THE ROLE OF PLATELET-MACROPHAGE INTERACTIONS IN INFLAMMATION AND WOUND HEALING

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ABSTRACT

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INFLAMMATION AND WOUND HEALING

(Under the direction of Dr. Thomas H. Fischer)

During the normal response to injury, platelets and the proteins of the coagulation cascade achieve hemostasis by forming a platelet-rich clot. Platelets are later removed from the wound site by macrophages. This phase of wound healing, known as the late inflammatory phase, also involves the release of many cytokines which amplify the leukocyte response. This inflammatory phase is dysfunctional in patients who suffer from impaired wound healing, which often predisposes them to infection and/or increased time to wound closure. A better understanding of how inflammation resolves during normal wound healing may help identify new methods of intervention for patients who suffer from impaired wound healing.

The overall goal of this dissertation is to better define the role of platelet-macrophage interactions in the resolution of inflammation and wound healing. We hypothesize that platelet-macrophage interactions enhance the inflammatory response during cutaneous wound healing. The first part of this work characterizes the mechanisms that regulate macrophage phagocytosis of

autologous platelets, including analysis of common surface proteins on the platelets and macrophages and their potential role for internalization of platelets. Using *in vitro* phagocytosis assays and analysis by microscopy, we analyzed the conditions necessary for platelet uptake, the role of platelet activation in this process, and the role of sulfated polysaccharides in inhibiting this process.

The second part of this work focuses on the inflammatory consequences of platelet-macrophage interactions. Cytokines secreted from resting and LPS-activated macrophages were analyzed during co-incubation of macrophages with either apoptotic cells or activated platelets. We also tested the *in vivo* role of one particular cytokine, IL-23, in cutaneous wound healing using both IL-23 knockout mice and their wild-type littermates.

The overall conclusions of this work suggest that macrophage phagocytosis of autologous platelets correlates with platelet activation, yet occurs independently of platelet surface P-selectin and phosphatidylserine. Secondly, platelet uptake enhances macrophage activation and pro-inflammatory cytokine secretion, which is in sharp contrast to the macrophage response following phagocytosis of apoptotic cells. Finally, *in vivo* studies suggest that in the absence of IL-23 expression, wound healing is slightly impaired.

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CHAPTER I

INTRODUCTION

THE PUBLIC HEALTH RELEVANCE OF WOUND CARE

Wound healing is a critical part of the resolution of many disease processes and medical interventions. Regardless of whether the tissue injury results from trauma, a disease process, or a medical intervention, the body must be able to mount a reparative response. The time required for proper wound healing can affect the length of hospital stay for any patient, from those in orthopedics receiving joint replacements to patients in the burn unit receiving skin grafts.

The inability to properly repair a wound can itself be the cause of hospitalization. Chronic wounds are characterized by either delayed repair or frequent recurrence as compared to normally repaired tissue (1, 2). These wounds are difficult to treat, frequently infected, and often lead to lower extremity amputation. Non-healing wounds continue to be the leading cause of non-traumatic amputation in the United States, resulting in more than 80,000 lower extremity amputations in the US alone in 2003 (3). Although impaired wound healing has several causes, such as malnutrition (4), immunodeficiency (5), and certain medications (6), the most common systemic cause is diabetes mellitus, a

condition which affects over 20 million Americans (3). The increasing prevalence of diabetes has resulted in a sharp rise in hospitalizations for chronic wounds, which are also often called ulcers. In 2003 alone, more than 870,000 hospitalized diabetic patients had some form of lower extremity disease, and more than 250,000 of them were hospitalized primarily for inflammatory ulcers or related complications (3). In 2007, the cost of treating chronic diabetic complications (including impaired wound healing) totaled more than $58 billion in the US, which was more than double the cost of direct treatment of diabetes ($27 billion) (7).

Despite the prevalence of complications associated with wound healing across so many disciplines, many questions on the basic principles of wound healing remain. From a biological standpoint, chronic wounds are characterized by excessive inflammation (2, 8, 9). The normal inflammatory processes that occur during wound healing are often delayed in initiation and/or prolonged beyond the normal time of resolution (1, 2, 8, 10). Some studies have identified defects in the cells that normally participate in wound healing, although the exact cause of these defects in wound repair is unclear (8, 10).

Clinical advances in wound care have attempted to address the issues of infection and inflammation in chronic wounds, and several therapies are currently being developed. These include negative pressure (vacuum assisted) devices (11), various growth factors and gels for topical application (12-15), and several dressings designed to enhance wound closure (16-18). Despite such advances, the complexity of non-healing wounds has presented many challenges for new

therapies. Research in this field is complicated by the fact that the exact mechanisms regulating inflammation in normal wound repair are also not completely understood (19).

OVERVIEW OF NORMAL WOUND REPAIR

The complicated process of normal wound repair is divided into several overlapping phases (20). In response to injury, platelets and the proteins of the coagulation cascade achieve hemostasis by forming a platelet-rich fibrin clot. Some of these proteins, such as thrombin, in addition to other plasma proteins such as activated complement, increase vascular permeability and attract neutrophils and monocytes to the wound site (21).

The clot provides a provisional matrix and a reservoir of growth factors for the inflammatory cells that arrive during the inflammatory phases (22). In the early inflammatory phase, the wound site is dominated by neutrophils which are drawn to the clot by the chemoattractant complement components C3a and C5a. In the absence of bacterial contamination, the neutrophil response generally subsides within a few days. Additionally, depletion of circulating neutrophils in aseptic experimental models of wound healing has demonstrated that neutrophils are not absolutely required for normal wound healing (23).

Apoptotic neutrophils, wound debris, and platelets are removed from the wound site by macrophages during the late inflammatory phase of wound healing. During this time (6-8 days after injury), the number of monocyte-derived cells in the wound space peaks and there is an additional burst of secreted

cytokines which amplify the leukocyte response (24). Although many of the cytokines have overlapping functions, others, such as IL-6, are absolutely required for normal wound repair (25). These cytokines and growth factors have autocrine effects which transition the monocytes into inflammatory macrophages. In addition to phagocytosis of wound debris and amplification of the monocyte response, macrophages also secrete additional cytokines and growth factors required for generating granulation tissue.

As the inflammation resolves, a remodeling phase begins in which fibroblasts and other cells migrate into the granulation tissue and angiogenesis begins, while epithelial cells complete the wound closure (19). T lymphocytes are also involved in normal remodeling, although the exact T cell subset is still being characterized (26-29). The process of remodeling and scar resolution, performed mainly by fibroblasts, may continue for weeks.

The mechanisms that regulate the transition from the inflammatory phase to the remodeling phase of wound healing are not well understood. A major focus of this work is the non-hemostatic effect of platelets on these inflammatory phases of wound repair.

PLATELETS IN HEMOSTASIS AND WOUND REPAIR

Platelet Function at Wound Sites:

Platelets are anucleate cells which are essential for normal hemostasis, and the hemostatic role of platelets has been studied extensively. Following injury, platelets adhere to wound sites by binding to exposed extracellular matrix

proteins, primarily von Willebrand Factor (VWF) (30, 31) and collagen (32, 33). Platelets also aggregate with one another and form a temporary plug with fibrin which prevents additional blood loss.

The process of platelet activation and aggregation results in many critical changes to the platelet surface. Integrins already on the surface of the platelet, such as the $\alpha_{IIb}\beta_3$ complex, undergo conformational changes to mediate adhesion and aggregation (34). Other proteins within the granules of the platelet, such as Factor V (35), P-selectin (36, 37), and CD40L (38-40), are translocated to the platelet surface where they interact with coagulation proteins and leukocytes. The plasma membrane of activated platelets also loses its phospholipid asymmetry during activation which results in surface exposure of phosphatidylserine (41, 42). Overall, the surface of the activated platelet provides a hemostatic surface for the propagation of the coagulation cascade and generation of large amounts of thrombin, which in turn cleaves fibrinogen into fibrin and forms a stable clot (43, 44).

Proteins within the platelet granules are not restricted only to the surface of the activated platelet. Platelet activation also causes the complete release of the contents of the secretory granules, which include adhesion molecules, chemokines, coagulation and fibrinolytic factors, and other growth factors (45). These proteins in turn can react with other proteins and cells within the wound environment.

Although the role of platelets in hemostasis has been well characterized, the platelet impact on inflammation and the later stages of wound healing has

only recently been addressed. In studies of dermal wound healing in mice, thrombocytopenia results in significantly higher numbers of macrophages and T cells within the wound, suggesting a role for platelets in regulating the inflammatory response (46). Additionally, there are several indirect lines of evidence for the impact of platelets on inflammation and wound healing. Platelets are present in the wound space from the initial coagulation phase through the late inflammatory phase, and they release a variety of proteins that may regulate inflammation and thrombosis (47). Several secretory products of platelets, such as platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β), stimulate a pro-inflammatory effect on other wound healing cells (48-50). Platelets can also activate a portion of their own secreted TGF-β, which then induces monocyte chemotaxis and growth factor production (51, 52). Interestingly, autologous platelets have been proposed as a therapeutic treatment for wound healing because they are such a rich source of these beneficial growth factors (53).

Platelet Clearance Mechanisms:

Platelets are normally cleared from circulation by macrophages either at wound sites or in the spleen or liver (54-58). Although the clearance mechanism of normal platelets has not been resolved, several studies have examined the mechanisms by which macrophages clear different types of platelets, such as opsonized platelets, chilled (blood-bank stored) platelets, and platelets aged at normal body temperature (59-62).

In the case of autoimmune response to autologous platelets, platelets become coated with IgG-containing autoantibodies, or opsonized, and are cleared more quickly than non-opsonized platelets via Fc-receptors on macrophages (63). The clearance of opsonized platelets is a central characteristic of conditions involving autoimmune thrombocytopenia, such as Anti-phospholipid Syndrome, Wiskott-Aldrich syndrome, and Systemic Lupus Erythematosus (64-67). These conditions can be worsened by additional bacterial infection because phagocytosis of opsonized platelets is also enhanced by platelet-bound LPS (68). Patients who are treated with the anticoagulant heparin can also develop autoantibodies against platelets, which specifically recognize a complex between heparin and Platelet Factor 4 on the platelet surface (69, 70).

Early storage protocols for platelets at the blood bank involved keeping platelets refrigerated to limit bacterial contamination. However, chilled platelets are rapidly cleared from circulation (59, 71). Chilled platelets contain surface clusters of GPIbα which triggers their phagocytosis by macrophages, primarily in the liver, via an $\alpha_M\beta_2$ integrin-dependent manner (59, 72-74). Removal of sialic acid, for example by neuraminidase during viral infection, also triggers integrin-dependend platelet removal by liver macrophages (75, 76). Blocking β-N-acetylglucosamine residues of the GPIbα protein preserves the *in vitro* function of chilled platelets (77), and modification of chilled platelets by glycosylation restores their *in vivo* circulation times, but only for platelets that have been chilled for two hours or less (78, 79).

The known effects of refrigeration on platelets have resulted in storage protocols in which isolated platelets are stored at room temperature, but only for 5-7 days due to loss of hemostatic function. This phenomenon is known as storage lesion, and is associated with platelet activation (80). Both stored platelet concentrates and individual *in vitro* studies have implicated platelet activation in platelet concentrates. Studies on how platelets normally age are complicated by the ability to track aged platelets *in vivo,* although one canine study has suggested that platelets express phosphatidylserine *in vivo* during aging (81). *In vitro* experiments have demonstrated that platelets aged at 37^{o}C for 24hrs become activated as determined by increased expression of phosphatidylserine and P-selectin (62). However, despite the increased expression of surface P-selectin, both *in vitro* and *in vivo* experiments have shown that platelet clearance occurs independently of P-selectin (62, 82, 83). Platelet aging has been compared to a programmed cell death process, and thrombin-activation of platelets triggers an apoptotic phenotype as characterized by phosphatidylserine exposure, mitochondrial inner transmembrane potential depolarization, expression of pro-apoptotic proteins Bax and Bak, and activation of the apoptosis marker caspase-3 (84). There is also evidence that platelet aging results in metalloproteinase-dependent degradation of platelet GPIbα, which reduces platelet function (85). Uptake of activated or aged platelets is thought to be mediated by scavenger receptors on the macrophage because it is inhibited by the polysaccharide (and savenger receptor ligand) fucoidan (62).

However, neither a specific molecule on activated or aged platelets nor a specific macrophage receptor for this process has been identified.

Despite the studies on chilled, opsonized, and aged platelets, no study has identified the mechanism by which macrophages interact with fresh platelets that have not been altered by *ex vivo* storage conditions or immune responses. Furthermore, no study has addressed the mechanism by which autologous human platelets interact with macrophages. These conditions are critically different from those mentioned above because, under normal conditions, autologous platelets do not form GPIbα clusters or become coated with antibodies. The most relevant work, therefore, may be the studies regarding clearance of aged platelets.

Importantly, the literature reviewed here has examined platelet clearance mechanisms accomplished by splenic or hepatic macrophages, or macrophages cultured *in vitro*. These mechanisms can be considered similar to what may happen during platelet clearance at wound sites, although there are no studies that specifically examine platelet clearance in tissues other than the liver or spleen. Phagocytic clearance of cells other than platelets is discussed in the fifth section of this chapter.

MONOCYTE-PLATELET INTERACTIONS IN THE CIRCULATION

The interaction between platelets and monocytes, the precursors of macrophages, has been studied far more extensively than that of platelets and terminally differentiated macrophages. Platelets and monocytes interact in

circulating blood under two circumstances: in the formation of platelet-monocyte aggregates and in the recruitment of leukocytes to inflamed endothelium. Platelet-monocyte aggregates are significant because activation of the monocyte ligand for P-selectin (PSGL-1) results in several pro-inflammatory downstream effects (47, 86). Additionally, the P-selectin interaction with PSGL-1 is required for monocyte recruitment to inflamed endothelium during a variety of conditions (87, 88).

Beyond the mechanism of platelet-monocyte binding, additional studies have identified some pro-inflammatory effects of platelet-leukocyte interactions. Binding to activated platelets increases monocyte expression of interleukins (ILs) and matrix metalloproteinases (MMPs), including IL-1β, IL-8, Monocyte Chemoattractant Protein-1 (MCP-1) and MMP-9 (89-94). Platelet binding also stimulates monocytes to release an increased amount of superoxide anion (95). Studies using fresh human monocytes have also demonstrated that phagocytosis of platelets, but not latex beads or zymosan, enhances monocyte survival and renders them refractive to FAS-ligand induced apoptosis (96).

Despite the previously characterized interactions between platelets and undifferentiated monocytes, the focus of the remainder of this dissertation is on how platelets interact with differentiated monocyte-derived macrophages. It is important, however, to be mindful of the ability of monocytes to become activated by engagement of PSGL-1, as well as the potential for platelets do induce pro-inflammatory cytokines in macrophage precursors.

MACROPHAGES AND PHAGOCYTOSIS IN WOUND REPAIR

Macrophage Function at Wound Sites:

Just as platelets are essential for normal hemostasis, monocyte-derived macrophages are also essential for normal wound repair. This fact has been demonstrated in macrophage-depleted animals that exhibit defective wound repair (97). Macrophages at the wound site maintain the ability to further amplify the inflammatory response because, unlike platelets, they have the ability to continually synthesize and secret growth factors and cytokines.

Monocytes, the precursors of macrophages, are recruited to wound sites by complement fragments (C5a) (98), β-chemokines (MCP-1, MIP-1α) (99, 100), and other growth factors such as PDGF and TGF-β (49). Monocytes quickly differentiate into macrophages, as characterized by increased levels of lysosomal enzymes, increased expression of complement receptors and scavenger receptors, and increased phagocytic capabilities (101). The first major function of macrophages is degradation of the extracellular matrix, which is accomplished by secreting proteinases such as MMPs, elastases, and acid hydrolases. Macrophage phenotype is also significantly influenced by the extracellular environment, and effective wound repair requires a critical balance of cytokines at the wound site. In the environment of such complex stimuli, macrophages are capable of functioning in both degradative and reparative processes in sequential fashion (102). Thus, macrophages perform a second, reparative, function by secreting many cytokines that recruit additional macrophages to the wound site and stimulate fibroblast proliferation and collagen synthesis.

A third major function of macrophages at the wound site is phagocytosis of wound debris. Senescent cells and matrix fragments are internalized and degraded within the lysosomal system. This wound debris includes apoptotic cells as well as platelets within the clot. Although these components are commonly grouped together and termed “wound debris,” there no studies which have demonstrated how macrophages recognize activated platelets as part of this debris.

Ligands and Receptors Involved in Phagocytosis:

Particle recognition and phagocytosis, as well as the resulting downstream effects, are unique to the precise conditions surrounding the phagocyte and target cell. As a result there are many overlapping mechanisms for recognition and internalization of other cells by macrophages. One component shared by all types of phagocytosis is the formation of a phagocytic cup following actin polymerization (103). The extent to which actin is utilized and the kinetics of particle uptake are partially dependent on the size of the target cell (104).

In the context of immunology, there are two overlapping mechanisms of macrophage phagocytosis: Complement-mediated and Fc-mediated (105-109). Complement-mediated phagocytosis occurs when components of the complement system, primarily C1q, bind to foreign cells and pathogens such that they can interact with macrophages, primarily through complement receptor 3 (CR3) (106, 110, 111). Fc-mediated phagocytosis refers to the interaction between the Fc-region of IgG-containing antibodies and one of several isoforms

of the Fc-receptor on macrophages (108). Because C1q can also interact with IgG and IgM, the same cell could be internalized solely by complement-mediated mechanisms, Fc-mediated mechanisms, or a combination of the two (112). Phagocytosis by either receptor, in turn, activates different signaling pathways for phagocytosis. For example, complement-mediated phagocytosis requires intact microtubules but not active protein tyrosine kinases (113). Fc-receptor-mediated phagocytosis, however, requires tyrosine kinases but not intact microtubules (113).

Despite the well-studied mechanisms of phagocytosis during immune responses, perhaps the body of work most relevant to this dissertation is the collection of studies regarding macrophage phagocytosis of apoptotic cells (in the absence of opsonization) because activated platelets share many characteristics with apoptotic cells. However, the features of apoptotic cell clearance are complex because so many variations exist in the type of phagocyte or target cell involved. Generally speaking, macrophage phagocytosis of apoptotic cells, which also requires actin polymerization, is regulated by intracellular cAMP levels, can be inhibited by oxidative stress, and often involves scavenger receptors (114-119).

There have now been several classes of scavenger receptors that have been identified (120). Collectively, these receptors are thought to recognize a broad range of molecular patterns on target cells. Thrombospondin, which was first identified on platelets, is known to form a bridge between platelets and the scavenger receptor CD36 (121, 122). In addition to scavenger receptors, the

Mer receptor tyrosine kinase is another important receptor for apoptotic cells, which is bridged via Gas6 (123-125). Phagocytosis of apoptotic cells can also be CD14-dependent, which can inhibit macrophage ability to respond to LPS (126). Loss of sialic acid on apoptotic cells results in exposed side-chain sugars that are recognized by lectin receptors on macrophages (127) and this mechanism is similar to the one described above regarding chilled platelets.

One of the most well-studied ligands for phagocytosis of apoptotic cells is phosphatidylserine (128, 129). However, a specific and unique macrophage receptor for phosphatidylserine has not been identified. Rather, cooperation between receptors, in addition to recognition of phosphatidylserine, is often the case. These cooperative effects often involve integrin signaling. For example, CD36 can cooperate with other integrins, such as the $\alpha_v\beta_3$ integrin, to facilitate uptake of apoptotic cells (130-132). Another integrin, $\alpha_v\beta_5$ also plays a role in recognition and internalization of apoptotic cells (133). A final example is that of CD44-mediated phagocytosis, which can also be blocked by antibodies against CD11b (the integrin αM) (134). In the context of wound healing, efficient phagocytosis of apoptotic neutrophils via β-2 integrins on macrophages is required for normal wound healing (135).

A macrophage receptor required for phagocytosis of fresh, autologous human platelets has not been identified. The exact surface changes on the activated platelet which are required for recognition and internalization by macrophages also have not been described.

Inflammatory Consequences of Phagocytosis:

Phagocytosis of apoptotic cells is critically important because of the delicate balance between proinflammatory and anti-inflammatory stimuli. For example, apoptotic cells can induce cholesterol efflux from macrophages, but if cells become apoptotic by means of external stress, such as cholesterol loading, the response from the phagocyte can be pro-inflammatory (136, 137). If the cells are not removed from tissues before becoming necrotic, the inflammatory response may be enhanced (138, 139). Additionally, phagocytosis of dying cells can have profound consequences in chronic inflammatory diseases, such as atherosclerosis (140, 141).

Based on how cells normally undergo apoptosis, macrophage phagocytosis of apoptotic cells is generally an anti-inflammatory event, which suppresses the secretion of pro-inflammatory cytokines such as IL-1β, IL-8, and TNF-α (114, 115, 142). Macrophage contact with apoptotic cells or phosphatidylserine inhibits the IL-12 family of cytokines, which includes IL-12, IL-23, and IL-27 (143, 144). This effect may also occur by simply ligating macrophages Fc-receptors (145, 146). Other studies have shown that although Phosphatidylserine serves as a marker for phagocytosis, it alone cannot trigger inflammatory suppression (147, 148). The immunosuppressive effect of phagocytosis of apoptotic cells is regulated at the transcriptional level and also occurs in conjunction with increased levels of TGF-β and PGE2 (114, 142, 149). The immunosuppressive effects of apoptotic cells occur through several signaling pathways such as the MAPK-ERK pathway and the SOCS-STAT pathway (147).

The effects of phagocytosis on macrophages also depend on both the classical or alternative pathways of macrophage activation (150, 151). Much of the immunosuppressive effects of apoptotic cells have been shown to occur in macrophages that are cultured in a pro-inflammatory “type 1” phenotype. Macrophages that exert a less inflammatory phenotype and express high levels of IL-10, or are treated with glucocorticoids actually exhibit enhanced phagocytosis of apoptotic cells (152, 153).

The only known work regarding platelet-macrophage interactions has been done in the field of atherosclerosis and suggests that phagocytosis of platelets increases lipid accumulation and exacerbates inflammation within advanced atherosclerotic lesions (55, 154, 155). The generation of foam cells has also been associated with platelet phagocytosis (156). However, the inflammatory effects of platelet phagocytosis by macrophages in normal conditions and wound healing are not well understood.

THE ROLE OF IL-23 IN INFLAMMATION

IL-23-mediated signaling:

As mentioned above, regulation of cytokine expression during wound healing is critical for resolution of the inflammatory response. Several cytokine families have been identified. The interleukins, a group of leukocyte-secreted cytokines are further divided by structure and function. The IL-6/IL-12 family of cytokines is of particular interest because they are produced by cells of the innate immune system but have profound influences on adaptive cell-mediated

immunity. IL-12 is the best characterized cytokine of this group. IL-23, a heterodimer that shares a subunit with IL-12, is a recently discovered member of this cytokine family (157, 158).

Until recently, $CD4^+$ T cells were categorized as either helper type-1 (Th1) or type 2 (Th2). Th1 responses occur primarily through the action of interferon-gamma (IFN-γ). For example, the IL-12/IFN-γ pathway induces cytotoxic factors important for the direct killing of microbes or infected cells. Th2 cells are thought to be involved in humoral immunity via secretion of IL-4, IL-5 and IL-13.

The effects of IL-23 are different from the classical Th1 and Th2 responses (159). It has recently been discovered that IL-23 acts on T cell function by inducing the production of IL-17. This new subset of T cells has been described as Th17 cells (160). IL-23-induced IL-17 production is considered pro-inflammatory and, in addition to its effects on fibroblasts, it acts on both epithelial and endothelial cells, as well as keratinocytes(157). The IL-23/IL-17 pathway is implicated in several processes of both normal and pathological inflammation (157, 161-166). It has also been demonstrated *in vivo* that IL-23 is a distinct regulator of the development of chronic inflammation (162, 163, 165).

The role of IL-23 in psoriasis:

Although IL-23 has not been identified in normal skin, it is strongly implicated in psoriasis. Psoriasis is a chronic inflammatory condition that occurs in the skin, and is characterized by excessive inflammation and hypertrophic scarring. This autoimmune disease is partially mediated by T lymphocytes.

However, the T-cell mediated tissue damage arises from macrophage-derived IL-23 production. A role for IL-23 has also been shown experimentally, which imiquimod (a TLR 7/8 ligand) was used to induce psoriasis. In this system, the disease is almost completely absent in mice deficient for the IL-23 receptor (167). Immunohistochemical analysis of human psoriasis lesions have shown expression of IL-23 by macrophages (and also dendritic cells) *in situ* and these cells also express high levels of IL-23 in *ex vivo* analysis (168). However, the exact cause for macrophage production of IL-23 in psoriasis has not been identified. Interestingly, IL-12/IL-23 inhibitors are being developed for the treatment of this disease (169).

Overall, macrophage-derived IL-23 plays a role in several autoimmune reactions, and its downstream effects lead to T-cell mediated inflammation, epidermal hyperplasia, and fibroblast proliferation (161, 170). When occurring in the skin, these effects manifest as psoriasis. However, a role for IL-23 in the normal wound response by macrophages has not been tested.

CONCLUSION

The work presented in this dissertation better defines how inflammation resolves during normal wound healing, which may help identify new points of intervention for patients who suffer from impaired wound healing. Chapter 2 of this dissertation details the experiments performed to determine how macrophage phagocytosis of autologous platelets is regulated. Chapter 3 focuses on the inflammatory consequences of the macrophage response to

platelets. Chapter 4 addresses the *in vivo* role of IL-23 in wound healing. Finally, Chapter 5 summarizes the major conclusions presented in this work and provides an overview for future research in this field. The research in this dissertation contributes to our understanding of how platelet-macrophage interactions may play a significant role in the inflammatory component of many pathologies.

CHAPTER II

PHAGOCYTOSIS OF AUTOLOGOUS PLATELETS BY HUMAN MACROPHAGES IS ENHANCED BY PLATELET ACTIVATION AND INHIBITED BY SULFATED POLYSACCHARIDES

INTRODUCTION

Phagocytic clearance of cells within the circulation and surrounding tissues occurs daily in both normal and pathological settings. Cells that have become apoptotic as part of their normal life cycle are recognized and cleared by phagocytosis in a non-phlogistic manner (171-173). Infected cells can also trigger clearance by phagocytosis by displaying immune recognition patterns on their membranes (174-176). The importance of phagocytosis is emphasized by common conditions involving impaired phagocytosis, which may manifest as persistent infections or chronic inflammatory lesions such as diabetic ulcers and atherosclerotic plaques (141, 177-180). Importantly, the mechanisms regulating a phagocytic event are unique to the particular phagocyte and target cell.

Although several different cell types are capable of phagocytosis, macrophages are the dominant phagocyte of the reticuloendothelial system (RES). Phagocytic clearance of infected or apoptotic cells occurs primarily in the spleen, but macrophages also maintain tissue homeostasis by clearing apoptotic cells and debris at sites of injury and inflammation (181). During their

differentiation from primary monocytes, macrophages acquire specialized receptors and machinery for recognizing and clearing both apoptotic and infected cells (182). For example, during immune responses macrophages utilize complement and Fc- receptors to recognize and clear cells that are opsonized by activated complement or autoantibodies (105, 107). In clearing apoptotic cells, macrophages use various other overlapping receptors such as scavenger receptors and integrins that function uniquely or in cooperation with each other depending on the exact target cell (116, 118, 126, 127, 130, 133, 138).

Platelets are anucleate cells which normally circulate in a discoid (resting) shape, but play an integral role in maintaining vascular integrity. These small anucleate cells adhere to activated endothelium or underlying basement membrane components and alter their shape and membrane to provide a surface upon which coagulation reactions can occur (32, 33, 183). Regardless of whether a vascular injury occurs in cutaneous tissue or internally, platelets are necessary to generate a stable clot. Within their 8-10 day lifespan, platelets can become activated either in the circulation or during adherence at a site of injury, and in this process they become targeted for destruction by the RES (62). The process of platelet activation involves several changes to the cell surface, such as expression of P-selectin and loss of membrane asymmetry (37, 41, 42). These changes in the platelet membrane may provide molecular signals to macrophages that trigger phagocytosis.

The precise mechanism by which macrophages recognize and phagocytose activated platelets remains to be identified. Circulation studies in

mice and *in vitro* using aged platelets have indicated that platelet clearance occurs independently of platelet-surface P-selectin, but may involve scavenger receptors on macrophages (62, 82, 83). Although the phagocytosis of opsonized or otherwise altered platelets has been well studied, the phagocytosis of freshly isolated, activated, autologous platelets has not been examined. Here we use a completely human *in vitro* system to examine the phagocytosis of fresh autologous platelets by human monocyte-derived macrophages (MDMs). We show here that phagocytosis of fresh autologous activated platelets correlates with platelet activation, yet occurs independently of P-selectin and phosphatidylserine exposure. We also examine the inhibition of platelet phagocytosis by fucoidan and show that inhibition of platelet uptake by polysaccharides is dependent on the sulfate content of the polymer.

MATERIALS AND METHODS

Monocyte-derived Macrophages

Human monocytes were isolated and cultured using techniques similar to those previously described (184, 185). Blood from healthy human donors was collected into citrate and Peripheral Blood Mononuclear Cells (PBMCs) were isolated by using Lymphoprep (Accurate Chemical) according to the manufacturer's instructions. Monocytes were further isolated by plating the PBMCs on gelatin-coated tissue culture flasks for 45min at 37deg followed by 10 washes with PBS to remove non-adherent lymphocytes. Monocytes (>95% purity as assessed by flow cytometry) were then detached from the flasks by

incubation in 10mM EDTA for 2min at 37deg. Monocytes (500ul @ 500,000/ml) were then plated in 24-well plates overnight in RPMI 1640 + 10% FBS + 10ng/ml recombinant human GM-CSF (R&D Systems). Monocytes were plated on glass coverslips for SEM analysis and plastic tissue-culture plates for all other experiments. Media was changed on day 2 and day 5. By day 7, this procedure yields 250,000 cells per well that are >95% CD14+ CD45+ CD68+ CD1a- as assessed by flow cytometry. There were no detectable platelets in the wells on day 7, when the monocyte-derived macrophages (MDMs) were used for co-incubation experiments.

Platelets

Platelets were isolated from whole blood collected in ACD from healthy human donors nd spun 15min at 500g to generate platelet-rich plasma (PRP). PRP was pelleted 10min at 800g at the platelet pellet was washed 2 times in citrated saline (pH 6.8). Some platelet samples were degranulated by incubating 1ml of platelets (250,000/ul in citrated saline) with 10ul of10uM calcium ionophore A21387 (Sigma) for 15min on rocker at room temperature, then washed three times with citrated saline. Platelets were fluorescently labeled with Cell Tracker Green CMFDA (invitrogen) as previously described.(186) After the final wash, platelets were resupsended in warm serum-free RPMI for 15min at 37deg.

For flow cytometric analysis, fresh or ionophore-treated platelets (250,000/ul) were incubated in either citrated saline or serum-free RMPI media

for 1hr at 37deg. A 10ul aliquot was stained with either FITC-anti-CD62P (Biolegend) or FITC-Annexin-V (Biolegend).

Phagocytosis Experiments

Thirty minutes prior to the start of each experiment, the MDMs were washed 3X with PBS and incubated with 500ul fresh RPMI. In some experiments the media was supplemented with 10% autologous human serum. A 25ul aliquot of fluorescently labeled platelets (250,000/ul) was added to each well of macrophages. Platelets and macrophages were co-incubated for 45min, then washed 3 times with PBS to remove free platelets. Warm trypsin-edta was then added to the macrophages and cells were incubated 15min at 37deg. Macrophages were then collected and fixed in 1% cold paraformaldehyde and analyzed by flow cytometry within 1 hour.

Phagocytosis inhibitors

As a broad phagocytosis inhibitor, some wells received latrunculin (1ug/ml final). To inhibit Fc-mediated phagocytosis, macrophages were treated with heat-aggregated IgG (100ug/ml final, aggregated by heating at 65deg for 20min and centrifuging 10min at 14,000g). P-selectin interaction with monocyte/macrophage PSGL-1 was blocked by incubating monocytes/macrophages with 10ug/ml anti-PSGL-1 (clone KPL-1, Biolegend) for 30min prior to the co-incubation experiments. Heparin sulfate, dextran suflate and fucoidan were from Sigma. GlcNac polymers were similar to those

previously described (187) and were kind gifts from Marine Polymer Technologies (Danvers, MA). Stock solutions of polysaccharides were dissolved in serum-free media to final concentrations of 25mg/ml.

RESULTS

Macrophage Phagocytosis of Autologous Platelets

To examine the interaction between human MDMs and autologous platelets, we utilized an *in vitro* co-culture system consisting of 7-day old MDMs to which we added freshly isolated autologous platelets. The use of autologous platelets excludes the possibility that platelet-macrophage interactions are the result of an immune response triggered by the recognition of platelets as 'foreign.' MDMs and platelets were first co-cultured in serum-free RPMI media and examined by SEM and TEM at various time points to visualize the interaction between these two different cell types. As shown in Figure 2-1A, we observed platelets interacting with MDMs within as little as 10min. Free platelets appeared activated as indicated by their irregular shape and extended pseudopodia. At the 40min time point, platelets near the macrophages had become entrapped by a network of macrophage filopodia. Although the macrophages were firmly attached to the coverslip and did not migrate, they appeared to direct groups of filopodia in the direction of nearby platelets that had settled on the dish. Visualization of these cultures suggests that the interaction between human

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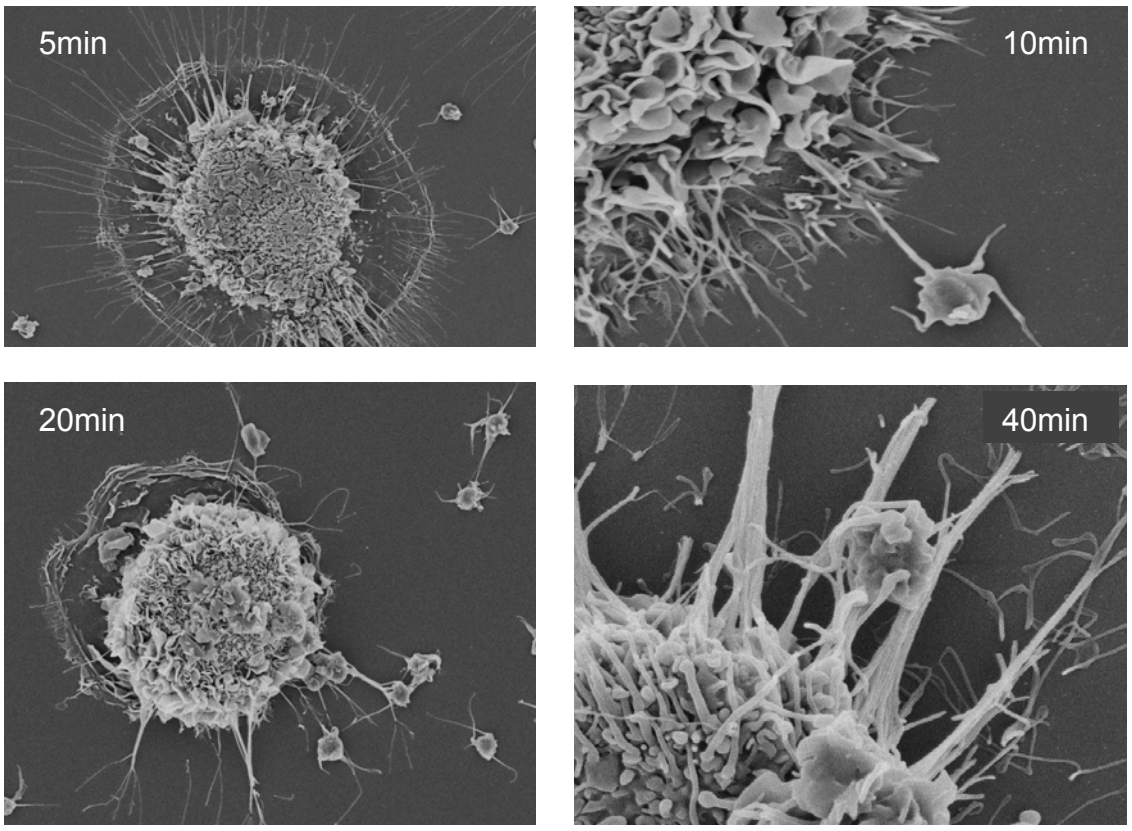

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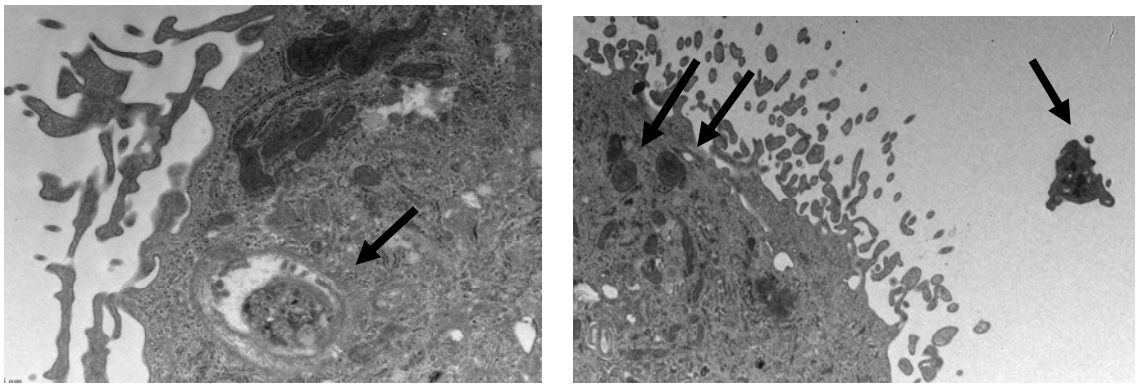

Figure 2-1. ***Electron Microscopy of Platelet-Macrophage Interactions.*** **(A)** MDMs cultured for 7 days on glass coverslips were washed 3 times with PBS and incubated in serum-free media. Freshly isolated platelets were added at a platelet:macrophage ration of 25:1. At various times the cells were washed and fixed for microscopy as described in "Methods." Total co-incubation time is indicated in each panel. Magnification is 7960X (5min), 21590X (10min), 7320X (20min), and 19060X (40min). **(B)** Arrows indicate platelets in TEM samples (30min co-incubation). Magnification is 12,500X (left) and 5,000 (right).

macrophages and activated, autologous platelets occurs *in vitro*, and that it occurs in the absence of additional serum factors. The SEM analysis, in addition to time-lapse confocal microscopy (not shown), revealed that entrapped platelets are drawn towards the macrophages, which suggests that the platelet-macrophage interaction results in phagocytosis.

Platelet phagocytosis was confirmed by TEM as shown in Figure 2-1B. Incubation in serum-free RPMI media did not cause platelet degranulation, and therefore both free and internalized platelets can be identified by their unique granular structure. Internalized platelets appear both outside and within macrophages in this time frame (Figure 2-1B). Most macrophages internalized more than one platelet.

Phagocytosis of autologous platelets was also analyzed using fluorescence microscopy and flow cytometry. Platelets were fluorescently labeled with CMFDA-Cell Tracker Green and co-incubated with MDMs for 45 minutes. As shown in Figure 2-2, we observed uptake of the fluorescent platelets as confirmed by the presence of punctate fluorescence within the macrophages. Adherent, but not internalized platelets were removed by treating the MDMs with trypsin at the end of the co-incubation period. Non-trypsinized cells contain both internalized and adherent platelets (Figure 2-2B), whereas trypsin treatment removed the adherent platelets (Figure 2-2C). As a negative control, and to confirm the role of actin polymerization in phagocytosis, MDMs were pretreated with the actin inhibitor latrunculin (Figure 2-2D). Treatment with latrunculin inhibited phagocytosis, but not binding, demonstrating that the

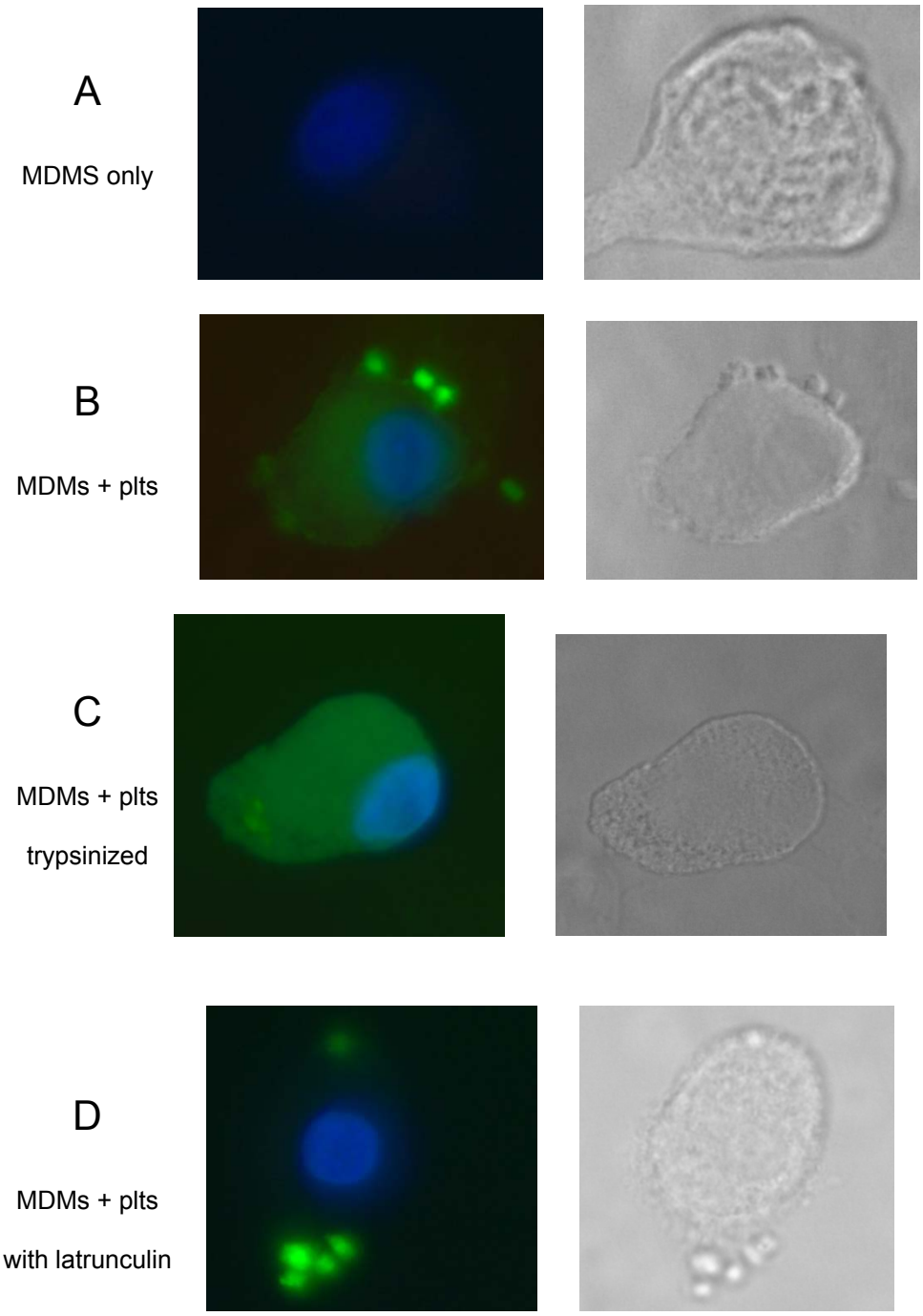

Figure 2-2. ***Analysis of Platelet-Macrophage Interactions by Fluorescence Microscopy.*** Representative cytospin preparations of MDMs co-incubated with platelets (45min in serum-free media) and fixed prior to collection (100X, light microscopy left panels and corresponding fluorescence image in right panels). Culture conditions were MDMs alone **(A)**, MDMs + platelets **(B)**, MDMs + platelets followed by treatment with trypsin **(C)**, MDMs + platelets + latrunculin **(D)**. Green fluorescence indicates the CMFDA label (platelets) and blue indicates DAPI staining (macrophage nucleus).

fluorescent dye is only transferred to the macrophages when the platelets are internalized.

Phagocytosis of autologous platelets occurs independently of phosphatidylserine and P-selectin

Having confirmed phagocytosis of freshly isolated autologous platelets in this *in vitro* system, we next examined the role of platelet activation in this process. When freshly isolated, washed human platelets are incubated in serum-free RPMI media they develop some shape change (see SEM micrographs, Figure 1), which suggests that some platelet activation has occurred in this *in vitro* system. We therefore used flow cytometry to more accurately examine platelet activation status in different culture conditions. Freshly isolated human platelets were incubated in warm serum-free RPMI media for 20min in the presence and absence of thrombin or the calcium ionophore A21387, and compared to fresh (resting) platelets incubated in citrated saline. Platelets were analyzed for expression of P-selectin, an alpha granule component expressed during early platelet activation, and phosphatidylserine, a membrane lipid exposed on the surface of completely (and irreversibly) activated platelets. Incubation in serum-free media alone resulted in a near ten-fold increase in P-selectin expression but did not induce surface expression of phosphatidylserine (Figure 2-3A). In fact, platelets incubated in serum-free RPMI for up to 2 hours did not express levels of phosphatidylserine higher than controls (not shown). Treatment of platelets with either thrombin or calcium ionophore A21387, agents known to cause complete

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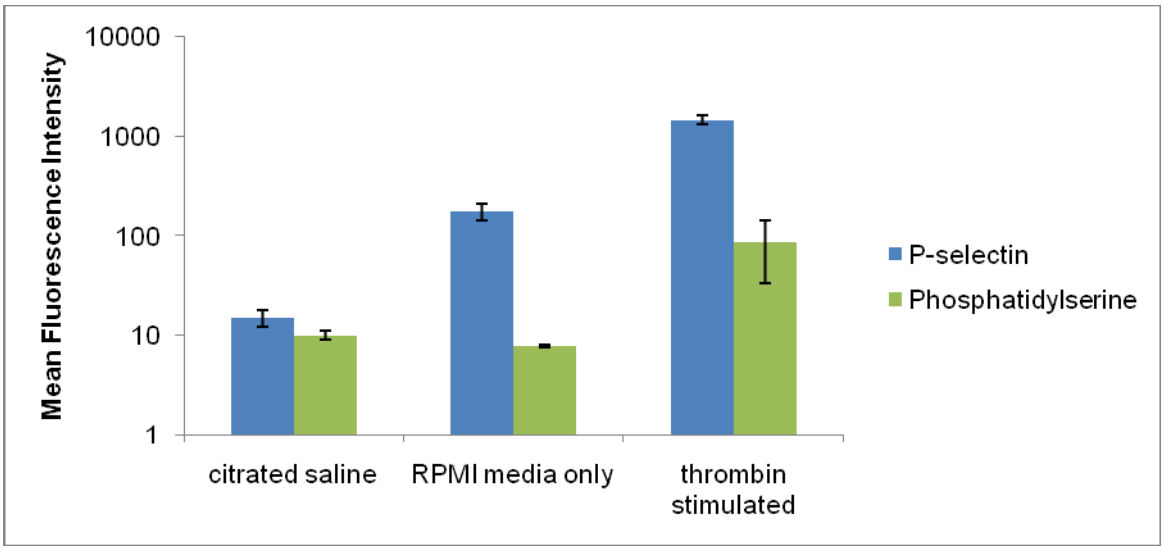

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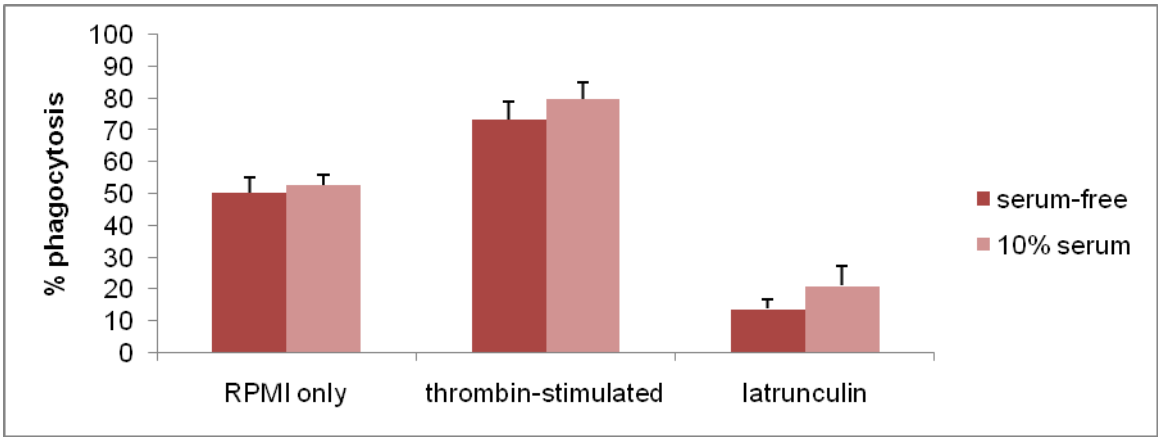

Figure 2-3. ***Flow Cytometry of Platelets and Macrophages*****.**
A. ***Platelet Activation in Culture Media.*** Platelets (1ml at 250,000/ul) were isolated from fresh human PRP and incubated for 1hr at 37deg in either citrated saline, RPMI media, or RPMI + 1 U thrombin. Following the incubation period, a 10ul aliquot of platelets was stained with FITC-anti-CD62P or FITC-annexin-V for 20min at room temp. Cells were then fixed and analyzed immediately by flow cytometry. Three independent platelet preparations were analyzed using unlabeled platelets, as CMFDA labeling is known to have no effect on platelet activation (186).
B. ***Phagocytosis of fresh autologous platelets depends on the platelet activation state, but does not require serum.*** Shown are the average of 5 independent experiments using a different platelet-monocyte donor for each experiment. Phagocytosis was determined by averaging the number of FL1+ macrophages (versus untreated control) in a 10,000 macrophage sample analyzed by flow cytometry.

degranulation and irreversible platelet activation (188), resulted in even higher levels of P-selectin and also increased surface expression of phosphatidylserine (Figure 2-3A).

Because trypsin treatment removes adherent platelets from the macrophages (Figure 2-2C), we were able to quantify phagocytosis by flow cytometry because only the macrophages that have fully internalized platelets generate a fluorescence signal. When freshly isolated platelets were incubated in serum-free RPMI media and added in excess to 7-day old macrophages, 50-60% of the macrophages internalized at least one platelet within 45min (Figure 2-3B). As expected, pretreatment of the MDMs with the actin inhibitor latrunculin almost completely blocked phagocytosis, confirming the role of actin polymerization that occurs in all cases of phagocytosis. The presence of 10% autologous human serum had no significant effect on phagocytosis, which is significant because it excludes the possibility that the platelet-macrophage interaction requires a soluble serum-bound "bridging" molecule, such as IgG, complement, or Gas6. Furthermore, the phagocytosis of platelets incubated only in RPMI media suggests that just mild platelet activation, in the absence of complete degranulation, is sufficient to trigger phagocytosis. Additionally, our flow cytometry results indicate that phagocytosis of autologous platelets occurs in the absence of phosphatidylserine exposure. Although phagocytosis was enhanced when the platelets did express phosphatidylserine, we conclude that surface exposure of phosphatidylserine is not an absolute requirement for phagocytosis of platelets.

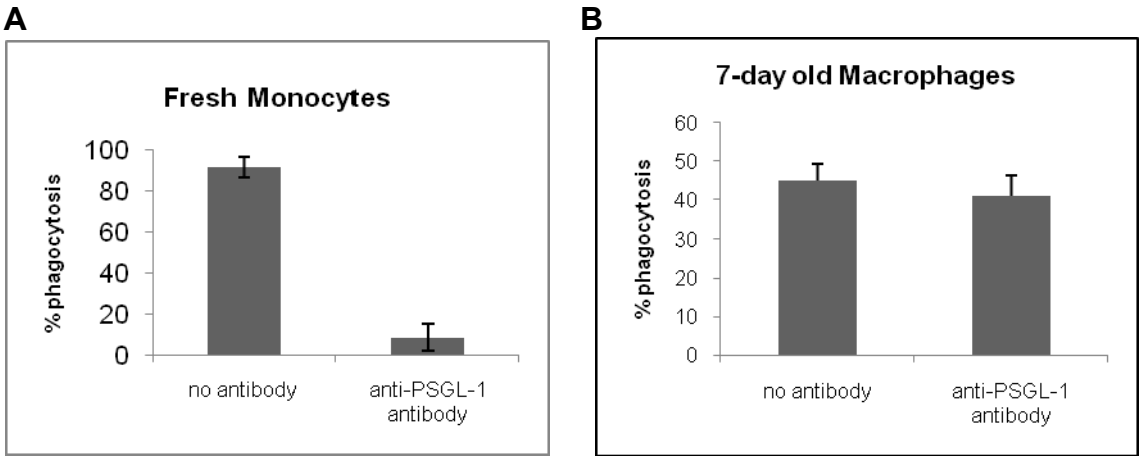

Figure 2-4. ***Phagocytosis of fresh autologous platelets by macrophages is P-selectin independent.*** Fresh monocytes or 7-day old MDMs were pre-incubated with a PSGL-1 blocking antibody (clone KPL-1, 1ug/ml final) for 30min at 37deg prior to incubation with fresh platelets for 1 hour. Monocytes were then fixed with 1% paraformaldehyde and analyzed immediately by flow cytometry (10,000 counts collect). Macrophages were treated with trypsin to remove adherent platelets, fixed in 1% paraformaldehyde and analyzed immediately by flow cytometry.

The amount of phagocytosis increased if the platelets were pre-stimulated with either calcium ionophore A23187 (not shown) or thrombin, suggesting that phagocytosis of platelets correlates with platelet activation. We next specifically tested the role of P-selectin in regulating phagocytosis because platelet uptake correlated with expression of P-selectin (Figure 2-3). As a comparative control, we used fresh autologous monocytes, which are known to bind activated platelets via interactions between P-Selectin on platelets and P-Selectin Glycoprotien-1 (PSGL-1) on monocytes (189). Fresh monocytes or 7-day old MDMs were pre-incubated with a PSGL-1 blocking antibody prior to incubation with fresh platelets. We chose to block the receptor on the macrophages, rather than P-selectin on the platelets, because the PSGL-1 blocking antibody is extremely effective in blocking this interaction, and because treating the platelets with an anti-P-selectin antibody could result in platelet opsonization, predisposing them to Fc-mediated uptake. As shown in Figure 2-4, the PSGL-1 blocking antibody inhibited the formation of platelet-monocyte complexes by almost 90%, but had no effect on platelet phagocytosis by 7-day old macrophages. These results suggest that phagocytosis of autologous activated platelets occurs independently of platelet P-selectin. Additionally, the interaction between platelet P-selectin and leukocyte PSGL-1 may only be significant for circumstances involving circulating monocytes, but not for differentiated macrophages.

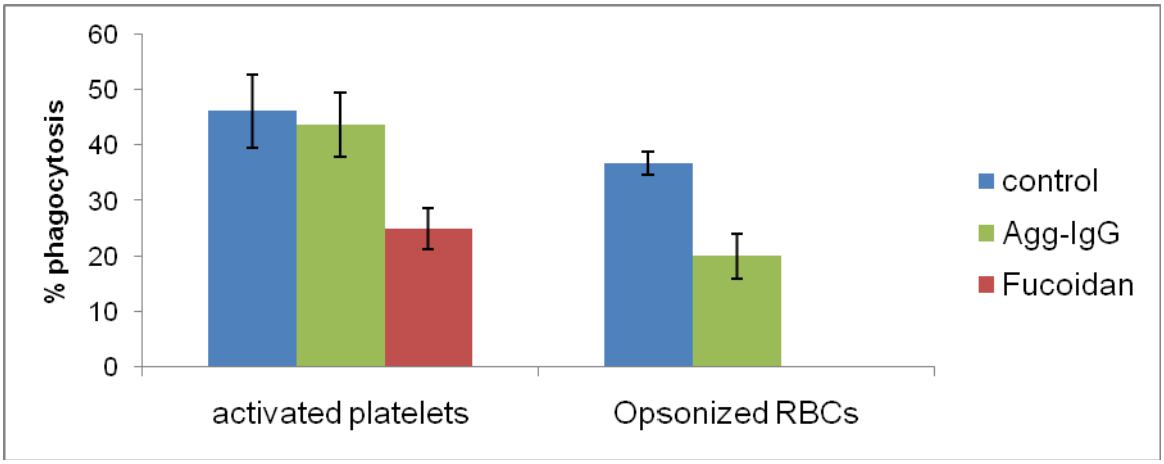

Figure 2-5. ***Platelet Uptake is Inhibited by Fucoidan, but not by Agg-IgG***. MDMs were washed and incubated with serum-free RPMI alone, RPMI + heat-aggregated IgG (1mg/ml), or RPMI + Fucoidan (250ug/ml) for 30min prior to the addition of CMFDA-platelets or CMFDA-opsonized-RBCs . After 45min co-incubation, cells were treated with trypsin, collected, and analyzed for fluorescence by flow cytometry. Shown are the average number of FL1+ macrophages in a given 10,000 macrophage sample for 3 independent experiments.

Phagocytosis of fresh platelets occurs independently of Fc-receptors, but may involve Scavenger Receptors

We next investigated the role of two common macrophage phagocytosis mechanisms: those mediated by Fc-Receptors and those mediated by Scavenger-Receptors. Activated platelets secrete IgG from their granules and contain surface-bound IgG which could be recognized by macrophage Fc-receptors (190). To test this possibility, macrophage Fc-receptors were blocked by treating the cells with heat-aggregated IgG, which down regulates Fc-mediated phagocytosis (107, 191). This treatment inhibited uptake of opsonized RBCs, but did not affect phagocytosis of freshly isolated platelets or degranulated platelets (Figure 2-5). These results suggest that macrophage phagocytosis of autologous platelets is not an Fc-mediated process.

Fucoidan is a sulfated polysaccharide and a known Scavenger Receptor ligand (192, 193). Incubation of macrophages with fucoidan inhibited platelet uptake by approximately 50% (Figure 2-5). This suggests that macrophage phagocytosis of autologous platelets may be mediated by type A Scavenger Receptors.

Platelet uptake is inhibited by sulfated polysaccharides

In addition to fucoidan, there are several other polysaccharides which exert varying effects on blood and platelets, although their specific interactions with macrophage phagocytosis receptors are less clear. We tested dextran sulfate and heparin sulfate for their ability to inhibit platelet phagocytosis. All

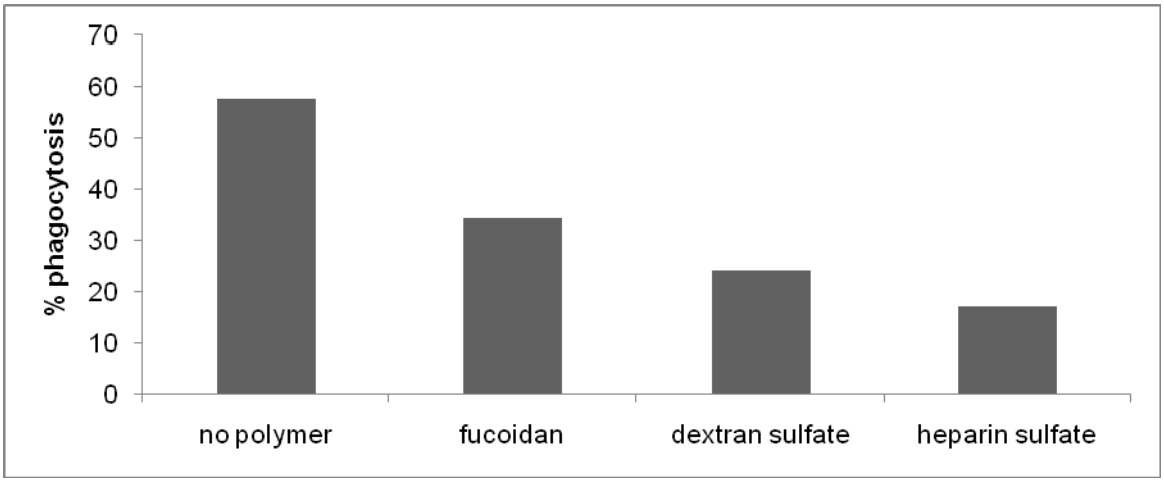

Figure 2-6. ***Inhibition of Platelet Phagocytosis by sulfated polysaccharides***. MDMs were washed with PBS and pre-treated with serum-free RPMI + 250ug/ml of either fucoidan, dextran sulfate, or heparin sulfate. After 30min of pre-treatment, an excess of freshly isolated activated autologous CMFDA-labeled platelets were added and the system incubated for an additional 45min. Macrophages were then analyzed by flow cytometry as described above.

three compounds are essentially composed of linked sugar residues which contain with various degrees of sulfate moieties attached to the sugars. Addition of equivalent amounts of fucoidan, dextran sulfate, and heparin sulfate to the *in vitro* system all resulted in significant inhibition of platelet phagocytosis (Figure 2-6). Heparin sulfate had the most profound effect on platelet phagocytosis, inhibiting phagocytosis by over 70%. An equivalent amount of the non-sulfated polysaccharide glycogen had no significant effect on platelet phagocytosis, which suggests that sulfation is a key component to the inhibition of phagocytosis by these polymers. To test the hypothesis that sulfate is a required component for inhibition of platelet uptake by polysaccharides, polymers of n-acetylglucosamine (GlcNac) were prepared with various degrees of sulfation, and were tested in comparison to polymers of the same molecular weight but having the sulfate moiety replaced by carboxylation or deacetylation. Only sulfated polymers were effective in inhibiting platelet phagocytosis, whereas the carboxylated and deacetylated polymers had no effect (Figure 2-7). These results suggest that polysaccharides only inhibit platelet phagocytosis if the polymer is sulfated, and that increased degree of sulfation leads to a stronger inhibitory effect of the polymer.

Because the sulfated polymers could be interacting with either the macrophages alone, the platelets alone, or both, we performed additional experiments to test the hypothesis that sulfated polysaccharides are interacting with macrophage receptors. When the macrophages were pretreated with polysaccharides, and then washed just prior to adding platelets to the system,

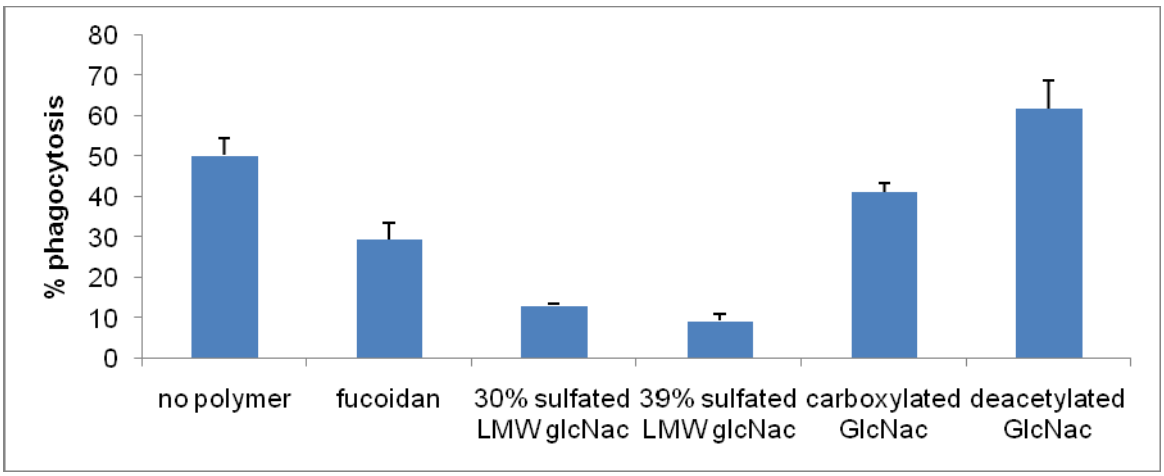

Figure 2-7. ***Effect of polymer sulfation on inhibition of platelet phagocytosis.*** MDMs were washed with PBS and pre-treated with serum-free RPMI + 250ug/ml of polymer. After 30min of pre-treatment, an excess of freshly isolated activated autologous CMFDA-labeled platelets were added and the system incubated for an additional 45min. Macrophages were then analyzed by flow cytometry as described above. Shown is the average of three independent experiments.

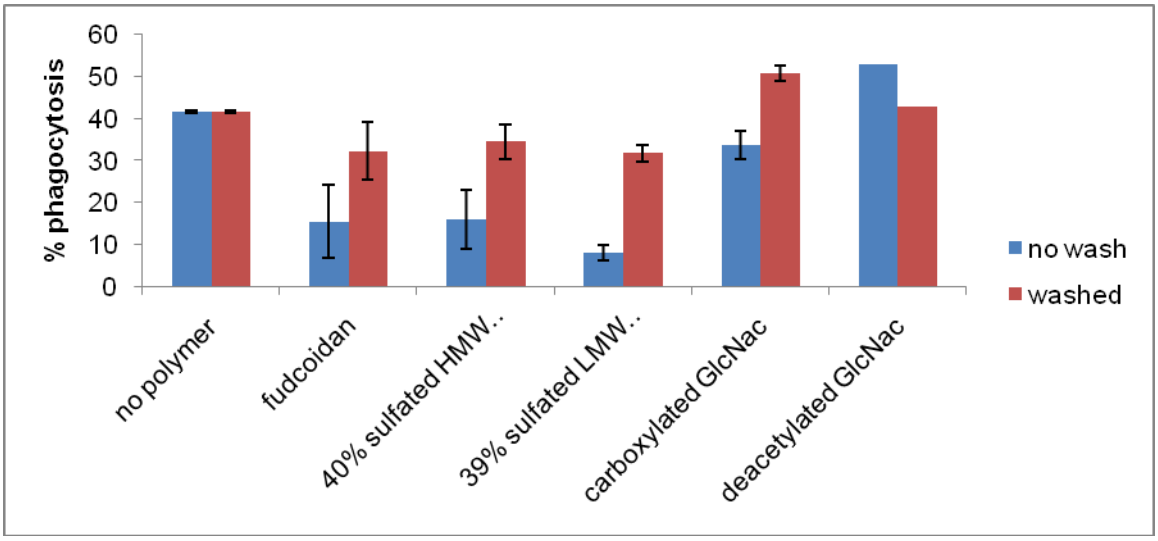

Figure 2-8. ***Effect of washing with polymer pre-treatment of MDMs.*** MDMs were washed with PBS and pre-treated with serum-free RPMI + 250ug/ml of polymer. Then, in half of the samples (shown in red), the macrophages were washed again 3X with PBS prior to adding the platelets. An excess of freshly isolated activated autologous CMFDA-labeled platelets were added and the system incubated for an additional 45min. Macrophages were then analyzed by flow cytometry as described above. Shown is the average of three independent experiments.

the inhibitory effect on phagocytosis was largely lost (Figure 2-8). However, flow cytometry experiments using FITC-labeled polysaccharides demonstrated that with polymers, washed, and then added to the macrophages (Figure 2-9). These results suggest that the polysaccharides must be present during the co- the FITC-polymers can bind to both macrophages and platelets (not shown). Phagocytosis was also not significantly affected if the platelets were pre-treated incubation in order to inhibit platelet uptake. The sulfated polysaccharides can be removed from both platelets and macrophages by vigorous washing.

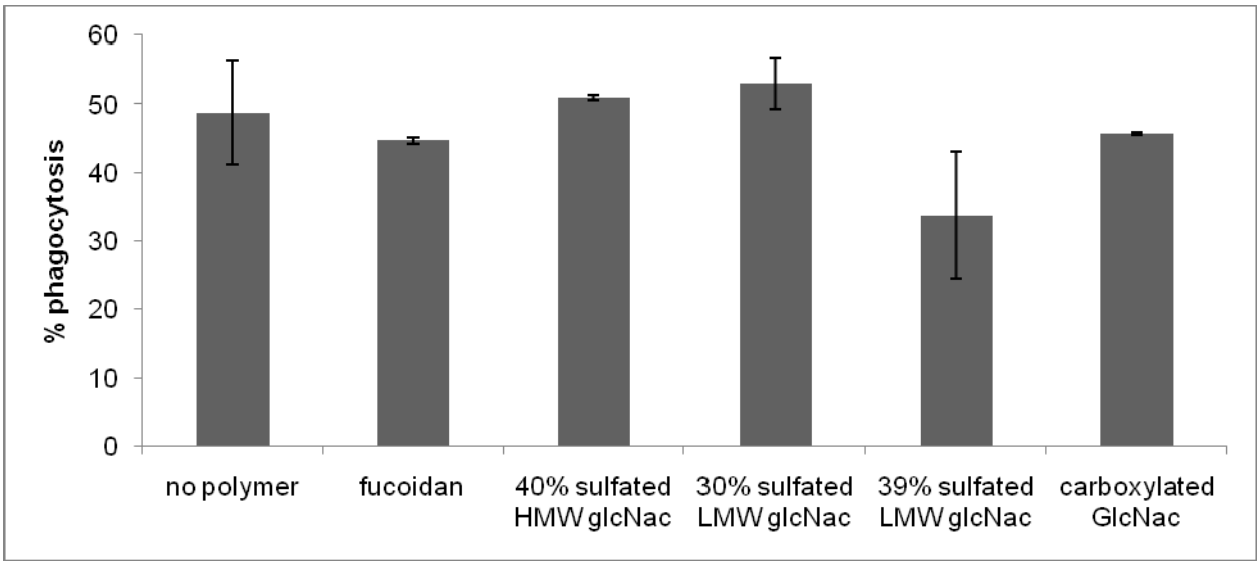

Figure 2-9. ***Pre-treatment of platelets with sulfated polysaccharides.*** Activated CMFDA-labeled platelets were incubated for 30min with polymers (250ug/ml) and then washed 3 times with citrated saline before adding to the macrophages. The phagocytosis assay was then performed as described above. Shown are the average of 3 independent experiments.

DISCUSSION

Phagocytosis is an important means of clearing both immunologically compromised cells as well as apoptotic cells. Monocyte-derived macrophages are efficient phagocytes in organs of the RES and within injured tissues. However, the process of platelet clearance by macrophages is poorly understood.

Platelets contribute to the maintenance of tissue homeostasis throughout the body, and in response to injury or senescence they become activated. During platelet activation, several changes in the cell membrane occur which may lead to recognition and clearance by macrophages. We therefore sought to determine the role of several common receptors and ligands that might be involved in this process. This work presents the first study examining the interaction between human monocyte-derived macrophages and fresh, autologous, activated platelets.

We have demonstrated phagocytosis of fresh autologous, activated, platelets by monocyte-derived macrophages using an entirely human *in vitro* system. Uptake of freshly isolated platelets is dependent on actin polymerization, but occurs independently of any soluble serum factors. Additionally, phagocytosis of platelets is enhanced with platelet activation.

Because phagocytosis correlates with platelet activation, we would expect no phagocytosis to occur in the presence of quiescent platelets. However, the use of RPMI culture media prevents such experiments from being done because RPMI media alone causes platelet activation (Figure 2-3A), probably due to the

presence of calcium and phosphate. Additionally, the use of platelet inhibitors such as EDTA or prostaglandin, which may have maintained the platelets in a resting state, also could not be used because they would also affect macrophage function. Thus, one disadvantage of our system is that the platelet-macrophage interaction using activated platelets could not be compared to an interaction in which the platelets were in a truly resting state. The interaction involving activated platelets, therefore, remained the focus of this work.

The fact that phosphatidylsine exposure is not a requirement for phagocytosis of activated platelets is somewhat surprising, because it is a well documented 'eat me' signal for the phagocytosis of many cells (194-197). Examples of phosphatidylserin-independent uptake of apoptotic cells have been reported, but are rare (198).

Although P-selectin binding to PSGL-1 is critical for platelet-monocyte interactions, we have shown that it does not play a role in platelet interactions with monocyte-derived macrophages (Figure 2-4). Our findings are supported by additional *in vivo* work that has shown that clearance of infused activated platelets in mice occurs independently of P-selectin (82). Interestingly, recent studies have demonstrated that platelets can be phagocytosed by resting neutrophils, but the process does involve phosphatidylserine and P-selectin (199).

Other work on platelet phagocytosis has focused on modified platelets such as chilled platelets, opsonized platelets, and aged platelets (59, 63-65, 72-74, 77), each of which involves distinct changes to the platelet surface. Aged

platelets most closely resemble freshly activated platelets because during aging platelets increase expression of phosphatidylserine and P-selectin (62). Interestingly, Brown et al. have shown *in vitro* that phagocytosis of aged platelets is not inhibited by P-selectin antibodies or Phospho-L-serine (62). Together these studies suggest that macrophages may recognize freshly activated platelets in the same way that they clear aged platelets.

Macrophages have evolved to use many surface receptors for phagocytosis such as complement receptors, Fc-receptors, scavenger receptors and integrins. We have shown here that platelet phagocytosis is neither complement, nor Fc-mediated (Figures 2-3 and 2-4). We have also shown inhibition of platelet phagocytosis by the scavenger receptor ligand fucoidan. However, the effect was largely lost when the fucoidan was washed out of the system. One possible explanation for this effect is that fucoidan binds to macrophage receptors, but with such a low affinity that the vigorous washing can disrupt the interaction. Other studies utilizing fucoidan to inhibit scavenger-receptor phagocytosis did not wash out the fucoidan prior to the phagocytosis assay (62).

We also observed a significant decrease in platelet phagocytosis when the macrophages were treated with dextran sulfate and heparin sulfate, which are ligands of different forms of scavenger receptors. Interestingly, the polysaccharides heparin sulfate and dextran sulfate have been associated with thrombocytopenia, in which a complex between the polymer and surface-bound Platelet Factor 4 is recognized by autoantibodies (70). Our results, however,

suggest that sulfated polysaccharides actually inhibit platelet phagocytosis, at least in the absence of antibodies against heparin-PF4. Furthermore, fucoidan is the only polymer of the three that is a known ligand of macrophage scavenger receptors, which supports the possibility that there are other receptors, or a combination of receptors, involved in the recognition and uptake of fresh, autologous, activated platelets.

We have shown that inhibition of platelet phagocytosis by sulfated polysaccharides also correlates with the degree of sulfation of the polysaccharide. Although flow cytometry analysis suggested that both platelet and macrophages can interact with sulfated polymers (not shown), the effect of washes after polymer treatment suggests that the interaction is not particularly stable. Sulfated polysaccharides such as fucoidan have also been shown to act as anticoagulants (200). The interaction of sulfated polysaccharides with both platelets and macrophages warrants further study.

Although a specific macrophage receptor and platelet ligand involved in platelet phagocytosis remain to be identified, the system presented here exhibits a completely human *in vitro* representation of the phagocytosis of fresh, autologous, activated platelets. We have demonstrated for the first time that phagocytosis of freshly isolated autologous platelets by MDMs occurs independently of phosphatidylersine and P-selectin. Additionally, we have shown that uptake of fresh activated platelets is inhibited by sulfated polysaccharides and may involve macrophage scavenger receptors. Future studies using this system may shed additional light on the mechanism of platelet phagocytosis as

well as identify novel polymers that can prolong platelet survival by inhibiting phagocytosis.

CHAPTER III

PHAGOCYTOSIS OF AUTOLOGOUS PLATELETS ENHANCES INFLAMMATORY RESPONSES BY HUMAN MACROPHAGES

INTRODUCTION

Macrophages are critical effector cells that regulate both normal and pathological inflammatory processes through cytokine production. During normal inflammatory processes, such as wound healing, the interaction of macrophages with damaged tissue and cells stimulates secretion of pro-inflammatory cytokines (20, 175, 201). Some of these macrophage-derived cytokines, such as TNF-α, IL-6, and IL-23, work to destroy microbial pathogens, amplify the macrophage response, or recruit additional leukocyte subsets (202, 203). As these processes occur, the inflammatory stimuli dissipate and macrophages begin to secrete different cytokines, such as IL-10 and TGF-β, which dampen the pro-inflammatory response and stimulate angiogenesis(51, 152, 202). The changing cytokine expression during normal inflammatory processes is a result of the changes in inflammatory stimuli detected by macrophages(204, 205).

In pathological settings such as diabetic ulcers and atherosclerotic plaques, macrophage cytokine production is improperly regulated and inflammatory stimuli are not adequately eliminated (8, 10, 54, 206). Macrophages in these settings produce an excess of pro-inflammatory cytokines,

which drives additional tissue injury and prevents resolution of the inflammation. The underlying mechanisms that explain why some inflammatory reactions resolve normally, and others do not, is an area of active investigation.

Several recent studies have focused on the interaction of macrophages with other cells within the inflammatory microenvironment. A major function of macrophages at sites of inflammation is to remove other cells from the lesion by phagocytosis, which can have profound effects on cytokine production by macrophages (144, 174). The response by macrophages following phagocytosis can vary depending on the condition of the cell being cleared (136, 142, 147). The present work is focused on the macrophage interaction with either apoptotic cells or platelets, both of which are found in a wide range of inflammatory settings.

The macrophage response following phagocytosis of apoptotic cells is immunosuppressive (105, 114, 144, 173). Macrophage phagocytosis of apoptotic cells down regulates pro-inflammatory cytokines such as IL-6 and IL-12 (114, 115, 143). It has also been shown in LPS-activated human macrophages that phagocytosis of apoptotic cells inhibits secretion of IL-1β, IL-8, and TNF-alpha (142). This process of immunosuppresion occurs in conjunction with increased expression of anti-inflammatory cytokines, such as IL-10, and growth factors such as TGF-β (207). The phagocytosis of apoptotic cells during wound healing is often considered a major turning point from the inflammatory phase of wound healing to the remodeling phase that involves angiogenesis.

Platelets, on the other hand, are an often overlooked part of the inflammatory milieu. Indeed, the primary function of platelets is to adhere to sites of injury and aggregate so that the coagulation cascade can propagate. However, the formation of a platelet-rich clot culminates in the release of many cytokines and growth factors which have profound effects on inflammation (48-51). Platelets remain at the wound site until the clot is separated from the skin (in the case of cutaneous wound healing) or components of the clot are cleared by phagocytes (208, 209). Phagocytosis of activated platelets occurs during the macrophage response to injury and also within inflammatory lesions such as atherosclerotic plaques and intestinal lesions of colitis patients (40, 54, 140, 154, 210).

It is unknown if the inflammatory effects of platelet phagocytosis are the same as the effects of phagocytosis of apoptotic cells. Activated platelets express CD40L, a pro-inflammatory molecule, and phosphatidylserine, an anti-inflammatory lipid that is also found on apoptotic cells (40-42). Although circulating platelets can exert a pro-inflammatory on circulating monocytes (47, 86), their effect on differentiated macrophages, particularly at sites of inflammation and repair, is not clear.

We show here that in human monocyte-derived macrophages, co-incubation with autologous platelets results in an inflammatory profile that is opposite to the macrophage response following phagocytosis of apoptotic cells. Platelets failed to induce the wound healing protein TGF-β, but enhanced secretion of pro-inflammatory cytokines TNF-alpha, IL-6, and IL-23.

Furthermore, we show that enhancement of cytokine levels is CD40L dependent and can be reversed if the platelets are loaded with the glucocorticoid dexamethasone. These results have wide ranging implications for both normal and pathological inflammation.

METHODS

Cells

To prepare MDMs, blood from healthy human donors was collected into citrate and Peripheral Blood Mononuclear Cells (PBMCs) were isolated by using Lymphoprep (Accurate Chemical) according to the manufacturer's instructions. Monocytes were further isolated by plating the PBMCs on gelatin-coated tissue culture flasks for 45min at 37deg followed by 10 washes with PBS to remove non-adherent lymphocytes. Monocytes (>95% purity as assessed by flow cytometry) were then detached from the flasks by incubation in 10mM EDTA for 2min at 37deg. Monocytes (500ul @ 500,000/ml) were then plated in 24-well plates overnight in RPMI 1640 + 10% FBS. On Day 2, fresh media + 10ng/ml recombinant human GM-CSF was added. Media was changed again on day 5. By day 7, this procedure yields 250,000 cells per well that are >95% CD14+ CD45+ CD68+ CD1a- as assessed by flow cytometry. There were no detectable platelets in the wells on day 7, when the monocyte-derived macrophages (MDMs) were used for co-incubation experiments.

To prepare platelets, blood from healthy human donors was collected into ACD and spun 15min at 500g to generate platelet-rich plasma (PRP). PRP was

pelleted 10min at 800g at the platelet pellet was washed 2 times in citrated saline (pH 6.8). Platelets were activated by resuspending in warm serum-free RPMI media for 15min at 37deg. For degranulated platelets, 1ml of platelets (250,000/ul) were incubated with 10ul of calcium ionophore A21387 (Sigma) for 15min on rocker at room temperature, then washed three times with citrated saline. Dexamethasone-loaded platelets were prepared by incubated 1ml of platelets (250,000/ul) in citrated saline with 5ul of dexamethasone (10mM in DMSO) for 15min on rocker at room temperature. Platelets were then washed three times with citrated saline to remove unbound dexamethasone.

To prepare apoptotic cells, PBMCs were isolated as above, and following monocyte-adherence to gelatin-coated flasks the non-adherent lymphocytes were collected. These cells were >99% CD14 positive as measured by flow cytometry. Cells were rendered apoptotic (Annexin-V positive, Propidium Iodide negative) by UV-irradiation for 10min followed by overnight incubation in RPMI + 10% FBS at 37deg + 5% CO2.

Co-incubation Experiments

Each well of MDMs was washed 3 times with PBS and incubated with fresh RPMI + 10% autologous human serum. Activated, degranulated, or dexamethasone-loaded platelets ($5x10^6$) were added to each well. Some wells also received LPS (100ng/ml). Cells that were treated with dexamethasone alone received an equivalent volume of dexamethasone as was added for platelet-loading.

After 24hrs, supernatants were collected, spun 10min at 14,000g, and frozen at -80deg. Cytokines were measured by ELISA using capture and detection antibodies (eBioscience) per the manufacturer's instructions. Cytokines were measured in duplicate and averaged.

In some experiments, the amount of protein secreted was normalized to the amount secreted by macrophages treated with LPS alone. Each experiment was performed 5 times using 5 different MDM donors. In each experiment, the platelets added were from the same donor as the MDMs.

RESULTS

Platelets do not induce cytokine secretion in resting macrophages

The precise culture conditions of macrophages *in vitro* can impact potential cytokine production, and resting macrophages usually require direct stimulation in order to produce detectable levels of any cytokine. To determine if platelets could provide an effective stimulus for cytokine production in resting macrophages, we added freshly isolated platelets to 7-day old autologous MDMs and co-cultured them for 24hrs. We then analyzed the supernatants for levels of TNF-α, IL-6, IL-23, and TGF-β and compared them to untreated macrophages and macrophages activated with LPS.

As shown in Table 3-1, none of the analyzed cytokines were produced by untreated macrophages. LPS stimulation, although variable between donors,

induced robust amounts of the pro-inflammatory cytokines TNF-α, IL-6, and IL-23. Co-incubation with platelets failed to induce secretion of any of the tested cytokines. Co-culture with apoptotic cells also failed to induce pro-inflammatory cytokine secretion, but did result in secretion of the wound healing growth factor TGF-β. These results suggest that under resting conditions *in vitro,* both platelets and apoptotic cells are relatively inert, and they are incapable of inducing an inflammatory response on their own.

Platelets enhance LPS-induced macrophage activation

Having analyzed the baseline activation of our resting MDM cultures, we next measured the effects of platelets on activated macrophages. We activated macrophages with LPS, which activates a variety of overlapping intracellular signaling pathways that are involved in the pro-inflammatory gene expression that occurs during wound healing (211). Two different types of platelets were used in the co-culture experiments: activated and degranulated. Activated platelets, prepared by incubating platelets in serum-free RPMI medium, are representative of circulating activated platelets and express markers of surface activation such as P-selectin and CD40L. Degranulated platelets, prepared by stimulation with the calcium ionophore A21387, are completely and irreversibly activated and maintain surface exposure of phosphatidylserine, in addition to P-selectin and CD40L.

The pro-inflammatory cytokines TNF-α, IL-6, and IL-23 were measured in macrophage supernatants after stimulation with LPS for 24hrs and co-culture with either apoptotic cells or autologous platelets. We have previously used this

	TNF-α	IL-6	IL-23	TGF-β
PLATELETS	ND	ND	ND	ND
APOPTOTIC CELLS	ND	ND	ND	120 +/- 78
LPS CONTROL	3682 +/- 1116	3253 +/- 1312	266 +/- 168	not analyzed
UNTREATED CONTROL	ND	ND	ND	ND

Table 3-1. ***Cytokine Expression in Resting Macrophages*.** Human MDMs ($2.5x10^5$) were co-incubated with autologous platelets ($5x10^6$) , apoptotic lymphoctyes ($5x10^6$) , or LPS (1ug/ml) for 24hrs in RPMI media + 10% autologous human serum. Cytokines were measured by ELISA and values are expressed in pg/ml. Results are the average of at least 3 independent experiments using different human donors. ND = none detected.

co-culture system and verified that the conditions described here are conducive to phagocytosis of platelets or apoptotic cells by macrophages. In order to account for inter-donor variability, in each experiment the cytokine levels were normalized to the amount of cytokine produced by LPS stimulation alone.

When compared to LPS stimulation alone, macrophage co-incubation with apoptotic cells inhibited LPS-induced secretion of all three pro-inflammatory cytokines (Figure 3-1). However, co-incubation with activated platelets enhanced macrophage secretion of TNF-α, IL-6, and IL-23 (Figure 3-1). Induction of pro-inflammatory cytokines in the presence of platelets was 20-60% higher than the levels obtained by LPS treatment alone. Furthermore, the macrophage cytokine secretion was enhanced to a similar degree after co-incubation with both types of platelets. These data suggest that both partially activated platelets (P-selectin and CD40L positive) and completely degranulated platelets (P-selectin, CD40L, and Phosphotidylserine positive) enhance LPS-induced macrophage activation. Platelet can therefore exert a pro-inflammatory effect on activated macrophages even after complete activation, which is a process that has been compared to apoptosis.

CD40L antibodies reduce platelet-dependent effects on LPS-activated macrophages

Activated platelets express CD40L, a known pro-inflammatory molecule that is known stimulate macrophages via their CD40 receptors. Therefore, we

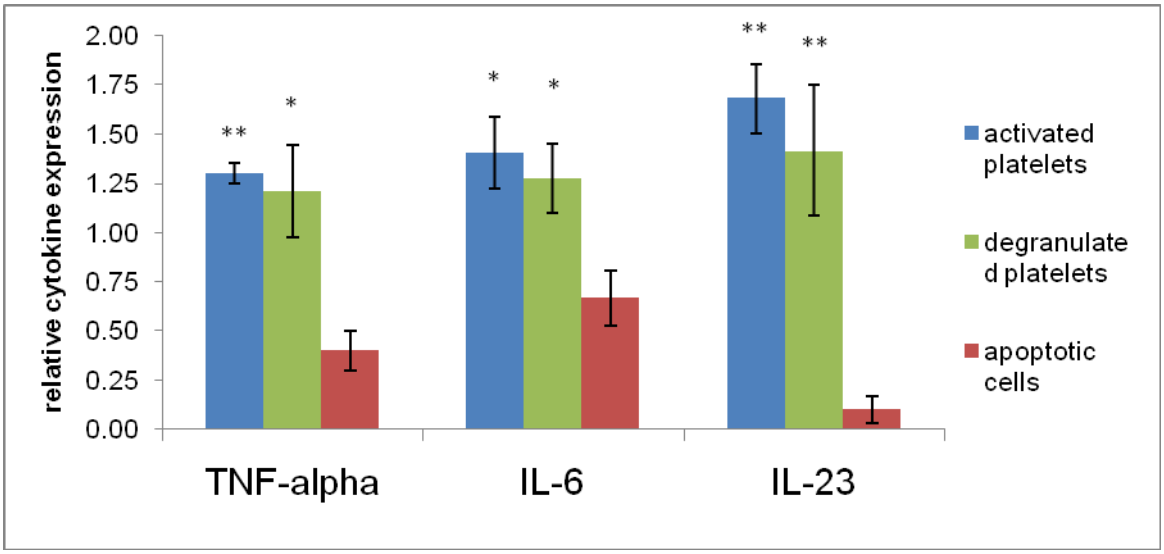

Figure 3-1. ***Platelets enhance, while apoptotic cells inhibit, LPS-induced cytokine secretion.*** Human MDMs were co-incubated with $5x10^6$ autologous platelets or apoptotic lymphocytes in the presence of 10% autologous human serum and 1ug/ml LPS. Cytokine levels were measured in the culture supernatant after 24hrs. In each experiment, cytokines were measured by ELISA and normalized to the amount of cytokine secreted by macrophages treated with LPS only (relative expression = 1). Shown are the average of 5 independent experiments. Asterisks indicate instances in which the relative cytokine expression from platelet co-incubation reached a statistically significant difference from co-incubation with apoptotic cells (Student's t-test, (*) = $p < 0.05$, (**) = $p < 0.0001$).

tested the hypothesis that CD40L on the platelet surface is responsible for the enhanced macrophage cytokine secretion observed during co-incubation with platelets. Fresh platelets were opsonized with either a control antibody (recognizing platelet CD42b) or an anti-CD40L blocking antibody. Although we did not observe enhanced IL-6 expression in the presence of anti-CD42-opsonized platelets, these control platelets did enhance macrophage secretion of TNF-α and IL-23 in a manner similar to activated platelets (Figure 3-2). The enhanced secretion of TNF-α and IL-23 was reduced when the platelets were opsonized with the CD40L-blocking antibody, and relative cytokine levels were similar to those obtained by treatment with LPS alone (Figure 3-2). These results suggest that CD40-CD40L signaling may be responsible for the enhancement of LPS-induced macrophage activation by platelets. In the absence of CD40-CD40L signaling, platelets do not have a significant impact on LPS-induced cytokine production.

Glucocorticoids reverse the platelet effect on macrophages

Although the platelet effect on macrophage cytokine expression may be a necessary part of normal wound healing, enhancement of macrophage activation by platelets might be detrimental to the resolution of inflammation. Enhancement of macrophage cytokine expression might be particularly counterproductive in inflammatory lesions where excessive inflammation is causing further tissue destruction and inflammation, such as in diabetic ulcers and atherosclerotic plaques. We therefore sought a treatment that would not

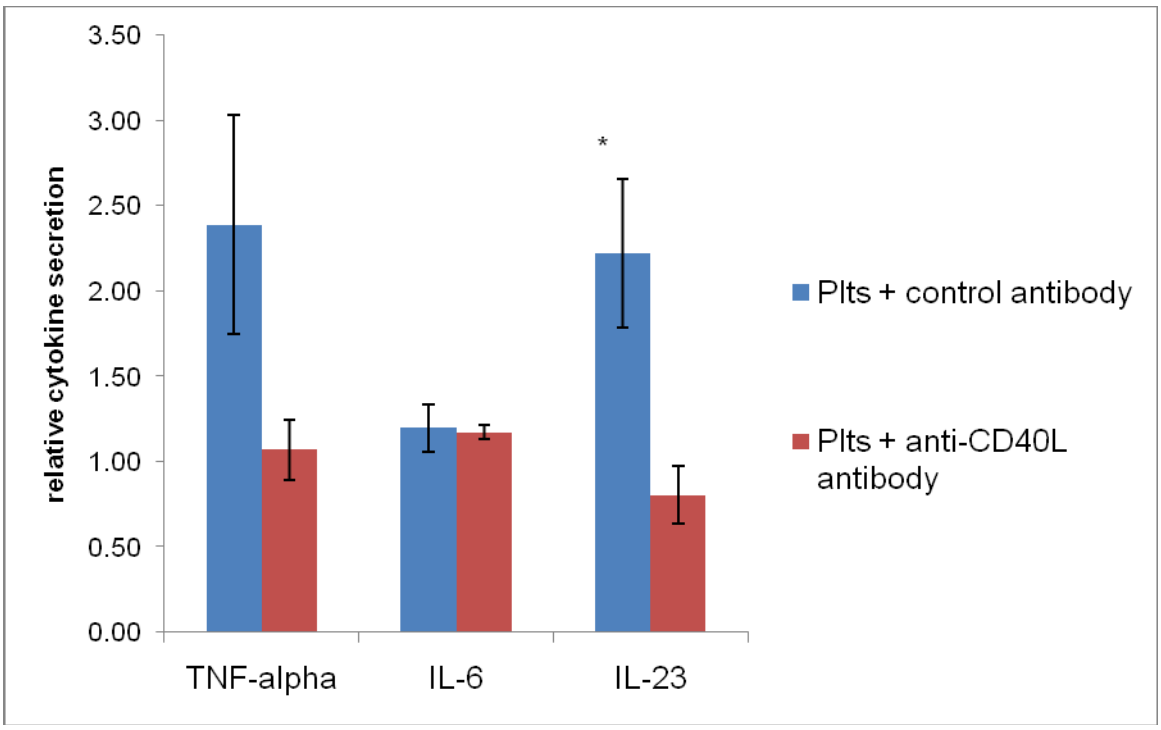

Figure 3-2. ***CD40L blockade reduces platelet enhancement of LPS-induced macrophage cytokine secretion.*** Fresh platelets (100ul @ 25,000/ul) were incubated in serum-free RPMI medium with 1ug of anti-CD42b or anti-CD40L antibody for 20min at 37deg prior to being added to 7-day old MDMs with 1ug/ml LPS. Supernatants were collected 24hrs later, cytokines measured by ELISA and values normalized to the amount of cytokine produced by LPS treatment alone (relative expression = 1). Shown are the average of 4 independent experiments. In each experiments, freshly isolated autologous platelets were used. Asterisk (*) indicates a statistically significant difference between control platelets and anti-CD40L platelets ($p < 0.05$).

only neutralize the platelet effect, but actually reverse it. In effect, we sought a method for rendering platelets to be more anti-inflammatory, much like apoptotic cells.

Glucocorticoids have powerful anti-inflammatory and immunosuppressive effects on macrophages. Based on the knowledge that platelets can bind glucocorticoids via glucocorticoid receptors (212), we tested the hypothesis that glucocorticoid-bound platelets would be less inflammatory than normal activated platelets. Pilot studies were performed to determine the kinetics and saturating concentration of dexamethasone loading into platelets (not shown). Dexamethasone-loaded platelets, from which excess dexamethasone had been washed, were added to LPS-activated macrophages in the same system as above. As shown in Figure 3-3, the levels of cytokines produced after co-culture with dexamethasone-loaded platelets were inhibited to 30-50% of the levels produced by stimulation with LPS alone. These results demonstrate that the effect of dexamethasone loading on the ability of platelets to enhance cytokine secretion goes beyond neutralization of the platelet effect. Rather, the dexamethasone-loaded platelets actually inhibit the LPS-induced cytokine production by macrophages.

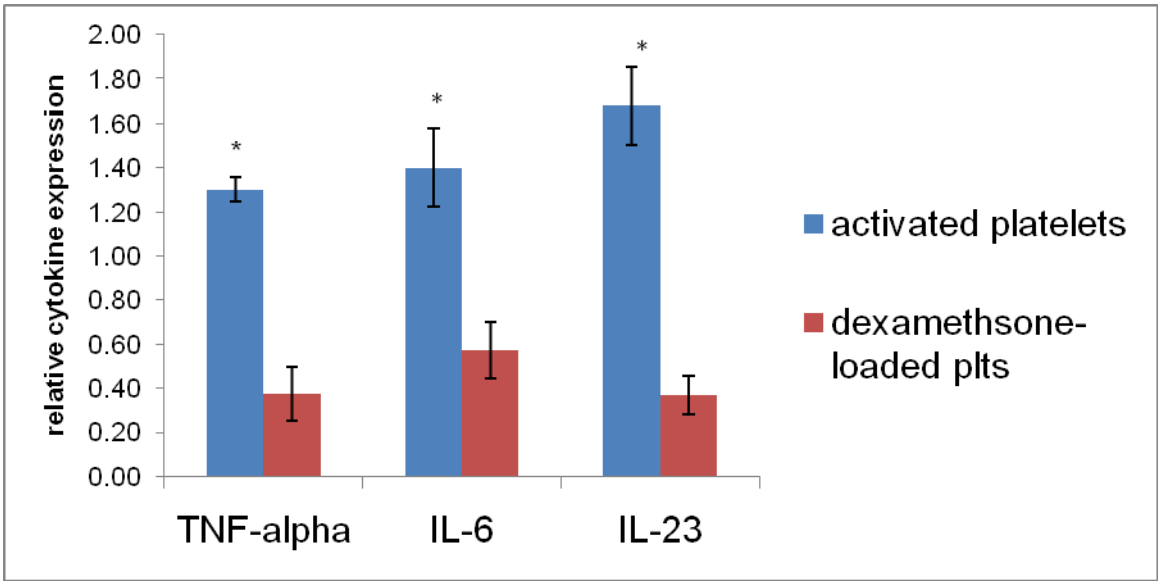

Figure 3-3. ***Dexamethasone-loaded platelets inhibit LPS-induced macrophage activation.*** Freshly isolated platelets were left untreated or loaded with dexamethasone as described in Methods. Platelets and LPS (1ug/ml) were added to 7-day old MDMs and supernatants collected 24hrs later. Cytokines were measured by ELISA and normalized to the cytokine amounts produced by macrophages stimulated with LPS only (relative expression = 1). Shown are the average of 5 independent experiments. Asterisks (*) indicate statistically significant differences ($p < 0.01$) between unmodified and dexamethasone-loaded platelets. Data for activated platelets, used for comparsion, is the same as in Figure 3-1.

DISCUSSION

Phagocytosis of surrounding cells by macrophages has important consequences for macrophage cytokine production. Under normal inflammatory conditions, clearance of apoptotic cells by macrophages triggers a transition from pro-inflammatory cytokine production to anti-inflammatory cytokine production.

Activated platelets are also a major component of the inflammatory environment and share many characteristics of apoptotic cells (62). Previous work suggests that macrophages phagocytose platelets in the context of wound repair, but the consequences of this interaction with respect to the inflammatory state of the macrophages were unknown. We have shown here that the inflammatory response by macrophages is not the same when interacting with activated platelets as it is for phagocytosis of apoptotic cells.

Our initial observations demonstrated that neither platelets nor apoptotic cells elicited an inflammatory response in resting macrophages. This was expected because *in vitro* cultured macrophages express very few cytokines and are overall very metabolically inactive. However, cell-cell interactions in wound healing do not involve 'resting' macrophages. During differentiation at the wound site, macrophages become far more activated than *in vitro* MDMs. The early populations of macrophages at wound sites are representative of 'classically' activated macrophages, as they are responding to the endogenous signals released primarily from neutrophils during the first stage of wound healing (213). Thus, in our subsequent experiments, we sought to mimic an activation state in our macrophage population *in vitro* that would recapitulate the phenotype of the

macrophages present in wound healing *in vivo*. To do so, we utilized bacterial LPS as a means of activating macrophages in our culture system. Although LPS is a bacterial component recognized specifically at the cell surface by TLR4 and CD14, it activates a variety of overlapping intracellular signaling pathways that are involved in pro-inflammatory gene expression (211). Thus LPS-activated macrophages are a good representation of classically activated macrophages found at wound sites.

In the present study, apoptotic cells inhibited production of pro-inflammatory cytokines by LPS-activated macrophages in co-culture. TNF-α, IL-6, and IL-23 were all inhibited. These results are in agreement with previous findings for TNF-α, IL-1β, IL-8, IL-12 (142-144), and are extended to now include IL-6 and IL-23. In contrast to the effect of apoptotic cells, activated platelets enhanced pro-inflammatory cytokine secretion from LPS-activated macrophages. These results extend previous studies which have highlighted the inflammatory effects of platelet interactions with circulating monocytes. Binding of primary monocytes to activated platelets increases monocyte expression of interleukins and matrix metalloproteinases (MMPs), including IL-1β, IL-8, monocyte chemoattractant protein-1 (MCP-1) and MMP-9 (89-92). We have shown here that differentiated activated macrophages also generate a pro-inflammatory response to platelets which suggests that the macrophage receptor(s) that trigger the inflammatory response to activated platelets is also present on primary monocytes.

We have also identified a potential role for platelet CD40L in the enhancement of macrophage activation by platelets. Platelets opsonized with a CD40L-blocking antibody did not exhibit pro-inflammatory cytokine levels that were significantly different from those produced by LPS treatment alone. Platelets opsonized with a control antibody (anti-CD42b) did induce secretion of TNF-α and IL-23 at levels much higher than those produced by LPS treatment alone.

The lack of enhanced IL-6 expression in response to anti-CD42-opsonized platelets was unexpected. One possible explanation for this result is that IL-6 expression is regulated differently from TNFα and IL-23. The IgG component of the opsonizing antibody could down regulate gene expression of IL-6 without affecting the other cytokines. Down regulation of cytokine expression after Fc-receptor ligation has been reported previously (145, 146).

In our completely human *in vitro* system, we observed high variability between individual donors and experiments. Donor variability in human monocytes in response to LPS has been observed in other studies (214, 215). Despite the variability in the actual cytokine levels, the trends observed in comparing co-culture experiments to LPS-activation alone were consistent and statistically significant.

Interestingly, autologous platelets and platelet gels have been indicated as a therapeutic for non-healing wounds (14, 53). The proposed mechanisms for this approach are based on the plethora of growth factors contained in platelets, and the ability of platelets and their secretory products to stimulate wound

healing cells *in vitro*. Given our results presented here, it is tempting to speculate that platelets might provide the necessary stimulation to correct deficient cytokine production. IL-6 is a required cytokine for wound closure (216), and we have demonstrated that its expression by activated macrophages is enhanced in the presence of activated platelets. However, chronic wounds are complex and often exhibit excessive and dysregulated inflammation. Therefore, stimulation of macrophages by platelets may not be effective if the wound site is already overburdened by inflammation. Furthermore, the inability of platelets to induce expression of TGF-β suggests that they may not be an ideal tool for mediating the transition from the inflammatory phase of wound healing to the remodeling and angiogenic phases. More investigation of the temporal and spatial requirements of cytokine expression in normal and chronic wounds is needed.

This work suggests that platelets may also play an important role in other inflammatory settings. Inflammatory lesions such as atherosclerotic plaques and intestinal colitis lesions are characterized by macrophage infiltrates and are exacerbated by CD40L-mediated inflammation (40, 210, 217, 218). From a therapeutic standpoint, the platelet effect on macrophage activation might be undesirable. We have shown here that platelets can be loaded with gluococorticoids and subsequently used to inhibit LPS-induced macrophage activation. This may provide a new therapeutic option for modulating aberrant inflammation.

CHAPTER IV

THE IN VIVO ROLE OF IL-23 DURING NORMAL WOUND HEALING

INTRODUCTION

IL-23 is a member of the IL-6/IL-12 family of cytokines, which are produced by cells of the innate immune system but have profound influences on both innate and adaptive cell-mediated immunity. IL-12, the best characterized cytokine of this group, is a heterodimer composed of one p40 subunit and one p30 subunit. IL-23 is also a heterodimer, and is composed of the same p40 subunit as IL-12, dimerized to a unique p19 subunit (157, 158).

IL-23 has important functions in both innate and adaptive immunity. However, the effects of IL-23 are different from the classical Th1 and Th2 responses (159). It has recently been discovered that IL-23 drives differentiation of a subset of T cells which produce IL-17, which are called Th17cells (160). IL-23-mediated Th17 function is considered weakly pro-inflammatory, but can stimulate proliferation in fibroblasts, keratinocytes, epithelial and endothelial cells (157). More important, perhaps, is the growing evidence that the IL-23/IL-17 pathway is an essential component in many cases of pathological autoimmune inflammation (157, 161-166). These include central nervous system encephalitis, arthritis, inflammatory bowel disease, and psoriasis.

Psoriais is a chronic inflammatory skin condition that is characterized by the presence of dry red, scaly plaques (219). These lesions are the result of excessive inflammation and proliferation of epidermal cells, although the exact triggering mechanism for formation of these plaques is unclear. A major component in the pathogenesis of psoriasis is the recruitment and activation of T lymphocytes (220). Furthermore, the T-cell mediated tissue damage arises from macrophage-derived IL-23 production (221). The requirement for IL-23 has been shown in experimental models of psoriasis in which disease was prevented in both IL-23 deficient animals, as well as animals deficient for the IL-23 receptor (167). Immunohistochemical analysis of human psoriasis lesions have shown expression of IL-23 by macrophages (and also dendritic cells) *in situ* (168). These macrophages and dendritic cells also express high levels of IL-23 when analyzed *ex vivo* (168). However, the exact cause for macrophage production of IL-23 in psoriasis has not been identified. Interestingly, IL-12/IL-23 inhibitors are being developed for the treatment of this disease (169).

Despite the known role of IL-23 in autoimmune inflammatory pathologies, there is no known role for IL-23 in normal inflammatory processes. Given the effects of IL-23 on so many cell types involved in wound repair, in addition to its essential role the pathogenesis of psoriasis, we have tested the hypothesis that IL-23 also plays a role in normal cutaneous wound healing. We show here that wound healing is slightly impaired in IL-23 deficient mice.

METHODS

Mice

Breeder mice that were heterozygous for the IL-23 p19 allele were obtained from the Mutant Mouse Regional Center (MMRRC, University of California - Davis). Mice were cross bred to generate mice that were either wild type or homozygous knockout for the p19 allele. Genotype was confirmed by PCR.

Wound healing experiments

All wound healing experiments were performed on 8-week old mice in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. For the entire surgery portion of the experiment, mice were maintained in an aseptic environment under inhaled anesthesia (Isoflurane) with supplemental oxygen. Once the animal was anesthetized, the hair on the back was shaved and depilated (using Nair®) to expose clean skin. After cleaning the entire area with 70% alcohol, a sterile 10mm punch biopsy was used to create a full-thickness wound in the center of the back of the mouse. Any bleeding was controlled immediately with sterile gauze. The animal was then removed from the anesthesia apparatus and allowed to regain consciousness before being returned to a clean cage. All mice were housed individually following surgery.

Beginning 24 hours after surgery, and every other day after, mice were briefly anesthetized and their wounds were photographed. Digitized images were used to calculate the wound area using the ImageJ software.

Histological analysis

At selected time points, animals were euthanized and tissues collected in formalin for histological processing. Paraffin-embedded skin tissues were cut in 5μm sections and stained with Hematoxylin and Eosin for analysis of cell infiltrates.

RESULTS

Rate of wound closure

To determine if IL-23 was necessary for wound healing *in vivo*, we performed wound healing experiments on both wild-type and IL-23 knockout mice. Large (10mm diameter) wounds on the backs were left untreated and allowed to heal normally over the course of 2-3 weeks. The average wound size created at the start of the experiment was the same for both genotypes. Beginning 24hrs after wounding, and every other day after that, the wounds were measured and the calculated wound size was compared to the original wound size for that particular animal. As shown in Figure 4-1, over the first 24hrs, the wound size decreased by about 20% in both normal and IL-23 -/- mice. During days 2-5 post-wounding, the size of the wounds on the IL-23 -/- mice did not significantly decrease, while the wild-type wounds progressed towards closure. The difference in the amount of wound area remaining was statistically different between the genotypes 5 days after wound healing. However, after day 5, the wound healing of both types of mice followed a similar rate and there was no statistically significant difference between the two groups.

IL-23 could possibly affect several aspects of wound healing, which we addressed by histological analysis. No difference between wild type and IL-23 knockout mice was seen histologically in unwounded skin (Figure 4-2). The first leukocytes to respond during normal wound healing are neutrophils, and IL-23 has been shown to regulate neutrophil homeostasis (222, 223). However, as shown in Figure 4-2, within 24hrs after wounding both genotypes exhibited similar neutrophil infiltrate into the clot. Since the proposed source of IL-23 in the skin is macrophages and dendritic cells (168), we also compared macrophage infiltration into the wound site across genotypes, yet no significant diference was detected (Figure 4-2). Overall, the loss of IL-23 expression had little effect on normal cutaneous wound healing in mice.

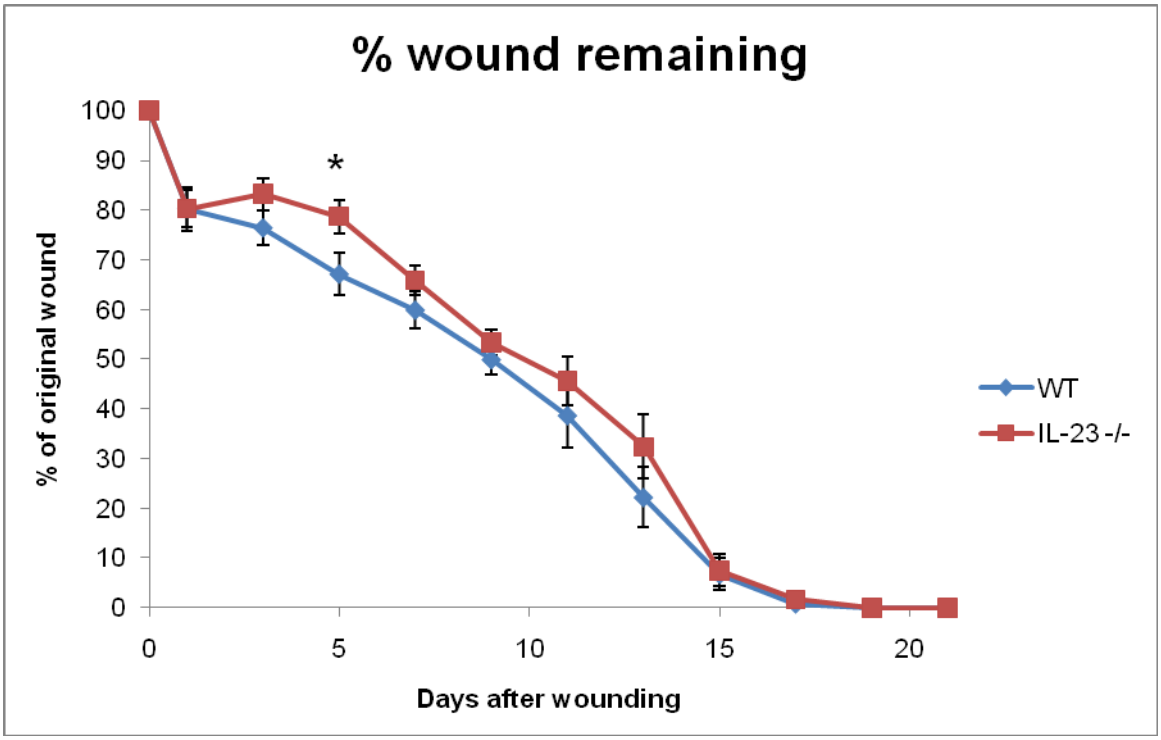

Figure 4-1. ***Wound closure is impaired in IL-23 -/- mice.*** Full thickness wounds were created on the backs of 8-week old mice and measured every other day. Asterisk (*) indicates that the difference between wild-type and IL-23 knockout wounds were statistically significant on day 5.

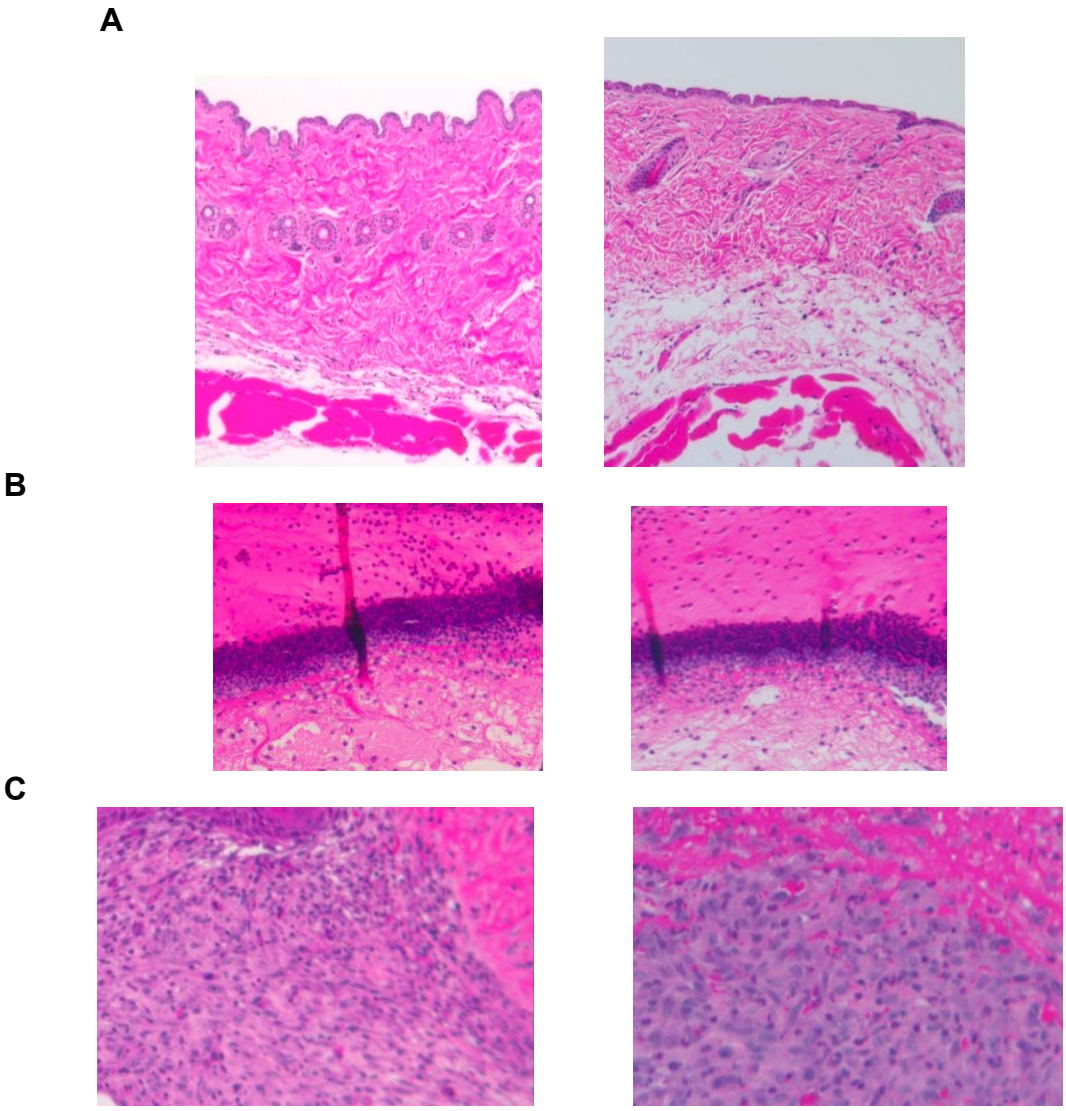

Figure 4-2. ***Histological analysis of wound healing in wild-type and IL-23 -/- mice.*** Histological sections of wild-type (left) and knockout (right) mice were analyzed before wounding (A), 3 days after wounding (B) and 5 days after wound (C). Arrows in (B) identify the band of neutrophils infiltrating the clot. Nuclei in (C) are indicative of macrophage morphology.

DISCUSSION

The cytokine IL-23 plays an important role in both innate and adaptive immunity (165). IL-23-mediated inflammation drives T-cell differentiation, as well as proliferation of fibroblasts, keratinocytes, and epithelial cells (157). Furthermore, IL-23 is highly expressed in the skin during the autoimmune conditions known as psoriasis. Because of the stimulatory effect of IL-23 on other wound healing cells as well as its expression by macrophages, we hypothesized that IL-23 expression was a part of the normal wound healing response.

There was no significant difference in wound healing between wild type and IL-23 deficient mice with respect to time required for wound closure. While only statistically significant at day 5 post-wounding, we did observe that mice deleted for IL-23 maintained larger wound area during the first 5 days of healing when compared to wild type controls. Histologically, however, there were no apparent differences in leukocyte infiltration during this period. Based on previous studies of IL-23 in the psoriasis model, we presume that macrophages are likely the primary source of IL-23 at the wound site in our mouse model, yet the effect on wound healing appeared to occur prior to, and during the initial stages of, macrophage influx at the wound site. This suggests that perhaps a different cell population within the wound space produces IL-23 in response to tissue injury. The lack of available antibodies for paraffin-embedded tissue sections prevented the identification of IL-23-expressing cells in the wild type mice.

The reason that loss of IL-23 did not have a significant effect on wound healing is probably due to overlapping functions of cytokines within the inflammatory environment. For example, both IL-23 and the inflammatory cytokine IL-6 are known to stimulate keratinocytes via phosphorylation of STAT3 (25, 224). In contrast to our results in the present study, IL-6 deficient mice suffer from impaired wound healing, which suggests that IL-6 is probably the main effector for stimulation of keratinocytes. Even in the absence of IL-23, the function of IL-6 is likely enough to ensure that keratinocyte proliferation and wound closure are not significantly affected. Although injection of IL-23 into the skin induces what amounts to a wound healing response (161), there does not appear to be a requirement for IL-23 in normal cutaneous wound healing. The functions of IL-23 remain restricted to autoimmune pathologies.

CHAPTER V

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

SUMMARY

The overall goal of this dissertation was to examine the role of platelet-macrophage interactions in inflammation and wound healing. We first determined how macrophage phagocytosis of autologous platelets is affected by platelet activation and sulfated polysaccharides. These experiments revealed several interesting findings about the role of common platelet activation markers in the recognition and internalization of platelets by macrophages. A distinct role for sulfonation was also identified in the inhibitory effects of polysaccharides in this process. Next, the inflammatory consequences of platelet phagocytosis by macrophages were studied and new details emerged about the pro-inflammatory effects of platelets. A new platelet-based therapeutic for the treatment of inflammatory diseases was also designed and tested. Finally, *in vivo* studies further investigated the role of IL-23 expression in wound closure. These overall conclusions, their impact on the current field, and recommended future studies are described in detail below.

MECHANISMS OF PLATELET PHAGOCYTOSIS BY MACROPHAGES

Platelets play a central role in maintaining vascular integrity, and they respond to injury by adherence, activation, and aggregation. The process of platelet activation has been well characterized and involves many changes to the surface of the cell (42). Of particular interest to the work described in this dissertation are the platelet activation markers phosphatidylserine, P-selectin, and CD40L, which support coagulation reactions, monocyte adherence, and leukocyte activation respectively (36-39, 183, 210). None of these three markers are expressed on the cell surface of resting platelets, yet they rapidly translocate to the platelet surface during activation. Regardless of whether platelets become activated by injury or senescence, they are targeted for clearance by the RES when they become activated (57, 62, 81).

We have shown in this dissertation that interactions of platelets with macrophages, the main phagocyte of the RES, can be recapitulated *in vitro,* establishing a model system in which we could durther explore mechanisms of platelet recognition and internalization by macrophages. Several previous studies have examined the clearance mechanism of altered platelets, such as chilled platelets or platelets affected by storage lesion (57, 59, 62, 67, 75, 77, 79, 81, 82). The work presented here, however, is the first to examine the interaction between freshly isolated, unmodified platelets with autologous macrophages. Our system was entirely comprised of human components and autologous cells so as to avoid confounding factors such as an immune responses by phagocytes or the recognition of platelets as ‘non-self.’

We first validated our model by determining that macrophage phagocytosis of autologous platelets occurs in an actin-dependent manner, which agrees with previous studies of many different types of phagocytosis (105). We also demonstrated that phagocytosis of autologous platelets occurs in serum-free conditions. The ability of macrophages to phagocytose platelets in the absence of serum excludes the possibility that normal platelet phagocytosis is an 'immune-mediated' function mediated by processes such as opsonization with complement or IgG, because such processes require serum factors (68, 104, 105, 107, 108, 110-112, 190, 225).

Phagocytosis of autologous platelets by macrophages also correlates with platelet activation. Our system was not capable of investigating platelet-macrophage interactions using true 'resting' platelets. However, macrophages internalized platelets that were only partially activated and had not reached the irreversible phase of phosphatidylserine exposure. This finding is significant because it suggests that platelets may be targeted to the RES after only slight activation, which can result from a variety of systemic conditions such as shock, inflammation, infection, or even some medications (226-230). Therefore, future studies should consider the possibility that partial platelet activation targets cells to the RES. The downstream effects of such interactions are discussed in the last two sections of this chapter.

Additionally, flow cytometric analysis of our assay conditions revealed that platelet phagocytosis is enhanced when platelets are fully activated. We therefore investigated the role of platelet activation markers in phagocytosis of

platelets and determined that neither the P-selectin-PSGL-1 interaction, nor phosphatidylserine exposure are required for platelet phagocytosis by macrophages. Nonetheless, some cell surface changes must occur on the platelet to trigger clearance by the RES, and future studies should focus on other cell surface changes that occur during platelet activation.

We also examined macrophage phagocytic receptors in the process of platelet phagocytosis. In agreement with the lack of an immune response to autologous platelets, neither complement nor Fc-receptors are needed for platelet uptake. In agreement with studies on aged platelets, we did identify a possible role for scavenger receptors in platelet uptake, because platelet phagocytosis was inhibited by the polysaccharide fucoidan, a known scavenger receptor ligand (192). Future studies should investigate the role of other macrophage receptors with tools such as function-blocking antibodies or RNAi.

We then further examined the inhibitory effect of sulfated polysaccharides on platelet phagocytosis by macrophages. A variety of sulfated polysaccharides inhibited platelet phagocytosis, and the degree of sulfation correlated with each polymer's ability to inhibit phaogyctosis. These results are significant because sulfated polysaccharides have been used as anticoagulant drugs yet can also induce thrombocytopenia (69, 70, 200, 231, 232). For example, heparin has been used extensively in the clinic to reduce the risk of thrombosis, yet it can be recognized by autoantibodies when bound to the platelet surface (70). A critical search continues, therefore, to identify drugs that can inhibit thrombosis without significantly raising the risk of bleeding. The results presented in this dissertation

suggest that sulfated polysaccharides may actually prevent platelet phagocytosis in the absence of autoantibodies. Therefore, this system can be used in conjunction with existing platelet assays to screen compounds for their effect on both platelet function and clearance under a variety of experimental conditions.

PLATELET-MACROPHAGE INTERACTIONS IN WOUND HEALING

The wound healing environment remains a complex and poorly understood system. Nonetheless, a non-hemostatic role for platelets in regulating skin inflammation is emerging (233). Our *in vitro* studies suggest that activated platelets are more than a simple component of the wound debris. Platelets enhance macrophage secretion of IL-6 (an important wound healing cytokine) and IL-23, which are important cytokines during the early phases of wound healing, prior to any confirmed platelet-macrophage interactions. Later, macrophages interact with activated platelets and this results in an inflammatory response that is opposite that which occurs when macrophages clear apoptotic cells. Specifically, apoptotic cells inhibit pro-inflammatory cytokine secretion while inducing secretion of anti-inflammatory and pro-wound healing cytokines IL-10 and TGF-β, while platelets do not.

Previous studies have suggested that autologous platelet gels can enhance wound healing (14, 53). Although many questions remain regarding the activation of macrophages during wound healing, our results suggest that use of autologous platelets in wound care should be carefully considered with respect to macrophage activation and cytokine production. Platelets might be beneficial in

circumstances where macrophage stimulation is desired, but detrimental in cases where macrophages are already hyperactive. More investigation of the temporal and spatial requirements of macrophage activation during wound healing is needed.

Finally, although IL-23 is essential for autoimmune skin inflammation and induces a wound healing response when injected into normal skin (161, 165, 167, 168, 221), a significant role for IL-23 in normal cutaneous wound healing does not appear to exist. This cytokine is more likely restricted to development of T-cell mediated inflammation and autoimmune disease.

PLATELET-MACROPAHGE INTERACTIONS IN INFLAMMATION

The most significant impact of this dissertation is related to the potential role of platelets in inflammatory diseases. A pro-inflammatory effect of platelets on circulating monocytes has been demonstrated previously, and circulating platelet-monocyte aggregates are a common indication of systemic inflammation (91, 93, 94, 96, 234). The present work has demonstrated that activated platelets also enhance LPS-induced macrophage activation by enhancing pro-inflammatory cytokines. This is in direct contrast to the effect of apoptotic cells, which inhibit macrophage pro-inflammatory cytokine secretion.

One possible source of the platelet effect on macrophage activation is platelet CD40L. This inflammatory molecule is a potent activator of macrophage inflammatory responses (235), and we have shown that it may be partly responsible for the observed enhancement of macrophage cytokine expression.

Several diseases such as atherosclerosis and sepsis have implicated a role for CD40L in macrophage activation (40, 218, 236). The role of CD40L in inflammatory pathologies is supported by evidence that an entire disease state of inflammatory bowel disease can be induced in mice simply by engaging CD40 receptors within the gut (40, 237). Nonetheless, it is important to consider the effect of not only CD40L, but also other platelet-derived mediators, such as sphingosine-1-phosphate that may impact the polarization of pro-inflammatory macrophages (238).

The pro-inflammatory cytokines analyzed in this dissertation (TNF-α, IL-6, IL-23) can directly exert pro-inflammatory effects on surrounding cells and tissues. Additionally, IL-6 and IL-23 regulate Th17 responses, which are characterized by T-cell mediated autoimmune inflammation (159). Many autoimmune diseases such as inflammatory bowel disease (IBD), lupus, sepsis, arthritis and psoriasis are characterized by Th17 responses (239-243). Another important characteristic of these pathologies is the presence of activated platelets (210, 236). We speculate, therefore, that platelet-macrophage interactions may represent a previously unidentified source for the generation of Th17-mediated autoimmune inflammatory diseases. Future studies should examine the role of platelet-macrophage interactions in animal models of Th17-mediated disease.

Current clinical approaches to many diseases of inflammation and Th17-autoimmunity have targeted macrophage activation and/or specific macrophage cytokines (153, 204, 244). One multifunctional, systemic approach is

immunosuppression with glucocorticoids (245). Glucocorticoids such as dexamethasone have powerful anti-inflammatory and immunosuppressive effects, yet they carry some risks exist with long-term systemic use. We have thus designed a method for preparing 'anti-inflammatory platelets,' which inhibit macrophage pro-inflammatory cytokine production by targeted delivery of glucocorticoids. These dexamethasone-loaded platelets are effective in inhibiting pro-inflammatory cytokine secretion by activated macrophages, which suggests that they might be useful in obtaining targeted, localized immunosuppressive effects. However, *in vivo* studies are needed to determine the efficacy of targeting high-dose delivery of glucocorticoids to inflammatory lesions. Nonetheless, the *in vitro* studies presented here are proof of principle and a promising start towards this potential new therapy.

The results of this dissertation suggest that when platelets and macrophages co-localize at sites of inflammation, platelets may actively participate in the polarization of macrophages. Given the plasticity of macrophage polarization, particularly during inflammatory processes, we further speculate that platelet -macrophage interactions represent a new link between the innate and adaptive immune response. Future work in this area should focus on identifying platelet-macrophage interactions *in vivo* so that the effect of platelets on macrophage activation can be directly assessed in inflammatory pathologies.

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