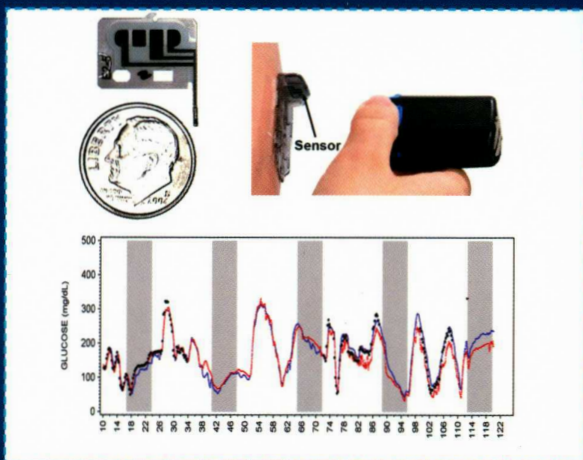


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J. D. Winefordner, Series Editor

In Vivo Glucose Sensing



Edited by
David D. Cunningham
Julie A. Stenken

IN VIVO GLUCOSE SENSING

Edited by

DAVID D. CUNNINGHAM

JULIE A. STENKEN



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Marisha L. Godek and David W. Grainger

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2.1 INTRODUCTION

Biosensor–tissue compatibility is a significant obstacle in the development of viable, enduring implantable biosensors.^{1,2} The clinical literature and product development files in industry are littered with biosensor failures in many different sites and

situations. Problems with the continuous interfacing of the sensing device with host physiology arise from the complex immune response of the host to the chemical and physical properties of the implanted device. Phenomena surrounding this response arise from both acute and chronic inflammatory host reactivities, often characterized under the general term, “biocompatibility.” Biocompatibility has been more formally defined as the ability of the implanted material to perform with an appropriate host response within a specific application or biomedical context and implies that the material must minimally perturb and be accommodated by the host at some acceptable level.^{3,4} Implanted biosensors have an additional requirement: the host environment must not interfere with the sensor performance.⁵

Inflammation is a normal response to homeostatic disruption of multiple possible origins. Wounding produces inflammation as part of normal sequelae to healing. Since device implantation (or any invasive procedure) produces wounding, inflammation is a natural and known host response to a biomaterial. These events initiate immediately upon device contact with host physiology: with the adsorption of host proteins, surface activation of blood-derived elements including coagulation proteins, complement, and platelets, and progression to the eventual recruitment of inflammatory cells, unresolved wound healing and granulation tissue, and eventual fibrosis. The foreign body reaction (FBR)—the ubiquitous host inflammatory response to implanted biomaterials—is the consequence of these numerous physiological responses, including aberrant wound healing and inflammation reactions in the presence of an implanted device (e.g., sensors), grossly characterized by chronic inflammation, compromised healing at the implant site, and in many cases, biomedical device failure.^{6,7} This response seems to be slightly dependent on device surface chemistry or topology, and more pronounced in soft tissues than in hard tissues, but nonetheless a recognized problem with substantial clinical implications and challenges in most device scenarios.⁸ For implantable biosensors, FBR inflammatory events and ensuing fibrosis often translate to a loss of performance. Significantly, with multiple molecular, cellular, tissue-specific and intersecting, integrated, or synergistic kinetic timelines responsible for the exceedingly complex FBR, the host reaction is poorly understood.^{9,10} Lack of understanding translates to lack of clinical control and inability to sustain reliable sensor reporting functions in many implant sites. Therefore, an understanding of the FBR at the cellular and molecular level is necessary for producing design parameters required for a new generation of devices that successfully suppress, avoid, or manipulate inflammation and associated fibrosis, and enhance desired tissue responses (e.g., angiogenesis and tissue integration). These design parameters must consider implant site-specific physiology, aspects of material-associated biocompatibility, and molecular and cellular components responding to the local trauma of sensor implantation.

2.2 THE IMPLANT SITE AS A WOUND SITE

Invasive or surgical placement of a biomedical device produces tissue trauma, resulting in the formation of a wound site. Normal wounds heal and remodel over time to restore tissue homeostasis in the absence of implanted materials. Interestingly, sites of long-term implants do not heal properly: the ensuing FBR prompted by the

presence of an implant represents an aberrant form of wound healing.⁷ Thus, despite surgical finesse, modern device design, sterility, and miniaturization, implant sites do not heal “normally,” instead producing a state of chronic irritation and inflammation that resolves to an unusual state of tolerance in some cases and outright rejection in others. Examination of what is currently known about the progression of the essential “normal” wound healing response is important for understanding components that distinguish the FBR. Diverse research efforts currently focus on the FBR, crossing immunology, pathology, surgery, cellular and molecular biology, materials science, and biomedical engineering. One effort focuses on physical and chemical aspects of the unique biointerface between the implant material and the body, often distilled to studies of “cells on surfaces.”^{11–13} At the cellular level, this response can be examined in terms of key immune cells known to interact with materials and facilitate FBR development, in particular monocytes and elicited or activated macrophages primarily sourced from circulating monocytes.⁷ These cells are implicated in acute and chronic inflammatory responses to foreign entities of various origins.^{7,14,15} Importantly, these cells produce complex molecular signaling cascades using soluble proteins and peptides. Hence, these “cell on surfaces” studies rapidly evolve into molecular biochemistry studies in the detective work associated with implant FBR physiology. Furthermore, communication and other relationships between monocytes, macrophages, and other host cells (e.g., fibroblasts and endothelial cells) found in the implant site are significant to biocompatibility of implanted materials. Macrophage cells at implant site use soluble molecular signals to recruit and influence the behavior of other cell types at this site, contributing to the development of specific physiological outcomes (e.g., inflammation, thrombosis, fibrosis, angiogenesis, and wound stabilization).^{7,10,15}

Given the molecular and cellular basis for wound healing and aberrant healing responses observed in implant sites, and the empirical correlation of macrophage activity in implant sites with FBR, this chapter focuses on the macrophage as a fundamental player in this context. Specifically, the chapter introduces basic monocyte and macrophage biology and currently accepted standards for classifying these cells within the mononuclear phagocyte system (MPS). This is important to understand their complex relationships with various cells present at a wound (implant) site. Further examination of aspects of monocyte and macrophage differentiation and macrophage activation responding to diverse wound-site stimuli, and the differences between classical, alternative, and innate activation are discussed. Elucidating the cellular basis for normal versus aberrant wound healing and similarities and differences between macrophages and dendritic cells (DCs) is critical, so roles of the macrophage in the FBR and origin of multinucleated cells—foreign body giant cells (FBGCs), osteoclasts, and Langhans cells—are also mentioned. The chapter closes with some review of the known effects of materials chemistry, topology, and porosity on cell response and the use of the cage implant system to examine *in vivo* components of the FBR.

2.2.1 The Mononuclear Phagocyte System as a Source of Wound-Site Monocytes and Macrophages

An appreciation for the complexity and diversity of cellular players believed to mediate the FBR at biomaterial implant sites is important to understanding the

cellular and molecular basis of the FBR. Significant to this challenge is the concurrent evolution of new understanding of the MPS and its constituents: bone marrow progenitor cells and circulating monocytes and macrophages, cells intimately tied to the FBR.¹⁶ Monocytes and macrophages are leukocytes that are part of a very adaptable, highly dynamic hematopoietically derived cellular response system, exhibiting vast heterogeneity with respect to maturity, morphology, and response to stimuli upon activation in peripheral tissue sites.¹⁷ Characterizing a plastic and locally recruited population such as this is not trivial. Classification within the MPS involves cellular origin (from bone marrow progenitors), cellular morphology and ultrastructural features, protein expression profiling (e.g., non-specific esterase and lysosomal hydrolase activities, and cytokine production), and cellular behaviors (e.g., phagocytosis).¹⁸ The significance of each of these characteristics to local wound healing and also implant-related responses is even more complex.

More generally, MPS cells can be classified by location, morphology, and cell surface markers. Cell appearance and size both change with their progression to fully differentiated, mature states. Monoblasts and promonocyte (MPS progenitor cells in bone marrow) are smaller and less complex in both appearance and function than either more mature monocytes or macrophages.¹⁸ Macrophage cells are derived from blood monocytes and bone marrow precursors, representing the chief differentiated MPS cell.¹⁹ These cells are distributed throughout the body, exhibiting a broad range of structural and functional diversity despite originating from common progenitors. Monocytes and neutrophils, another important wound-site leukocyte, share a common myeloid marrow-based progenitor, the granulocyte-macrophage colony-forming unit (GM-CFU).¹⁸ Monocytes likely remain in the bone marrow for less than 24 h before entering the peripheral blood, where they circulate for several days before entering tissues to replenish resident macrophage populations.^{20,21} After transition, monocytes do not return to circulation, but reside in the organ, tissue, or implant site for several months as mature, terminally differentiated macrophages.¹⁸ Previously, it was accepted that approximately 95% of tissue-resident macrophages were derived from monocytes, and the remaining 5% derived from local division of mononuclear phagocytes within the tissues that have yet to complete cell division.²² However, recent studies suggest that significant local macrophage proliferation and renewal do occur, especially under steady-state conditions.^{16–18,23} This circulating residency is species dependent: monocytes exhibit a half-life of 17.4 h in mice, up to 70 h in humans, and constitute between 5% and 10% of peripheral blood leukocytes in humans.^{17,20,24} The circulating monocyte exhibits heterogeneity; monocyte morphology, size, nuclear morphology, and granularity vary. Interestingly, monocyte heterogeneity is conserved in humans and mice, and is believed to reflect monocyte developmental stages correlating to distinct physiological roles such as tissue homing or response to inflammatory signals.¹⁷ This is important to many preclinical *in vivo* models of inflammation, wound healing, and modeling of the FBR, where equivalence of murine and human cell origins and phenotypes in tissue sites is important and where knockout murine models of wound healing are increasingly implemented experimentally. Circulating monocyte populations are frequently harvested as a source of progenitor inflammatory cells, and differentiated to macrophage *in vitro*

for experimental exploitation, to serve as a “primary culture” in diverse experimental models.

Monocyte cells vary expression of their cell surface markers related to their physiological roles. “Classic” monocytes were described as $CD14^{hi}CD16^{-}$ cells, and differential expression of these cluster of differentiation (CD) markers and chemokine receptor (CCR) profiles allowed further classification of monocytes (for a detailed review of monocyte subsets and surface markers, see Ref. 17).²⁵ Generally, monocytes are $F4/80^{+} CD11b^{+}$ cells that can also be classified based on the expression of specific surface receptors (i.e., CCR2, CD62L (L-selectin), and CX₃C chemokine receptor 1).²⁶ One subset, the “inflammatory monocytes,” expresses CCR2, targeting migration toward CC-chemokine ligand 2 (i.e., $CCL2^{+}$ or $MCP-1^{+}$), important to attracting cells to wound sites.^{27,28} Importantly, different subsets of monocytes are thought to differentiate to specific cell types: inflammatory monocytes are thought to differentiate to macrophages, aid in the clearance of pathogens and/or apoptotic cells, and facilitate inflammatory resolution, whereas *in vitro* experiments suggest that $CD14^{+}CD16^{+}$ monocytes are more likely to become dendritic cells.^{14,17} Furthermore, monocyte subpopulations that share common traits with more mature cells have been described; $CD14^{+}CD16^{+}CD64^{+}CD86^{hi}HLA-DR^{hi}$ monocytes share characteristics with dendritic cells.²⁹

Monocytes are one of the two cell types known to differentiate to dendritic cells (Figure 2.1), the most competent antigen-presenting cells known and efficient stimulators of B and T cells.^{30,31} *In vitro*, this maturation process is facilitated by the addition of GM-CSF and interleukin-4 (IL-4), known to push $CD14^{+}$ monocytes toward a dendritic cell phenotype.^{30,32,33} Like macrophages, dendritic cells exhibit phenotypic and functional heterogeneity (e.g., interstitial dendritic cells, lymphoid dendritic cells, and Langerhans dendritic cells); furthermore, dendritic cells and activated macrophages exhibit similar stellate morphologies.³⁴ Dendritic cells can be distinguished from macrophages by their ability to activate naïve T cells and by altered expression profiles of specific markers (e.g., the β_2 integrin CD11c).^{16,35}

In vivo, dendritic cells are migratory cells found in the blood and lymph (veiled cells), in secondary lymphoid tissues (interdigitating dendritic cells), in organs (interstitial dendritic cells), and in the epidermis and mucous membranes (Langerhans cells). Collectively and constitutively, dendritic cells express more major histocompatibility complex (MHC) class II and costimulatory molecules required for antigen presentation to T cells than macrophages, and this is one of the main distinctions between these cell types. Similar to macrophages, dendritic cells are a plastic population modulated by cytokines present in the local environment. Dendritic cells play a role in wound healing by responding to tissue damage and directing effector cells to tissue damage sites. In addition, they are capable of secreting copious amounts of proinflammatory cytokines at their precursor stage.³⁴

As monocytes emigrate from blood vessels to tissues, they develop (differentiate) into highly specialized cell types (e.g., dendritic cells and osteoclasts) and macrophages (Figure 2.1); examples include alveolar macrophages (lung), Kupffer cells (liver), Langerhans cells (epidermis), and brain microglia and osteoclasts (bone).¹⁹ Although the development and differentiation pathways remain poorly understood *in vivo*, efforts to determine branch points within the developmental

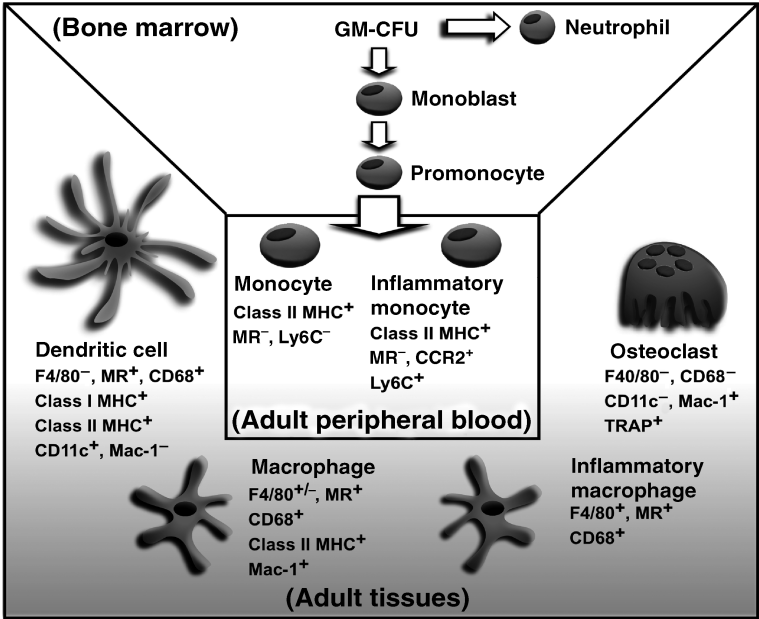


Figure 2.1 Monocyte (MC) derivation from precursors and maturation to highly specialized cell types: macrophages, dendritic cells, and osteoclasts. Neutrophils and monoblasts derive from a common precursor, the GM-CSF. Promonocytes precede MCs in maturity. MCs originate in the bone marrow and pass into the blood and tissues, where they mature to dendritic cells or macrophages (MΦs) in response to local environmental cues. Figure design by Dr. Marisha Godek; graphics by Dr. Gregory Harbers.

pathways have been undertaken.^{16,17,19,35–37} Differentiated macrophage morphology is varied: cells typically possess a single, spherical concentric nucleus containing several nucleoli, large numbers of mitochondria and cytoplasmic vacuoles, a ruffled margin, and many fine cytoplasmic granules.³⁸ Macrophage heterogeneity is often associated with “immunocompetency,” and specialization of function correlates to localization in different tissues.^{18,19,39} Furthermore, small subpopulations of macrophages with distinct characteristics exist within specific tissues (e.g., spleen), suggesting a role for the microenvironment in phenotypic regulation.^{17,19} Developing macrophages can be traced to early embryonic forms; these cells express macrophage markers including the β_2 integrin CD11b, mannose receptor (MR or MMR), and FMS (macrophage colony-stimulating factor 1 receptor precursor; CSF-1-R).^{40–43} Importantly, it is still unclear whether tissue macrophages arise from lineage-committed precursors or randomly from the monocyte pool.¹⁷

Significantly, the implanted biomaterial provokes a spectrum of macrophage differentiation and activation profiles locally at the site, depending on this broad cell type heterogeneity, tissue site physiology, and device properties. It is also likely that local activation and acute wound healing at the implant site provide chemotactic cues that recruit other monocytes and immature MPS cells to the implant site.

2.3 MONOCYTE AND MACROPHAGE ACTIVATION AND INFLAMMATORY RESPONSE IN LOCAL TISSUE SITES

As a major immune system sentinel, macrophages play a critical role in host defense against a diverse group of environmental challenges.^{44,45} Immune cell “activation” is frequently referred to, often described simply as an upregulation of a variety of signaling pathways resulting in altered gene expression and increased biochemical and functional activity, particularly receptor expression and macrophage phagocytic activity.¹⁹ Much of this cell inflammatory activity is locally derived: implant sites provide considerable macrophage activation. Monocyte and macrophage heterogeneity converges in the host response to inflammation, where inflammatory monocyte-derived macrophages arise from a variety of stimuli.¹⁷ Monocytes that enter tissues can mature into nonspecifically stimulated macrophages (“elicited” macrophages); alternatively, monocytes may be influenced by cytokines and mature into “activated” macrophages with enhanced functionality.⁴⁶ Generally, “activated” macrophages display more class II MHC, are more phagocytic, and release more cytokines (e.g., IL-1) than their unactivated counterparts.⁴⁷ Extreme activation leads to the overproduction of chemical mediators (e.g., cytokines, reactive oxygen intermediates (ROIs), and reactive nitrogen intermediates (RNIs)), which may in turn result in systemic imbalances often leading to tissue damage/necrosis, anaphylactic shock, and death. Once macrophage cells differentiate within a tissue, it is not known if they retain some degree of functional plasticity, or rather if they are “terminally differentiated.”¹⁷ However, tissue-resident macrophages are known to undergo “reactivation” in response to inflammatory stimuli, with enhanced expression of antigenic markers that are otherwise undetectable or present at very low levels.⁴⁶

Inflammatory monocytes (defined as $CCR2^{+}Ly6C^{+}$) are recruited by cytokines and chemokines and undergo chemotaxis to inflammatory tissue sites where they differentiate to macrophages, likely under the local influence of further cytokine signals.²² *In vitro* studies designed to simulate specific inflammatory stimuli have led to the delineation of different types of macrophage activation: classical, alternative, and innate. Furthermore, some stimuli (e.g., transforming growth factor, TGF- β , IL-4, IL-10) serve to “deactivate” macrophages (i.e., interfere with the process of macrophage activation or inhibit the cytotoxic functions of activated macrophages).^{17,45} These processes are directly relevant to understanding roles of macrophage in the FBR: methods to reversibly activate or attenuate macrophage reactivity to implanted materials could prove fruitful in alleviating severity and duration of inflammation that leads to implant-based fibrosis complications.

2.3.1 Soluble Signaling Molecules Involved in Inflammatory Activation

A plethora of signaling molecules—acting in a tailored, concerted fashion—are involved in directing cellular activities and cell-to-cell communication, affecting a wide variety of cell behaviors including migration, adhesion, activation, proliferation, differentiation, and fusion—all important toward the establishment of a FBR *in vivo*. Chemokines are a superfamily of polypeptides and a subset of the larger class of

cytokines that selectively control leukocyte activation, adhesion, and chemotaxis and typically have multiple effects on their target cells.^{48,49} Cytokines are low molecular weight regulatory proteins or glycoproteins secreted by numerous cell types involved in cell-to-cell communication, but particularly potent macrophage activators capable of modulating macrophage function.⁵⁰ Infections, acute wounding, and tumors all elicit cytokine production as the basis for producing inflammation, but the types, kinetics, and cellular targets are important regulators of specific biological responses since most cytokines have short half-lives. Importantly, while cytokines direct macrophage behaviors, macrophages also produce many cytokines that act on various cell types including other macrophages. Inflammatory cytokine production may be the consequence of wounding, exposure to bacterial endotoxins (e.g., lipopolysaccharide (LPS)), microbes and viral particles, or priming factors (e.g., interferon- γ (IFN- γ)).⁵¹ A list of cytokines produced by macrophages and their biological actions can be found in Table 2.1. Importantly, cytokines are pleiotropic: their specific action depends upon the physiological context in which they are present and cells on which they might act. Significantly, macrophage cytokine secretory profiles are likely affected by cell maturity and local environmental cues. This is perhaps the most important challenge in controlling of the local tissue reaction to implanted biosensors: guidance of the macrophage cytokine expression and reactivity profiles in the presence of the implant.

In vitro, stimulants are routinely employed to push monocyte/macrophage cells to an “activated” state. These include LPS, a potent mitogen, and phorbol esters (e.g., phorbol 12-myristate 13-acetate (PMA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA), and others) that act as differentiating agents.⁵² LPS is an endotoxin present in Gram-negative bacterial cell walls, often associated with systemic bacterial infections, that leads to macrophage activation and subsequent production of inflammatory cytokines.^{51,53} LPS induces cytokine gene expression via numerous transcription factors including members of the AP-1, C/EBP, Ets, and NF- κ B/rel families.⁵⁴ Other bacterial components including peptidoglycans, trehalose diesters, lipoteichoic acid, and lipomannans are all recognized by and activating to macrophages.⁵⁴

Phorbol esters are tumor promoters capable of binding to and activating protein kinase C (PKC), one critical component in T cell activation.^{55,56} PMA is a structural analogue of diacylglycerol (DAG), an allosteric activator of PKC. PKC activation via phosphorylation leads to calcium release, resulting in a cascade of cellular responses including rapid proliferation.⁵⁶ Experimentally, phorbol esters have been used as chemoattractants and to differentiate nonadherent monocytes to adherent macrophage cultures.^{55,57–60}

“Classical macrophage activation” can be achieved by stimulation with cellular cytokine IFN- γ and microbial activators (pyrogens, LPS), resulting in amplified microbicidal activity, production of reactive oxygen species (ROS) and proinflammatory cytokines, and increased antigen presentation. In contrast, “alternative activation” is the result of cell exposure to cytokines IL-4 and IL-13, associated with tissue repair and humoral immunity. Alternative activation enhances phagocyte endocytosis, heightened expression of MR, and increased cell growth and tissue repair. “Innate activation,” typically associated with infections, involves stimuli (e.g., pathogen-derived LPS, peptidoglycans, and lipoteichoic acid) that act through

TABLE 2.1 Selected Macrophage Cytokine Products and Their Effects

Cytokine	Stimulus for production	Biological action	Induces production of	Cellular target(s)
G-CSF	IL-1, LPS	Granulocyte colony stimulation, terminal differentiation of myeloid cells, enhanced neutrophil function	—	Granulocytes, myeloid stem cells, neutrophils
GM-CSF	IL-1, TNF, LPS, retroviral infection	Granulocyte, eosinophil and macrophage colony stimulation, enhanced neutrophils/eosinophil function	IL-1, TNF, PGE ₂ , O ₂	Macrophages, eosinophils, granulocytes
M-CSF	IL-1, LPS	Macrophage colony stimulation, antiviral	IL-1, IFN- γ , TNF- α , PGE ₂ , PA	Macrophages
TNF- α	IL-1, IL-2, GM-CSF, LPS	Tumor necrosis, endotoxic shock-like syndrome, cachexia, fever, acute-phase protein response	IL-1, IL-6, GM-CSF, MHC I, MHC II	Macrophages
IL-1	IL-2, TNF, GM-CSF, Ag presentation	Fever, acute-phase protein response, hypotension, increased ICAM-1 expression (endothelial cells)	IL-2, IL-4, IL-6, TNF, PGE ₂ , collagenase	Macrophages, endothelial cells
IL-6	IL-1, TNF, PDGF	Hemopoietic cell proliferation, fever, acute-phase protein response	IL-2R (T cells), IgG (B cells)	Macrophages, T cells, B cells
IL-10	T and B cell activation, LPS	Inhibitory to T cell proliferation and cytokine production	MHC II (B cells)	Macrophages, T cells, B cells
IL-12	T cell activation, LPS	Promotion of cell-mediated immunity, activated T and NK cell proliferation	IFN- γ	T cells
TGF- β	—	Fibrosis and wound healing <i>in vivo</i> , influences integrin expression and differentiation	—	Macrophages, T cells, B cells, epithelial cells
FGF	—	Angiogenesis <i>in vivo</i> , endothelial cell chemotaxis and growth	IFN- γ	Endothelial cells, myoblasts

(continued)

TABLE 2.1 (Continued)

Cytokine	Stimulus for production	Biological action	Induces production of	Cellular target(s)
PDGF	LPS, lectins, zymosan, thrombin coagulation	Neutrophil activation, collagen synthesis augmentation, mesenchymal cell proliferation and chemotaxis	IL-1, IL-1R, IFN- β , IFN- γ , PGE ₂	Neutrophils, mesenchymal cells
EGF	—	Angiogenesis, wound healing, proliferation/differentiation of basal epithelial cells	—	Epithelial cells
MIP-1 α	—	Neutrophil recruitment, regulation of hemopoiesis	—	Neutrophils

Other macrophage cytokine products include IFN- α , IL-8, IL-15, IL-16, and IL-18. Abbreviations: colony-stimulating factor (CSF), interleukin (IL), transforming growth factor- β (TGF- β), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), epidermal growth factor (EGF), macrophage inflammatory protein-1 α (MIP-1 α), lipopolysaccharide (LPS), antigen (Ag), tumor necrosis factor (TNF), intracellular adhesion molecule-1 (ICAM-1), prostaglandin E₂ (PGE₂), major histocompatibility complex (MHC), and interferon (IFN). Sources for this information can be found in Refs 47,132–141.

ligation of the toll-like receptors (TLRs), whereas cell deactivation can be achieved by IL-10, TGF- β , and steroids.¹⁷

In addition to cytokine and chemokine production, macrophages and other MPS cells are known to produce a wide range of substances (over 100) varying in size (32–440 kDa) and biological activity, affecting virtually every aspect of cell behavior from cell growth to cell death.⁴⁵ Enzymes such as lysozyme, proteases, and lipases, as well as enzyme inhibitors, complement components, adhesive proteins (e.g., fibronectin), ROIs and RNIs, and coagulation factors are all known secretory products of macrophages.^{18,45} Furthermore, macrophages are known to modulate implant-associated angiogenesis and fibrogenesis by producing numerous factors (see below).

2.3.2 Macrophage Receptors as Phenotypic Markers of Activation States

Macrophage presence and reactivity in tissue sites are elicited through different combinations of effector molecules (e.g., cytokines, chemokines) acting on specific receptors; thus, the expression and presence of given membrane receptors on macrophage cell surfaces correlate to specific (acquired) functions and activation profiles.¹⁸ Furthermore, macrophage maturity and/or differentiation state is/are often characterized based on the presence or absence of specific surface receptors (e.g., F4/80⁺, Mac-1⁺; Figure 2.1).⁶¹ Receptor profiles vary between cells of different maturity, primary and secondary derivation, and between cell lines.⁶²

Early membrane phenotypic markers identified on macrophages were receptors for the fragment constant (Fc) portion of IgG antibodies and for the complement protein C3.^{63,64} Other important receptors include lipoprotein, fibronectin, laminin, fibrinogen, hormone, chemokine, lectin-like, adhesion and migration, and advanced glycosylation endproducts.^{18,29} The macrophage mannose receptor (MMR) is a classic phagocytic receptor highly upregulated by IL-4 and implicated in IL-4-induced fusion to FBGC (see below).⁶⁵ Cytokine receptors include numerous interleukins (IL-1, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-13, IL-16, IL-17), macrophage-colony stimulating factor (M-CSF/CSF-1) and GM-CSF, interferons (IFN- α , IFN- β , IFN- γ), and many others.¹⁸ A diverse array of chemokine receptors have also been identified: CCR1, CCR2, CCR5, CCR8, CCR9, CXCR1, CXCR2, CXCR4, and CX3CR1.¹⁸ Significantly, no single marker is known to reliably distinguish macrophages from dendritic cells.¹⁶ Phenotypic characterization of macrophage in wound and implant sites has focused on determination of the qualitative (presence or absence) and semiquantitative expression levels (up- or downregulation) of these characteristic receptors using diverse methods including flow cytometry, immunoassays, and staining and genetic expression assays (e.g., polymerase chain reaction (PCR) and its variants, microarray assays).

2.4 ROLE OF THE MACROPHAGE IN NORMAL WOUND HEALING

The normal wound healing process is a concerted effort between multiple cell types including leukocytes (monocytes, macrophages, platelets, granulocytes, and

lymphocytes), fibroblasts, endothelial cells, and keratinocytes.¹⁴ Macrophages play an instrumental role in wound healing; the absence of wound-site macrophages has been correlated to impaired wound healing.⁶⁶ Under normal circumstances (i.e., the absence of a biomaterial) wound healing progresses through four stages: hemostasis, inflammation, proliferation, and resolution.¹⁴ Hemostasis (vasoconstriction and clot formation) occurs initially, followed by the inflammatory stage, beginning within hours of injury and persisting for several days. The inflammatory stage is marked by the influx of leukocytes; tissue macrophages in particular play a critical role in wound healing.⁶⁷ “Proliferation” refers to the regenerative stage where protein synthesis, cellular differentiation or replication occurs, and the healing process begins. During the resolution stage, wound remodeling, accompanied by regression of newly formed capillaries, takes place.¹⁴

The sequence of events related to normal wound healing involves several essential cellular players. Neutrophils, comprising 60–70% of the circulating leukocyte population, arrive at the wound site first but are quickly replaced by monocytes.¹⁸ Monocytes are attracted by a variety of chemokines, platelet-derived factors, and complement split products produced locally and transiently.¹⁴ Monocytes are recruited throughout the inflammatory stage, and once at the injury site differentiate to macrophages, becoming the predominant cell type in the wound by day 5 and persisting for up to months.^{7,18} Activated macrophages amplify the inflammatory response by producing chemoattractants for monocytes and other cell types, resulting in further cellular infiltration of the injury site. Macrophage-derived factors play a role in angiogenesis, extracellular matrix deposition, and fibroproliferation requisite for wound repair.^{10,15,68} Furthermore, macrophage presence at the wound site is essential for removal of necrotic tissue and apoptotic cells.⁶⁷

Primary macrophage functions in normal wound sites are (1) phagocytosis of apoptotic cells (e.g., neutrophils) and extracellular debris and the modulation of (2) angiogenesis and (3) fibrogenesis for wound-site homeostasis.^{14,67} Phagocytosis of senescent neutrophils is believed to be mediated by specific subsets of lectin (monosaccharide specific), integrin (thrombospondin specific), and lipid (phosphatidylserine specific) receptors found on wound-site macrophages.⁶⁹ Numerous studies report wound-site macrophage stimulation of capillary growth (angiogenesis) required to supply nutrients for tissue regeneration.¹⁴ *In situ* studies indicate that these macrophages produce various proangiogenic factors including transforming growth factors- α and - β (TGF- α , TGF- β), vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), IL-8, and basic fibroblast growth factor (bFGF), a potent endothelial cell proliferative agent that has been shown to be angiogenic *in vivo*.^{14,70–75} In addition to producing proangiogenic factors, monocytes and macrophages are known to produce and secrete thrombospondins, as well as several other poorly defined antiangiogenic factors, suggesting an additional counter role in vascular regression and the resolution phase of wound healing.⁷⁶

Similar to their modulation of angiogenesis, wound-site macrophages exhibit both pro- and antifibrotic functions required for acute and chronic wound stasis and remodeling.⁷⁷ Profibrotic functions include the ability of macrophages to produce extracellular matrix (ECM) components such as fibronectin, thrombospondins, and

proteoglycans.⁴⁵ Furthermore, macrophage-derived growth factors such as TGF- β and PDGF directly stimulate collagen synthesis and fibroblast proliferation.¹⁵ In contrast, macrophages produce numerous effectors of ECM degradation including metalloproteinases and proteinase activators.⁷⁸ These findings suggest a role for the wound macrophages in ECM remodeling. These same macrophage functions are also important in implant-associated fibrosis and encapsulation responses. Two chemokines that affect the participation of both monocyte and macrophage in wound healing—MCP-1 and macrophage inflammatory protein (MIP)-1 α —are known to exhibit distinct patterned expression throughout the course of wound repair. MIP-1 α levels peak concomitant with maximum macrophage levels within the wound site. Furthermore, MIP-1 α antibody neutralization was shown to decrease macrophage numbers at the wound site, suggesting that MIP-1 α is crucial for macrophage recruitment during wound repair.^{79,80}

Limited studies have demonstrated that wound-site macrophages are phenotypically distinct from other macrophage populations, and these altered phenotypes have both temporal- and location-dependent components.^{81,82} Based on the changing role of the wound macrophage in angiogenesis alone, a phenotypic shift from “reparative/proliferative” to “regression/antiproliferative” should occur.¹⁴ However, a wound-specific phenotype is currently unknown and rigorous investigation toward this end has not been attempted; this type of study is complicated by the heterogeneous wound macrophage population, evolving phenotypes, the pleiotropic nature of wound macrophage function, and the overlapping phases of (and functional shifts assumed to accompany) wound healing.

2.5 THE FOREIGN BODY REACTION

The FBR is often described as an altered, abnormal, or aborted wound healing process. The most obvious distinction between normal and abnormal in this context is that wound healing in the presence of a foreign entity (e.g., a biomaterial or biomedical device) does not progress to or through the normal resolution stage (e.g., a scar). The FBR is often associated with unresolved local acute inflammatory events, chronic inflammation, continued and prolonged presence of monocytes, macrophages and lymphocytes, limited angiogenesis, and excessive fibrogenesis.⁷ Chronic inflammation may also result from implant-associated infection, inherent chemical and physical properties, or motion of the implant.⁷ Chronic inflammation is generally localized and usually short lived, but may persist for years in the presence of a foreign body.⁷ Generally, prolonged, protracted inflammation, wound healing processes, and the ensuing FBR are all facets of the host response to tissue injury that occurs upon placement of a biomaterial or biomedical device.⁷ In the context of implanted biomaterials, the FBR with the development of granulation tissue, chronic inflammation, and fibrosis is considered to be the normal wound healing response.⁷

The FBR to biomaterial implants physically comprises the implant, FBGC and surrounding granulation tissue comprising macrophages, fibroblasts, and capillaries, in varying amounts.⁷ Surface chemical and physical properties of the implant (e.g., composition, surface roughness, general shape, and physiological placement)

influence to some extent the intensity of the FBR reaction, its physiological structure, and thickness of the encapsulation tissue.⁷ Notably, no materials or device designs reported to date completely attenuate the FBR, indicating a basal, generic level of host reactivity that may be impossible to eliminate. Most, but not all, implanted materials in soft tissue end up extensively fibrosed (abnormal formation of fibrous tissue) and physically isolated from the adjacent host tissue via an avascular fibrous capsular sheath (i.e., densely compacted collagen matrix).^{83,84} Altered cellular phenotype(s) within the wound site, perhaps mediated by local inflammatory macrophage populations, may lead to overproduction of collagen and ECM components that contribute to fibrosis and fibrous encapsulation.⁷ The FBR fibrous capsule provides an adherent, avascular, impermeable barrier often responsible for biomedical device failure or malfunction.^{8,85} This constitutes one of the hallmarks of end-stage “healing” in response to a biomaterial, distinct from that of normal healing.⁷ Relatively flat, uniform surfaces may have a FBR sheath containing only one to two macrophage-occupied strata, while rough or angular, edged surfaces exhibit a thicker cellular layer directly adjacent to the implant material, with varying degrees of granulation tissue subadjacent to the cellular layer.⁷ End-stage healing (remodeling) at the implant site may involve either tissue regeneration (new tissue synthesis by parenchymal cells of the same type) or dysfunctional replacement (connective tissue, fibrous capsule formation).⁸⁶ The underlying tissue anatomical and physiological framework is critical for successful regeneration; without it, fibrosis occurs but normal function is not restored. The persistent inflammatory state surrounding implanted materials, elicited by several cellular players and correlated with macrophage presence, is likely to be responsible for the departure of wound healing mechanisms from normal prohealing and remodeling to excess matrix and fibrotic reactions observed around sensors. It is likely that macrophages within the biomaterial/implant wound site exhibit altered molecular repertoires compared to “normal” wound healing macrophage populations and that the molecular profile of biomaterials-associated macrophages lies somewhere between the “normal” wound healing macrophage populations and that of a giant cell. Unfortunately, none of these cell populations is well characterized at this time, and substantial effort must be undertaken to progress toward a thorough characterization of these cell types to understand the progression from normal to abnormal wound healing.

2.5.1 The Role of the Wound-Site Macrophages in the Foreign Body Reaction

In addition to terminal differentiation into macrophage phenotypes, MPS cells are known to exhibit an alternative terminal state in specific microenvironments within the host: the fusion product of multiple cells to produce a multinucleated giant cell (Figure 2.2).¹⁸ The first reported observation of these cells dates back to 1868 by Langhans and later reports (1912, 1925, and 1927, respectively) by Lambert and Lewis.^{10,87,88} Three types of giant cells are most commonly identified: osteoclast, Langhans cell, and FBGC. Each is distinct in location and appearance, but all share common traits: (1) derivation from MPS cells, (2) a role in tissue remodeling and/or immune defense, and (3) an association with disease, lesions, and tumors.⁸⁹

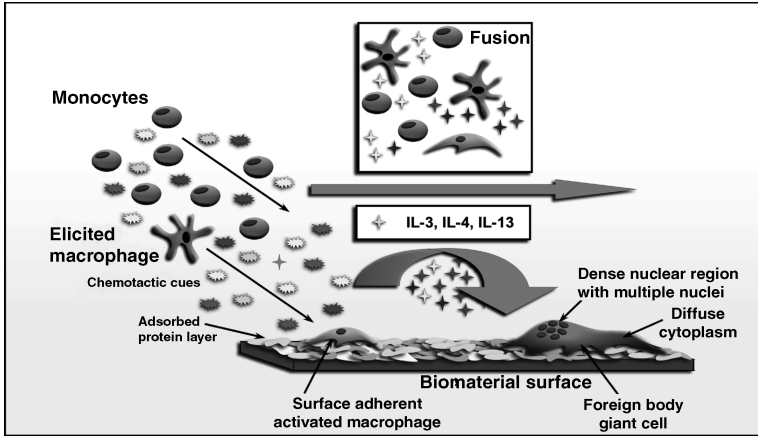


Figure 2.2 FBGC formation at the biomaterial surface. Circulating MCs and tissue-resident macrophages, MΦs, are elicited/attracted to the wound site (biomaterial) by chemotactic cues (e.g., MCP-1). MCs mature at the wound site to MΦs that may adhere to the protein adsorbed biomaterial. Adherent and/or activated MΦs produce soluble chemical signals (i.e., cytokines, chemokines) that mediate further influx of MCs, MΦs, and other cell types to the wound site. In the presence of specific cytokine signals (e.g., IL-3, IL-4, IL-13), MCs and MΦs may fuse into large multinucleated cells, FBGC, which may contain over 100 nuclei in their dense central nuclear region. *Note:* Cartoon is not to scale. Figure design by Dr. Marisha Godek; graphics by Dr. Gregory Harbers.

These cells are well known to be the products of fusion, not cell division in the absence of cytokinesis.^{45,89–92}

Osteoclasts are multinucleated cells found attached to the bone that they actively resorb and critical for normal bone homeostasis—they play a key role in normal bone turnover and replacement. Normally, osteoclasts are recruited and stimulated by strain-induced signals coming from local regions of microdamaged, mechanically stressed bone. Osteoclastic activity resorbs small patches of bone as a normal prerequisite to bone neogenesis. However, they are also active in bone tumors, bone transplant rejection, and the pathogenesis of osteoporosis.⁸⁹ Langerhans cells (not to be confused with the epithelial dendritic Langerhans cell) are examples of giant cells frequently encountered in diseased states involving granulomatous lesions such as tuberculosis, syphilis, sarcoidosis, and deep fungal infections.^{87,93} These cells exhibit a nuclear arrangement that is horseshoe or circular in shape, and the nuclei are found at the periphery of the cells.

Foreign body giant cells, the large multinucleated cells characteristic of the FBR surrounding implant materials and devices, comprise many fused monocyte and macrophage cells in response to a diseased state and/or the presence of foreign entities such as implanted biomaterials.^{7,10,87} Residing in direct proximity to the tissue–implant interface, FBGCs may persist for the lifetime of the implant.^{7,10} The exact nature of their cellular state (metabolically active, phagocytic, quiescent, senescent) at extended times *in vivo* remains unknown.⁷ Furthermore, the exact molecular signals that regulate FBGC formation and FBR development remain undefined.¹⁰

FBGCs may contain over a hundred nuclei, reflecting massive fusion of mononuclear MPS cells.^{7,94} In contrast to the peripheral nuclear arrangement of the Langhans cell, the nuclei of the FBGC are found in a dense central location.⁹⁵ FBGCs are not highly phagocytic, possibly due to the relatively large size of the foreign entity that results in a FBR.⁹⁶ Despite the fact that FBGC do not phagocytize the implant directly, they do exhibit increased lysosomal and respiratory enzyme activity, and are reported to exhibit fewer cell surface and complement receptors, suggesting a change to a task-oriented phenotype directed toward dismantling and removing the insulting entity.^{7,97} In fact, upon fusing to a giant cell phenotype, the macrophage is thought to redirect its functional role from endocytosis to establishment of a powerful external lysosome.⁸⁹ Both giant cells and osteoclasts adhere strongly to substrates through a “sealing zone” into which lysosomal enzymes and protons are secreted, allowing powerful extracellular bioactivity within a closed compartment adjacent to the surface.⁹⁸ FBGC presence always appears to correlate with an FBR. Importantly, such an association could be only correlative and not causative. That the FBGC contributes functionally to the FBR, further inflammatory mediation, or prolongation of abnormal fibrotic reactions remains to be proven.

Monocytes, the immature circulating progenitor form of the macrophage, differentiated macrophages, and activated macrophages all play important roles in the development of the FBR *in vivo*.^{10,18} Figure 2.2 outlines key events associated with biomaterials placement in the body. The sequence begins with the rapid and immediate adsorption of wound-site host proteins from blood, lymph, or other extracellular fluids (complex biological milieu, thousands of possible proteins).⁶ The biomaterials implant is likely to be completely covered with adsorbed proteins, many in nonnative conformations and also in dynamic states of exchange with other soluble species. Hence, the biomaterials surface chemistry is likely to be masked by a poorly controlled, poorly defined protein layer by the time host cells interrogate the implant site. Host neutrophils are the first cells to arrive at the site upon acute injury—their presence is very transient (neglected for simplicity in this diagram) as they are rapidly replaced by circulating monocytes that differentiate *in situ* in response to endogenous wound healing factors into mature, activated macrophages, often implant adherent. Subsequently, these macrophages provide soluble signals for further monocyte/macrophage infiltration, and, in response to cytokine cues, some macrophages may fuse to form FBGC. Whether only fully differentiated, mature, or inflammatory macrophage populations are exclusively involved in FBGC formation is not known. Furthermore, implant site-resident macrophages modulate angiogenesis and fibrogenesis as a wound healing response.^{10,15}

Foreign body giant cell formation (i.e., macrophage fusion) is influenced and enhanced by specific cytokines including IL-3, IL-4, and IL-13.^{95,99–101} IL-4 is known to upregulate expression of the MMR, also known as CD206, CCR2 (the receptor for monocyte chemoattractant protein-1, MCP-1), a classic phagocytic receptor implicated in fusion to FBGC.⁶⁵ MCP-1 has also been implicated in monocyte recruitment to biomaterials implanted in the peritoneal cavity and has been deemed requisite for macrophage fusion to FBGC.¹⁰ Furthermore, MCP-1 has been detected on FBGC at implant sites 2–4 weeks after implantation, suggesting its role in

recruitment of additional monocytes/macrophages and maintenance or progression of the FBR.¹⁰ The fusion process is thought to be mediated by the interaction of transiently expressed cell surface proteins, although the detailed mechanism remains unclear.⁸⁹

Several molecular markers that play a role in fusion have been identified: MMR, the macrophage fusion receptor (MFR), comprising CD44 and CD47, and the dendritic cell-specific transmembrane protein (DC-STAMP).^{45,89,102,103} *In vivo* and *in vitro* macrophage studies performed by Vignery et al. have demonstrated that all cells tested, regardless of origin (species, organ/tissue), express the same functional markers, a characteristic of osteoclasts.^{98,104–107} To date, no single molecule has been identified with restricted expression to either giant cells or osteoclasts, although it has been postulated that multinucleated macrophages acquire a tissue-specific molecular repertoire.⁸⁹

In addition to altered or enhanced cell *surface* protein expression, distinct matricellular proteins (secreted macromolecules that interact with ECM proteins as modulators of cell–matrix interactions that serve no structural role) mediate elements of the FBR: inflammation, angiogenesis, and collagenous encapsulation. Members of the matricellular protein family (i.e., osteopontin, tenascins, thrombospondins, SPARC) are upregulated during inflammatory events and play a role in mediating cell–matrix interactions.^{108–110} For example, Hevin and SPARC, two members of the SPARC family that are highly homologous, mediate different aspects of the FBR; Hevin plays a role in biomaterial-induced inflammation, whereas SPARC mediates collagen-based capsule formation.^{110,111} Thrombospondin 2 (TSP2) has been shown to influence the development of the FBR capsule; experiments performed in TSP2-null mice demonstrated a highly vascularized thick capsule with abnormally shaped collagen fibers as compared to normal mice.¹⁰⁹

2.5.2 Modulating the FBR *In Vivo*: Approaches to the “Pink Capsule”

The FBR appears to be a ubiquitous, graded response to all implanted materials at least in soft tissue, present to some extent regardless of device chemical composition or physiological placement. Frustration in addressing the clinical problems of the FBR with biomaterials chemistry alone has prompted alternative strategies. In this regard, both pharmacotherapies (i.e., drug delivery) and biomaterials interfacial physical modifications including topological and topographical cues, textures and surface-accessed porosity, and physical barrier strategies have been employed to evade or attenuate the FBR *in vivo*.¹¹² A primary goal has been to locally modulate the properties of resulting fibrosis and encapsulation *in vivo*—to achieve the “pink capsule”—a vascularized, perfused, and readily diffusive fibrous end point rather than the impenetrable barrier of the typical fibrous sheath encapsulating most devices. The “pink capsule” goal is a clinical resignation to the host tissue’s persistent fibrotic response but with the compromise to produce angiogenesis sufficient to provide adequate blood flow for oxygen and analyte transport across the capsule to and from the device surface. This objective then necessarily has correlated production of the

host inflammatory response with methods for prompting neovasculogenesis locally *in situ* adjacent to the implant. In fact, the correlated biology of wound healing and implant-associated inflammatory macrophages with follow-on angiogenesis can be exploited in this regard.¹¹³

Implant-associated coupling of local inflammation control and angiogenesis has been achieved with both local drug release and biomaterials physical cues, altering rates and histology of capsule formation, and associated angiogenesis. Dexamethasone, a well-known glucocorticoid anti-inflammatory drug active against macrophages, has been released for over a decade from commercial pacemaker leads in humans and more recently deployed in vascular stents to diminish inflammatory and fibrotic activities.^{114–116} As dexamethasone might also attenuate angiogenesis, its local release is coupled with co-release of a vasculogenically active molecule (e.g., growth factors VEGF, PDGF, or bFGF) to induce new blood vessel production into the device coating or surface, resulting in a combination drug therapy at the implant site. Release of VEGF alone from implanted devices subdermally may not provide effective angiogenesis, as shown in the abnormal edematous vascular product from a subdermal VEGF releasing sensor coating in rats.^{117,118} However, delivery of the VEGF transgene to an *ex ova* chorioallantoic membrane model provided new vascularity sufficient to improve acetaminophen biosensor performance for 8 days in this model.¹¹⁹ Rats exposed to dual release of dexamethasone and VEGF from degradable microspheres exhibit attenuated inflammatory and fibrotic response and enhanced vessel growth.¹²⁰ However, another recent study has shown that combination local release of dexamethasone and VEGF from a subdermal sensor hydrogel coatings in rats alters several inflammatory responses but not in mutually additive, beneficial ways that might reliably produce the “pink capsule” or attenuated capsule performance objectives. Once drug release was exhausted, normal foreign body reactions occurred.¹²¹

Biomaterials surface texture, porosity, and microtopology have a long history of producing varied inflammatory, histological, and angiogenic responses *in vivo*. Intuitively, tissue inflammatory and angiogenic responses must be correlated effects from this influence but are largely empirically observed and without a clear and predictable mechanism or enduring FBR influence.^{122–125} In one of the many examples, neovascularization at the interface of microporous polymer membranes and tissues was shown to occur in several implanted membranes with pore sizes sufficiently large to permit complete penetration by host cells (0.8–8 μm pore size). Larger pore membranes had 80–100-fold more vascular structures at the membrane–tissue interface for 5 μm pore size polytetrafluoroethylene (PTFE) membranes compared to 0.02 μm pore size PTFE membranes and maintained for 1 year subcutaneously in rats. Nonetheless, this strategy only transiently suppresses certain components of the FBR without complete, satisfactory resolution or clinical solution.¹²⁶ This suggests a cellular effort toward “biointegration” of these textured or porous membranes, advantageous to implant systems or devices where nutrient supply, oxygenation, and exchange of other soluble factors are critical to device function as well as tissue integration. How this might be promoted longer term to more permanently stabilize the implant site toward perfused, integrated tissue or a vascularized capsule remains to be achieved.

2.5.3 Foreign Body Giant Cell Formation *In Vitro*

To further characterize the role of the macrophage and significance of the FBGC in the FBR, much effort has been devoted to establishing reliable cell fusion protocols for *in vitro* studies. Most efforts focus on pooled human blood monocytes, matured in cultures using GM-CSF and subsequently stimulated with IL-4 and IL-13 to produce fused cells.^{65,95,100,101} Numerous factors play into achieving successful *in vitro* fusion: cell source (primary versus secondary derived (immortalized) cells), cell maturity (monocyte versus macrophage lineages), serum source (autologous versus nonautologous, complement containing), cell age (passage number for secondary cells), and the cytokine cocktail employed to facilitate fusion. Human primary cells represent an expensive and short-lived cell source to form FBGCs *in vitro*; reliable use of secondary-derived immortalized monocyte-macrophage cell lines to produce relevant FBGC cultures would be greatly beneficial in terms of a readily available cost-effective cell source. To date, only limited fusion studies have been performed using cell lines, and the high fusion rates typical for primary-derived cells have not been achieved in cell lines.^{65,127} This means that FBGC studies focus largely on *in vivo* implant models with both the benefits and the challenges of the full *in vivo* spectrum of cellular and molecular host response to dissect in order to tease out FBR mechanisms.

2.6 *IN VIVO* TECHNIQUES TO INTERROGATE CRITICAL COMPONENTS OF THE FBR

In vitro duplication of the FBR with much physiological fidelity is seemingly complicated by its relative simplicity compared to the *in vivo* implant wound healing scenario. Full cascades of cell-cell signaling responses, multiple cell types, and the phased kinetics of various cell-based inflammatory responses are completely missing from most models reported to date. Therefore, *in vivo* animal implant healing models have been developed in attempts to get more accurate information closer to the actual situation in human FBR.

2.6.1 The Cage Implant System

Historically, *in vivo* biocompatibility has been qualitatively assessed by histological evaluation of explanted devices.⁶ The presence or absence of specific inflammatory and fibrotic cell types and the degree and duration of the incidence of these cells at the implant site can be correlated to a relative degree of success or failure in terms of tissue compatibility. However, this common “implant, sacrifice, explant, and evaluate” single-time-point-per-animal approach is quite limited in assessment of time-dependent phenomena (e.g., direct assay of molecular and cellular mediators) associated with the progression of the inflammatory response and the development of the FBR.

The “cage implant” model developed and described by Marchant et al.¹²⁸ was designed to examine synthetic materials and proximal physiological response(s) to them in real time *in vivo*. This system allows qualitative and quantitative assessments

of the inflammatory response to materials of diverse composition *in vivo*.¹²⁸ Specifically, material changes and fibrous capsule formation/progression can be monitored, and associated inflammatory exudate can be collected and analyzed. Material changes can be further characterized by bulk physical analyses (molecular weight, stress-strain, FTIR) and by modern biomaterials relevant surface analytical techniques.⁸ From inflammatory exudates, quantitative nonadherent leukocyte counts and phenotypic profiling can be obtained, as well as evaluation of extracellular exudates (e.g., alkaline and acid phosphatases, chemokines, cytokines, ECM production, etc).¹²⁸ The system is unique in its ability to internally control samples, provides real-time sampling and different types of inflammatory information from the implant site, and translation across different species and in different tissue sites.

2.7 FUTURE DIRECTIONS

Numerous previous studies of implantable biomaterials employ monocyte and macrophage cells to examine cytotoxicity, inflammatory response, and general “biotolerance.” Primary deficiencies in these studies surround the lack of accuracy of the experiments compared to the problem, inability to collect the dimensionality of data necessary to accurately model MPS and inflammatory complexity, and lack of understanding of the cell mechanisms involved either *in vitro* or *in vivo*. The selection of the specific cells employed to this end is often limited by what is available, inexpensive, and practical, often excluding cells of primary derivation. Multiple immortalized model cell lines of monocyte and/or macrophage origin from human and murine sources are available, each with different morphologies, adhesion patterns, and growth requirements. No one has compared these cell sources using any basis for MPS fidelity. Thus, comparison of results for cell types arising from different species, of varied maturity and with different growth, subculture, and harvesting requirements is problematic. A thorough characterization of each cell type would support some comparison, but consensus is lacking in terms of what markers are appropriate for distinguishing maturity and differentiation, especially between closely related macrophages and dendritic cells. Characterization via surface marker detection is commonly employed, but often reliance is on relative levels of expression rather than presence or absence of markers to distinguish between cells of the MPS. In addition, most cellular and molecular phenotyping tools are currently focused on mouse markers, and cellular and *in vivo* inflammatory and healing model focus is therefore primarily murine derived. Attractive knock-in and knockout genetic models are also primarily murine. Many other animal molecular details are limiting, based on the narrow range of toolkits available. As murine physiology and healing mechanisms are likely distinct from larger animal models, the relevance of much of the data to human extrapolation might be suspected.

This begs the question—are these MPS cells truly different from each other, or rather, specialized adaptive cell states?¹⁶ No single marker can be used to define if a cell is a macrophage. It has been suggested by Hume that, based on new insight provided by detailed characterizations of cells of the mononuclear system in recent years, a revised system for classifying immune cells of this lineage is overdue.¹⁶

Considering many different types of cell culture supports and their effects on the macrophage, it is evident that there is no unambiguous culture substrate to which other materials can be compared.¹²⁹ The activating nature of the surface may result from denatured or partially denatured proteins adsorbed to the surface that act as attractants and/or provide macrophage activating cues. Previous reports indicate that denatured non-ECM proteins serve as nonspecific attractants for granulocytes.⁵⁵ Recently, efforts to develop and employ new materials that mimic the three-dimensional environment more natural to cells have led to commercial synthetic support production (e.g., Ultra-Web[®] Synthetic ECM, SurModics/Donaldson[®]) for use in static cultures.¹³⁰ Culture methods have seen some advances, examples include roller bottle systems (designed to more closely represent cell growth in the presence of shear forces) and spinner flasks (designed to support suspended nonadherent cell growth). Other technique and technology advances seek to more accurately mimic *in vivo* conditions for *in vitro* studies.¹³¹ Additional considerations for appropriate design of *in vitro* studies require the use of serum, given that serum proteins play determinant roles in cell interactions with surfaces *in vivo*. Macrophage studies can be also complicated by the presence of contaminating levels of endotoxins produced by bacteria. Therefore, all studies that employ macrophages should also describe measures taken to test for and reduce endotoxin contamination.

Advances in the quest for improved biocompatible materials rely on a thorough understanding of the FBR. This necessitates a detailed understanding of macrophage phenotype and function in this unique context. With the advent of new molecular characterization techniques and the likely discovery of novel macrophage markers, the relative relationships between cells of the MPS should be clarified. This should assist in elucidating relationships between these cells on biomaterials. In addition, understanding of the role of relatively new markers related to macrophage fusion (e.g., CD47 (MMR), MCP-1) and comparison to macrophage-specific (maturity) markers should provide a more detailed picture of the macrophage phenotype found at biomaterials-induced wound sites.^{10,45,65}

Given that cell lines are certain to remain in use for *in vitro* purposes, improved phenotypic characterization should aid in the selection of specific cell lines that most faithfully represent primary cells within the experimental context. Specific validation guidelines for appropriate experimental design (serum inclusion, endotoxin testing, etc.) in the context of *in vitro* models for testing of biomaterials must be followed to collect meaningful data for comparisons and standardization. Relevant to *in vitro* work, characterization and comparison of immature and mature (aged) primary-derived cells to macrophage cell lines with respect to integrin expression on both standard tissue culture supports and model biomaterial surfaces would provide insight into the suitability of employing (aged, immortalized) adherent macrophage cultures that may be “prebiased” with respect to integrin expression based solely on their culture selection methods. *In vitro* studies must be carefully designed to be relevant to FBR progress *in vivo*; more relevant *in vitro* modeling of isolated aspects of the FBR requires an understanding of relationships between specific subsets of monocyte/macrophage and macrophage interaction with other cell types (e.g., fibroblasts, lymphocytes). Future work should address more thorough characterization of all macrophage phenotypes present at implant sites (adherent, activated, or fused

macrophage) and duration of each phenotype using validated markers. This could be attempted *in situ* using specific markers directed toward surface antigens or soluble chemokine/cytokine products, or using techniques that allow cell harvest and sorting based on size and various surface markers. Prudent combinations of techniques with new methods for integration and interpretation of dynamic systems will likely yield the most convincing results in terms of characterizing FBR event sequelae. To conclude, better understanding of the molecular orchestration of events underlying the FBR should allow for design principles to provide improved device biocompatibility through modification and modulation of both materials and host inflammatory responses. New tools including mutant animal models, new molecular biomarkers, and new experimental techniques, including coculture and tissue cultures, should help provide more and better information. However, understanding the FBR will depend on new, coordinated improvements in molecular tools, wound healing characterization standards, cell phenotyping and standardized *in vitro* models, preclinical testing validation, new pharmacological control and modulating agents, and methods to integrate complex data sets from multiple cell types, signaling pathways, and feedback loops involved in inflammation.

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DAVID D. CUNNINGHAM, PhD, is a Volwiler Associate Research Fellow at Abbott Diagnostics.

JULIE A. STENKEN, PhD, is Professor and 21st Century Chair of Proteomics at the University of Arkansas

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