

**GLYCOPROTEIN-MEDIATED INTERACTIONS OF DENDRITIC CELLS
WITH SURFACES OF DEFINED CHEMISTRIES**

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**GLYCOPROTEIN-MEDIATED INTERACTIONS OF DENDRITIC CELLS
WITH SURFACES OF DEFINED CHEMISTRIES**

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SUMMARY

Ongoing advances in the field of regenerative medicine towards the development of tissue-engineered (TE) replacements of donor organs for transplantation have been accompanied by advances in the design of suitable biomaterials for use in combination products. These biomaterials may be used either as scaffolds with incorporated biological components such as autologous, allogeneic or xenogeneic cell, protein or DNA sources which finally progress into healthy, functional tissue, or as carriers in vaccine delivery applications (Langer 1993; Atala 2004; Kulig 2004; Tseng 2005). In addition to optimizing the surface and bulk properties of these biomaterials to meet their specific applications, several groups have focused on overcoming the non-specific host inflammatory response generated against the biomaterials, which impedes implant functionality during its *in vivo* lifetime (Llull 1999; Lavik 2004). Besides eliciting a non-specific host inflammatory response, the biomaterial may also augment the specific host adaptive immune response against the biological component in the combination product (Babensee 1998a), thereby functioning as an adjuvant. Although biomaterials having strong adjuvant potentials would be useful as vaccine carriers where the biomaterial-mediated enhancement of host immune responses is advantageous, in contrast, the opposite effect is required for TE applications, where a minimal host response is necessary for successful implant performance, vascularity and integration with host tissues. Therefore, it is important to investigate the abilities of biomaterials to potentiate diverse adjuvant effects, so that biomaterials having controlled and consistent adjuvant effects may be used for varied applications.

The aim of this thesis research was to examine host immune and inflammatory responses to biomaterials, focusing on the roles of biomaterials in supporting dendritic cell (Tabares 2006) maturation. Since DCs ‘bridge’ innate and adaptive immunity and DC maturation results in adaptive immunity or tolerance, the hypothesis underlying this work is that DC maturation upon biomaterial contact *in vitro* would be representative of the adjuvant effects of the biomaterial *in vivo*, since adjuvants function by inducing DC maturation. Co-delivery of poly (lactic-*co*-glycolic acid) (PLGA) boosted antibody levels specific against the antigen introduced *in vivo* (Ertl 1996; Walker 1998; Raghuvanshi 2001; Matzelle 2004). Furthermore, PLGA triggered DC maturation *in vitro* (Yoshida 2004). Not all biomaterials support DC maturation; contact with agarose elicited lower DC maturation as compared to PLGA exposure *in vitro* (Yoshida 2006a) and biomaterial form (microparticles or film) may regulate differential DC responses as well (Bennewitz 2005). It was hypothesized that differential biomaterial surface properties such as chemistry, charge and hydrophobicity/hydrophilicity would regulate the adsorption of distinct DC ligands, that would therefore sustain unique bi-directional interactions with DCs and DC receptors, ultimately resulting in varied DC responses and maturation.

Following preliminary characterizations of model self-assembled monolayers (SAM) endgroups presenting different chemistries, characterization of the profiles of carbohydrates ligands of DC Pattern Recognition Receptors (PRRs) namely C-type Lectin Receptors (CLRs) associated with adsorbed human serum or plasma proteins were performed using enzyme linked lectin assays (ELLAs). The differential carbohydrate profiles observed on different chemistries indicated that distinct ‘biomaterial associated molecular patterns’ may exist on biomaterials, analogous to the conserved pathogen

associated molecular patterns (PAMPs) that are recognized by DCs and stimulate DC maturation. Specifically, the NH₂ SAM endgroups were associated with highest complex mannose, the CH₃ SAM with least sialylated groups, and COOH SAM with higher α -galactose than CH₃ or OH SAMs. These presented carbohydrates may perform individual pro- or anti- inflammatory roles in the context of biomaterials, which remain to be elucidated.

As an initial step towards further characterizing the serum or plasma protein sources of glycosylations, the adsorbed proteins eluted from different SAM endgroups were identified by immunoblotting as part of collaboration with Dr. John Brash and Rena Cornelius, from McMaster University, Canada. Lower band intensity of pro-inflammatory protein fibrinogen was detected in eluates from CH₃ SAM endgroups compared to other SAM endgroups following pre-adsorption with filtered plasma, as well as lower amounts of fibrinogen adsorbed to CH₃ SAM compared to COOH or NH₂ SAM from radiolabeling assays. Also, lower band intensity of adhesive protein vitronectin or anti-inflammatory albumin were also measured in eluates from CH₃ SAM endgroups while no bands could be detected for complement-3 or vitronectin and faint bands for albumin in eluates from OH SAM endgroups. On the other hand, pre-incubation with filtered serum resulted in absence of bands corresponding to Factor XI, Factor XII, prekallikrein or α_1 -anti-trypsin in CH₃ SAM eluates compared to other SAM endgroups, implying differential profiles of adsorbed serum or plasma proteins on different SAM chemistries. Although these differences in protein adsorption may be regulated by surface chemistry and hence imply that CH₃ SAM endgroups were least inflammatory due to least presence of pro-inflammatory proteins, the possibility of non-uniform protein

elution from the different endgroups cannot be ignored, and was assessed by radioactive labeling of fibrinogen to measure protein adsorption and elution from different SAM endgroups. Furthermore, types of incubating medias were important in determining the profiles of adsorbed proteins: filtered versus unfiltered; serum versus plasma. To circumvent the technical difficulty of handling all proteins in serum or plasma simultaneously, future directions include focusing in on a panel of upto five glycosylated serum or plasma proteins that are commonly adsorbed onto biomaterials, clearly distinguishable and with known fragmentation profiles on gels, to investigate the profiles and protein sources of detected carbohydrates that are eluted from different SAM endgroups.

The differential effects of different SAM chemistries on DC responses were examined by evaluating DC maturation following plating of cultures that were characterized as CD4⁺ DCs, CD3⁺/CD4⁺ T lymphocytes and CD19⁺/CD24⁺ B lymphocytes. These studies demonstrated that DCs cultured on CH₃ SAM endgroups displayed rounded morphologies, similar to immature DCs (iDCs) and exhibited lower allostimulatory abilities. These results implied that while OH, COOH or NH₂ SAM endgroups were modest stimulators of DCs and triggered higher expression of maturation markers as compared to iDCs, CH₃ SAM endgroups triggered lowest DC maturation. In contrast, measurement of levels of pro-inflammatory cytokines tumor necrosis factor- α (TNF- α) or interleukin-6 (IL-6) in DC supernatants, indicated that highest levels of both cytokines were present in supernatants on CH₃ SAM endgroups, that were hitherto thought to be least maturing to DCs. This inconsistency was further investigated in the study presented herein, where it was revealed that DCs on CH₃ SAM expressed highest

levels of anti-inflammatory apoptosis-inducing HLA-DQ and were also undergoing highest apoptosis, possibly initiated by high levels of TNF- α . This is significant as phagocytosis of apoptotic DCs has potent immunosuppressive effects (Skoberne 2005b; Wallet 2005a; Chen 2006b). Furthermore, both DCs and autologous T lymphocytes on CH₃ SAM significantly upregulated expression of cytotoxic T lymphocyte associated antigen receptor-4 (CTLA-4), responsible for T cell inhibition and anergic effects (Walunus 1998; Oosterwegel 1999; Orabona 2006), compared to other SAM endgroups. Taken together, these results imply possible tolerogenic mechanisms that may have been responsible for lower DC maturation on CH₃ SAM. For these studies, loosely adherent DCs alone were examined for all treatments, since the expression profiles for maturation markers following biomaterial exposure were similar to adherent DCs with the exception of CD80 expression which was upregulated in the adherent DCs, presumably as a result of DC stimulation during the detachment procedure.

Collectively, these results demonstrate that carbohydrate constituents of ‘biomaterial associated molecular patterns’ presented on biomaterial surfaces may vary based on surface properties. In addition, these results establish the differential DC maturation effects of different SAM chemistries and elucidate the mechanisms responsible for their varied abilities to support DC maturation. Finally, the assays developed in this research and the mechanisms examined here may be transferred from model systems to natural or polymeric biomaterials that have been employed in combination products, with the ultimate goal of matching biomaterials that facilitate optimal degrees of adjuvancy with their specific applications.

CHAPTER 1

INTRODUCTION

Following the genomics and proteomics eras, the field of glycomics involving the analysis of glycan structure and function and the decoding of the ‘glycome’ or the comprehensive set of glycosylated moieties associated with cells, tissues or organs has recently gained prominence (Turnbull 2007). To further this field, several sophisticated tools have emerged including mass spectrometry (MS) (Chalabi 2006; Comelli 2006) and/or high performance liquid chromatography (HPLC) techniques (Tabares 2006) that are specially optimized for performing glycan studies. Other novel strategies include the development of glycan or glycan-binding protein arrays (Feizi 2004; Palma 2006), glycan libraries, the generation of synthetic glycoconjugates (Gama 2006; Turnbull 2006), glycan-binding lectin microarrays (Kuno 2005) as well as the establishment of large-scale databases for the successful amalgamation of varied data types (Goldberg 2005; Turnbull 2006). Glycans constitute the major type of post-translational modification of proteins and invest proteins with immense structural and functional diversity. Additionally, as glycans are present in intracellular regions of most tissues and organs as well as on cell exteriors (Varki 1999; Shriver 2004), they play significant roles in controlling angiogenesis (Liu 2002a), tissue-repair (Shriver 2002), development (Ioffe 1994; Perrimon 2000) and even immunity (Baum 2002; Crocker 2002). Hence an improved understanding of carbohydrate forms and functions would provide a huge impetus towards launching carbohydrate-based biomedical applications. The identification of glycosylated markers would aid in the diagnosis of diseases including liver sclerosis,

cancer (Smorenburg 2001; Liu 2002b; Wahrenbrock 2003), schizophrenia or in inflammation (Turnbull 2007). Furthermore, purified recombinant engineered glycoforms may have therapeutic uses in biological drugs (Turnbull 2007).

However, the field of glycomics faces tremendous technical challenges due to the intrinsic complexity and diversity of glycan structures (Raman 2005). Although, considerable progress has been made, there remains an immense potential for further advances. A similar lacking is also reflected in the field of biomaterials science and in the study of fundamental biomaterial inflammatory responses, where the roles of carbohydrates have not been previously considered. The thesis presented herein represents initial attempts to characterize and elucidate the role of a novel class of biomolecules, the carbohydrates, that are present either in the local milieu or associated with adsorbed proteins on biomaterial surfaces, in mediating inflammatory cell interactions with the biomaterials.

Glycans regulate multiple biochemical pathways and coordinate multicellular biological responses (Iozzo 2001; Shriver 2004). Likewise, glycans may be critical in mediating host immune/inflammatory responses to biomaterials. Glycosylated moieties may be associated with adsorbed proteins on biomaterial surfaces and influence cellular responses to biomaterials, but these interactions have not been sufficiently examined thus far. An understanding of the glycan-based interactions between carbohydrates presented by adsorbed proteins on implant surfaces with the glycoproteins present in solution and with glycosylations on the surfaces of immune cells recruited to sites of implantation or tissue damage, may elicit a superior level of comprehension of the mechanisms underlying host responses against tissue-engineered (TE) devices used in regenerative

medicine. Specifically, the generation of TE devices composed of a biomaterial scaffold within which biological components such as autologous, allogeneic or xenogeneic cells, proteins or DNA are incorporated (Langer 1993; Atala 2004; Kulig 2004; Tseng 2005), has evolved in an attempt to resolve the scarcity of healthy donor organs that are available for transplantation in the treatment of organ failure. The ultimate goal would be to successfully integrate these devices with host tissue and achieve complete implant functionality. Among the tremendous challenges in meeting this objective, overcoming the host inflammatory and immune responses that severely restrict device usage remains significant (Babensee 1998a; Llull 1999; Lavik 2004). With combination products such as these, in addition to the non-specific inflammatory response mounted against the biomaterial component, a specific immune response may also be mounted against the biological component facilitated by the biomaterial (Babensee 1998a). Biomaterial adjuvancy and underlying mechanisms, from the cellular perspective, are still unknown. Understanding this relationship will provide design guidelines for biomaterials for a multitude of applications where a broad spectrum of adjuvant potentials are desired, such as in TE where minimal biomaterial adjuvancy is optimal or in biomaterial vaccine delivery systems where a high adjuvant effect that would enhance the host response is desired for enhanced vaccine potency. The adjuvant effect of biomaterials has been observed both *in vivo* and *in vitro* and has been demonstrated to be mediated by critical antigen presenting cells (APCs) such as dendritic cells (DCs) that mature upon adjuvant stimulation to present peptide antigen to adaptive immune cells and form the pivotal link between innate and adaptive immunity. In the innate immune response, DCs recognize conserved pathogen associated molecular patterns (PAMPs) on pathogen surfaces using

pattern recognition receptors (PRRs). Since DCs typically recognize and respond to complex carbohydrates and lipid patterns in pathogens, the objective here, is to characterize the presence of carbohydrates in the adsorbed protein layer, as possible mediators of DC interactions with biomaterials in support of DC maturation. Furthermore, the emphasis is on investigating a new family of bio-moieties which can mediate inflammatory cell/biomaterial interactions and which have hitherto not been investigated.

The objective of this thesis research is to elucidate the ‘biomaterial-associated molecular patterns’ in the adsorbed proteins on biomaterials of defined and different chemistries and correlate their influence on resultant DC responses. In particular, the presence of ligands of calcium-dependant C-type lectin receptors (CLRs), a carbohydrate-binding family of DC PRRs associated with adsorbed proteins on biomaterials and the roles of these carbohydrates in mediating DC maturation will be investigated. **It was hypothesized that DCs recognize and respond to biomaterials either indirectly through the adsorbed protein layer, specifically through carbohydrate modifications of these proteins, or through carbohydrates inherent in the biomaterial chemistry, using PRRs to initiate an immune response.** Although the various stages of the biomaterial - host response, including but not restricted to, non-specific host protein adsorption, complement activation, coagulation, inflammatory and immune cell recruitment and activation and wound healing processes have been examined, the adjuvant effects of biomaterials remains to be thoroughly characterized. Furthermore, the roles of glycosylated moieties in enhancing or mitigating the DC-mediated responses to biomaterials still need to be elucidated. Biomaterials such as poly

(lactic-*co*-glycolic acid) (PLGA) acted as adjuvants by causing enhanced host responses against co-delivered antigens (Newman 2002; Stivaktakis 2005), or by increasing antigen-specific host antibody production (Matzelle 2004) in a biomaterial form-dependant manner (Bennewitz 2005). Differential adjuvant potentials of biomaterials were demonstrated; while PLGA enhanced DC maturation *in vitro*, agarose did not (Yoshida 2006a). Self assembled monolayers (SAM) presenting different endgroup chemistries (-CH₃, -OH, -COOH or -NH₂) are a well-characterized model system and have been used to study effects of surface chemistry, charge, hydrophobicity/hydrophilicity on host responses by several groups (Silver 1995; Mrksich 1996; Lindbald 1997; Harder 1998; Sigal 1998; Kalltorp 1999; Luk 2000; McClary 2000; Tegoulia 2000; Shen 2001; Keselowsky 2003; Michael 2003; Schwendel 2003; Barbosa 2004; Dadsetan 2004; Barbosa 2005a; Sperling 2005; Barbosa 2006). Since biomaterials function as adjuvants *in vivo* by causing DC maturation and enhancing their ability to present antigen to adaptive immune cells, characterization of the DC ligands (with emphasis on carbohydrates) on SAM surfaces, their effects on stimulating DC maturation and elucidating the mechanisms underlying these responses, are critical for optimal design of biomaterials having controlled adjuvancy for varied applications.

To address the above central hypothesis, two specific aims were pursued.

Specific Aim 1: Elucidate the profile of carbohydrates associated with proteins adsorbed to model biomaterial surfaces of defined chemistries and examine the role of carbohydrate ligands in supporting DC maturation in the context of model biomaterial surfaces.

The working hypothesis for this specific aim was that contact of SAMs presenting different endgroup chemistries would direct a differential profile of carbohydrates associated with adsorbed proteins. The SAM surfaces were characterized by X-ray photoelectron spectroscopy (XPS), contact angle measurements and chromogenic substrate endotoxin assay to evaluate the elemental composition, hydrophobicity/hydrophilicity and endotoxin content of the surfaces, respectively. An Enzyme Linked Lectin Assay (ELLA) was developed using lectin probes to detect the presence of carbohydrate ligands of DC CLR associated with adsorbed proteins on SAM surfaces that were pre-incubated with 1% or 10% human serum in phosphate buffered saline (PBS) or 1% or 10% human plasma in PBS, similar to % serum/plasma used for DC culture. The ELLA absorbances were normalized against amounts of mean adsorbed total human immunoglobulin (IgG) or human serum albumin (HSA) measured using Enzyme Linked Immunosorbent Assays (ELISAs). To distinguish the proteins adsorbed to different SAM chemistries, bound proteins were eluted with sodium dodecyl sulfate (SDS)/dithioerithritol (DTE) and characterized using polyacrylamide gel electrophoresis (PAGE), with immunoblotting to identify the presence of specific proteins.

Specific Aim 2: Characterize DC responses to model biomaterial surfaces having different chemistries and elucidate the mechanisms underlying these responses.

The working hypothesis for this aim was that DCs would interact differently with biomaterial SAM surfaces having different chemistries and hence undergo differential morphological, phenotypic and functional responses as a function of biomaterial surface properties. For this aim, DCs derived from peripheral human blood mononuclear cells were cultured on these SAMs surfaces and the types and compositions of cells present in culture were characterized. The extent of maturation was determined as compared to negative control of immature DCs (iDCs) and positive control of mature DCs (mDCs) induced with lipopolysaccharide (LPS) treatment. The phenotype of DCs was examined by morphology in Giemsa-stained cytopins and expression of activation markers by flow cytometry. The allostimulatory capacities of DCs cultured on different SAM endgroups were assessed using a mixed lymphocyte reaction (MLR). Secretion of pro- and anti-inflammatory mediators were measured using ELISAs and cellular apoptosis was determined using a variety of assays.

Taken together, these studies are an initial step towards understanding the mechanisms involved in differential DC responses to biomaterials of well-defined and different chemistries. Results presented here include novel approaches to measure carbohydrates moieties associated with proteins presented by biomaterials and to investigate their immune and inflammatory roles. Furthermore these results represent in-depth analyses of DC phenotypic and functional responses to different biomaterials, essential in ultimately having controlled and predictable host responses to combination

products where biomaterials having well-understood host responses may potentiate a broad range of adjuvant effects *in vivo*.

CHAPTER 2

RESEARCH SIGNIFICANCE

Glycosylations are present ubiquitously as post-translational modifications of proteins in the extracellular matrix (ECM), on cellular surfaces and within cells (Varki 1999; Shriver 2004). Although glycans have been implicated in multiple biological phenomena including in development (Ioffe 1994; Perrimon 2000), coagulation (Jin 1997) and in mediating bacterial as well as viral infections (Fu 2003), these biological moieties have been comparatively unexploited in developing novel therapeutic agents (Shriver 2004). Several promising studies have suggested critical roles for glycans in regulating many diseases including cancer (Smorenburg 2001; Liu 2002b; Wahrenbrock 2003) and cardiovascular disease (Shriver 2002), thereby making them ideal targets for biomedical interventions. The enormous motivation for developing glycan-based therapeutic drugs has been enhanced by their small size, increased stability, high specificity and potentially lower immunogenicity compared to other biomolecules (Shriver 2004; Sioud 2004). However, the immense heterogeneity and complexity of glycan structures and associated linkages that allows them to participate in varied biochemical processes (Iozzo 2001) also renders their study extremely challenging. This difficulty is exacerbated by the lack of currently available methods to amplify glycans for analysis (Shriver 2004), as well as the weak interactions between glycans (Yang 2002).

Another area in which glycans may play vital roles is in the orchestration of the host immune/inflammatory responses to biomaterials. The adsorbed protein layer that masks implanted devices almost instantaneously upon implantation may be composed of glycan post-translational modifications of proteins. The interactions of glycosylated

biomolecules associated with implant surfaces as well as those present in solution and on surfaces of recruited immune cells may be important mediators of host responses, but have not been previously studied. This thesis research attempts to characterize a novel class of biomolecules which not been formerly investigated, namely carbohydrates and examine their roles in regulating inflammatory cell/biomaterial interactions such as those associated with tissue engineered (TE) devices.

The vast numbers of people awaiting organ transplants in the United States (94,086 at the end of 2005, Transplant Statistics: National Reports, Scientific Registry of Transplant Recipients) has lead to the emergence of TE alternatives to transplant organs and tissue. In both TE devices and biological drug delivery systems, the combination products are composed of biomaterial components that impart physical and structural integrity to the biological components such as cells, proteins, DNA or RNA (Langer 1993; Atala 2004; Kulig 2004). The recipient immune and inflammatory responses generated against the combination product may however substantially limit its integration with host tissue and its efficacy *in vivo* (Babensee 1998a; Llull 1999; Lavik 2004). The host response is expected to be composed of a specific adaptive response against the biological components as well as a non-specific inflammatory response against the biomaterial scaffold (Babensee 1998a). At present, several efforts are underway to advance the field of biomaterials to synthesize novel biomaterials and modify existing ones to meet biocompatibility requirements. An important step in furthering this process would be to extensively examine biomaterial interactions with host immune and inflammatory systems, when biomaterials are present as combination products.

Unforeseen device failure may occur as a result of the synergism between the immune and inflammatory responses between the biological and biomaterial components, respectively. Biomaterials may augment the specific immune response developed against the biological component due to their adjuvant effects (Babensee 1998a). This property of biomaterial adjuvancy may be critical in selecting appropriate biomaterial constituents of combination products and hence needs to be investigated in depth. In the research presented herein, the ability for biomaterials to support the *in vitro* maturation of professional antigen presenting dendritic cells (DCs) that form the pivotal link between innate and adaptive immunity has been investigated, with especial emphasis on carbohydrates in supporting DC maturation. The ultimate goal would be to use this *in vitro* measure of DC activation to predict the *in vivo* adjuvancies of these biomaterials.

Although the inflammatory potentials of biomaterials have been examined in TE, their interactions with DCs and capacities to stimulate DC maturation have not yet been comprehensively explored. The goal of this thesis research was to characterize the DC ligands that were presented by different functional chemistries on the extensively studied model biomaterial monolayer systems, the effects of these ligands on stimulating DC maturation and the elucidation of DC receptors and cellular mechanisms involved in mediating these DC responses. This research project is unique and different from other studies that have examined biomaterial-DC interplay in order to evaluate biomaterials for specific end applications; such as in TE where low adjuvancy is required or in vaccine delivery where high adjuvancy is desired to enhance the host response, in that the objective here is to not only screen the abilities of biomaterials to stimulate DC maturation but also to understand ensuing DC responses in the context of the biomaterial

associated host ligands, specifically carbohydrates. This information would be useful in designing suitable biomaterials with a wide spectrum of adjuvant potentials for diverse applications.

In meeting this goal, differential effects of model self assembled monolayers (SAM) that present different functional chemistries on DC maturation were examined and the underlying cellular processes were probed; while contact with OH, COOH or NH₂ SAM endgroups triggered modest DC maturation, in contrast, contact with CH₃ SAM endgroups resulted in lowest DC maturation, augmented by the presence of anti-inflammatory DC apoptosis and T cell immunosuppressive mechanisms on this surface. The differential profiles of DC carbohydrate ligands associated with proteins presented by different SAM chemistries implied that there may exist a correlation between carbohydrate ligands and DC maturation. Since DCs play a central role in mediating host responses, establishing a clear understanding of DC maturation in response to biomaterials *in vitro* would be expected to be representative of the adjuvant effects of these biomaterials *in vivo*.

On a separate note, either DCs, or macrophages, another class of professional antigen presenting cells (APCs), could be derived *in vitro* from primary peripheral blood mononuclear cells (PBMCs) when cultured with or without DC differentiation cytokines respectively and could be distinguished morphologically as well as phenotypically. Macrophages have been studied extensively in terms of their responses to biomaterials, since they are the primary immune cell type recruited to implant sites, are present in close proximity with biomaterial implants throughout their presence *in vivo* and are key regulators of the intensity and length of host responses, by secreting cytokines,

chemokines and growth factors (Anderson 1988; Brodbeck 2002a; Labow 2005; Xia 2006; Kao 2007). Both macrophages as well as DCs are professional APCs (Sallusto 1994; Triozzi 1997) and have the capacities to stimulate adaptive immune responses against implant associated pathogens. Macrophages are the major mediators of inflammatory responses to biomaterials (Anderson 1988), while DCs are more efficient at eliciting adaptive immune responses (Setum 1991; Sallusto 1994; Triozzi 1997). Hence, DCs may be involved in initiating cellular and humoral immunity against implant associated antigens (Babensee 1998a), thereby playing distinctive roles from macrophages. While macrophages have formed the focus for previous studies that examined host responses to biomaterials, this work compares DCs with macrophages and extends that focus to DCs by examining DC-mediated host responses.

Finally, information from this attempt to explore the interconnections between innate/inflammatory and adaptive immunity, with a focus on DCs, in the context of host responses to TE devices may then be used to allow for a biomaterial-centered approach to generate predictable and controlled host responses. This thesis work is significant as it aims to ultimately provide design criteria for biomaterials, based on an understanding of the mechanisms involved in biomaterial-induced DC maturation, thereby regulating DC phenotype at the cellular level, and advancing host acceptance and tissue integration of a TE graft, long-term.

CHAPTER 3

LITERATURE REVIEW

Innate and adaptive immunity

Innate immunity

The initial response against host invasion either by pathogen ingestion or by receptor-mediated recognition of conserved pathogenic molecules, is mounted by innate immunity comprising host white blood cells including phagocytic macrophages or dendritic cells (DCs), eosinophils, basophils, mast cells, lymphocytes, neutrophils and natural killer (NK) cells (Janeway 2001). Recognition of foreign agents by these cells triggers the secretion of pro-inflammatory cytokines including interleukin-1 (IL-1), IL-12 or IL-8 which may act locally, or systemic activators such as tumor necrosis factor- α (TNF- α), to generate a potent and non-specific protective host response against the target pathogens. Neutrophil activation leads to the initiation of inflammation accompanied by increased vascular permeability to allow for increased leukocyte recruitment to the infected area (Janeway 2001).

Professional antigen presenting cells (APCs) including DCs or macrophages express pattern recognition receptors (PRRs) that recognize conserved pathogen associated molecular patterns (PAMPs), leading to their enhanced abilities to stimulate naïve adaptive immune cells such as T and B cells to cause directed antigen lysis or synthesize antigen-specific antibodies (Medzhitov 1997). Since DCs form the critical link between a non-specific innate response and a specific adaptive response, they are referred to as ‘bridging’ the two arms of immunity (Banchereau 1998a).

Innate immunity includes the complement system, a set of plasma proteins that exist in an inactive form, but yield active inflammatory fragments upon enzymatic cleavage that are recognized by innate immune cells (Janeway 2001). The three known pathways of complement activation; complement 1(C1)-complex stimulation and antibody or pathogen-bound C1q that gives rise to C3- convertase, or C3 hydrolysis, or binding of mannan binding lectin (MBL) to mannose on pathogens causing protease activation and the generation of C4b and C2 from C4 and C2 respectively, all trigger the formation of C3-convertase, that ultimately leads to the establishment of the C5bC6C7C8C9 membrane attack complex (MAC) that ruptures pathogenic membranes and induces target cell lysis (Janeway 2001).

Adaptive immunity

The adaptive immune system primarily comprises T and B lymphocytes that are activated by interactions between T cell receptor (TCR) or B cell receptor (BCR) complexes respectively, with major histocompatibility complex (MHC) molecule counterparts on APCs such as DCs or macrophages. The APC activation is associated with upregulated MHC expression and increased presentation of peptide antigen to lymphocytes in the context of MHC (Janeway 2001). This TCR-mediated antigen recognition event leads to a complex cascade of intracellular events that drives the clonal expansion of T lymphocytes in an IL-2-mediated manner. During this process of clonal expansion and differentiation of naïve T lymphocytes into effector cells which occurs slowly and confers target specificity and memory on adaptive immunity, T lymphocytes migrate from lymphoid organs where the APC binding event takes place, into the lymph nodes, where they come into contact with the antigen. The effector T lymphocytes

function either as CD8⁺ cytotoxic T (T_c) lymphocytes that act by directly killing specific antigen or as CD4⁺ helper T lymphocytes (T_h) that provide other B lymphocyte activation signals that are necessary for their transformation into mature terminally differentiated plasma cells, capable of secreting antibodies. In addition to the primary MHC-TCR signal required for activation, a secondary signal provided by co-stimulatory molecules that are upregulated on activated APCs such as DCs or macrophages (Banchereau 1998a) involving CD40 receptor expressed on APCs and CD40 ligand (CD40 L) on T lymphocytes that stimulates B-7 family co-stimulatory receptor interactions are necessary for lymphocyte stimulation (O'Sullivan 2003). A tertiary signal for activation is provided by release of cytokines and chemokines (Banchereau 1998a). Although many effector cells finally undergo apoptosis and are cleared from the system, a small number remain in circulation as memory effector cells and counter subsequent attacks by the same antigen (Janeway 2001).

Immunity is tightly regulated, since the signals required for T lymphocyte stimulation: antigen peptide presentation by MHC and co-stimulation by APC can only be provided by professional APCs (Janeway 2001). The presentation of intracellular cytosolic (viral or other) antigens to CD8⁺ T lymphocytes by APCs is mediated by MHC class I receptors, which respond by secreting perforins or granzymes that result in target lysis or apoptosis. On the other hand, endocytosed extracellular antigens are processed and presented via MHC II to CD4⁺ T lymphocytes which assist CD8⁺ T and/or B lymphocyte functions. In certain cases 'cross-presentation' may occur wherein exogenous antigens may be endocytosed and broken down in the cytosol and then loaded onto MHC I molecules in the endoplasmic reticulum (ER) (Banchereau 1998a; Rock 2005).

Alternatively, as reviewed in (Rock 2005) antigen lysis and loading onto MHC I may occur in the endosome. As a result of cross presentation, both CD4 as well as CD8+ T lymphocytes may be activated or undergo tolerization for the specific antigen (Rock 2005). The CD4+ T lymphocytes may be T_{h1} which stimulate the secretion of interferon- γ (IFN- γ) and promote macrophage bactericidal functions and B lymphocyte synthesis of antigen-coating antibodies that are recognized and destroyed by 'cellular immunity', or T_{h2} which stimulate IL-4 secretion and humoral immunity via B lymphocyte production of neutralization antibodies. While humoral immunity comes into contact with pathogens in blood and extracellular fluids, cellular immunity has access to tissues as well. In addition to T lymphocyte IL-2-mediated proliferation regulated by APCs, inhibition of T lymphocytes has been linked to high affinity interactions between the B-7 family of co-stimulatory molecules on APCs and cytotoxic T lymphocyte associated antigen receptor-4 (CTLA-4) expressed by activated T lymphocytes via signaling cascades that are activated upon CTLA-4 ligation. This inhibitory mechanism limits T lymphocyte proliferations and hence by extension, the adaptive response. Finally, immune tolerance against antigens may be generated by MHC-TCR signaling in the absence of co-stimulation, resulting in T_{h3} and anergic regulatory T lymphocytes that act through anti-inflammatory mediators including IL-4, IL-10 and the potently immunosuppressive transforming growth factor- β (TGF- β) (Janeway 2001).

Dendritic cells

Immature dendritic cells and capture of antigens

Dendritic cells originate from bone marrow progenitors and circulate the body through blood to ultimately reach peripheral tissues (Banchereau 1998a). Here, the DCs search for pathogens which they detect, process, degrade and present as foreign to effector cells, thereby initiating primary responses and may also orchestrate rapid secondary responses by inducing immunological memory. Immature DCs capture pathogens via phagocytosis, an efficient means of antigen uptake, pinocytosis by forming large vesicles to partake of fluids or via receptor-mediated endocytosis of picomolar and nanomolar amounts of antigen (Sallusto 1995; Banchereau 1998a). Recently DCs have been shown to phagocytose biomaterial microparticles as well (Reece 2001; Walter 2001; Lutsiak 2002; Newman 2002).

Immature DCs (iDCs) express intracellular and extracellular germline-encoded PRRs such as Toll-like receptors (TLRs) (Kaisho 2001; Medzhitov 2001), C-type lectin receptors (CLRs) (Sallusto 1995; Geitjenbeek 2000; Brown 2001a; Figdor 2002), scavenger receptors (SRs), surfactant proteins (SPs) and complement receptors (CRs) as recently reviewed in (Brown 2006), that bind conserved PAMPs and may trigger the release of cytokines and other inflammatory mediators depending on the specific stimulus, upon ligand uptake. Immature DCs also express chemokine co-receptors, F_C antigen-uptake receptors, and exhibit low levels of IL-2R α /CD25, adhesion molecules [intercellular adhesion molecule-1 (ICAM-1)/CD54, lymphocyte function-associated antigen-3 (LFA-3)/CD58], co-stimulatory receptors (B7-2/CD86, B7-1/CD80), MHC and CD40 receptors (Banchereau 1998b).

The type I transmembrane TLRs encode an extracellular leucine-rich repeat (LRR) and an intracellular Toll/IL-1 (TIR) domain and trigger different responses to varied stimuli (Medzhitov 1997; Iwasaki 2004). So far, over ten different toll-like receptors have been identified; although more may exist in mammals. Among known ligands, TLR2 recognizes bacterial (Henderson 1996) and mycoplasmal lipopeptides (Aliprantis 1999; Brightbill 1999; Hirschfeld 1999; Lien 1999) and peptidoglycans (PGN) (Schwander 1999; Yoshimura 1999); TLR3 recognizes poly (I:C) groups (Alexopoulou 2001); TLR4 recognizes bacterial lipopolysaccharides (LPS) (Poltorak 1998; Lien 2000; Poltorak 2000); flagellin is recognized by TLR5 (Hayashi 2001); TLR6 recognizes mycoplasmal lipopeptides (Takeuchi 2001); TLR7 recognizes imidazoquinolines (Hemmi 2002); TLR9 recognizes CpG DNA (Hemmi 2000). Signaling via several TLRs involves recruitment of myeloid differentiation primary response gene (MyD88), nuclear translocation of nuclear factor- κ B (NF- κ B) and triggering of mitogen activating protein (MAP) kinases such as c-Jun N-terminal protein kinase (c-JNK) and p38 (Simon 2003).

The transmembrane CLRs may be type I or II lectins based on the outward or inward orientation of their N termini with respect to the cytoplasm, respectively (Figdor 2002). The type I lectins possess multiple carbohydrate recognition domains (CRDs) while type II lectins contain one CRD, specific for a carbohydrate moiety, each (Figdor 2002). The CLR family has been broadly classified as mannose or galactose binding (McGreal 2004) – the mannose receptor (MR) (Sallusto 1995), DC-specific ICAM-3 grabbing non-integrin (DC-SIGN) (Geitjenbeek 2000), Dectin-1 (Brown 2001a), are examples of CLRs that bind mannose moieties, while asialoglycoprotein receptor

(ASGPR) (Valladeau 2001) and macrophage galactose N-acetyl-galactosamine (GalNAc) specific lectin 1 (MGL) bind α - and/or β -galactose or GalNAc (Valladeau 2001; Figdor 2002; van Kooyk 2003). The carbohydrate-binding specificities of different CLRs vary and have been reviewed in (Figdor 2002; van Kooyk 2003); while MR recognized terminal mannose, fucose, sialylated groups or N-acetylglucosamine (GlcNAc), DC-SIGN recognized mannose-enriched internal residues. Binding of carbohydrates occurs via coordination bonds with Ca^{2+} and hydrogen bonds formed between acid and amide side groups which also bind the Ca^{2+} (Figdor 2002). Certain CLRs are also expressed on Langerhans cells, plasmacytoid DCs, monocytes, macrophages, polymorphonuclear cells (PMNs), B cells and thymic endothelial cells (Figdor 2002) and are involved in a variety of functions including antigen uptake, T-cell interaction and migration (Jiang 1995; Sallusto 1995; Ariizumi 2000a; Ariizumi 2000b; Ariizumi K. 2000; Geijtenbeek T. B. 2000; Geijtenbeek 2000; Geitjenbeek 2000). Dectin-1 receptor that possesses an intracellular ITAM (immunoreceptor tyrosine-based activation motif) and binds β -glucans (Brown 2000; Brown 2001a; Gantner 2003; Brown 2006) has been the only DC CLR implicated in Syk-mediated signaling (Rogers 2005), enhancement of TLR2-based NF- κ B activation (Gantner 2003). Also, binding of mannose-capped lipoarabinomannan (ManLAM) to DC-SIGN skewed the response from a pro-inflammatory T_{h1} response to a T_{h2} response (van Kooyk 2003; Geitjenbeek 2005). Furthermore, galactose/GalNAc has been associated with enhanced presence of TLR2 mRNA, implying a link between CLRs and TLRs. Others studies have demonstrated dectin-1 cross-talk with TLR2 signaling pathways, suggesting linked synergistic responses to antigens from the two PRR families (Gantner 2003; Dillon 2006). On the other hand, dendritic cell immunoreceptor (DCIR)

which has been associated with inhibitory functions possesses an ITIM motif (Kanazawa 2002).

Maturation of dendritic cells and migration to secondary lymphoid organs

The transition from iDCs to mature DCs (mDCs) is triggered by “danger signals” such as PAMPs, damage-associated molecular patterns (DAMPs), pro-inflammatory cytokines or necrotic cells. During this process, iDCs transition into mDCs, transforming from mediating antigen capture functions to antigen presentation functions and may direct either immune or tolerogenic outcomes (Banchereau 1998a). Mature DCs exhibit fewer endocytic vesicles and Fc receptors implying limited antigen uptake and phagocytic capabilities and high levels of CD54, CD58, CD80, CD86 receptors that facilitate communication with T lymphocytes (Banchereau 1998a; Banchereau 2000; Adams 2005), enhanced expression of MHC and co-stimulatory complexes to display antigen to T lymphocytes and reorganization of actin cables to facilitate migration from sites of pathogen encounter to T lymphocyte areas of the lymphatic system (Banchereau 1998a). Following maturation, DCs alter their abilities to respond to microenvironmental chemokines, by upregulating macrophage inflammatory-3 β (MIP-3 β) and secondary lymphoid organ chemokine (SLOC) chemokines that drive DC and T lymphocytes to secondary lymphoid organs, thereby assisting in the stimulation of adaptive immunity (Banchereau 2000). The signaling mediators that are critical in regulating DC maturation include NF- κ B, mitogen-activated protein kinase (MAPK), p38 and c-JNK, that are also involved in cell responses to inflammation and stress (Banchereau 2000; Kriehuber 2005b).

Dendritic cell subsets

Dendritic cells exist as different subsets and lineages and exhibit considerable flexibility in terms of function, hence differentially modulating the final host response (Liu 2001). Common myeloid progenitors (CMP) in the bone marrow (Patwa 2006) differentiate into CD34⁺ CD11c⁺ CD1a⁺ iDCs (Strunk 1997) that develop into skin Langerhans cells (LCs), while CD34⁺ CD11c⁺ CD1a⁻ iDCs give rise to interstitial DCs in tissues (Ito 1999). These two types of DCs vary in their expression of cell surface receptors and also exhibit functional differences; interstitial DCs but not LCs uptake antigen and stimulate naïve B cells in a CD40 and IL-2-dependant manner. Besides this classification, two types of pre-DCs also exist; monocytes (pre-DC1) or plasmacytoid cells (pre-DC2) (Liu 2001), that are different from iDCs in that they are less migratory in culture, exhibit fewer dendritic extensions of the plasma membrane and have reduced abilities to stimulate naïve T cells, in agreement with their lower expression of co-stimulatory receptors. While pre-DC1s are different from pre-DC2s, both are involved in mediating microbial immunity (Spits 2000; Bendriss-Vermare 2001; Liu 2001). In culture pre-DC1s differentiate into immature myeloid DC1s (im-DC1) upon treatment with granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 or following bacterial contact, CD40 stimulus or endotoxin (Cella 1997b), derived by similar methods as DCs used in this thesis research. Alternatively, pre-DC2s generate im-DC2s in response to IL-13 in culture or following viral activation. The im-DC1s potentiate IL-12 mediated T_{h1} responses, while im-DC2s direct IL-4-responsive T_{h2} responses (Rissoan 1999). Finally, lymphoid DC subsets were identified, that generated DCs from early

thymic T cell precursors (Shortman 2000), which are CD8⁺ in contrast to CD8⁻ myeloid DCs and are distinguished by their abilities to trigger T_{h2} and T_{h1} responses, respectively (Pulendran 2001), although the presence of CD8 on lymphoid DCs is controversial (Brasel 2000; Traver 2000). Others have demonstrated that pre-DC2s may qualify for classification as ‘lymphoid’. Cumulatively, DCs are remarkably flexible in their abilities to adapt themselves based on environmental stimuli and may undergo modifications based on the specific stimulus to be countered (Liu 2001).

Adjuvants

Adjuvants have been used in vaccine applications, to enhance the mild protective response elicited against the antigen. Adjuvants may be particulate or immunostimulatory. While particulate antigens function by APC- mediated endocytosis and stimulation, the latter are derived from pathogens and include LPS (Singh 1999) that functions as an adjuvant by binding TLR4 and inducing TLR4-mediated stimulation of DC maturation, the activation of NF- κ B and antigen presentation via MHC complexes (Akira 2001; Medzhitov 2001; An 2002). Inorganic agents such as aluminum hydroxide, aluminum phosphate (Baylor 2002) potassium alum (Glenny 1926) and calcium phosphate have been used clinically as adjuvants. Complete Freund's adjuvant (CFA) has common applications in animal models (Alving 2002). Natural adjuvants include streptococcal nucleic acids (Bültmann 1975), bacterial peptidoglycans (Nauciel 1974) necrotic products, liposomes (Smith Korsholm 2007), double-stranded (ds) RNA, single-stranded (ss) DNA (Busconi 2006), unmethylated CpG DNA (Verthelyi 2006) and bacterial cell walls – all of which are TLR ligands. In pharmacologic applications, caffeine, by itself a minimal adjuvant, acts as a potent adjuvant when dispensed in

combination with acetaminophen (Zhang 2001). On the other hand, virosomes have been shown to generate adjuvant effects with nominal inflammatory responses by mediating fusion, uptake and antigen processing by host APCs (Gluck 2005). Adjuvants are modified to induce mitigated host responses that increment responses to co-delivered antigens by imitating, but not reaching the full potential as in normal infections.

Host response associated with biomaterial implants

Protein adsorption

The introduction of medical devices induces the host response against the implant itself as well as against the injury sustained by the host as a result of implantation (Anderson 1988). The initial stages of this host response include the non-specific adsorption of host proteins onto the implant biomaterials surface, directed by surface properties such as porosity, topography, chemistry, hydrophobicity/hydrophilicity and charge (Mrksich 1996; Shen 2001; Thiele 2003a). Prominent among the host proteins found adsorbed to surfaces are albumin, fibrinogen, fibronectin, vitronectin, plasminogen, immunoglobulins (Ig) and complement proteins, several of which have been implicated in immune and inflammatory consequences (Brash 1987; Tang 1993b). Host immune and other cells interact therefore with the adsorbed protein biofilms (Tang 1993b) and not with the bare biomaterial itself, since protein adsorption precedes the recruitment of immune or inflammatory cells to the biomaterial site (Tang 1993b). However, the amounts, profiles or conformations of adsorbed proteins vary based on surface properties, implying that characterization of these variations would provide initial clues regarding the bi-directional interplay between cells and cell-modified biofilms. In most cases, hydrophobic surfaces allow higher protein adsorption as compared to hydrophilic

surfaces, which are able to hydrogen bond with water molecules, therefore making it energetically more favorable for proteins to adsorb to hydrophobic materials (Collier 1997; Otto 2003). Polyethylene terephthalate (PET) surfaces that were pre-incubated with albumin had greater passivating effects and permitted lower cell adhesion compared to uncoated PET. On the other hand, PET surfaces pre-incubated with pro-inflammatory proteins such as fibrinogen triggered higher inflammatory responses (Tang 1993a). Adsorption of fibrinogen on biomaterial surfaces, a major component of adsorbed proteins (Pankowsky 1990; Nimieri 1994), has also been linked to promoting monocyte adhesion (Shen 2001), while others have shown that the conformation in which fibrinogen adsorbed is significant (Hu 2001; Keselowsky 2003), as modifications in conformation may trigger the complex cascade of events leading to the inflammatory response. Furthermore, the adsorption of other serum or plasma proteins such as thrombin, anti-thrombin III and plasminogen have been linked to coagulation and thrombus formation and presence of C3 on biomaterials surfaces may be an important link to complement activation, as discussed next.

Complement activation

As discussed earlier, the classical, alternative and mannose-binding lectin-mediated pathways of complement activation unite at C3 convertase, which ultimately leads to the formation of MAC (Ratner 1996). The prevalent view in biomaterial literature is that biomaterial activation of complement primarily occurs via the alternative pathway of spontaneous C3b binding to nucleophiles on hydroxyl or amine terminal surfaces, in contrast to non-activators such as anionic terminal carboxyl or heparinized

materials, that promote the existence of inactive form of C3b bound to Factor H, even though polyacrylamides activate complement in a non-nucleophile-mediated manner (Ratner 1996; Gorbet 2004). Others have suggested that biomaterial complement activation may occur via the classical pathway as observed by delayed onset of activation in the absence of C4 components *in vivo* (Tulunay 1993; Lhotta 1998; Videm 1999), while others have shown its involvement *in vitro* as well (Nilsson 1993; Thylen 1996; Sefton 2000). The possibility that the two pathways are linked either by the same biomaterial or by each other, has also not been ruled out (Gorbet 2004). Furthermore, while certain biomaterials generate elevated levels of C3b and MAC, others may trigger augmented levels of the former but not of the latter, as reviewed (Gorbet 2004). In terms of the mannose-binding lectin pathway of complement activation, direct evidence of its involvement in biomaterial-based activation has not yet been demonstrated, but this pathway may play a role particularly in polysaccharide-based polymers.

Connections have been made between complement activation and non-specific protein adsorption, or with leukocyte activation, since low levels of MAC are sufficient to activate leukocytes, or even with platelet activation in a multiple ways; since C1q activates platelets to secrete glycoprotein (GP)IIb/IIIa, a mediator of platelet attachment to biomaterials, expression of P-selectin on platelets that is important in mediating platelet interactions with endothelial cells (ECs), among others (Gorbet 2004). Also, while inhibitors of classical complement had similar inhibitory effects on platelet activation (Bing 1970; Rinder 1999), the effects of terminal complement mediators have been less clear (Larsson 1997). Taken together, this implies that not only is it likely that multiple factors link complement and platelets, but also that a model is emerging,

wherein a complex network of activating and inhibitory interactions exist between several of these players in transitioning between homeostasis and inflammation (Gorbet 2004).

Pre-adsorption of C3b onto cellulose acetate (CA) beads triggers interactions with monocytes and granulocytes, in a calcium-dependant manner and may be mediated by leukocyte complement receptors such as CR3 (Hiraishi 2003), while others have shown that pre-incubation of C3a or C5a on CA beads may regulate granulocyte-mediated anti-inflammatory effects (Nishise 2006), implying a role of complement in biomaterial-mediated host immune/inflammatory responses. More recently, the specific type of polysaccharide; dextran, dextran sulfate or chitosan and the accessibility of their hydroxyl moieties was linked to the conversion of C3 in serum to C3b (Bertholon 2006). Finally, another study demonstrated that the effects of poly(styrene sulfonic acid) (PSSa) on activating the alternative serum complement pathway varied as a function of PSSa concentration, stimulating complement activation at low concentrations in a factor H-mediated manner, or inhibiting complement at higher PSSa concentrations via interactions with factor D (Murakami 2005). Furthermore, the current approach to limit blood complement activation upon exposure to artificial biomaterials by maintaining an unactivated endothelial cell layer on these biomaterials, indicates the complex and dynamic nature of biomaterial-complement-cell interactions (McGuigan 2007). While the effect of complement on leukocyte adhesion and activation may vary for different biomaterials and biocompatibility issues may be overcome by blocking complement, this was found to be true only for certain biomaterials, further highlighting the complexity of these interactions (Gorbet 2004).

Acute and chronic inflammation

The inflammatory processes accompanying implantation of biomaterials have been classified as acute or chronic based on the different cell types involved and the timelines during which these processes occur (Anderson 1988; Tang 1995). During acute inflammation, which occurs within minutes to days after injury, phagocytic neutrophils are recruited to sites of implants by chemokine factors by extravasation through activated endothelium via interactions mediated by chemokine, integrin and selectin receptors on neutrophils (Ratner 1996). Neutrophil adhesion and interaction with biomaterial surfaces occurs through the adsorption of proteins such as IgG or fibrinogen or via complement proteins (Tang 1993a). Neutrophils uptake biomaterials and trigger the release of organic or inorganic reactive oxygen species (ROS) that damage biomaterials including polyurethanes (PU) (Labow 2001) via secretion of hydrolytic or proteolytic mediators. Alternatively, neutrophils may undergo 'frustrated phagocytosis' when they fail to uptake larger biomaterials (Henson 1971). The release of ROS, also secreted during stress, has degradative effects on not only the biomaterial but also has cytotoxic effects on bystander cells.

On the other hand, chronic inflammation, which occurs within a day of implantation, is mediated by monocytes, macrophages and lymphocytes and is characterized by increased vascularization and presence of connective tissue (Ratner 1996). Macrophages penetrate the tissue surrounding biomaterial implants and are the principal cell type associated with them as observed on surfaces of medical devices (Anderson 1988) and tissue engineering scaffolds (Brodbeck 2002a; Xia 2006). Besides their ability to respond to a wide range of materials including metals (Takebe 2003),

polymers (Solheim 2000; Labow 2005), collagen (Khouw 1998; Xia 2006), monocytes and macrophages are 'professional phagocytes' similar to neutrophils and uptake particulate wear debris (Tondravi 1997), partly responsible for the slackening of joint replacements, associated bone degradation, that ultimately results in unsuccessful joint replacements (Garrigues 2005). Although it is clear that macrophages respond to biomaterials by releasing cytokines, chemokines, angiogenic factors and even regulate tissue restoration, the link between biomaterial properties, adsorbed proteins and complement fragments and their interactions with macrophage receptors such as TLRs (Akira 2001; Gordon 2002), SRs (Palecanda 2001; Peiser 2002) and CLR (Zamze 2002), as well as integrin molecules (Berton 1999; Phillips 2005) that mediate extracellular matrix (ECM) interactions via restructuring of the cytoskeletal elements of the plasma membrane (Kwiatkowska 1999) that mediate recognition and antigen uptake, remains to be fully elucidated. Following the recognition of pathogens or biomaterials, macrophages may release extracellular superoxide, hydrogen peroxide, nitric oxide (Strvrtinova 1995), potent pro-inflammatory cytokines, chemotactic factors growth factors, proteases, arachidonic acid metabolites and coagulation factors (Ratner 1996; Bosca 2005), to regulate reparative wound healing processes as well as inflammatory mediators such as bioactive lipids, acid hydrolases and inducers of tissue damage (Xia 2006) and direct responses of multiple cell types.

Granulation tissue

The formation of granulation tissue that takes place about a day after biomaterial implantation marks the initiation of wound healing (Ratner 1996). Fibroblasts and

vascular endothelial cells migrate towards the site of implantation in response to soluble factors secreted by macrophages. Endothelial cells proliferate and are organized into capillary tubes towards the formation of newly sprouted blood vessels (neovascularization) or vessels that arise from pre-existing ones (angiogenesis) (Ratner 1996). On the other hand, fibroblasts multiply and initially generate proteoglycans, subsequently followed by the formation of collagen. Typical vascularized granulation tissue is therefore characterized by the presence of fibroblasts, myofibroblasts that assume a smooth muscle-like phenotype and usually also by macrophages. In special cases where a significant loss of tissues and cells has occurred which cannot be completely regenerated, increased amounts of granulation tissue are formed, leading to scar formation (Ratner 1996). Therefore, depending on the specific characteristics of the injury site, granulation tissue formation occurs in a process initiated upto about 3 days after implantation and lasting until 5 days after.

In the context of biomaterials, neovascularisation and granulation tissue formation may vary according to the biomaterial. In particular, expression of vascular endothelial growth factor (VEGF) mRNA, critical for endothelial cell survival, migration and hence angiogenesis, increased in macrophages but not fibroblasts exposed to hydroxyethylmethacrylate (HEMA), implying the complex nature of these interactions (Mantellini 2006). Also, poly(DL-lactide-co-glycolic acid) (PLGA) that was processed to exhibit nanostructured roughness enhanced fibronectin adsorption and adherence (Miller 2006) and proliferation (Miller 2004) of vascular cells as well as endothelial cells (Miller 2004). Concurrently, other studies showed that adhesion of smooth muscle cells also

increased on polymers that were processed to present nanostructured topographies (Thapa 2003).

Foreign body reaction

Granulation tissue and foreign body giant cells (FBGCs) are the primary components of the foreign body reaction (Anderson 1988). The large multinucleated FBGCs are formed when monocytes or macrophages fuse on biomaterial surfaces in an effort to uptake larger biomaterials and remain at implant sites throughout their *in vivo* durations (Ratner 1996; Shen 2001). The exact role of FBGCs at host-implant interfaces remains unclear. In certain cases FBGCs completely isolate the implant from the local microenvironment, as part of a protective mechanism for either the host or implant. On the other hand, FBGCs that are in contact with biomaterial surfaces may be secreting hydrolytic enzymes and other mediators that are responsible for biomaterial degradation. For instance, stress cracking as observed with poly(etherurethane urea) (PEUU) surfaces (Kao 1994). Biomaterial properties such as chemistry, form, surface topography, and implant surface area to volume ratios are critical in directing the foreign body response as characterized by macrophage fusion (Collier 1997; Shen 2001; Collier 2004; Dadsetan 2004). Porous biomaterials that have higher such ratios attract more macrophages and foreign body giant cells and are hence associated with increased fibrosis as compared to smooth surfaces (Ratner 1996). Furthermore, flat smooth surfaces have been associated with one to two cell thick macrophages (Anderson 1988; Anderson 2000), while rougher poly(tetrafluoroethylene) PTFE surfaces have macrophages as well as FBGCs present at the surface (Ratner 1996). In addition, the foreign body response is directed by the

differential amounts and profiles of host proteins that commonly adsorb to biomaterials having different surface properties (Collier 1997; Kao 1999; Jenney 2000; Collier 2002) as well as differential cytokine, chemokine and growth factors present in the local milieu (Kao 1995). It was observed *in vitro* that FBGCs could be generated from macrophages that were cultured in the absence of any cytokines (McNally 1995; McNally 2002), or with IL-4 (McInnes 1988; McNally 1995) and IL-13 (DeFife 1997). The IL-4 stimulated fusions of these macrophages were regulated by key receptor-mediated interactions, including those of β 1, β 2 integrins (McNally 2002) and CD206/macrophage mannose receptor (MMR) (McNally 1996), CC chemokine ligand 2 (CCL2)/ macrophage chemo attractant protein-1 (MCP1) (Kyriakides 2004) and α -tocopherol (McNally 2003), but limited by inhibitors of diacylglycerol kinase (McNally 2003). In contrast, polymer networks composed of polyacrylamide and poly(ethylene glycol) (PEG) did not support macrophage fusion into FBGCs, but retained macrophages as monocytes aggregates (Collier 2004). Finally, macrophage induction with GM-CSF and IL-4 (Kyriakides 2004) is similar to methods for DC induction (Romani 1996) used in this thesis work with a starting population of peripheral blood mononuclear cells (PBMCs).

Fibrosis

The formation of fibrous tissues surrounding implants indicates the final phase of the regenerative response (Ratner 1996). The extent of fibrosis depends on the site of implantation, the proliferative ability of the surrounding cells, site vascularity and the degree to which the tissue framework has been destroyed by injury. The greater this

disruption, the lower is the likelihood of original parenchymal tissue restoration in injured implant sites and higher the possibility of fibrosis occurring (Ratner 1996).

Differential biomaterial chemistries have been associated with differentially regulating the properties of associated fibrous capsules (Barbosa 2006). Specifically, methyl terminal implants elicited the formation of thicker fibrous capsules as compared to carboxyl or hydroxyl chemistries, demonstrating an effect of biomaterial chemistry on host wound healing responses (Barbosa 2006). Furthermore, the presence of fibrous capsules may interfere with controlled release of drugs from biomaterial-based carrier systems (Anderson 1981), while other studies have addressed the issue of drug release despite fibrous capsule formation in response to radiofrequency ablation to tumor sites, by locally releasing anti-inflammatory agents such as dexamethasone that has been shown to reduce capsule thickness (Blanco 2006). In certain cases, formation of a mature fibrous capsule for thin, macroporous condensed PTFE (cPTFE) with low polymer surfaces areas that contact cells may be associated with increased tissue integration in comparison to expanded PTFE and low or high weight polypropylene and may hence be beneficial depending on the specific application (Voskerician 2006).

Biomaterials in combination products

Biomaterials have been used in tissue engineering (Hubbell 1995) as 3-dimensional scaffolds for adhesion and delivery of viable cells for restoring or renewing tissue or organ function (Ratner 1996). The biomaterial component is critical in steering cell adhesion, proliferation, differentiation, survival, migration and vascularization and should therefore have good biocompatibility, mechanical and physical properties and controllable degradable properties. Furthermore, the convenient large scale production of

biomaterials having consistent properties which are unaffected by sterilization processes required for *in vitro* and *in vivo* applications, are other important criteria (Peppas 1994; Ratner 1996; Langer 2004).

The ongoing thrust in the field of biomaterials is to advance the development of synthetic biomimetic materials that seek to combine the key properties of natural biomaterials such as purified proteins including collagen which encode ligands of cellular receptors responsible for critical cellular processes and undergo material restructuring in response to cellular proteases, implying a beneficial two-way communication between the cells or tissues and the materials, along with advantageous properties of synthetic biomaterials (Lutolf 2005). In an effort to create an ECM-like cellular microenvironment, micro- (Oberpenning 1999) and nano-fibrillar materials have been synthesized mimicking ECM properties of complex fine, fibers, non-fibrillar hydrogels using hydrophilic polymers which mimic the properties of viscosity, elasticity and diffusivity of ECM (Menger 2002; Zhang 2003; Estroff 2004), into which biological factors may be incorporated (Niece 2003), novel protease or matrix metalloproteinase (MMP)-responsive hydrogels that allow for cell-directed remodeling (Basbaum 1996), coating of critical ECM components such as laminin, fibronectin, collagen and glycosaminoglycans (GAGs) as whole proteins or specific peptide sequences of these proteins, essential for cell and tissue survival and regeneration (Hersel 2003; Shin 2003), growth factor localization and controlled temporal release by biomaterials (Mahoney 2001) representative of similar release mechanisms provided by ECM components, are underway (Lutolf 2005).

Self-assembled monolayers (SAM)

Self-assembled monolayers (SAM) presenting robust, well-controlled surface properties have been used extensively as model biomaterial systems, to examine the effects of varying biomaterial surface chemistry, charge and hydrophobicity/hydrophilicity on host inflammatory and immune responses (Maoz 1988; Ulman 1991; Whitesides 1991; Ratner 1996). The SAM chemistries focused on in this thesis research presented terminal methyl (-CH₃), hydroxyl (-OH), carboxyl (-COOH) or amine (-NH₂) and have been previously characterized as hydrophobic, neutral hydrophilic, anionic hydrophilic or cationic hydrophilic respectively. The SAMs are composed of alkanethiols [e.g., CH₃(CH₂)_nSH] that firmly attach gold-coated substrates via functional thiol bonds. Different SAM chemistries have been shown to differentially direct host protein adsorption as well as immune and inflammatory cell recruitment, proliferation, adhesion and survival. In particular, higher amounts of pro-inflammatory fibrinogen (Fg) was adsorbed to OH SAM than to CH₃ SAM (Tegoulia 2000), although others have found otherwise (Shen 2001; Dadsetan 2004; Barbosa 2005a). It was also observed that higher amounts of pro-inflammatory C3 (Hirata 2003) was measured on OH SAM than on CH₃ SAM, although highest amounts of human IgG were detected on CH₃ SAM compared to OH, COOH or NH₂ SAM (Silin 1997). In contrast, adsorption of anti-inflammatory protein albumin was higher on CH₃ SAM compared to either OH or COOH SAM (Martins 2003). More recently, the adsorption of human fibrinogen was demonstrated to decrease with increased presence of OH in the monolayer (Rodrigues 2006). Along with this decrease, the ability of platelets to adhere and be stimulated decreased with greater hydrophilicity (increased -OH groups) (Rodrigues 2006). On the

other hand, pre-adsorbed Fg activated platelets, in contrast with pre-adsorbed albumin that had anti-inflammatory effects on –OH monolayers but not on –CH₃ monolayers (Rodrigues 2006). In agreement with this finding, others have shown that OH SAMs allow high leukocyte adhesion linked to their abilities to support high complement activation regulated by C3b binding to –OH terminal monolayers that was proportional to the numbers of acidic sites on the endgroup, while the –CH₃ endgroups allowed least leukocyte adhesion (Sperling 2005). The exact opposite was seen for platelet adhesion (Sperling 2005).

Different SAM chemistries have also varied in their recruitment of immune/inflammatory cells such as monocytes and PMNs (Barbosa 2005a), activated Mac-1 positive leukocytes *in vivo* to implanted materials in mice air pouch cavities (Barbosa 2005b). Although the CH₃ SAM endgroups were observed as recruiting highest PMNs, these surfaces least supported cell adhesion of neutrophils or leukocytes, but allowed platelets to adhere (Sperling 2005). As discussed previously, the CH₃ SAM endgroups also supported the formation of thicker fibrous capsules (Barbosa 2006), hence implying that these may support highest inflammatory responses among the SAM endgroups tested, that are similar to those used in this thesis research. Others have demonstrated that differential monolayer chemistries direct the conformations of adsorbed fibronectin and hence cell integrin-mediated interactions and cell adhesion (Keselowsky 2003). Finally, sophisticated protein adsorption (Mrksich 1996) or cell adhesion (Mrksich 1997) studies have been performed using microcontact printing to deposit different SAM chemistries on the same surface, that also exhibit variations in surface topology.

Summary

Significant efforts are ongoing in the field of biomaterials science to advance our understanding of the host responses to biomaterials, especially in the area of host protein adsorption and biomaterial-mediated inflammatory responses. However, the roles of biomaterials in combination products remain to be fully elucidated. It is possible that the inflammatory response against the biomaterial have synergistic effects with the host adaptive response specific against the biological components, resulting in potent and at present, insufficiently characterized host responses. It is crucial therefore, to examine the interactions between these two arms of the immune system in the context of biomaterials, with emphasis on DC-orchestrated ‘bridging’ of innate and adaptive responses as well as to characterize DC ligands present in adsorbed ‘biofilms’ on biomaterials, towards ultimately designing optimal materials for different *in vivo* applications.

CHAPTER 4

COMPARATIVE CHARACTERIZATION OF CULTURES OF PRIMARY HUMAN MACROPHAGES OR DENDRITIC CELLS FOR BIOMATERIAL STUDIES

INTRODUCTION:

The success of tissue engineered (TE) substitutes of organs for transplantation depends on transplanted cell viability and functionality *in vivo*. Furthermore, to achieve successful integration of the implanted device with the host system, it is important that the implant should trigger negligible host inflammatory as well as immune responses. Currently, primary causes leading to implant failure include the foreign body response, fibrosis, scar tissue formation and device degradation mediated by host immune cells (Babensee 1998a).

In combination products such as those used in TE, the host response may consist of a non-specific inflammatory response against the biomaterial component as well as a specific immune response directed against the biological component. The biomaterial component may boost the immune response against the cellular component by enhancing the recruitment of innate immune antigen presenting cells (APCs) such as dendritic cells (DCs) or macrophages and supporting their activation and hence ultimately augmenting adaptive immunity as well (Matzelle 2004). In this process, biomaterials may act as adjuvants that augment the specific immune response towards incorporated or shed biological constituents of combination products (Matzelle 2004). This enhancement of the specific immune response against device-associated antigens may finally result in device malfunction (Babensee 1998a).

Professional APCs such as DCs or macrophages bridge innate and adaptive

immunity (Banchereau 1998). Macrophages are recognized as playing arguably the most important role in mediating host foreign body responses to implanted materials. Macrophages are crucial regulators of inflammation and wound regeneration (Anderson 2001; Brodbeck 2002a; Kao 2007; Van den Beucken 2007). Biologically active macrophages at implant or tissue damage sites are the major phagocytes of biomaterial particles and debris, adhere to biomaterial surfaces via interactions with adhesive proteins fibronectin and vitronectin and secrete a wide gamut of cytokines, chemokines, growth factors and matrix metalloproteases (MMPs) (Kao 2007; Van den Beucken 2007(Dinnes 2007). Persistent activation of macrophages triggers their fusion and the formation of foreign body giant cells (FBGCs) that remain associated with implants until explantation (Kao 2007). Macrophages are widely distinguished as pivotal controllers of the strength and extent of the host response (Van den Beucken 2007). Additionally, macrophages present antigen and activate adaptive immunity (Anderson 2004; Kao 2007). While macrophages are key mediators of inflammatory responses, DCs are more potent inducers of immune responses and may act by stimulating lymphocyte responses against foreign antigens (Setum 1991; Sallusto 1994; Triozzi 1997; Banchereau 2000; Janeway 2001), even those that are associated with implants (Babensee 1998a). Taken together, macrophages and DCs function centrally in regulating fundamental biomaterial inflammatory responses, although the distinctness of their roles has not yet been fully appreciated. These APCs detect conserved distinct moieties on pathogens using pattern recognition receptors (PRRs) that trigger DCs to mature and result in efficient presentation of antigens by DCs or macrophages (Janeway 1998). Since adjuvants function by stimulating APCs as demonstrated by increased secretion of pro-

inflammatory cytokines by DCs and upregulated expression of major histocompatibility complex (MHC) and co-stimulatory receptors (Banchereau 1998a; Singh 1999; Tsuji 2000), biomaterials may act as adjuvants by regulating APC responses. Since APCs form the critical link between innate and adaptive immunity, their responses to biomaterials *in vitro* have formed the focus for several studies.

Macrophages are the primary infiltrating cells associated with implanted biomaterials during the majority of their *in vivo* lifetimes and are involved in immune, inflammatory and foreign body responses as reviewed in (Xia 2006). Primary macrophages have previously been derived from monocytes isolated from peripheral blood mononuclear cells (PBMCs) in the absence of any cytokines, generating an assortment of fusing macrophages and FBGCs (McNally 1995; McNally 1996; McNally 2002). The interleukin-4 (IL-4) stimulated fusions of these macrophages were regulated by key receptor-mediated interactions, including those of β 1, β 2 integrins and CD206/macrophage mannose receptor (MMR) (McNally 1995; McNally 1996; McNally 2002). Macrophage adhesion, fusion and apoptosis on differentially modified polyurethanes were dependant on the specific surface modification, leading to the formation of fused adherent FBGCs with IL-4 stimulation, or apoptosis in the absence of IL-4 (Jones 2004). Furthermore, albumin-coated polyester terephthalate (PET) surfaces decreased macrophage recruitment and adherence, while naturally adsorbed fibrinogen (Fn) or Fn-coated PET surfaces increased macrophage recruitment (Tang 1993a).

Dendritic cells play diverse roles *in vivo* and their differentiation has been induced through a variety of methods *in vitro*. Typically, blood monocytes or CD34+ hematopoietic stem cells (HSCs) are induced to differentiate into DCs by exposure to

granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 or in certain cases with tumor necrosis factor- α (TNF- α), or certain interleukins and/or Hepatocyte Growth Factor (HGF), or even in the absence of GM-CSF (Permonti 1995; Xu 1995; Bender 1996; Brossart 1998; Zou 2002; Sprague 2005; Tang 2005). Primary human DCs have been cultured *in vitro* from PBMCs by several groups since their initial characterization (Thiele 2001). In our laboratory DCs were cultured as per methods described in (Romani 1996) starting with PBMCs isolated from healthy donors, selecting for monocytes via plastic adherence and inducing the differentiation of DCs by culturing with GM-CSF and IL-4. It has been reported that the culture of immature DCs (iDCs) in DC media with 75:25 poly(lactic-*co*-glycolic acid) (PLGA) microparticles (MP) or films increased expression of DC maturation markers and induced phenotypic changes indicative of maturation, indicating that biomaterials acted as adjuvants by enhancing the immune response to co-delivered antigens (Yoshida 2004; Babensee 2005; Yoshida 2006b; Yoshida 2006c). It was observed that the form of PLGA (MPs or film) affected maturation since PLGA MPs triggered higher allogeneic T cell proliferation in a mixed lymphocyte reaction (MLR) and a greater delayed type hypersensitivity (DTH) response than the response triggered by PLGA films. It has also been demonstrated that DCs mature differentially upon contact with different biomaterials; although PLGA caused DC maturation, culture with agarose did not (Yoshida 2006a). Furthermore, it has been shown that biomaterials modulate immune responses to co-delivered antigens *in vivo*, thereby explaining in part the biomaterial adjuvant effect observed (Bennewitz 2005). Recently, DCs were shown to mature in response to biomaterial contact *in vitro*, providing in part an explanation for the biomaterial adjuvant effects observed *in vivo*

(Newman 2002; Yoshida 2004; Babensee 2005; Bennewitz 2005; Stivaktakis 2005; Yoshida 2006a; Yoshida 2006b).

Although macrophages and DCs are both professional APCs, macrophages expressed adhesion and co-stimulatory molecules at earlier time points during culture; however, the increases in CD1a or MHC II, CD86, CD80, CD40 maturation markers expression was sustained until later time points for DCs (Pickl 1996; Triozzi 1997; Santin 1999) and may account for their enhanced efficiencies in presenting soluble protein antigens to naïve T cells and initiating adaptive immune responses (Setum 1991; Sallusto 1994; Triozzi 1997). Macrophages are more proliferative (Santin 1999) and phagocytic (Sousa 1993; Aderem 1999; Banchereau 2000; Kiama 2001; Thiele 2001; Thiele 2003b) than DCs, differ in their mechanisms of antigen loading onto MHC II complexes (Bryant 2004) and are better able to degrade whole bacteria (Guidos 1987). While DCs and macrophages may activate differential pathogen-stimulated signaling cascades (Visintin 2001; Hornung 2002; Werling 2004), both types of APCs have been implicated in the induction of tolerance (Guidos 1984; Finkelman 1996; Hoves 2006). In this study, the *in vitro* cultures of macrophages derived from monocytes in the absence of cytokines (McNally 1995; McNally 1996; Brodbeck 2002a; McNally 2002; Jones 2004; Brodbeck 2005) were compared with those of DCs derived from monocytes using GM-CSF and IL-4 (Newman 2002; Yoshida 2004; Babensee 2005; Stivaktakis 2005; Yoshida 2006a; Yoshida 2006b) at relevant time points during APC-biomaterial studies, such as isolation of adherent PBMCs, harvesting of DCs or macrophages for biomaterial plating and after biomaterial exposure. Our findings were evaluated in the light of relevant literature that compares DCs versus macrophages based on phenotype and function both *in vitro* and *in*

vivo.

It was hypothesized that the relative compositions of the different cell types present in the DC or macrophages cultures would change with time and that cultures containing primary DCs or macrophages would be phenotypically distinct albeit starting from the same PBMC population. In this way, the distinct effects of the culture systems would be elucidated in deriving *in vitro* primary cultures of macrophages or DCs for further studies in the inflammatory response to biomaterials. Therefore, the objective of this work was to present a systematic study comparing DCs and macrophages in terms of morphologies and phenotype at significant time points during biomaterial studies under culture conditions known to induce their derivation while starting with the same PBMC population. Furthermore, the goal was to characterize the various other cell types present in cultures of primary DCs or macrophages derived from monocytes isolated from PBMCs, which may be important in driving the differentiation of these APCs and mediating cell-cell and cell-biomaterial immune/inflammatory outcomes. These studies characterize lymphocytes and DCs or macrophages in situations that are analogous to those occurring upon biomedical device implantation and the commencement of the host foreign body response.

METHODS:

Blood Collection and Isolation of Adherent PBMCs

Peripheral human blood was collected from consenting donors using heparin (333 U/ mL blood) (Baxter Healthcare Corporation, Deerfield, IL) as an anticoagulant. This procedure was performed by phlebotomists at the Georgia Tech Student Health Center in

accordance with the Georgia Institute of Technology's Institute Review Board (IRB)-approved protocol # H05012. All subjects enrolled in this research responded to an Informed Consent which was approved by the IRB of Georgia Institute of Technology. The PBMC layer was separated using lymphocyte separation medium (LSM) (Cellgro MediaTech, Herndon, VA) by differential gradient centrifugation [400 g, 30 minutes, room temperature (RT)] (Thermo Fisher Scientific Inc., Waltham, MA) (Model # 5682, Rotor IEC 216). The PBMC layer was collected and erythrocytes lysed [155 mM ammonium chloride, 10 mM potassium bicarbonate (both from Sigma, St. Louis, MO) and 0.1 mM ethylene diamine tetra-acetic acid (EDTA) (Gibco, Grand Island, NY)] and washed twice with sterile phosphate buffered saline (PBS) (Gibco) at RT as described in (Yoshida 2004). The PBMCs were resuspended at 5×10^6 cells/ mL in media containing RPMI-1640 having 25 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid] and L-glutamine (Gibco), 100 U/ mL penicillin/streptomycin (Cellgro) and 10% (v/ v) fetal bovine serum (FBS) (Invitrogen, Carlsbad, CA) and 10 mL of the cell suspension was plated onto tissue culture (TC) plates [Falcon 353803 (Primaria) 100 mm x 20 mm (Becton Dickinson, Franklin Lakes, NJ)] and incubated for adherence (2 hrs) at 37°C with 95% relative humidity and 5% CO₂ to select for adherent monocytes. Adherent PBMCs that remained following three washes with pre-warmed media were collected for further analysis as described below. Dendritic cells or macrophages were each derived three times from three different donors, using a different set of three donors for DCs or for macrophages.

Human PBMC-derived DC Culture

Dendritic cells were derived *in vitro* from human PBMCs using a previously described method (Romani 1996) as reported elsewhere (Sallusto 1994; Yoshida 2004). For culture to derive DCs, adherent PBMCs that remained following three washes with pre-warmed media were cultured in fresh media supplemented with GM-CSF (1000 U/ mL) and IL-4 (800 U/ mL) (both from Peprotech, Rocky Hill, NJ) (DC media) for 5 days. On the fifth day of culture, loosely adherent and non-adherent cells containing DCs were gently harvested by centrifuging at 1100 rpm for 10 minutes and plated at 1.5×10^6 cells/ well in 3 mL/ well in DC media into 35 mm X 10 mm polystyrene TC treated sterile suspension culture dishes (Corning, Corning, NY) and left untreated (negative control) to yield iDCs, or treated with 1 μ g/ mL lipopolysaccharide (LPS) (*E.Coli* 055:B5) (Sigma) (positive control) to yield mature DCs (mDCs). On day 5 of culture or on day 6 of culture following treatment with or without LPS, both the loosely adherent and adherent cell fractions containing DCs were collected for further analysis as described below.

Human PBMC-derived macrophage culture

Macrophages were derived from human PBMCs based on modifications of a previously published method (McNally 1995; McNally 1996; McNally 2002). To derive macrophages, adherent cells that remained following three washes with pre-warmed media were resuspended at 5×10^6 cells/ mL in macrophage media [RPMI-1640 containing 25 mM HEPES and L-glutamine (Gibco), 100 U/ mL penicillin/streptomycin (Cellgro) and 25% (v/ v) filter sterilized autologous human serum]. To obtain autologous human serum, non-heparinized human blood was centrifuged at 3000 rpm for 10 minutes

at RT. The supernatant was collected to remove red blood cells. Clots were pushed down manually using a sterile pipette tip, where necessary. Autologous human serum was then prepared by allowing clotting at RT for 90 minutes in the TC hood. Clear serum was obtained by centrifugation at 3000 rpm for 15 minutes at RT after gently pushing down residual clots/precipitates and was filter-sterilized. Ten mL of this cell suspension was plated onto a 100 mm TC plate [Falcon 353803 (Primaria) 100 mm x 20 mm (Becton Dickinson)] and incubated for adherence (2 hours) at 37°C with 95% relative humidity and 5% CO₂ at a concentration of 5x10⁶ cells/mL with pre-warmed macrophage media to select for adherent monocytes. After the incubation, plates were washed 3 times with warm media to remove non-adherent cells and cultured without cytokines. On day 3 of culture, media was replaced with warmed macrophage media. On day 6 of culture, media was replaced with warmed media containing 25% (v/v) filter sterilized autologous heat inactivated serum (heat-inactivated at 56°C for 30 minutes). On days 6 and 10 of culture, both the loosely adherent and adherent cell fractions containing macrophages were collected for further analysis as described below.

Morphology of adherent PBMCs, DCs or Macrophages

Initial monocytes in the adherent PBMC population and DC or macrophage morphologies as a function of time were examined throughout the culture duration by phase contrast microscopy. Cells were processed for Cytospin preparations as described earlier by us and others (Sallusto 1994; Yoshida 2004) (Cytospin Cytocentrifuge, Thermo Shandon, Pittsburg, PA) and stained with Differential Hematology Stain (Astral

Diagnostics, West Deptford, NJ). Three images were taken at three different regions for each cytospin and a representative image was chosen.

Characterization of adherent PBMCs and DC or Macrophage culture systems

The levels of initial adherent PBMC and DC or macrophage surface markers expression were monitored at relevant time points in biomaterial culture studies, in both adherent and loosely-adherent fractions; on day 0 (adherent PBMCs), for DC cultures on day 5 (as for harvesting of loosely-adherent DCs for 24-hour biomaterial culture) and 6 (as for harvesting of loosely adherent DCs after 24-hour biomaterial culture) and for macrophage cultures on day 6 (as for macrophage media change – replacement with heat-inactivated serum) and 10 (harvesting of loosely-adherent macrophages for biomaterial studies), using a flow cytometric technique as described previously by us and others (Sallusto 1994; Yoshida 2004), using cell type identification markers as listed in Table 1. Table 1 was prepared with reference to technical product sheets. Loosely adherent and adherent cells that were removed using cell dissociation solution (CDS) (Sigma), were collected and resuspended in Hank's HEPES buffer (120 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 10 mM glucose, 30 mM HEPES) (all from Sigma) containing 1% (v/v) human serum albumin (HSA) (EMD Biosciences, San Diego, CA) and 1.5 mM CaCl₂ (Sigma) and stained with mouse anti-human mAb CD11c (clone 3.9; IgG₁), CD19 (clone SJ25-C1; IgG₁), CD14 (clone UCHM-1; IgG2a) (all from Southern Biotech, Birmingham, AL), CD1c (clone AD5-8E7; IgG2a), (Miltenyi Biotec, Auburn, CA), CD3 (clone SK7; IgG1 κ), CD123 (clone 9F5; IgG1 κ), CD4(clone L200; IgG1 κ), CD45RO (clone UCHL1; IgG2a κ), CD25 (clone 2A3; IgG1 κ), CD64 (clone 10.1; IgG1 κ), CD15 (clone H198;

IgM κ), CD24 (clone ML5; IgG2 $\alpha\kappa$), CD8 (clone RPA-T8; IgG1 κ), CD206 (clone 19.2; IgG1 κ) (all from Becton Dickinson Biosciences, San Jose, CA) for 1 hour at 4°C maintained on ice and in the dark. Samples were strained via cell strainers (40 μ m nylon pore) (Becton Dickinson) into 200 μ l of flow cytometry buffer and analyzed immediately using BD LSR flow cytometer (Becton Dickinson) and 5000 events were collected per sample. Autofluorescence was used as negative controls. Analysis was performed using BD LSR flow cytometer (Becton Dickinson), based on gating cell clusters from previously identified positions on forward scatter (FSC) versus side scatter (SSC) dot plots (Setum 1991; Banchereau 1998a; Thiele 2003b). Data analysis was performed using BD FACS DIVA v. 4.1.2 (Becton Dickinson) and using WinMDI 2.8 (Scripps Research Institute, La Jolla, CA).

Table 1: Cell sources and expression profiles of leukocyte and lymphocyte cell surface markers labeled for using flow cytometry

Receptor	Cell sources
CD4	Present on cytotoxic and suppressor T cells, thymocytes and a subset of NK cells
CD8	T cytotoxic/ suppressor populations, peripheral blood lymphocytes, thymocytes
CD3	Present on normal PBMCs, thymocytes, Purkinje cells in cerebellum
CD45RA	Normal human lymphocytes
CD45RO	Present on Thymocytes, activated T cells, memory T cells, granulocytes, monocytes, only certain resting T cells
CD25	Subset of peripheral blood lymphocytes
CD19	All stages of B cell differentiation except terminal plasma cells. Also present on follicular DCs
CD24	Present on B cells, granulocytes
CD14	Monocytes, macrophages, DCs and Langerhans cells
CD15	Present on granulocytes including neutrophils, eosinophils, varying extents on monocytes, not on lymphocytes or basophils
CD64	Present on monocytes and macrophages but not on lymphocytes or resting granulocytes
CD206 (MMR)	Human macrophages, cultured DCs, not on resting monocytes
CD11c	NK cells, a subset of B and T cells, granulocytes, monocytes macrophages and DCs
CD123	High expression in peripheral blood on plasmacytoid DCs and basophils, low on monocytes, eosinophils, myeloid DCs and subsets of hematopoietic progenitor cells
CD1c	A subset of B and T cells, granulocytes, monocytes, macrophages, DCs and NK cells

RESULTS:

PBMCs:

Initial adherent PBMC morphologies

Initial adherent PBMC populations were composed of monocytes and lymphocytes both between 6-8 μm in diameter. Cytospin images of the initial adherent PBMCs are shown in Figure 4-1a indicate that both populations were small and rounded.

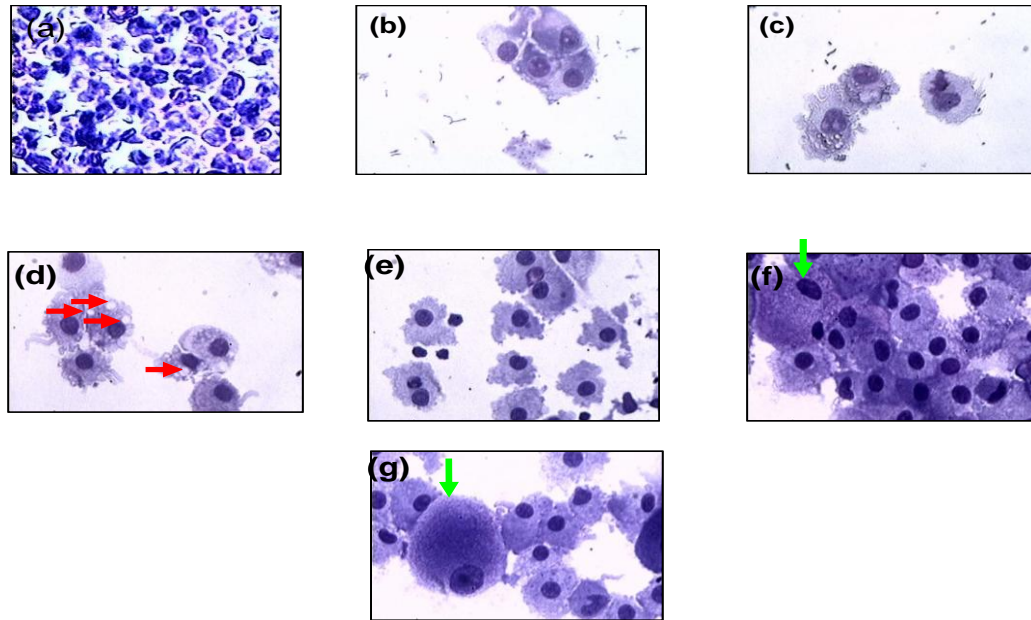


Figure 4-1: Immature DCs are rounded in morphology while LPS-matured DCs exhibit cellular processes and exist as distinct clusters of singly nucleated cells. Conversely, macrophages/FBGCs exhibit fewer membrane projections, exist as fused clusters of cells with indistinct membranes and form large, firmly adherent multinucleated FBGCs with extensive cytoplasm.

Initial adherent PBMCs were isolated from human blood at day 0 (a). Immature DCs derived from human PBMCs were cultured in the presence of GM-CSF and IL-4 until Day 5 and then plated on polystyrene surfaces without (b) or with (c) LPS. On Day 6 of culture, LPS-treated mDCs exhibited extensive dendritic processes in contrast to iDCs that exhibited fewer processes. Macrophages cultured on polystyrene surfaces in the adherent fraction from day 6 (d), the loosely adherent fraction from day 10 (e) and the adherent fraction from day 10 (f, g) exhibited fewer membrane projections as compared to mDCs, endocytosed lipid droplets and fused to form large multinucleated FBGCs. Original magnification: 40x. Geimsa-stained cytopins were prepared for three donors and a representative set of images for one donor has been shown. Horizontal arrows in red indicate the presence of lipid droplets and vertical arrows in green indicate the presence of very large macrophages/FBGCs that were multinucleated in certain cases.

Differential cell types in initial adherent PBMCs population

The adherent cell fraction derived from PBMCs on day 0 (2 hours after plating for plastic adherence in DC media without GM-CSF and IL-4 cytokines) consisted of two populations; a monocyte population which was MMR+, CD11c+, CD14+, CD123+

CD64^{low} and a lymphocyte population which was comprised of CD3+, CD8+, CD45RO+ T lymphocytes and CD24+ CD19+ B lymphocytes (Table 2).

To examine the hypothesis that the relative compositions of cells in culture changed with time, the percentages of each cell type present were measured at all time points and in all fractions. The adherent cell fraction derived from PBMCs on day 0 was comprised of 38 ± 19% CD14+ monocytes, about 62 ± 15% CD3+ T lymphocytes or 52 ± 4% CD24+ or CD19+ B lymphocytes (Table 2). In the FSC/ SSC flow cytometry dot plots, T cells were clustered in the upper half of lymphocyte gate while B lymphocytes were present throughout this gate.

Table 2: Cell populations, expression of cell surface markers, and percentages of each cell type present in the initial adherent PBMC population (day 0)

Day 0 (Adherent PBMCs)
Monocytes: CD11c+, CD123+, CD14+, MMR+ (38 ± 19% CD14+)
T cells: CD3+, CD8+, CD45RA+, CD45RO+ (62 ± 15% CD3)
B cells: CD24+, CD19+ (52 ± 4% CD24+)

Dendritic cells:

DC morphologies

Dendritic cells were about 15 µm in diameter by day 5 of culture with GM-CSF and IL-4 and remained as such until the final day 6 time point tested. Based on cytopsin

images, the morphology of iDCs was smooth and rounded (Figure 4-1b), while maturation induced by LPS treatment resulted in mDCs that exhibited several dendritic processes of the plasma membrane (Figure 4-1c). Dendritic cells were loosely adherent on days 5 and 6 of culture and existed as clusters of individual cells both as iDCs (Figure 4-1b) or mDCs (Figure 4-1c). Light microscopy as well as cytopspin images revealed the presence of DC-DC clusters or of DCs clustered with several smaller lymphocytes (about 6-8 μm in diameter).

Differential cell types in cultures during differentiation of DCs

To further examine this hypothesis, the percentages of different cell types present in DC cultures were measured at all time points and in both adherent and non-adherent fractions for DCs when cultured in DC media with GM-CSF and IL-4 with or without maturation stimuli. At day 5 of culture with DC differentiation cytokines, the loosely adherent cell fraction was composed of a DC population which was CD14+, CD11c+, CD123+, CD1c+, CD4+, MMR+ and a lymphocyte population which was comprised of CD3+, CD4+, CD45RO+ T lymphocytes and CD24+ CD19+ B lymphocytes (Table 3). Upon treatment with the maturation stimulus, LPS, for one day, as compared to iDCs, on day 6, the same DC and lymphocyte populations were observed as at day 5 (Table 3). Cells positive for CD1c, which is known to be expressed on both on B cells and DCs (Cao 2002; van der Wel 2003), also expressed DC markers CD123 and CD11c by day 6 (Table 3). The adherent cell fractions on days 5 and 6 of culture had similar expression of DC and lymphocyte cell surface markers as the loosely adherent cell fraction (data not shown).

The percentages of different cell types present in the DC cultures varied at different time points during culture. By day 5 of culture, the loosely-adherent DC cultures were comprised of $43 \pm 10\%$ CD1c+ DCs and $29 \pm 11\%$ of a mixture of CD3+ T lymphocytes and $24 \pm 8\%$ CD24+ or CD19+ B lymphocytes. By day 6 of culture, loosely-adherent iDC or mDC cultures were comprised of about $60 \pm 5\%$ or $57 \pm 6\%$ CD1c+ DCs, respectively and $51 \pm 14\%$ or $34 \pm 10\%$ CD3+ T lymphocytes or CD19+ or CD24+ B lymphocytes or $50 \pm 5\%$ or $40 \pm 10\%$ T or B lymphocytes, respectively (Table 3).

Table 3: Cell populations, expression of cell surface markers, and percentages of each cell type present in the loosely adherent DC population (day 6)

Day 5 (Loosely adherent)	Day 6 (Loosely adherent)
CD4+ Dendritic cells: CD11c+, CD1c+, CD14+, CD123+, MMR+ ($43 \pm 10\%$ CD1c+)	CD4+ Dendritic cells (iDCs or mDCs): CD11c+, CD123+, CD14+, MMR+ ($60 \pm 5\%$ CD1c+ iDCs) ($57 \pm 6\%$ CD1c+ mDCs)
T cells: CD3+, CD8+, CD45RA+, CD45RO+ ($29 \pm 11\%$ CD3+)	T cells: CD3+, CD8+, CD45RA+, CD45RO+ ($51 \pm 14\%$ CD3+ with iDCs) ($50 \pm 5\%$ CD3+ with mDCs)
B cells: CD24+, CD19+ ($24 \pm 8\%$ CD24+)	B cells CD24+, CD19+ ($34 \pm 10\%$ CD24+ with iDCs) ($40 \pm 10\%$ CD24+ with mDCs)

Macrophages:

Macrophage morphologies

Macrophages and/or FBGCs were about 15-20 μm in diameter. Furthermore, cytospin images revealed that huge FBGCs and several giant cells with widespread

cytoplasms, similar to those described in (McNally 1995), were present in both non-adherent and adherent fractions of macrophage cultures as shown for adherent cells on days 6 (Figure 4-1d) and 10 (Figures 4-1 e and f, g respectively) of culture, especially by the latter time point. Based on cytospin images, these cells were less rounded than adherent PBMCs but more rounded than DCs and contained several lipid droplets, possibly internalized by phagocytosis (Figure 4-1d). Macrophages mostly adhered to the polystyrene substrate especially by day 10 of culture and adhered in large clusters of cells having fused plasma membranes (Figures 4-1 f, g). In certain cases, multinucleated giant cells were observed (Figure 4-1 g). Light microscopy revealed the presence of highly granular macrophages in both non-adherent and adherent fractions.

Differential cell types in cultures during differentiation of macrophages

The percentages of different cell types present in macrophage cultures were measured at all time points and in all fractions. At day 6 of macrophage culture, the loosely adherent (data not shown) and adherent fractions of cells were composed of a macrophage population which was MMR+, CD64+, CD14+, CD11c+ and a lymphocyte population which was comprised of CD3+, CD45RO+ and CD4^{low} T lymphocytes and CD24+ and/or CD19+ B lymphocytes (Table 4). By day 10, the final time point of culture, both the loosely adherent (data not shown) and adherent cell fractions (Table 4) were composed of macrophages and T or B lymphocytes having similar profiles of cell surface markers as on day 6 of culture.

The percentages of different cell types present in the macrophage culture system varied at different time points and in different cell fractions during culture. By day 6 of culture, the loosely adherent fraction were comprised of $33 \pm 15\%$ CD64+ macrophages with associated $62 \pm 14\%$ CD3+ T or $59 \pm 13\%$ CD24 or CD19+ B lymphocytes present as a separate population in the dot plot. The adherent fraction yielded a higher percentage of about $53 \pm 21\%$ CD64+ macrophages with associated lymphocytes and about $43 \pm 22\%$ T or $45 \pm 23\%$ B lymphocytes (Table 4). By day 10 of culture, the loosely adherent fraction was composed of $36 \pm 18\%$ CD64+ macrophages with associated lymphocytes and $60 \pm 18\%$ T or B lymphocytes (data not shown). By day 10 of culture, the adherent fraction was composed of $61 \pm 18\%$ CD64+ macrophages with associated T lymphocytes and about $26 \pm 14\%$ T or $27 \pm 15\%$ B lymphocytes (Table 4).

Table 4: Cell populations, expression of cell surface markers, and percentages of each cell type present in the adherent macrophage population (day 10)

Day 6 (Adherent)	Day 10 (Adherent)
Macrophages: CD11c+, CD64+, CD14+, MMR+ ($53 \pm 21\%$ CD64+)	Macrophages: CD11c+, CD64+, CD14+, MMR+ ($61 \pm 18\%$ CD64+)
T cells: CD3+, CD4+, CD45RA+, CD45RO+ ($43 \pm 22\%$ CD3+)	T cells: CD3+, CD4+, CD45RA+, CD45RO+ ($26 \pm 14\%$ CD3+)
B cells: CD24+, CD19+ ($45 \pm 23\%$ CD24+)	B cells: CD24+, CD19+ ($27 \pm 15\%$ CD24+)

DISCUSSION:

To summarize, the initial adherent PBMCs present at day 0 of human blood

collection were comprised of $38 \pm 19\%$ monocytes and $62 \pm 15\%$ associated T or $52 \pm 4\%$ B lymphocytes (Table 2). Treatment of these initial populations with DC differentiation cytokines GM-CSF and IL-4 induced the differentiation of monocytes into loosely adherent DCs by day 5 of culture along with $29 \pm 11\%$ associated T and $24 \pm 8\%$ B lymphocytes (Table 3). By day 6 of culture, about $60 \pm 5\%$ iDCs or $57 \pm 6\%$ mDCs were present along with associated lymphocytes (Table 3). Alternatively, culture of the initial adherent PBMCs on day 0 in the absence of any cytokines led to the differentiation of monocytes into $53 \pm 21\%$ adherent macrophages by day 6 along with $43 \pm 22\%$ associated T or $45 \pm 23\%$ B lymphocytes (Table 4). At day 10 of macrophage culture $61 \pm 18\%$ adherent cells were macrophages and $26 \pm 14\%$ associated T or $27 \pm 15\%$ B lymphocytes were present (Table 4). Adherent PBMCs were 6-8 μm in diameter at day 0, loosely adherent DCs were approximately 15 μm at day 6 and adherent macrophages were 15-20 μm at day 10. While mDCs exhibited multiple dendritic processes, macrophages and FBGCs exhibited fewer processes (Figure 4-1).

Dendritic cells or macrophages derived from the same initial adherent PBMC populations could be distinguished phenotypically. The DCs were CD14+, CD64-, MMR+ (Table 3) and the firmly adherent macrophages were CD14+, CD64+, MMR+ (Table 4). The presence of MMR detected on monocytes is not usually observed on resting monocytes, but may have been detected on these monocytes due to their activation during the isolation procedure. Both myeloid (CD11c+) and lymphoid (CD123+) DC subsets were present in the DC cultures (Table 3) in agreement with previous findings (Donaghy 2001). It has been previously shown that DCs cultured with GM-CSF and IL-4 were positive for CD40, CD80, CD83, CD86, HLA-DQ, HLA-DR

and maturation induced by LPS treatment resulted in an upregulation of these maturation markers (Sallusto 1994; Yoshida 2004). However, no differences were observed between iDCs and mDCs for the cell identification markers tested in this study (Table 3). From the initial cell population composed of about $38 \pm 19\%$ monocytes (Table 2), the final harvest yielded about $60 \pm 5\%$ or $57 \pm 6\%$ loosely adherent iDCs or mDCs respectively (Table 3) or about $61 \pm 18\%$ adherent macrophages (Table 4) and about $36 \pm 18\%$ macrophages in the non-adherent fraction (data not shown). To summarize, the profiles of the key cell type identification markers were as follows: CD14⁺, CD64⁺, MMR⁺ macrophages were distinct from CD14⁺, CD64⁻, MMR⁺ DCs and from, CD14⁺, CD64^{low}, MMR⁺, monocytes. The percentage of DCs reported in this study is lower than in another study that reported a 95% DC yield from PBMC-derived monocytes, possibly due to differences in donors and reagents (Pickl 1996).

Dendritic cells or macrophages derived from the same initial adherent PBMC populations could also be distinguished based on morphology. Loosely adherent iDCs (Figure 4-1b) exhibited fewer dendritic extensions as compared to loosely adherent LPS-treated mDCs (Figure 4-1c) on day 6 of culture, consistent with earlier findings (Sallusto 1994; Yoshida 2004). Macrophages presented fewer processes compared to DCs and adhered in groups of cells having fused plasma membranes (Figures 4-1f, g), in contrast to DCs that existed as clusters of loosely adherent distinct cells (Figure 4-1c). These clusters may be composed of DCs and/or T and B lymphocytes or macrophages surrounded by multiple lymphocytes (Figure 4-1e) as observed by us in cytopspin images and other groups that have reported the formation of DC: B cell (Kushnir 1998) and DC:T cell (Inaba 1987) clusters *in vitro*. As expected, macrophages and FBGCs were

larger than DCs (15 μm for DCs versus about 15-20 μm for macrophages and/or FBGCs). In agreement with our observations, other studies have observed that macrophages had higher adherence to plastic than DCs and also had consistently higher expression of adhesion receptor lymphocyte function-associated antigen-1 (LFA-1) (Santin 1999).

Another goal was to present a systematic study of the different cell types present in the adherent and non-adherent fractions during different time points in cultures of macrophages or DCs. It was observed that CD3+, CD4+ T lymphocytes and CD19+, CD24+ B lymphocytes were present until day 6 in the DC cultures in both fractions (Table 3). Similarly, both T and B lymphocytes were present for longer, until day 10 in the macrophage cultures in the loosely adherent fraction (data not shown). Lymphocytes were observed in the monocytes/macrophages or monocytes/DCs clusters at different time points throughout the culture, possibly supplying differentiation and other signals to these cells. The percentages of T and B lymphocytes remained between 24-51% of all cells present for DC cultures (Table 3) and about 60% of all adherent cells for macrophage cultures (Table 4) and between 26-45% of all non-adherent cells (data not shown) for macrophage cultures.

While it has been well-established that DCs play a major role in directing B and T cell activation and function, the reverse is also true. Increasingly, the cross-talk between DCs and B cells (Bayry 2004; Bayry 2005) and/or T cells (Latour 2001; Johansson 2003) both in *in vitro* and *in vivo* settings are thought to play a role in regulating the differentiation, migration and maturation of DCs. B cells play an important role in regulating the ability of DCs to present antigen both *in vitro* and *in vivo* and produce

cytokines and chemokines that direct DC migration (Bayry 2005). The MHC-T cell receptor (TCR) (Banchereau 1998a), CD40-(CD40L and CD80/86 – CD28 (Van Gool 1999) interactions trigger DC-mediated T cell activation. In contrast, others including CD80/86 – cytotoxic T lymphocyte associated antigen receptor-4 (CTLA-4) result in T cell anergy (Lenschow 1993). In addition, other interactions between DCs and T cells may direct the activation states of both. For instance, the interaction between CD47 on T cells and signal-regulator protein- α (SIRP- α) on myelomonocytic cells prevents DC maturation and lowers T cell sensitivity to IL-12 (Latour 2001). Another study observed that the presence of lymphocytes *in vitro* enhanced the adhesion and fusion of monocytes into macrophages even in the absence of cell-cell contact and also that the proliferation of lymphocytes increased while in co-culture with macrophages (Brodbeck 2005). Despite these indications that B and T lymphocytes present *in vitro* may regulate DC or macrophage differentiation and responses to biomaterials, the presence of these cell types in *in vitro* culture systems have not been well-characterized previously, to the best of our knowledge, except when intentionally added to specifically examine cross-talk (MacEwan 2005).

It is important to characterize the various cell types present in the two well-established protocols for generating macrophages or DCs *in vitro* and to measure the purity of these cultures. These results also suggest time points and cell fractions that may be optimal for isolation of specific cell types from this culture. The optimal time point for isolation of macrophages is on day 10 of culture (~ 60% macrophages) and from the adherent fraction (Table 4). This observation may partly account for the longer lifetimes of lymphocytes in macrophage cultures. Similar to these observations, others have

demonstrated that macrophages stimulate lymphocyte proliferation throughout co-culture durations (Brodbeck 2005). While it may be possible to separate the cell types, the DC, B and T cell interactions may be important in guiding DC differentiation. In the MLR assay, DCs are co-cultured with allogeneic T cells from day 6 of culture to examine their ability to stimulate naïve T cells. The lifetimes of these different cell types *in vitro* may be significant in evaluating the contributions of antigen-presenting B cells in DC cultures in enhancing allogeneic T cell responses mediated by DCs. While previous studies have demonstrated LPS as being a strong maturation stimulus for DCs as exemplified by an upregulation of co-stimulatory and MHC II molecules (Sallusto 1994; Yoshida 2004), an effect of LPS stimulation was not observed on the expression of T or B lymphocyte markers measured in this study (Table 3). Future work may include further understanding the interactions between the different cell types present in culture that are critical in guiding DC or macrophage differentiation *in vitro*.

Characterization of the DC culture derived from PBMCs may also be important in areas where PBMCs have therapeutic applications. Dendritic cells are important regulators of host responses against viral diseases such as human immunodeficiency virus (Brodbeck) (Wu 2006), different forms of cancers (Fazle Akbar 2006), parasitic infections, tuberculosis (Rubakova 2007), rheumatoid arthritis (Zvaifler 1985), multiple sclerosis (Duddy 2001), bipolar disorder (Knijff 2006) and the strategies involving DCs to combat these diseases include adoptive transfer of *ex vivo* modified-stimulated DCs. Therapeutic approaches may commence with *ex vivo* cultures of DCs or even PBMCs that are similar to those characterized here. This study therefore, is a first step towards characterizing the other cell types in the co-culture, in order to ultimately better account

for their contributions in directing DC or macrophage differentiation and responses.

Although previous groups have described primary DC or macrophage cultures, this study is unique as it performs a thorough characterization both of primary DCs or macrophage as well as other immune cell types present in the cultures which are used to study the inflammatory cell responses to biomaterials *in vitro*. As a result, immune responses at different time points during biomaterial exposure would be considered not only from the perspective of the APC alone but also taking into account the effects of lymphocytes in culture, which may play a role in regulating APC differentiation, survival, adherence and immune/inflammatory outcomes. Furthermore, the absence of neutrophils, cellular mediators of host inflammatory responses, at all time points during either DC or macrophage cultures, implies that this DC culture system would be optimal to examine the effects of biomaterials in mediating DC-orchestrated specific adaptive immune responses. Finally, this physiologically representative DC culture system provides a robust platform to assess the abilities of biomaterials that are present in combination products along with biological components, similar to those used in TE or vaccine delivery applications, to support DC maturation *in vitro*. This *in vitro* readout would therefore be an initial step towards correlating the *in vivo* adjuvant effect of biomaterials in potentiating a broad spectrum of adaptive immune responses. These specific immune responses are directed against the biological component in combination products such as those used in TE applications and are distinct from the non-specific host inflammatory responses elicited against the biomaterial. The biomaterial adjuvant effect could ultimately be tuned to meet the immune/inflammatory criteria desired in the specific end application(s).

CHAPTER 5

GLYCOPROTEIN-MEDIATED INTERACTIONS OF DENDRITIC CELLS WITH BIOMATERIALS. PART 1. DENDRITIC CELL RESPONSES TO SAM ENDGROUPS AND UNDERLYING MECHANISMS.

INTRODUCTION:

Tissue engineering (TE) has arisen as an attractive alternative to the field of organ transplantation, due to the scarcity of healthy organ donors. Along with the development of TE devices composed of biomaterials scaffolds that provide structural integrity to the incorporated biological components such as autologous, allogeneic or xenogeneic cell, protein or DNA sources that ultimately develop into well-integrated, functional tissue, the science of advancing biomaterial surface and bulk properties to meet these needs has progressed rapidly (Langer 1993; Atala 2004; Kulig 2004; Tseng 2005). Furthermore, the non-specific inflammatory responses mounted against these biomaterials that impede their functioning have been examined extensively in order to engineer biomaterials with suitable *in vivo* properties (Anderson 1988; Ratner 1996; Llull 1999; Stock 2001; Lavik 2004). In addition to the non-specific inflammatory response, the biomaterial component in combination products can boost the specific host immune response against the biological component, indicating that biomaterials may play the role of adjuvants (Babensee 1998a). The adjuvant effect of biomaterials may be desired in biomaterial vaccine delivery systems to augment the host response; however, for TE applications, biomaterials with nominal to no adjuvant potential are ideal. The adjuvant effects of biomaterials need to be completely described before biomaterials having predictable and fine-tuned adjuvant effects can be applied in diverse situations.

The objective of this study was to assess the immune and inflammatory roles of

biomaterials, with especial emphasis on their abilities to initiate dendritic cell (Tabares 2006) maturation. The maturation of professional antigen presenting cells (APCs) such as DCs is accompanied by increased expression of Major Histocompatibility Complex (MHC) or co-stimulatory molecules such as CD40, CD80/B7-1 and CD86/B7-2 (Kurts 1996; Banchereau 1998a; Janeway 1998; Aderem 2000; Guyton 2000; Akira 2001; Brown 2001a; Medzhitov 2001; Janeway 2002). Since DCs are the link between innate and adaptive immunity and DC maturation leads to enhanced antigen presentation for an adaptive immune response, the underlying hypothesis is that DC maturation, in response to biomaterials *in vitro* would correlate with the adjuvant effects of these biomaterials *in vivo*. Dendritic cells have been shown to undergo differential activation in a biomaterial-dependent manner *in vitro*. Dendritic cells treated with chitosan or poly (lactic co-glycolic acid) PLGA films increased expression of CD86, CD40 or HLA-DQ (MHC II molecule) while alginate or hyaluronic films had the opposite effects (Babensee 2005). The adjuvancy of biomaterials such as PLGA has been demonstrated *in vivo*, where co-delivery of antigen with PLGA resulted in increased antigen-specific antibody levels (Ertl 1996; Walker 1998; Raghuvanshi 2001; Matzelle 2004; Bennewitz 2005) which has correlated with the observed *in vitro* maturation of DCs upon treatment with PLGA (Yoshida 2004). However, biomaterials such as agarose demonstrated weak abilities to stimulate DC maturation *in vitro* (Yoshida 2006a). It was hypothesized that DCs would interact differentially with diverse biomaterials that presented distinct DC ligands as a function of surface properties such as chemistry, charge and hydrophobicity/hydrophilicity, leading to differential DC responses and maturation.

The overall goal is to examine the interconnections between innate/inflammatory

and adaptive immunity, with emphasis on the role of DC receptors and cellular mechanisms involved in regulating these interactions, in the context of host responses to combination products. Particularly, the objective was to examine the effect of biomaterial chemistry on DC maturation using well studied model self-assembled monolayers (SAM) presenting different endgroup chemistries (Silver 1995; Mrksich 1996; Lindbald 1997; Harder 1998; Sigal 1998; Kalltorp 1999; Luk 2000; McClary 2000; Tegoulia 2000; Shen 2001; Keselowsky 2003; Michael 2003; Schwendel 2003; Barbosa 2004; Dadsetan 2004; Barbosa 2005a; Sperling 2005; Barbosa 2006). The broad hypothesis is that these materials presumably interact with DCs through the adsorbed host protein layer including associated carbohydrate post-translational modifications, that may be analogous to pathogen associated molecular patterns (PAMPs) which are recognized by DC pathogen recognition receptors (PRRs) (Banchereau 1998a; Janeway 2001) such as Toll-like receptors (TLRs) (Akira 2001; Medzhitov 2001) or carbohydrate-binding C-type lectin receptors (CLRs) (Geitjenbeek 2000; Brown 2001a; Figdor 2002; van Kooyk 2003), resulting in DC maturation. Based on an understanding of the mechanisms involved, the ultimate goal is to provide design criteria for biomaterials thereby regulating DC phenotype at the cellular level, and advancing host acceptance and tissue integration of a tissue engineered graft, long-term.

METHODS:

Self-Assembled Monolayer preparation

Self-assembled monolayer endgroups were assembled on 35 mm X 10 mm tissue culture (TC)-treated polystyrene dishes (Corning, Corning, NY) used for DC culture or

on 16-well glass chamber slides (*LAB-TEK*, Nalge Nunc International, IL) for measurement of endotoxin content (Keselowsky 2003). Clean substrates were sequentially coated with 50 Å Ti followed by 150 Å Au using an electron beam evaporator (CVC Products/ Veeco, Rochester, NY) and stored under vacuum at room temperature (RT) up to two weeks until used. The following alkanethiols were used as received from commercial sources (1 mM in absolute ethanol): 1-dodecanethiol (SH-(CH₂)₁₁-CH₃) (CH₃ SAM), 11-mercapto-undecanol (HS-(CH₂)₁₁OH) (OH SAM), 11-mercaptopundecanoic acid (HS-(CH₂)₁₀-COOH) (COOH SAM), (Aldrich Chemical, Milwaukee, WI) and 11-amino-1-undecanethiol, hydrochloride (C₁₁H₂₆ClNS) (NH₂ SAM) (Dojindo Laboratories, Gaithersburg, MD). The SAM endgroups were allowed to assemble by incubation for 12 hrs at RT by immersing the Ti/Au-coated slides or coverslips in alkanethiol solutions, after which the dishes or slides were opened and air-dried for ten minutes, both under sterile conditions. Prior to cell culture, SAM-coated dishes and lids or slides were washed once with absolute ethanol (Sigma, St. Louis, MO) and then five times with filter sterilized (0.22 µm) (Corning) phosphate buffered saline (PBS) (Gibco, Grand Island, NY) at RT and used fresh for each experiment. From other studies, CH₃ SAMs were highly hydrophobic, while OH SAMs were least hydrophobic (Keselowsky 2003).

Chromogenic Endotoxin Assay

Endotoxin content of biomaterials was measured using a chromogenic *Limulus* Amebocyte Lysate (LAL) assay (QCL-1000 Chromogenic LAL Endpoint Assay, Cambrex, Walkersville, MD) using the 96-well microplate method. Standards in 96-well

flat-bottomed microplates and sample wells in 16-well chamber slides coated with different SAM endgroups were treated with endotoxin-free water (Cambrex) in triplicate. Since the LAL assay was designed to be performed on samples in solution, freshly prepared SAM endgroups in endotoxin-free water were used as samples to detect endotoxin present on SAM endgroups wherein LAL was added and incubated (10 minutes, 37°C) in the presence of the biomaterials. Next, the chromogenic substrate (Ac-Ile-Glu-Ala-Arg-pNA) was added to each well, including the SAM surfaces, and incubated (6 minutes, 37°C), thereby allowing the LAL-substrate reaction to proceed as indicated by color development. Glacial acetic acid (25% v/ v) (J.T.Baker, Phillipsburg, NJ) at RT was added as a stop solution to standard or sample wells and the mixture in SAM-coated sample wells was transferred into 96-well flat-bottomed microplates to avoid interference with the reading by the SAM surfaces. Absorbances were measured immediately using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA) at 405 nm. Endotoxin content in the samples was read off standards generated from the manufacturer's kit. For all SAM endgroups, endotoxin amounts were below detection (detection limit: 0.1 endotoxin units (EU)/ mL; i.e., absorbance readings below blank sample) and well below the approved FDA endotoxin limit for implantable devices; 0.5 EU/ mL.

Human PBMC-derived dendritic cell culture

Dendritic cells were derived from human peripheral mononuclear cells (PBMC) using a previously described method (Romani 1996) as reported elsewhere (Sallusto 1994; Romani 1996; Yoshida 2004). Briefly, peripheral human blood was collected from

consenting donors using heparin (333 U/ mL blood) (Baxter Healthcare Corporation, Deerfield, IL) as an anticoagulant. This procedure was performed by phlebotomists at the Georgia Tech Student Health Center in accordance with the Georgia Institute of Technology's Institute Review Board (IRB)-approved protocol # H05012. All subjects enrolled in this research responded to an Informed Consent which was approved by the IRB of Georgia Institute of Technology. The PBMC layer was separated using lymphocyte separation medium (LSM) (Cellgro MediaTech, Herndon, VA) by differential gradient centrifugation (400 g, 30 minutes, RT) (Thermo Fisher Scientific Inc., Waltham, MA) (Model # 5682, Rotor IEC 216). The PBMC layer was collected and erythrocytes lysed [155 mM ammonium chloride, 10 mM potassium bicarbonate (both from Sigma) and 0.1 mM ethylene diamine tetra-acetic acid (EDTA) (Gibco)] and washed twice with sterile PBS. The PBMCs were resuspended at 5×10^6 cells/ mL in DC media [RPMI-1640 containing 25 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid]] and L-glutamine] (Gibco), 100 U/ mL penicillin/streptomycin (Cellgro) and 10% (v/ v) FBS and 10 mL of the cell suspension was plated onto TC plates [Falcon 353803 (Primaria) 100x20mm (Becton Dickinson, Franklin Lakes, NJ)] and incubated for adherence (2 hrs) at 37°C with 95% relative humidity and 5% CO₂ to select for adherent monocytes. Adherent cells that remained following three washes with pre-warmed media were cultured in fresh media supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) (1000 U/ mL) and interleukin-4 (IL-4) (800 U/ mL) (both from Peprotech, Rocky Hill, NJ) (DC media) for 5 days. On day 5 of culture, loosely adherent and non-adherent cells containing DCs were gently harvested by centrifuging at 1100 rpm for 10 minutes, and plated at 1.5×10^6 cells/ well in 3 mL/ well in

DC media into 35 mm X 10 mm sterile suspension culture dishes and left untreated (negative control) to yield immature DCs (iDCs), or treated with 1 µg/ mL lipopolysaccharide (LPS) (*E.Coli* 055:B5) (Sigma) (positive control) to yield mature DCs (mDCs) or treated with the different SAM endgroup surfaces. On day 6, cells were harvested for further analysis.

Dendritic cell morphology

Dendritic cell morphology was examined throughout the culture duration by phase contrast microscopy. Dendritic cells cultured in DC media were processed for Cytospin preparations as described earlier (Yoshida 2004) (Cytospin Cytocentrifuge, Thermo Shandon, Pittsburg, PA) and stained with Differential Hematology Stain (Astral Diagnostics, West Deptford, NJ). Giemsa-stained cytospin images were obtained on day 5 or on day 6 for loosely-adherent iDCs, mDCs or DCs treated for 24 hrs with different SAM endgroup surfaces. Three images were taken at three different regions for each cytospin and a representative image was chosen.

Flow cytometry for determination of maturation marker expression

To examine the effects of DC culture on the different SAM endgroups, the levels of DC surface marker expression were measured on day 6 using a flow cytometric technique as described previously (Yoshida 2004). Loosely adherent cell fractions containing DCs for iDCs, mDCs or DCs treated with different SAM endgroup surfaces were collected and resuspended in Hank's HEPES buffer (120 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 10 mM glucose, 30 mM HEPES) (all from Sigma) containing 1% Human

Serum Albumin (HSA) (EMD Biosciences, San Diego, CA) and 1.5 mM CaCl₂ (Sigma) and stained with saturating concentrations of mouse anti-human monoclonal antibody against CD14 (clone UCHM1; IgG2 α), CD40 (clone B-B20; IgG1 κ), CD80 (clone BB1; IgM κ), CD86 (clone BU63; IgG1 κ), CD19 (clone SJ25-C1; IgG₁), CD14 (clone UCHM-1; IgG2 α) (all from Southern Biotech, Birmingham, AL), CD83 (clone HB15a; IgG2b) (IO Test Immunotech Beckman Coulter, Marseille, France) HLA-DQ (clone TU169; IgG2 α), HLA-DR (clone TU36; IgG2 α), CD3 (clone SK7; IgG1 κ) (all from Becton Dickinson Pharmingen, San Diego, CA) or cytotoxic T lymphocyte associated antigen receptor-4 (CTLA-4-Ig) (clone 48815; IgG_{2b}) (R&D Systems, Minneapolis) while maintained on ice and in the dark. Samples were strained via cell strainers (40 μ m nylon pore) (Becton Dickinson, San Jose, CA) into 200 μ l of flow cytometry buffer and analyzed immediately using BD LSR flow cytometer (Becton Dickinson) and 5000 events were collected per sample. Autofluorescence was used as negative controls. Analysis was performed using BD LSR flow cytometer (Becton Dickinson). Data analysis was performed using BD FACS DIVA v. 4.1.2 (Becton Dickinson Biosciences) or WinMDI 2.8 (Scripps Research Institute, La Jolla, CA).

In order to demonstrate that typically harvested loosely adherent DCs for further analysis were representative of the entire DC population in a well (loosely adherent/nonadherent and adherent), in some experiments, the adherent cell fractions were removed from cell culture dishes using pre-warmed cell dissociation solution (CDS) (Sigma). Dendritic cells or associated lymphocytes present in DC cultures were identified based on size separation and counted using Coulter Multisizer III (Beckman Coulter) for both fractions, for all treatments. Data has been presented as % adherent DCs of the total

DCs present for different treatments. Furthermore, adherent cell fractions were also analyzed by flow cytometry as described above. Both DCs as well as associated lymphocytes remained adherent to TC dishes at the 24 hr timepoint measured, under iDC or mDC treatments ($28 \pm 18\%$ or $35 \pm 21\%$ mean adherent DCs of total DCs present in cell culture wells, respectively) or different SAM treatments; CH₃, OH, COOH or NH₂ SAM ($45 \pm 32\%$, $54 \pm 19\%$, $34 \pm 28\%$ or $53 \pm 25\%$ mean adherent DCs of total DCs present in cell culture wells, respectively), as measured using six different donors. However, both loosely adherent fractions as well as adherent fractions on all treatments, as determined from three different donors, were composed of similar cell types; CD3+ T cells, CD19/CD24+ B cells as well as CD1c+ DCs and were therefore not distinct populations. Furthermore, although no significant differences in expression were observed for maturation markers CD83, CD86, HLA-DQ or HLA-DR between the DCs in the loosely adherent or adherent cell fractions, higher expression of CD80 was observed for DCs present in the adherent fraction as compared to DCs in the loosely adherent fraction. This was observed with multiple treatments and may be as a result of the DC maturation induced while instigating the detachment of trigger-sensitive DCs from well bases. Hence, throughout this study, loosely adherent DCs [similar to loosely adherent migratory blood-resident DCs (Banchereau 2000)] were collected and examined using different assays.

Mixed lymphocyte reaction (MLR)

The allostimulatory capacities of DCs treated with different SAM endgroups were assessed using a mixed lymphocyte reaction (MLR) as per methods in (Yoshida 2004)

using an allogeneic MLR. On day 6 of DC culture, allogeneic T cells were isolated from PBMCs by negative selection using Pan T cell magnetic isolation (Miltenyi Biotech, Auburn, CA) according to manufacturer's protocols. These cells were used as responder cells. The T cells were resuspended in RPMI-1640 supplemented with 25 mM HEPES and L-glutamine (Gibco BRL) with additional 100 U/ mL penicillin- streptomycin (Cellgro) and heat inactivated filter sterilized (0.22 μ m) (Corning) 10% human AB serum [RPMI-10AB media] and plated at a concentration of 10^5 cells/ well in a 96- well flat-bottomed plate (Corning) in triplicate for treatment groups or controls. Dendritic cells cultured in DC media with or without maturation stimulants (LPS) or treated with different SAM endgroup surfaces were resuspended at 1.6×10^5 cells/ mL, and treated with 25 μ g/ mL mitomycin C (Sigma) for 30 minutes at 37°C to prevent their proliferation. Upon extensive washing with RPMI-10AB media, DCs were counted, resuspended and added to responder cells in triplicates at graded DC: T cell ratios. Cells were co-cultured for 4 days at 37°C, with the addition of 10 μ M 5-bromo-2-deoxyuridine (BrdU) for the last 24 hrs of culture. Dendritic cell-induced T cell proliferation was measured using BrdU colorimetric cell proliferation ELISA (Roche Applied Science, Indianapolis, IN) according to manufacturer's directions.

Cytokine secretion per DNA amount in cell culture supernatants

Levels of pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) or IL-6 or anti-inflammatory mediators such as IL-10, transforming growth factor- β (TGF- β) or prostaglandin E-2 (PGE-2) or other factors such as shed soluble TNF Receptor Inhibitor (sTNFR I) normalized to DNA amount present in the cell culture supernatants

were detected using appropriate sandwich ELISAs (R&D Systems) and DNA amounts were measured using PicoGreen DNA kit (Molecular Probes, Oregon, USA) for both loosely adherent cells and the non-adherent cell fractions (removed using trypsin/ 0.05% EDTA) (Sigma), following manufacturer's protocols. Levels of shed CTLA-4 per DNA amount was measured using ELISA (Bender Med Systems, Burlingame, CA). Supernatants were collected by swirling cell culture dishes clockwise, anti-clockwise, from left to right and from right to left, ten times in each direction on Day 6 of culture following 24-hr treatment with different SAM endgroup surfaces, cleared by centrifugation at 1100 rpm for 10 minutes and stored at -20°C for analysis. Amounts of pro-inflammatory cytokines were presented normalized against total DNA amounts for each treatment group.

Detection of apoptosis

Apoptotic cells were identified by staining using Annexin V labeled with Fluorescein Isothiocyanate (FITC) for 1 hr at 4°C in the dark and on ice, and adding Phycoerythrin (PE) -propidium iodide in the dark immediately prior to analysis (both from BD Pharmingen). Apoptotic controls of staurosporine (Sigma)-treated apoptotic DCs incubated for 1, 3 or 6 hrs [optimal concentration of 10 mM in Dimethylsulfoxide (DMSO (Sigma)) determined by dose response] and of 70% ethanol- treated necrotic DCs incubated at 4°C for 1 hr were stained with FITC-Annexin V alone, or PE-Propidium iodide alone, or both and were used to identify populations by defining the positions of horizontal and vertical quadrant axes. To further elucidate mechanism, the levels of active Caspase-8/FLICE (Molecular Probes, California, USA) or active

Caspase-3 (R&D Systems) that were present in the cell lysates of DCs and associated T cells as a result of different SAM treatments were measured. Finally, apoptotic DNA fragmentation induced by cell culture on the different SAM endgroups was measured by Terminal transferase dUTP nick end labeling (TUNEL) staining (R&D Systems) for DCs or associated T cells.

Statistical Analysis

Statistical analysis was performed using two-way mixed model analysis of variance (Young 2001), setting variance due to treatments as the fixed factor and variance due to donors or due to different draws performed for the same donor as the random factors (for pooled data) and performing pairwise comparisons between treatments with a Tukey post-test using Minitab software (Version 13.20, Minitab Inc., State College, PA). To observe the effects of different treatments compared to iDCs, one-sided Student's T Test was performed (Microsoft Excel v. 2003). The p value of less than or equal to 0.05 was considered significant.

RESULTS:

Human PBMC-derived DCs exhibit rounded morphology when treated with CH₃ SAM endgroups or dendritic morphologies when treated with other SAM endgroups

Dendritic cells were treated with the different SAM surfaces in DC media and their morphology compared with that of iDCs or LPS-treated mDCs. Mature DCs showed extensive cellular processes in contrast to the smooth iDCs. Among SAM endgroups, DCs treated with CH₃ SAM in DC media demonstrated rounded cellular morphology

similar to iDCs while DCs treated with OH, COOH or NH₂ SAM which exhibited dendritic processes similar to mDCs (Figure 5-1).

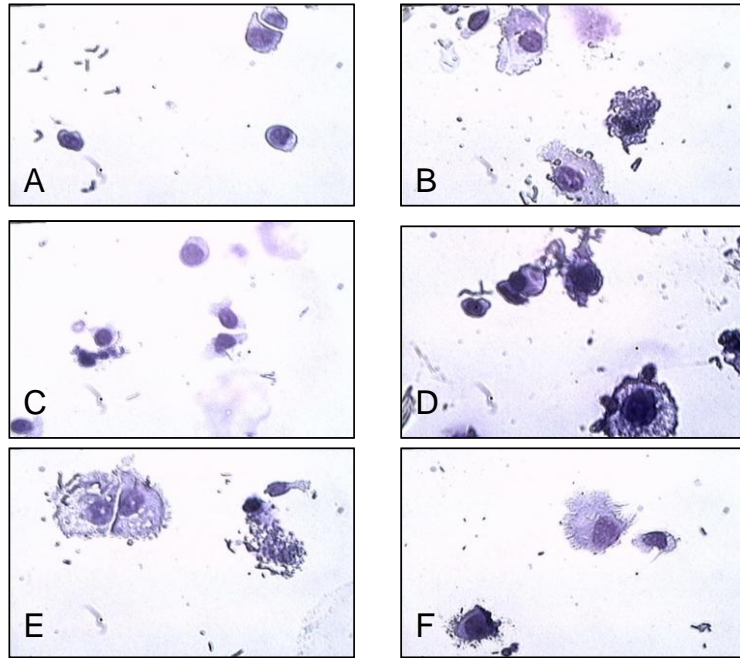


Figure 5-1: Dendritic cells treated with different SAM endgroups show variations in cell morphology.

Immature DCs derived from human PBMCs were cultured in the presence of GM-CSF and IL-4 until Day 5 and then plated on polystyrene surfaces without (a) or with (b) LPS or on CH₃ (c), OH (d), COOH (e), or NH₂ (f) SAM endgroups for 24 hrs. Giemsa-stained cytopins taken on Day 6 of culture indicated that DCs treated with OH, COOH or NH₂ SAM endgroups exhibited dendritic processes similar to positive control LPS-treated mDCs and unlike those treated with CH₃ SAM endgroups which were similar to iDCs. Original magnification: 40x. Cytopins were prepared for six donors with similar results, and representative results are shown.

Dendritic cells treated with CH₃ SAM endgroups are less allostimulatory than iDCs while DCs treated with all other SAM endgroups are more allostimulatory than iDCs at different DC: T cell ratios

The allostimulatory capacities of DCs treated with the different SAM endgroups in DC media were assessed using an MLR. In Figure 5-2, the Y-axis represents the

absorbances associated with induced T cell proliferation as measured using a colorimetric BrDU assay and the X-axis represents the different DC:T cell ratios. As expected, mDCs were more allostimulatory than iDCs at lower DC:T cell co-culture ratios. Among SAM endgroups, DCs treated with NH₂, COOH or OH SAM endgroups in DC media were more allostimulatory than iDCs at different DC: T cell ratios. In contrast DCs treated with CH₃ SAM endgroups in DC media were less allostimulatory than iDCs at higher DC: T cell ratios. At the DC: T cell ratio of 1:3.125, DCs treated with COOH SAM triggered higher T cell proliferation than DCs treated with CH₃ SAM.

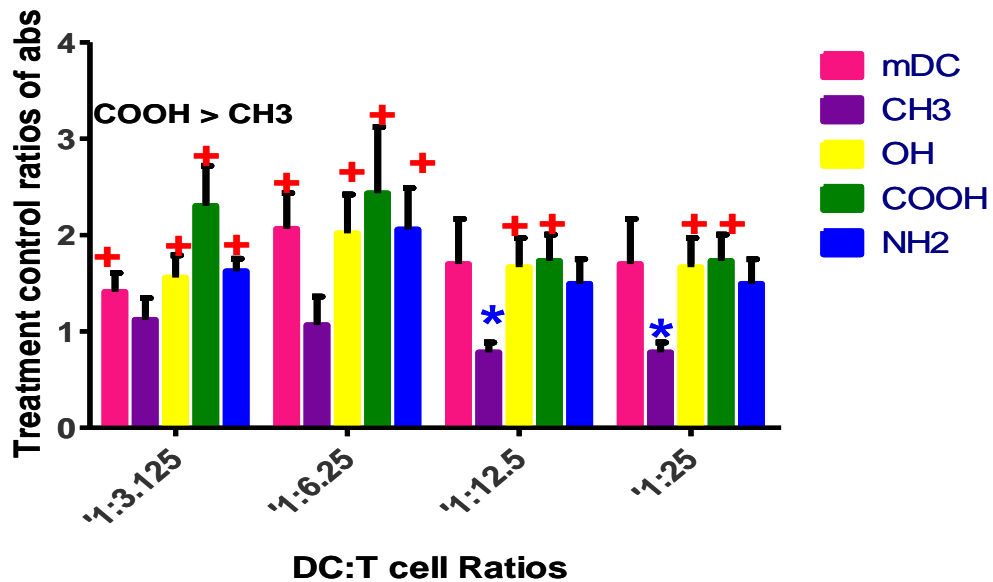


Figure 5-2: Dendritic cells treated with different SAM endgroups exhibit differential allostimulatory profiles in an MLR.

Mature DCs were significantly more allostimulatory than iDCs at lower DC: T cell ratios. Dendritic cells treated with NH₂, COOH or OH SAM endgroups were significantly more allostimulatory than iDCs at different DC: T cell ratios. In contrast, DCs treated with CH₃ SAM endgroups were less allostimulatory than iDCs at higher DC: T cell ratios. This experiment was repeated six times with different donor combinations. Data represented as treatment control ratios of the absorbances associated with induced T cell proliferation for each treatment revealed statistical significance of the findings; mean \pm SEM, n=3 wells. '+' indicates greater than iDC, '*' indicates less than iDC, differences between SAM endgroups in words, $p \leq 0.05$.

Dendritic cells exhibit differential expression of maturation markers when treated with SAM endgroups having different surface chemistries

The expression levels of various markers of DC phenotype were assessed using flow cytometry. As shown in Figure 5-3, generally, iDCs expressed low levels of these markers and maturation induced by LPS treatment resulted in an increase in the expression of these markers, as observed with CD80, CD83 or HLA-DQ. Among SAM endgroups, DCs treated with OH, COOH or NH₂ SAM in DC media had higher expression of maturation markers CD80, CD83 or CD86 than iDCs. In contrast DCs treated with CH₃ SAM in DC media had higher expression of HLA-DQ receptor, that has strong anti-inflammatory apoptosis-inducing effects upon ligation (Leverkus 2003; Zang 2005a; Zang 2005b), compared to iDCs. Furthermore, mature DCs had higher expression of CD80 compared to all SAM chemistries as well as iDCs (Figure 5-3).

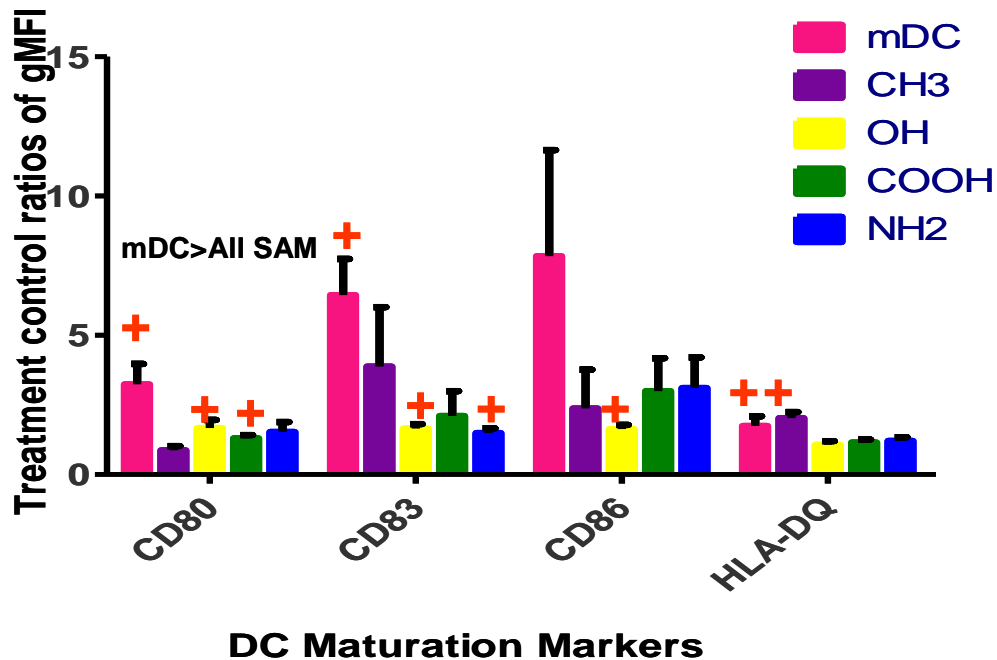


Figure 5-3: Dendritic cells treated with different SAM endgroups exhibit differential expression levels of DC maturation markers.

Dendritic cells matured with LPS exhibited higher expression of CD80, CD83 or HLA-DQ compared to iDCs. Dendritic cells treated with OH, COOH or NH₂ SAM endgroups increased expression of CD80, CD83 or CD86 as compared to iDCs, but to a lower extent than DCs treated with LPS as observed with CD80 expression. In contrast, DCs treated with CH₃ SAM endgroups had higher expression of HLA-DQ as compared to iDCs. This experiment was performed using six independent donors in triplicate. Data represented as treatment control ratios for each treatment revealed statistical significance of the findings; mean ± SEM, ‘+’ indicates increase over iDC, differences between SAM endgroups in words, $p \leq 0.05$.

Human PBMC-derived DCs secrete high TNF- α or IL-6 in response to CH₃ SAM contact

As another method of evaluating DC responses to different SAM endgroups, levels of pro-inflammatory cytokines such as TNF- α or IL-6 present in cell culture supernatants of iDCs, mDCs or DCs treated for 24 hrs in DC media with different SAM endgroups were measured using ELISAs and the amounts were normalized against total cellular DNA amount. For TNF- α , as expected, LPS-treated mature DCs secreted significantly higher levels of TNF- α than iDCs, across combined values for three

different donors performed in triplicate (Figure 5-4a) or as indicated for a representative donor (Figure 5-4b). Among SAM endgroups however, surprisingly, it was observed that DCs treated with CH₃ SAM in DC media secreted highest levels of pro-inflammatory TNF- α and DCs treated with OH SAM in DC media instigated higher TNF- α secretion than DCs treated with COOH or NH₂ SAM (Figure 5-4b).

Similar results were obtained for amounts of IL-6 secreted by DCs treated with different SAM endgroups in DC media normalized against total DNA amounts. As expected, LPS-treated mature DCs secreted significantly higher levels of IL-6 than iDCs, across pooled values for three different donors performed in triplicate (Figure 5-4c) or as indicated for a representative donor (Figure 5-4d). As shown in Figure 5-4d, DCs treated with CH₃ or OH SAM endgroups in DC media were associated with higher secreted IL-6 levels compared with DCs treated with other SAM endgroups.

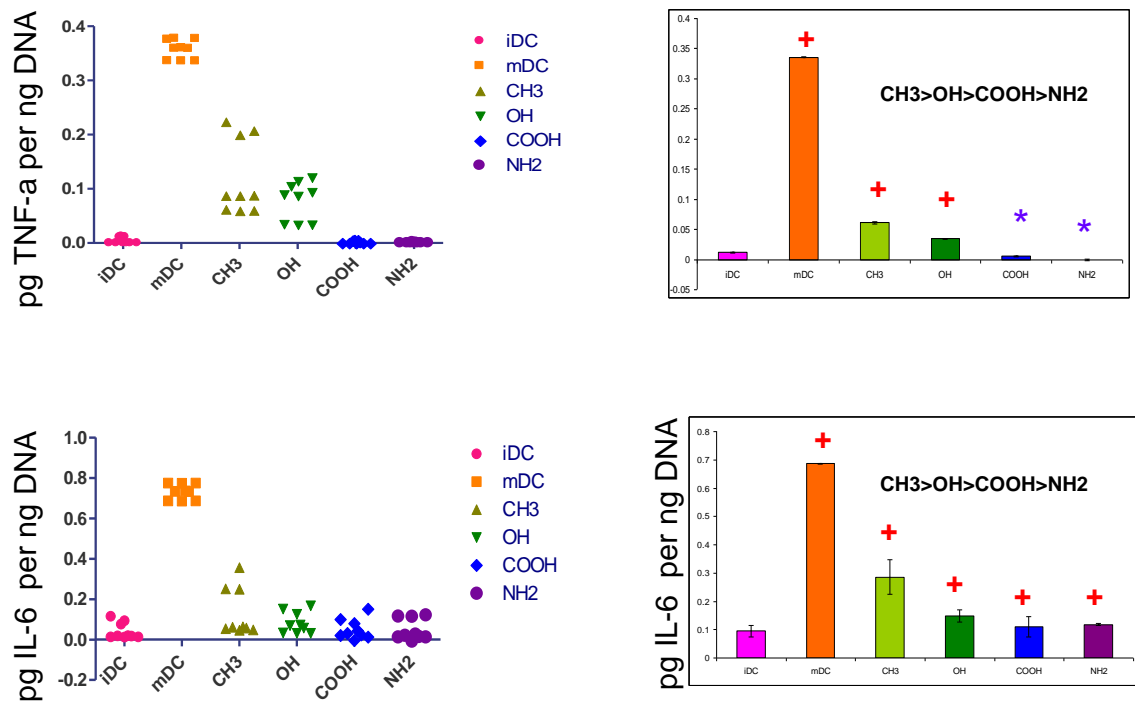


Figure 5-4: Contact with CH₃ SAM induces highest secretion of pro-inflammatory cytokines from human PBMC-derived DCs compared with other SAM endgroups. Supernatants from iDCs, LPS-matured DCs, or DCs treated with different SAM endgroups were collected and TNF- α (a, b) or IL-6 (c, d) content was measured and shown normalized against DNA content. The figures for TNF- α (a) or IL-6 (c) indicate combined data from all three different donors in triplicate and hence a representative figure from one donor has been shown for TNF- α (b) or IL-6 (d). The DCs treated with LPS secreted high levels of both pro-inflammatory cytokines with all donors. Among SAM endgroups, contact with CH₃ SAM induced highest TNF- α or IL-6 secretion by DCs. For (b) or (d); mean \pm S.D, ‘+’: indicates greater than iDCs; ‘*’: indicates less than iDC, differences between SAM endgroups in words, $p \leq 0.05$.

Secretion of anti-inflammatory mediators – IL-10, TGF- β or stimulators of anti-inflammatory alternatively activated DCs (AA-DCs) – PGE₂

To assess levels of anti-inflammatory mediators that may be present in the media of DCs treated with CH₃ SAMs in DC media and counteract the effects of high pro-inflammatory cytokine levels and account for lower DC maturation observed on this surface, ELISAs were performed to measure levels of IL-10, TGF- β or PGE₂ in the DC

supernatants per total DNA amount. Undetectable levels of IL-10 were present in supernatants of all SAM endgroups (data not shown). No clear trends were observed in levels of TGF- β or PGE₂ in supernatants from different SAM endgroups (data not shown).

Human PBMC-derived DCs undergo high apoptosis in response to CH₃ SAM contact

To examine if more DCs on CH₃ SAM were undergoing apoptosis and causing DC immunosuppression, since phagocytosis of apoptotic DCs has strong anti-inflammatory effects on DCs (Ferguson 2002; Clayton 2003; Duffield 2003; Newton 2003; Skoberne 2005a; Wallet 2005a; Chen 2006b), the percentages of live, early apoptotic, late apoptotic or necrotic, or non-viable DCs were assessed by flow cytometry by staining for annexin V and propidium iodide as presented as treatment control ratios. Interestingly, contact with CH₃ SAM induced higher early apoptosis of DCs in comparison to iDCs. Furthermore, contact with CH₃ SAM induced lowest levels of live DCs as compared both to mDCs as well as to all other SAM chemistries (Figure 5-5).

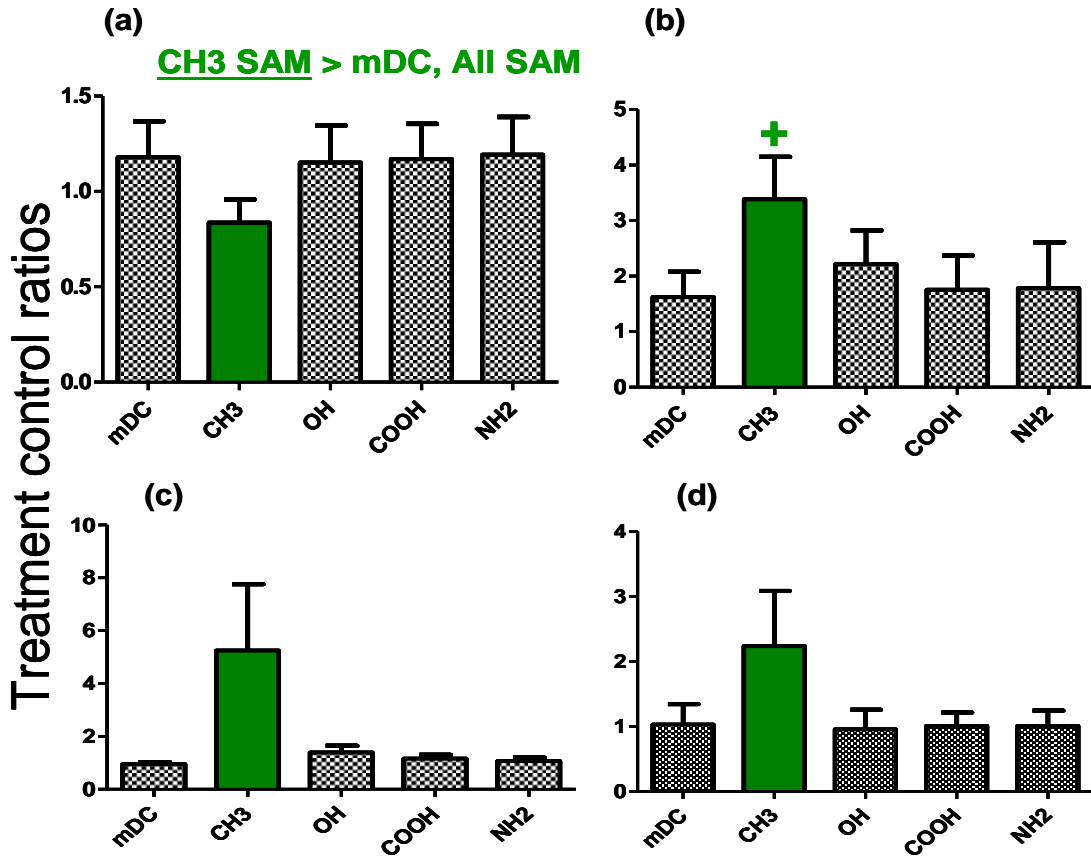


Figure 5-5: Contact with CH₃ SAM induces fewer live human PBMC-derived DCs than all other treatments and enhances early apoptosis of DCs compared to iDCs.

Cells were harvested following 24 hrs of biomaterial culture and stained for annexin V and propidium iodide. Data is presented as treatment control ratios of cells present in each group; Annexin V/ propidium iodide -/-, +/-, +/+, -/+ indicating cells that were live (a), early apoptotic (b), late apoptotic or necrotic (c) or non-viable (d) respectively. Contact with CH₃ SAM resulted in least live DCs compared to mDCs and to all other SAM endgroups as well as higher early apoptosis of DCs as compared to iDCs. This experiment was repeated six times with six different donors; mean \pm S.D, '+' indicates greater than iDC, differences between SAM endgroups in words, $p \leq 0.05$.

Human PBMC-derived DCs undergo high caspase- 8 - mediated apoptosis in response to

CH₃ SAM contact

To investigate if the mechanism of apoptosis observed with cells on SAM endgroups was mediated by cytosolic caspase proteins, ELISAs were performed to

measure amounts of active caspase-8 or active caspase-3 present in cell lysates. Contact with CH₃ SAM induced highest levels of active cellular caspase-8 compared to iDCs, mDCs and DCs treated with OH or NH₂ SAM chemistries (Figure 5-6). Undetectable amounts of caspase-3 were detected in DC lysates for all SAM treatments (data not shown).

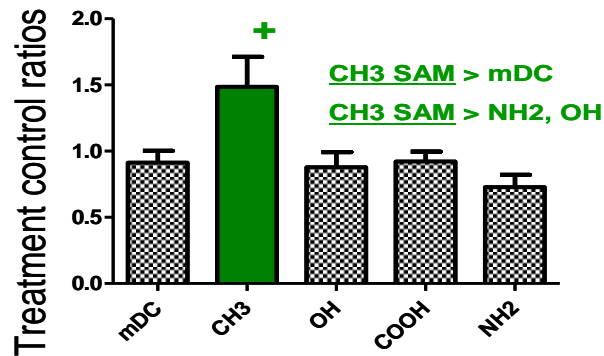


Figure 5-6: Contact with CH₃ SAM induces higher levels of active caspase-8 from lysed cells compared with mDC treatment or compared to cells treated with OH or NH₂ SAM endgroups.

Following 24 hrs of biomaterial culture, cells were lysed and active caspase-8 content was measured. Contact with CH₃ SAM induced higher levels of active caspase-8 compared with cells treated with OH or NH₂ SAM endgroups or cells exposed to LPS. This experiment was performed six times with six different donors; mean \pm S.D, ‘+’: indicates greater than iDCs; differences between SAM endgroups in words, $p \leq 0.05$.

Human PBMC-derived autologous T cells exhibit higher TUNEL positive staining in the presence of mDCs or DCs treated with CH₃ SAM or NH₂ SAM

DNA fragmentation associated with end stage apoptosis was measured by quantification of TUNEL positive cells. The autologous T cells present in the DC culture under LPS, CH₃ SAM or NH₂ SAM treatments had higher TUNEL positive staining as compared to T cells cultured in the presence of iDCs. Among treatment groups, no

differences were observed in numbers of TUNEL positive DCs (Figure 5-7).

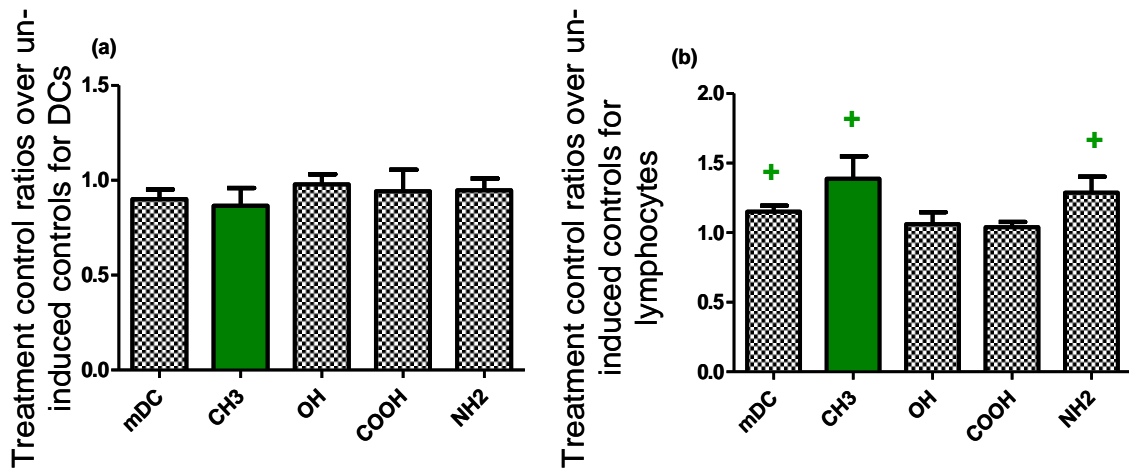


Figure 5-7: Associated T cells present in DC cultures treated with different SAM endgroups exhibit differential TUNEL staining.

Dendritic cells treated with different SAM endgroups exhibited no differences in TUNEL staining (a). However, higher TUNEL positive staining was observed for associated T cells present in DC cultures with certain treatments including for those on CH₃ SAM as compared to iDCs (b). This experiment was performed six times with six different donors; mean \pm S.D, '+' indicates greater than iDCs, $p \leq 0.05$.

Human PBMC-derived DCs shed similar amounts of sTNFRI on different SAM endgroups

The levels of shed sTNF RI that negatively regulates TNF- α -mediated apoptosis by competitively binding soluble TNF- α were measured (Brodbeck 2002b). However, no differences were observed in sTNFRI levels in supernatants from different SAM endgroups (data not shown) across all donors tested.

Human PBMC-derived DCs or T cells demonstrate high positive staining for CTLA-4 in response to CH₃ SAM contact; undetectable levels of shed CTLA-4 with all SAM endgroups

To identify if CTLA-4 expressed by activated T cells that binds the B-7 family of

co-stimulatory receptors on DCs and causes T cell inhibition, was involved in immunosuppression observed on CH₃ SAM (Walunus 1998; Oosterwegel 1999; Orabona 2006), levels of shed or bound CTLA-4 were measured for DCs or T cells treated with the different SAM endgroups in DC media. Interestingly, highest bound CTLA-4 was measured for DCs treated with CH₃ SAM endgroups in DC media compared to all other treatments (Figure 5-8a) or for T cells treated with CH₃ SAM endgroups compared to iDCs, mDCs or DC treated with OH or NH₂ SAM (Figure 5-8b). No differences were observed in levels of shed CTLA-4 in supernatants from different SAM endgroups (data not shown).

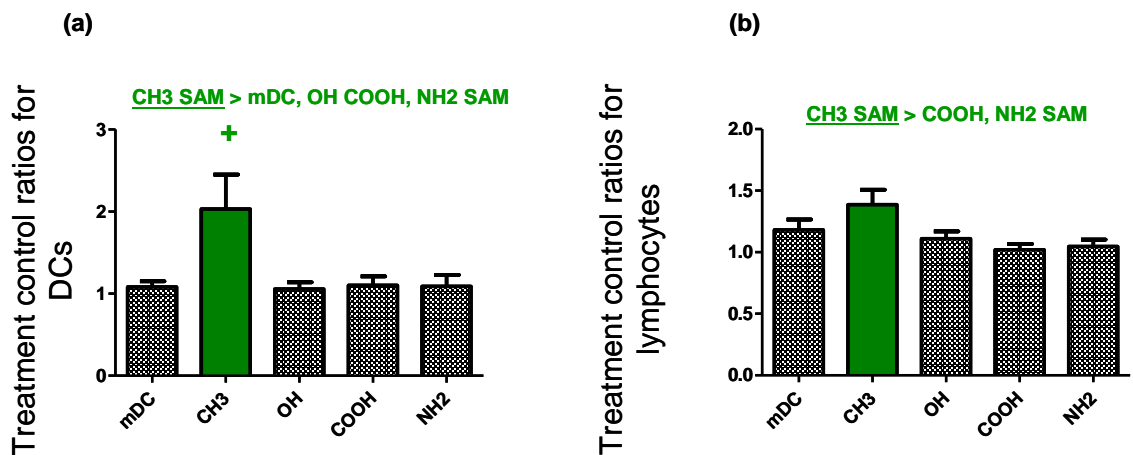


Figure 5-8: Dendritic cells and associated T cells treated with CH₃ SAM endgroups increase expression of bound CTLA-4; no differences in shed CTLA-4 levels among SAM endgroups.

Contact with CH₃ SAM endgroups triggered increases in expression of CTLA-4 for DCs as compared to all other treatments (a). Furthermore, contact with CH₃ SAM endgroups triggered increases in expression of CTLA-4 for associated T lymphocytes present in DC cultures as compared to iDC treatment or to DCs exposed to OH, COOH or NH₂ SAMs (a) or to T cells exposed to COOH or NH₂ SAM endgroups (b). This experiment was repeated six times with six different donors; mean \pm S.D, '+' indicates greater than iDCs; differences between SAM endgroups in words, $p \leq 0.05$.

DISCUSSION:

Dendritic cells treated with different SAM endgroups showed differential levels of maturation as assessed by examining morphology, maturation marker expression and allostimulatory capacities. Based on morphology, DCs treated with CH₃ SAM in DC media were least mature (Figure 5-1). In a functional MLR assay, DCs treated with CH₃ SAM endgroups in DC media were also less allostimulatory than iDCs at different DC: T cell ratios while DCs treated with other SAM endgroups were more allostimulatory (Figure 5-2). Furthermore, from maturation marker expression, DCs treated with OH, COOH or NH₂ SAM endgroups in DC media showed modestly higher activation than iDCs (Figure 5-3). In contrast, DCs treated with CH₃ SAM endgroups in DC media expressed highest levels of HLA-DQ receptor (Figure 5-3), which upon ligation induces immunosuppressive DC apoptotic death (McClary 2000; Keselowsky 2003; Barbosa 2004). To summarize, OH, COOH or NH₂ SAM endgroups caused modest DC maturation while CH₃ SAM endgroups caused lower DC maturation.

The interesting contradiction observed was that DCs treated with CH₃ SAM endgroups in DC media were less mature than those treated with other SAM endgroups, but were so in the presence of highest levels of pro-inflammatory cytokines (Figure 5-4). An analogous situation was observed *in vivo* using a rodent air pouch model where CH₃ SAM endgroups showed low cell adhesion density even though OH or CH₃ SAM implants recruited highest inflammatory cells (Barbosa 2004). To probe the mechanism by which DCs remained less mature while being exposed to high levels of pro-inflammatory cytokines in our system, the levels of immunosuppressants such as IL-10, TGF-β or stimulators of AA-DCs such as PGE₂ in media on CH₃ SAM endgroups that

may counteract the effects of pro-inflammatory cytokines and cause lower DC maturation were assessed, but found to not be responsible. Low DC maturation on CH₃ SAM may be explained by highest presence of apoptotic DCs and high presence of apoptosis-induced non-viable autologous T cells on these surfaces. High T cell apoptosis on CH₃ SAM may have been induced by high TNF- α in the microenvironment or by ligation of highly expressed HLA-DQ (Leverkus 2003; Zang 2005a; Zang 2005b). It is worth noting however that although both DCs and autologous T cells on CH₃ SAM were undergoing apoptosis, autologous T cells were TUNEL+ indicative of end stage apoptosis, while DCs were positive for caspase-8 and Annexin V/ propidium iodide, which are markers of early apoptosis (Figures 5-7). If lower sTNFR1 levels had been observed in CH₃ SAM supernatants, thereby accounting for the high measured soluble TNF- α on these surfaces, this mechanism of triggering TNF- α -mediated apoptosis may have been implicated. Taken together, these results explain in part why lower DC maturation was observed on CH₃ SAM endgroups and also shed light on the possible role played by high secreted TNF- α in the microenvironment, which acts not only as a DC maturation stimulus (Bertho 2001), but also plays a role as a potent inducer of DC apoptosis (Bertho 2001), as previously described in the literature.

These findings are supported by recent literature that implicates apoptosis as a mechanism for DC-mediated tolerance (Ferguson 2002; Clayton 2003; Duffield 2003; Newton 2003; Skoberne 2005a; Wallet 2005b; Chen 2006c). Apoptotic cell uptake by DCs results in downregulation of DC maturation markers and allogeneic T cell responses (Ferguson 2002; Clayton 2003; Duffield 2003; Newton 2003; Skoberne 2005a; Wallet 2005b). This is cell-type specific. Although the engulfment of neutrophils or other DCs

causes DC tolerization, phagocytosis of autologous T or B cells does not, hence only DCs have been gated for Figure 5-5. Finally, high bound CTLA-4 was measured on DCs (Figure 5-8a) or autologous T cells (Figure 5-8b) treated with CH₃ SAM endgroups in DC media. Binding of CD86 on DCs to CTLA-4 on T cells acts as an inhibition signal for T cells via decreased IL-2 synthesis and triggers a tolerogenic response (Walunus 1998; Oosterwegel 1999; Orabona 2006). The presence of CTLA-4 on DCs which normally do not express the receptor may represent an instance of transfer of cell surface proteins between different cell types of which only one type characteristically expresses the protein, but then bestows it upon the other cell type. This suggestion stems from a recent review (Davis 2007) that proposes a mechanism whereby proteins channeled from anergic T cells to APCs, possibly mediated by exosomes, may then induce APC tolerance against the agonist peptide. Although the nature of the agonist remains unclear for biomaterials, a similar mechanism of regulatory T cell-induced DC tolerization may occur on CH₃ SAM. These findings are supported in part by studies that have demonstrated that the presence of lymphocytes in the DC culture may be significant in providing necessary DC differentiation signals. Specifically, cell-cell communication between DCs and associated B (Bayry 2004; Bayry 2005) or T cells (Latour 2001; Johansson 2003) has been shown to affect DC maturation as well as migration events. Furthermore, these results suggest that the presence of associated lymphocytes may also be involved in influencing DC-mediated host responses to biomaterials. This study is novel as it further elucidates the mechanisms responsible for DC immunosuppression on model SAM biomaterials.

Interestingly, both the loosely adherent as well as adherent cell fractions for all

treatments comprised similar cell types, namely CD3⁺ T cells, CD19/CD24⁺ B cells as well as CD1c⁺ DCs. Dendritic cells present in both fractions exhibited similar levels of most maturation markers: CD83, CD86, HLA-DQ or HLA-DR, but demonstrated significantly increased levels of CD80 expression following induced detachment from multiple biomaterial treatments using CDS. Therefore, in order to focus solely on the abilities of the different biomaterials to support DC maturation and ignore the contributions of other effects, the gently-harvested DCs present in the loosely adherent fraction alone was considered in this study. Future work may include the characterization of the expression of integrin receptors including CD50, CD54, CD58, CD11b or CD11c that mediate cell adhesion and are also known to have differential expression on iDCs and mDCs and mediate maturation or apoptotic functional events in DCs (Janeway 1998; Nasr 2006; Skoberne 2006), to gain additional insights regarding mechanisms behind DC adherence and their significance if any, in regulating DC responses.

The pro-inflammatory nature of OH SAM endgroups observed here is supported in the literature. The OH SAMs have been associated with high leukocyte adhesion linked to high complement activation (Tegoulia 2000) and significantly higher amounts of pro-inflammatory fibrinogen (Fn) than hydrophobic SAM (Silver 1995), although others have found otherwise (Lindbald 1997; Kalltorp 1999; Shen 2001). Furthermore OH SAMs have been associated with higher adsorbed pro-inflammatory complement-3 (C3) or immunoglobulin G (IgG) amounts than CH₃ SAM endgroups (Sigal 1998). Furthermore, the cationic polysaccharide chitosan when co-administered with antigens, caused greater mucosal and systemic immune responses, as demonstrated by an upregulation of MHC class II receptors on OX62⁺ DCs and triggering CD3⁺ T cell

activation in the spleen (Bivas-Benita 2004), in agreement with these findings that demonstrate NH₂ SAM as a modest stimulator of DC maturation and with other studies (Porporatto 2005; Wang 2006).

The literature regarding the pro- or anti-inflammatory nature of CH₃ SAM endgroups is conflicting. Although it was concluded based on *in vitro* and *in vivo* studies (Lindbald 1997; Barbosa 2004; Barbosa 2005a; Barbosa 2005b; Barbosa 2006) that CH₃ SAM endgroups caused an acute inflammatory response as exemplified by increased recruitment of inflammatory cells to CH₃ SAM endgroups, higher presence of Mac-1⁺ cells as well as thicker fibrous capsules, lowest cell adhesion has been observed to CH₃ SAM endgroups (Barbosa 2003; Barbosa 2004; Barbosa 2005a; Barbosa 2005b; Barbosa 2006). It is unclear if upregulated Mac-1 integrin (CD11b/ CD18) [also Complement Receptor-3 (CR3)] on CH₃ SAM, which has been implicated in internalization of apoptotic cells and DC tolerization (Coxon 1996; Whitlock 2000; Verbovetski 2002; Skoberne 2006), potentiates anti-inflammatory effects on CH₃ SAM, in accordance with our observations. These results suggest that –NH₂, OH or COOH terminal materials may be suitable for applications where a modest immune response is desired, although the mechanisms underlying DC activation may be different, while CH₃ SAM may be better suited for less immunostimulatory applications. The ultimate goal would be to better understand the mechanisms underlying differential DC responses to biomaterials to better design suitable biomaterials for diverse applications.

CHAPTER 6

GLYCOPROTEIN-MEDIATED INTERACTIONS OF DENDRITIC CELLS WITH BIOMATERIALS. PART 2. PROFILES OF CARBOHYDRATES LIGANDS ASSOCIATED WITH ADSORBED PROTEINS ON SAM ENDGROUPS.

*INTRODUCTION:

An understanding on the host-biomaterial interactions accompanying the implantation of combination products composed of a biomaterial component and biological components (tissues, cells, proteins or DNA) is important in ascertaining the success of these implants. Besides the non-specific inflammatory response against the biomaterial, the biomaterial may act as an adjuvant, beneficially, by enhancing the desired specific immune response against the biological component in vaccine delivery systems, but this enhancement may be disadvantageous when biomaterials are utilized in tissue engineering applications, where implant functioning is impaired by fibrous capsule and scar tissue formation as part of the host protective response, or even undergo cell-mediated implant degradation (Langer 1993; Tang 1995; Babensee 1998a; Babensee 2000; Rihova 2000; Stock 2001). A controlled host response to implanted biomaterials is therefore desirable. Although the non-specific host inflammatory response to biomaterials has been examined extensively (Anderson 1988; Langer 1993; Tang 1995; Ratner 1996; Babensee 1998a; Babensee 2000; Rihova 2000; Stock 2001; Wettero 2002), the adjuvant effects of biomaterials have only been recently described (Yoshida 2004; Babensee 2005; Bennewitz 2005; Yoshida 2006a; Yoshida 2006b). The co-delivery of the biomaterial poly (lactic-*co*-glycolic acid) (PLGA) along with model shed antigen (Ag) ovalbumin

* Galactose data was provided by Inn Inn Chen during her time as an Undergraduate Research Scholar at the Babensee laboratory in Georgia Tech, Atlanta, USA.

(OVA) increased the levels of antibodies generated again OVA, demonstrating the adjuvant effects of biomaterials *in vivo* (Ertl 1996; Walker 1998; Bennewitz 2005). Furthermore, since Ag presenting cells (APCs) such as dendritic cells (DCs) mature in response to adjuvants and upregulate their expression of co-stimulatory and major histocompatibility class II (MHC II) molecules and have reduced endocytic and phagocytic functions (Sallusto 1994; Zhao 1996; Cella 1997a; Winzler 1997; Banchereau 1998a; Singh 1999; Banchereau 2000; Sun 2003), the effects of biomaterials in causing DC maturation have been studied *in vitro*. While certain biomaterials such as PLGA caused DC maturation, others such as agarose were less effective at triggering the transition from immature DCs (iDCs) to mature DCs (mDCs) *in vitro*, and would thereby presumably act as weaker adjuvants (Yoshida 2004).

Dendritic cells are professional APCs that connect innate and adaptive immunity and therefore play a vital role in directing the immune response. Upon encountering pathogens or ‘danger signals’ including heat-shock proteins and necrotic cells indicative of tissue injury, iDCs, that express high levels of Pathogen Recognition Receptors (PRRs) that recognize Pathogen Associated Molecular Patterns (PAMPs) transform into mDCs that have increased expression of CD80, CD86 co-stimulatory molecules and MHC II complexes and present internalized processed Ags on MHC complexes to naïve T or B lymphocytes (Zhao 1996; Cella 1997a; Winzler 1997; Babensee 1998a; Singh 1999; Banchereau 2000; Sun 2003). The conserved moieties recognized in PAMPs include bacterial lipopolysaccharide (LPS) (Banchereau 1998a; Gallucci 1999; Banchereau 2000; Figdor 2002; Janeway 2002; van Kooyk 2003; Pulendran 2004), N-acetylglucosamine (GlcNAc) and N-acetylmuraminic acid (MurNAc) in peptidoglycans

(Banchereau 1998a), β (1-3) glucans in zymosan (Brown 2001a; Brown 2003; Brown 2006) and mannose-containing bacterial pili (Silverblatt 1983). Toll-like receptors (TLRs) and C-type lectin receptors (CLRs) are PRRs expressed by DCs that recognize conserved epitopes on PAMPs (Bhardwaj 1994; Lotze 1999; Aderem 2000; Guyton 2000; Termeer 2000; Akira 2001; Brown 2001a; Medzhitov 2001; Okamura 2001; Smiley 2001; Valladeau 2001; Engering 2002; Geitjenbeek 2002; Termeer 2002; Gantner 2003; McGreal 2004; Rogers 2005; Dillon 2006). While the importance of TLR-PAMP interactions in stimulating DC maturation has been well characterized due to (nuclear factor- κ B) NF- κ B mediated regulation of pro-inflammatory/immunoregulatory genes (Termeer 2000; Akira 2001; Medzhitov 2001; Smiley 2001; Termeer 2002), the role of CLR-mediated recognition, uptake, processing and presentation of carbohydrate Ags has emerged recently (Brown 2001a; Valladeau 2001; Engering 2002; Geitjenbeek 2002; Gantner 2003; McGreal 2004; Rogers 2005; Dillon 2006). The CLRs and their carbohydrate ligands in the context of biomaterials have been poorly understood thus far and form the focus of this work.

The CLR family has been broadly classified as mannose or galactose binding (McGreal 2004). Examples of CLRs that bind mannose moieties are mannose receptor (MR), DC-specific ICAM-3 grabbing non-integrin (where ICAM-3 is intercellular adhesion molecule 3) (DC-SIGN) (van Kooyk 2003), and Dectin-1 (Rogers 2005; Brown 2006). Examples of CLRs that bind α - and/or β -galactose or GalNAc moieties are asialoglycoprotein receptor (ASGPR) (Valladeau 2001) and macrophage galactose N-acetyl-galactosamine (GalNAc) specific lectin 1 (MGL) (van Villet 2005). The carbohydrate binding specificities of different CLRs vary and have been reviewed in

(Figdor 2002; van Kooyk 2003). For example, MR recognized terminal mannose, fucose, sialylated groups or GlcNAc and DC-SIGN recognized mannose-enriched internal residues (Geitjenbeek 2002). Carbohydrate recognition by certain CLR has been linked recently with endocytosis and processing of cargo (Jiang 1995; Mahnke 2000; Cobb 2004), activation of intracellular signaling mediators and differential downstream immune consequences via ITAM (immunoreceptor tyrosine-based activation motif) (Jiang 1995; Gantner 2003; Cobb 2004; Rogers 2005; Brown 2006; Dillon 2006) or ITIM (immunoreceptor tyrosine-based inhibitory motif) (Kanazawa 2002) residues. Dectin-1 that possesses an ITAM motif and binds β -glucans, has been implicated in the activation of Syk-mediated signaling pathways (Rogers 2005; Brown 2006). Furthermore, Dectin-1 has been shown to upregulate TLR 2-mediated activation of NF- κ B and exhibit synergism in the regulation of IL-1 and tumor necrosis factor- α (TNF- α) (Gantner 2003).

It is therefore important to characterize the DC carbohydrate ligands present on biomaterial surfaces that may be recognized by CLR and hence mediate DC interactions. The roles of glycans in controlling fundamental immune/inflammatory responses to biomaterials have been poorly studied thus far. It is hypothesized that DCs may respond to biomaterials by recognizing 'biomaterial associated molecular patterns', analogous to PAMPs, through the adsorbed protein layer, specifically through carbohydrate modifications of these proteins using PRRs to initiate an immune response. To characterize the presence of carbohydrate ligands of DC CLR, self-assembled monolayers (SAMs) with $-\text{CH}_3$, $-\text{OH}$, $-\text{COOH}$, or $-\text{NH}_2$ endgroups that present a broad spectrum of surface properties such as charge, hydrophobicity or hydrophilicity,

topography, surface energy and chemistry and have been thoroughly characterized in (Mrksich 1996; Lindbald 1997; Harder 1998; Sigal 1998; Kalltorp 1999; Luk 2000; McClary 2000; Tegoulia 2000; Shen 2001; Barbosa 2003; Keselowsky 2003; Michael 2003; Schwendel 2003; Dadsetan 2004) were chosen. These SAM surfaces were used as model biomaterials to observe the effect of distinct surface properties on directing protein adsorption and carbohydrate presentation on the surface. We have previously characterized DC responses to different SAM chemistries, and observed that while OH, COOH or NH₂ SAM triggered modest DC maturation, least maturation was observed on CH₃ SAM. This was likely due to highest immunosuppressive DC apoptosis upon CH₃ SAM contact (CHAPTER 5). The importance of understanding differential DC responses on different chemistries from the perspective of distinct DC ligands regulated by biomaterial chemistry formed the motivation for this paper.

METHODS:

Self-assembled monolayer preparation

Self-assembled monolayers presenting –CH₃, –OH, –COOH or –NH₂ chemistries were assembled on 16-well glass chamber slides (*LAB-TEK*, Nalge Nunc International, Rochester, NY) for carbohydrate or protein measurement assays or on 9 mm X 9 mm glass coverslips for material characterization assays (Bellco Glass, Inc., Vineland, NJ) (Keselowsky 2003) as described previously (CHAPTER 5) The SAM endgroups were allowed to assemble by 12 hr incubation at room temperature (RT) by immersing the Ti/Au-coated slides or coverslips in alkanethiol solutions, following which the chamber slides were washed with 95% ethanol (Sigma, St. Louis, MO), dried with N₂ gas (Airgas

South, Chamblee, GA) for 10 minutes in a fume hood, equilibrated with Phosphate Buffered Saline (PBS) (Gibco, Grand Island, NY) for 5 minutes at RT and used fresh.

X-ray photoelectron spectroscopy

Characterization of surface chemistries of the SAM endgroups was performed by low resolution survey scans (spot size 400 μm) or high resolution C1s spectra (spot size 200 μm) x-ray photoelectron spectroscopy (XPS) using a Surface Science model SSX-100 (Surface Sciences Laboratories, Mountain View, CA), with monochromatized $\text{Al}_{K\alpha}$ X-rays at 10 kV, at the Microelectronic Research Center at Georgia Institute of Technology. Atomic percentages of elements were obtained from low resolution scans and high resolution scan curves were fit based on software provided by the manufacturer, with placement of the hydrocarbon peak at 284.6 eV. Different takeoff-angles of 0° , 20° , 55° or 70° were used (angle between the beam and the normal to the surface) to permit measurements at varying depths from the film surface. The detection limit for angle resolved XPS is ~ 0.1 atom% in composition and 10-250 \AA in depth (Yoshida 2006a), with maximum and minimum depths attained at takeoff angles of 0° and 90° respectively. Scans were taken on two spots per sample per take-off angle. Prior to XPS, freshly-prepared SAM samples were washed with 95% ethanol and dried with N_2 and analyzed immediately. Theoretical values were determined based on known chemical structure.

Contact angle measurements

Advancing contact angles between freshly-prepared flat SAM and 5 μm drops of de-ionized (DI) H_2O were determined in ambient air using a Rame-Hart model # 100-00

goniometer (Mountain Lakes, NJ). For each surface, contact angles were measured for three water droplets, on two sides of each droplet and the values presented denote the average and standard deviations for three measurements in total.

Preparation of human serum or human plasma

Human blood was obtained from healthy volunteers with informed consent, according to a protocol approved by the Institute Review Board (IRB) # H05012. All subjects enrolled in this research signed an Informed Consent which was approved by the IRB of Georgia Institute of Technology. Peripheral human blood was collected using sterile 60 mL syringes (Becton Dickinson, Franklin Lakes, NJ) and needles (Becton Dickinson) using heparin (333 U/ mL blood) (Baxter Healthcare Corporation, Deerfield, IL) as an anticoagulant. The clear yellowish human plasma (HP) layer was separated using lymphocyte separation medium (LSM) (Cellgro MediaTech, Herndon, VA) by differential gradient centrifugation of blood diluted 1:1 with sterile PBS (400 g, 30 minutes, RT) (Thermo Fisher Scientific Inc., Waltham, MA) (Model # 5682, Rotor IEC 216), filtered sterilized (0.22 μ m) (Corning, Corning, NY) and heat inactivated for 30 minutes in a water bath pre-warmed to 56°C, aliquotted and stored at -20°C. A stock solution of pooled HP from three donors was used for experiments.

For preparation of human serum (HS), peripheral human blood was collected without heparin, so that HS could be isolated from clotting factors. The HP was isolated from non-heparinized blood by centrifugation of blood at 3000 rpm for 10 minutes, RT, after clots were pushed down manually using a sterile pipette tip, where necessary. The HS was then prepared from non-heparinized HP by allowing the HP to remain at RT for

90 minutes in the tissue culture (TC) hood and cleared by further centrifugations at 3000 rpm for 15 minutes after gently pushing down residual precipitates and was filter sterilized (0.22 μm) (Corning) and heat inactivated for 30 minutes in a water bath pre-warmed to 56°C, aliquotted and stored at -20°C. A stock solution of pooled HS from the same three donors as used for HP was used for experiments.

Enzyme linked lectin assay for detection of carbohydrates associated with adsorbed proteins on SAM endgroups or control polystyrene

Enzyme Linked Lectin Assays (ELLAs) were performed on SAM endgroups having pre-adsorbed HS or HP proteins using a previously described method with some modifications (Leriche 2000). The SAM endgroups were incubated with 60 μl / well of serial half dilutions of HS or HP in PBS starting from 20%, down to 2.5% and then from 1% down to 0.25% (v/ v) or with PBS alone (1 hr, 37°C) in duplicate wells in two chamber slides leading to a total of 4 wells per HS or HP concentration. After aspirating out HS, HP or PBS samples from wells, wells were blocked with 0.5 mg/ mL of freshly prepared Bovine Serum Albumin (BSA) (Sigma) in PBS (block buffer; 1 hr, 37°C), washed three times for 5 minutes each with block buffer at RT and incubated with biotinylated lectin [*Narcissus pseudonarcissus* (NPA; 12.5 μg / mL), *Sambucus nigra* (SNA-1; 12.5 μg / mL), *Ulex europeaus I* (UEA-1; 50 μg / mL), *Ulex europaeus II* (UEA-2; 12.5 μg / mL), *Pisum sativum* (PEA; 25 μg / mL), *Hippeastrum hybrid* (HHA; 50 μg / mL), *Peanut agglutinin* (PNA; 12.5 μg / mL), *Artocarpus interifolia* (AIA; 25 μg / mL) or *Bauhinia purpurea* (BPA; 25 μg / mL)] with the carbohydrate detection specificities indicated in Table 5 (all from EY Laboratories, Inc., San Mateo, CA)] in block buffer (2

hrs, 37°C). For UEA-2 lectin, 1.0 mg/ mL heat inactivated BSA (56°C, 30 minutes) in PBS was used in a sensitive blocking step and for PEA or BPA lectins, 1.0 mg/ mL of BSA in PBS was used. Following incubation with the biotinylated lectin, wells were washed and incubated with 10 µg/ mL avidin/alkaline phosphatase (AV/AP) (EY laboratories Inc.) in block buffer (1 hr, 37°C) for detection of bound lectin. The concentration of AV/AP used was 1.25 µg/ mL for SNA-1, PEA or HHA lectins or 20 µg/ mL for AIA or BPA lectins. Finally, wells were washed and incubated with 1.0 mg/ mL p-nitrophenylphosphate (pNPP) (Sigma) substrate for detecting AV/AP (1 hr, 37°C). The solutions were transferred to wells of a clear flat-bottomed TC-treated 96-well plate (Corning) to avoid interference with the reading by the SAM endgroups, reaction stopped at RT with 40 µl of 0.4 M NaOH (Sigma) and absorbance read immediately at 405 nm using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Positive control glycoprotein standard dilutions in PBS (1:1) were run in parallel on TC-treated polystyrene (Phillips) 96-well plates (control) for each lectin; α-2-macroglobulin (from 100 – 12.5 µg/ mL) or (1000 – 3 µg/ mL) for NPA (van Leuven 1993) or HHA (Balzarini 2005) respectively, glycophorin A (100 – 3 µg/ mL) for SNA-1 (Shibuya 1987), lactoferrin (1000 – 62.5 µg/ mL) or (1000 – 15 µg/ mL) for UEA-1 (De Carlos 2002) or PEA respectively, bovine fetuin (1000 – 3 µg/ mL) for UEA-2, asialofetuin (5 – 0.6 µg/ mL) or (31.25 – 1.9 µg/ mL) for PNA (Balu 2001) or BPA (Sueyoshi 1988) respectively and immunoglobulin A (IgA) (50 – 1.5 µg/ mL) for AIA lectin (Roque-Barreira 1985) (all from Sigma), and based on lectin probe specificity data from (van Damme 1998). An identical set of samples (HS or HP) as those run on SAM were also run on PS for each experiment.

Table 5: Dendritic cell C-type lectin receptors, their corresponding carbohydrate ligands and carbohydrate specificities for plant lectins used as probes

C-type Lectin Receptor Abbreviations: ASGPR, asialoglycoprotein receptor; MGL, macrophage galactose N-acetyl-galactosamine specific lectin 1; MR; mannose receptor; DC-SIGN, DC-specific ICAM-3-grabbing non-integrin (where ICAM-3 is intercellular adhesion molecule 3).

Plant Lectin Abbreviations: AIA, Artocarpus integrifolia; PNA, Arachis hypogaea; BPA, Bauhinia pupurea; NPA, Narcissus pseudonarcissus; UEA-1, Ulex europaeus; SNA-1, Sambucus nigra; UEA-2, Ulex europaeus-2; PEA, Pisum sativum; HHA, Hippeastrum hybrid.

Plant Lectin specificity information from (Brown 2001b; McGreal 2004).

C-Type Lectin	Carbohydrate Specificity	Lectin Probe Specificity	Lectin Probe
ASGPR (Van Damme 1998) MGL (Akira 2001; Roque-Barreira 1985)	Galctose Gal/N-acetyl-D-galactosamine (GalNAc) (Van Damme 1998; Cambi 2005; Higashi 2002)	α -galactose	AIA
		β -galactose	PNA
		GalNAc	BPA
MR/MMR (Akira 2001; Roque-Barreira 1985)	Mannose (Akira 2001; Roque-Barreira 1985) Fucose (Akira 2001) Sialyl Lewis X [sLe(x)], N-acetyl-D-glucosamine (GlcNAc) (Hitchen 1998)	Mannose	NPA
		α -L-fucose	UEA-1
		NANA (Neu5Ac α (2,6)Gal/GalNAc)	SNA-I
		GlcNAc β (1,4) GlcNAc	UEA-II
DC-SIGN (Akira 2001; Roque-Barreira 1985)	Mannose Complex mannose residues sLe(x) (Akira 2001; Roque-Barreira 1985)	methyl-D-mannopyranoside, D-mannose	PEA
		mannose α (1,3) and α (1,6) mannose	HHA
		NANA (Neu5Ac α (2,6)Gal/GalNAc)	SNA-I
Dectin-1 (Akira 2001; Roque-Barreira 1985)	β -glucans (Akira 2001; Roque-Barreira 1985)	GlcNAc β (1,4) GlcNAc.	UEA-II

Enzyme linked immunosorbent assay (ELISA) for detection of total human immunoglobulin (IgG) or human serum albumin (HSA)

An enzyme linked immunosorbent assay (ELISA) was performed to measure

amounts of total human IgG or total human serum albumin (HSA) adsorbed from 10% HS (v/ v) onto SAM endgroups. The SAM endgroups were incubated with 60 μ l of 1% or 10% (v/ v) HS in PBS (1 hr, 37°C), followed by blocking with 1.0 mg/ mL of BSA in PBS for HSA ELISA or with 0.5 mg/ mL of BSA in PBS for IgG ELISA (both for 1 hr, 37°C) and incubating with 1:10000 dilution of monoclonal mouse anti-HSA (clone HSA-11; IgG2a) (no cross reaction with BSA) or 1:1000 affinity isolated AP-conjugated polyclonal goat anti-human IgG (IgG γ) (both from Sigma) (2 hrs, 37°C), for detection of HSA or IgG respectively. Wells were washed three times for 5 minutes each with PBS at RT. For the IgG ELISA, wells were incubated with 1.0 mg/ mL pNPP (30 minutes, RT) after incubating with AP-conjugated primary antibody. For detection of HSA, wells were incubated with 1:1000 dilution of affinity isolated AP-conjugated goat anti-mouse IgG (IgG) (Sigma) (2 hrs, 37°C), washed and incubated with pNPP substrate (1 hr, RT). The reactions were stopped with 40 μ l of 0.4 M NaOH (Sigma) at RT and the solutions transferred to 96-well clear-bottomed plates to avoid interference with SAM and absorbance read at 405 nm using a SpectraMax Plus 384 plate reader (Molecular Devices). Standard curves were generated with 1:10 dilutions of purified IgG or HSA (both from Sigma) in PBS (starting from 100 μ g/ mL of IgG or 1 mg/ mL for HSA) on 96-well PS control surfaces. To confirm that the varying carbohydrate profiles observed on different SAM endgroups were in fact different, the ELLA absorbances were normalized to amounts of total human IgG or HSA and the ratios were compared.

Statistical analysis

Statistical analysis was performed using general linear model analysis of variance

(Young 2001) with Minitab software (Version 13.20, Minitab Inc., State College, PA) using pairwise comparisons between SAMs, with a Tukey post-test and a p value of less than or equal to 0.05 was considered significant.

RESULTS

Contact angle and XPS characterizations of Self-assembled monolayer surfaces endgroups show close agreement with established theoretical values

The SAMs presenting endgroup chemistries $-\text{CH}_3$, $-\text{OH}$, $-\text{COOH}$, or $-\text{NH}_2$ represented a broad spectrum of surface properties including charge, hydrophobicity/hydrophilicity and chemistry. The CH_3 SAMs are uncharged, hydrophobic and the OH SAM are neutral, hydrophilic. Under physiological pH conditions (7.2-7.4), the COOH or NH_2 SAM are both hydrophilic, and are negatively or positively charged, respectively (Mrksich 1996; Lindbald 1997; Harder 1998; Sigal 1998; Kalltorp 1999; Luk 2000; McClary 2000; Tegoulia 2000; Shen 2001; Barbosa 2003; Keselowsky 2003; Michael 2003; Schwendel 2003; Dadsetan 2004). The SAM endgroups were characterized by XPS analysis (Table 6) and contact angle determination (Table 7). The low resolution XPS survey scans showed agreement with estimated theoretical values for these surfaces and previously published values (Keselowsky 2003) and indicated modification of Au/Ti surfaces with the alkanethiols with appropriate changes in C 1s and O 1s percentages as expected, for glass surfaces that were coated with Au/Ti and assembled with alkanethiols presenting different functional endgroups. Carbon was the predominant element present for all samples. On control samples coated with Au/Ti alone, no carbon was detected. The percentages of different elements

measured were similar to expected values based on theoretical estimates for all samples using take-off angles of 0°, 20°, 55° and 70° for surface analysis at different depths. Overall the SAMs were composed of alkanethiols that were anchored to the substrates at the furthest end from the surface and that presented functional endgroups at the end closest to the surface (Table 6).

Table 6: Elemental composition of SAM endgroups presenting different chemistries using variable-angle XPS where angle is calculated from the surface plane

SAM	Angle (°)	C1s (%)	O1s (%)	N1s (%)	S2p (%)
CH3	0	89.54 ± 3.88			12.85 ± 7.27
	20	86.97 ± 7.02			13.02 ± 7.03
	55	94.64 ± 2.31			5.35 ± 2.32
	70	95.19 ± 0.65			4.8 ± 0.65
	Theoretical	92.3			7.7
OH	0	83.67 ± 3.01	11.4 ± 3.18		4.84 ± 0.16
	20	84.23 ± 5.98	11.84 ± 5.09		3.92 ± 0.91
	55	85.49 ± 0.3	8.03 ± 1.64		6.45 ± 1.34
	70	83.73 ± 7.39	12.27 ± 2.44		3.95 ± 4.89
	Theoretical	84.6	7.7		4.1
COOH	0	82.5 ± 6.4	15.4 ± 4.77		2.21 ± 1.81
	20	83.65 ± 0.78	9.6 ± 4.35		6.73 ± 6.55
	55	80.17 ± 1.01	14.62 ± 1.21		5.2 ± 2.23
	70	81.68 ± 0.01	13.69 ± 1.88		4.61 ± 1.86
	Theoretical	79	14		7
NH2	0	77.31 ± 3.86		8.60 ± 1.56	14.01 ± 2.02
	20	87.29 ± 1.38		8.55 ± 1.13	4.14 ± 2.51
	55	83.35 ± 7.23		10.98 ± 3.72	5.67 ± 3.64
	70	80.35 ± 1.44		10.71 ± 6.13	8.92 ± 7.58
	Theoretical	86		7	7

Advancing contact angles were measured (on both sides of the drop) between 5- μ m drops of DI water and glass coverslips coated with Au/Ti, on which the different

SAM endgroups were allowed to assemble, to measure the degree of hydrophobicity/hydrophilicity of the surface. The contact angle measurements indicated that 11-mercapto-1-undecanol with a terminal hydroxyl group was the most hydrophilic surface and that 1-dodecanethiol, with a terminal methyl group, was the most hydrophobic (Table 7) (Tanahashi 1997; Sigal 1998; Franco 2000; Keselowsky 2003).

Table 7: Advancing contact angles between different SAM endgroups and water droplets in ambient air

SAM	Advancing angle (°)
CH3	108 ± 2
OH	24 ± 5
COOH	31 ± 3
NH2	35 ± 4

Differential ELLA absorbances on different SAM endgroups or control PS

The ELLA assays were performed using lectin probes mentioned in Table 5 to characterize the carbohydrates ligands of DC CLR associated with the adsorbed HS or HP proteins on different SAM chemistries. On 96-well TC-treated PS surfaces treated as control, linear standard curves were generated for each lectin-glycoprotein (Figure 6-1a-h) to confirm assay reproducibility. In general, absorbances corresponding to the presence of specific carbohydrates decreased when PS surfaces were pre-incubated with decreasing HS dilutions in PBS (1:1 dilutions between 20% and 2.5% and between 1% and 0.25%) when probed with AIA (β -galactose) or BPA (GalNAc) galactose-family lectins (Figure 6-2 a-d). In contrast, pre-incubation of PS surfaces with decreasing HS

concentrations resulted in increasing absorbances when probed with NPA (mannose) or UEA-2 (GlcNAc) mannose-family lectins or unchanged absorbances with different HS dilutions for UEA-1 (fucose) or SNA-1 (sialylated groups) (Figure 6-3 a, c-f). For PEA (complex mannose) high absorbances were detected when PS surfaces were pre-incubated with concentrations of 2.5% or 1% HS (intermediate among the range of HS concentrations tested) (Figure 6-3b).

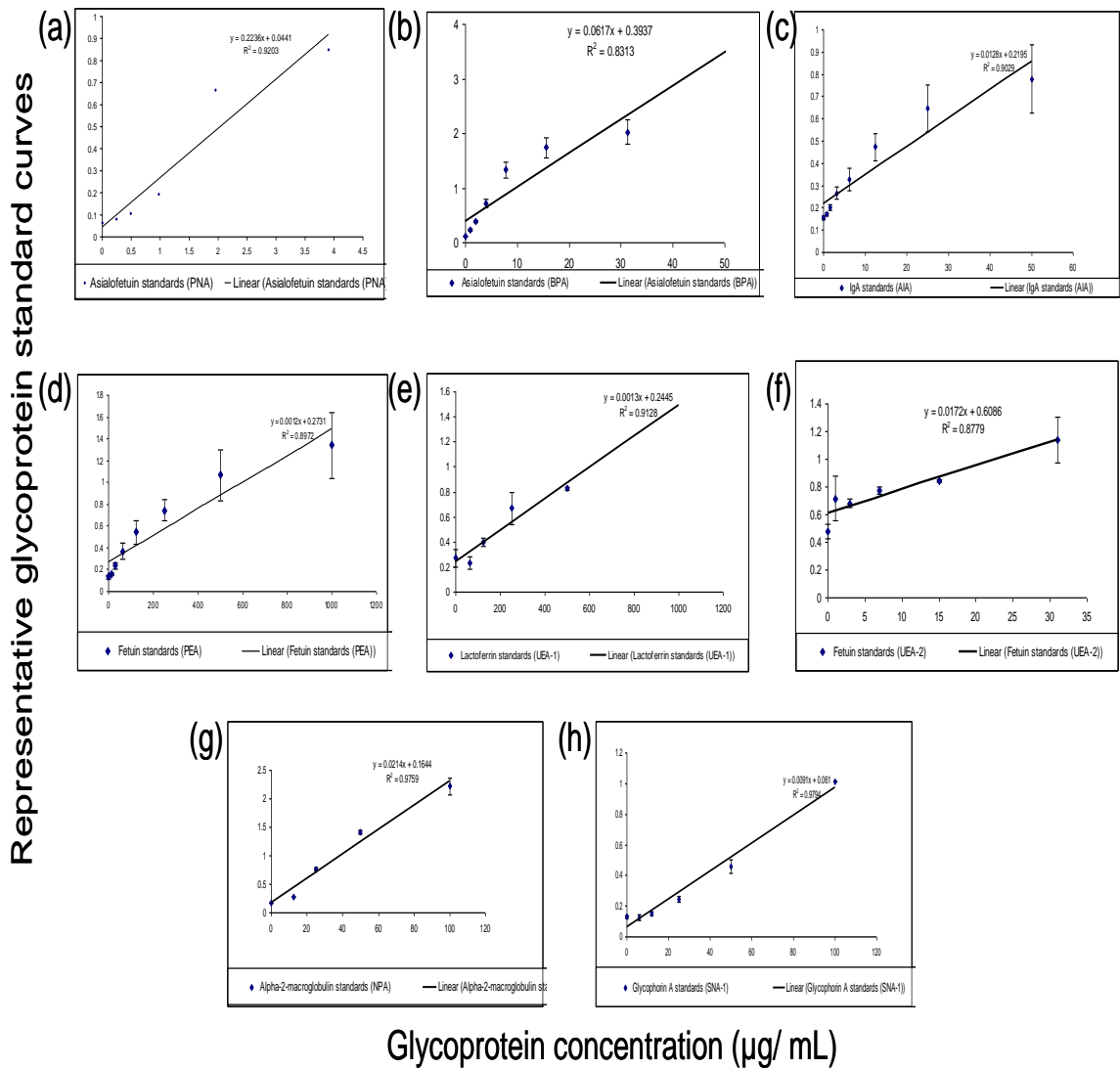


Figure 6-1: ELLA standard curves for lectin probes using positive control adsorbed glycoproteins on control polystyrene surfaces.

Enzyme linked lectin assays were performed using pairs of lectin probe: serial half-dilutions of glycoprotein in PBS on polystyrene surfaces; PNA: asialofetuin (a), BPA: asialofetuin (b), AIA: IgA (c), PEA: bovine fetuin (d), UEA-1: lactoferrin (e) UEA-2: bovine fetuin (f), α -2-macroglobulin: NPA (g), glycophorin A: SNA-1 (h), to confirm assay reproducibility and sensitivity. Standard curves were resolved using linear curve fitting routines. mean \pm SD, representative results of n = 6 independent determinations.

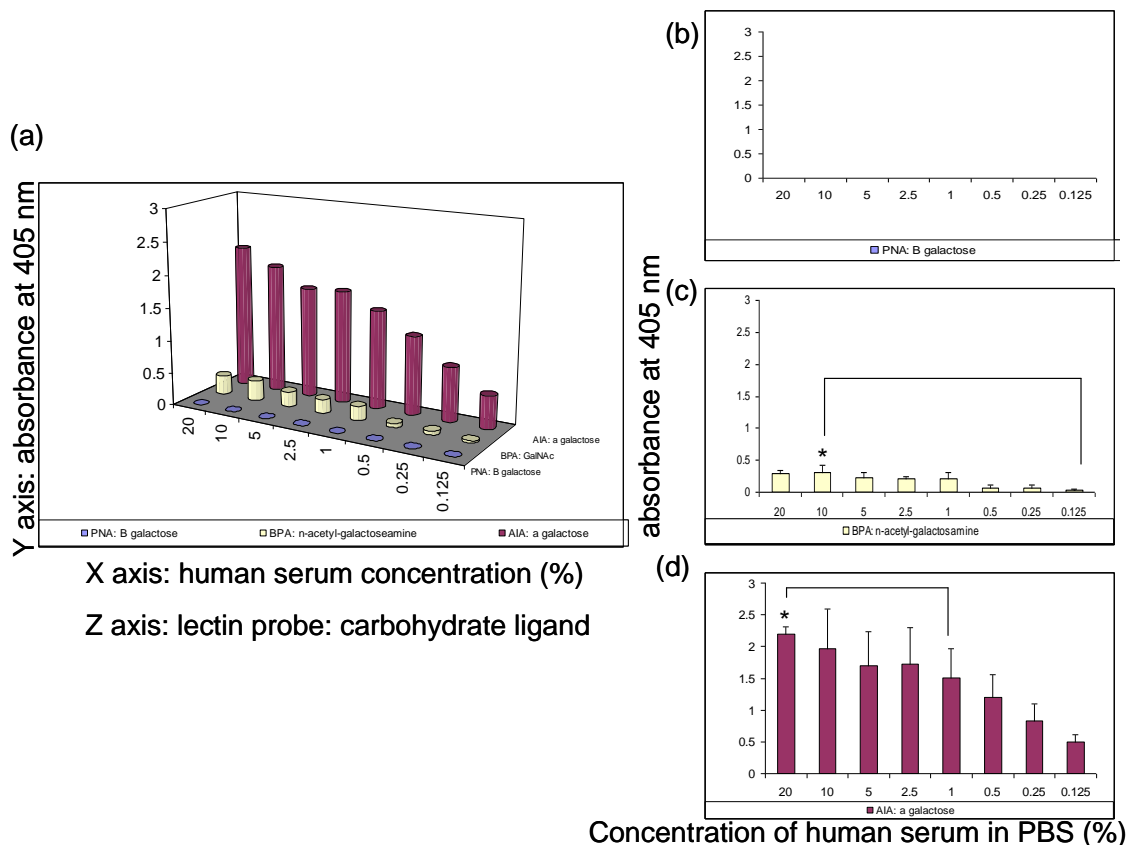


Figure 6-2: Trends of α -galactose or GalNAc presence in the adsorbed protein layer on polystyrene surfaces with decreasing concentration of pre-incubating HS in PBS. Enzyme linked lectin assays were performed using plant lectins probes for carbohydrate ligands of ASGPR or MGL, as shown for all lectins together in (a) or separately for each lectin namely β -galactose (PNA) (b), n-acetyl-galactosamine (GalNAc) (BPA) (c) or α -galactose (AIA) (d) associated with proteins adsorbed on SAM pre-incubated with varying concentrations of HS in PBS. Absorbances corresponding to presence of GalNAc (BPA) or α -galactose (AIA) decreased with decreasing concentration of pre-incubating HS. Presence of β -galactose (PNA) was undetectable with all HS concentrations tested. mean \pm SD, n = 3 independent determinations, ‘*’: significantly different from HS concentration, p \leq 0.05.

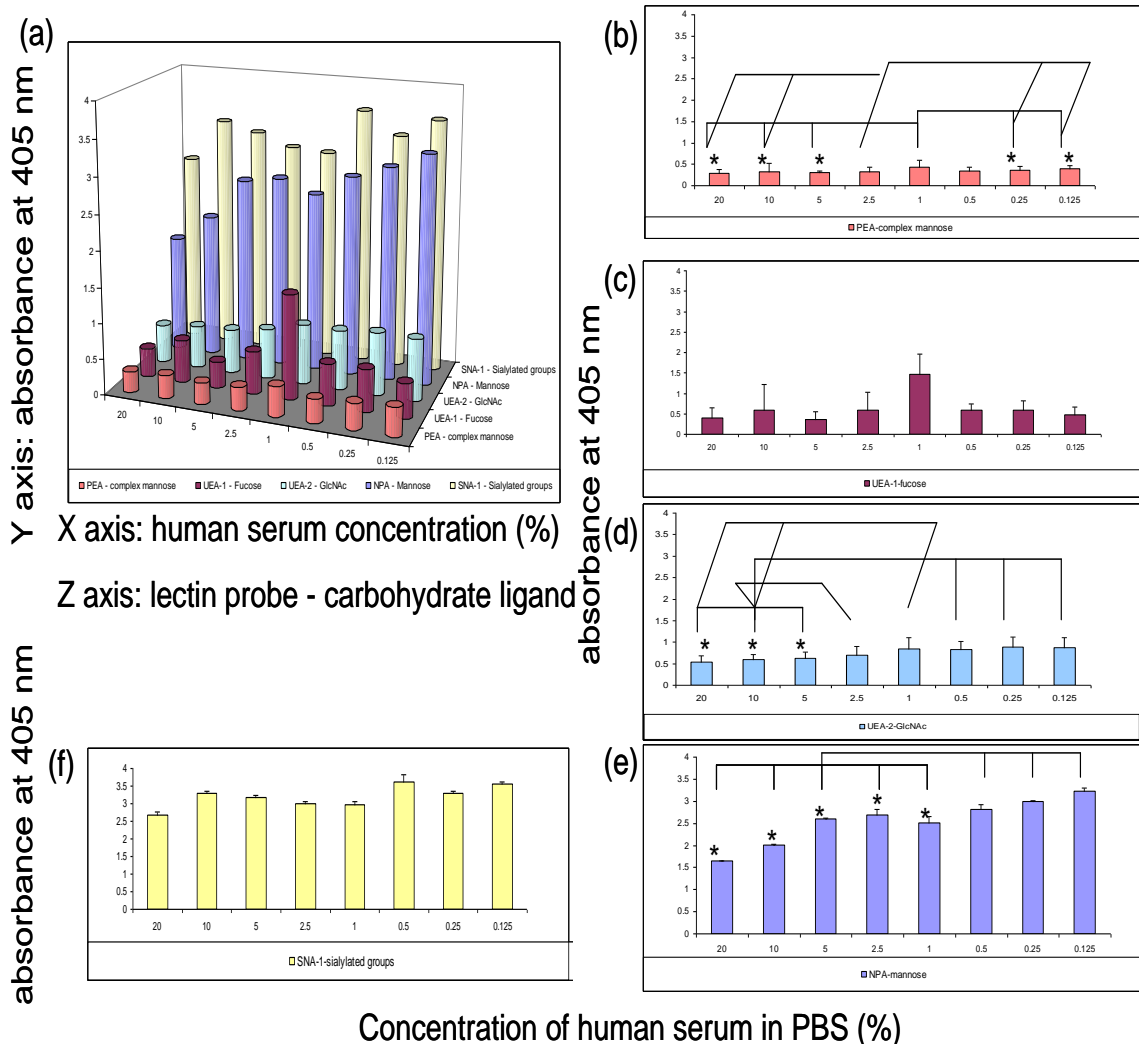
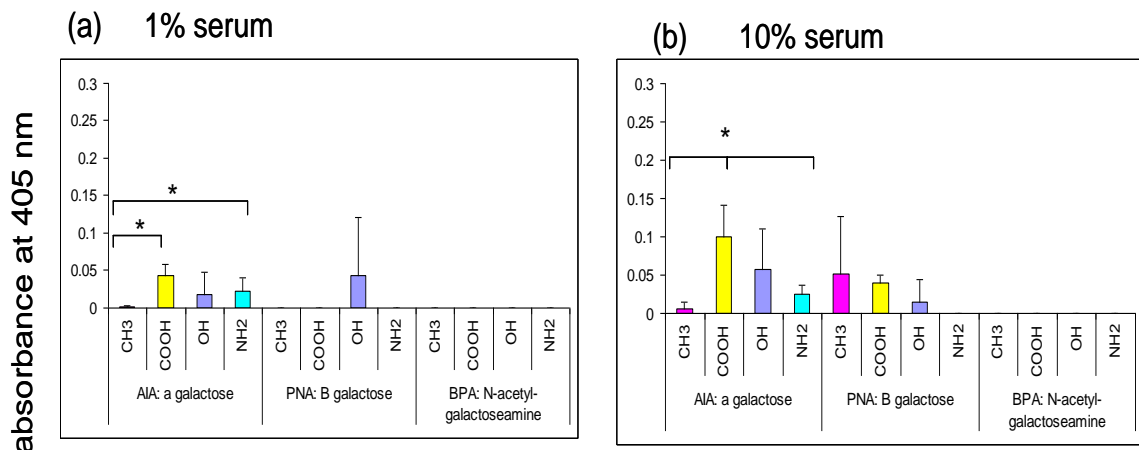


Figure 6-3: Trends of mannose or sialylated groups presence in the adsorbed protein layer on polystyrene surfaces with decreasing concentration of pre-incubating HS in PBS.

Enzyme linked lectin assays were performed using plant lectins probes for carbohydrate ligands of MR, DC-SIGN or Dectin-1, as shown for all lectins together in (a) or separately for each lectin namely complex mannose (PEA) (b), α -fucose (UEA-1) (c), GlcNAc (UEA-2) (d), mannose (NPA) (e) or sialylated groups (SNA-1) (f) associated with proteins adsorbed on SAM pre-incubated with varying concentrations of HS in PBS. Absorbances corresponding to presence of mannose (NPA), or GlcNAc (UEA-2) increased with decreasing concentration of pre-incubating HS. Absorbances corresponding to the presence of sialylated groups (SNA-1) or α -fucose (UEA-1) remained unchanged across HS dilutions. Absorbances corresponding to presence of complex mannose (PEA) was highest at intermediate concentrations of HS tested. mean \pm SD, n = 3 independent determinations, ‘*’: significantly different from HS concentration, $p \leq 0.05$.

Among SAM endgroups, pre-incubation with 1% HS resulted in absorbances that indicated the higher presence of α -galactose on COOH or NH₂ compared to CH₃ SAM (Figure 6-4a). On the other hand, pre-incubation with 10% HS used for DC culture, resulted in absorbances that implied higher levels of α -galactose on COOH SAM than on NH₂ or CH₃ SAM (Figure 6-4b). For SAMs that were pre-incubated with 1% or 10% HS in PBS, highest presence of complex mannose was detected on NH₂ SAMs and also OH SAM had higher absorbances compared to CH₃ SAM (Figure 6-5). Lowest absorbances corresponding to the presence of sialylated groups were detected on CH₃ SAM (Figure 6-5). In another set of experiments SAM endgroups were incubated with 1% or 10% HP and similar results were obtained as observed with incubating solutions of 1% or 10% HS, however trends were less defined with HP with both mannose or galactose family lectin probes (data not shown). No differences in ELLA absorbances for sialylated groups were observed following either 1 hr or 24 hrs incubations of 1% or 10% HS on different SAM (data not shown). Similar results were obtained when 1% or 10% HP was incubated on different SAM (data not shown).



lectin probe: carbohydrate ligand of galactose-family DC CLR

Figure 6-4: Differential presence of α -galactose on different SAM endgroups pre-incubated with 1% or 10% HS.

Enzyme linked lectin assay was performed using plant lectins probes for carbohydrate ligands of ASGPR or MGL namely β -galactose (PNA), n-acetyl-galactoseamine (GalNAc) (BPA) or α -galactose (AIA) associated with proteins adsorbed on SAM pre-incubated with 1% or 10% HS in PBS. Among SAM endgroups, pre-incubated with 1% HS, absorbance corresponding to α -galactose showed trends: COOH or NH₂ greater than CH₃ SAM (a) or with 10% HS in PBS, absorbance corresponding to α -galactose showed trends COOH greater than NH₂ or CH₃ SAM (b), mean \pm SD, n = 9 independent determinations; ‘*’: significantly different from indicated SAM endgroup, p \leq 0.05.

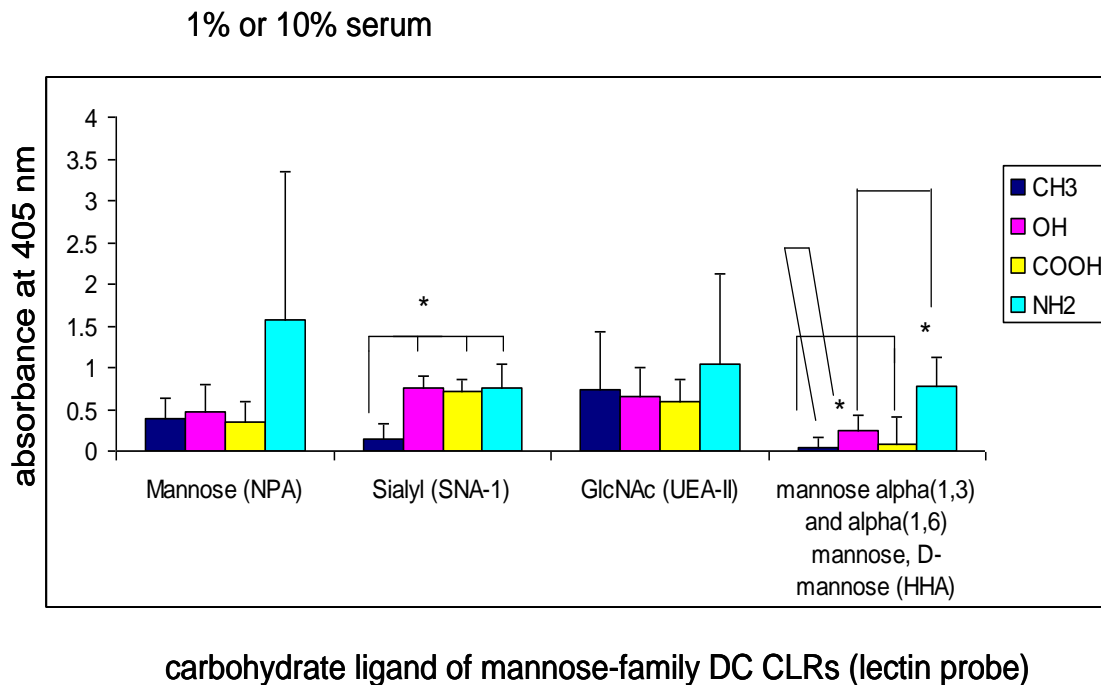


Figure 6-5: Differential presence of sialylated groups or complex mannose on different SAM endgroups pre-incubated with 1% or 10% HS.

Enzyme linked lectin assay was performed using plant lectins probes for carbohydrate ligands of MR, DC-SIGN or Dectin-1, namely complex mannose (PEA), α -fucose (UEA-1), GlcNAc (UEA-2), mannose (NPA) or sialylated groups (SNA-1) associated with proteins adsorbed on SAM pre-incubated with 1% or 10% HS in PBS. The CH₃ SAM endgroups were associated with absorbances corresponding to least presence of sialylated groups and the NH₂ SAM were associated with absorbances corresponding to highest presence of complex mannose. mean \pm SD, n = 9 independent determinations; ‘*’: significantly different from indicated SAM endgroup, p \leq 0.05.

Normalization of ELLA absorbances to protein amounts

The absorbances measured using the ELLA assays were normalized to adsorbed protein amounts (human IgG or HSA) to investigate if the differences in glycosylation profiles across SAM treatments persisted after accounting for variations in amounts of adsorbed proteins (data not shown). The CH₃ SAM had significantly higher levels of adsorbed IgG compared with COOH SAM with 10% HS. Normalized ratios of ELLA absorbance over mean IgG amount indicated similar trends as those observed from ELLA

absorbances for both mannose and galactose family lectins, although significance was not reached. No significant differences were observed for amounts of adsorbed HSA on different endgroups with HS since HSA was not detected on all SAM endgroups and hence normalizations of ELLA absorbances over HSA amounts could not be performed.

DISCUSSION

Differential carbohydrate absorbance profiles were observed on different SAM endgroups, suggesting that different biomaterial chemistries may drive the formation of distinct ‘biomaterial associated molecular patterns’ composed of proteins and carbohydrates. In this study, incubating concentrations of 1% or 10% pooled heat-inactivated filtered HS or HP were chosen, to allow for comparisons with the *in vitro* DC culture on SAM with media (CHAPTER 5) containing 10% heat-inactivated filtered fetal bovine serum (FBS) or 1% autologous heat-inactivated filtered HP. When pre-incubated with 10% HS, COOH or NH₂ SAM were associated with higher absorbances corresponding to the presence of α -galactose than CH₃ SAM and undetectable levels of GalNAc (Figure 6-4b). Pre-incubation with 1% HS resulted in higher α -galactose on COOH SAM than on CH₃ or NH₂ SAM and still undetectable levels of GalNAc (Figure 6-4a). With incubating solutions of 1% or 10% HS, the NH₂ SAM were associated with highest presence of complex mannose as well as higher complex mannose on OH versus CH₃ SAM. Finally, the CH₃ SAM surfaces were associated with least presence of sialylated groups (Figure 6-5). These results have been summarized in Table 8 and imply that the profiles of presented carbohydrates varied with different types and concentrations of incubating solutions, probably as a result of differential protein adsorption from these

solutions.

Table 8: Summary of results for enzyme-linked lectin assay on polystyrene or SAM endgroups for mannose or galactose family lectins

Carbohydrate Moiety	Polystyrene		SAM	
	Serum	Plasma	10% Serum	Plasma
Mannose	<u>Increasing</u> absorbance trend with decreasing serum %		No trend	No trend
GlcNAc	<u>Increasing</u> absorbance trend with decreasing serum %		No trend	No trend
Sialylated groups	Increasing absorbance trend with decreasing serum %		<u>Lowest on CH3 SAM</u>	No trend
α -Fucose	No absorbance trend with decreasing serum %		No trend	No trend
Complex mannose	<u>No absorbance trend</u> with decreasing serum %		<u>NH2 SAM > All SAM</u> <u>OH > CH3 SAM</u>	No trend
α -Galactose	<u>Decreasing</u> absorbance trend with decreasing serum %		<u>COOH > CH3, NH2</u>	No trend
β Galactose	Not Detectable		No trend	No trend
GalNAc	<u>Decreasing</u> absorbance trend with decreasing serum %		Not Detectable	Low levels

In an attempt to quantify the differences in absorbances, and to confirm that the differential carbohydrate profiles were in fact different and not solely due to differences in amounts of adsorbed HS or HP proteins, ELLA absorbances were normalized to mean adsorbed IgG or HSA amounts. Although, significance was not reached in comparisons between ratios on different SAM endgroups (data not shown), trends were similar to those observed from ELLA absorbances. Furthermore, since several proteins in HS or HP are glycosylated, in certain cases with multiple glycosylations and linkages, normalization against single proteins such as IgG or HSA (negligible glycosylations) performed in this study, alone, may not be sufficient. A more optimal normalization

method for future studies would be to quantify adsorption of a panel of glycosylated proteins that are commonly adsorbed to biomaterials.

In this study, SAMs having $-CH_3$, OH, $-COOH$ or $-NH_2$ chemistries were used as model biomaterials to examine the profiles of carbohydrates associated with adsorbed HS or HP proteins. The XPS (Table 6) and contact angle (Table 7) characterizations of the SAM endgroups in this study revealed close agreement with those described by others (Tanahashi 1997; Sigal 1998; Franco 2000; Keselowsky 2003) and showed a broad spectrum of surface properties. Differences in amounts and conformations of adsorbed proteins have been observed on different SAM. Variations in antibody binding affinities against the cell binding domain of fibronectin (Fn) were observed on different SAM (McClary 2000; Capadona 2003). Furthermore, significantly higher amounts of pro-inflammatory fibrinogen (Fg) were adsorbed to OH SAM than to CH_3 SAM (Silver 1995), although others have found otherwise (Lindbald 1997; Kalltorp 1999; Shen 2001; Rodrigues 2006). It was observed that higher amounts of pro-inflammatory complement-3 (C3) (Hirata 2003) was measured on OH SAM than on CH_3 SAM, although highest amounts of human IgG were detected on CH_3 SAM compared to OH, COOH or NH_2 SAM (Silin 1997). In contrast, adsorption of anti-inflammatory protein albumin was higher on CH_3 SAM compared to either OH or COOH SAM (Martins 2003).

Similar to the pro- or anti-inflammatory roles ascribed to differed HS or HP proteins such as C3 or albumin respectively, it may be worth speculating that carbohydrates associated with these proteins may also have pro- or anti-inflammatory effects. On 96-well TC-treated PS surfaces that served as experimental controls, several carbohydrates probed for were associated with differential absorbance patterns following

pre-incubations with different concentrations of HS or HP in PBS (1: 1 dilutions between 20% and 2.5% and 1% and 0.25%) (Figures 6-2 and 6-3). In general, trends indicated increasing absorbances for certain mannose group carbohydrates (GlcNAc or mannose) on PS (Figure 6-3) with decreasing HS concentrations, suggesting that higher presentation of these carbohydrates occurred with decreasing levels of other ‘masking’ proteins. A notable contrast was observed for galactose group carbohydrates (α -galactose or GalNAc) on PS (Figure 6-2), where increasing absorbances corresponded to increased presence of these carbohydrates with increasing HS concentrations. These results offer indirect support to literature that has implied opposing roles for mannose or galactose family proteins in stimulating immune responses. In tumor glycans that have high levels of exposed galactose moieties following de-sialylation of O-glycans, the galactose molecules were recognized by MGL, leading to the induction of tumor tolerance in the absence of TLR signaling (van Villet 2005). While galactose binding to CLR has been linked to anti-inflammatory effects, ligand binding to MR may result in pro-inflammatory effects, since blocking of MR inhibited the fusion of macrophages to form foreign body giant cells (FBGCs) (McNally 1996), although others have reported observing both pro- and anti-inflammatory effects depending on the nature of the MR ligand (Chieppa 2003). It may therefore be of importance to further characterize the pro/anti-inflammatory roles of the carbohydrate ligands presented by biomaterials, given these findings.

It may also be important to characterize a panel of commonly adsorbed proteins on biomaterial surfaces that are likely sources of associated carbohydrate moieties. For instance, this panel of proteins may be composed of IgG, fibronectin, vitronectin, fibrinogen, complement-3 and IgA. The known glycosylations associated with these

proteins vary: N-linked glycans with fucose are present on IgG (Jefferis 1990) and fibronectin contains one N-glycosylation and IgA includes terminal sialic acid, galactose and GalNAc groups (Xu 2005). Complement-3 contains glucosylated N-linked glycans (Crispin 2004), while fibrinogen possesses N-linked glycans (Henschen-Edman 2001) and vitronectin possesses N-linked glycans and sialylations (Uchibori-Iwaki 2000).

The CLR family of DC PRRs recognize carbohydrates on pathogens primarily via Ca-dependant interactions (formation of co-ordinate and hydrogen bonds) (Weis 1996) and possess single (type II) or multiple (type I lectin) carbohydrate recognition domains (Figdor 2002; McGreal 2004). The Ags endocytosed by different CLRs were processed via MHC class II pathways as reported in (Cobb 2004) but reached different cytosolic compartments. For instance although MR and DEC-205 (homologous to MR) (Jiang 1995; Engering 2002; Chieppa 2003) targeted Ags to early endosomes away from MHC II complexes and were less efficient at Ag delivery (Mahnke 2000; Hawiger 2001; Chieppa 2003), ASGPR (Valladeau 2001) targeted Ags to late endosomes (Mahnke 2000; Hawiger 2001; Chieppa 2003). So far, Dectin-1 receptor that possesses an intracellular ITAM and binds β -glucans (Rogers 2005; Brown 2006) has been the only DC CLR implicated in Syk-mediated signaling (Keystone 1977; Yokota 2001; Hoebe 2003; Rogers 2005; Brown 2006; Dillon 2006). Furthermore, yeast zymosan-dectin-1-mediated enhancement of NF- κ B activation by ligand binding to TLR 2 has also been implicated (Young 2001; Gantner 2003; Brown 2006). While zymosan-treated DCs had reduced abilities to stimulate CD4⁺ T lymphocytes and co-delivery of zymosan with OVA in mice resulted in anti-inflammatory interleukin-10 (IL-10) secretion and in suppression of immune responses to OVA, even in the presence of LPS (Dillon 2006) and binding of

mannose-capped lipoarabinomannan (ManLAM) to DC-SIGN skewed the response from a pro-inflammatory T_{helper1} (T_{h1}) response to a T_{h2} response (Jiang 1995; van Kooyk 2003), in contrast, other CLRs such as MR, that play a significant role in mediating the fusion of macrophages and the formation of FBGCs (McNally 1996), may be associated with pro-inflammatory effects. This is supported by findings that certain CLRs possess intracellular ITAM (Keystone 1977; Yokota 2001; Hoebe 2003; Rogers 2005; Brown 2006; Dillon 2006) motifs, while others possess ITIM residues (Kanazawa 2002). Taken together, the emerging model of PRRs and their ligands indicates that the specific carbohydrate ligand bound is important, since the same or different carbohydrate ligands may target a different complex network of DC CLRs and other PRRs and hence trigger differential DC responses.

A clear link also exists in the literature between altered glycosylations and disease progression (Johnson 2000; Kanoh 2004; Patwa 2006). While normal host proteins have relatively low terminal mannose, fucose or GlcNAc (Lee 1991; McGreal 2004), disease states are associated with alterations in protein glycosylations. Tumors have been associated with increased sialylation, mannosylation, fucosylation (Tabares 2006) and reduction in O-glycan lengths (Turner 1992; Brockhausen 1999). Liver disease is associated with lowered sialylation and increased glycan branching (Turner 1992; Tabares 2006) and rheumatoid arthritis by increased fucosylation and presence of sialyl Lewis X on alpha-1-acid glycoprotein (Elliott 1997; Havenaar 1998). Finally, it was observed that iDCs expressing DC-SIGN, but not mDCs, interacted with carcinoembryonic antigen (CEA) having higher Lewis x or Lewis y Ag levels on colon epithelial tumor cells than on CEA of normal cells (Okuyama 1998; Cambi 2005; van

Gisbergen 2005). Another line of speculation therefore, is that if the proteins adsorbed to the biomaterial have undergone conformational changes as reported previously (Keselowsky 2003) and hence present an altered profile of carbohydrates, the biomaterial may appear analogous to diseased states to circulating immune cells, including migrating DCs.

These results suggest that different chemically defined biomaterials differentially modulate carbohydrate presentation on the adsorbed protein layers, possibly due to differences in types or amounts or both of adsorbed proteins. Glycosylations in PAMPs or altered glycosylations in diseases initiate DC recognition, and in the same way, glycosylations presented by biomaterials may impact DC maturation in a CLR-mediated manner. This study is significant as it provides another class of biomolecules which may mediate cellular interactions with a biomaterial and can influence the host response to the material.

CHAPTER 7

IMMUNOBLOT ANALYSIS OF PROTEIN ELUATES FROM SELF-ASSEMBLED MONOLAYER SURFACES

INTRODUCTION:

New advances in the field of tissue engineering (TE) include the generation of TE combination products. These implants that are composed of biomaterial as well as biological components are designed to ultimately overcome the current scarcity of organs available for transplantation. The field of biomaterials has made rapid strides to meet the growing demands for synthesizing biomaterial components of TE products. The biomaterial component is crucial in regulating cell adhesion, proliferation, differentiation, survival, migration and vascularization. Besides, it is important that the biomaterial be biocompatible, have strong physical and mechanical properties and exhibit controlled degradability *in vivo* (Peppas 1994; Hubbell 1995; Ratner 1996; Langer 2004).

The success of TE devices depends to a large extent on the utilization of optimal biomaterials for the specific application, the functionality of the biological component *in vivo* and the capacity of the combination product to be well-assimilated into the host immune/inflammatory system (Ratner 1996). With combination products, a non-specific inflammatory response may be elicited against the biomaterial component, as has been widely studied by several groups (Anderson 1988; Langer 1993; Tang 1995; Ratner 1996; Sefton 2000). This phenomenon includes a series of processes initiating from the non-specific adsorption of host proteins almost instantaneously onto the surfaces of the biomaterial (Brash 1987). Subsequently, phagocytic cells such as neutrophils are attracted to implant or damage sites and recruited monocytes mature into antigen presenting

macrophages. These macrophages direct lymphocyte responses and mediate key phases of the foreign body response and the formation of the fibrous capsule, while themselves remaining throughout at implant sites and eventually fusing to form foreign body giant cells (FBGCs) (McNally 1996; Anderson 2000; Brodbeck 2002a; MacEwan 2005; Xia 2006; Kao 2007; Van den Beucken 2007). The host inflammatory response is eventually replaced by a wound healing phase that is accompanied by the formation of fibroblast-mediated scar tissue formation (Ratner 1996).

In addition to the non-specific inflammatory response generated against the biomaterial component, the biological component may trigger a specific adaptive immune response (Babensee 1998a). This immune response may be exacerbated or lessened by the biomaterial component, implying a biomaterial-mediated adjuvant effect (Babensee 1998a). Based on their specific surface and bulk properties, biomaterials may stimulate a broad range of immune responses. As adjuvants operate by triggering dendritic cell (Tabares 2006) maturation, biomaterials may regulate DC responses and hence facilitate DC-orchestrated host responses (Babensee 1998a; Yoshida 2004; Babensee 2005). Furthermore, DCs, another class of antigen presenting cells (APCs) such as macrophages, are pivotal in connecting innate and adaptive immunity and are an important immune cell type to focus on for studying fundamental host-biomaterial interactions (Cella 1997b; Medzhitov 1997; Banchereau 1998a; Janeway 2002). While previous studies have confirmed a central role for macrophages in controlling the host inflammatory response (Anderson 2001; Anderson 2004), emerging studies have suggested a role of DCs, that are more powerful initiators of adaptive immunity than macrophages (Setum 1991; Sallusto 1994; Triozzi 1997; Banchereau 2000; Janeway

2001), as principal directors of host immune responses. An exacerbation of the host response against combination devices due to bi-directional interactions between biomaterials and DCs may ultimately lead to device failure and diminish implant performance (Babensee 1998a). Hence, a clear understanding of the effects of biomaterials on host responses is critical.

In this work, model self-assembled monolayers (SAM), a robust, well-described system with controlled surface properties (Mrksich 1996; Lindblad 1997; Harder 1998; Sigal 1998; Kalltorp 1999; McClary 2000; Tegoulia 2000; Shen 2001; Barbosa 2003; Keselowsky 2003; Schwendel 2003; Dadsetan 2004) were used to examine the profiles and compositions of spontaneously adsorbed host proteins associated with SAM surfaces that presented different terminal endgroup chemistries. Since protein adsorption represents an initial, significant event in the host response and is directed by surface properties including chemistry, hydrophobicity/hydrophilicity, and charge, the effects of varying these characteristics were assessed in this study. The types of proteins adsorbed are important as they may differentially allow or inhibit immune/inflammatory cell adhesion, survival and differentiation and hence influence the ensuing response (Brash 1987; Ratner 1996; Collier 1997; Kao 1999; Jenney 2000; Sefton 2000; Collier 2002; Gorbet 2004). Furthermore, the presence of distinctly pro- or anti-inflammatory proteins may activate or passivate adherent cells and hence indirectly control recruitment of other cells (Anderson 1988; Tang 1993a; Tang 1995; Ratner 1996).

In this study, proteins associated with different SAM chemistries were characterized using sodium dodecyl sulfate (SDS) - polyacrylamide gel electrophoresis (PAGE) and immunoblotting following the *in vitro* incubation of either 10% (v/v)

filtered heat inactivated human serum (HS) or 1% (v/ v) filtered heat inactivated human plasma (HP) proteins. Identical serum/plasma preparations were used in previous studies characterizing DC responses to the same set of SAM chemistries and were hence used here as pre-adsorbing medias. The proteins present in HP or HS were probed using anti-human polyclonal antibodies and specifically included cell-adhesive proteins, complement or coagulation proteins, pro-inflammatory proteins and those present in abundance such as albumin or immunoglobulin (IgG). Briefly, differential protein profiles were observed in eluates from different SAM chemistries following exposure to filtered HS or HP or from unfiltered HS or HP controls. Notably, weak band intensities from gels as well as lower amounts of adsorbed protein from radiolabeling experiments implied least presence of certain pro-inflammatory mediators of host cell activation such as fibrinogen on CH₃ SAM endgroups compared to OH, COOH or NH₂ SAM with filtered HP. With filtered HS, several clotting factors that were observed on OH, COOH or NH₂ SAM, were absent on CH₃ SAM. Finally, the types of medias, concentrations and filtration effects employed were found to be extremely important in determining the profiles of adsorbed proteins on the different biomaterial surfaces.

METHODS:

Self-Assembled Monolayer Preparation

Self-assembled monolayer (SAM) surfaces were assembled on 35 mm X 10 mm sterile, tissue culture (TC)- treated, non-pyrogenic polystyrene (Phillips 2005) dishes (Corning, Corning, NY) (Keselowsky 2003) used for HS or HP adsorption and elution assays, conducted at Georgia Tech. Alternatively, alkanethiol monolayers were

assembled on both sides of 9 mm X 9 mm glass coverslips (Bellco Glass, Inc., Vineland, NJ) for protein quantification assays. Clean dishes or coverslips were coated sequentially with 50 Å Ti followed by 150 Å Au films using an electron beam evaporator (CVC Products/ Veeco, Rochester, NY) on the bases of the dishes as described in (Keselowsky 2003), or on both sides of the coverslips and stored under vacuum at room temperature (RT) until two weeks until used. The following alkanethiols were used as received from commercial sources (1 mM in absolute ethanol): 1-dodecanethiol (SH-(CH₂)₁₁-CH₃) (CH₃ SAM), 11-mercapto-undecanol (HS-(CH₂)₁₁OH) (OH SAM), 11-mercaptoundecanoic acid (HS-(CH₂)₁₀-COOH) (COOH SAM), (Aldrich Chemical, Milwaukee, WI) and 11-amino-1-undecanethiol, hydrochloride (C₁₁H₂₆ClNS) (NH₂ SAM) (Dojindo Laboratories, Gaithersburg, MD). The SAMs were allowed to assemble by 12 hr incubation at RT by immersing the Ti/Au- coated dishes in alkanethiol solutions. Following this, the dishes were washed with 95% ethanol (Sigma, St. Louis, MO) dried with N₂ gas (Airgas South, Chamblee, GA) for 10 minutes in a fume hood, equilibrated with Phosphate Buffered Saline (PBS) (Gibco, Grand Island, NY) for 5 minutes and used fresh. For coating both sides of the coverslips in alkanethiol solutions, the coverslips were immersed in polypropylene vials having outer diameter of 12.5 mm (VWR International, San Diego, CA) containing 2 mLs of 1 mM alkanethiol solution each, such that both Au/Ti-coated sides were constantly exposed to solution and the edges were in contact with the vial walls. Each vial containing coated coverslips was sealed with parafilm (VWR) individually and shipped as such at RT to Dr. John Brash's laboratory at McMaster University, Canada, and used immediately for radiolabeled fibrinogen adsorption and elution experiments as described below. Upon removal of the coverslips

coated on both sides from the coating SAM solutions, the coverslips were air-dried for 5 minutes, equilibrated with PBS at RT for 5 minutes in separate vials and then placed in PBS in 24-well plates for 10 minutes at RT. Advancing contact angles of CH₃, OH COOH or NH₂ SAM surfaces were measured to be $108 \pm 2^\circ$ C, $24 \pm 5^\circ$ C, $31 \pm 3^\circ$ C or $35 \pm 4^\circ$ C, respectively. Contact angle determinations were performed at Georgia Tech. Unmodified surfaces of TC-PS were used as controls.

Radiolabeling of fibrinogen

A solution of human fibrinogen (Enzyme Research Laboratories Inc, South Bend, IN, USA) in isotonic Tris-buffered saline (TBS) (50 mM Tris base, 150 mM NaCl; pH 7.4) was prepared, aliquotted and stored at -70°C. The iodine monochloride method was employed to label fibrinogen with ¹²⁵I (ICN Pharmaceuticals Inc, Radiochemical Division, Irvine, CA, USA). Following this, radiolabeled fibrinogen was flowed through an anion exchange column packed with AG-1-X4 resin (BioRad, Richmond, CA, USA) for removal of unbound radioactive iodide. The amount of unbound radioactive iodide that still remained in the labeled protein solution was measured to be less than 1%. This quantification was performed on the supernatant obtained following fibrinogen precipitation with trichloroacetic (Fisher Scientific, ON, Canada) (Gluck 2005) solution of 20% w/ v in water. In the final solution used, the ratio of labeled to unlabeled fibrinogen was maintained at 10%.

Quantification of amounts of adsorbed or eluted fibrinogen

The SAM-coated glass coverslips were first equilibrated in PBS-NaI solutions for

15 minutes for surface hydration and then pre-incubated in 1 mL of ^{125}I -labeled fibrinogen solution (0.5 mg/ mL) comprised of 10% of label and less than 1% free iodide for 60 minutes at RT and maintained throughout under static conditions (Mulzer 1989; Cornelius 1994; Babensee 1998b). For these adsorption studies, PBS solution comprising 5% NaI was used, since the presence of low levels of non-radioactive iodide was observed to reduce non-specific binding of ^{125}I - to Au surfaces. This may be significant, as Au-I interactions may result in the formation of complex ionic species including AuI_2^- and AuI_4^- , that may then interfere with protein adsorption.

The surfaces were then rinsed three times for 2 minutes each with 3 mLs of PBS-NaI at RT to remove residual non-adsorbed fibrinogen. After this, the surfaces were wicked onto filter paper (Bio-Rad) to remove any remaining buffer. The surface radioactivity was then measured in conjunction with calibration solutions, as necessary. This procedure was followed on four coated coverslips per SAM chemistry, per experiment. All observations were recorded in triplicate. To elute the adsorbed protein, the surfaces were incubated in 10% SDS/ 2.3% dithioerithritol (DTE) (both from Sigma) eluting solution in de-ionized (DI) water on a shaker at RT for 24 hours. The surfaces were then rinsed three times with PBS to remove traces of the eluting solution. Finally the radioactivity associated with the eluate as well as with the surfaces was determined. Again, four replicates were analyzed per surface chemistry in each experiment and all experiments were performed in triplicate.

Preparation of human plasma (HP) or human serum (HS)

Human blood was obtained from healthy volunteers with informed consent,

according to a protocol approved by the Institute Review Board (IRB) # H05012. All subjects enrolled in this research signed an Informed Consent which was approved by the IRB of Georgia Institute of Technology. Peripheral human blood was collected using sterile 60 mL syringes (Becton Dickinson, Franklin Lakes, NJ) and needles (Becton Dickinson) using heparin (333 U/ mL blood) (Baxter Healthcare Corporation, Deerfield, IL) as an anticoagulant. The clear yellowish HP layer was separated using lymphocyte separation medium (LSM) (Cellgro MediaTech, Herndon, VA) by differential gradient centrifugation of blood diluted 1:1 with sterile PBS (400 g, 30 minutes, RT) (Thermo Fisher Scientific Inc., Waltham, MA) (Model # 5682, Rotor IEC 216), filtered sterilized (0.22 μ m) (Corning) and heat inactivated for 30 minutes in a water bath pre-warmed to 56°C, aliquotted and stored at -20°C. Alternatively, plasma was heat inactivated but left unfiltered in order to examine the effects of sterile filtering on the plasma proteins. A stock solution of pooled HP from three donors was used for experiments for either filtered HP or for control unfiltered HP.

On the other hand, for preparation of HS, peripheral human blood was drawn in the absence of heparin, to allow for fibrin clot formation. The non-heparinized blood was centrifuged (3000 rpm, 10 minutes, RT) after clots were compressed manually with a sterile pipette tip, as needed and maintained at RT for 90 minutes in the TC hood. Resultant HS was collected and cleared by further centrifugations at 3000 rpm for 15 minutes. Again, as necessary, residual precipitates were pressed down and the HS was filter sterilized (0.22 μ m) and heat inactivated for 30 minutes in a water bath pre-warmed to 56°C, aliquotted and stored at -20°C. Alternatively, serum was heat inactivated but left unfiltered as with plasma. A stock solution of pooled HS from three donors was used for

experiments for either filtered HS or for control unfiltered HS.

Adsorption and elution of HS or HP proteins from biomaterial surfaces

The SAM surfaces were pre-incubated with 3 mLs/ dish of solutions of 10% filtered heat-inactivated HS or with 1% filtered heat-inactivated HP in PBS at RT for 1 hr. After this, the HS or HP solutions were removed and the dishes were washed three times for 5 minutes each with PBS at RT to remove residual non-adsorbed HS or HP proteins. To remove adsorbed proteins the SAM-coated dishes were then incubated in 1 mL/ dish of 10% SDS/2.3% DTE eluting solution in DI water on a shaker at RT for 24 hours. Samples of pre-adsorbing HS or HP solutions prior to and after incubation and of protein eluates from each SAM chemistry were collected, stored in low retention, 1.7 mL PS vials (VWR) at -20°C at Georgia Tech and shipped overnight on dry ice to McMaster University for further analysis.

Polyacrylamide gel electrophoresis and immunoblot analysis of eluates from different biomaterial surfaces

Polyacrylamide gel electrophoresis and immunoblot analyses were performed to characterize the profiles of HS or HP proteins in eluates from different SAM chemistries or from control PS surfaces, based on methods described elsewhere (Mulzer 1989; Cornelius 1994; Babensee 1998b). Briefly, solutions composed of SDS - proteins from serum, plasma or eluate samples (protein concentration 0.4-0.5 mg/ mL; 1 to 150 μ L, depending on the protein concentration) were reduced with β -mercaptoethanol (Bio-Rad) at 95°C for 5 minutes. Then, reduced samples were loaded onto 12% (w/ v)

polyacrylamide separating gels and a 4% (w/ v) stacking gel, prepared based on previously described protocols. After proteins were separated based on size by SDS-PAGE (200 V, 45-50 minutes), the proteins were electrophoretically transferred onto an Immobilon PVDF membrane (Millipore, Bedford, MA, USA). The transferred proteins were blocked for 1 hour with 0.3% Tween 20 (Bio-Rad) in TBS and then rinsed with water. Unbound sections of the membrane were stained overnight using Protogold solution (Bio-cell Research Laboratories, Cardiff, UK, distributed by Cedarlane Laboratories, Hornby, ON, Canada) to identify positions of protein bands.

Immunoblot analysis was also performed based on previously established protocols (Mulzer 1989; Cornelius 1994; Babensee 1998b). Alternatively, after proteins were separated based on size by SDS-PAGE (200 V, 45-50 minutes) and transferred, the membrane was cut into 3 mm strips and blocked with a 5% (w/ v) solution of blocking buffer [nonfat dry milk in TBS] for 1 hour. The strips were then treated with 1:1000 dilutions of desired primary antibodies generated in goat (Table 9) in solutions of 1% (w/ v) nonfat dry milk and 0.05% (v/ v) Tween 20 in TBS for 1 hour, then rinsed three times for five minutes each with 0.1% (w/v) nonfat dry milk in TBS. Bound antibodies were detected using 1:1000 dilutions of secondary antibodies (Table 10) conjugated with alkaline-phosphatase (AP) in 1% (w/ v) nonfat dry milk and 0.05% (v/ v) Tween 20 in TBS and 5-bromo-4chloro-3-indonyl phosphate (BCIP) and nitro-blue tetrazolium (NBT) (both from Bio-Rad). This experiment was run in parallel with pre-stained molecular weight standards (Bio-Rad).

Table 9: Proteins probed for on immunoblots along with corresponding hosts and commercial sources of antibodies

Antibody	Host	Source
Factor XI	Goat	Cedarlane Laboratories, Hornby, ON, Canada
Factor XII	Goat	Cedarlane Laboratories, Hornby, ON, Canada
Prekallikrein	Sheep	Cedarlane Laboratories, Hornby, ON, Canada
HMWK	Goat	Cedarlane Laboratories, Hornby, ON, Canada
Fibrinogen	Goat	Cedarlane Laboratories, Hornby, ON, Canada
Plasminogen	Goat	Sigma, St. Louis, MO, USA
ATIII	Sheep	Cedarlane Laboratories, Hornby, ON, Canada
Complement Factor C3	Goat	Calbiochem, Behring Diagnostic, La Jolla, CA, USA
Transferrin	Goat	Sigma, St. Louis, MO , USA
Alpha-1-antitrypsin	Goat	Cedarlane Laboratories, Hornby, Ontario, Canada
Fibronectin	Rabbit	Cedarlane Laboratories, Hornby, Ontario, Canada
Albumin	Goat	Cedarlane Laboratories, Hornby, Ontario, Canada
IgG	Goat	Sigma, St. Louis, MO, USA
Beta-lipoprotein	Goat	Sigma, St. Louis, MO, USA
Alpha-2-macroglobulin	Goat	Sigma, St. Louis, MO, USA
Vitronectin	Sheep	Cedarlane Laboratories, Hornby, Ontario, Canada
Complement Factor B	Goat	Calbiochem, Behring Diagnostic, La Jolla, CA
Complement Factor H	Goat	Calbiochem, Behring Diagnostic, La Jolla, CA
Complement Factor I	Goat	Calbiochem, Behring Diagnostic, La Jolla, CA
Apolipoprotein A1	Goat	ESBE, Markham, ON, Canada
Prothrombin	Sheep	Cedarlane Laboratories, Hornby, Ontario, Canada
Hemoglobin	Rabbit	Sigma, St. Louis, MO, USA

Table 10: Alkaline-phosphate conjugated secondary antibodies used in immunoblotting and corresponding commercial sources

Secondary Antibody	Source
Rabbit Anti Goat IgG Alkaline Phosphatase Conjugate	Sigma Chemical Co., St. Louis, USA
Rabbit Anti Sheep IgG Alkaline Phosphatase Conjugate	Bethyl Laboratories Inc., Montgomery, TX, USA
Goat Anti Rabbit IgG Alkaline Phosphatase Conjugate	Bio-Rad Laboratories, Hercules, CA, USA

Statistical analysis

Statistical analysis was performed using general linear model analysis of variance (Young) with Minitab software (Version 13.20, Minitab Inc., State College, PA) using pairwise comparisons between SAMs and a Tukey post-test. A p value of less than or equal to 0.05 was considered as significant.

RESULTS:

Gold-stained gels of proteins present in filtered or unfiltered HS or HP

Differential presence of proteins bands were observed between gold-stained gels of filtered or unfiltered HS and filtered or unfiltered HP solutions used for pre-incubation on control PS surfaces or on different SAM chemistries (Figure 7-1). In general, more protein was detected in unfiltered HS (Figure 7-1b) or unfiltered HP (Figure 7-1d) compared to filtered HS (Figure 7-1a) or filtered HP (Figure 7-1c), respectively, given that the protein loading was kept constant between these samples.

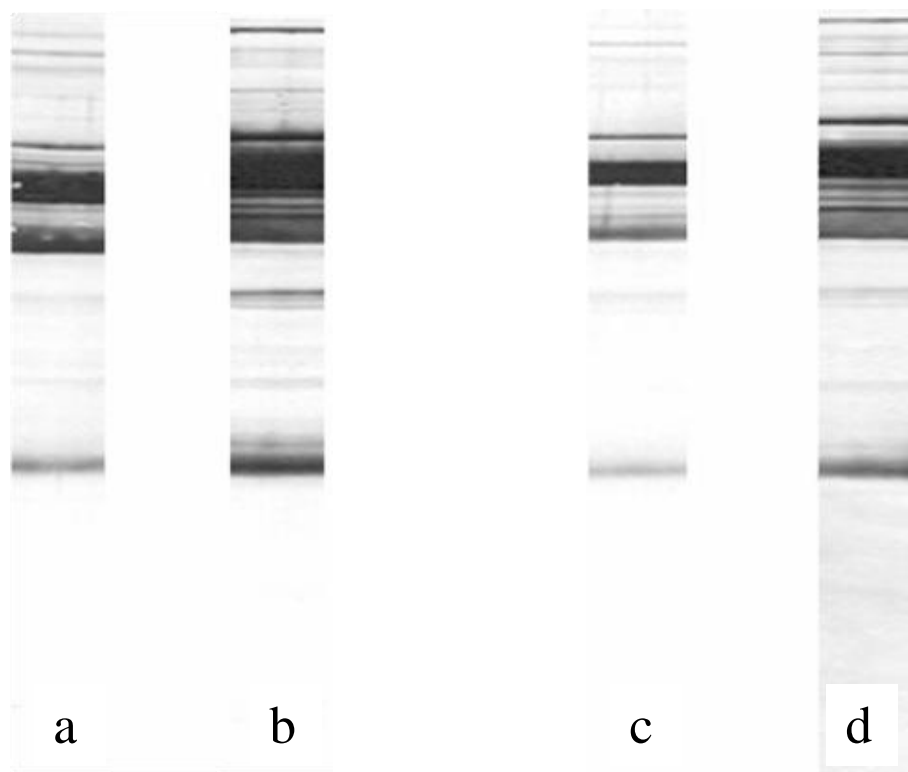


Figure 7-1: Gold stained gels of proteins present in filtered or unfiltered HS or filtered or unfiltered HP.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate filtered (a) or unfiltered (b) HS or filtered (c) unfiltered (d) HP proteins. These proteins were then transferred to a PVDF membrane and gels were stained with gold.

Gold-stained gels of proteins eluted from control PS surfaces following pre-adsorption with either filtered or unfiltered HS or HP

Differential presence of proteins bands were observed between gold-stained gels of eluates from control PS surfaces that were pre-adsorbed with either filtered or unfiltered HS versus filtered or unfiltered HP solutions (Figure 7-2).

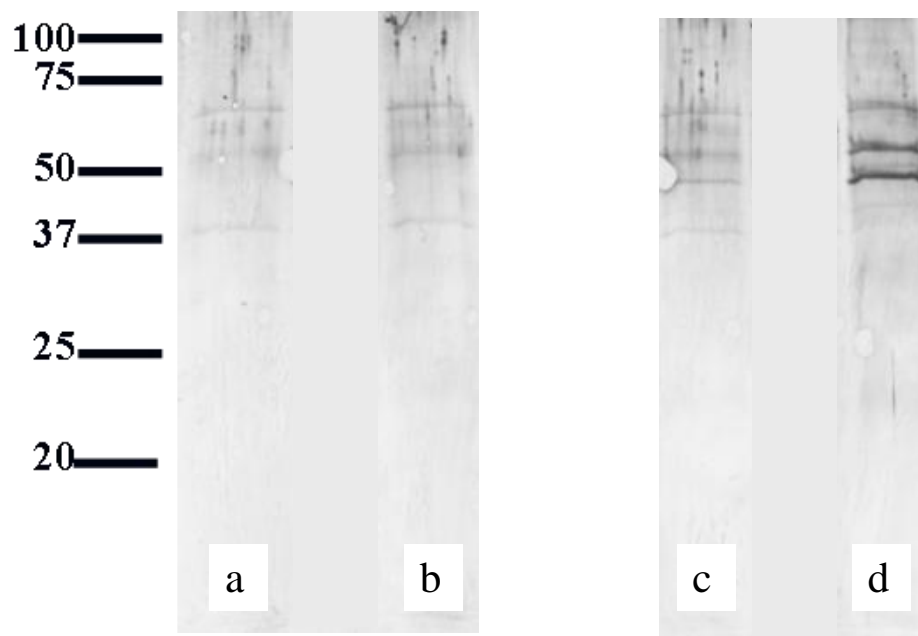


Figure 7-2: Gold stained gels of proteins eluted from control PS surfaces following exposure to filtered or unfiltered HS or filtered or unfiltered HP.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate filtered (a) or unfiltered (b) HS or filtered (c) unfiltered (d) HP proteins present in eluates from control PS surfaces. These proteins were then transferred to a PVDF membrane and gels were stained with gold.

Gold-stained gels for protein eluates from different SAM chemistries

The proteins associated with control PS surfaces or with different SAM chemistries were detected using gold-stained gels (Figure 7-3). Proteins bands of different molecular weights were observed in eluates from all biomaterials examined, following pre-incubation with either filtered HS (Figure 7-3a) or filtered HP (Figure 7-3b) or with unfiltered HP (data not shown).

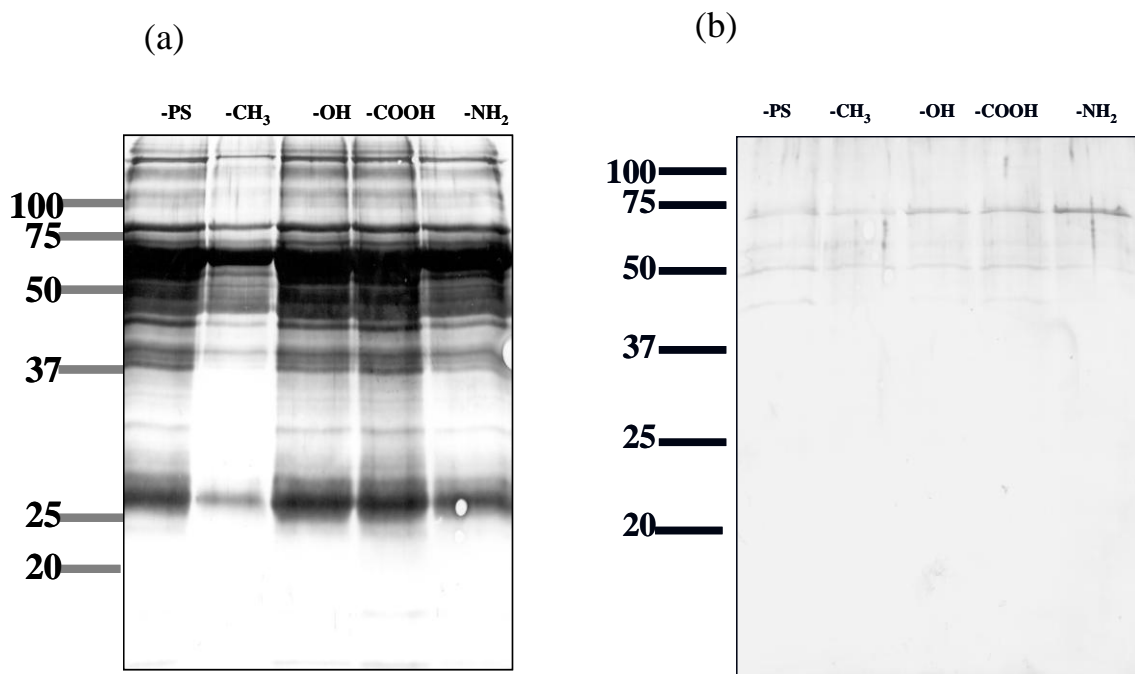


Figure 7-3: Gold stained gels of proteins eluted from control PS surfaces or from different SAM chemistries following exposure to either filtered HS or filtered HP.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins eluted from control PS or from different SAM chemistries following exposure to either filtered HS (a) or to filtered HP (b). [PS, CH₃ SAM, OH SAM, COOH SAM or NH₂ SAM] (bandwise from left to right). These proteins were then transferred to a PVDF membrane and gels were stained with gold.

Amounts of adsorbed, eluted and bound fibrinogen on different SAM chemistries

To examine the possibility that the differential presence of proteins associated with different SAM chemistries was due to differential elutability of proteins, the amounts of adsorbed, eluted or bound radiolabeled fibrinogen associated with the different surfaces were assessed. Significantly higher protein was adsorbed to COOH or NH₂ SAM surfaces as compared to protein adsorbed to CH₃ or OH SAM surfaces. Also, significantly higher bound protein remained on COOH or NH₂ SAM surfaces after elution as compared to CH₃ or OH SAM surfaces (Figure 7-4a). The percentage of adsorbed fibrinogen eluted from CH₃ SAM was $86 \pm 6\%$, from OH SAM was $91 \pm 3\%$,

from COOH SAM was $67 \pm 8\%$, and from NH_2 SAM was $77 \pm 8\%$, (Figure 7-4b). Overall, higher elution of fibrinogen was achieved with OH or CH_3 SAM surfaces than with NH_2 or COOH surfaces.

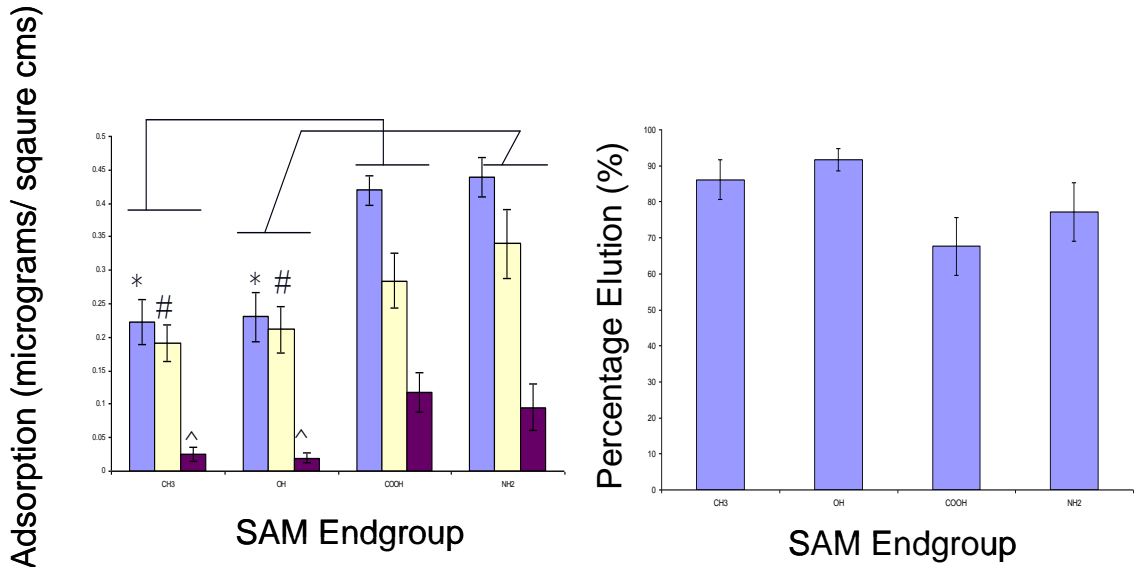


Figure 7-4: Higher amounts of adsorbed, eluted or bound fibrinogen were associated with COOH or NH_2 SAM endgroups compared to OH or CH_3 SAM endgroups. However, higher elution of adsorbed fibrinogen could be achieved on CH_3 or OH SAM endgroups.

Amounts of adsorbed, eluted or bound fibrinogen on different SAM chemistries are shown in (a) and percentages of eluted proteins from different chemistries in (b). Data presented as mean \pm S.D, n=12 wells revealed statistical significance of findings; ‘*’ indicates lower levels of adsorbed fibrinogen, ‘#’ indicates lower levels of eluted fibrinogen, ‘^’ indicates lower levels of bound fibrinogen post-elution compared to other SAM endgroups, $p \leq 0.05$.

Immunoblot analysis of proteins present in pre-incubating unfiltered or filtered HS or HP

As expected, several HS or HP proteins were detected in serum or plasma samples used for incubation with control PS or SAM surfaces. For filtered HS (Figure 7-5a, Table 11), these included strong as well as faint bands in certain cases corresponding to presence of intact or degraded fragments of Factor XI, Factor XII, pre-kallikrein,

HMWK, plasminogen, antithrombin, C3, transferrin, α_1 -anti-trypsin, fibronectin, albumin, IgG, α_2 -macroglobulin, vitronectin, Factor B or Factor H, Factor I, apolipoprotein A1 or prothrombin. Certain proteins including β -lipoprotein or hemoglobin were non-detectable (n.d) (Figure 7-5a, Table 11). Fibrinogen although detected ~ 67 kDa, was present as very faint bands. Bands of fibrinogen at ~ 58 , ~ 47 kDa were n.d.

As expected a differential profile of proteins were present in filtered HP solutions used for pre-adsorption. To summarize, the protein profiles in filtered HP included strong as well as/or faint bands in certain cases corresponding to presence of intact or degraded fragments of fibrinogen, plasminogen, antithrombin, C3, transferrin, albumin, IgG, α_2 -macroglobulin, vitronectin, Factor B, Factor H, Factor I or apolipoprotein A1. Certain proteins including fibronectin, α_1 -anti-trypsin or β -lipoprotein were n.d (Figure 7-5b, Table 12). On the other hand, although the profiles of plasma proteins that were detectable in filtered HP were similar to those in unfiltered HP, certain differences did exist. To summarize, the protein profiles in unfiltered HP (Table 13) included strong as well as faint bands in certain cases corresponding to presence of intact or degraded fragments of Factor XI, Factor XII, pre-kallikrein, HMWK, fibrinogen, plasminogen, antithrombin, transferrin, α_1 -anti-trypsin, albumin, IgG, C3, vitronectin, Factor B or Factor H, Factor I, apolipoprotein A1 or α_2 -macroglobulin. Certain proteins including fibronectin or β -lipoprotein were non-detectable (n.d) (Table 13).

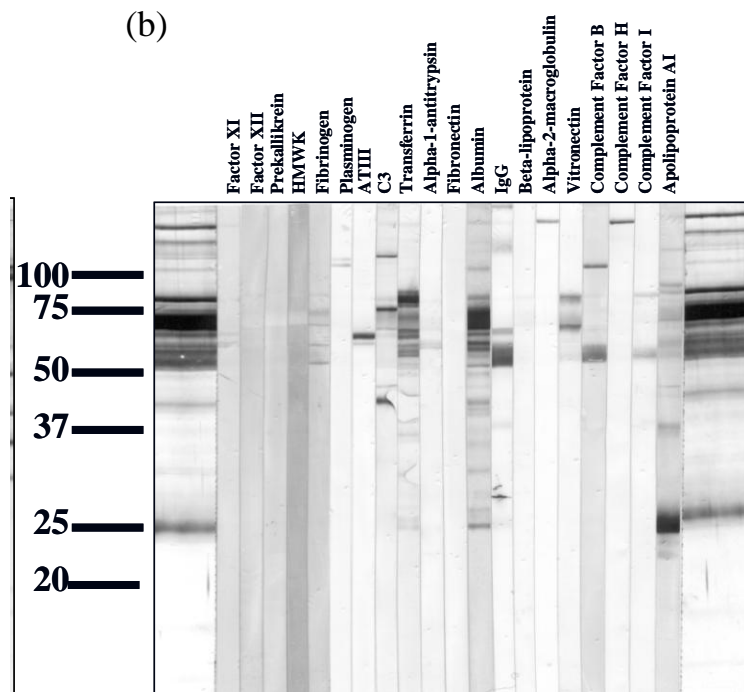
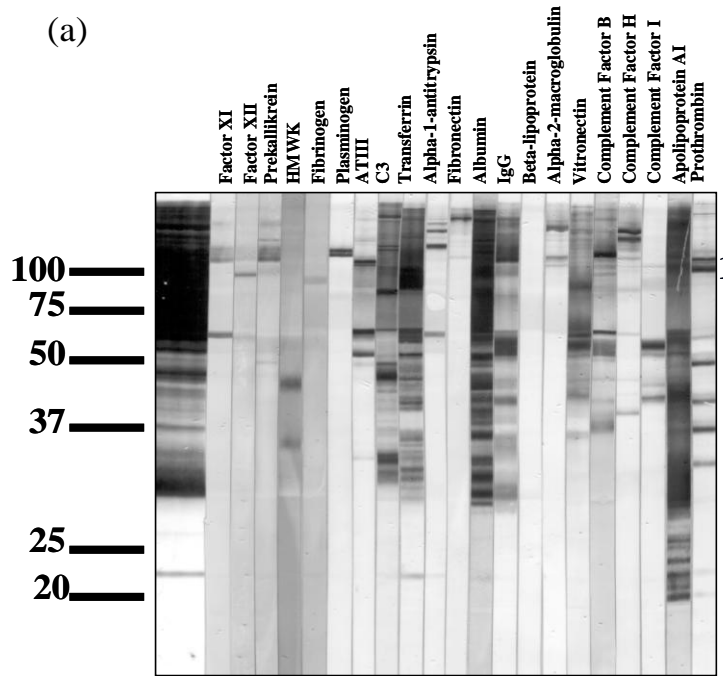


Figure 7-5: Presence of bands corresponding to several major HS or HP proteins in either filtered HS or filtered HP as expected, from immunoblotting experiments. Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins in either filtered HS (a) or filtered HP (b). Prestained molecular weight standards, shown on the right side, were used to estimate the molecular weight of the bands. Serum or plasma concentration, intact molecular weights and intensities of major bands are listed in Table 11 for filtered HS or Table 12 for filtered HP.

Immunoblot analysis of proteins present in eluates from control PS surfaces pre-incubated with either filtered or unfiltered HS or HP

Certain HS or HP proteins that were detected in pre-adsorbing filtered plasma or serum (Figure 7-5) were also detected in eluates from control PS surfaces (Figure 7-6). For instance, bands corresponding to the presence of Factor XI, Factor XII, fibrinogen (again only at ~67 kDa and very faint), plasminogen, C3, antithrombin, transferrin, α_1 -anti-trypsin, albumin, IgG, α_2 -macroglobulin, vitronectin, Factor B, Factor H, Factor I, apolipoprotein A1 or prothrombin were present in eluates from control PS surfaces (Figure 7-6a, Table 11) that were pre-incubated in HS. Presence of proteins such as prekallikrein, HMWK, hemoglobin or β -lipoprotein was n.d (Figure 7-6a, Table 11).

Pre-adsorption of control PS surfaces with filtered HP led to the following profile of adsorbed proteins including, fibrinogen, C3, transferrin, albumin, IgG, vitronectin, apolipoprotein A1, antithrombin or prothrombin (Figure 7-6b). In contrast, certain other proteins such as plasminogen, α_2 -macroglobulin, Factor B or Factor H that were present with unfiltered HP eluates (Table 13) were not present in filtered HP eluates (Figure 7-6b, Table 12).

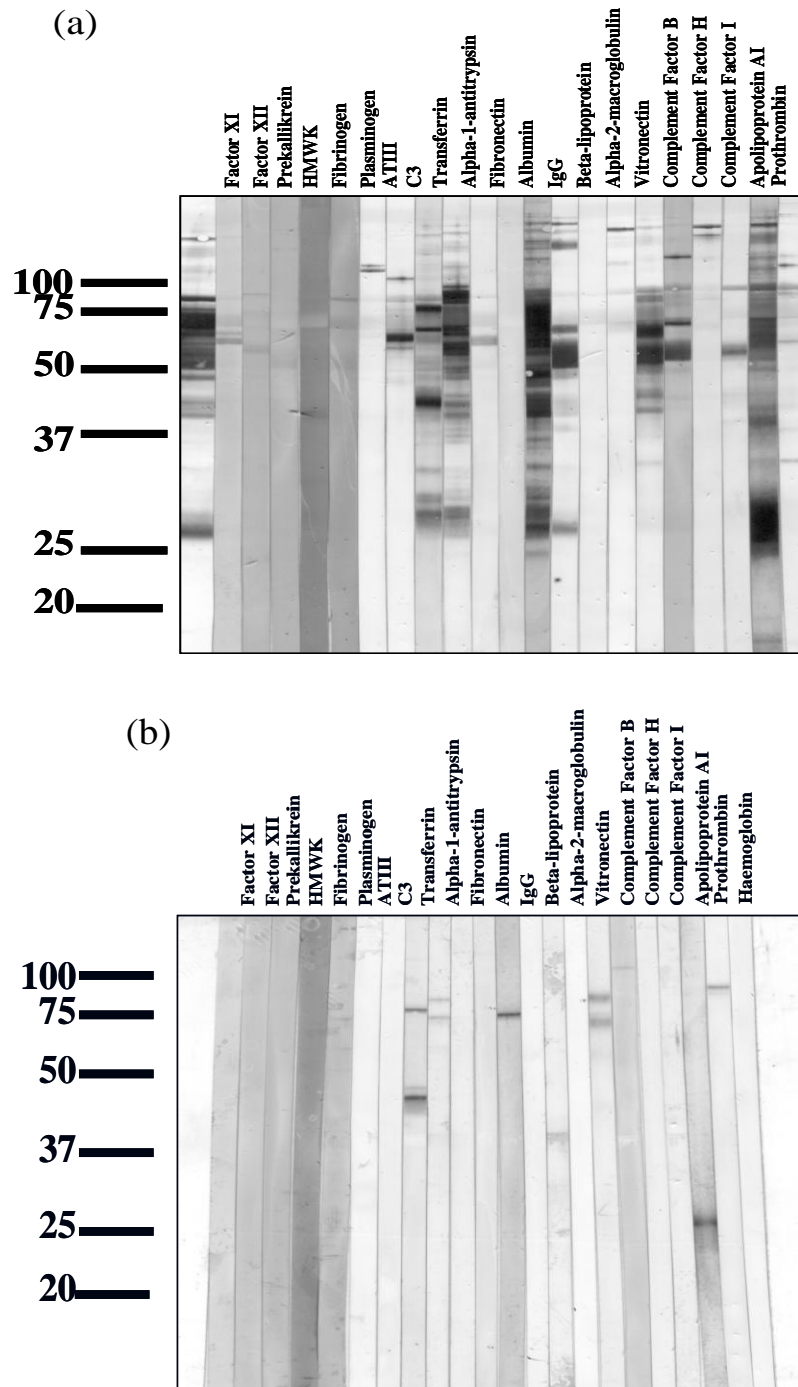


Figure 7-6: Immunoblot of HS or HP proteins present in eluates from control PS surfaces preadsorbed with either filtered HS or filtered HP proteins.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins present in eluates from control PS surfaces that were preadsorbed with either filtered HS (a) or filtered HP (b). Prestained molecular weight standards, shown on the right side, were used to estimate the molecular weight of the bands. Serum or plasma concentration, intact molecular weights and intensities of major bands are listed in Table 11 for filtered HS or Table 12 for filtered HP.

Comparisons of Immunoblot analyses of proteins present in eluates from different SAM surfaces pre-incubated with either filtered or unfiltered HS or HP

Interestingly, differential profiles of adsorbed proteins were observed in eluates from different SAM chemistries; CH₃ (Figure 7-7), OH (Figure 7-8), COOH (Figure 7-9) or NH₂ (Figure 7-10) as summarized below and shown in Tables 11 or 12 following exposure to 10% filtered HS or to 1% filtered HP. With filtered HS, bands at ~60 kDa (Factor XI), ~80 kDa (Factor XII), ~55 kDa (pre-kallikrein), fibrinogen, ~95, 96 kDa (plasminogen), ~57 kDa (α_1 -anti-trypsin) or ~93 kDa (prothrombin) were detected in eluates from OH, COOH or NH₂ SAM or in PS eluates, but not in CH₃ SAM eluates. Also, multiple protein fragments corresponding to the presence of hemoglobin were present in eluates from OH, COOH or NH₂ SAM, but could not be detected on CH₃ SAM or in PS eluates. Finally, bands corresponding to the presence of antithrombin, C3, transferrin, albumin, IgG, α_2 -macroglobulin, vitronectin, Factor B, Factor H, Factor I, apolipoprotein A1 were detected in eluates from all SAM chemistries (Figures 7-7a to 7-10a, Table 11), similar to control PS (Figure 7-6a), while HMWK and β -lipoprotein were n.d on any surface.

With pre-adsorbing filtered HP as well, subtle differences in intensities of bands for plasma proteins were observed based on chemistry. Lower band intensities were observed for pro-inflammatory fibrinogen present as three distinct bands at ~ 67, ~ 58 and ~ 47 kDa (absent versus very faint) in the eluates from CH₃ SAM surfaces (Figure 7-7b) compared with eluates from other SAM surfaces or control PS surfaces (Figure 7-6b to 7-10b, Table 12). In addition, lower band intensities corresponding to presence of C3 fragments at ~ 75 and ~ 55 kDa (faint versus strong) were present in the eluates from CH₃

SAM surfaces (Figure 7-7b) compared with eluates from COOH or NH₂ SAM surfaces or control PS (Figure 7-6b, 7-9b to 7-10b, Table 12). It is likely that the low molecular weight fragments of C3 were generated during heat-inactivation of plasma. Bands corresponding to the presence of adhesive protein vitronectin at ~ 75, ~ 65 kDa (very faint versus faint) were also weaker in eluates from CH₃ SAM (Figure 7-7b, Table 12) as compared to COOH or NH₂ SAM (Figure 7-9b to 7-10b) or compared to control PS (v. faint versus strong) (Figure 7-6b). Finally, lower band intensity of anti-inflammatory protein albumin at ~ 66kDa (faint versus strong) was observed for eluates from CH₃ SAM (Figure 7-7b, Table 12) or OH SAM (Figure 7-8b) as compared to other endgroups or control PS (Figure 7-6b). With eluates from OH SAM, no bands corresponding to the presence of C3 or vitronectin were detected and faint bands for albumin (Figure 7-8b, Table 12). Strong bands corresponding to the presence of apolipoprotein A1 were detected in eluates from all SAM endgroups (Figure 7-7b to 7-10b, Table 12).

Finally, with unfiltered HP, faint bands at ~95, 96 kDa corresponding to presence of plasminogen, faint bands at ~110 kDa corresponding to antithrombin as well as faint bands at ~150 kDa corresponding to Factor H were detected only in eluates from COOH SAM, similar to on control PS (Table 13). In addition, faint bands at ~55 kDa (IgG) or ~93 kDa (Factor B) were detected in eluates on CH₃ or COOH SAM, similar to on control PS (Table 13), but not on the other SAMs. Also, faint bands at ~80 kDa (transferrin) or bands for prothrombin were detected in eluates of OH or COOH SAM, or on OH, COOH, NH₂ SAM, respectively (Table 13). Finally, bands corresponding to the presence of fibrinogen, C3, albumin, vitronectin, apolipoprotein A1 were detected in eluates from all SAM chemistries similar to control PS (Table 13).

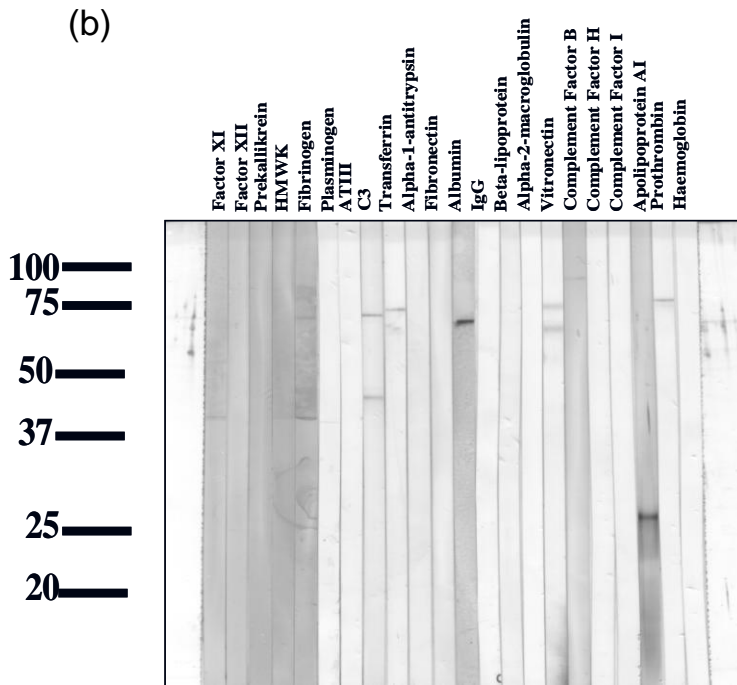
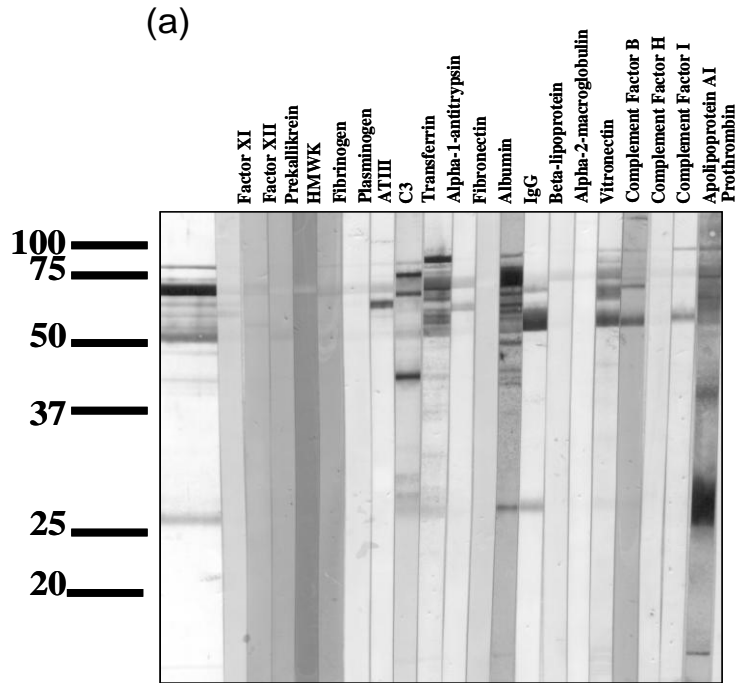


Figure 7-7: Immunoblot of HS or HP proteins present in eluates from CH₃ SAM surfaces preadsorbed with either filtered HS or filtered HP proteins.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins present in eluates from CH₃ SAM surfaces that were preadsorbed with either filtered HS (a) or filtered HP (b). Prestained molecular weight standards, shown on the right side, were used to estimate the molecular weight of the bands. Serum or plasma concentration, intact molecular weights and intensities of major bands are listed in Table 11 for filtered HS or Table 12 for filtered HP.

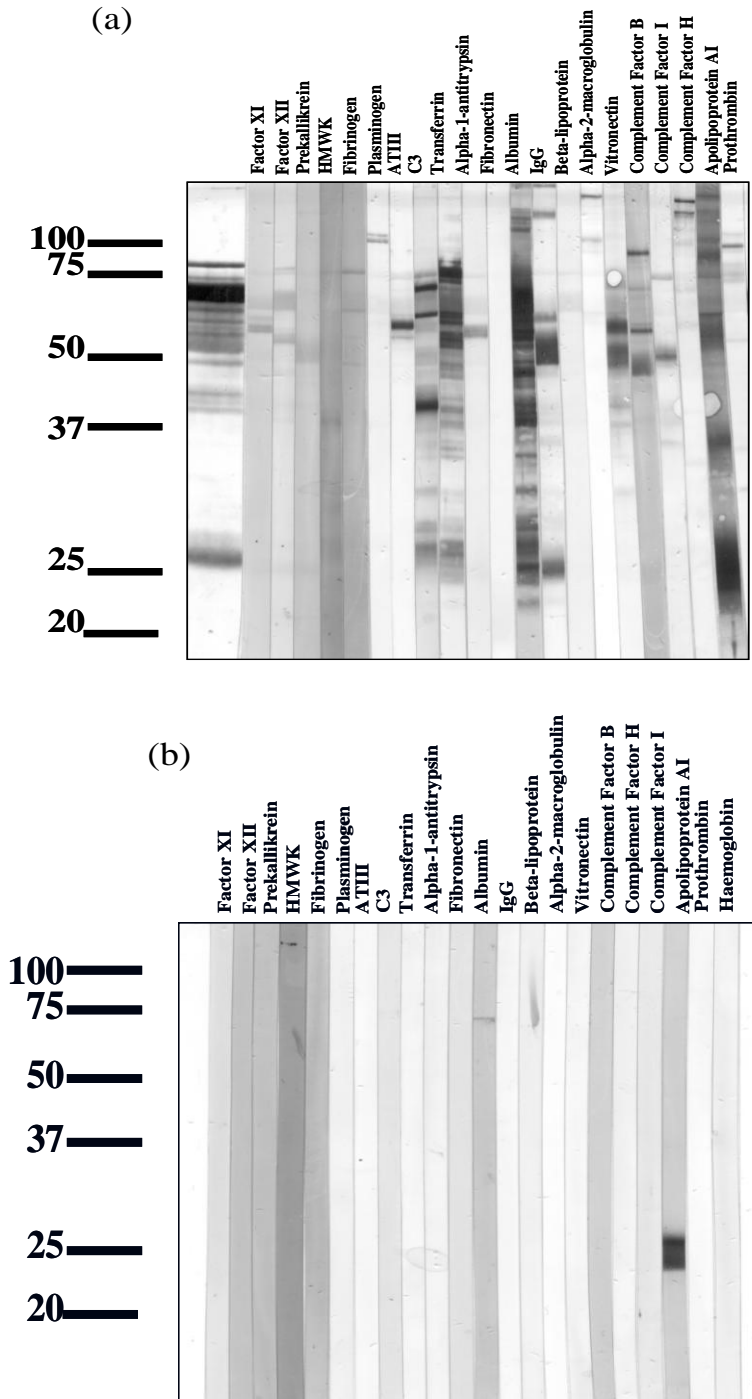


Figure 7-8 Immunoblot of HS or HP proteins present in eluates from OH SAM surfaces preadsorbed with either filtered HS or filtered HP proteins.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins present in eluates from OH SAM surfaces that were preadsorbed with either filtered HS (a) or filtered HP (b). Prestained molecular weight standards, shown on the right side, were used to estimate the molecular weight of the bands. Serum or plasma concentration, intact molecular weights and intensities of major bands are listed in Table 11 for filtered HS or Table 12 for filtered HP.

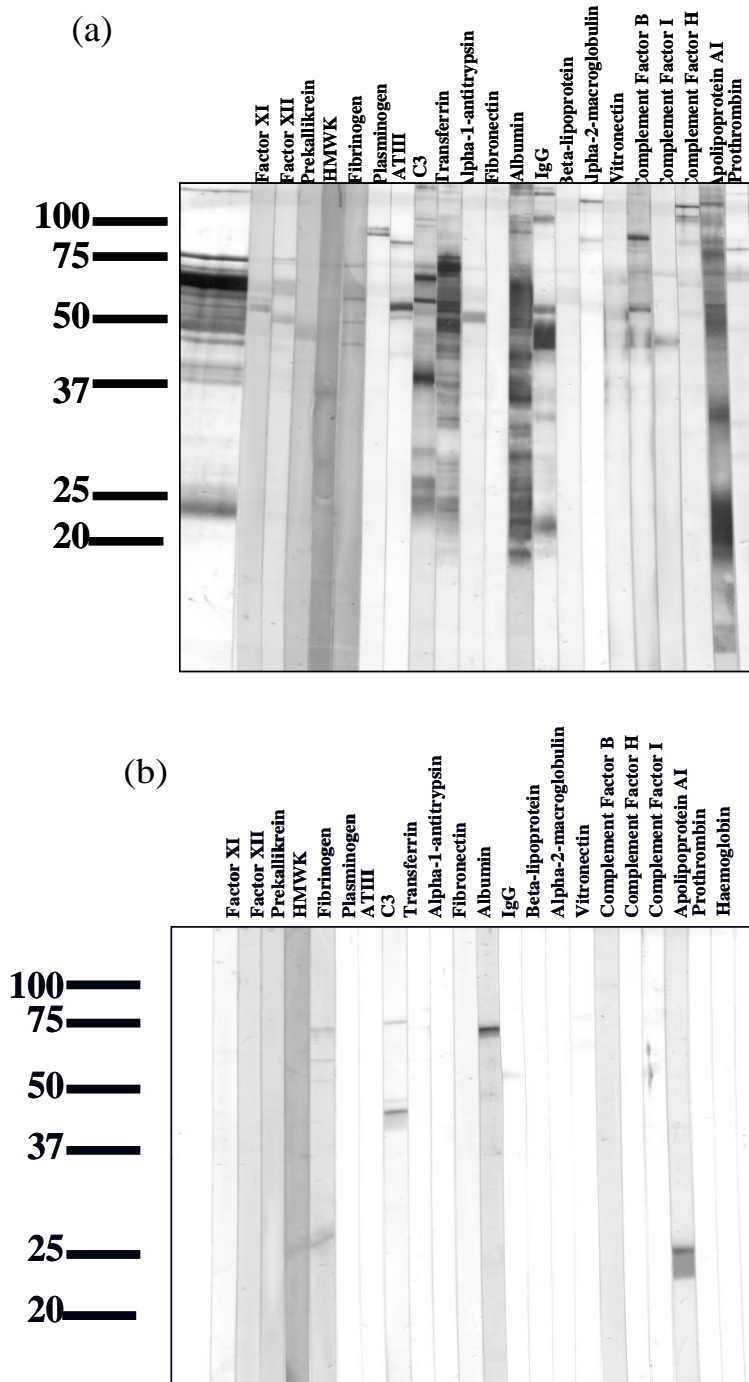


Figure 7-9: Immunoblot of HS or HP proteins present in eluates from COOH SAM surfaces preabsorbed with either filtered HS or filtered HP proteins.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins present in eluates from COOH SAM surfaces that were preadsorbed with either filtered HS (a) or filtered HP (b). Prestained molecular weight standards, shown on the right side, were used to estimate the molecular weight of the bands. Serum or plasma concentration, intact molecular weights and intensities of major bands are listed in Table 11 for filtered HS or Table 12 for filtered HP.

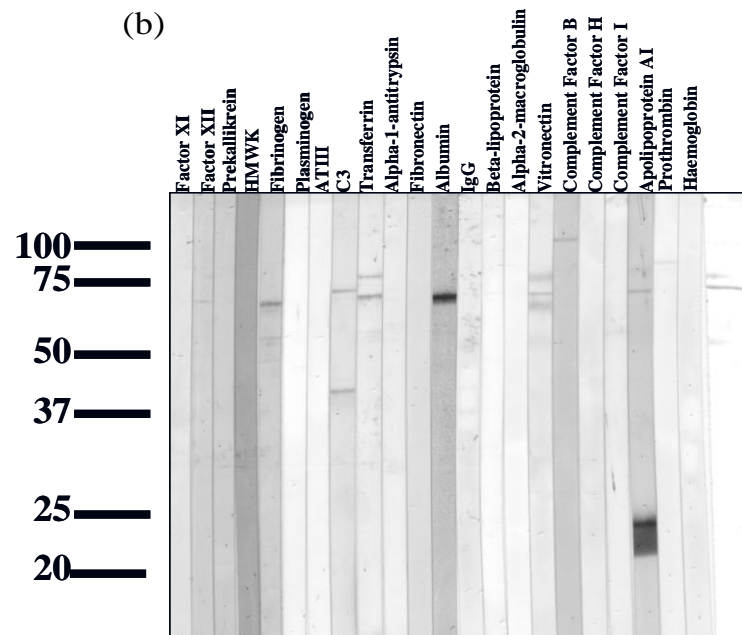
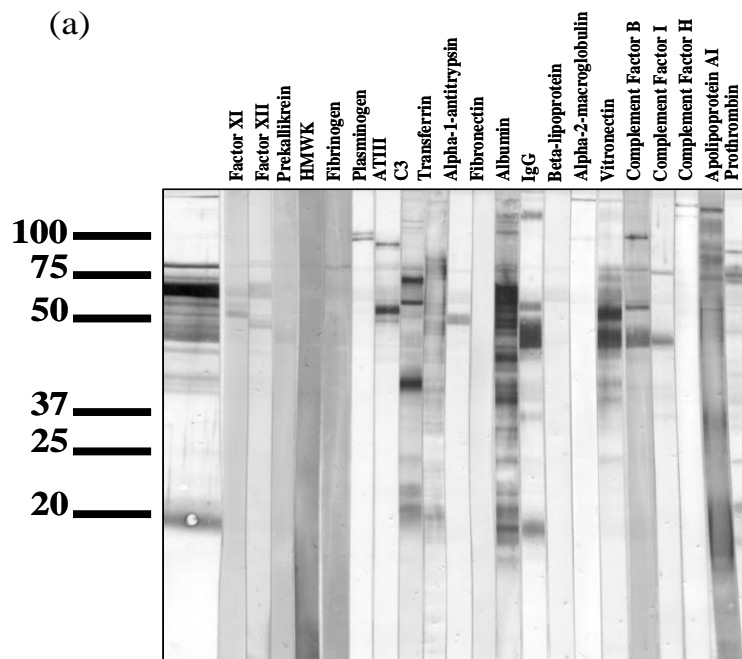


Figure 7-10: Immunoblot of HS or HP proteins present in eluates from NH₂ SAM surfaces preadsorbed with either filtered HS or filtered HP proteins.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate proteins present in eluates from NH₂ SAM surfaces that were preadsorbed with either filtered HS (a) or filtered HP (b). Prestained molecular weight standards, shown on the right side, were used to estimate the molecular weight of the bands. Serum or plasma concentration, intact molecular weights and intensities of major bands are listed in Table 11 for filtered HS or Table 12 for filtered HP.

Table 11: Serum concentration, intact molecular weights and intensities of major bands in the immunoblots of eluates from different SAM chemistries (filtered HS proteins)

Protein	Plasma Conc.* (ug/mL)	Intact MW** (kD)	Filtered h.i Serum	Eluate from PS	Eluate from CH3	Eluate from OH	Eluate from COOH	Eluate from NH3
Factor XI	5	83	83, 60	60		60	60	60
Factor XII	15-45	80	80	80 faint, 55		80 faint, 55	80 faint, 55	80 faint, 55
Prekallikrein	35-45	85	85, 55-faint			50	50	
HMWK	30-90	120	45-faint					
Fibrinogen	3000-4000	67, 58, 47	67 v. faint			58, 47	67, 58, 47	
Plasminogen	200	94	95, 96	95, 96-faint		95, 96-faint	95, 96-faint	95, 96-faint
Antithrombin	150	57	50, 57, 93-all strong bands	57, 93	57	57	57, 93	57, 93
C3	1100	75, 110	75-v. strong Lots of LMW bands present.	75-strong 60-strong, 38	75-faint 60-faint 38	75 60 38	75 60 38 LMW bands	75 60 38 LMW bands
Transferrin	2000-3200	80	80- strong, Range of other bands, LMW bands	80 LMW bands	80	80-faint LMW band	80 strong, LMW bands	80 strong, LMW bands
α -1-antitrypsin	2900	57	57, 87	57		57	57	57
Fibronectin	300	200	200					
Albumin	45000-80000	66	66-strong band Num. other bands.	66-strong band Num. other bands.	66-strong band Num. other bands.	66-strong band Num. other bands.	66-strong band Num. other bands.	66-strong band Num. other bands.
IgG	8000	55, 27	55, 27-strong band, faint other bands.	55, 27, other bands	55, 27 faint	55, 27, other bands	55, 27, other bands	55, 27, other bands
Betalipoprotein α -2-macroglobulin	2400	185	185- thin, strongt band.	185-thin, faint band	185, thin, faint	185, thin, faint	185, thin, faint	185, v. faint
Vitronectin		75, 65	55, 65, band, 75 faint diffuse band	55, 65 very strong, LMW bands.	55, 65 strong, LMW bands.	55, 65 strong.	55, 65, 75 faint diffuse bands	55, 65 strong.
Factor B	200	93	93 thin sharp band	93, 50 LMW bands	93, 50	93, 50	93, 50	93, 50
Factor H	500	150	150 thin sharp bands.	150-faint	150 v. faint	150 v. faint	150 v. faint	150 v. faint
Factor I	34	50, 38	50, 80, LMW bands.	50, 80, LMW bands.	50, 80	50, 80	50, 80	50, 80
Apolipoprotein A1		28	28 very strong band, some other HMW, LMW bands.	28 v. strong band	28 v. strong band	28 v. strong band	28 v. strong band	28 thin sharp band
Prothrombin	120	68	35, 91, 93 thin strong band. Other bands	35, 91, 93, other bands.		93-faint	93-faint	93-v. faint
Hemoglobin	-	64, 32, 16	ND	ND	ND	64 heavy, 36, 48 faint	64 heavy, 36, 48 faint	64 heavy, 36, 48 faint

Table 12: Plasma concentration, intact molecular weights and intensities of major bands in the immunoblots of eluates from different SAM chemistries (filtered HP proteins)

Protein	Plasma Conc.* (ug/mL)	Intact MW** (kD)	Filtered h.i. Plasma	Eluate from PS	Eluate from CH3	Eluate from OH	Eluate from COOH	Eluate from NH3
Factor XI	5	83						
Factor XII	15-45	80						
Prekallikrein	35-45	85						
HMWK	30-90	120						
Fibrinogen	3000-4000	67,58,47	67, 58, 47 Faint bands	v. faint 67, 58, 47		v. faint 67, 58, 47	v. faint 67, 58, 47	v. faint 67, 58, 47
Plasminogen	200	94	95, 96-faint					
Antithrombin	150	57	57-v. strong					
C3	1100	75,110	110-v. faint 75-strong LMW bands present.	75- strong 55- strong	75-faint 55-faint		75- strong 55- strong	75- strong 55- strong
Transferrin	2000-3200	80	80- strong, few LMW bands	80				80
α -1-antitrypsin	2900	57	n. d.					
Fibronectin	300	200	n. d.					
Albumin	45000-80000	66	66-strong band Num. other bands.	66- strong band	66-faint band	66-faint band	66- strong band	66- strong band
IgG	8000	55, 27	55, 27- strong band, faint other bands.	27-faint				
Betalipoprotein			n. d.					
α -2-macroglobulin	2400	185	185- thin, very faint band.					
Vitronectin		75, 65	75, 65 faint bands	75, 65 strong	75, 65 v. faint		75, 65 faint	75, 65 faint
Factor B	200	93	93 thin sharp band, 55 thick band.	93				93
Factor H	500	150	150 thin faint band.					
Factor I	34	50, 38	50 faint band, 78 faint band.					
Apolipoprotein A1		28	28 very strong band, some faint HMW bands.	28 strong band	28 strong band	28 strong band	28 strong band	28 strong band
Prothrombin	120	68		80	80			80
Hemoglobin	-	64, 32, 16						

Table 13: Plasma concentration, intact molecular weights and intensities of major bands in the immunoblots of eluates from different SAM chemistries (unfiltered HP proteins)

Protein	Plasma Conc.* (ug/mL)	Intact MW** (kD)	Unfiltered h.i. Plasma	Eluate from PS	Eluate from CH3	Eluate from OH	Eluate from COOH	Eluate from NH3
Factor XI	5	83	60					
Factor XII	15-45	80	50					
Prekallikrein	35-45	85	85, 55-faint					
HMWK	30-90	120	55,45-faint					
Fibrinogen	3000-4000	67,58,47	67, 58, 47 LMW-faint bands	67, 58, 47 LMW-faint bands	67, 58, 47-faint	67	67, 58, 47	67, 58-v. faint
Plasminogen	200	94	95, 96	95, 96-faint			95, 96-faint	
Antithrombin C3	150 1100	57 75,110	57-v. strong 110-v. faint 75-strong LMW bands present.	110-faint 75-strong 55-strong	75- .v.faint 55- v.faint	75- v.faint 55- v.faint	110-faint 75 55 LMW band	75-v faint 55-v. faint
Transferrin	2000-3200	80	80- strong, LMW bands	80 LMW band		80-faint	80 LMW band	
α -1-antitrypsin	2900	57	57					
Fibronectin	300	200						
Albumin	45000-80000	66	66-strong band Num. other bands.	66-thin strong band	66-thin strong band	66-thin strong band	66-thin strong band	66-thin strong band
IgG	8000	55, 27	55, 27-strong band, faint other bands.	55-faint	55-v. faint		55-faint	
Betalipoprotein								
α -2-macroglobulin	2400	185	185- thin, strongt band.	185-thin, faint band				
Vitronectin		75, 65	75, 65 bands LMW bands	75, 65 strong LMW bands.	75, 65 strong LMW bands.	75, 65 strong.	75, 65 strong LMW bands.	75, 65 strong.
Factor B	200	93	93 thin sharp band	93	93		93	
Factor H	500	150	150 thin sharp band.	150-faint			150-faint	
Factor I	34	50, 38	50 faint band, 78 faint band.					
Apolipoprotein A1		28	28 very strong band, some faint HMW bands.	28 v. strong band	28 v. strong band	28 v. strong band	28 v. strong band	28 thin sharp band
Prothrombin	120	68	80 thin strong band.	80 55-faint		80	80 55-faint	80-v. faint
Hemoglobin	-	64, 32, 16						

DISCUSSION:

Gold-stained gels, immunoblot and PAGE analysis of protein eluates from control PS surfaces or from different SAM surfaces were performed to characterize the profiles of HS or HP proteins adsorbed to the different biomaterial surfaces. To summarize the results from the gold-stained gels, differential presence of protein bands were detected in pre-adsorbing solutions of filtered or unfiltered HS (Figure 7-1a or 7-1b) or in filtered or unfiltered HS eluates from all biomaterial surfaces (Figures 7-2a, b or 7-3a), compared to filtered or unfiltered HP solutions (Figure 7-1c or 7-1d) or corresponding filtered HP eluates (Figures 7-2c,d or 7-3b), respectively. From this study, it is evident that the types of incubating medias used strongly influence the profiles of proteins adsorbed to different biomaterial surfaces. It is also worth noting that the results, or interpretation of results, may be skewed depending on the media used. In addition, the effects of filtration should be taken into account, since it has been well-established that filtration via a 0.22 μm pore filter results in the removal of certain serum/plasma proteins. Lastly, the concentrations of incubating medias may also influence the results; higher concentration of proteins in 10% HS may account for higher presence of proteins as compared to results shown for 1% HP (Figure 7-3). With lower media concentrations, the possibility that highly abundant plasma/serum proteins are more easily detected than less abundant plasma/serum proteins cannot be excluded and may partly be responsible for faint/n.d presence of certain proteins especially with 1% HP. However, it should be emphasized that proteins adsorbed to biomaterial surfaces from 10% filtered heat inactivated HS or 1% filtered heat inactivated HP are identical to serum/plasma used in DC media and hence very similar to biofilms that are exposed to DCs and that regulate DC responses.

In terms of protein adsorption amounts, higher protein was adsorbed to COOH or NH₂ SAM endgroups compared to CH₃ or OH SAM (Figure 7-4a). Also, higher protein remained bound to COOH or NH₂ SAM endgroups after elution with SDS/DTE compared to CH₃ or OH SAM (Figure 7-4a). While elutability of proteins from CH₃ or OH SAM was comparable ($86 \pm 6\%$ or $91 \pm 3\%$), the percentage of protein that could be successfully eluted from COOH or NH₂ SAM surfaces was lower and about $67 \pm 8\%$ or $77 \pm 8\%$ respectively (Figure 7-4b). These results implied that the lower presence of various protein bands in CH₃ SAM eluates compared to other SAM endgroups upon contact with filtered or unfiltered HS or HP solutions (Figure 7-7, Tables 11-13) was not due to lower protein elutability from these surfaces.

The proteins characterized by immunoblotting in this study represent a subset of all the proteins in fact present in HS or HP. Since it is not feasible to test all HS or HP proteins, those proteins that are commonly associated with biomaterials were tested here. Immunoblot analyses for proteins present in filtered HS or in filtered or unfiltered HP have already been completed and presented here, while analyses for unfiltered HS are ongoing and will be included in the manuscript version of this chapter to be submitted for publication. Proteins have been eluted using SDS in several other studies (Babensee 1998b), since SDS is not expected to disrupt material surfaces. The caveat to these results is that the proteins that were analyzed were SDS-elutable proteins and there may be proteins on the surface with which the cell can interact which are not SDS elutable and were hence not detected herein. The majority of proteins expected to be present in filtered pre-adsorbing HS or HP solutions were detected in Figures 7-5a or 7-5b. Specifically, in filtered HS, these proteins included intact or degraded fragments of Factor XI, Factor XII,

pre-kallikrein, HMWK, plasminogen, antithrombin, C3, transferrin, α_1 -anti-trypsin, fibronectin, albumin, IgG, α_2 -macroglobulin, vitronectin, Factor B or Factor H, Factor I, apolipoprotein A1 or prothrombin (Figure 7-5a, Table 11), although other proteins such as β -lipoprotein or hemoglobin were not present. In contrast, Factor XI, Factor XII, pre-kallikrein, HMWK or α_1 -anti-trypsin were not detectable in filtered HP solutions (Figure 7-5b, Table 12), but could be detected in unfiltered HP (Table 13). Fibronectin was n.d in both plasma preparations (Tables 12, 13). In addition, in pre-adsorbing filtered HS, multiple bands were detected for antithrombin III, possibly implying the formation of antithrombin III-complex (~93 kDa) (Figure 7-5a). For prothrombin also, multiple bands were present, not as intact prothrombin (e.g. at ~75 kDa), but as thrombin (~32 kDa) (Figure 7-5a).

To summarize the immunoblot results for filtered HS eluates from control PS surfaces or from different SAM chemistries, several HS proteins that were adsorbed to control PS were also found associated with all SAM chemistries (Figures 7-6a to 7-10a, Table 11). This list comprised proteins such as antithrombin, C3, transferrin, albumin, IgG, α_2 -macroglobulin, vitronectin, Factor B, Factor H, Factor I or apolipoprotein A1. Additionally, certain clotting factors or other proteins that were present in control PS eluates were detected only in certain SAM eluates; Factor XI, Factor XII, pre-kallikrein, plasminogen, α_1 -anti-trypsin or prothrombin were present in OH, COOH or NH₂ SAM eluates or in PS eluates, but not in CH₃ SAM eluates. Furthermore, although vitronectin bands were detected at ~55 kDa and ~65 kDa in filtered HS eluates (Figures 7-6a to 7-10a), vitronectin was detected at ~65 kDa and ~75 kDa in plasma eluates, implying that degradation may have occurred in filtered HS eluates. Taken together, these results

suggest that the profiles of proteins on certain SAM endgroups more closely resembled those present on hydrophilic control PS surfaces; notably profiles of proteins present on hydrophobic CH₃ SAM surfaces were least like those on PS surfaces or the other SAM surfaces (Figures 7-6a to 10a, Table 11).

Focusing next on the profile of adsorbed proteins on control PS surfaces that were pre-incubated with filtered HP, fibrinogen, C3, transferrin, albumin, Factor H, IgG, vitronectin, apolipoprotein A1 or prothrombin (Figure 7-6b) were present similar to results observed for unfiltered HP (Table 13). However, plasminogen, α_2 -macroglobulin, Factor B or Factor H were no longer detected adsorbed to control PS, following filtration of pre-adsorbed HP. On the other hand, several unfiltered HP proteins that were adsorbed to control PS were also found associated with all SAM chemistries (Table 13). This list comprised proteins such as fibrinogen, C3, albumin, vitronectin, apolipoprotein A1. Additionally, certain proteins that were present in control PS eluates were detected only in certain SAM eluates; plasminogen or Factor H were only in COOH SAM eluates, Factor B or IgG was in both CH₃ and COOH SAM eluates, while transferrin or prothrombin were present in OH or COOH SAM eluates or in OH, COOH or NH₂ SAM eluates, respectively. Taken together, these results suggest that for unfiltered HP, proteins present on anionic hydrophilic COOH SAM surfaces closely resembled those present on hydrophilic control PS surfaces, notably (Table 13).

The SAM chemistries used in this study have been previously characterized by us (CHAPTER 6) and others (Mrksich 1996; Kalltorp 1999; Tegoulia 2000; Shen 2001; Capadona 2003; Schwendel 2003), (Maoz 1988; Ulman 1991; Whitesides 1991). The SAM chemistries focused on here presented terminal methyl (-CH₃), hydroxyl (-OH),

carboxyl (-COOH) or amine (-NH₂) and were shown to be hydrophobic, neutral hydrophilic, anionic hydrophilic or cationic hydrophilic respectively. The SAMs are composed of alkanethiols [e.g., CH₃(CH₂)_nSH] that are covalently attached to Au/Ti-coated substrates and hydrophobic chains composed of about 10-12 C atoms tightly packed together to exclude water. Profiles, conformations and amounts of adsorbed proteins have been shown to vary as a function of surface chemistry. Specifically, more pro-inflammatory fibrinogen was associated with OH SAM as compared to CH₃ SAM (Tegoulia 2000), though this result was controversial (Shen 2001; Dadsetan 2004; Barbosa 2005a). More recently, the adsorption of human fibrinogen was demonstrated to decrease with increased presence of OH in the monolayer (Rodrigues 2006). In this study, weaker band intensities corresponding to fibrinogen were detected on CH₃ SAM, among filtered HP eluates (Table 12) or fibrinogen bands were absent on CH₃ SAM with filtered HS (Table 11), as well as lower amounts were present on CH₃ SAM from radiolabeling experiments (Figure 7-4a). Furthermore, higher presence of pro-inflammatory C3 (Hirata 2003) was measured on OH SAM than on CH₃ SAM. Although lower band intensities corresponding to C3 were detected in CH₃ SAM eluates in agreement with this finding, non-detectable levels were present in OH SAM eluates, following pre-adsorption with filtered HP (Table 12), possibly due to the high affiliation of C3b for OH SAM (Ratner 1996). Also, highest amounts of human IgG were detected on CH₃ SAM compared to OH, COOH or NH₂ SAM (Silin 1997). Interestingly, although bands indicating IgG could be detected on control PS, CH₃ or COOH SAM eluates with unfiltered HP (Table 13), the presence of IgG could only be detected on control PS surfaces (Figure 7-6b) but not on any of the SAM surfaces (Figures 7-7b to 7-10b) with

filtered HP (Table 12). Also, adsorption of anti-inflammatory protein albumin was higher on CH₃ SAM compared to either OH or COOH SAM (Martins 2003). Finally, bands corresponding to the presence of apolipoprotein A1 were detected in eluates from different SAM treatments. The significance of this finding may be further examined with reference to the profiles of lipid or carbohydrate post-translational modifications present on apolipoprotein A1 (Dayal 2002; Zannis 2006) that may act as ligands for DC C-type lectin receptors (CLRs) or scavenger receptors (SRs), with different chemistries.

Extending this discussion of protein profiles to resultant responses of immune/inflammatory cells to different SAM chemistries, platelet adhesion and activation was reduced with greater hydrophilicity (increased –OH groups) (Rodrigues 2006). Furthermore, pre-incubated fibrinogen resulted in platelet stimulation, while pre-adsorption with albumin elicited anti-inflammatory effects on –OH monolayers but not on –CH₃ monolayers (Rodrigues 2006). In further support of this observation, others have shown that OH SAMs permit increased leukocyte binding. This result agrees with the capacities for OH SAM to trigger high complement activation as measured by binding of C3b to –OH terminal monolayers. This binding was found to be proportional to the numbers of acidic sites on the endgroup. On the other hand, –CH₃ endgroups supported lowest leukocyte adhesion (Sperling 2005). The opposite was true for effects of OH SAM on platelet adhesion (Sperling 2005).

Different SAM chemistries have demonstrated differential recruitment of immune/inflammatory cells including monocytes, polymorphonuclear (PMNs) cells (Barbosa 2005a) and activated Mac-1 positive leukocytes *in vivo* to implant sites in mice air pouch cavities (Barbosa 2005b). Although the CH₃ SAM endgroups facilitated

maximum PMN recruitment, these surfaces were associated with least presence of adherent neutrophils or leukocytes, even though platelets were still observed to adhere (Sperling 2005). Additionally, the CH₃ SAM endgroups were associated with thicker fibrous capsules (Barbosa 2006), raising the possibility that these surfaces may support highest inflammatory responses among the SAM endgroups tested. Others have shown that differential monolayer chemistries direct different conformations of adsorbed fibronectin and therefore distinct cell integrin-mediated surface adhesion interactions (Keselowsky 2003). Overall, studying the ability of biomaterials to support DC maturation *in vitro* represents a significant initial step towards ultimately examining the potential of the biomaterials to stimulate DC-regulated adjuvant effects under *in vivo* conditions.

Recent studies have suggested a vital role for glycans in mediating multiple biological processes such as in development (Ioffe 1994; Perrimon 2000), coagulation (Jin 1997) and in controlling bacterial or viral diseases (Fu 2003). Since glycan post-translational modifications of proteins exist on cell surfaces, in intracellular cytosolic compartments, in the extracellular space between cells and tissues and in serum or plasma proteins, it is becoming increasingly evident that they direct multiple phenomena. Approaches to target diseases such as cancer (Smorenburg 2001; Liu 2002b; Wahrenbrock 2003) and cardiovascular disease (Shriver 2002), include the use of glycan-directed strategies. However, their importance in mediating fundamental immune/inflammatory responses in the context of biomaterials remains to be elucidated. Glycosylations may be present associated with adsorbed proteins on biomaterial surfaces and may play critical, hitherto undefined roles in facilitating cell attachment, survival,

differentiation and other key cell responses. Future directions may include concentrating on a select group of relevant glycosylated serum or plasma proteins that are generally adsorbed onto biomaterials, obviously discernible and with established degradation profiles on gels, to scrutinize the profiles and protein sources of detected carbohydrates that are eluted from different SAM endgroups, thereby adding another dimension to these studies. Characterization of carbohydrates present associated proteins in SAM eluates would be performed using two-dimensional gel electrophoresis and lectin blotting, using a panel of lectins with specificities similar to those described in Table 5 in CHAPTER 6. The ultimate goal would be towards the design of optimal norms for synthesizing biomaterials that have an extensive spectrum of adjuvancies for wide-ranging applications in regenerative medicine.

CHAPTER 8

CONCLUSIONS AND FUTURE WORK

The host response elicited against implanted combination products may comprise a non-specific inflammatory response against the biomaterial component as well as a specific adaptive immune response against the biological component, which may be enhanced by the biomaterial adjuvant effect. Since adjuvants function by supporting DC maturation *in vivo*, this hypothesis was tested by examining the effects of different biomaterials in sustaining DC maturation *in vitro*. With this objective, attempts have been made in this thesis research to compare the morphologies, phenotypes and functional characteristics of DCs following contact with different biomaterials. Since DCs orchestrate the adaptive immune response or may mediate immune tolerance, DC responses to biomaterials may predict the nature of the host adaptive response to biomaterial implants. The adaptive immune response may be synergistic with the host inflammatory response and therefore be important in determining the tendency of the biomaterial to stimulate adaptive immunity against associated biological components and hence cause graft rejection.

The *in vitro* assays conducted in this thesis research were designed to evaluate DC responses to different biomaterial surface chemistries, as an *in vitro* measure of their adjuvant potentials *in vivo*. These assays examined several key parameters of DC responses and underlying mechanisms, as well as characterized likely DC ligands in the context of biomaterial responses. The key areas that may be included in future studies have been described here.

While experiments conducted in this thesis work have included characterizations of DC cultures and of the other cell types that were present in cultures derived from human PBMCs such as autologous lymphocytes (CHAPTER 4), the importance of DC-lymphocyte interactions in maintaining the differentiated DC status and in influencing DC immune responses and the receptors mediating these interactions should be further investigated. Furthermore, the cytokines and chemokines secreted both by DCs and lymphocytes should be assessed. A more complete characterization of the proportions of different DC subsets (lymphoid, myeloid, pre-DC1s, pre-DC2s) (Liu 2001) isolated using PBMCs as a starting population should be identified. The types of DC subsets collected may influence the ensuing T_{h1}/T_{h2} cytokine balances and hence this information may be helpful in optimizing DC isolation protocols to drive the T_h cell responses based on desired end applications. In addition, more thorough analyses of the mechanisms underlying differential DC responses to biomaterials should be conducted. The temporal variations of DC maturation responses should be assayed by examining other timepoints besides 24 hours such as 6 and 12 hours. The 6-hour timepoint may be especially relevant for studies characterizing the expression of PRRs such as TLRs or CLRs, which are upregulated on iDCs and may be downregulated by the 24-hour timepoint. Furthermore, blocking studies should be performed to identify the roles of specific PRRs molecules, both at the protein level using function blocking antibodies (Akashi 2000), as well as using RNA interference approaches involving short hairpin RNA (Arrighi 2004; Cekaite 2007). More emphasis may also be placed on exploring the key signaling pathways triggered by activation of different PRRs or combinations of multiple PRRs. For instance, these approaches should include investigations into TLR-mediated signaling via MyD88

and MAPK/ IKK pathways or Trif-mediated activation of interferon regulatory factors (IRF) pathways (Kawai 2006) in response to biomaterial associated antigens, preferential signaling via these pathways for certain TLRs and the balance between NF- κ B (maturation, differentiation, survival) and AP-1 pathways (apoptotic death) (Kriehuber 2005a) in the presence of different biomaterials. Finally the link between activation of certain TLRs/CLRs by antigens related to biomaterials and the ensuring regulation of T_{H1}/T_{H2} cytokine balances should be probed (Pulendran 2005). The endotoxin levels associated either with the biomaterials or with specially processed FBS used in DC media have been vigilantly monitored throughout this work. Although the endotoxin levels associated with SAM surfaces were negligible (CHAPTER 5), high endotoxin was present in de-glycosylated FBS (Figures A1-1 to A1-3 and Figure A2-1, APPENDIX), probably as result of the extensive processing steps. At present, the current strategies available to purge proteins of endotoxin are not applicable for protein concentrations as high as that present in FBS and hence cannot be used. Future studies may however, focus on overcoming this issue towards examining the role of carbohydrates in supporting biomaterial-centered DC maturation.

Among the model SAM chemistries utilized in this thesis research, we concluded that CH_3 SAM surfaces supported lowest DC maturation, since cells cultured on these surfaces were rounded, similar to iDCs and were undergoing high apoptosis, which may partly explain the DC immunosuppression observed, since phagocytosis of apoptotic DCs is a potent inducer of anti-inflammatory effects on DCs (Skoberne 2005b; Wallet 2005b; Chen 2006c) (CHAPTER 5). Although others have concluded from *in vitro* (Lindbald 1997; Barbosa 2003) and *in vivo* studies (Lindbald 1997; Barbosa 2003; Keselowsky

2003; Schwendel 2003; Barbosa 2005a) that CH₃ SAM surfaces caused an acute inflammatory response as exemplified by increased recruitment of inflammatory cells to CH₃ SAM surfaces, higher presence of Mac-1⁺ cells and thicker fibrous capsule, some inconsistencies do still exist since both this group and others (Schwendel 2003) observed that cells still adhered least to CH₃ SAM surfaces. Since Mac-1 (CD11b/CD18) is an integrin receptor and these cells show least adhesion to the CH₃ SAM surface, it may indicate that Mac-1 receptor may play another role on this surface. Several groups (Coxon 1996; Whitlock 2000; Verbovetski 2002; Morelli 2003; Skoberne 2005b) have shown that Mac-1 receptor (also CR3), plays a role in internalizing apoptotic cells and causing DC tolerance. In human DCs, opsonization by Mac-1 ligand iC3b, resulted in tolerized DCs having downregulated MHC II and CD86 and upregulated CCR7 expression (Verbovetski 2002). In the splenic marginal zone, DCs were found to undergo apoptosis resulting in DC tolerance that was mediated by CR3 (CD11b/Cd18) receptor (Morelli 2003). Furthermore, $\alpha_M\text{-}\beta_2$ -mediated leukocyte apoptosis and apoptosis of neutrophils should be blocked using CD11b/CD18 antibodies (Coxon 1996). These results could be interpreted in a different manner, taking into account this recent literature. The CH₃ SAM surfaces that have high Mac-1⁺/CR3⁺ cells may cause increased cellular apoptosis and render cells anti-inflammatory. This leads to several interesting questions and future directions, including if CH₃ SAM implant surfaces would recruit more cells, allow lower cell adhesion, support higher apoptosis and hence mediate immune tolerance, thereby reflecting our *in vitro* results (CHAPTER 5) *in vivo*. The effect of apoptosis in mediating immunosuppression *in vivo* should be tested by conducting blocking studies to inhibit the function of Mac-1/CR3 to observe if this

renders recruited cells less apoptotic, in connection with the literature implicating Mac-1 in inducing DC apoptosis. These and other unanswered questions may be investigated using model SAM surfaces *in vivo*, once issues concerning the stability of these monolayers *in vivo* have been resolved, although the use of short term implants have been shown to be successful.

Another significant factor in determining biomaterial biocompatibility is the nature of the host protein biofilm that forms very rapidly on the biomaterial surfaces and with which host immune cells interact. This interaction may be bi-directional and may influence immune cell responses as well as induce cellular protease-directed biofilm remodeling (Hubbell 1995). Hence, the characterization of the biofilm may be important towards the elucidation biomaterial surface properties that render biomaterials activating or passivating and may be of interest in future studies, as an extension of the work performed in (CHAPTER 6).

As an initial approach to characterize the adsorbed protein layer and associated post-translational modifications such as carbohydrates or lipids, mass spectrometry (MS) should be performed on SAM surfaces that have been pre-incubated with human serum or plasma. Firstly, MS should be performed directly on SAM surfaces pre-coated with serum/plasma (37°C, 1hr). Secondly, proteins should be eluted from SAM surfaces prior to MS [trypsin/ SDS and MS analysis should be performed by matrix-assisted laser desorption ionization (MALDI)/ electrospray ionization (ESI)-time of flight (TOF) (Chalabi 2006; Comelli 2006). Finally, enzyme-based cleavage of lipids or carbohydrates associated with adsorbed proteins should be performed and MS analysis should be conducted to characterize variations if any, in the adsorbed biofilms on different SAM

surfaces. There are however, several technical issues associated with performing MS that need to be overcome, prior to these analyses. These include successfully removing proteins off the surface and into the analyzer, which may be compounded if multiple serum or plasma proteins were adsorbed. Furthermore, lipase/glycosidase cleavages would occur at random positions and methods to confirm that cleavages have occurred and the positions they have occurred in would first need to be identified. Also, importantly, the cleavage of only the presented sugars that the cells interact with on the surface should not be controlled. Finally, several proteins in serum are glycosylated, with multiple types of glycosylations and sugar linkages, making data interpretation difficult. Although several groups have performed MS to characterize proteins, those experiments were performed on samples in solution and considerable expertise may be required to transition to characterizing adsorbed biofilms on biomaterial surfaces.

As another approach to characterizing the biofilm layer, immunoblot analysis of protein eluates from different SAM chemistries have already been examined in this thesis work, as part of collaboration with Dr. Brash and his laboratory at McMaster University, Canada (CHAPTER 7). Furthermore, in order to elucidate the profiles of carbohydrates associated with proteins eluted from different SAM chemistries, as well as the protein sources of these glycosylations, a protocol to perform lectin blotting using NPA, UEA-1, UEA-2, SNA-1 or HHA lectins that are specific for different carbohydrate moieties has been optimized and has been tested on purified single proteins including anti-thrombin III, fibrinogen, albumin, vitronectin, plasminogen (Figure A3-1b, APPENDIX). In addition, lectin blotting using NPA, UEA-1, UEA-2, SNA-1 or HHA lectins was performed using plasma or serum samples, following SDS-polyacrylamide gel

electrophoresis (PAGE) (Figure A3-1a, APPENDIX). However since several lectins bind the same protein and vice-versa, clear trends were not distinguishable. Finally, to circumvent the technical difficulty of handling all proteins in serum or plasma simultaneously, future directions include focusing in on a panel of up to five glycosylated serum or plasma proteins that are commonly adsorbed onto biomaterials, clearly distinguishable and with known fragmentation profiles on gels, to investigate the profiles and protein sources of detected carbohydrates that are eluted from different SAM endgroups. Specifically, this panel of proteins on interest could include IgG, fibronectin, vitronectin, fibrinogen, complement-3 and IgA. While IgG has been characterized as having N-linked glycans with fucose (Jefferis 1990) and galactose moieties on mannose $\alpha(1-3)$ or $\alpha(1-6)$ (Takahashi 1987), fibronectin possesses N-glycosylations at one site each on its β and γ chains, IgA contains terminal sialic acid, galactose and GalNAc (Xu 2005), complement-3 has glucosylated N-linked glycans (Crispin 2004), fibrinogen possesses N-linked glycans (Henschen-Edman 2001) and vitronectin has N-linked glycans and sialylations (Uchibori-Iwaki 2000). N-glycosylations are typically present on asparagine and typically include N-acetylneuraminic acid (NeuNAc), galactose, GlcNAc and mannose. Alternatively, O-glycosylations occur on serine or threonine and comprise NeuNAc, galactose, GlcNAc and GalNAc (Voet 2004). Besides this set of proteins, additionally, other glycosylated serum/plasma proteins such as apolipoprotein A1 (Dayal 2002; Zannis 2006) may be examined. Alternatively, proteins having known specific glycosylations such as α_1 -antitrypsin that contains di and tri-antennary N-glycans with alpha-neuraminic acid (Kolarich 2006), transferrin (Bryrne 2006), haptoglobin (He 2006) or high density lipoprotein (HDL) (Vinals 2003) that comprise N-linked glycans either

inherently or following the induction of mutations (Isordia-Salas 2003), α_2 -macroglobulin that has oligomannose glycans (Arnold 2006), that have been probed for using immunoblot analyses as described in CHAPTER 7, may be investigated.

Following the characterization of the profiles of proteins and their post-translational modifications presented in adsorbed biofilms on different biomaterials either by MS or SDS-PAGE and lectin blotting, the next step should be directed towards describing the roles of these modifications in directing DC-mediated host responses to biomaterials. These strategies may include using conjugates composed of carbohydrate ligand - BSA such as mannose-BSA for instance, that would specifically block carbohydrate-binding abilities of DC receptors, or lectin-BSA conjugates to specifically block certain glycosylations presented by adsorbed proteins on biomaterials, or, finally, receptor blocking using mannan, β -glucans (Rogers 2005), ManLAM (Geitjenbeek 2005) or other ligands mentioned in the literature. Caveats to these suggestions are the host immune responses generated against BSA or the lectin itself. Also, any one carbohydrate-BSA or lectin-BSA conjugate used alone may not successfully block the functionality of type I CLR (such as MMR) that have multiple carbohydrate recognition domains to bind distinct glycosylated ligands. In such cases, approaches involving combinations of these should be utilized.

Another potential future direction would be towards the development of sophisticated, high-throughput biomaterial screening systems, utilizing techniques such as DNA microarrays (Ahn 2002; Edwards 2003), proteomics (Richards 2002), genomics (Hashimoto 1999) as readouts of DC responses, on combination libraries of different biomaterials. Modifications of the chemical vapor deposition (CVD) system established

in Dr. Jorge Lahann's laboratory at the University of Michigan, at Ann Arbor (Chen 2006a) may be utilized as one such example high-throughput systems to examine if different proteins and/or carbohydrates ligands of DC PRRs immobilized on polydimethylsiloxane (PDMS) substrates either alone or as combinations, support differential DC maturation. Multi-well surfaces in which each well is modified with any one known carbohydrate DC ligand (mannose, fucose, NeuNAc, GalNAc, galactose, GlcNAc) (Figdor 2002) or if available, with one known lipid ligand of scavenger receptors (SR) [acetylated low density lipoprotein (acLDL) (Chen 2006d), oxidized LDL (Yoshida 2006c), high density lipoprotein (Tserentsoodol 2006), apolipoprotein A-1 (Cavelier 2006)] or other SR ligands including advanced glycation end products (Nagai 2007), gp96 (Berwin 2003), careticulin (Berwin 2003), bacteria, heat shock proteins (HSPs) (Facciponte 2005), phosphatidylserine (Greenberg 2006) should be used to observe effects on DC maturation. As a next step towards gradually building up the complexity of the system to resemble adsorbed biofilms and based on the results obtained from pre-immobilized carbohydrate or lipid-modified surfaces alone, surfaces may then be modified with both lipids and carbohydrates, either as random amalgamations or as combinations that mimic PAMPs as LPS that has a complex chemistry composed of both lipid and sugar components. Dendritic cells would be cultured on these surfaces and both DC maturation as well as expressions of TLRs such as TLR2, TLR4, TLR5 (that bind carbohydrate and/or lipid ligands), CLR's such as Dectin-1, MMR, DC-SIGN and ASGPR and SRs of interest that bind these ligands should be examined at different timepoints, to identify the PRRs on DCs that mediate biofilm recognition. A similar system comprising glycoprotein arrays used to examine carbohydrates that are presented in the context of

proteins has also been developed (personal communication with Dr. Dan Ratner). Other sophisticated tools such as those involving the incorporation of sugar labeling groups to visualize and follow glycoconjugates within cells (Hsu 2007) or *in vivo* (Sawa 2006) or even lectin microarrays (Kuno 2005) could be employed. Next, ELISA-based quantization of binding and immunohistochemical staining by biochemical cross-linking of bound receptors, previously achieved on SAMs (Keselowsky 2003) should be performed. This assay involves the removal of other cellular components while leaving behind cell receptors still attached to ligand. However, there are still some issues that need to be overcome prior to conducting this assay. The exact structure of the receptors of interest would need to be known, since specific biochemical linking steps need to be performed. The advantages of using this adaptation of the CVD system compared to using SAM surfaces presenting different chemistries are that this would be a more controlled system where cell responses should be attributed to the presence of specific adsorbed sugar/lipid ligand of PRRs. This system may be an initial method of addressing the roles of relevant carbohydrate or lipid modifications of serum/ plasma proteins, systematically. However a significant level of characterization would first need to be performed on these surfaces, prior to their usage. Endotoxin levels of these surfaces would also need to be carefully monitored.

This thesis starts to address the roles of biomaterials as adjuvants in the context of combination products used *in vivo*, by examining the effects of biomaterials in supporting DC maturation *in vitro*. This work along with future studies that systematically examine the physical and chemical properties of biomaterials and their associated adsorbed biofilms in supporting DC-centric host responses may ultimately lead to the development

of critical criteria for designing biomaterials that have fine-tuned adjuvancies for use in regenerative medicine

APPENDIX 1

ROLES OF CARBOHYDRATE LIGANDS IN SUPPORTING DENDRITIC CELL MATURATION IN THE CONTEXT OF SELF-ASSEMBLED MONOLAYERS

INTRODUCTION:

Controlling the non-specific host responses associated with implanted tissue-engineered (TE) devices is critical for their success (Anderson 1988; Langer 1993; Tang 1995; Ratner 1996; Babensee 1998a; Babensee 2000; Rihova 2000; Stock 2001; Wettero 2002). More recently, it was also observed that the specific immune response generated against the biological constituent of combination products could be enhanced by the biomaterial component, thereby causing the biomaterial to act as an adjuvant (Babensee 1998a). Adjuvants function by stimulating antigen (Ag) presenting cells (APCs) such as dendritic cells (DCs) and causing DC maturation as exemplified by the upregulation of co-stimulatory and major histocompatibility II (MHC II) molecules that facilitate activation and presentation of peptide Ag to adaptive immune cells, as well as the secretion of pro-inflammatory cytokines (Sallusto 1994; Zhao 1996; Cella 1997a; Winzler 1997; Banchereau 1998a; Singh 1999; Banchereau 2000; Sun 2003). The biomaterial adjuvant effect was demonstrated *in vivo*, when delivery of poly (lactic-co-glycolic acid) (PLGA) along with ovalbumin (OVA) resulted in the production of higher levels of antibodies that were specific against OVA (Bennewitz 2005). Furthermore, treatment of immature DCs (iDCs) with PLGA resulted in DC maturation (Yoshida 2006a). In contrast, iDCs matured to a lesser extent when treated with agarose (Yoshida 2006a), demonstrating that while certain biomaterials may have potent adjuvant effects *in vitro*, others may facilitate modest immune responses against the biological component

(Ertl 1996; Walker 1998; Yoshida 2004; Babensee 2005; Bennewitz 2005; Yoshida 2006a; Yoshida 2006b).

The differential ability of biomaterials to stimulate immune and inflammatory responses is a critical parameter in the design of suitable biomaterials for diverse applications. For instance, lower adjuvant potency is ideal for biomaterial scaffolds used in TE applications, while the opposite is required for materials that are used to deliver protein vaccines, where an augmented immune response is desired to ensure vaccine efficacy. It is well known that biomaterial surface properties such as chemistry, hydrophobicity/hydrophilicity and charge, as well as bulk properties such as porosity, hardness, among others, are important criteria in determining host-biomaterial associations including the amounts, profiles and conformations of non-specifically adsorbed host proteins, cell adhesion, survival, differentiation, proliferation, secretion of growth factors and cytokines and in directing cell-cell communications (Anderson 1988; Langer 1993; Tang 1995; Ratner 1996; Babensee 1998a; Babensee 2000; Rihova 2000; Stock 2001; Wettero 2002). The types of adsorbed proteins (regulated by surface properties) could be important; for instance, while adsorbed albumin (Martins 2003) has been linked to having a passivating effect, adsorbed proteins such as complement-3 (C-3) (Hirata 2003) or fibrinogen (Fn) (Silver 1995) are associated with pro-inflammatory effects. For the purposes of this study, well-characterized, robust self-assembled monolayers (SAM) (Mrksich 1996; Lindbald 1997; Harder 1998; Sigal 1998; Kalltorp 1999; Luk 2000; McClary 2000; Tegoulia 2000; Shen 2001; Barbosa 2003; Capadona 2003; Keselowsky 2003; Michael 2003; Schwendel 2003; Dadsetan 2004) having (-CH₃), (-OH), (-COOH) or (-NH₂) endgroups were used model biomaterial surfaces to study the

interactions between surface chemistry and immune cells, specifically DCs. The broad hypothesis underlying this work is that DCs may interact differentially with adsorbed host proteins on different surface chemistries, specifically with the glycosylations that are associated with adsorbed host proteins and mature differentially, hence triggering distinct adaptive immune responses. Although glycosylations may play major roles in mediating host responses to biomaterials, these have hitherto been largely unstudied. However, with the arrival of the glycomics era and associated novel strategies to probe carbohydrate structure and function (Shriver 2004; Turnbull 2007), the roles of these biomolecules in maintaining key biological processes are gaining in prominence.

Immature DCs recognize conserved protein-carbohydrate-lipid-based pathogen associated molecular patterns (PAMPs) using pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Aderem 2000; Akira 2001; Medzhitov 2001) or carbohydrate-binding C-type lectin receptors (CLRs). This recognition event may trigger the iDCs to transition into mature DCs (mDCs) that present processed internalized antigen via MHC complexes to T or B lymphocytes or result in DC tolerance, thereby linking innate and adaptive immune immunity (Matzinger 1994; Cella 1997a; Banchereau 1998a; Gallucci 1999; Banchereau 2000; Heath 2001; Medzhitov 2001; Engering 2002; Figdor 2002; Janeway 2002; Sun 2003; van Kooyk 2003). The CLRs include mannose receptor (MR) that binds terminal mannose, fucose, sialylated groups or GlcNAc (Sallusto 1995), DC-specific ICAM-3 grabbing non-integrin (where ICAM-3 is intercellular adhesion molecule 3) (DC-SIGN) that binds mannose-enriched internal residues (Geitjenbeek 2000), Dectin-1 (Brown 2000; Brown 2001a; Brown 2006) that binds β -glucans, asialoglycoprotein receptor (ASGPR) (Valladeau 2001) and macrophage

galactose N-acetyl-galactosamine (GalNAc) specific lectin 1 (MGL) that bind α - and/or β -galactose or GalNAc. The carbohydrates internalized via CLR binding events have been shown to reach different intracellular compartments, may be differentially presented on MHC complexes, stimulate the secretion of pro- or anti-inflammatory mediators in combination with TLRs (Gantner 2003; Rogers 2005; Dillon 2006) or individually, upon ligand binding (Gantner 2003; Rogers 2005) and induce CLR-mediated augmentation of TLR-2-induced activation of transcription factors such nuclear factor- κ B (NF- κ B) (Brown 2006) that mediates critical cellular events such as DC maturation.

Carbohydrate moieties that are similar to those present in PAMPs and that are ligands for DC CLRs may play a role in supporting DC maturation in the context of biomaterials. It was previously observed that differential profiles of carbohydrate ligands of DC CLRs were present on different SAM chemistries (CHAPTER 6). In this study, a first step has been taken towards examining the roles of these carbohydrates in causing DC maturation, by comparing DCs that were cultured in DC media with fetal bovine serum (FBS) that was selectively de-glycosylated of these carbohydrate ligands, versus DCs that were cultured in DC media with non-processed FBS. While several groups have previously focused on the inflammatory roles of adsorbed proteins, this study is significant as it focuses on the effects of glycosylated ligands of DCs presented by biomaterials surfaces in stimulating DC-mediated immune responses.

METHODS:

Self-Assembled Monolayer Preparation

Self-assembled monolayer surfaces were assembled on 16-well glass chamber

slides (*LAB-TEK*, Nalge Nunc International, Rochester, NY) for carbohydrate or protein measurement assays or on 35 mm X 10 mm sterile, tissue culture (TC)- treated, non-pyrogenic polystyrene (Phillips) dishes (Corning, Corning, NY) used for DC culture (Keselowsky 2003) as described in CHAPTERS 4 and 5. Clean chamber slides or dishes were coated sequentially with 50 Å Ti followed by 150 Å Au films using an electron beam evaporator (CVC Products/ Veeco, Rochester, NY) as described in (Keselowsky 2003) and stored under vacuum at room temperature (RT) until two weeks until used. The following alkanethiols were used as received from commercial sources (1 mM in absolute ethanol): 1-dodecanethiol (SH-(CH₂)₁₁-CH₃) (CH₃ SAM), 11-mercapto-undecanol (HS-(CH₂)₁₁OH) (OH SAM), 11-mercaptoundecanoic acid (HS-(CH₂)₁₀-COOH) (COOH SAM), (Aldrich Chemical, Milwaukee, WI) and 11-amino-1-undecanethiol, hydrochloride (C₁₁H₂₆ClNS) (NH₂ SAM) (Dojindo Laboratories, Gaithersburg, MD). The SAMs were allowed to assemble by 12 hr incubation at RT by immersing the Ti/Au-coated slides or dishes in alkanethiol solutions, following which the chamber slides were washed with 95% ethanol (Sigma, St. Louis, MO) dried with N₂ gas (Airgas South, Chamblee, GA) for ten minutes in a fume hood, equilibrated with Phosphate Buffered Saline (PBS) (Gibco, Grand Island, NY) for 5 minutes at RT and used fresh. The SAM-immersed dishes used for DC culture were opened under sterile conditions in the TC hood, washed once with absolute ethanol (Sigma), followed by five washes with sterile-filtered (0.22 μm) (Corning, Corning, NY) PBS and used immediately for cell culture assays. Based on previous characterizations CH₃ SAMs were most hydrophobic and OH SAMs were most hydrophilic. Also, x-ray photon spectroscopy (XPS) analysis of surface elements showed close agreement with calculated

theoretical values (CHAPTER 6).

Selective de-glycosylation of Fetal Bovine Serum (FBS)

Fetal bovine serum (Invitrogen, Carlsbad, CA) (50 mLs) was thawed and treated with 1 μ l of N- or O-glycosidases (PNGase F, Sialidase, β -galactosidase, glucosaminidase and O-glycosidase) sequentially, in no specific order, as per manufacturer's instructions (QA bio, San Mateo, CA) in the absence of denaturing proteins and incubated for 24 hours at 4°C. Next, to separate the cleaved N- or O-linked carbohydrates from the protein mixture, FBS was flowed sequentially through lectin gel columns (gravity separation) that were specific for carbohydrate ligands of DC CLR, similar to those used in Enzyme Linked Lectin Assays (ELLA) (CHAPTER 6) and cleaved moieties were separated by affinity chromatography at RT as per manufacturer's instructions. The effluent de-glycosylated FBS was collected. Carbohydrates retained via binding to lectin gel probes were eluted and the eluates were collected separately. Lectin columns used included *Narcissus pseudonarcissus* (NPA) (probe for mannose), *Sambucus nigra* (SNA-1) (sialylated groups), *Ulex europeaus I* (UEA-1) (fucose), *Ulex europeaus II* (UEA-2) (GlcNAc), or *Peanut agglutinin* (PNA) (α -galactose) (all from EY Laboratories, Inc., San Mateo, CA). To elute the bound carbohydrates from the columns, the following eluates were used: 0.2 M mannose in Tris Buffered Saline (TBS) (for NPA), 0.1 M lactose in PBS (for SNA-1), 0.1 M fucose in PBS (for UEA-1), 0.1 M GluNAc in PBS (for UEA-2), 0.2 M galactose (for PNA) in PBS or 0.05 M GalNAc in TB (all from EY laboratories lectin gel kit), respectively. Lectin columns were regenerated with 1.4 M NaCl (Sigma) and conditioned with PBS or TBS (only for NPA

or PNA) prior to flowing FBS through. To measure the total protein recovered in processed FBS (hereafter referred to as proc-FBS) a BCA assay (Sigma) was performed on non-processed FBS (hereafter referred to as FBS) or proc-FBS samples following manufacturer's instructions. A glycoprotein carbohydrate estimation assay (Pierce, Rockford, IL) was used to measure % carbohydrate content of FBS or proc-FBS after selective de-glycosylation, following manufacturer's instructions. About 75% of the total protein in FBS was recovered in proc-FBS and the % carbohydrate content decreased from about 10% in FBS to below detection (detection limit: 0.1% carbohydrate content; i.e., absorbance readings below blank sample) in proc-FBS.

Chromogenic Endotoxin Assay

Endotoxin content of FBS or sterile-filtered (0.22 μm) proc-FBS was measured using a chromogenic Limulus Amebocyte Lysate (LAL) assay (QCL-1000 Chromogenic LAL Endpoint Assay, Cambrex, Walkersville, MD) as previously described in CHAPTER 5. Standards or samples (triplicate) in endotoxin-free water were added to TC - treated 96-well PS plates (Corning). Next, LAL was added and incubated for 10 minutes at 37°C after which, the chromogenic substrate (Ac-Ile-Glu-Ala-Arg-pNA) was added to each well and incubated for 6 minutes. Finally, glacial acetic acid in endotoxin free water (25% v/ v) (J.T.Baker, Phillipsburg, NJ) was added as a stop solution and the absorbance associated with color development in the substrate corresponding to the endotoxin present in the sample was measured at 405 nm. Endotoxin content in the samples was read off standards generated from endotoxin standards. For FBS, endotoxin amounts were below detection (detection limit: 0.1 endotoxin units (EU)/ ml; i.e., absorbance

readings below blank sample) and well below the approved FDA endotoxin limit for implantable devices; 0.5 EU/ ml. However, for proc-FBS, endotoxin levels detected were above 10 ng/ ml or 100 EU/ ml.

Enzyme Linked Lectin Assay (ELLA) for detection of carbohydrates associated with adsorbed proteins on SAM

To further characterize the processed FBS and to measure remaining carbohydrates from processed FBS associated with adsorbed proteins on biomaterial surfaces that may act as ligands for DC CLR, ELLAs were performed on SAM using a previously described method with some modifications (Leriche 2000), as described in CHAPTER 6. Briefly, the SAM were incubated with 60 μ l/ well of 10% (v/ v) FBS or proc-FBS in PBS or with PBS alone (1 hr, 37°C). After aspirating out FBS, proc-FBS or PBS samples from wells, wells were blocked with 0.5 mg/ mL of Bovine Serum Albumin (BSA) (Sigma) in PBS (block buffer; 1 hr, 37°C), washed three times for five minutes each with block buffer at RT and incubated with biotinylated lectin [*Narcissus pseudonarcissus* (NPA; 12.5 μ g/ mL), *Sambucus nigra* (SNA-1; 12.5 μ g/ mL), *Hippeastrum hybrid* (HHA; 50 μ g/ mL), *Peanut agglutinin* (PNA; 12.5 μ g/ mL), or *Bauhinia purpurea* (BPA; 25 μ g/ mL) for which differential trends were previously observed in CHAPTER 6 depending on the SAM endgroup] with the carbohydrate detection specificities indicated in Table 5 from CHAPTER 6 (all from EY Laboratories, Inc., San Mateo, CA) (94) in block buffer (2 hrs, 37°C). For BPA lectin, wells were blocked with 1.0 mg/ mL of BSA in PBS. Following incubation with the biotinylated lectin, wells were washed and incubated with 10 μ g/ mL avidin/alkaline phosphate

(AV/AP) (EY laboratories Inc.) in block buffer (1 hr, 37°C) for detection of bound lectin. The concentration of AV/AP used was 1.25 µg/ mL for SNA-1 or 20 µg/ mL for BPA lectin. Finally, wells were washed and incubated with 1.0 mg/ mL p-nitrophenylphosphate (pNPP) (Sigma) substrate for detecting AV/AP (1 hr, 37°C). The solutions were transferred to wells of a clear flat-bottomed TC-treated 96-well plates to avoid interference with the reading by the SAM, reaction stopped with 40 µl of 0.4 M NaOH (Sigma) and absorbance read at 405 nm using a SpectraMax Plus 384 plate reader (Molecular Devices, Sunnyvale, CA). Positive control glycoprotein standard dilutions in PBS (1:1) were run in parallel on TC-treated PS 96-well plates (control) for each lectin; α-2-macroglobulin (from 100 – 12.5 µg/ mL) or (1000 – 3 µg/ mL) for NPA (van Leuven 1993) or HHA (Balzarini 2005) respectively, glycophorin A (100 – 3 µg/ mL) for SNA-1 (Shibuya 1987), asialofetuin (5 – 0.6 µg/ mL) or (31.25 – 1.9 µg/ mL) for PNA (Balu 2001) or BPA (Sueyoshi 1988) (all from Sigma), and based on lectin probe specificity data from (van Damme 1998). An identical set of samples (FBS or proc-FBS) as those run on SAM were also run on control PS for each experiment.

Human Peripheral Blood Mononuclear Cell-derived Dendritic Cell Culture

To investigate the roles of carbohydrate ligands of DC CLR associated with FBS proteins in supporting DC maturation, proc-FBS that was selectively de-glycosylated of DC CLR ligands was used in DC media on different SAM chemistries. Dendritic cells were derived *in vitro* from human peripheral blood mononuclear cells (PBMCs) using a previously described method (Romani 1996) as reported elsewhere (Yoshida 2004) and in CHAPTER 5. Briefly, peripheral human blood was collected from consenting donors

using heparin (333 U/ mL blood) (Baxter Healthcare Corporation, Deerfield, IL) as an anticoagulant. This procedure was performed by phlebotomists at the Georgia Tech Student Health Center in accordance with the Georgia Institute of Technology's Institute Review Board (IRB)-approved protocol # H05012. All subjects enrolled in this research responded to an Informed Consent which was approved by the IRB of Georgia Institute of Technology. Briefly, the PBMC layer was separated using lymphocyte separation medium (LSM) (Cellgro MediaTech, Herndon, VA) from whole blood using centrifugation. The PBMCs were resuspended at 5×10^6 cells/ mL in media{RPMI-1640 containing 25 mM HEPES [4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid) and L-glutamine] (Gibco), 100 U/ mL penicillin/streptomycin (Cellgro MediaTech, Herndon, VA)} and 10% (v/ v) FBS or proc-FBS and 10 mL of either of these cell suspensions were plated onto a TC plates and incubated for adherence (2 hrs) at 37°C with 95% relative humidity and 5% CO₂ to select for adherent monocytes. Adherent cells that remained following three washes with pre-warmed media were cultured in fresh media supplemented with granulocyte macrophage colony-stimulating factor (GM-CSF) (1000 U/ mL) and interleukin-4 (IL-4) (800 U/ mL) (both from Peprotech, Rocky Hill, NJ) (DC media) for 5 days. On the fifth day of culture, DCs were collected and plated at 1.5×10^6 cells/ well in 3 mL/ well in DC media with FBS or proc-FBS, supplemented with the cytokines and left as untreated iDCs, or as lipopolysaccharide LPS-treated mDCs or treated with different SAM endgroups. On day 6 of culture, the loosely adherent cell fractions containing DCs were harvested for DCs cultured in DC media with FBS or proc-FBS for the various treatment groups, for further analysis.

Dendritic cell morphology

Dendritic cell morphology was examined throughout the culture duration by phase contrast microscopy. Dendritic cells cultured in DC media with FBS or proc-FBS were processed for Cytospin preparations as described earlier (Yoshida 2004) (Cytospin Cytocentrifuge, Thermo Shandon, Pittsburg, PA) and stained with Differential Hematology Stain (Astral Diagnostics, West Deptford, NJ). Giemsa-stained cytopspin images were obtained on day 6, following 24 hour SAM treatment, for loosely adherent cell populations containing DCs for iDCs, mDCs or DCs treated with different SAM endgroups in DC media with FBS or proc-FBS. Three images were taken at three different regions for each cytopspin and a representative image was chosen.

Flow cytometry for determination of maturation marker expression

To examine the effects of DC culture in DC media with proc-FBS, the levels of DC surface marker expression were measured on day 6 using a flow cytometric technique as described previously (Yoshida 2004). Loosely adherent cell fractions containing DCs that were cultured in DC media with FBS or proc-FBS were collected and resuspended in Hank's HEPES buffer (120 mM NaCl, 10 mM KCl, 10 mM MgCl₂, 10 mM glucose, 30 mM HEPES) (all from Sigma) containing 1% Human Serum Albumin (HSA) (EMD Biosciences, San Diego, CA) and 1.5 mM CaCl₂ (Sigma) and stained with saturating concentrations of mouse anti-human monoclonal antibody against CD14 (clone UCHM1; IgG2 κ), CD40 (clone B-B20; IgG1 κ), CD80 (clone BB1; IgM κ), CD86 (clone BU63; IgG1 κ) (all from Southern Biotech, Birmingham, AL), CD83 (clone HB15a; IgG2b) (IO Test Immunotech Beckman Coulter, Marseille, France) HLA-DQ (clone TU169;

IgG2 κ), or HLA-DR (clone TU36; IgG2 κ) (both from Becton Dickinson Pharmingen, San Diego, CA), while maintained on ice and in the dark. Samples were strained via cell strainers (40 μ m nylon pore) (Becton Dickinson, San Jose, CA) into 200 μ l of flow cytometry buffer and analyzed immediately using BD LSR flow cytometer (Becton Dickinson, San Jose, CA) and 5000 events were collected per sample. Autofluorescence was used as negative controls. Analysis was performed using BD LSR flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was performed using BD FACS DIVA v. 4.1.2 (Becton Dickinson Biosciences, San Jose, CA) and using WinMDI 2.8 (Scripps Research Institute, La Jolla, CA).

Secretion of Tumor Necrosis Factor - α (TNF- α) or (IL-6) per DNA amount

The release of pro-inflammatory cytokines, tumor necrosis factor- α (TNF- α) or IL-6 normalized to DNA amounts, by DCs during their culture in DC media with FBS or proc-FBS on SAM endgroups was assessed in cell culture supernatants. Briefly, supernatants were collected by swirling cell culture dishes clockwise, anti-clockwise, from left to right and from right to left, ten times in each direction on day 6 of culture. This was performed following treatment of DCs for 24 hrs in DC media containing FBS or proc-FBS, with the different SAM chemistries. The supernatants were cleared by centrifugation at 1100 rpm for 10 minutes and stored at -20°C for analysis, using a method as described previously (Yoshida 2004). Concentrations of TNF- α or IL-6 per DNA amount were measured using ELISAs (R&D Systems, Minneapolis, USA) in accordance with manufacturer's instructions. The DNA amounts were measured by pooling both loosely adherent cells and the non-adherent fraction, [removed using

trypsin/ 0.05% EDTA (Sigma)] using a PicoGreen DNA kit (Molecular Probes, Oregon, USA) following manufacturer's instructions. Amounts of pro-inflammatory cytokines were normalized against total DNA amounts for each treatment group.

Statistical Analysis

Statistical analysis was performed using two-way analysis of variance (Young 2001), setting variance due to treatments as the fixed factor and variance due to donors as the random factor and performing pairwise comparisons between treatments with a Tukey post-test using Minitab software (Version 13.20, Minitab Inc., State College, PA). To observe the effects of different treatments compared to iDCs, one-sided Student's T Test was performed (Microsoft Excel v. 2003). The p value of less than or equal to 0.05 was considered significant.

RESULTS

Characterization of selectively proc-FBS using ELLAs

The ELLA assays were performed following pre-incubation of different SAM chemistries with either FBS or proc-FBS to characterize the profiles of carbohydrates associated with adsorbed proteins. No significant differences were observed for carbohydrate associated with proteins adsorbed to different SAM chemistries with either FBS or with proc-FBS due to the process of de-glycosylating of the serum (data not shown).

Morphology of DCs cultured in DC media with FBS or proc-FBS

For DCs cultured in DC media with FBS, iDCs or DCs on CH₃ SAM were rounded and presented fewer dendritic extensions as compared to mDCs or DCs on OH, COOH or NH₂ SAM endgroups (Figure A1-1a-f). In contrast, for DCs cultured in DC media with proc-FBS, iDCs, mDCs or DCs treated with all SAM endgroups exhibited dendritic processes (Figure A1-1g-l).

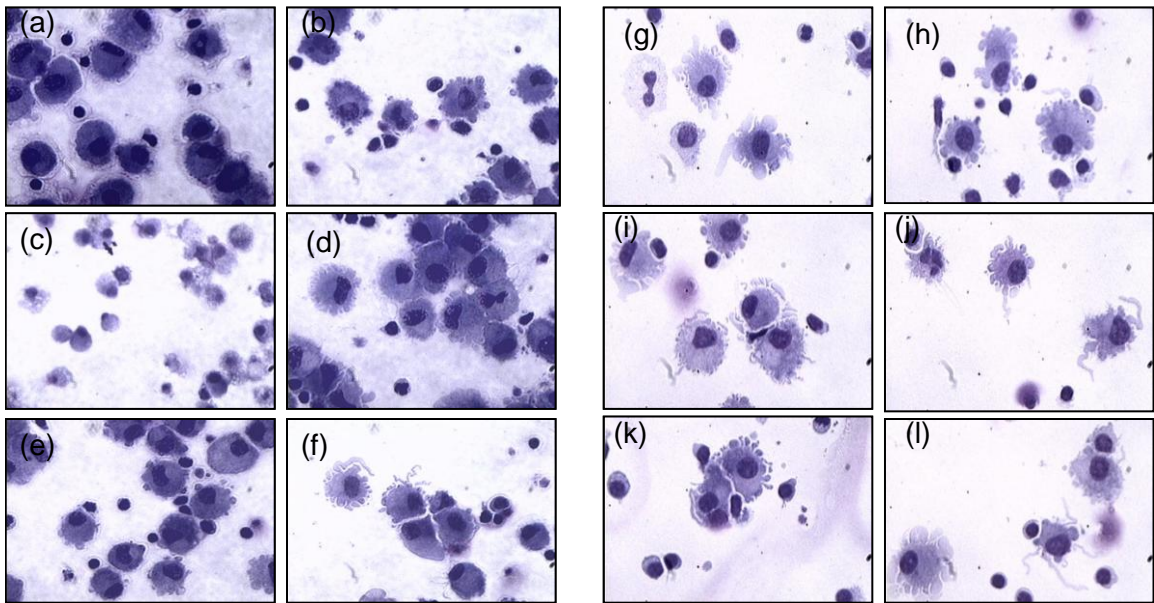


Figure A1-1: Dendritic cells cultured in DC media with FBS or proc-FBS demonstrate variations in cell morphology.

Immature DCs derived from human PBMCs were cultured in DC media with FBS (a-f) or with proc-FBS (g-l) in the presence of GM-CSF and IL-4 until Day 5 and then plated on polystyrene surfaces. Cells were cultured in DC media without (a or g), or with LPS (b or h), or on CH₃ (c or i), OH (d or j), COOH (e or k), NH₂ (f or l) SAM endgroups for 24 hours with FBS or proc-FBS, respectively. Giemsa-stained cytopins taken on Day 6 of culture indicated that DCs treated with OH, COOH or NH₂ SAM endgroups exhibited dendritic processes similar to positive control LPS-treated mDCs and unlike those treated with CH₃ SAM endgroups which were similar to iDCs when cultured in DC media with FBS. However, DCs cultured in DC media with proc-FBS exhibited membrane processes for all treatments similar to mDCs. Original magnification: 40x. Cytopins were prepared for six donors with similar results, and representative results are shown.

Maturation marker expression for DCs cultured in DC media with FBS or proc-FBS

Lipopolysaccharide treatment resulted in an upregulation of CD40, CD83, CD86, HLA-DQ or HLA-DR expression on mDCs as compared to iDCs only for DCs that were cultured in DC media with FBS (Figure A1-2a) but not for DCs that were cultured in DC media with proc-FBS (Figure A1-2b). Dendritic cells cultured in DC media with FBS on CH₃ SAM exhibited highest expression of anti-inflammatory apoptosis inducing HLA-DQ receptor as compared to iDCs or to DCs treated with all other SAM endgroups, in agreement with previous results (Figure A1-2a and CHAPTER 5). Other modest differences were observed for DCs cultured in DC media with FBS on different SAM chemistries (Figure A1-2a). In contrast, DCs treated with different SAM endgroups with proc-FBS exhibited no differences in maturation marker expression among SAM endgroups (Figure A1-2b). Furthermore, for DCs cultured in DC media with proc-FBS no differences were observed in the levels of expression of markers with different SAM treatments as compared to mDCs (Figure A1-2b). On the contrary, for DCs cultured in DC media containing FBS, mDCs exhibited higher levels of CD40, CD80 and CD83 as compared to different SAM treatments (Figure A1-2a).

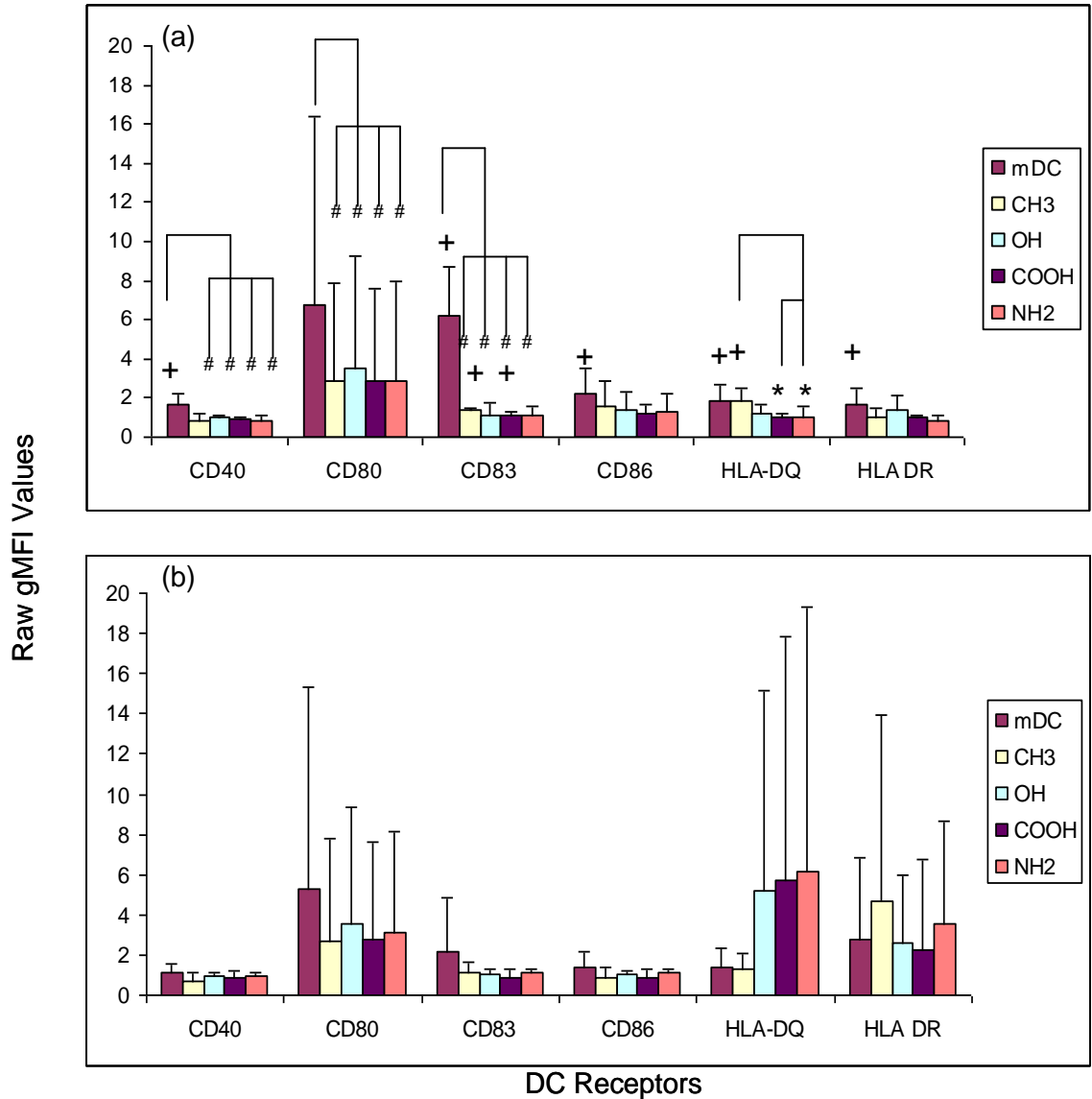


Figure A1-2: Dendritic cells cultured in DC media with FBS exhibit increases in expression of CD40, CD83, CD86, HLA-DQ or HLA-DR when matured using LPS while DCs cultured in DC media with proc-FBS do not.

Immature DCs cultured in DC media with FBS exhibited higher expression of maturation markers in response to 24 hour exposure to LPS (a), in contrast to DCs cultured in DC media with proc-FBS which did not exhibit significant increases in expression (b). Furthermore, any differences in marker expression observed for DCs on different SAM chemistries with FBS (a) were absent with proc-FBS (b). This experiment was performed six times with six different donors. Data represented as treatment control ratios of gMFI values revealed statistical significance of the findings; mean \pm S.D, '+': indicates increase over iDC, '*': indicates less than SAM endgroup, '#': indicates less than mDCs, $p \leq 0.05$.

Secretion of TNF- α or IL-6 by DCs cultured in DC media with FBS or proc-FBS

As expected, for DCs cultured in DC media with FBS, LPS-treated mDCs secreted significantly higher levels of TNF- α than iDCs. Among SAM endgroups, DCs treated with CH₃ SAM secreted higher levels of pro-inflammatory TNF- α compared with DCs treated with all other SAM endgroups. Also, DCs treated with OH SAM instigated higher TNF- α secretion than DCs treated with COOH or NH₂ SAM (Figure A1-3a) as reported in CHAPTER 5. On the other hand, for DCs cultured in DC media with proc-FBS, mDCs or DCs treated with the different SAM endgroups all secreted similar levels of TNF- α that were significantly higher as compared to TNF- α secreted by iDCs and no differences in TNF- α levels were observed among DCs treated with different SAM endgroups (Figure A1-3b). Similar results were observed when amounts of IL-6 secreted by the different treatment groups were measured (data not shown).

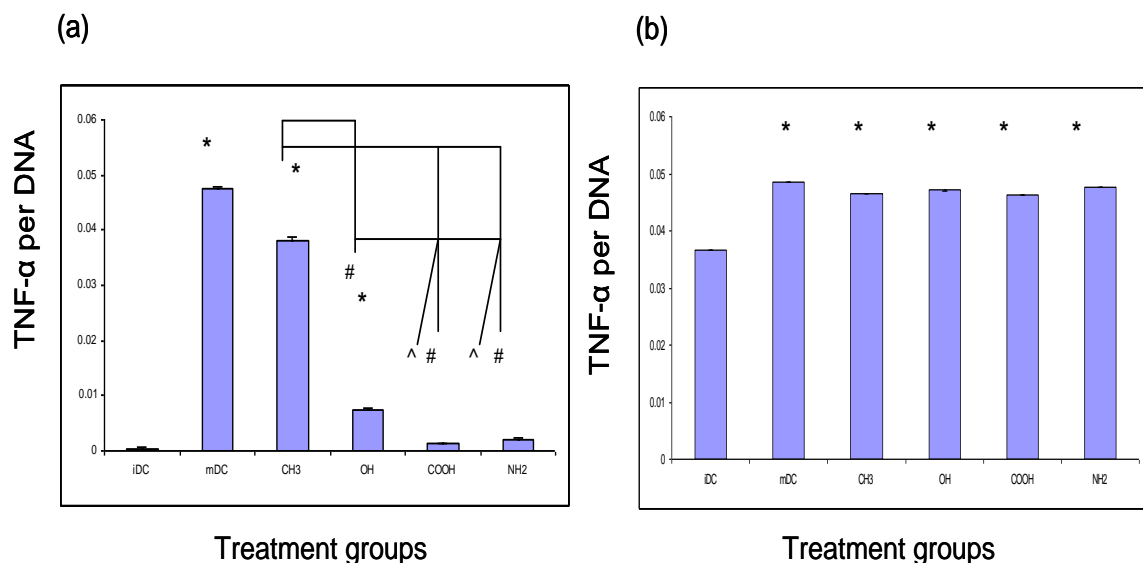


Figure A1-3: Differential profiles of TNF- α secretion for DCs cultured in DC media with FBS or with proc-FBS.

Supernatants from iDCs, LPS-matured DCs, or DCs treated with different SAM endgroups were collected and TNF- α content was measured and shown normalized against DNA content for culture in DC media with FBS (a) or proc-FBS (b). Dendritic cells cultured in DC media with proc-FBS, with all treatments, secreted high TNF- α similar to levels secreted by mDCs cultured in DC media with FBS. Among SAM endgroups, contact with CH₃ SAM induced highest TNF- α secretion by DCs cultured in DC media with FBS (a). No differences were observed in levels of TNF- α secreted by DCs treated with different SAM endgroups with proc-FBS (b). This experiment was performed three times with three different donors; mean \pm S.D, ‘*’: greater than iDCs; ‘#’ or ‘^’: indicates less than SAM endgroup, $p \leq 0.05$.

DISCUSSION

This study focused on investigating the roles of carbohydrate ligands of DC PRRs associated with adsorbed proteins on biomaterial surfaces in supporting DC maturation. As an initial step towards elucidating their roles, the FBS used in the DC media was selectively de-glycosylated of carbohydrates which are known ligands for DC CLRs. This proc-FBS was used in the DC media with which to treat DCs with different

SAM endgroups and these DCs were compared morphologically, phenotypically and functionally against DCs that were cultured in DC media using non-processed FBS (FBS) to assess the role of protein glycosylations. Overall, these results demonstrated that culture in DC media with proc-FBS slightly increased background iDC maturation, but strikingly lowered LPS maturation effects and for the most part overwhelmed any biomaterial effects on maturation marker expression. Based on morphology, DCs cultured in DC media with proc-FBS exhibited several dendritic extensions of the plasma membrane indicative of maturation, irrespective of whether they were treated with or without LPS, or on different SAM endgroups (Figure A1-1b), notably distinctive from iDCs or DCs on CH₃ SAM which were less mature following culture in DC media with FBS (Figure A1-1a). As shown in Figure A1-2a, LPS treatment caused DC maturation as exemplified by an upregulation of CD40, CD80, CD86 and HLA-DQ only when DCs were cultured in DC media with FBS (Figure A1-2a) but not with proc-FBS (Figure A1-2b). Finally, culture in DC media with proc-FBS also resulted in significantly higher secretion of TNF- α (Figure A1-3) for iDCs as well as for all treatments as compared to culture in DC media with FBS. Taken together, these results imply that although proc-FBS caused higher basal maturation of iDCs, the removal of glycosylations lowered the ability of DCs to mature in response to LPS stimulus and may imply a significant role for protein glycosylations as DC maturation stimuli in the context of biomaterials.

There are several interpretations as well as caveats for these results. Firstly, in proc-FBS, the lowered levels of DC carbohydrate ligands may have counteracted the LPS maturation stimulus and lowered expression of DC maturation markers. This may imply that serum glycosylations are an important stimulus for DC maturation. Secondly,

selective removal of glycosylations in proc-FBS may have exposed other DC ligands or removal of glycosylations may be incomplete or proteins that are critical to the host response may have been removed, thereby yielding higher basal maturation with iDCs. Lastly selective de-glycosylation of FBS may have a more potent effect on DC maturation that masks the weaker effects due to the biomaterial. However, elevated levels of endotoxin were present in proc-FBS and introduce an important caveat to these results. Also, results from ELLA assays did not indicate differences in profiles of presented carbohydrates on different SAM chemistries with either FBS or proc-FBS incubating solutions, implying that the carbohydrate profiles on the biomaterials after FBS de-glycosylations were not significantly altered. Other considerations include that no trends in carbohydrate profiles were observed even with FBS, different from results in CHAPTER 6, where pooled human serum was used instead of FBS. Furthermore, although profiles of presented carbohydrates were not modified with de-glycosylation, % carbohydrate content decreased.

Dendritic cells exhibit impaired abilities to respond to a second LPS challenge (high-dose – 10 ng/ ml for human DCs) (Jotwani 2003; Saito 2006) following exposure to the first dose of LPS (low dose – 0.2 ng/ ml for human DCs). This LPS-unresponsiveness of DCs is also referred to as LPS tolerance or endotoxin desensitization. Results from other groups characterizing LPS unresponsive DCs are summarized below in Table 14.

Table 14: Summary of phenotypic and functional characteristics of endotoxin-desensitized DCs from the literature

Flow Cytometry	Cytokines (TNF- α , IL-6)	MLR	Conclusions	References
Acquisition of CD83 expression was inhibited	Induction of TNF- α was inhibited	Ability to stimulate allogeneic T cell proliferations was high	LPS tolerance abrogated terminal human DC maturation (defined as TNF- α , IL-12 secretion and CD83 neo-expression) However desensitization was not total deactivation since able to stimulate T cells	(Rieser 1998)
Reduced CD83 expression	Inhibited IL-12, TNF- α , IL-10 and lowered IL-6 secretion		LPS tolerance lowered the ability of human DCs to mature	(Cuellar 2004)
Reduced expression of MHC class II molecules and inhibited MHC-restricted antigen presentation		Lowered ability to stimulate allogeneic T cells and induced Th2 type responses	Endotoxin tolerance inhibits alloimmune responses in donor animals (DCs from mice)	(Ishiyama 2006)

The objective in performing this comparative analysis of results was to observe if the reduced DC maturation observed with proc-FBS in this study was due to the presence of endotoxin contaminant and not due to carbohydrate removal alone. The analysis suggests that although some of the findings in this study agree with DC responses typically induced by endotoxin (i.e. inability to upregulate maturation marker expression in response to LPS) (Figure A1-2), other results do not [CD83 expression, TNF- α , IL-6 secretion (Figure A1-3), allostimulation] and in fact were exactly opposite to well-documented characteristics of endotoxin-exposed DCs. The DC responses observed here

therefore, strongly contrasted with those normally seen due to endotoxin contamination.

Taken together, these results suggest that although endotoxin was present, it does not fully explain our results and may need to be further investigated. The dosage of endotoxin present may be important; here the concentration of endotoxin present was higher than that of other studies and may explain the atypical DC responses to endotoxin observed here. The other possibility is that the removal of carbohydrates from FBS counteracted the endotoxin effect resulting in DCs that were phenotypically similar to endotoxin-exposed DCs, but functionally opposite (pro-inflammatory cytokine secretion, allostimulation). Therefore, the dual presence of endotoxin as well as de-glycosylated FBS may have rescued DCs from the deactivation state that is characteristic of endotoxin treatment. Hence DCs may respond to LPS re-challenge more effectively in the absence of other glycosylated ligands of DC C-type lectin receptors, as part of a complex interplay of factors that are responsible for DC responses. Considering the effects due to biomaterials, the dual presence of endotoxin and de-glycosylated FBS overwhelmed the weaker effects of biomaterials presenting different endgroup chemistries on DC responses.

In summary, a novel protocol has been developed for selective partial removal of carbohydrate ligands of DC CLR of interest from FBS. Although serum remained glycosylated after processing (as expected since only selective removal of all glycosylations in serum was planned) successful removal of carbohydrates did occur as indicated by high levels of carbohydrate in eluates (data not shown) as well as the decrease in carbohydrate content from 10% for FBS to below assay detection limits for proc-FBS. Furthermore, the glycosylations remaining in proc-FBS may include

carbohydrates that are not ligands of DC CLR. Although no differences were observed in profiles of presented carbohydrates on different SAM chemistries with proc-FBS versus FBS from ELLAs, this may have been due to the fact that no trends were observed between biomaterials for FBS itself. The effect of complement activation in causing basal iDC maturation is not expected to be a factor since commercially available heat-inactivated FBS was used. This protocol may be further optimized to enable higher protein recovery from columns, to ascertain complete removal of glycosylations of interest and to better control the removal of only carbohydrates of interest. In future studies, attempts may be made to decontaminate the FBS of high endotoxin contaminant levels that were likely acquired during the multiple processing steps, using commercially available strategies that are inapplicable at present due to the high protein concentration in FBS, but may become available once concentration issue are overcome. Other strategies to probe the effects of carbohydrates or glycoproteins on DC maturation may include high-throughput carbohydrate, glycoprotein (Feizi 2004; Palma 2006) or lectin microarrays (Kuno 2005). These as well as other future directions towards linking the roles of carbohydrates in regulating DC responses using novel analytical tools from the glycomics era have been outlined in CHAPTER 8.

In conclusion, this study represents an ongoing effort to examine the roles of carbohydrate ligands of DC PRRs towards supporting DC maturation. Due to the indefinite nature of certain results, this work would need to be re-examined prior to drawing any definite conclusions and has therefore been relegated to the appendix section.

APPENDIX 2

A.2 Maturation marker expression for DCs cultured using regular FBS or de-glycosylated FBS on different SAM chemistries

The expression levels of the various markers of DC phenotype including CD80 and CD86 co-stimulatory molecules, HLA-DQ and HLA-DR MHC II molecules, CD83, a dendritic cell marker and CD40 were assessed by using flow cytometry. A representative set of histograms are presented here. Generally, iDCs expressed lower levels of these markers and maturation induced by LPS treatment resulted in upregulated expression on mDCs as seen with CD40, CD80 and CD83. Differential levels of expression of maturation markers were observed for different SAM chemistries as reported in CHAPTER 5, implying differential DC responses to different SAM chemistries (Figure A2-1a).

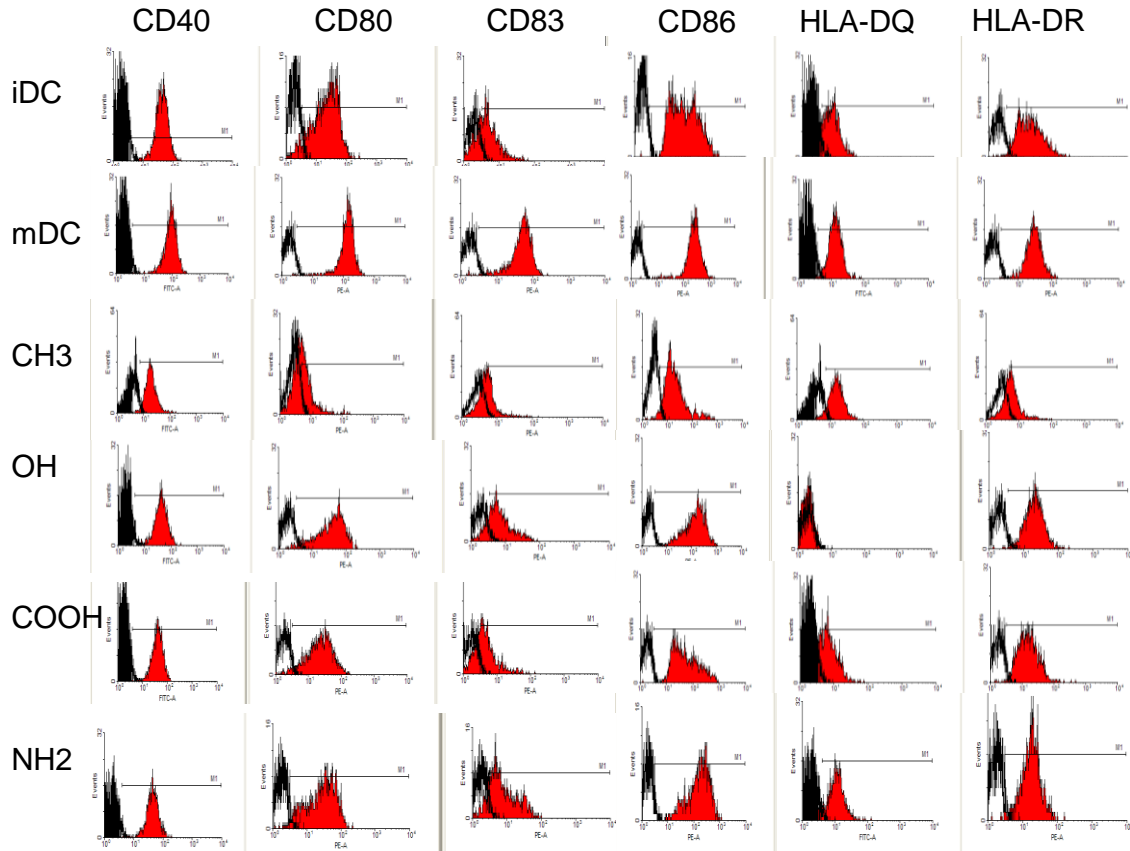


Figure A2-1a: Dendritic cells treated with various SAM chemistries differentially modulate their expression of co-stimulatory and MHC class II molecules when cultured with regular FBS.

Immature DCs expressed lower levels of CD40, CD80 and CD83 as compared to mature DCs that were treated with LPS. Dendritic cells treated with -CH₃, -OH, -COOH, or -NH₂ SAM chemistries exhibited differential levels of different maturation markers. Black unfilled histograms represent autofluorescence and red filled histograms represent fluorescence due to marker. This experiment was repeated six times with similar results, and representative results are shown.

Next the expression levels of the various maturation markers were assessed for DCs that were cultured on the different SAM chemistries, in the presence of processed de-glycosylated FBS, prepared as per methods described in APPENDIX 1. It was observed that in the presence of de-glycosylated FBS, no differences were observed for different SAM treatments as compared to culture using regular FBS, where a differential expression was obtained with different SAM treatments (Figure A2-1a). Furthermore, in

the presence of de-glycosylated FBS, similar levels of most maturation markers were observed even for iDCs and LPS-treated mDCs (Figure A2-1b).

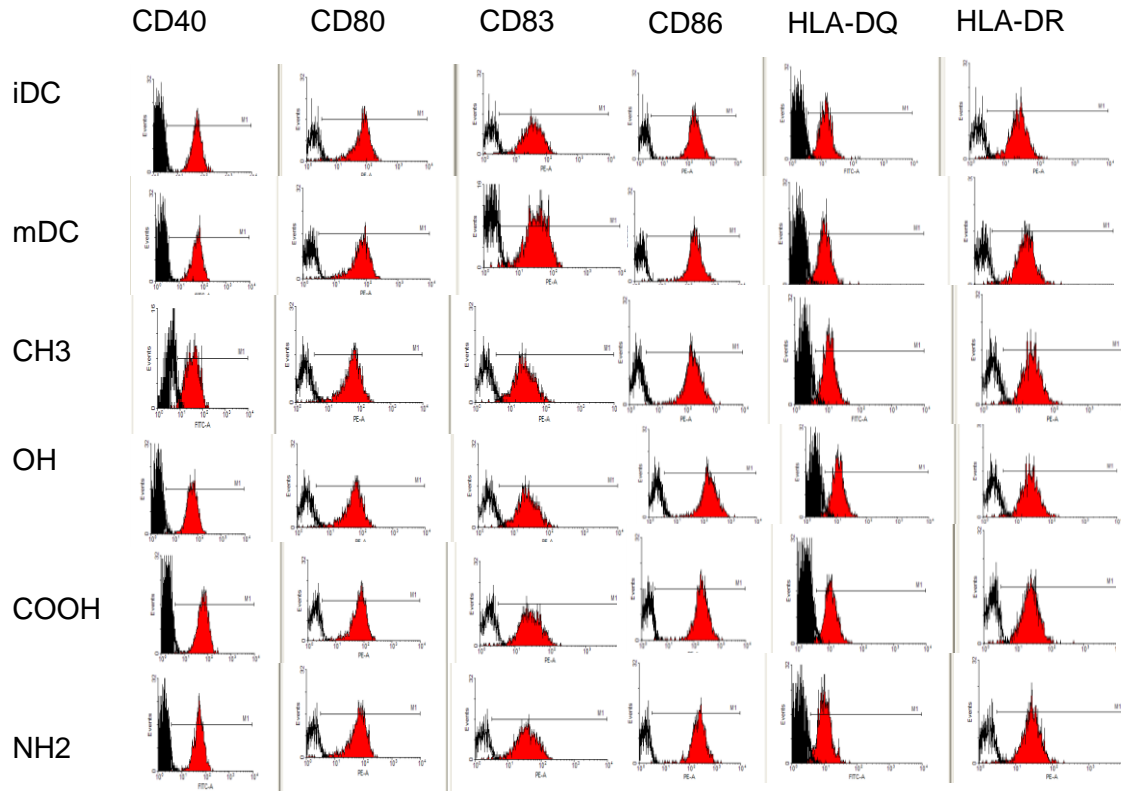


Figure A2-1b: Dendritic cells treated with various SAM chemistries exhibit similar expressions of costimulatory and MHC class II molecules in the presence of de-glycosylated FBS.

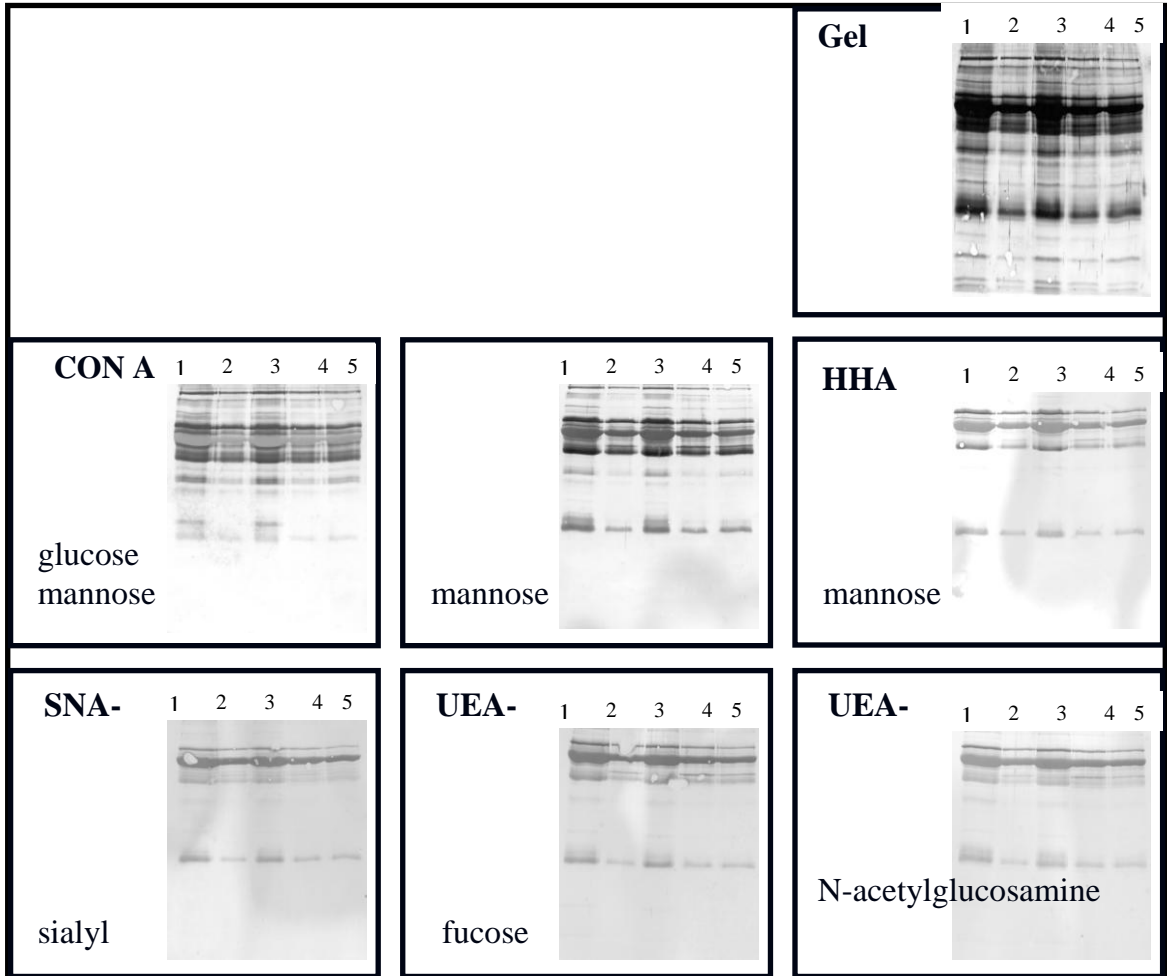
Immature DCs did not express lower levels of CD40, CD80 and CD83 as compared to mature DCs that were treated with LPS, when cells were cultured with processed de-glycosylated FBS. Dendritic cells treated with -CH₃, -OH, -COOH, or -NH₂ SAM chemistries exhibited similar levels of different maturation markers. Black unfilled histograms represent autofluorescence and red filled histograms represent fluorescence due to marker. This experiment was repeated six times with similar results, and representative results are shown.

APPENDIX 3

A.3 Lectin blotting on serum, plasma or single purified protein samples

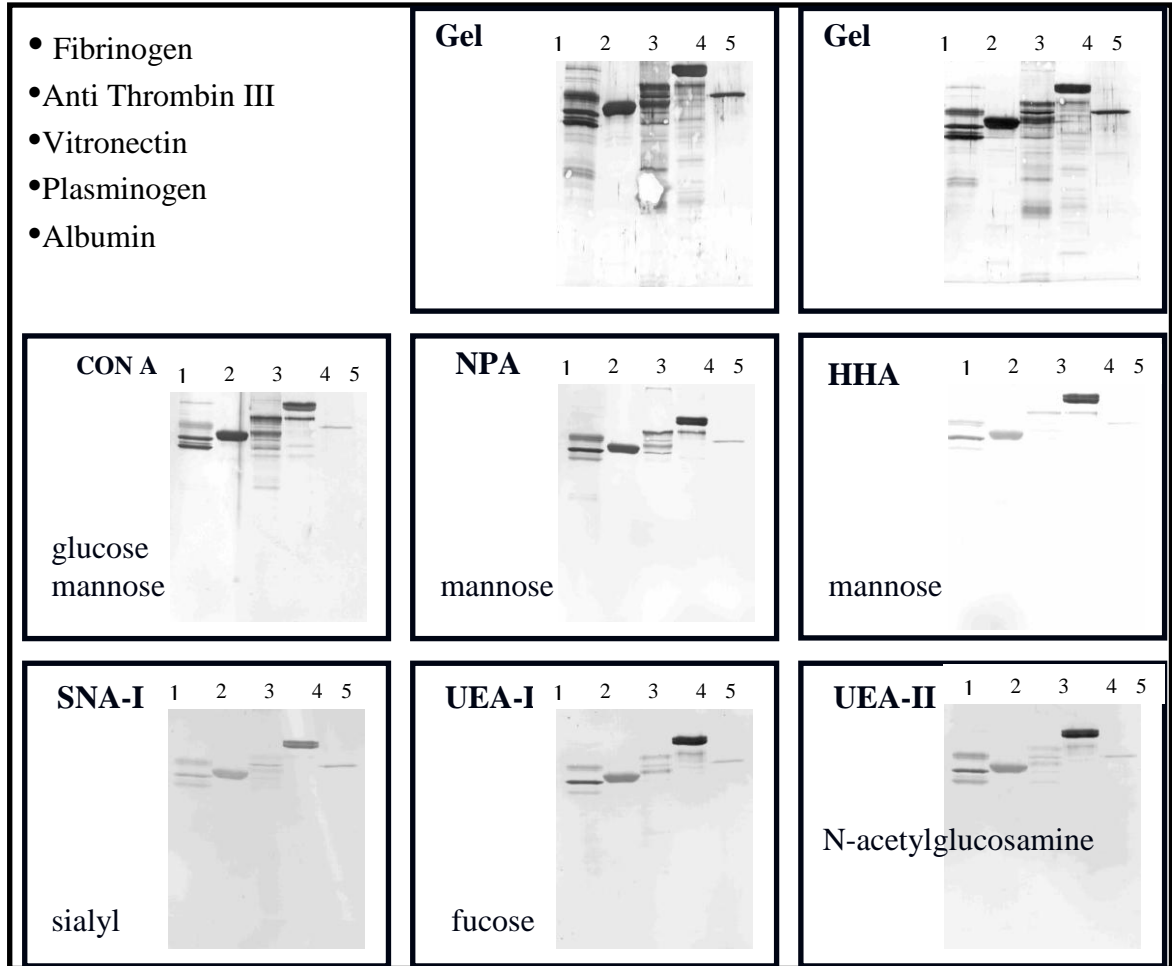
This work was performed in collaboration with Dr. John Brash and Rena Cornelius from McMaster University in Canada. The overall goal of this study is to characterize the carbohydrates modifications of proteins present in eluates from different SAM chemistries and to identify the specific proteins that were associated with these glycosylations. As a first step, lectin blotting was performed using NPA, UEA-1, UEA-2, SNA-1 or HHA lectins either on pre-absorbing plasma or serum samples including 10% filtered serum, 1% filtered heat-inactivated plasma, 10% unfiltered serum, 1% unfiltered heat-inactivated plasma and 1% filtered non-heat-inactivated plasma (Figure A3-1a) or on purified single proteins including anti-thrombin III, fibrinogen, albumin, vitronectin, plasminogen (Figure A3-1b). Heat inactivation was performed by maintaining samples for 30 minutes at 56°C. Plasma, serum or purified proteins were initially run using SDS-PAGE and then the proteins were transferred to polyvinylidene fluoride (PVDF) membranes for lectin blotting. For lectin blotting, membranes were first blocked using Tween/Tris buffered saline (TBS) (RT, 2.5 hrs) then incubated with the lectin (RT, 2 hrs), washed 3 times for 5 minutes each using block buffer, then incubated with avidin alkaline phosphatase (RT, 15 minutes), washed and color was developed with NBT/BCIP in alkaline phosphatase buffer (RT) on a shaker. Although the protocol for performing lectin blotting was successfully optimized, it was observed however that since several lectins bind the same protein and vice-versa, clear trends were not distinguishable (Figure A3-1a). However, distinct trends were observed when single glycosylated proteins were used, in agreement with their known glycosylations. Fibrinogen or

vitronectin are controls for N-glycans including sialylated groups (NeuNAc), GlcNAc or mannose (Uchibori-Iwaki 2000; Henschen-Edman 2001). On the other hand, albumin is composed of trace glycosylations. As expected, positive staining was obtained for most N-linked glycans tested on fibrinogen or vitronectin, while in general, negligible to non-existent carbohydrate levels were detected for albumin (Figure A3-1b).



Figures A3-1a: Absence of clearly distinguishable trends from lectin blotting experiments performed using different preabsorbing serum or plasma samples.

Sodium dodecyl sulfate- polyacrylamide gel electrophoresis was performed to separate serum or plasma proteins [10% filtered serum (1), 1% filtered heat-inactivated plasma (Wallet), 10% unfiltered serum (3), 1% unfiltered heat-inactivated plasma (4) or 1% filtered non-heat-inactivated plasma (Wallet)] (bandwise, 1-5 from left to right) (row 1). These proteins were then transferred to a PVDF membrane and lectin blotting performed with Con A (glucose, mannose), NPA (mannose), HHA (mannose) (all in row 2, from left to right), SNA-1 (sialylated groups), UEA-I (fucose) or UEA-II (GalNAc) (all in row 3, from left to right). No clear trends could be obtained using different lectin probes.



Figures A3-1b: Lectin blotting protocol has been optimized for lectins having different specificities on purified single protein samples.

Sodium dodecyl sulfate - polyacrylamide gel electrophoresis was performed using different single purified proteins [fibrinogen (1), antithrombin III (Wallet), vitronectin (3), albumin (4), plasminogen (Wallet)] (bandwise, 1-5 from left to right) (both in row 1). These proteins were then transferred to a PVDF membrane and lectin blotting performed with Con A (glucose, mannose), NPA (mannose), HHA (mannose) (all in row 2, from left to right), SNA-1 (sialylated groups), UEA-I (fucose) or UEA-II (GalNAc) (all in row 3, from left to right).

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