (A) Images are representative of CD11b data. Blue stain is dapi, green is antibody fluorescence (FITC) (B).



CHAPTER 5: *IN VIVO* MOLECULAR AND CELLULAR ASPECTS OF FOREIGN BODY RESPONSE DEVELOPMENTS IN A MURINE MINIMALLY INVASIVE IMPLANT MODEL DEPEND ON IMPLANT MATERIAL

This chapter was written by Lisa M. Chamberlain, and edited by David W. Grainger and Mercedes Gonzalez-Juarrero, and submitted to *Biomaterials*

5.1 Abstract

The foreign body response is a chronic inflammatory process resulting from the implantation of any material into host tissue. In soft tissue, this most often produces implant encapsulation by a thick avascular collagenous layer. Few *in vitro* studies provide reliable and predictable hallmarks of this response *in vivo*. To provide new data for *in vivo* molecular mechanisms behind this response, non-degradable (nylon mesh film and Teflon-AF® films) as well as degradable (lactide-based polyester films) biomaterials were subcutaneously implanted into mice using a minimally invasive needle injection method and analyzed at explant from 1 to 21 days. Immunohistochemistry techniques on sectioned tissue demonstrated biomaterial-dependent trends for macrophage recruitment, and cytokine, and chemokine production. Additionally, foreign body giant cells co-localized with all markers tested, with material-dependent rates of co-localization.

5.2 Introduction

The foreign body response (FBR) is an inflammatory host reaction that occurs following implantation of any material into living tissues.[1] The FBR often compromises implanted medical device functions or pre-disposes the patient to increased risk of adverse events (e.g., infection, fibrosis, adhesions), requiring device removal.[2] With acute and chronic phases involving complex cascades of multiple host cell types and interacting inflammatory and healing components, the molecular etiology behind the FBR is not well characterized. Understanding and controlling the FBR in the context of implants is critical to improving biomaterials performance. In particular, directly observing FBR development in vivo in various implant systems,[3-5] and then accurately recapitulating the complex dynamics of molecular and cellular events in vitro have both proven difficult.[5-10] Few in vivo models have produced clear FBR mechanisms and definitions of the process, and few in vitro assays faithfully capture and duplicate these processes. Hence, a better understanding of events contributing to FBR formation, directly correlating and connecting current in vitro and in vivo experiments, is important.

Initiation of the FBR is prompted by the normal wound healing process initiated immediately after insertion of an implant into tissue most often through a surgical incision.[11] Within seconds following implantation, the implant surface is barraged with blood- and tissue- derived proteins and extra cellular matrix debris, forming a layer on the implant surface. As wound healing continues, recruited cells -- granulocytes, neutrophils, and monocytes enter the lesion and release further cascades of cytokines and chemokines locally that seek to control inflammatory processes and recruitment of other cell types to the lesion. Over time, recruited monocytes differentiate into macrophage cells to surround and adhere to the implant

surface. This process leads to macrophage cell activation with the purpose of eliminating the foreign body by normal processes of phagocytosis. Because the macrophages are often unable to remove the implant physically and chemically despite continuous efforts, it is believed that activated macrophages and monocytes (often referred to as being in a state of "frustrated phagocytosis") fuse to form foreign body giant cells (FBGC) as a terminal implant-centric wound healing phenotype. This process also results in activation of local fibroblast cells, leading to extensive proliferation and collagen production at the implant site. Ultimately, this produces a largely avascular, dense, collagenous capsule surrounding soft tissue implants and associated FBGCs, and analogous acellular fibrous tissue formation around implants in other tissue types.[11]

Few studies have used *in vivo* animal models to directly assay, interrogate or address the FBR formation across short and longer-term implant time scales *in situ*.[12,13] New information on the temporal and physical dynamics of co-localization of various cell types contacting the implant and participating in the FBR is needed. Furthermore, co-localization and identification of soluble factor cascades (e.g., cytokines and chemokines) within the FBR resulting from implantation of different biomaterials, and their connections to key cellular events requires further elucidation. The influx of monocytes and macrophages to implant sites is a commonly studied part of the foreign body phenomenon, with studies generally exhibiting high recruitment of macrophages to an implant site at early time points (3-7 days) which attenuates out to day 21.[12,14]

Recently, a new murine implant model has been reported for study of the FBR using a minimally invasive syringe needle subcutaneous deployment of flexible polymer

films and fibers that avoids surgical incision or general anesthetic. (REF). Explantation of the biomaterial at necrospsy, and histology and immunohistochemistry of the implant site harvest allows ready determination of cell and molecular composition, identification and localization, and architecture of the tissue directly adjacent to the polymer film implant. While immunohistochemistry is semi-quantitative staining technique, and is more capable of revelaing the presence of a protein rather than its production sources, it reveals wound-site localization within the tissue and association with specific cell types in the lesion, providing previously unknown information regarding the FBR. Additionally, the minimally invasive implant placement method reduces wound site trauma, healing artifacts from surgery and animal morbidity, and allows multiple implants per animal, as well as short-term and long-term implant/explant comparisons to capture wound site dynamics. Finally, the method could approximate similar implantation conditions to needle-based subcutaneous glucose sensors that suffer numerous FBR-associated problems.[15] The first murine in vivo study using this method demonstrated this utility, exploring the spatial relationships of cells in the FBR, and demonstrating the presence of several cytokines at the site.[16]

This current study utilizes several model biomaterials as implants with this new murine implant model. Two non-degradable materials, sterile flexible microbiological filter membranes (a pyrogen-free commercial nylon cell strainer material), and Teflon-AF® films, as well as bioresorbable PLLA films, were selected as common polymer materials readily available with some history of in vitro use.[6,7,17] Additionally, these polymer materials exhibit different bulk and surface chemistries.[17-20] These materials were implanted via subcutaneous needle "injections" into murine subcutaneous tissue and analyzed as explanted tissues at times as early as 1 day

and as long as 21 days. With this approach, we demonstrate material-based differences in the initial and later cellular and molecular stages of FBR development in vivo.

5.3 Materials and Methods

5.3.1 Materials preparation

Nylon microbiological-grade filter material was excised from sterile, pyrogen-free cell strainers (product # 352350, 70μM mesh, BD Biosciences, Santa Clara, CA) using autoclaved razor blades, and cut into squares of approximately 0.5cm by 0.5cm for insertion into deployment needles. Teflon-AF® (DuPont Fluoroproducts) films were prepared by solvent casting a 0.01% v/v Teflon-AF®/FC-40 solvent solution onto a clean 100mm Fluoroware™ dish (Entegris, Billerica, MA). Fluoroware dishes were cleaned by washing with hot DI water, rinsing extensively with DI water and methylene chloride. Polymer solution (10ml) was added to the Fluoroware dish and solvent was removed by vacuum in an oven at 60℃ fo r several hours. This process was repeated with subsequent layers of Teflon-AF® until a total of 50mL of solution had been added to the fluroware dish and dried into a robust flexible film. Teflon-AF® films were excised from Fluroware dishes by cutting squares of approximately 0.5cm by 0.5cm and peeling them from the surface carefully using autoclaved razor blades and dissection equipment. PLLA materials were prepared similarly to Teflon-AF® materials in these dishes. A 0.1% solution (10 ml) of 100kDa PLLA in methylene chloride was solvent cast onto clean Fluoroware dishes, and solvent was removed while loosely covered in a fume hood at room temperature for several hours, and this was repeated with subsequent layers of PLLA until a total of 50mL of solution had been added to the Fluroware dish. PLLA films were excised from Fluoroware dishes by cutting squares of approximately 0.5cm by 0.5cm and peeling

them from the surface using autoclaved razor blades and dissection equipment. The nylon filter, Teflon-AF®, and PLLA film squares (approximate thickness of 0.01mM) were inserted into 16.5 gauge medical grade syringe needles in a sterile hood by folding them several times and using tweezers to place them inside the open (sharp) end. All materials were tested for the presence of contaminating endotoxin using a Pyrogene™ Assay kit (Cambrex, East Rutherford, NJ), and endotoxin levels were determined to be below the kit detection limit (0.02 EU/mL).

5.3.2 Animal care and surgery

Specific-pathogen-free female mice (6-8 weeks old, Jackson Laboratory, Bar Harbor, ME). were maintained in the biosafety level 2 facilities at Colorado State University, and given sterile water, mouse chow, bedding, and enrichment for the duration of the experiments. Animal guidelines for the care and use of laboratory animals have been observed, all experimental protocols used in this study were approved by the Animal Care and Use Committee of Colorado State University. Mice were anesthetized by intraperitoneal injection of ketamine (30mg/kg) prior to implant insertion. One implant of each material was placed into an anesthetized mouse using the film pre-loaded 16.5 gauge syringe needles, using a thin sterile plunger to physically push the films into three physiologically distinct subcutaneous dorsal tissue sites on the back of each mouse. The needle was inserted subcutaneously into the tissue location, and the implant was pushed under the skin with the plunger. Sixteen mice were implanted dorsally, each with nylon filter, Teflon-AF®, and PLLA flexible film implants. At 1, 3, 7, and 21 days following implantation, four mice were euthanized using CO_2 asphyxiation. Tissue samples containing the polymers were collected by surgical excision around the implanted films, and processed for histology and immunostaining.

5.3.3 Tissue fixation and processing

Implants were removed from mice at necropsy and immediately stored in zinc fixative solution (BD Pharmigen) for 48 hours. Tissue samples were sent to pathology services at Colorado State University for paraffin embedding and sectioning.

5.3.4 Immunohistochemistry

Paraffin was removed from slides by a two-step incubation in EZ-dewax (BioGenex, San Ramon, CA) for 10 minutes each. Tissue samples were re-hydrated by 5 minute incubations in a series of ethanol solutions (95%, 95%, 70%, and 30%, followed by pure water). Endogenous peroxidases in the tissue were blocked by incubation in a methanol solution with 0.3% hydrogen peroxide for 10 minutes. Slides were rinsed with PBS containing 0.05% tween-20, and then blocked by incubating in PBS containing 0.05% tween-20 and 3% bovine serum albumin (blocking solution) for 30 minutes. Primary antibodies were then diluted in blocking solution and incubated with samples overnight at 4°C. Samples were then rinsed copiously with blocking solution prior to incubation with secondary biotinylated antibodies diluted in blocking solution for one hour at room temperature. Antibodies and dilutions used are listed in table 1. Samples were rinsed once more with blocking solution, and an ABC kit (Vector Labs, PK6100) was used to attach avidin-conjugated horseradish peroxidase to secondary antibodies. Staining of slides was completed by using an AEC kit (Vector Labs, PK6100) for 3-5 minutes. Slides were then rinsed in DI water for 3 minutes to stop the AEC kit. Two to three drops of Hematoxilin QS (Vector Labs, SK4200) was then added to slides and rinsed immediately with tap water. Liquid mounting media was then applied to the surface

of each slide (Biomeda Crystal/Mount™ cat. M03) and slides were allowed to dry overnight at room temperature prior to imaging.

5.3.5 Microscopy

Samples were imaged using DIC microscopy on a Nikon Eclipse TE 2000-U microscope (Nikon Inc., Torrance, CA), a QImaging Retiga Exi camera (QImaging, Surrey, BC Canada), and QCapture software (QImaging, Surrey, BC Canada). Ten random fields containing approximately 25% polymer material were selected for use as images, with half being on the skin side and half being on the muscle side of the implant.

5.3.6 Quantitation of protein expression

Expression of protein was quantified as the number of positive-staining cells per protein marker per field. Images containing an average of approximately 25% implant material were used, and cells positively stained were counted from at least 10 random fields at each time point. Positive cells were determined visually by comparison to negative control slides (sections treated with secondary but not primary antibody). Additionally, the number of FBGCs per field and the total number of positively stained FBGCs per field were counted at day 21. FBGC protein co-localization at day 21 was quantified as a percentage of all FBGC staining positive due to low FBGC numbers per field.

5.3.7 Statistics

Significance was determined by Anova followed by post-hoc t-tests. P values less than or equal to 0.05 were considered to be significant.

5.4 Results

5.4.1 Macrophage recruitment and cytokine expression in the FBR varies depending on the type of biomaterial implanted.

As the macrophage is central to the proposed development mechanism of the FBR, the number of macrophages over time was assessed by observing the presence of the macrophage marker F4/80. The number of cells positive for F4/80 macrophage marker increased over time in all implants. However, PLLA surfaces appeared to initiate this process earlier than nylon filter and Teflon-AF® films. Thus, PLLA materials exhibited significant increases in the number of F4/80 positive cells by day 3 post-implantation, whereas Teflon-AF films showed only significant increases in the number of these cells at day 7. Furthermore, at 21 days post-implantation, all surfaces had recruited the same number of cells (Figure 5.1).

Tumor necrosis alpha (TNF-alpha) is a very potent inflammatory cytokine responsible for activating expression of chemokines that in turn recruit a number of cell types to the inflammatory site.[21] The number of positive cells expressing TNF-α adjacent to each type of implant was determined. Few differences from surface to surface were observed, with the exception of nylon filter membrane having significantly lower numbers of positive cells per field at days 3 and 21 (Figure 5.2). IL-6 is also a commonly assayed inflammatory cytokine.[21] A large amount of IL-6-positive cells were seen from day 3 on all surfaces. However, PLLA surfaces exhibited the highest number of positive cells per field on days 7 and 21 (Figure 5.3).

MCP-1, a chemokine that recruits monocytes and macrophages to a wound healing site, has been shown to be expressed upon macrophage adhesion to surfaces *in*

vitro.[9,22] The number of MCP-1 positive cells were significantly higher in samples obtained from implanted nylon filter surfaces at day 1, and for PLLA surfaces on day 3, but there were no significant differences at days 7 or 21 (Figure 5.4). IL-4 is a TH2 cytokine demonstrated in the literature to be vital for macrophage fusion, and is therefore considered to be an important signaling molecule in the FBR.[23-26] The number of cells per frame positive for IL-4 were significantly higher for Teflon-AF® implants at day 3, and PLLA implants at day 21 (Figure 5.5). TGF-β is a blanket term for a large family of cytokines with strong capacity to activate fibroblasts, induce proliferation and induce collagen production. TGF-β1, TGF-β2, and TGF-β3 are involved in different types of cell differentiation, tissue growth, development, and repair.[27] An antibody that binds with all three types of TGF-β allowed comprehensive viewing of its presence in the explanted tissue samples. The number of positive cells for TGF-β were scant at the first three time points, a trend that continued to day 21 for PLLA. However, at day 21 there were significantly higher numbers of positive cells for this cytokine per frame on samples obtained after implantation of nylon filter and Teflon-AF® films (Figure 5.6).

5.4.2 FBGCs stain positive for all tested antigens at day 21 with no differences in numbers of foreign body giant cells per frame.

No significant differences in the number of FBGCs were observed at day 21 among all film implant types (Figure 5.7). All FBGC appeared to be attached to implanted films. Co-localization of cytokines and macrophage markers with FBGCs was variable, with no defined patterns of expression between different FBGC samples. Thus, some FBGCs stained positive whereas other were negative for the same marker on the same slide for a given implant material. FBGCs positive for F4/80, TNF-α, IL-6, MCP-1, IL-4, and TGF-β were seen on all films at day 21 (Figure 5.8).

However, the percent of positive FBGCs varied for MCP-1 which was significantly less on Teflon-AF® films and for TGF-β which was significantly lower on PLLA surfaces (Figure 5.9).

5.5 Discussion

Temporal and tissue spatial location of wound healing and inflammatory cytokines, chemokines, and macrophages within a foreign body lesion is not well-studied in the context of implanted materials. This study reports changes observed in tissue explants in the levels of several relevant proteins over time following material implantation. Previous work has shown quantitative RT-PCR data that FBGC in rats produce many cytokines at the mRNA level, several which overlap with this study, including IL-6, TNF-α, MCP-1, and TGF-β.[28] Figures 5.8 and 5.9 demonstrate the presence of these molecules at the protein level both in FBGC and other cells near implanted film materials.

Macrophage recruitment to the site of a foreign body is considered to be an essential step in the foreign body response.[29] The F4/80 macrophage marker was used to track macrophage recruitment to the implant site (Figure 5.1).[30] The time course design of biomaterial and tissue explantation allowed demonstration that macrophages are recruited differently to implant sites depending on the implant material. Specifically, in this syringe-deployed subcutaneous system, macrophages are recruited earlier to PLLA film implants than those comprising Teflon-AF®. However, all materials appeared to attract nearly the same number of macrophages by day 21. Interestingly, at day 21 post-implantation many cells stain positive for MCP-1, a chemokine that recruits monocytes and macrophages. This suggests that macrophage recruitment may not be complete even 3 weeks following biomaterial

implantation with this model. Previous studies of macrophage recruitment to foreign bodies in rats using an in vivo cage model observed an opposite trend: a decrease in the number of macrophages over time in the cage system, although MCP-1 was not measured.[12]

Recent *in vitro* studies using macrophage cultures of several phenotypes and sources on similar biomaterials have shown a common initial burst of cytokine production followed by a reduction to stable, basal levels over time in short-term and extended (21-day) cultures.[9,22] However, these *in vivo* data disagree with certain *in vitro* trends (Figures 5.1-5.6). IL-6 and TGF-β appear to exhibit an opposite trend to each other, with the numbers of positive cells increasing over time in vivo, regardless of implant material (Figures 5.3 and 5.6). This observation may be related to the recruitment of macrophage cells to the site of the implant. As noted above, figure 5.1 indicates a continuing recruitment of F4/80 positive cells (macrophages) to the site over time. If each macrophage initiates a similar burst of cytokine production following implant contact and adhesion, continual adhesion events may cause a slow build-up of excess cytokine presence at the local site. Interestingly, 21 days has often been quoted as time for maturation and completion of the foreign body response *in vivo*. Yet the continued presence of cells expressing chemokine MCP-1 is consistent with a continued recruitment of macrophage cells to the implant area (figure 5.4). Additionally, a continued inflammatory cascade represented by sustained levels of inflammatory cytokines TNF-α and IL-6 (figures 5.2 and 5.3), evidence for a continued alternative pathway of macrophage activation at the site (IL-4, figure 5.5),[31] and continued stimulation of collagen production (TGF-β, figure 5.6) are seen at this time point. Collectively, these support a difference between needle-based implant deployment of films and FBR development subcutaneously versus

other implantation models that cause larger wounds using surgical methods.[5] In contrast, some similarities for *in vivo* results with this new model and previous reports are also evident. Other studies using cage implants have also demonstrated the observed increase in TGF-β over time,[32] and a significantly lower amounts of TGF-β present near hydrophilic surfaces.[13] Comparisons of *in vivo* data to those from *in vitro* models indicates that an *in vitro* model using one cell type does not emulate the complexity of the *in vivo* scenario where several cell types and multiple immunological and physiological factors participate in the FBR.

Encapsulation of an implant within a collagenous capsule is considered to be the resolution of the foreign body response. The signaling molecule, TGF-β, is known to induce collagen expression by fibroblasts, and correlates strongly with various wound healing responses. Interestingly, our data indicates that the film materials non-degradable over the experimental time frame (e.g., nylon filter and Teflon-AF®) recruit significantly more TGF-β-positive cells at day 21 than degradable PLLA films, a trend repeated in the percent of FBGCs noted positive for TGF-β in these sites. A further distinction between degradable and non-degradable materials is observed by the presence of IL-6-positive cells, which are significantly higher at days 7 and 21 near PLLA implants than nylon filter and Teflon-AF®.

5.6 Conclusions

Initial macrophage recruitment to subcutaneous polymer film implant sites in a murine minimally invasive implant model has some correlation to the material used. Cytokine and chemokine presence near the polymer implants is also shown to be material-dependent. Additionally, differences in macrophage recruitment timelines between this new implant model and that of the previously reported cage implant

system are shown. Our data indicate little correlation between *in vitro,* and *in vivo* models of the FBR. Additionally, discrepancies of macrophage recruitment times between this implant model and other *in vivo* models imply that the method of device implantation is more impacting to the observed cellular and molecular aspects of FBR than previously reported, and application-specific models should be used where possible in the study of new materials involvement in producing the FBR.

5.8 References

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Table 5.1. Antibodies used for immunohistochemistry protocols, their sourcing, and their dilutions.

Antibody	Company	Catalog #	Dilution
Rabbit anti-mouse TGF-β	Santa Cruz	SC-7892	1:75
Goat anti-mouse IL-4	Santa Cruz	SC-1260	1:50
Rat anti-mouse F4/80	Serotec		1:25
Goat anti-mouse IL-6	R&D Systems	AF-406-NA	1:100
Goat anti-mouse TNF-α	Santa Cruz	SC-1348	1:50
Goat anti-mouse MCP-1	Santa Cruz	SC-1784	1:50
Anti-Rabbit biotinylated	Caltag	L43015	1:200
Anti-Goat biotinylated	Dako	E0466	1:200
Anti-Rat biotinylated	Santa Cruz	SC-2041	1:200

Figure 5.1: Representative images and collated statistics for the number of cells per frame positive for F4/80 as a function of film implant time and polymer chemistry. *In vivo* expression of the macrophage marker F4/80 indicates a steady influx of macrophage cells to all surfaces over time (* indicates p≤0.05).

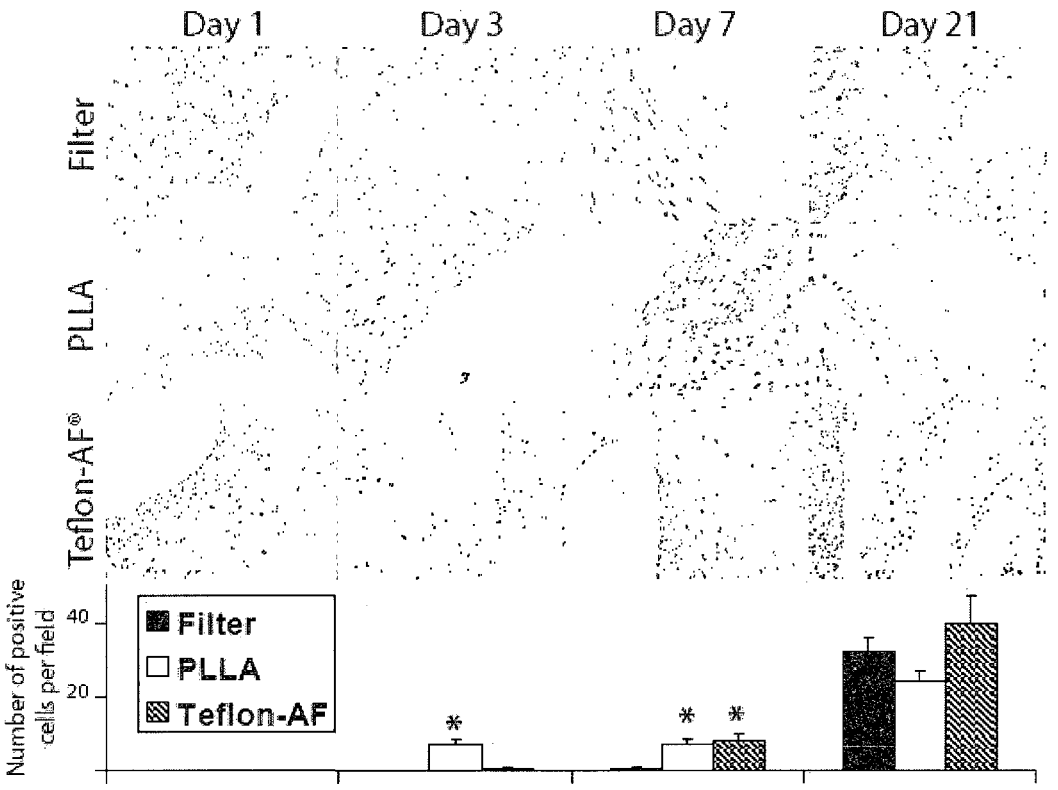


Figure 5.2: Representative images and collated statistics for the number of cells per frame positive for TNF-α as a function of film implant time and polymer chemistry. TNF-α positive cells are more numerous in lab-created materials at day 3, and in degradable materials at day 21 (* indicates p≤0.05).

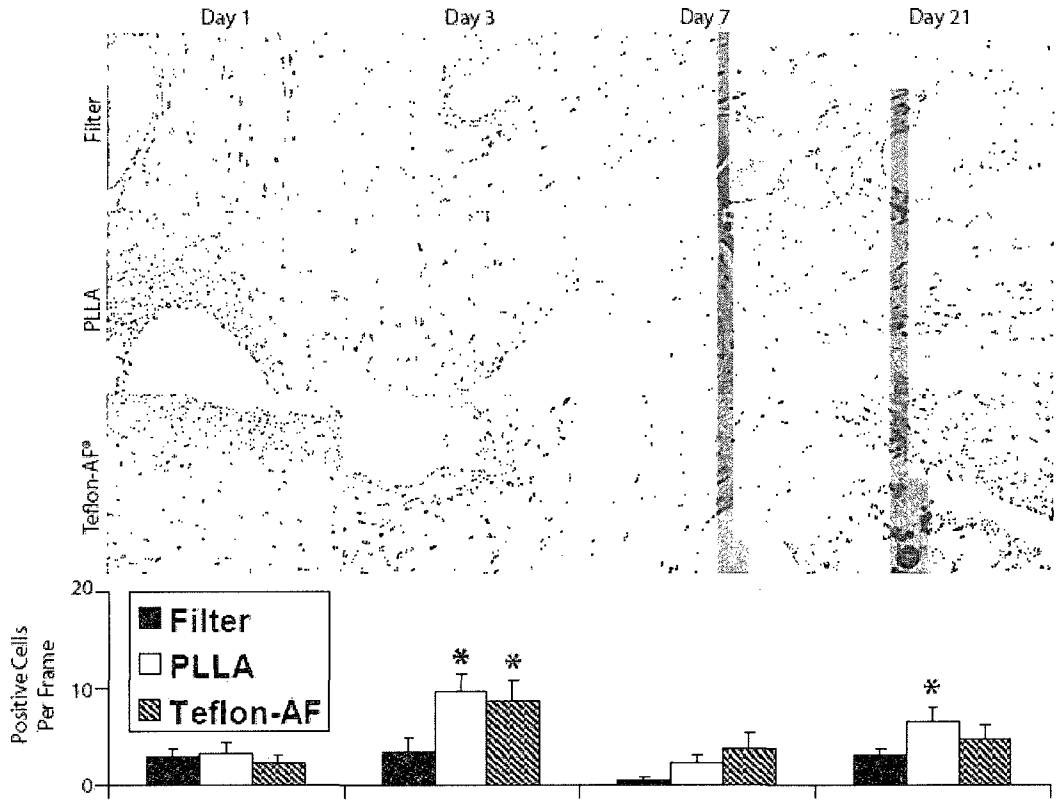


Figure 5.3: Representative images and collated statistics for the number of cells per frame positive for IL-6 as a function of film implant time and polymer chemistry. The presence of IL-6 positive cells is significantly higher for PLLA implants at days 7 and 21 (* indicates p≤0.05).

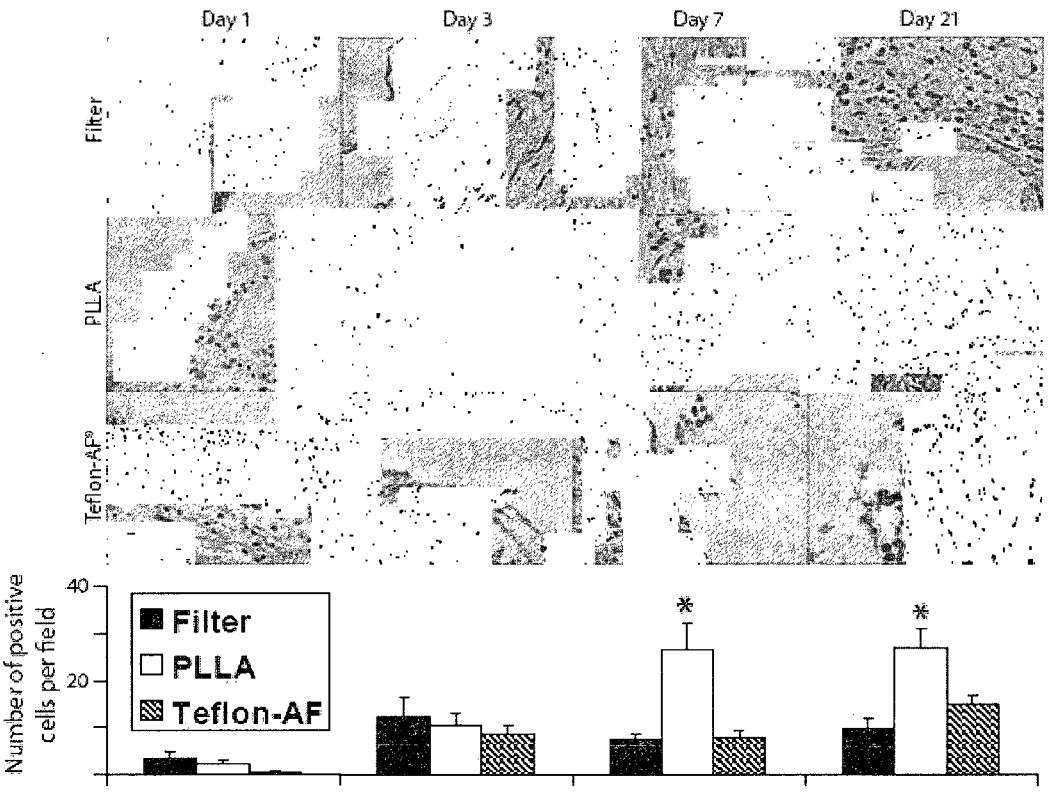


Figure 5.4: Representative images and collated statistics for the number of cells per frame positive for MCP-1 as a function of film implant time and polymer chemistry. The presence of MCP-1 positive cells remains largely steady over time. However at day 1 nylon filter implants recruit more cells, while at day 3, PLLA implants recruit more cells (* indicates p≤0.05).

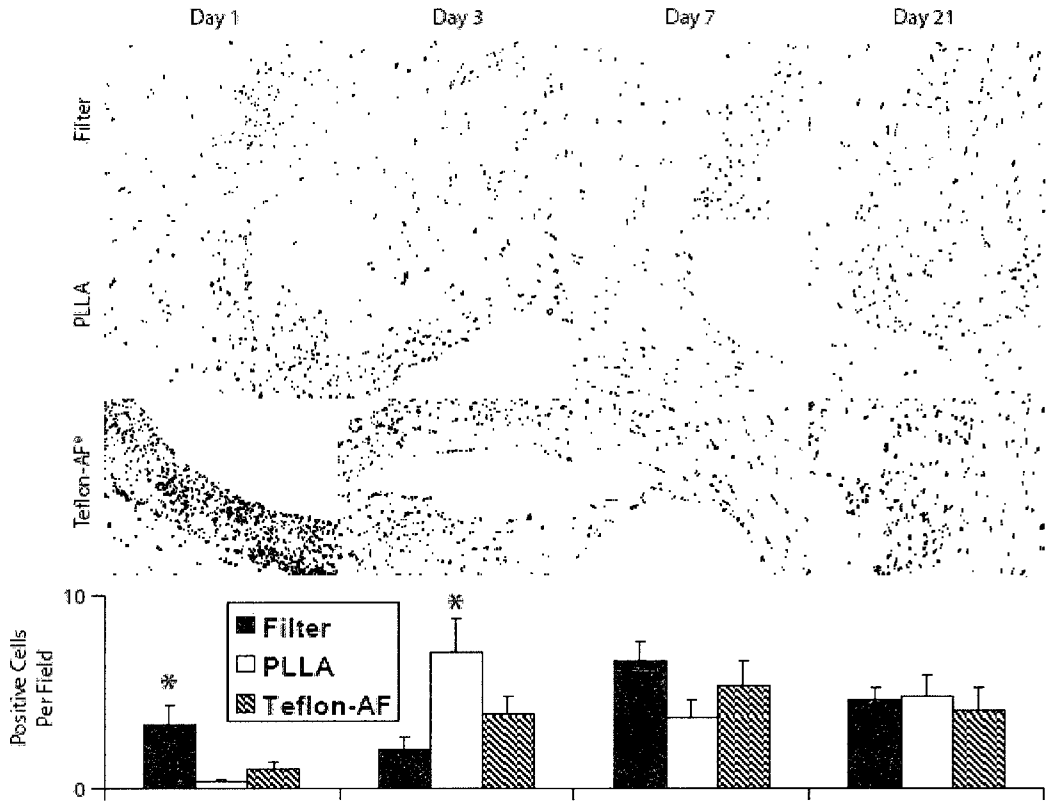


Figure 5.5: Representative images and collated statistics for the number of cells per frame positive for IL-4 as a function of film implant time and polymer chemistry. Significantly higher numbers of IL-4 positive cells are seen near Teflon-AF® implants at day 3, and near PLLA implants at day 21 (* indicates p≤0.05).

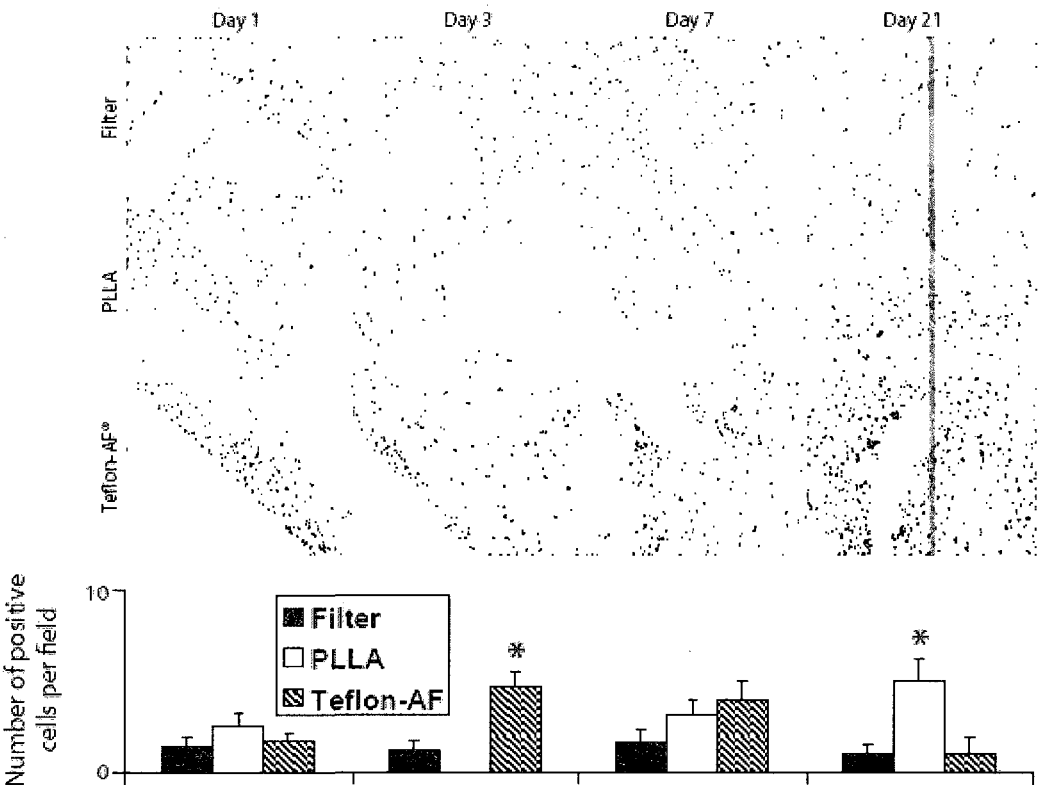


Figure 5.6: Representative images and collated statistics for the number of cells per frame positive for TGF- β as a function of film implant time and polymer chemistry. The presence of TGF-β-positive cells is very low until day 21. PLLA implants have significantly fewer cells than nylon filter or Teflon-AF® (* indicates p≤0.05).

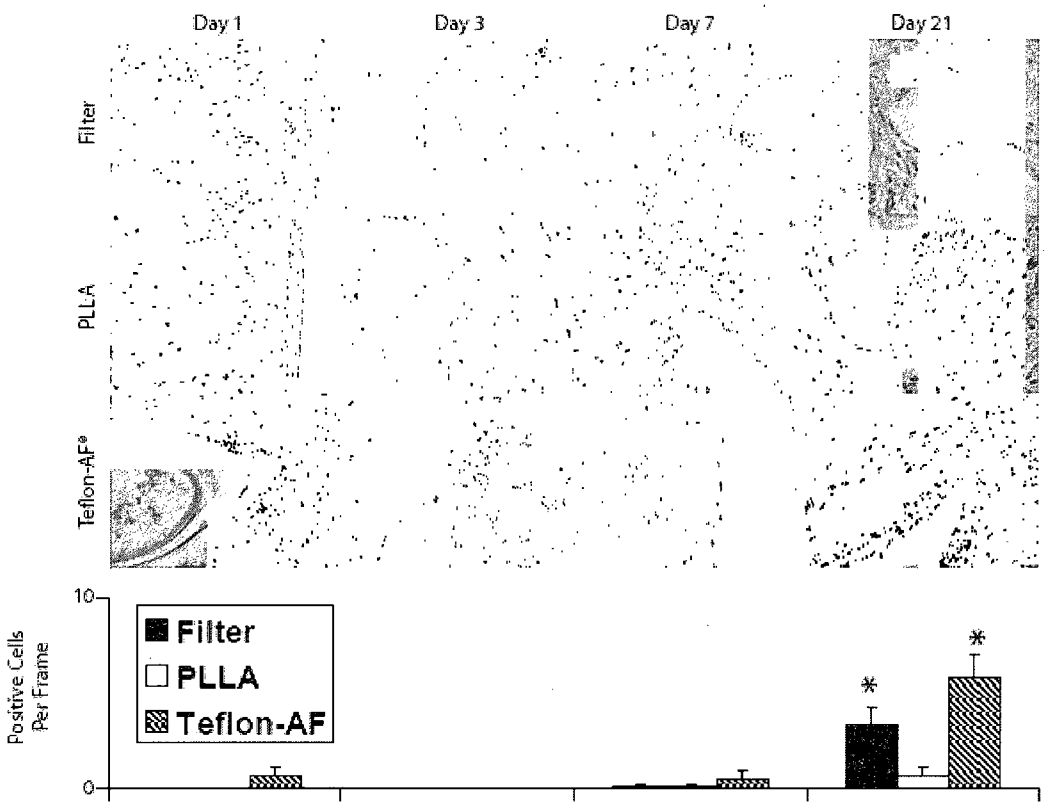


Figure 5.7: Number of foreign body giant cells per field. The density of foreign body giant cells is independent of implant material. At day 21, no differences in the number of foreign body giant cells per field were found among Filter, PLLA, and Teflon-AF® materials.

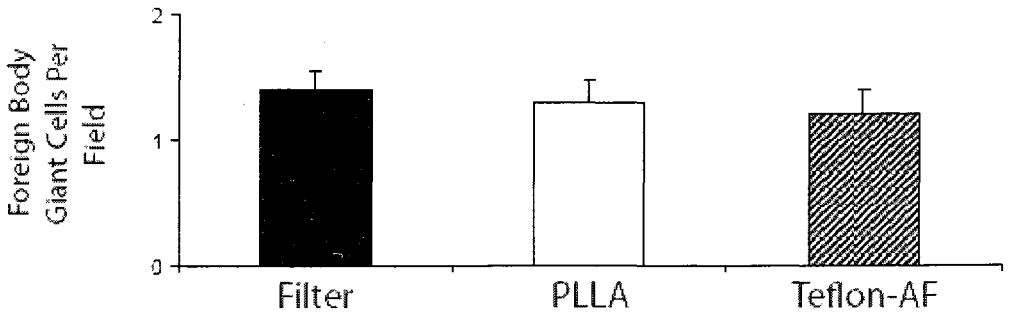


Figure 5.8: Micrographs of stained foreign body giant cells. All antigens were expressed by foreign body giant cells on all implant materials.

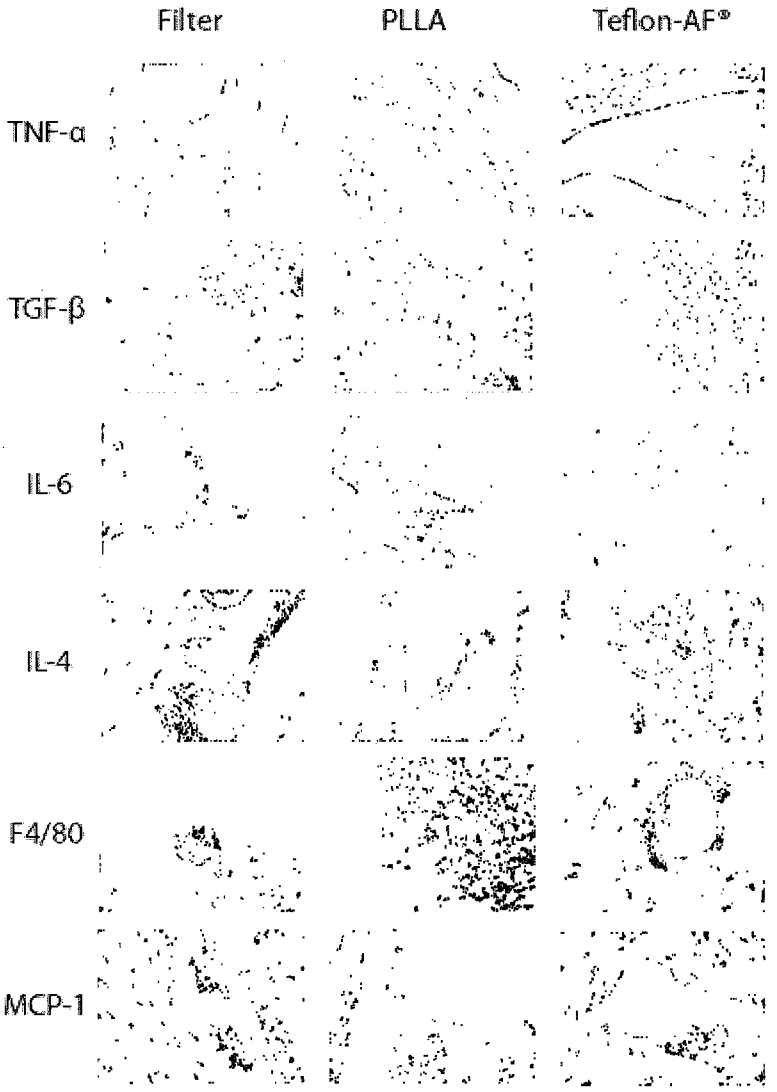
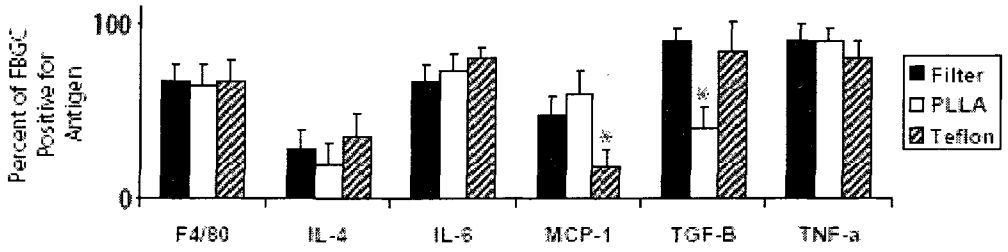


Figure 5.9: The percent of foreign body giant cells staining positive for each antigen on each surface at day 21. The number of foreign body giant cells positive for antigens are largely independent of implant material, with the exceptions of Teflon AF® exhibiting significantly less MCP-1-positive foreign body giant cells, and PLLA exhibiting significantly fewer TGF-β-positive cells (* indicates p≤0.05).



CHAPTER 6: SUMMARY AND FUTURE DIRECTIONS

This chapter was written by Lisa M. Chamberlain and edited by Mercedes Gonzalez-Juarrero and David Grainger.

6.1 Summary and Conclusions

The goal of this research was to expand knowledge on the role of macrophages in the immune response against implanted materials both *in vitro* and *in vivo*. To meet this goal, several common materials used in the making of medical devices have been used for both *in vivo* and *in vitro* studies.

We have cultured macrophage cells on biomaterial surfaces for time periods ranging from 8 hours to 21 days. In some instances at early time points these cells received an additional chemical stimulus along with the physical stimulus generated by simply culturing these cells on a surface. Within this set of experiments, the morphology, cytokine and chemokine release, surface marker expression and proliferation were observed and compared between different cell culture systems. In other experiments these cultures were fed with cytokines known to promote cell fusion.

Our *in vitro* studies demonstrated that the data obtained from immortalized cell lines and primary-derived cells are not equivalent. Thus, when cultures of cell lines and primary cells were compared based on their morphological characteristics, cytokines released in

response to physical and chemical stimuli, the strength of response to physical and chemical stimuli, and fusion potential there were several differences. Our *in vitro* data also showed evidence of a burst of cytokine release by macrophage cells immediately following adhesion to a material, followed by a lack of cytokine production and changes in the activation pattern of the cells that drove these cells towards an alternatively state of activation. Also, fusion of macrophages under our protocols resulted in significant decreases in several cell surface markers.

In vivo studies were performed using a murine subcutaneous injection model with some of the materials used for *in vitro* studies. We used immunohistochemistry techniques, and microcoscopic observation in tissues sections from tissue samples obtained at the implant site at day 1 to 21 post implantation. These studies allowed us to determine the number of macrophage recruited at the site of the implant as well as the number of positive cells for several cytokines, as well as the numbers of fused cells over time after implantation of the materials. The data obtained from samples taken at several time points post implantation revealed there were immune responses elicited after materials implantation that were dependent on the type of material used to prepare the implanted film. In our studies, the recruitment of macrophages at the implant site was slower to that previously reported in other in vivo studies.

In this body of work, we observed not only a lack of equivalence between primary derived and immortalized macrophage cells, but also a lack of equivalence between *in vivo* and *in vitro* studies. Our conclusions and recommendations are as follow: 1.- *in vivo* studies are the most reliable methodology to study inflammatory responses against implanted materials; 2.-if in vitro studies are chosen these should be performed using primary derived cells rather than immortalized cells. Additionally, differences in results

between our *in vivo* experiments and among other published *in vivo* studies of the foreign body response (FBR) suggest that the method of implantation of a material greatly affects the outcome of a study. Thus, the site in the body chosen for implantation of the materials as well as the methodology used for these procedures should be taken into account when extrapolating results between studies.

6.2 Future Work

Based on these conclusions, future work will need to focus on *in vivo* techniques and primary-derived macrophages for *in vitro* experimentation. Continuing our use of needle implantation methods and possibly branching out into other published methods will allow for more comparison to current literature and will expand our ability to study this phenomenon. Several studies for the future are described below.

6.2.1 Effects of implantation method on the foreign body response

Our *in vivo* study indicates a slower FBR with needle-injection implantation model compared to incision-pocket implantation models, as well as differences in macrophage recruitment to the implant site and the timing of cytokine/chemokine production near the implant when compared to cage-implant models.[1] The FBR is central to implant failure and we are seeing differences in this response that may be based on implantation method, therefore the utility of a material may depend on its method of use, method of surgical insertion, and possibly location of implantation. The effects of implantation method on the ensuing FBR, and comparisons of *in vivo* methods will help to optimize pre-clinical models and allow for better representation of inflammatory responses to different types of implants as well as giving insight to the optimum use of a material in the body.

Discrepancies in macrophage infiltration times between our in vivo work and previous studies may stem from our use of a different macrophage marker to identify incoming cells (F4/80). We used this marker as it is found to be expressed at a high level on macrophages of different maturities, however other studies have used a variety of other markers to label macrophages.[1-3] Therefore, studies of implantation effects on temporal events of the foreign body response will be studied, including the presence of key cytokines, as well as different cell types. Using markers for macrophages and monocytes previously used in the literature will help to determine the cause of discrepancies between our current studies and those previously done by others.

Model biomaterials (e.g. PLLA, Teflon-AF®) will be implanted via multiple methods and into multiple sites, including surgical creation of a subdermal pocket,[4] implantation via deployment needle (i.e. chapter 5), and supra-epicardially.[5] To track macrophage infiltration to the site, F4/80 and CD68 should both be used for facile comparisons to previous studies. Cytokine and chemokine production can be observed using the same methods as in Chapter 5 of this dissertation, looking at IL-6, TNF-α, MCP-1, IL-4, and TGF-β. Other cytokines can be observed as well.

6.2.2 Effects of inflammatory cytokines on creating 'M1' macrophages at an implant site and reducing fibrosis

Inhibiting the creation of a collagenous capsule around implants is an important goal in studies of the FBR. TGF-β is an important signal in the initiation of collagen production by fibroblasts, our *in vivo* studies detailed in Chapter 5 demonstrated a significantly lower number of cells staining positive for TGF-β around poly-L-lactide materials which

was accompanied by a significantly higher presence of cells staining positive for IL-6. This inverse relationship implies that the presence of IL-6, an inflammatory cytokine may decrease collagen deposition at the site. Further *in vivo* studies observing the effects of exogenous IL-6, as well as other inflammatory cytokines (e.g. IFN-γ, TNF-α) on the production of collagen around the implant will increase our understanding of this process.

It has been suggested that macrophages in the foreign body response are closer to an M2 or alternatively activated phenotype.[6,7] The use of these inflammatory cytokines may force these macrophages towards an M1 or classically activated phenotype, which could significantly change the type of inflammation at the implant site.

For this experiment, mice would be implanted with a polymer or filter and would have regular (e.g. daily) injections of exogenous cytokine. IL-6 and IFN-γ are of particular interest as we have demonstrated a trend of low TGF-β around implants with high IL-6, and IFN-γ is a strong inductor of M1 macrophages. If these initial experiments reduce the number of TGF-β positive cells, and reduce fibrosis surrounding these implants, then releasing the cytokines from polymer implants to observe similar responses would follow.

6.2.3 Effects of hypoxic environment on the foreign body response

It has been suggested that macrophages in hypoxic environments share the M2 macrophage phenotype involved in the FBR.[8] This has been largely explored in tumors, however it would be expected that a similar hypoxic setting may occur during wound healing and the ensuing FBR around an implant. An exploration of the amount of

oxygen present during a foreign body response to assess the *in situ* situation may allow us to draw similarities between the FBR and tumor-resident macrophages, allowing for greater understanding of both processes.

Here we would use a dyer probe, or other method of measuring oxygen saturation *in situ,* to assess the extent of hypoxia at an implant site over time. Further correlations to proteins encoded by hypoxia-sensitive genes (e.g. VEGF, PDGF) would also be completed.

6.2.4 A comparison of foreign body giant cell surface markers in vivo and in vitro

While *in vivo* models are optimal for studying the FBR to materials, the use of immortalized cells is a much cheaper and faster method to gain information about inflammatory responses to materials. Our observation of a cytokine burst by macrophages following adhesion to biomaterials may be useful in determining toxicity or inflammatory potential of a material. Additionally, the possibility of *in vitro* models utilizing multiple cell types to further explore how macrophages interact *in vivo* with other cells present in an implant site may work to expand the potential for *in vitro* studies. The co-culture of macrophages with fibroblasts, mesenchymal stem cells, and even macrophages of different maturities is likely to change the inflammatory signals released in response to materials.

The presence of fused macrophages (Foreign Body Giant Cells, FBGC) is a hallmark of the FBR.[9] Our understanding of the role of the FBGC cells at the interface of self/not-self is important in the understanding of the FBR. Our observations of decreased macrophage surface proteins suggest a deviation of FBGC away from the macrophage

phenotype. Previous research has shown FBGC to be less phagocytic than their macrophage counterparts,[10] and demonstrated their importance in the progressive degradation process occurring in implanted materials.[4] Other multinucleated cells, particularly osteoclasts, are involved in degrading materials as well, observing markers for these cells may show similarities. Further studies of cell surface marker expression both *in vitro* as well as *in vivo* will help to elucidate the role of the FBGC in the FBR.

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