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PhD Thesis

Inflammatory Responses to Biomaterials: Interaction between Leukocytes and Self-Assembled Monolayers

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Abstract

Whatever the type and application of the biomaterial used, once it is implanted in the human body, it interfaces with the living tissues. The very act of implantation means that there is tissue trauma, which will lead to a physiological healing reaction, consisting of two essential components: inflammation and repair processes. Leukocytes are a central cell type in directing host inflammatory and immune processes; thus, their response to biomaterials is extremely important in understanding biomaterial-host interaction.

The aim of the present thesis was to advance the understanding of the inflammatory response to biomaterials. In view of this objective, self-assembled monolayers (SAMs) of alkanethiols on gold with the terminal functionalities of OH, COOH and CH_3 were used as model surfaces, to investigate the interactions of inflammatory cells and the surface chemistry of biomaterials.

In the present work, both *in vitro* and *in vivo* studies of the interactions of SAMs with inflammatory cells were performed.

The *in vitro* inflammatory response was investigated by studying the adhesion of human mononuclear and polymorphonuclear leukocytes to the elected model surfaces. Our results indicate that human leukocytes adhere in greater numbers to methyl-covered surfaces than to hydroxyl and carboxyl-covered ones. It has also been concluded that pre-activation of leukocytes resulted in a general increase of cell adhesion to the surfaces.

The *in vivo* studies were performed using a rodent air-pouch model of inflammation. The main focus was on the initial acute inflammatory reaction. The different model surfaces were implanted in the mice air-pouches and afterward the inflammatory exudates and implants were retrieved and analyzed. Methyl-covered surfaces recruited the highest number of leukocytes to the air pouches. Higher numbers of cells adhere to the OH-coated and gold surfaces in comparison with COOH and with CH_3-coated ones. Methyl-covered SAMs induced the migration of large numbers of inflammatory cells into the air pouches but were associated with low numbers of adherent cells on the surface. This phenomenon was observed at 24, 48 and 72 hours after implantation.

The number of activated cells among the recruited inflammatory cells, expressing the adhesion molecule Mac-1 has also been determined. After 24 hours

of implantation, in sham-operated animals the number of Mac-1^{+} cells was low, and after the implantation of the different SAMs there was a significant increase in the number of these cells. The methyl-terminated surfaces induced a significant increase in the number of activated leukocytes. In addition, the inflammatory exudates induced by methyl-terminated SAMs were monitored 4, 24, 48 and 72 hours after implantation, and revealed that methyl-terminated surfaces induced a significant recruitment of phagocytes expressing the integrin Mac-1.

The end stage of the healing response to biomaterials was assessed by the study of the fibrous capsule formed one week after implantation. An increase in the thickness of the fibrous capsule was seen around implants coated with methyl groups, and also in gold surfaces, in comparison with sham-operated mice and COOH- and OH-covered surfaces.

Taken together the results obtained in the different parts of this study, indicate that the CH_3-covered surfaces seem to have the highest pro-inflammatory behavior of the three tested surfaces since they are responsible for (i) the *in vitro* adhesion of higher numbers of human leukocytes (ii) the *in vivo* recruitment and activation of larger numbers of inflammatory cells and also (iii) for the formation of thick fibrous capsules.

Resumo

Independentemente do tipo e da aplicação do biomaterial utilizado, uma vez implantado no corpo humano interage com os tecidos. O processo de implantação implica a existência de um ferimento nos tecidos, o que leva à activação de uma reacção fisiológica de cicatrização, que consta de dois componentes essenciais: inflamação e processos de reparação. Os leucócitos são um tipo de células que desempenham um papel fundamental na resposta inflamatória do hospedeiro e em processos imunológicos; consequentemente, a sua resposta à presença de um biomaterial é muito importante para a compreensão das interacções entre o hospedeiro e o biomaterial.

O objectivo da presente tese foi alargar a compreensão da resposta inflamatória a biomateriais. Tendo em conta este objectivo, foram utilizadas monocamadas auto-estruturadas (*self-assembled monolayers*, SAMs) de alcanotióis em ouro, com os grupos funcionais OH, COOH e CH_3 como superfícies modelo na investigação das interacções entre células inflamatórias e as características químicas da superfície de biomateriais.

No presente trabalho foram realizados estudos *in vitro* e também *in vivo* das interacções entre SAMs e células inflamatórias.

A resposta inflamatória *in vitro* foi investigada através do estudo da adesão de leucócitos humanos mononucleares e polimorfonucleares às superfícies modelo escolhidas. Os resultados indicam que os leucócitos humanos aderem em maior número às superfícies recobertas por grupos metilo, em comparação com as recobertas com grupos hidroxilo e carboxilo. Conclui-se que a pré activação dos leucócitos originou um aumento generalizado da adesão das células às superfícies estudadas.

Os estudos *in vivo* foram realizados utilizando um modelo animal de inflamação que consiste na formação de uma bolsa de ar subcutânea. O estudo foi mais direccionado para a resposta inflamatória aguda inicial. As diferentes superfícies modelo foram implantadas nas bolsas de ar dos animais e subsequentemente, os exsudados inflamatórios e os implantes foram recolhidos e analisados. As superfícies cobertas por grupos metilo atraíram o número mais elevado de leucócitos para as bolsas de ar. Um elevado número de células aderiu às superfícies recobertas por grupos OH e às superfícies de ouro, em comparação com

as superfícies recobertas por grupos COOH e CH_3. SAMs recobertas por grupos metilo induzem a migração de um grande número de células inflamatórias para as bolsas de ar, mas estão associados a um baixo número de células aderentes à superfície. Esta ocorrência foi observada 24, 48 e 72 horas após a implantação.

O número de células activadas entre as células inflamatórias recrutadas que expressam a molécula de adesão Mac-1 foram também estudadas. Em animais sem qualquer material implantado, após 24 horas o número de células Mac-1^+ era baixo. Após a implantação das diferentes SAMs verificou-se um aumento significativo no número destas células. As superfícies terminadas em grupos metilo induziram um aumento significativo de leucócitos activados. Adicionalmente, os exsudados inflamatórios induzidos por SAMs terminadas em grupos metilo foram examinados 4, 24, 48 e 72 horas após a implantação e revelaram que estas superfícies atraíram um número significativo de fagócitos que expressam a integrina Mac-1.

A fase final da resposta de cicatrização a biomateriais foi investigada através do estudo da formação da cápsula fibrosa uma semana após a implantação. Foi observado um aumento da espessura da cápsula fibrosa observada à volta de implantes recobertos com grupos metilo e também em superfícies de ouro, em comparação com animais sem material implantado e com superfícies cobertas com grupos COOH e OH.

Reunindo os resultados obtidos nas diferentes partes deste estudo, pode dizer-se que as superfícies com grupo terminal CH_3 apresentam o comportamento pró inflamatório mais acentuado de todas as superfícies estudadas, uma vez que são responsáveis pela (i) adesão *in vitro* do maior número de leucócitos humanos (ii) atracção e activação do maior número de células inflamatórias *in vivo* e também (iii) pela formação de cápsulas fibrosas mais espessas.

Résumé

Indépendamment du type et de l'application du biomatériau utilisé, une fois implanté dans le corps humain celui-ci interagit avec les tissus. Le processus de l'implantation implique l'existence d'une blessure au niveau des tissus qui conduit à l'activation d'une réaction physiologique de cicatrisation basée sur deux étapes essentielles : l'inflammation et le processus de réparation. Les leucocytes sont des cellules qui jouent un rôle fondamental dans la réponse inflammatoire du tissu hôte et par conséquent au cours du processus immunologique leur réponse à la présence d'un biomatériau est très importante pour la compréhension des interactions entre le tissu hôte et le biomatériau.

L'objectif de ce travail a été de mieux comprendre la réponse inflammatoire au biomatériau. Tenant en compte de cet objectif, des monocouches auto structurées (*self-assembled monolayers, SAM*) de alkanethioles en or, avec des groupes fonctionnels OH, COOH et CH_3 ont été utilisé comme modèles de superficies afin d'analyser des interactions entre cellules inflammatoires et les caractéristiques chimiques de la superficie du biomatériau.

Dans ce travail des études d'interaction des SAM et des cellules inflammatoires ont été réalisée *in vitro* et *in vivo*.

La réponse inflammatoire in vitro a été étudié à travers des études d'adhésion de leucocytes humains mononuclés et polymorphonuclés aux modèles de superficies choisis. Les résultats montrent que les leucocytes humains adhèrent en plus grand nombre aux superficies couvertes de groupe méthyle en comparaison avec celles recouvertes de groupes hydroxyle et carboxyle. Il a été également conclut que la pré-activation des leucocytes est á l'origine d'une augmentation généralisée de l'adhésion des cellules aux superficies étudiées.

Les études in vivo ont été réalisé en utilisant un modèle animal de l'inflammation qui consiste en la formation d'une poche d'air soucutanée. Cette étude a été focalisée sur la réponse inflammatoire aigue initiale. Les différents modèles de superficies ont été implanté dans la poche d'air des animaux et par la suite, les exsudant inflammatoires et les implants ont été collecté et analysé.

Les superficies couvertes de groupe méthyle attirent un nombre plus élevé de leucocytes au sein de la poche d'air. Un nombre élevé de cellules adhère aux

superficies recouvertes de groupes OH et aux superficies d'or, en comparaison avec les superficies couvertes de groupes COOH et CH_3. Les SAMs couvertes de groupes méthyle induisent la migration d'un grand nombre de cellules inflammatoires dans la poche d'air. Cependant le nombre de cellules adhérentes à la superficie est faible. Ce phénomène, a été observé 24, 48 et 72 heures après l'implantation.

Le nombre de cellules activées au sein des cellules inflammatoires recrutées, exprimant la molécule d'adhésion Mac-1 a été étudié. Chez les animaux sans matériau implanté, après 24h le nombre de cellules Mac-1^+ est faible. Cependant, après l'implantation des différents SAMs une augmentation significative de ces cellules a été observée. Les superficies avec des terminaisons méthyle induisent une augmentation significative des leucocytes activés. De plus, les exsudants inflammatoires induits par les SAMs avec des terminaisons méthyle ont été examinées à 4, 24, 48 et 72 heures après l'implantation. Les résultats montrent que ces superficies attirent un nombre significatif de phagocytes exprimant l'integrin Mac-1.

La phase finale de cicatrisation a été étudié a travers l'analyse de la formation de la capsule fibreuse une semaine après implantation des matériaux. Il a été observé une augmentation de l'épaisseur de la capsule fibreuse présente autour des implants recouverts de groupes méthyle et aussi des superficies d'or, en comparaison avec celles recouvertes de groupes COOH ou OH ainsi que les animaux sans matériau implanté.

Dans cette étude, l'ensemble des résultats obtenus indique que les superficies avec des terminaisons en groupe CH_3 présentent un comportement pro-inflammatoire plus accentué que les autres superficies étudiées étant donné qu'elles sont responsables de (i) l'adhésion *in vitro* d'un grand nombre de leucocytes humains, (ii) de l'attraction et l'activation d'un nombre élevé de cellules inflammatoires un vivo et également (iii) de la formation de capsules fibreuses épaisses.

CHAPTER I

INTRODUCTION

1. Biomaterials Science

1.1. General Aspects

Biomaterials Science may be defined as the physical and biological study of materials and their interaction with the biological environment [1]. A biomaterial is a material intended to interface with biological systems to evaluate, treat, augment or replace any tissue, organ or function of the body [2]. Biomaterials and medical devices are now commonly used as prosthesis in cardiovascular, orthopedic, dental, ophthalmologic, and reconstructive surgery, in interventions such as angioplasty and hemodialysis, in surgical sutures or bioadhesives, and as controlled drug release devices [1].

Diverse materials have been developed for implants and medical devices. Biomaterials can be polymers, metals, ceramics and natural materials. Also, different types of materials can be combined together into a composite material. Biomaterials are commonly integrated into devices or implants, and not used as isolated materials. Table 1 presents some examples of medical devices routinely used in modern medicine, their application and the types of materials that can be used in their production.

Biocompatibility is defined as the ability of a material to perform with an appropriate host response in a specific application [2]. This definition encompasses elements of physical and chemical properties, as well as the ideas of biological reaction to the material. The biological reaction to synthetic materials implanted in living organisms may be different according to the application whether in soft tissue, hard tissue or in the cardiovascular system (blood compatibility). Two different outcomes that might be discussed are blood compatibility, an acceptable performance in the blood stream, and healing, a process that occurs around the implant and observed in soft and hard tissues [1, 3].

Table 1
Some examples of medical devices: applications and types of materials used [1,4-7].

Application	Medical Device	Types of Materials
Biomaterials for Soft Tissue	Intraocular lens	Poly(methyl methacrylate), silicone rubber, hydrogel
	Contact lens	Silicone-acrylate, hydrogel
	Skin repair template	Silicone-collagen composite
	Breast implants	Silicone, Poly(dimethylsiloxane)
	Unresorbable sutures	Polypropylene, nylon
	Bioresorbable sutures	Poly(glycolic acid), chitin, chitosan
Biomaterials for Hard Tissue	Joint Replacement (hip, knee)	Titanium, Ti-Al-V alloy, stainless steel, polyethylene
	Bone plate for fracture fixation	Stainless steel, cobalt-chromium alloy
	Bone cement	Poly(methyl methacrylate)
	Bony defect repair	Hydroxylapatite
	Artificial tendon and ligament	Teflon, Dacron
	Dental implants	Titanium, alumina, calcium phosphate
Blood-Contacting Biomaterials	Blood vessel prosthesis	Dacron, Teflon, polyurethane
	Heart valve	Reprocessed tissue, stainless steel, carbon
	Catheter	Silicone rubber, Teflon, polyurethane
	Artificial heart	Polyurethane
	Stents	Stainless steel, tantalum, gold, nitinol
	Cardiopulmonary bypass	Polypropylene, polyethylene, polyvinylchloride
	Blood bags	Polyvinylchloride

Whatever the origin of materials used, once they are implanted into the human body they interface with living tissues [8]. The very act of implantation means that there is tissue trauma, which in turn induces a physiological healing reaction, consisting of two essential components, inflammation and repair processes, which represent a spectrum of interdependent pathomecanisms, in which inflammatory mediators act in a concerned mode to initiate and control cellular response. Success in the field of tissue integration of biomaterial implants will depend on the ability to

mimic physiological responses such as repair processes following injury and to control reactions like inflammation [9].

2. Biological Interactions with Biomaterials

2.1. General Aspects

The reaction of the human body to implantable materials is diverse and complex. The process of implantation results in injury to tissues or organs. It is this injury and the subsequent perturbation of homeostatic mechanisms that lead to healing. The response to injury is dependent on multiple factors such as the extent of injury, blood-material interactions and the extent of the inflammatory response [10]. Blood-implant contact is an inevitable and early occurrence during almost all implantation procedures in biological tissue [11]. Blood-material interactions include protein adsorption, platelet and leukocyte activation/adhesion, and the activation of complement and coagulation systems (Figure 1). All these phenomena are highly interlinked, and the control of these mechanisms may aid in the improvement of the biological behavior of biomaterials [12].

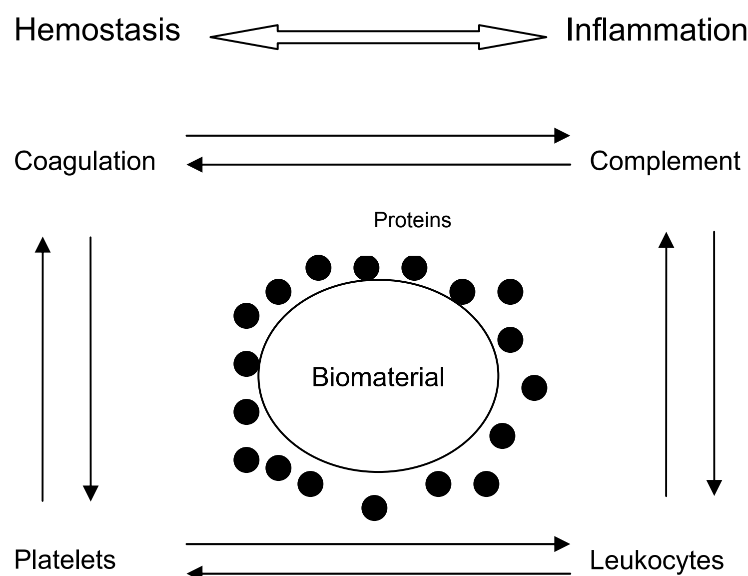

Figure 1
Overview of blood-material interactions. Adapted from Gorbet and Sefton [12].

The rapid adsorption of protein onto a biomaterial surface is regarded as the first major event induced by blood-biomaterial contact, and the adsorption of contact phase proteins may result in activation of the coagulation cascade. Also, blood contact with an artificial surface generally induces adhesion and aggregation of platelets. Thrombus formation results in an interaction between platelets and the coagulation system. Leukocytes adherent to the biomaterial surface contribute to platelet recruitment and fibrin formation. Contact of blood with biomaterials can induce complement activation that may also influence leukocyte adhesion to biomaterials [13].

2.2. Protein Adsorption

2.2.1. Fundamentals of Protein Adsorption

A protein is a complex molecule comprised of amino acid chains that interact with each other to give the molecule a three-dimensional structure. Each amino acid contributes to the chemical and physical properties of the protein. There are 20 different amino acids and of these 8 have non-polar side chains, 7 have neutral polar side chains, and 5 have charged polar side chains. Protein structure has been described on four different scales. Primary structure refers to the order and number of amino acids in the chain. Secondary structure results from hydrogen bonding associated with the amine linkages in the chain. Tertiary structure results from association within chains, including hydrogen bonding, ionic and hydrophobic interactions, salt bridges, and disulfide bonds. Quaternary structure results from associations between chains. It is often this structure that dictates how a protein interacts with surfaces and cells [6, 14, 15].

The adsorption processes are complicated. Even in the simplest case, where a single, well-defined protein adsorbs to a uniform, well-defined surface, a substantial range of processes is usually involved (Figure 2). These processes are complicated further by a range of conformationally altered and/or denatured states accessible to the adsorbed protein and by the many different microenvironments at the surface created by heterogeneities in the surface and the presence and conformations of other proteins [16].

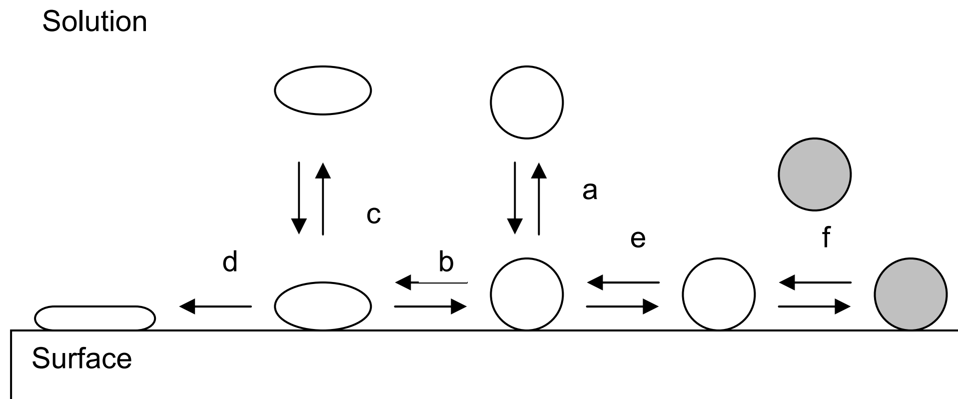

Figure 2
Complexity associated with protein adsorption to a solid surface. (a) Adsorption of a protein to the surface and dissociation from the surface to return to solution; (b) reversible denaturation and changes in conformation of the protein; (c) dissociation from the surface of the denaturate protein; (d) denaturation of the protein that results in irreversible adsorption; (e) lateral mobility of the adsorbed protein that results in changes of orientation; (f) exchange of the protein with another when the solution has more than one protein. Adapted from Mrksich and Whitesides [16].

2.2.2. Protein Adsorption to Biomaterials

The interaction of proteins with solid surfaces is not only a fundamental phenomenon but is also a key to several important applications such as nanotechnology, biomaterials and biotechnological processes. In the biomaterials field, protein adsorption is the first step in the integration of an implanted device or material with tissue [17]. Biological effects on a biomaterial surface such as thrombosis, complement activation, inflammatory reactions, cell adhesion and infections are closely associated with protein adsorption phenomena at the solid surface [18].

In as short a time as can be measured after implantation in a living system, proteins are already observed on biomaterials surfaces. In seconds to minutes, a monolayer of proteins adsorbs to most surfaces. The protein adsorption event occurs well before cells arrive at the surface. Therefore, cells see primarily a protein layer, rather than the actual surface of the biomaterial [19-23]. It is believed that cell-biomaterial interactions elicit immune reactions when these endogenous proteins are adsorbed in a conformation that makes the cell recognize the surface as foreign [24].

The structure and composition of the protein layer is mainly governed by the physicochemical surface properties of the biomaterial such as surface hydrophilicity/hydrophobicity, surface charge density, hydrogen bonding properties,

chain mobility on the surface and others. These properties determine the adsorption profile of plasma proteins and subsequent cell adhesion and activation processes [25, 26]. Among proteins that most readily adsorb to biomedical implants, albumin, immunoglobulin G (IgG) and fibrinogen usually predominate [20, 27-29].

Albumin dominates the adsorption phenomena on medical implants in the first stage of contact with body fluids, since it is the most abundant protein in serum and plasma and has a high mobility [30]. Albumin is often used as a passivating agent because it prevents the adsorption of other blood proteins and thus prevents the formation of thrombus [31, 32]. Albumin also blunts inflammatory responses [33], and reduces the adhesion of platelets [34].

Immobilized IgG activates the complement system [33] and promotes the adhesion of monocytes to the surface of biomaterials [35, 36]. Surfaces which exhibit a low adsorption of IgG generally prove more biocompatible than those displaying a high adsorption of this protein [37, 38].

Adsorbed fibrinogen is most important in mediating the short-term accumulation of inflammatory cells on implanted biomaterials [27]. Fibrinogen has been shown to mediate a pro-inflammatory effect at implant surfaces, mainly by causing an increased recruitment and adhesion of leukocytes in inflammation and tissue repair at implant surfaces [35, 39, 40]. Also, fibrinogen is capable of stimulating thrombosis, platelet adhesion and activation inflammation and bacterial colonization [34, 41].

2.3. Complement System

The complement system provides a rapid and efficient means to protect the host from invasive microorganisms. Biomaterial surfaces in any medical device used in contact with blood will also activate the system. Due to its diverse biological activities, complement is a key mediator of inflammation, a natural response of the host tissue to any injury. Inappropriate or excessive activation of the complement system can lead to harmful, potentially life-threatening consequences due to severe inflammatory tissue destruction [42, 43].

The complement system is a host defense system comprising more than 20 plasma proteins, acting within a cascade-like reaction sequence where the proteins are enzimatically activated. Complement activation can occur by three distinct pathways, the classical pathway, initiated by immune complexes, the alternative pathway that represents a non-specific means of recognizing foreign surfaces, and a recently described lectin pathway [44]. The complement components are designated by numerals (C1-C9) and by letter symbols (e.g., factor D). The peptide fragments formed by activation of a component are denoted by small letters. The early steps in complement activation can occur by one of the three pathways and culminate in the formation of C5b. Biomaterials activate the complement by the alternative pathway through the binding of C3b to the biomaterial surface. The final step, which is the same in all the pathways, involves C5b, C6, C7, C8 and C9, and leads to the formation of the membrane-attack complex (MAC, C5b-9) (Figure 3) [42, 45-48].

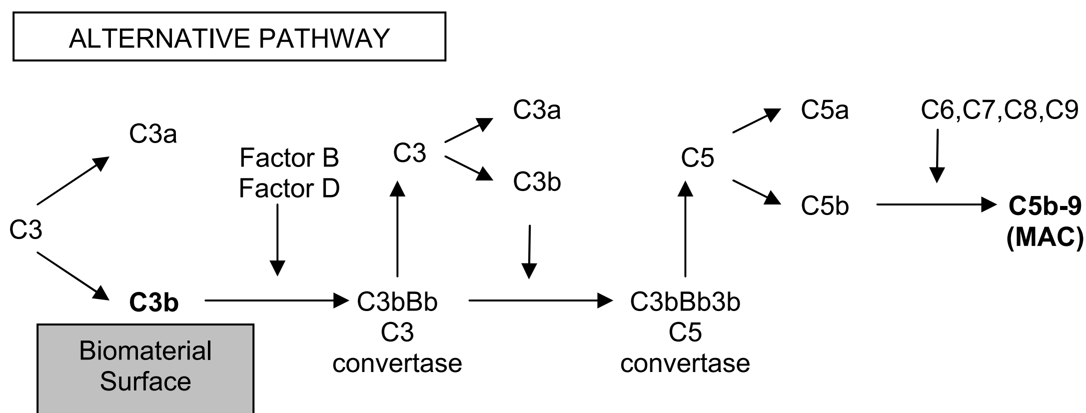

Figure 3
Simplified schematic representation of complement activation by biomaterials. Adapted from Kirschfink [42].

Surface groups such as amine (NH_2) and hydroxyl (OH) present at the surface of some biomaterials react with and eventually bind to the internal thioester in complement factor 3 (C3). A covalent amide or ester linkage is thereby supposed to form between C3b and the surface itself [33, 49, 50]. Recently, Wetterö et al. [51] suggest that complement upon activation with solid surfaces primarily associates to

other adsorbed proteins. They propose a more pronounced role for C3 association to other adsorbed proteins.

2.4. Leukocytes

2.4.1. General Aspects

White blood cells or leukocytes are a diverse group of cells (Table 2), found in the peripheral blood, whose major function is protection from environmental pathogens. These cells are broadly subdivided into two functional groups: those with responsibility for the ingestion and destruction of foreign particles – phagocytes – and those with responsibility for initiating specific immune responses against foreign antigens. Types of phagocytes include neutrophils, monocytes, eosinophils, and basophils. The various types of phagocytes have different functions and each behaves as a related but independent system. The immune effectors are lymphocytes. These cells are also subdivided into functionally specialized groups, each with a distinct role in the immune defense system [52].

Table 2
Normal leukocyte differential in adults. Adapted from Van Wynsberghe et al. [53].

Type of Leukocyte	Percentage
Neutrophils	50 – 70%
Lymphocytes	20 – 40%
Monocytes	1 – 10%
Eosinophils	± 3%
Basophils	± 1%

2.4.2. Leukocyte Adhesion

The adhesion of leukocytes to tissues or foreign materials is a phenomenon that can be seen as one of the hallmarks of inflammatory processes. The sorting of leukocytes out of vessels and their entry into sites of injury or infection requires

molecular mechanisms which enable the leukocyte to recognize such sites from within the vasculature and to form contact with the vascular lining, i.e. the endothelium, in order to exit and migrate through the blood vessel wall. Cell recognition as well as contact formation depends on several cell adhesion molecules which are involved in a sequential manner in concert with regulatory mediators such as the chemokines. The cell adhesion molecules that are involved in this process belong to three gene families which are: the selectins, the integrins and the immunoglobulins superfamily (Table 3) [54-56].

Selectins are the surface molecules that initiate cell contact between leukocytes and the endothelial cells. This docking of leukocytes to blood vessels that is made in combination with blood flow leads to a characteristic rolling movement of leukocytes on the endothelial surface. These rolling cells become capable of sensing signals from the endothelium which stimulate them to adhere more firmly to the vascular lining. Such signals can be given by chemokines or by other mediators of inflammation. Their stimulatory effect causes activation of leukocyte integrins that bind to the molecules of the IgG-superfamily present on the endothelial cell surface. The phenomenon triggers firm adhesion of the leukocytes to the endothelium and induces the leukocytes to actively migrate first on the blood vessel wall along gradients of chemotactic factors and after through the layer of the endothelium and of the underlying basement membrane (Figure 4) [54, 57].

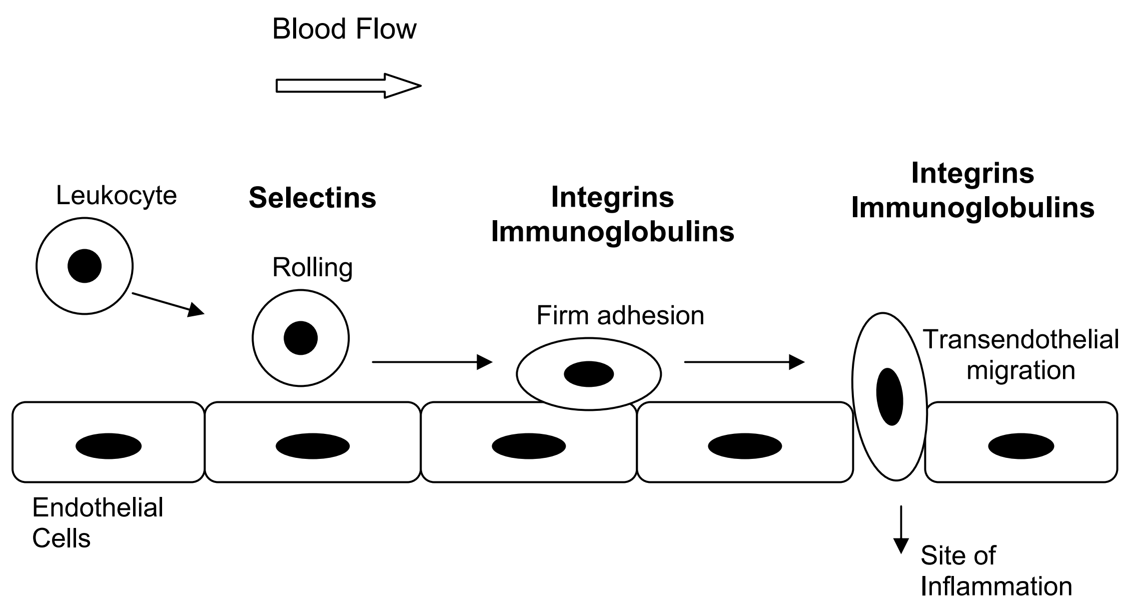

Figure 4
Adhesion of leukocytes to endothelial cells under conditions of blood flow. Adapted from Springer [59] and Brown [60].

Table 3
Leukocyte adhesion molecules involved in leukocyte endothelial cell adhesion. Adapted from Krieglstein and Granger [58].

Adhesion Molecule	Localization	Ligand	Function
	Selectin Family		
L-selectin	All leukocytes	P-/E-selectin MAdCAM-1	Rolling
P-selectin	Endothelial cells and platelets	L-selectin	Rolling
E-selectin	Endothelial cells	L-selectin	Rolling
	Integrin Family		
CD11a/CD18 (LFA-1)	All leukocytes	ICAM-1 ICAM-2	Adherence/emigration
CD11b/CD18 (Mac-1)	Granulocytes and monocytes	ICAM-1 C3b, fibrinogen	Adherence/emigration
CD11c/CD18	Granulocytes and monocytes	C3b	Adherence
CD11d/CD18	Macrophages	ICAM-3 ICAM-1	Adherence
CD49d/CD29 (VLA-4)	Lymphocytes, monocytes, eosinophils and basophils	VCAM-1	Adherence
CD49d/β_7	Lymphocytes	VCAM-1 MAdCAM-1	Adherence
	Ig-Supergene Family		
ICAM-1	Endothelium and monocytes	CD11a/CD18 CD11b/CD18	Adherence/emigration
ICAM-2	Endothelium	CD11a/CD18	Adherence/emigration
VCAM-1	Endothelium	CD49d/CD29	Adherence
PECAM-1	Endothelium, leukocytes and platelets	PECAM-1	Adherence/emigration
MAdCAM-1	Endothelium	L-selectin	Adherence/emigration

CAM = cell adhesion molecule; ICAM = intercellular CAM; VCAM = vascular endothelial CAM; PECAM = platelet endothelial CAM; MAdCAM = mucosal addressin CAM; LFA = lymphocyte function-associated antigen; VLA = very late antigen.

2.4.3. Leukocyte Activation

The direct contact between blood cells and implanted materials triggers a complex series of events. Material-induced leukocyte activation plays an important role in material failure. Circulating leukocytes express adhesion molecule receptors, which are up regulated in many inflammatory states, such as in inflammation induced by insertion of foreign materials, and this allows leukocyte binding to the endothelial adhesion molecules [61]. Leukocyte activation results in alterations in surface membrane receptors, such as CD11b/CD18 (Mac-1) up regulation, leading to an increased adhesiveness of white blood cells to artificial and biological surfaces and in the release of reactive products by the leukocytes [62].

Upon activation, leukocytes enhance their surface expression of the CD11b/CD18 integrin receptor [62]. Mac-1 is expressed at the surface of activated monocytes, macrophages, granulocytes and natural killer cells. This receptor can bind to the intercellular adhesion molecule 1 (ICAM-1), the complement factor C3b, fibrinogen and coagulation factor X [63-65]. Leukocytes from sites of active inflammation have been shown to increase surface density of CD11b/CD18 compared with leukocytes from non-inflamed tissues [61]. Mac-1 up regulation has been taken as an elective parameter to assess the potential risk of inflammation due to biomaterials before their clinical application [66, 67].

Cell activators have been used in several studies to assess the response of activated blood cells to biomaterials. Lectins such as PMA (phorbol 12-myristate 13-acetate) and PHA (phytohemagglutinin) activate human leukocytes *in vitro*. Lectins are proteins of non-immune origin that agglutinate cells and/or precipitate complex carbohydrates [62, 68-70].

Cell activation, such as that produced by PHA and PMA, is associated with molecular changes in the surface of leukocytes. These changes involve molecules that mediate intercellular aggregation and adhesion to vascular or other surfaces [71, 72]. PMA leads to an enhanced expression of adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular cell adhesion molecules-1 (ICAM-1) [73, 74]. PHA increases the density of several surface receptors of leukocytes (e.g. CD18, CD11a, CD54, CD58, CD44, CD49d, CD25) [75].

2.4.4. Leukocyte Adhesion to Biomaterials

The host responds with an inflammatory reaction to any injury, namely to the presence of foreign objects in the body. Leukocytes are the main cells that direct host inflammation and are central to the immune processes that may follow the initial inflammatory response. Thus, the response of white blood cells to biomaterials is of great importance in the understanding of material-host interaction [76]. The adhesion of leukocytes to artificial surfaces is an important phenomenon in the evaluation of biomaterials because the number of adherent leukocytes is often related to the extension of inflammatory response after implantation [77-79]. Adhesion of leukocytes to solid surfaces is known to depend on different factors that include surface free energy, surface hydrophilicity, surface chemistry, surface charge, protein adsorption, complement activation and adhesion of other cells such as platelets [77, 78, 80].

It must be reminded that the initial leukocyte interaction with biomaterials is potentially a limited one. This is because of the lack of various adhesion ligands and receptors on artificial surfaces compared with the vascular endothelium. Nevertheless, following exposure of plasma to an artificial surface, such as the one of a biomaterial, rapid deposition of plasma proteins creates the possibility for cell-surface interactions since many potential ligands for leukocyte receptors are also available on plasma proteins. For example, leukocytes bind to fibrinogen via CD11b/CD18 (Mac-1), to fibronectin via VLA-5 and to complement factor C3b via CD35 [81].

Leukocyte adhesion to biomedical devices is known to occur during many types of processes involving blood-material interactions, such as haemodialysis, haemofiltration, cardiopulmonary bypass and artificial hearth implantation [78, 82, 83]. In consequence to leukocyte adhesion, several reactions may be initiated, such as leukocyte spreading, formation of microthrombi through platelet-platelet and platelet-leukocyte interactions, detachment of thrombi by action of leukocyte proteases, detachment of adherent platelets and adsorbed proteins by leukocytes, and also because of release of leukocyte products which may give rise both to local and systemic vascular reactions, inflammatory responses that are mediated by leukocytes, and promotion of fibroblast ingrowth onto prosthetic materials [77].

3. Inflammation and Healing Response

3.1. General Aspects

The inflammatory process normally aims at the protection of the body from invasion by foreign organisms. However, it can also produce unwanted effects that involve tissue damage. This may result in frustrating various attempts to improve health, or contribute directly to disease by causing massive tissue injury. For example, implantation of a biomaterial may lead to a chronic inflammatory response that can destroy the foreign material [84].

The early intimate coupling between implant surface chemical properties, protein adsorption and inflammatory cell activation may influence the outcome of the *in vivo* integration process [85-87]. It is well known that the magnitude and duration of the inflammatory process has a direct impact on the stability and compatibility of biomaterials, hence affecting the efficacy of biomedical devices [48, 76].

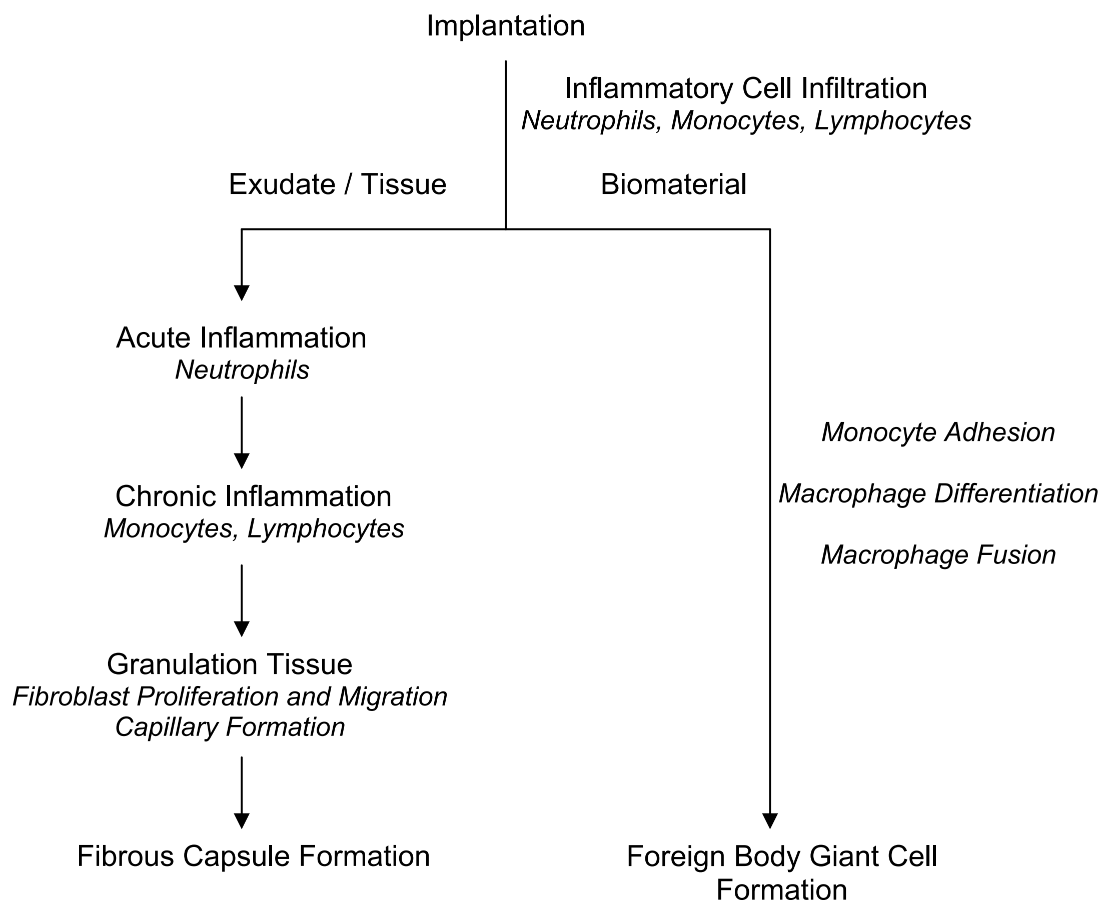

Figure 5
Sequence of events involved in inflammatory and wound healing response leading to foreign body giant cell formation. Adapted from Anderson [10].

The process of implantation of a biomaterial, prosthesis, or medical device results always in some injury to tissues or organs of the host. It is this injury and the subsequent alteration of the homeostatic mechanisms that lead to the cellular cascade of inflammation and wound healing. The implantation process initiates a sequence of events starting with an acute inflammatory response and leading in some cases to a chronic inflammatory response and/or granulation tissue development, a foreign body reaction and the formation of a fibrous capsule around the implant (Figure 5) [88, 89].

3.2. Acute Inflammation

Acute inflammation is defined as a biological response that involves white blood cells and is of relative short duration, lasting from minutes to days, depending on the extent of the tissue injury. The main features of acute inflammation are the exudation of fluid and plasma proteins – edema – and the emigration of leukocytes, predominantly neutrophils from the blood vessels into the affected tissues [10, 88, 90, 91]. Neutrophils, monocytes and other motile leukocytes move from inside small blood vessels to the implant site [92, 93].

The major roles that are considered for neutrophils in acute inflammation is to ingest microorganisms or foreign particles and to inactivate them through enzyme digestion. Although biomaterials are not generally suitable of being ingested by neutrophils or macrophages because of their large size, certain events are likely to occur. The process of recognition and attachment of leukocytes to the implant is accelerated when the inflammation-associated biomaterial is coated by naturally occurring serum factors called opsonins. The two major opsonins are IgG and complement factor C3b. Both are known to adsorb to the surface of biomaterials, and neutrophils and macrophages have corresponding cell membrane receptors for these proteins. These receptors may also play a role in the activation of the neutrophil or macrophage that became attached to the surface of the implant [10].

The host protein of greatest importance in acute inflammatory responses to implanted materials appears to be fibrinogen. Materials pre-coated with fibrinogen elicit large numbers of phagocytic cells [20]. Fibrinogen has been shown to mediate

a pro-inflammatory effect at implant surfaces, mainly by causing an increased recruitment and adhesion of leukocytes [39].

3.3. Chronic Inflammation

Persistence of an inflammatory stimulus leads to what is known as chronic inflammation which is characterized by the change in the type of predominant leukocyte. In chronic inflammation, the presence of macrophages, monocytes and particularly lymphocytes and plasma cells is often observed, and it also involves proliferation of blood vessels and of connective tissue. The chronic inflammatory response is usually confined to the implant site [88].

The macrophage is probably the most important cell in chronic inflammation because of being present in large numbers, and the great number of biological active products that it produces, namely reactive oxygen metabolites and chemotatic factors [10].

3.4. Granulation Tissue

On average, within one day after implantation of a biomaterial (injury), the healing response is initiated by the action of monocytes and macrophages. This is followed by proliferation of fibroblasts and proliferation also of vascular endothelial cells at the implant site, leading to the formation of granulation tissue, the hallmark of healing inflammation. Granulation tissue derives its name from the pink, soft granular appearance on the surface of healing wounds. Depending on the extent of injury, granulation tissue may be seen as early as three to five days following implantation of a biomaterial [10, 92].

3.5. Foreign Body Reaction

The foreign body reaction is composed of foreign body giant cells and the components of granulation tissue, that consist of macrophages, fibroblasts, and capillaries in varying amounts apposed to the surface of the biomaterial [88]. The

form and topography of the implanted material modulates the composition of the cells that are involved in the foreign body reaction. The foreign body reaction, consisting mainly of macrophages and/or foreign body giant cells, may persist at the tissue-implant interface for the lifetime of the implant [10].

Tissue macrophages, derived from circulating blood monocytes, may unite to form multinucleated foreign body giant cells. Very large foreign body giant cells containing large numbers of nuclei are typically present on the surface of biomaterials (Figure 6). Although these foreign body giant cells may persist for the lifetime of the implant, it is not known if they remain activated [10, 94, 95].

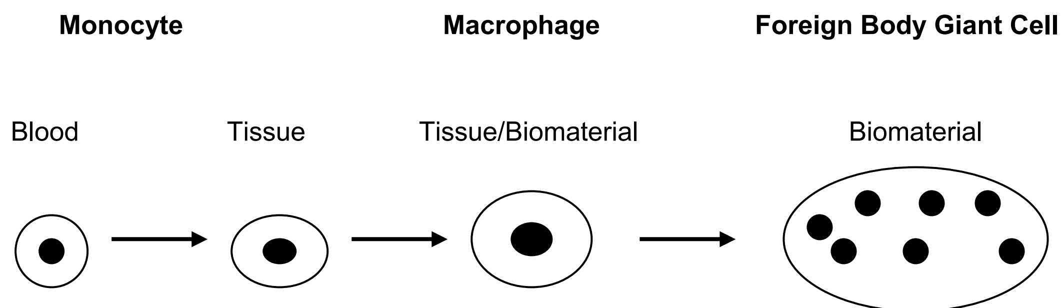

Figure 6
Transition from blood-borne monocyte to foreign body giant cell. Adapted from Anderson [10].

3.6. Fibrous Capsule Formation

The end stage of the healing response to biomaterial implantation is usually fibrosis or fibrous encapsulation, i.e., proliferation of fibroblasts with intense production of collagen fibers. Type I collagen often predominates and forms the fibrous capsule that surrounds the implant [10]. Generally, fibrous encapsulation surrounds the biomaterial or implant with its interfacial foreign body reaction, isolating the implant and foreign body reaction from the local tissue environment [92].

3.7. *In Vivo* Model of Inflammation

In our *in vivo* studies using mice we have adopted the air pouch model of inflammation (Figure 7). This is a well-known experimental approach to investigate the inflammatory response by creating a sterile subcutaneous cavity where the biomaterial can be implanted. The murine air pouches were generated according to the method of Sedgwick et al. [96]. This model has the advantage of using the cavity not only to insert a biomaterial but mostly for collecting inflammatory cells recruited by the presence of the implant [97, 98].

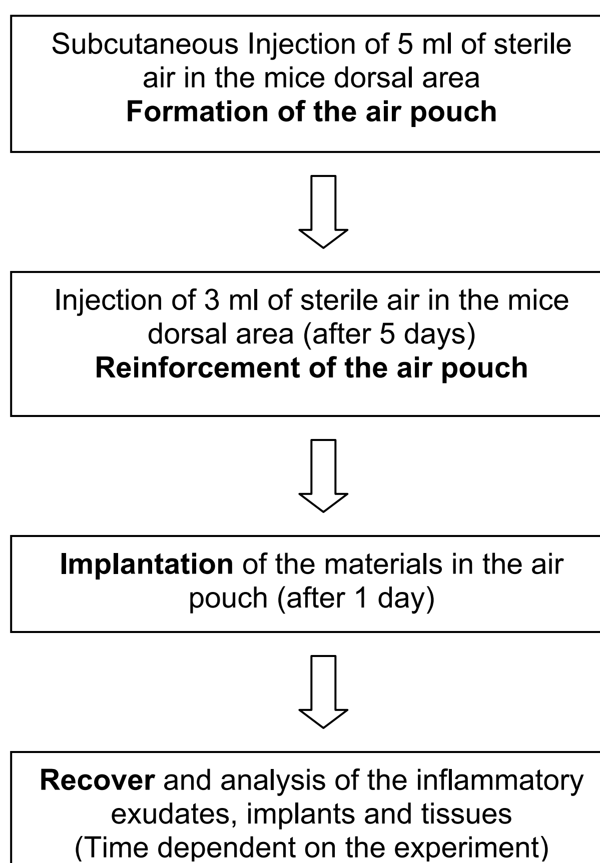

Figure 7
Schematic representation of the air pouch model of inflammation.

Previous investigations on inflammation produced by implantation of biomaterials have already adopted the air-pouch model [99], and it has been clearly demonstrated that this animal model is sensitive to the implant composition [100].

The main advantages of this method are the interaction between the implant and the subcutaneous lining of the air-pouch cavity, the ability to obtain a reliable quantification of inflammatory cells, and the possibility to carefully control the experimental conditions [101]. It has been used before in several studies assessing inflammatory responses to biomaterials such as, expanded poly(tetrafluoroethylene) (ePTFE) and silicone [97, 102], ultra-high molecular weight polyethylene (UHMWPE) [97, 103] and polymethylmethacrylate (PMMA) [100].

3.8. Analysis of Exudates and Implants

Several techniques can be used to study inflammatory exudates associated with implants and tissues surrounding implanted materials. We have predominantly used microscopy techniques, and also flow cytometry and histology.

Of all senses, the scientist relies most heavily on vision; thus the advent of light microscope is among the earliest and arguably most powerful tools in the history of science. Biomaterial scientists make extensive use of both light and electron microscopes to help fabricate and characterize new materials, and to study the behavior of cells and tissues at the biomaterials interface [1].

In our studies, we have used light microscopy to analyze the inflammatory exudates in terms of cell count and cell type and scanning electron microscopy to study the surface of implanted materials with regards to observation and quantification of adherent cells.

Flow cytometry allows the measurement of physical characteristics of cells based on the principle of laser beam diffusion. As each cell passes through the laser beam, a flash of scattered and/or fluorescent light is emitted, depending on cell characteristics. Upon reaching different photodetectors, the emitted light is converted into an electrical signal, giving information on cell size, granularity and/or fluorescence. Flow cytometry has several advantages for biocompatibility testing: The analysis is conducted at the single cell level, large numbers of cells are analyzed at a rapid rate, all measurements are quantitative and objective, and multiple cell parameters can be evaluated simultaneously [104].

We have analyzed the retrieved inflammatory exudates by flow cytometry, to quantify the exudates cellular composition and to assess CD11b/CD18 (Mac-1)

activation as a parameter of leukocyte activation. Flow cytometry is particularly well suited for studying exudates cell composition [105].

Histology techniques apply microscopy to understand the microanatomy of cells, tissues and organs [106]. We have analyzed histological sections of the tissues surrounding the implanted model surfaces to evaluate the fibrous encapsulation response.

4. Self-Assembled Monolayers

4.1. General Aspects

Self-assembled monolayers (SAMs) are molecular assemblies that are formed spontaneously by the immersion of an appropriate substrate into a solution of an active surfactant in an organic solvent (Figure 8). From the energetic point of view, a self-assembling surfactant molecule can be divided into three parts. The first part is the head group with higher affinity to the substrate surface (chemisorption). The very strong molecular-substrate interactions result in an apparent pinning of the head group to a specific site on the surface through a chemical bond. The second molecular part is the alkyl chain, and Van der Waals interactions between chains are the main forces in this case. The third molecular part is the terminal functionality [107].

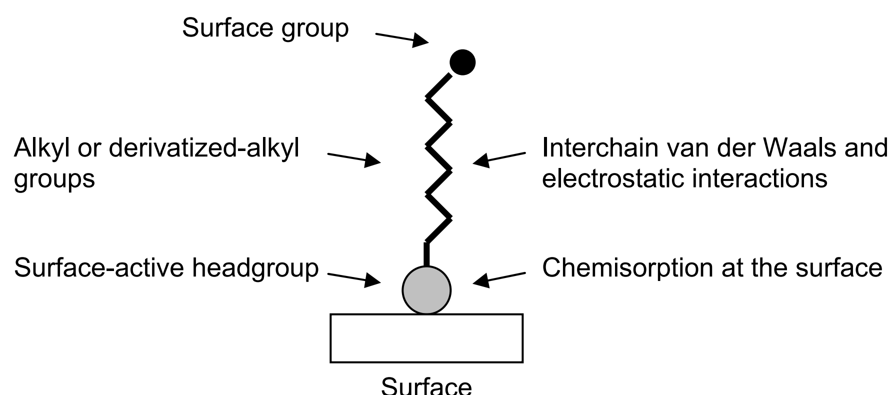

Figure 8
Schematic representation of the forces in a self-assembled monolayer. Adapted from Ulman [107].

There are several types of self-assembled monolayers [107, 108]:

- Monolayers of fatty acids: Formed by the spontaneous adsorption of long-chain n-alkanoic acids ($C_nH_{2n+1}COOH$). This is an acid-base reaction, and the driving force is the formation of a salt between the carboxylate anion and a surface metal cation.

- Monolayers of organosilicon derivatives: SAMs of alkylchlorosilanes, alkylalkoxysilanes, and alkylaminosilanes require hydroxylated surfaces as substrates for their formation. The driving force for this self-assembly is the in situ formation of polysiloxane, which is connected to surface silanol groups (-SiOH) via Si-O-Si bonds. These monolayers have been successfully prepared in substrates such as silicon oxide, aluminum oxide, quartz and glass.

- Monolayers of alkanethiols on gold.

Conventional biomaterial surfaces such as those formed by different biomedical polymers may possess a large degree of surface heterogeneity with regard to the type and distribution of functional groups, presence of hydrophilic and hydrophobic domains, surface roughness etc. SAMs have been successfully used to tailor material surfaces to obtain control over the molecular composition and the resulting properties of the surface [109].

4.2. Self-Assembled Monolayers of Alkanethiols on Gold

Self-assembled monolayers of alkanethiolates on gold (Figure 9) form on the adsorption of a long chain alkanethiol [$X(CH_2)_nSH$, n=11-18] to a gold surface. Sulphur has a very strong affinity for gold, and self-assembly proceeds rapidly to give well-defined surfaces.

The structure of these monolayers is well established. The sulphur atoms coordinate to the gold surface to give a close-packed array of alkyl chains. These chains are *trans*-extended and tilted approximately 30º, and present the terminal functional group X at the surface; these exposed groups determine the properties of

the interface. Even alkanethiols that are substituted with complex groups assemble into well-ordered monolayers that present these groups at the interface; alternatively, groups can be introduced onto the surface after the SAM is formed. The properties of SAMs can be controlled further by formation of “mixed” SAMs from solutions of two or more alkanethiols. SAMs on gold are stable in air or in contact with water for periods of months. The monolayers do undergo desorption at temperatures greater than 70ºC or when irradiated with ultraviolet (UV) light in the presence of oxygen [110-112].

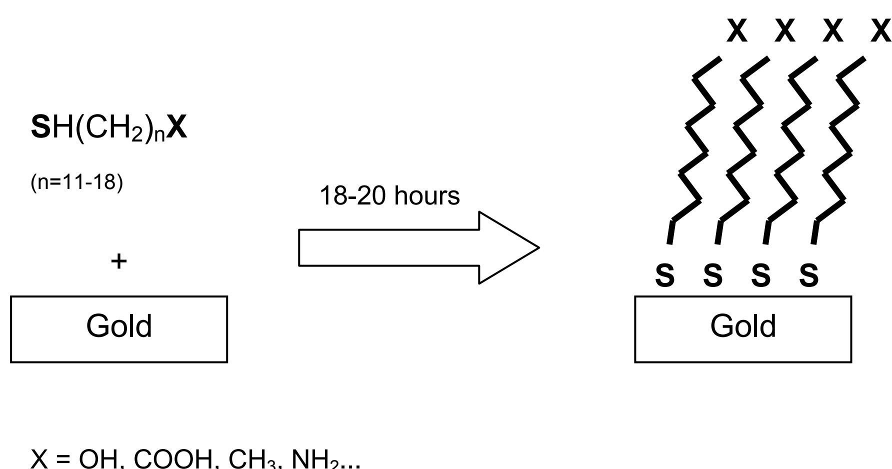

Figure 9
Schematic representation of the formation of a self-assembled monolayer of alkanethiols on gold. Adapted from Mrksich [16].

SAMs of alkylsiloxanes are more stable thermally than alkanethiolates on gold and do not require evaporation of a layer of metal for preparation of substrates. The siloxane monolayers are limited, however, in the range of functional groups that can be displayed at the surface. They are also not as ordered as monolayers of alkanethiols on metal surfaces [16]. It has been proposed that self-assembled, chemisorbed monolayers of alkanethiols on metal substrates open exciting new possibilities of engineering smooth surfaces with their chemical properties fine-tuned at the molecular level [107].

4.3. Characterization of Self-Assembled Monolayers

Self-assembled monolayers can be characterized by a wide range of analytical techniques. Some examples of routinely used methods for SAMs characterization are herein described.

Ellipsometry is the most common method of thickness analysis, which is one of the most important characteristic of a surface layer. This is a non-destructive optical method based on the fact that the state of polarization of the light reflected from a coated surface depends on the thickness and refractive index of the coating [18, 107, 113, 114].

IRAS - infrared reflection-absorption spectroscopy provides data on the chemical composition of gold-thiol monolayers. The IR beam is reflected at a small (grazing) angle from the monolayer surface, and the intensity of the reflected beam recorded. This makes it possible to directly obtain the IR spectrum of a monolayer [107, 113, 114].

X-ray photoelectron spectroscopy (XPS) measures the energy of the inner shell electrons ejected when the surface is irradiated with an X-ray beam in ultra-high vacuum. This energy is specific for every chemical element, and effectively XPS provides an elemental analysis of the monolayer [107, 113, 115].

Wettability measurements provide the simplest method for studying monolayers, yet give important information on the surface structure. A drop of liquid (usually water) is placed with a syringe on the monolayer surface, and the contact angle between the drop and the monolayer surfaces is measured. The value of the contact angle depends on the hydrophobicity of the outermost functions of the monolayer-air interface [107, 113].

4.4. Applications of Self-Assembled Monolayers

Table 4
Some examples of biological interactions with self-assembled monolayers.

Type of SAMs (Terminal Functionality)	Application	References
Alkanethiols on gold (OH, $COOH$, CH_3)	Primary human osteoblasts attachment and proliferation Influence of preadsorption of fibronectin and albumin	[116, 117]
Alkanethiols on gold (OH, $COOH$, CH_3, PO_3H_2, $PO_3(C_2H_5)_2$, SO_3H)	Platelet adhesion studies	[118, 119]
Alkanethiols on gold and of alkylsiloxanes (CF_3, NH_2, $COOH$, CH_3)	Adhesion and proliferation of corneal epithelial cells	[120]
Alkanethiols on gold (OH, $COOH$, CF_3, CH_3)	Bacterial cell attachment	[121]
Alkanethiols on gold (OH, $COOH$, CO_2CH_3, CH_3)	Endothelial cell growth Influence of preadsorption of fibronectin and albumin	[122]
Alkanethiols on gold (Oligo(ethylene glycol), $COOH$, CH_3)	Adhesion of 3T3 mouse fibroblasts Influence of preadsorption of fibronectin	[123-125]
Alkanethiols on gold (OH, $COOH$, CHO, NH_2, CH_3)	Immobilization of proteins: Albumin, Lysozyme and Immunoglobulin G	[30, 126]
Alkanethiols on gold ($COOH$, CH_3)	Growth and proliferation of human keratinocytes	[127]
Organosiloxanes (NH_2, CF_3)	Attachment and proliferation of skeletal myoblasts	[128]
Alkanethiols on gold (OH, $COOH$, CH_2OH, CH_3)	Adhesion of polymorphonuclear leukocytes Influence of preadsorption of fibrinogen	[78]

Self-assembled monolayers – particularly those formed by the adsorption of long-chain alkanethiols on gold – are a class of organic surfaces that are well suited for studying interactions of surfaces with proteins and cells [16]. SAMs of alkanethiols on gold are stable in a variety of organic and aqueous media, which makes them particularly useful model systems for investigating mechanisms of protein adsorption and cell adhesion [78].

The biological response to SAMs has been investigated using different chemically defined surfaces and several proteins and cell types. Table 4 presents some examples of application of self-assembled monolayers in these investigations.

5. Aim and Structure of the Thesis

The aim of this study is to analyze the inflammatory response to model surfaces, self-assembled monolayers (SAMs), with different terminal functional groups. In view of this objective, self-assembled monolayers of alkanethiols on gold with the terminal functionalities of OH, COOH and CH_3 were used. Initially, the *in vitro* adhesion of human leukocytes to these surfaces was investigated, and also the effect on adhesion of cells that have been submitted to pre-activation. Subsequently, *in vivo* studies using a rodent air-pouch model of inflammation were performed. Parameters such as inflammatory cell recruitment, activation and adhesion and also the fibrous capsule formation were investigated.

Chapter II deals with the *in vitro* adhesion of human mononuclear and polymorphonuclear leukocytes to self-assembled monolayers with OH, COOH and CH_3 terminal functional groups. Also, the human leukocytes were pre-activated with the lectins PMA and PHA, in order to study the influence of the activation of the white blood cells in their adhesion to biomaterials surfaces.

In Chapters III, IV, V and VI the *in vivo* inflammatory studies using the rodent air pouch model are described.

In Chapter III, SAMs with OH, COOH and CH_3 terminal functional groups were used to evaluate the inflammatory cell recruitment and adhesion after 24 hours of implantation. The inflammatory exudates were recovered and analyzed in terms of cell count and cell differential, and the number of adherent inflammatory cells was

quantify trough the study of the retrieved implants. Due to the nature of the results obtained with the methyl-covered surfaces, this study was performed for longer periods (24, 48 and 72 hours) with this surface and the results obtained are described in chapter IV.

In Chapter V methyl-covered surfaces continue to be investigated by the analysis of the inflammatory exudates by flow cytometry. The exudates were removed 4, 24, 48 and 72 hours post-implantation of the model surfaces. Leukocyte activation was investigated by the activation of the CD11b/CD18 (Mac-1) adhesion molecule.

Chapter VI describes the use of SAMs with OH, COOH and CH_3 terminal functional groups to investigate the formation of the fibrous capsule one week after implantation. In this chapter the initial inflammatory reaction (24 hours after implantation) was assessed by flow cytometry, with the aim of comparing it with the healing response (fibrous capsule formation).

Chapter VII presents a general discussion of the results described in the previous chapters and also some suggestions of future work in this research area. We have decided to present a general discussion instead of a detailed discussion because the latter is presented in each of the preceding chapters.

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CHAPTER II

Adhesion of Human Leukocytes to Biomaterials: An *In Vitro* Study Using Alkanethiolate Monolayers with Different Chemically Functionalized Surfaces

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ABSTRACT

The adhesion of human leukocytes to self-assembled monolayers (SAMs) of well-defined surface chemistry was investigated *in vitro*. Polymorphonuclear (PMN) and mononuclear leukocytes were isolated from human blood by centrifugation techniques. The effect on adhesion of cell activation produced by pre-incubation of leukocytes with phytohemagglutinin (PHA) and phorbol 12-myristate 13-acetate (PMA) was also studied. Gold substrates were modified by treatment with alkanethiols with three different terminal chemical groups: COOH, OH and CH_3. After incubation with the two subpopulations of leukocytes, the monolayers were washed, treated with fixative, stained with a Giemsa method and observed by light microscopy to quantify the number of attached leukocytes. Comparative quantification of the density of leukocyte adhesion to the three types of SAMs was determined. The hydrophobic surface expressing CH_3 was found to be the one that induced the highest adhesion density of leukocytes, both of PMN and mononuclear cells. *In vitro* activation of both mononuclear and PMN leukocytes further increased cell adhesion to the chemically defined monolayers that were used. This enhancement was higher

for PHA-activated than for PMA-stimulated mononuclear cells, whereas PMA treatment of neutrophils resulted in a higher rate of adhesion of these cells than PHA stimulation.

INTRODUCTION

The inflammatory response of the host is a key factor in determining the outcome of biomaterials implanted into the human body. In fact, the inflammation that usually protects the body from invasion by foreign microorganisms can also produce unwanted effects that may frustrate therapeutical attempts to improve human health. For example, the implantation of biomaterials may lead to an inflammatory response that can go as far as to destroy the foreign material [1]. Leukocyte adhesion to artificial surfaces is an important phenomenon in the evaluation of biomaterials since adherent leukocytes are often related to the inflammatory response seen after implantation [2-4]. Leukocytes have a central role in host inflammatory and immune processes; thus, their response to biomaterials is vital in understanding material-host interactions [5]. Leukocyte adhesion to biomaterials is known to occur during many different phenomena involving blood-biomaterial interactions such as haemodialysis, hemofiltration, cardiopulmonary bypass, and implantation of heart-assisting devices. The spectrum of events associated with leukocyte-biomaterial interactions include formation of microthrombi through aggregation of platelets, detachment of the thrombi by the action of proteases from white blood cells, release by leukocytes of active molecules that change local and systemic vascular reactions [6]. The adhesion of leukocytes to solid surfaces depends on many different factors such as surface chemistry, charge or hydrophilicity and protein adsorption [7, 8].

Biomaterials of well-defined surface chemistry were the elective model used to investigate leukocyte interaction with implants, as well as to understand the basic mechanisms of leukocyte physiology and pathology. Self-assembled monolayers (SAMs) are a recently developed class of organic surfaces that are well-suited for studying interactions of chemically defined surfaces with proteins and cells. SAMs of alkanethiolates on gold can be prepared by immersing a clean film of gold into a solution of terminally substituted alkanethiols [9-12]. SAMs are structurally the best ordered interfaces currently available for studying the interactions of cells and proteins with substrates of different surface chemistries [13-17].

With this system we have produced thiol-modified monolayers with the terminal functionalities of COOH, OH, CH_3. These preparations were obtained by the

treatment of gold-covered surfaces respectively with the chemicals 16-mercaptohexadecanoic acid, 11-mercapto-1-undecanol and 1-hexadecanethiol. We report here on the different adhesion densities of human PMN and mononuclear leukocytes to these SAMs. Experiments using *in vitro* pre-activation of these leukocyte subpopulations with PMA and PHA were also performed. Our data suggest that hydrophobic surfaces with the CH_3 functional group will cause greater adhesion density of both leukocyte subpopulations than the hydrophilic surfaces OH and COOH as their terminal functional groups. We found that *in vitro* pre-activation of human leukocytes also caused enhancement of adhesion density of leukocytes to SAMs.

MATERIALS AND METHODS

Substrate Preparation

The gold substrates were prepared with an automated load locked ion beam deposition system (Nordiko N3000). The 5 nm chromium adhesion layer and the 25 nm gold layer were deposited by ion beam sputtering from gold and chromium targets (99,9% purity) on silicon wafers (AUREL, Gmbh). Deposition rates used were 0,050 nm/s for chromium and 0,033 nm/s for gold. Deposition pressure was $3,5x10^{-5}$ Torr. The wafers were coated with 1,5 µm of photoresist (PFR7790EG, JSR Electronics), which is soluble in acetone, to protect the film surface. The wafers were then cut into 1 x 1 cm squares using a DISCO DAD 321 automated saw.

SAM Preparation

Before being used the gold substrates were cleaned with acetone and immersed in the so-called “piranha solution” (7:3 volume ratio of concentrated H_2SO_4 and 30% H_2O_2) for 10 minutes. The substrates were then washed with absolute ethanol, water and absolute ethanol again. The gold-coated substrates were immersed for 24 hours in a 1 mM ethanolic solution [18] of 11-mercapto-1-undecanol ($HS(CH_2)_{11}OH$) (97% purity), 1-hexadecanethiol ($HS(CH_2)_{15}CH_3$) (92%) and 16-mercaptohexadecanoic acid ($HS(CH_2)_{15}COOH$) (90%) all from Aldrich. Upon the removal from the thiol solutions, the monolayers were rinsed in absolute ethanol, ultrasonically cleaned in absolute ethanol for 2 minutes, rinsed with absolute ethanol again and dried in a stream of pure argon. The surfaces were stored in nitrogen until

used. The adhesion assays were performed within two days of the preparation of the SAMs.

Contact Angle

Contact angles were measured by the sessile drop method using deionized water with conductivity not greater than 1 μS/cm. The measuring system was an Optical Contact Angle Device OCA 15, with an electronic syringe (Dataphysics) and connected to a video camera. The software SCA20 was used for image analysis and for the calculation of the contact angles. The measurements were performed at 25ºC, in a thermostat-controlled environmental chamber saturated with water. The samples were dried in a vacuum oven for 1 hour before the contact angles were determined. Eight measurements on each surface were recorded.

Leukocyte Separation

Human leukocytes were isolated from fresh whole blood anticoagulated with citrate phosphate dextrose, using a centrifugation technique. 3 mL of Histopaque 1119 and 3 mL of Histopaque 1077 (Sigma) were introduced into a 15 mL tube, 6 mL of blood was gently added on top and the tube was centrifuged at 700g for 30 minutes at room temperature. The PMN or the mononuclear cells layer was transferred to a clean tube, washed with phosphate buffered saline (PBS) and centrifuged at 200g for 10 minutes. The cell pellet was washed twice and resuspended in 5 mL of PBS. The cell concentration was adjusted to 10^6 cells/mL for the adhesion experiments.

***In Vitro* Activation of Leukocytes**

In vitro activation of mononuclear and PMN cells was performed before the adhesion assay, with phytohemagglutinin (PHA) at 10 μg/mL (Sigma) and with phorbol 12-myristate 13-acetate (PMA) at 1 μg/mL (Sigma). The activators were added to the leukocyte suspension which consisted of 10^6 cells/mL in RPMI (Sigma) supplemented with 10% of foetal bovine serum (Gibco) and 2 mM of L-Glutamine (Gibco) and these preparations were incubated for 1 hour at 37ºC.

Adhesion Assay

For the adhesion assay the model surfaces were placed at the bottom of a 24 well plate, 1000 μL of RPMI (Sigma) supplemented with 10% of foetal bovine serum (Gibco) and 2 mM of L-Glutamine (Gibco) and 400 μL of the leukocyte suspension

(either non activated, activated with PMA or activated with PHA) were added. The adhesion assay was performed at 37^{0}C for 30 mins. Three replicates of each model surface were used.

Cell Count

After the adhesion assay the preparations were submitted to fixation in formol/ethanol and stained by the Giemsa method. The quantification of cells was done by light microscopy at a magnification of 100x using an Olympus PM3 reflection microscope. Ten areas were counted per sample.

Statistical Analysis

Data from the experiments are expressed as mean values and standard deviation. The significance of differences between group averages was assessed by Student's t-test. Significance was defined at $p<0.05$.

RESULTS

Contact Angle

Table I summarizes the results of contact angle measurements. The methyl-terminated surface (CH_3) provided an extremely hydrophobic substrate, with contact angles exceeding 100º. On the contrary, the hydroxyl (OH) and the carboxyl (COOH) terminated surfaces provided hydrophilic substrates. The non-thiol-coated gold layer is hydrophobic. These results are in accordance with published studies [3, 19, 20, 25], although the contact angles for the OH and COOH SAMs were a little higher than expected.

Table I

Contact angle data of SAMs with different terminal functional groups

Monolayer	Contact Angle (º)
Au	75.8 ± 1.9
$HS(CH_2)_{11}OH$	31.9 ± 3.6
$HS(CH_2)_{15}COOH$	47.7 ± 2.4
$HS(CH_2)_{15}CH_3$	108.0 ± 0.7

SAMs expressing CH_3 are hydrophobic, whereas those expressing OH and COOH are hydrophilic.

Leukocyte Adhesion Assay

We have compared the number of PMN and mononuclear leukocytes observed on gold monolayers treated with alkanethiols that cover the exposed surface with three well-defined chemical groups (COOH, OH, CH_3). The leukocytes were previously isolated from whole human blood and incubated *in vitro* with contact with the surface of the different monolayers during 30 minutes. The leukocyte preparations were then fixed and stained. Quantification of cells was done by light microscopy at a magnification of 100x.

Figures 1 and 2 show the number of cells counted for the same area of different preparations belonging to the three types of chemically distinct monolayers. The data show that adhesion density of leukocytes to gold surfaces is low and that it increases when any of the three functional groups is bound to the gold surface. The presence of any of the three chemical groups (COOH, OH, CH_3) on the gold monolayer induces a statistically significant increase in the number of PMN and also mononuclear leukocytes seen on the surfaces. The hydrophobic surfaces coated with CH_3 were found to cause the highest degree of adhesion of leukocytes to SAMs.

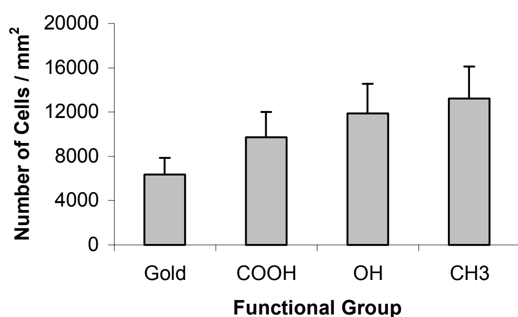

Figure 1

Adhesion of mononuclear leukocytes to SAMs with different functional groups; average number of cells per mm^2 and standard deviation are shown. Values for all of the distinct functional groups are significantly different ($p<0.05$) from each other and from controls (gold).

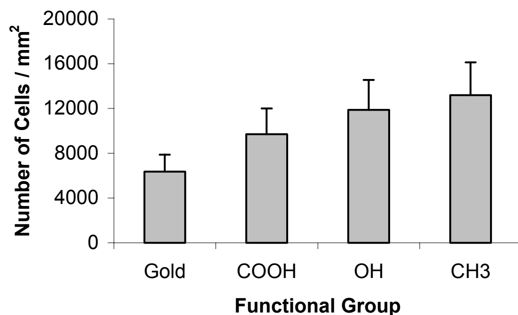

Figure 2

Comparison of adhesion of PMN leukocytes to SAMs with different functional groups; average number of PMN per mm^2 and standard deviation are shown. All values are significantly different ($p<0.05$) from each other and from controls (gold).

Effect on Cell Adhesion of Pre-Activation of Leukocytes

The influence on cell adhesion of the *in vitro* pre-activation by PHA or PMA of human neutrophils and mononuclear cells was also studied. In both instances, cell activation caused an increased density of leukocytes attached to the monolayers. The PHA treatment led to higher numbers of adherent mononuclear cells than the PMA stimulation, and the opposite was seen for neutrophils (Figures 3 and 4).

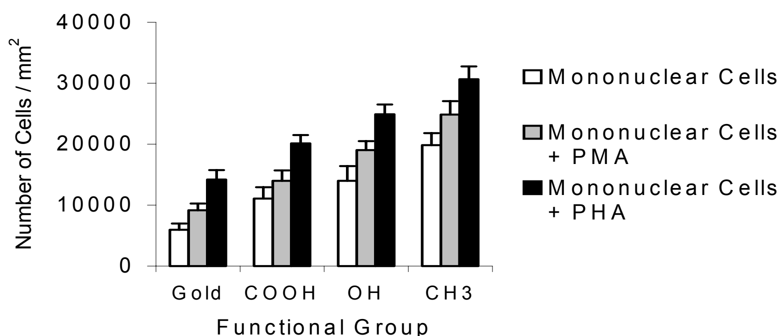

Figure 3

Adhesion of mononuclear cells with and without *in vitro* pre-activation. *In vitro* activation was performed using phytohemaglutinin (PHA) and also phorbol 12-myristate 13-acetate (PMA). Average number of mononuclear cells and standard deviation are shown. In each group, all values are significantly different ($p<0.05$) from each other.

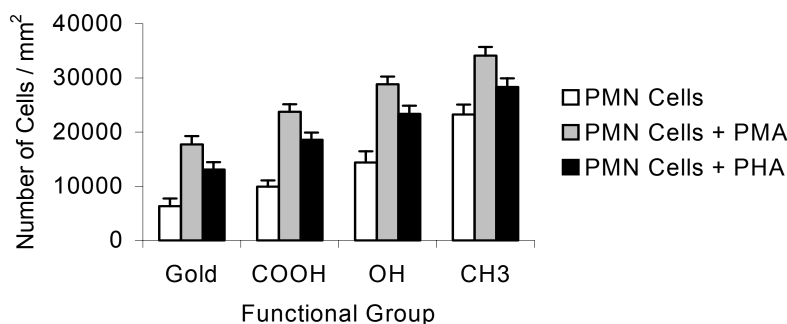

Figure 4

Adhesion of PMN cells with and without *in vitro* pre-activation. *In vitro* activation was performed using phytohemaglutinin (PHA) and also phorbol 12-myristate 13-acetate (PMA). Average number of PMN cells and standard deviation are shown. In each group, all values are significantly different ($p<0.05$) from each other.

DISCUSSION

In this study, the adhesion of human PMN and mononuclear leukocytes to self-assembled monolayers (SAMs) was quantified. The SAMs used were obtained by covering a gold surface with distinct chemical groups. This model allowed the comparison of the relative adhesion of PMN and mononuclear cells to gold surfaces coated with COOH, OH and CH_3 groups. Our numerical data indicate that leukocytes show adhesion in greater numbers to CH_3 groups than to OH and COOH groups. In addition, we show that pre-activation of leukocytes resulted in a general increase in attachment of the cells to the self-assembled monolayers.

For the isolation of the human leukocytes we have used a method of centrifugation of whole blood cells through a density gradient made with Histopaque (Sigma), which allows the harvesting of a rich cell band after a single centrifugation. This method has been recognized as an appropriate procedure to obtain PMN and mononuclear cells from whole blood and to keep their physiological features intact [21].

For the *in vitro* pre-activation of human leukocytes we have used the lectins PHA and PMA. Lectins are proteins of non-immune origin that agglutinate cells and/or

precipitate complex carbohydrates. These activators have been used in several studies to assess the response of activated blood cells to biomaterials [22-27].

We found that PMA and PHA caused an increase in the number of leukocytes that were seen attached to the SAMs. This result indicates that the adherence of leukocytes to the SAMs depends on the physiology of the cells: activation of leukocytes, a phenomenon commonly seen in inflammatory reactions, may lead to an enhancement of the attachment of these cells to monolayers. The difference between the effect of PHA and PMA in the degree of adherence of neutrophils and mononuclear cells to SAMs is in agreement with the fact that PMA is an elective activator of neutrophils, whereas mononuclear cells are better activated by treatment with PHA [28-30].

Cell activation, such as that produced by PMA or PHA, is associated with molecular changes in the surface of leukocytes. These alterations involve molecules that mediate intercellular aggregation and adhesion to vascular or other surfaces [31-33]. In fact, PMA directly activates the intracellular protein kinase C (PKC) [34] and this leads to an enhanced expression of adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular cell adhesion molecules-1 (ICAM-1) [35-36]; and also of a sulfatide receptor [37]. The chemoattractant activity of leukocytes is augmented by PHA [38]; this lectin increases the density of several surface receptors of leukocytes (e.g. CD18, CD11a, CD54, CD58, CD44, CD49d, CD25) [39]. Therefore our findings concerning a relative increase in the density of neutrophilic or mononuclear leukocytes on SAMs after PMA or PHA-triggered activation may be the result of these cell surface alterations.

The effect of chemical functionality on the biological compatibility of biomaterials has recently been addressed by researchers using the alkanethiol SAM technique. In the comprehensive review by Tengvall et al. [40], the importance of both ellipsometric and infrared spectroscopic techniques in the study of interactions between various plasma proteins and SAMs is stressed; their major conclusion was that hydrophobicity plays a key role in the adherence of proteins to SAMs.

The response of cells to SAMs has been investigated using different chemically defined surfaces and several cell types. Tidwell et al. [41] explored the effect of end terminal functional groups on the growth of endothelial cells and on the characteristics of protein adsorption and elution. They found that the best cell growth substrate was the COOH terminated SAM. Interestingly, the COOH terminal group was also described as leading to the highest amount of adhesion density of platelets [42], human corneal epithelial cells [43] and human osteoblast-like cells [44]. These results are in contrast with our data on human leukocytes, which were found to

adhere in higher numbers to CH_3 than to COOH coated SAMs; the difference in results suggests that the adhesion of cells to SAMs depends not only on the chemical characteristics of the monolayer but also on the cell type. It is conceivable that this phenomenon may be related to the different expression of surface proteins in the distinct cell types that have been investigated. For instance, human immunoglobulin G (IgG), which is present on the surface of B lymphocytes, was found by Silin et al. [45] to adsorb more efficiently to CH_3 than to COOH-terminated SAMs.

Our investigation shows that the chemical nature of the surface of a biomaterial modulates the degree of adhesion of leukocytes *in vitro*. This is in agreement with recent data obtained by Tegoulia and Cooper [3] who have investigated neutrophil adhesion under flow to chemically characterized monolayers. Our data indicate that thiol-modified SAMs with different terminal functional groups are able to cause distinctly different adhesion densities of PMN and mononuclear cells. We also report that the adherence of leukocytes to SAMs depends on the degree of activation of the cells. Some inferences regarding practical aspects of the selection of implants are suggested by our work. For instance, if attachment of leukocytes to the surface of the implant is to be avoided, then CH_3 groups should not be expressed on the surface of the biomaterial. Hydrophilic groups such as COOH and OH appear to be more appropriate if an inflammatory response of the host to the biomaterial is unwanted.

ACKNOWLEDGEMENTS

The authors acknowledge Cristina L. Martins for her help in the preparation of self-assembled monolayers, the Blood Bank of the Hospital Geral de St. António for donating the blood used in the leukocyte separation, Doctor Paulo Freitas and Doctor Susana Freitas (INESC) for the preparation of the gold samples, and the Portuguese Foundation for Science and Technology for awarding Judite N. Barbosa a scholarship.

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CHAPTER III

Inflammatory Responses and Cell Adhesion to Self-Assembled Monolayers of Alkanethiolates on Gold

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ABSTRACT

The acute inflammatory response and the adhesion of cells to self-assembled monolayers (SAMs) of well-defined surface chemistry was studied *in vivo* using a rodent air-pouch model of inflammation. SAMs with three different terminal functional groups (OH, COOH and CH_3) were implanted in subcutaneous air-pouches induced in BALB/c mice. After 24 hours, inflammatory cells were recovered from the air-pouches and the implants were removed and prepared for observation by scanning electron microscopy (SEM). The implants coated with OH and CH_3, were found to cause the highest recruitment of inflammatory cells into the subcutaneous pouches. Polymorphonuclear (PMNs) leukocytes predominated over mononuclear cells in inflammatory exudates of SAMs-coated implants, the opposite being found in uncoated implants (controls). CH_3-coated implants induced the highest number of inflammatory cells and also the largest percentage of PMNs seen in the subcutaneous pouches. Control and OH-covered implants presented the higher densities of attached inflammatory cells detected by SEM. In contrast, the CH_3-coated implants showed a very low density of cells adherent to the implant surface. We conclude that the chemical nature and the degree of hydrophobicity of the

surface of implants modulate both the local acute inflammatory reaction and the adhesion of leukocytes.

INTRODUCTION

Implanted biomaterials often trigger inflammatory responses, characterized by accumulation of a large number of phagocytes, specifically polymorphonuclear neutrophils (PMN) and macrophages/monocytes [1]. The magnitude and duration of the inflammatory process have a direct impact on biomaterial biostability and biocompatibility, hence affecting the efficacy of biomedical devices. The degree of inflammation caused by implants depends on the surgical trauma and on the chemical nature of the biomedical material [2]. Leukocytes such as PMN, monocytes, macrophages and lymphocytes are central in directing host inflammatory and immune processes; therefore, leukocyte response to biomaterials is a key parameter to evaluate material-mediated host reaction [3]. The initial inflammatory response to a biomaterial may determine how the healing process will evolve. The intensity of acute inflammation depends, namely, on the chemical composition, surface free energy, surface charge, porosity and roughness of the implant [4-6].

We have compared the acute inflammatory response to different self-assembled monolayers (SAMs). SAMs are a class of organic surfaces that are well-suited for studying interactions of chemically-defined surfaces with proteins and cells. SAMs of alkanethiolates on gold can be prepared by the immersion of a clean surface of gold into a solution of terminally substituted alkanethiols [7-9]. Different end-groups can be immobilized on the implant surface in a controlled way and this allows the evaluation of the influence of surface chemistry on the biological response to implants [10]. We have produced thiol-modified monolayers with the terminal functionalities COOH, OH and CH_3.

The aim of this study was to analyze *in vivo* the acute inflammatory response to chemically distinct SAMs that were implanted in a subcutaneous air-pouch produced in mice. We report here on the different degrees of cell recruitment to inflammatory air-pouches that are induced by these SAMs, and also on the adhesion of inflammatory cells to the surface of SAMs.

MATERIALS AND METHODS

Preparation of the Implants

Gold substrates were prepared with an automated load locked ion beam deposition system (Nordiko N3000). A 5 nm chromium adhesion layer and a 25 nm gold layer were deposited by ion beam sputtering from chromium and gold targets (99,9% purity) on silicon wafers (AUREL, Gmbh). Deposition rates used were 0,050 nm/s for chromium and 0,033 nm/s for gold. Deposition pressure was $3{,}5 \times 10^{-5}$ Torr. The silicon substrates are coated with gold on one side. The gold -coated surfaces act as a gold plus silicon control. The wafers were coated with 1,5 μm of photoresist (PFR7790EG, JSR Electronics), soluble in acetone, to protect the film surface. The wafers were then cut into 0,5 x 0,5 cm squares using a DISCO DAD 321 automated saw.

The gold substrates were pre-cleaned with acetone and immersed in "piranha solution" (7:3 volume ratio of concentrated H_2SO_4 and 30% H_2O_2) for 10 minutes. The substrates were then washed with absolute ethanol, water and absolute ethanol again. The gold-coated substrates were immersed for 24 hours in a 1 mM ethanolic solution [11] of 11-mercapto-1-undecanol ($HS(CH_2)_{11}OH$) (97% purity), 1-hexadecanethiol ($HS(CH_2)_{15}CH_3$) (92%) and 16-mercaptohexadecanoic acid ($HS(CH_2)_{15}COOH$) (90%), all from Aldrich. Upon the removal from the different thiol solutions, the monolayers were rinsed in absolute ethanol, ultrasonically cleaned in absolute ethanol for 2 minutes, rinsed again in absolute ethanol. The surfaces were then rinsed in three baths of sterile phosphate buffered saline (PBS), and put in sterile PBS until use. The subcutaneous insertions of the implants in the mice were performed within 2 hours. On insertion, the SAM-coated samples were oriented so that the coated surface stays facing the air-pouch cavity and the non-coated side will attach itself to the deeper wall of the inflammatory space.

Characterization of the Implants

The implants were characterized by contact angle measurements, after washing and sterilization with absolute ethanol, by the sessile drop method using deionized water with conductivity not greater than 1μS/cm. The measuring system was an Optical Contact Angle Device OCA 15, with an electronic syringe (Dataphysics), and was connected to a video camera. The software SCA20 was used for image analysis and for the calculation of the contact angles. The

measurements were performed at 25°C, in a thermostat-controlled environmental chamber saturated with water. The samples were dried in a vacuum oven for 1 hour before the contact angles were determined. Eight measurements on each surface were recorded.

Mouse Animal Model

In each set of experiments, 7 male BALB/c mice (Charles River, Spain) were used at 8 weeks of age. Air-pouches were generated according to the method of Sedgwick et al. [12], as adapted before by one of us [13]. Ether-anaesthetized mice were injected subcutaneously in the dorsal area with 5 mL of sterile air that caused the formation of an air pouch. A second subcutaneous injection of 3 mL of sterile air into the air pouch was performed 5 days later. This two-injection variant of the air-pouch model favors the formation of lining cells, resembling a sinovial membrane, that increase the reactivity of the air-pouch cavity [14]. Each animal received a single implant.

Implantation of the Biomaterials

One day after the second subcutaneous injection, the mice were anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4,0-8,0 mg/Kg of weight) and xilazine (Rompum, Bayer Co., Portugal; 0,8-1,6 mg/Kg) and the skin covering the air pouch area was shaved and cleaned with betadine. A surgical incision was made, the materials were placed inside the air pouch, and the incision was sutured. The same technique was performed for control animals, except that no material was implanted inside the air pouches.

Inflammatory Exudates

The exudates were recovered from the mouse air-pouches 24 hours after the implantation. The mice were ether-anaesthetized and sacrificed. Harvesting of inflammatory exudates was done by washing the air pouch cavities with 2 mL of PBS supplemented with 3% of saccharose (Panreac, Spain), followed by recovery of the lavage fluid. The total number of cells collected from the air pouches was determined with an automatic cell counter (Seac Cell Counter S2013, Metronik). For quantification of inflammatory cells, the exudates were centrifuged onto microscope slides (Cytospin3, Shandon), fixed in formol/ethanol and stained by the Giemsa method. The cells were counted by light microscopy at a magnification of 1000x, and the percentage of the different cell types was determined.

Implants

Explantation occurred immediately after the recovery of the inflammatory exudates. The sutures were cut, and the wound edges separated; the implants were then carefully removed from the pouches and fixed for scanning electron microscopy, as described below.

Scanning Electron Microscopy

The materials were removed from the pouches and fixed in 1,25% glutaraldehyde, dehydrated in a graded series of ethanol and subjected to critical-point drying from 100% ethanol. The samples were sputter-coated with gold and observed with a JEOL JSM-6301F scanning electron microscope. The acceleration voltage used was 15KeV.

Statistical Analysis

Data from the experiments are expressed as mean values and standard deviation. The significance of differences between group averages was assessed by Mann-Whitney test. Significance was defined at $p<0,05$.

RESULTS

Contact Angle

The methyl-terminated surface (CH_3) provided an extremely hydrophobic substrate, with a contact angle of 108.0º ± 0.7. The hydroxyl (OH) and the carboxyl (COOH) terminated surfaces provided hydrophilic substrates (contact angles of 31.9º ± 3.6 and 47.7º ± 2.4, respectively). The gold surface (control) is hydrophobic, presenting a contact angle of 75.8º ± 1.9. These data are in accordance with those cited in previously published studies [15,16].

Inflammatory Exudates Induced by SAMs Implantation

We have counted the total number of inflammatory cells, and their two main subpopulations (polymorphonuclear and mononuclear cells), that were collected from subcutaneous air-pouches of mice after 24 hours of implantation of different SAMs (Figures 1 and 2). A low number of leukocytes were seen in air-pouches of control animals (i.e., mice with sterile air-pouches with no implanted materials). After insertion of the implants there was a significant increase in inflammatory cells. The implants coated with OH and CH_3 caused the highest recruitment of inflammatory

cells to the air-pouch. Table I documents the different proportion of mononuclear and polymorphonuclear cells harvested from exudates of control and gold surfaces, and compares the values with those obtained for surfaces covered with COOH, OH and CH_3. Mononuclear cells (lymphocytes and monocyte/macrophage) were more numerous than PMNs in air-pouches with control implants (uncoated gold surfaces). The opposite phenomenon was seen after insertion of SAMs in the air-pouches: PMNs were more numerous than mononuclear cells in the induced inflammatory exudates. CH_3-coated implants triggered the highest number of inflammatory cells with the largest percentage of polymorphonuclear cells found in all air-pouches that were studied (figure 2).

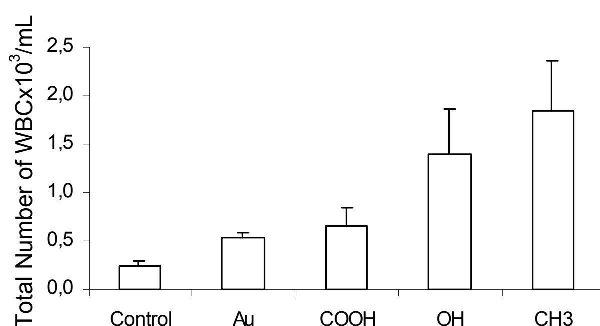

Figure 1
Total number of white blood cells (WBC) present in the inflammatory exudates recovered 24 hours after the implantation of the different materials. The differences between gold and COOH-covered surfaces and between OH- and CH_3-covered surfaces are not statistically significant; all other values are significantly different ($p<0,05$) from each other.

Table I
Percentage of mononuclear and polymorphonuclear cells present in the inflammatory exudates retrieved from the air pouches 24 hours after the implantation of the materials.

	Mononuclear Cells (%)	Polymorphonuclear Cells (%)
Control	85 ± 5	15 ± 4
Au	62 ± 4	38 ± 3
$HS(CH_2)_{15}COOH$	44 ± 4	56 ± 3
$HS(CH_2)_{11}OH$	39 ± 2	61 ± 4
$HS(CH_2)_{15}CH_3$	37 ± 7	63 ± 6

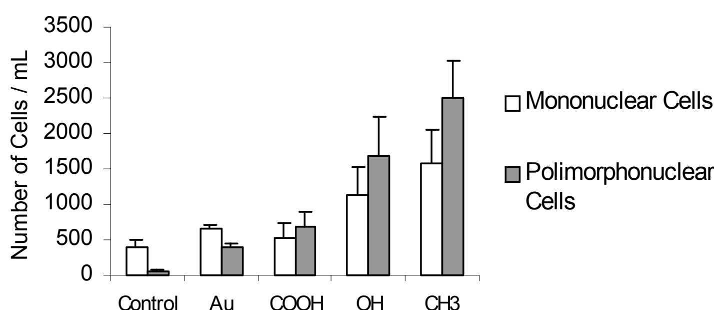

Figure 2
Number of mononuclear and polymorphonuclear cells recovered from the inflammatory exudates 24 hours after the implantation of the materials. The differences between gold and COOH-covered surfaces and between OH- and CH_3-covered surfaces are not statistically significant; all other values are significantly different (p<0,05) from each other.

Cell Adhesion to SAMs

Quantification of cells adherent to SAMs was obtained by scanning electron microscopy (SEM) using a magnification of 1000x. We found that the number of adherent inflammatory cells was quite low on CH_3-covered surfaces. This was in contrast with the high degree of cell adhesion on all other three types of surfaces. The OH-covered surfaces presented the highest density of adherent cells (figure 3).

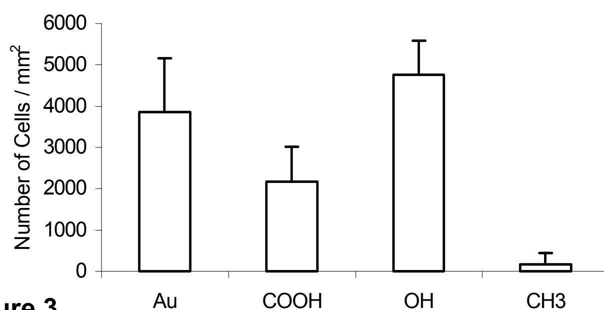

Figure 3

Adhesion of inflammatory cells to the SAMs implanted in the mouse air pouches and retrieved after 24 hours; average number of cells per mm^2 and standard deviation are shown. All values are significantly different (p<0,05) from each other and from controls (gold).

Scanning Electron Microscopy of the SAMs

Adherent cells showed two major types of surface morphology, as shown by SEM: i. flat cells with a large area of attachment to the surface of SAMs; ii. smaller and round-shaped cells with rough plasma membranes. The first type corresponds mainly to macrophages (although the presence of a few fibroblasts, although unlikely, can not be excluded), whereas the latter one corresponds to mononuclear cells

(lymphocytes, monocytes) or to granulocytes. These cells are illustrated in the scanning electron micrographs presented in figure 4.

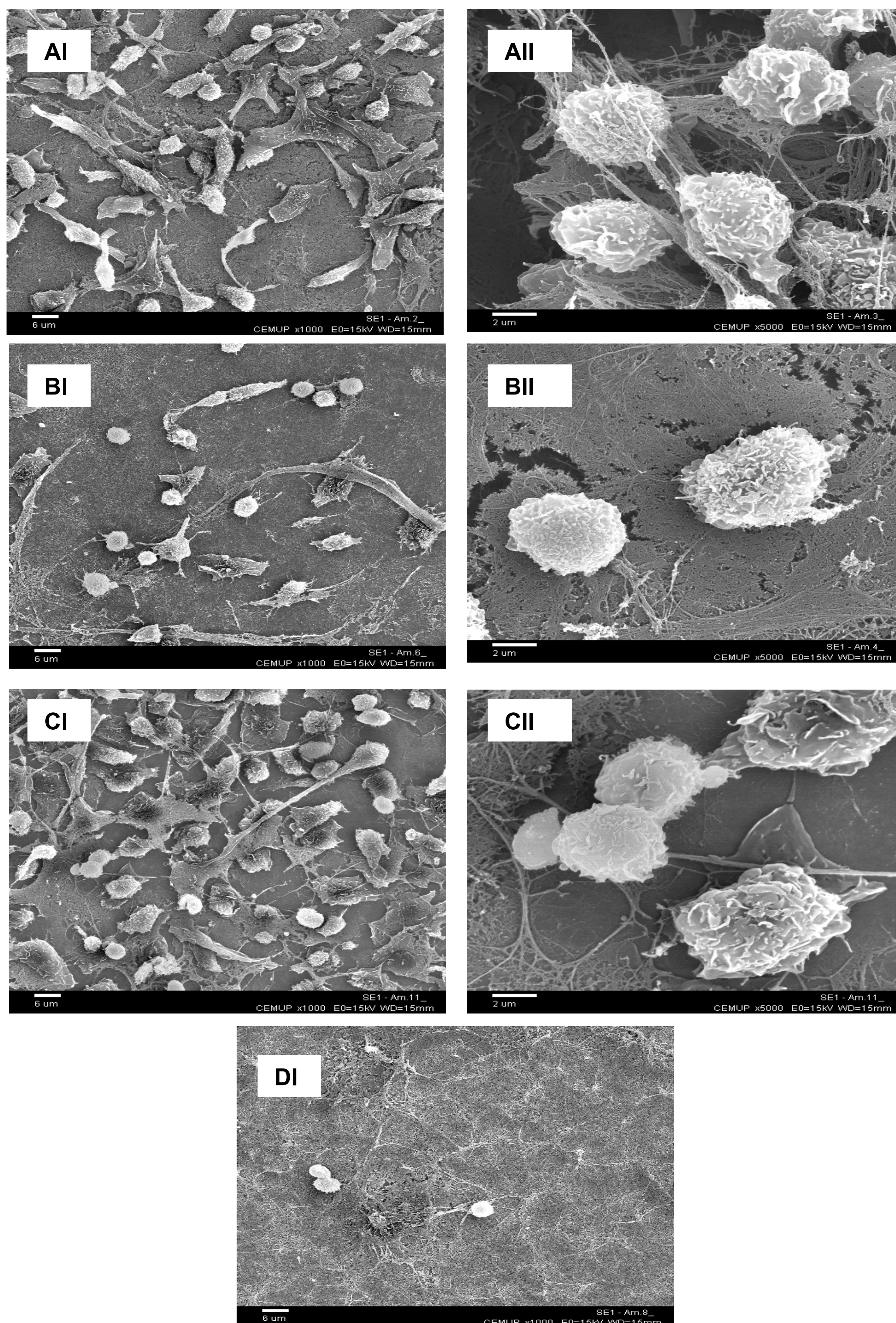

Figure 4
Scanning electron microscopy micrographs of cells adherent to various SAMs surfaces after 24 hours of implantation of materials inside the air-pouches of mice. (A) Gold surface; (B) COOH-covered surface; (C) OH-covered surface; (D) CH_3-covered surface; (I. 1000x; II. 5000x). Large flat cells (macrophages) predominate on implant surfaces of controls (fig 4A I, uncoated gold) and on OH-coated surfaces (fig 4C I). Very few cells were seen attached to CH_3-coated implants (fig 4D). Mononuclear cell and fibrin are illustrate in figure 4A II.

DISCUSSION

We document here that insertion of SAMs-coated implants in subcutaneous air-pouches of mice causes acute inflammatory reactions that are different according to the surface chemistry and the degree of hydrophobicity of the implant. This conclusion was based on the evaluation of cell recruitment to the air pouches and attachment to the implants surface that was observed during the early inflammatory response induced by SAMs having different terminal functional groups (COOH, OH and CH_3). Our data indicate that hydrophobic surfaces (such as CH_3-coated ones) induce a more intense acute inflammatory response and a lower degree of cell adhesion than hydrophilic surfaces.

We used here the air pouch model of inflammation [12]. This model involves the formation of a sterile subcutaneous cavity that can be used to insert a biomaterial and to study the inflammatory reaction caused by the implant [17,18]. The rodent air pouch has been used in the evaluation of the animal response to biomaterials [19], and it has been demonstrated to be sensitive to differences in implant composition [20]. The distinct advantages of this method are its intimacy with the tissues surrounding implants, the ability to quantitate inflammatory cells, and the chance to carefully control the experimental conditions [21]. It has been used before in several studies assessing inflammatory responses to biomaterials such as, expanded poly(tetrafluoroethylene) (ePTFE) and silicone [17,22], ultra-high molecular weight polyethylene (UHMWPE) [18,23] and polymethylmethacrylate (PMMA) [18].

We found that the *in vivo* cell recruitment to the air pouches was influenced by the different surface functionalities of the SAMs. Methyl surfaces attracted the highest numbers of leukocytes to the inflammatory cavity. This elective phlogistic activity of CH_3-coated surfaces may be derived from the hydrophobic nature of these SAMs. Our finding is in agreement with those reported by Lindblad et al. [24] who found higher numbers of inflammatory cells in the fluid space around the methylated implants than around hydroxylated or gold implants. We have observed that PMNs predominated over mononuclear cells (monocyte/macrophage and lymphocyte) in the exudates induced by all of the three types of SAMs (CH_3, OH and COOH); this was in contrast with what we detected in exudates caused by gold-coated surfaces. The finding is different from the data of Lindblad et al. [24] since they reported that mononuclear cells were predominant over PMN in subcutaneous implants of SAMs. Conceivably, the difference may come from the distinct kind of inflammatory cavities that they produced subcutaneously since they did not trigger a sinovial-like cavity as we have done with air pouching.

Several molecular parameters may be considered to explain the different phlogistic activity of the terminal functional groups of the herein investigated SAMs. Complement activation is known to enhance chemotaxis and aggregation of inflammatory cells: *in vitro* assays performed by Tengvall et al. [25] and Liu and Elwing [26] using SAMs have shown that uncharged hydroxyl groups have a higher capacity to activate the complement cascade in sera, via the alternative pathway, that either carboxylic groups or uncoated gold. Furthermore, depletion of complement by cobra venom factor was reported by Tang et al. [27] to result in inhibition of the inflammatory response caused in the peritoneal cavity of mice by the implantation of SAMs with surface-exposed hydroxyl groups. Our data are consistent with these previous studies since we found that hydroxyl-coated SAMs attracted significantly higher numbers of leukocytes to the subcutaneous inflammatory air-pouches than carboxylic SAMs or gold surfaces. However, it should be noted that Källtorp et al. [28] have concluded that there was not a clear relationship between *in vitro* activation of complement and *in vivo* recruitment of inflammatory cells by SAMs implants. Other immune proteins, such as the pro-inflammatory cytokines (IL-1α, IL-1β and TNFα) appear not to be altered by the implantation of either CH_3 or OH-coated SAMs.

We have found that higher numbers of cells adhere to OH-coated and gold surfaces (control), than to COOH and CH_3-terminated SAMs. The number of adherent inflammatory cells on the hydrophobic CH_3-coated surfaces was quite low.

Methylated surfaces induced the migration of larger numbers of inflammatory cells into the air pouches than hydroxylated or gold surfaces but were associated with a low density of cells attached to the implant surface. This phenomenon was described before by Lindbat et al. [24] and Källtorp et al. [28] and, thus, strengthens these observations.

Implanted materials quickly acquire a layer of host proteins well before the arrival of inflammatory cells therefore phagocytes interact with the spontaneously adsorbed proteins rather than with the material itself. Fibrinogen is important in mediating the short-term accumulation of inflammatory cells on implanted materials. Fibrinogen adsorbs spontaneously and denaturates on implant surfaces and becomes pro-inflammatory, mainly by causing an increased recruitment and adhesion of leukocytes in inflammation and tissue repair at implant surfaces [2, 29, 30]. SAMs with hydrophobic terminal functional groups such as CH_3 are known to present significantly lower fibrinogen deposition than surfaces composed of hydrophilic groups such as OH [31]. These results may explain the low number of adherent cells to the CH_3-coated surfaces.

A note of caution is pertinent in comparing our data with those of Silver et al. [31]. In fact, they reported on thrombogenic response of various SAMs of alkylsiloxanes, and their samples were in contact with canine blood rather than with mouse inflammatory leukocytes as it occurred in our experiments. Futhermore, several authors have studied the adsorption of fibrinogen to SAMs and obtained different results. Fibrinogen adsorption on gold-coated surfaces was higher than adsorption on all other SAMs, and adsorption to the CH_3-coated surfaces was higher than adsorption to the other SAMs [15, 24, 28]. The presence of fibrinogen in the surface layer of adsorbed proteins attracts more inflammatory cells to the surface than when fibrinogen is absent [32, 33], the low number of cells adherent to the CH_3-covered surfaces may seem contradictory. Lindblat et al. [24] postulated that is possible that fibrinogen binds to the hydrophobic surface and undergoes degradation before the arrival of the recruited cells, thus offering a lower number of accessible cell binding sites. The presence of soluble fibrinogen degradation products in the fluid space has been considered to promote a localization of inflammatory cells, for instance via a binding to soluble fibrin [24]. In our SEM observations (illustrated in figure 4) an extensive network of adsorbed fibrin-like proteins can be seen covering the CH_3-coated samples. However in our SEM preparations, it is not possible to specifically identify fibrinogen.

Källtorp et al. [28] have also tested the effects of C3, IgG and other proteins on cell adhesion to SAMs. The OH-covered surfaces adsorbed more IgG than CH_3 and gold-covered surfaces. C3 was detected on the surface of gold and OH-covered surfaces. The methyl surface presented very low or non-measurable amounts of C3 and IgG. In summary, the OH surface deposited C3 and IgG which were not found on the CH_3 surface. A possible reason for the higher numbers of cells found adherent to the OH-covered surface may be the activation of the complement system as observed through the surface bound of C3 and/or IgG [34-36].

Finally, the herein *in vivo* results are different from our recent data on *in vitro* adhesion of human leukocytes to SAMs. In fact, with cultured human leukocytes, we have found that they adhere in higher numbers to CH_3-coated SAMs than to OH, COOH and gold surfaces [37]. Taken together, our data suggest that both *in vivo* and *in vitro* investigations should be performed in order to fully understand the interaction between biomaterials and leukocytes.

CONCLUSIONS

The acute inflammatory response to SAMs with different terminal functional groups (COOH, OH and CH_3) was evaluated. We used the air pouch model of inflammation that involves the formation of a sterile subcutaneous cavity that is used to implant the biomaterial and allows the study of cell recruitment to the air pouch and adhesion to the implants. The *in vivo* cell recruitment was influenced by the different surface functionalities of the SAMs. Methyl-terminated surfaces attracted the highest number of leukocytes to the inflammatory cavity. Higher numbers of cells adhere to OH-coated and gold surfaces, than to COOH and CH_3-terminated SAMs. The number of cells adherent to the CH_3-coated surfaces was low. Methylated surfaces induced the recruitment of larger numbers of inflammatory cells but were associated with a low density of cells attached to the implant surface.

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CHAPTER IV

Inflammatory Cell Recruitment and Adhesion to Methyl-Terminated Self-Assembled Monolayers: Effect of implantation time

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ABSTRACT

The contribution of methyl groups in implant-triggered inflammation was investigated *in vivo* using self-assembled monolayers (SAMs) of alkanethiols on gold. The CH_3-coated implants were inserted in an air-pouch cavity induced in BALB/c mice. The *in situ* inflammatory response was monitored 24, 48 and 72 hours later. Inflammatory cells recovered from the air pouches were counted and observed by light microscopy. The cellularity of the implant surfaces was defined by scanning electron microscopy (SEM). In comparison with gold implants, the CH_3-coated SAMs recruited a significantly higher number of inflammatory cells. Polymorphonuclear leukocytes (PMN) were more numerous than mononuclear cells (Mo) in the exudates recovered from the air pouches with CH_3-coated SAMs. The opposite PMN/Mo proportion was observed in air pouches of the two control groups (mice receiving gold implants or sham-operated animals). Low density of adherent cells was seen on CH_3-coated implants, with no significant quantitative differences during the time course of the study. In contrast, the gold-coated surfaces were covered with numerous cells during all of the 3 days of the inflammation. In conclusion, implants with CH_3 surfaces are likely to induce PMN-dominated local acute inflammation but

these surfaces are not associated with a significant adherence of leukocytes to the implant.

INTRODUCTION

Host inflammatory reaction is a normal response to injury and to the presence of foreign bodies [1]. Leukocytes are central players in directing host inflammatory and immune processes; thus, their response to biomaterials is important in the understanding of material-host interactions [2]. Adhesion of leukocytes to solid surfaces, also a key phenomenon in biocompatibility, depends on different factors, such as surface chemistry [3,4]. The early intimate interaction between implant surface chemical properties, protein adsorption and inflammatory cell activation is known to influence the outcome of the *in vivo* integration process [5]. It is therefore of interest to advance our knowledge of the role of surface chemistry of implants in the regulation of tissue response to biomaterials.

We have used here model surfaces of well-defined surface chemistry to investigate *in vivo* leukocyte interaction with implants. For that, self-assembled monolayers (SAMs) were adopted; they are a class of organic surfaces that are well-suited for studying interactions of chemically defined surfaces with cells and proteins. SAMs of alkanethiolates on gold can be prepared by immersing a clean film of gold into a solution of terminally substituted alkanethiols [6-8]. SAMs are structurally the best ordered interfaces currently available for studying the interactions of cells and proteins with substrates of different surface chemistries [9-11].

We have recently found that methyl-terminated SAMs induce an early migration of high numbers of inflammatory cells to the implant area [12]. The aim of the current study was to investigate *in vivo* the kinetics of inflammatory cell recruitment caused by methyl-terminated SAMs, as well as the adhesion of leukocytes to this type of surface. For that, we have used the air-pouch model of inflammation [13], an *in vivo* system that involves the formation of a sterile subcutaneous cavity in mice that is appropriate for the insertion of an implant [14, 15]. The advantage offered by the air-pouch model comes from allowing a precise quantification of inflammatory cells. This was the main reason why we have adopted this approach. In fact, the repeated subcutaneous injection of air leads to the formation of an organized and well-lined space where the implants can be easily inserted and also exudate leukocytes can be collected and counted.

MATERIALS AND METHODS

Preparation of the Implants

Gold substrates were prepared with an automated load locked ion beam deposition system (Nordiko N3000). A 5 nm chromium adhesion layer and a 25 nm gold layer were deposited by ion beam sputtering from chromium and gold targets (99,9% purity) on silicon wafers (AUREL, Gmbh). Deposition rates used were 0,050 nm/s for chromium and 0,033 nm/s for gold. Deposition pressure was $3{,}5\times10^{-5}$ Torr. The silicon substrates are coated with gold on one side. The gold -coated surfaces act as a gold plus silicon control. The wafers were coated with 1,5 μm of photoresist (PFR7790EG, JSR Electronics), soluble in acetone, to protect the film surface. The wafers were then cut into 0,5 x 0,5 cm squares using a DISCO DAD 321 automated saw.

The gold substrates were pre-cleaned with acetone and immersed in a solution of 7:3 volume ratio of concentrated H_2SO_4 and 30% H_2O_2 for 10 minutes. The substrates were then washed with absolute ethanol, water and absolute ethanol again. The gold-coated substrates were immersed for 24 hours in a 1 mM ethanolic solution [16] of 1-hexadecanethiol ($HS(CH_2)_{15}CH_3$) (92% purity) from Aldrich. Upon the removal from the thiol solutions, the monolayers were rinsed in absolute ethanol, ultrasonically cleaned in absolute ethanol for 2 minutes and rinsed again in absolute ethanol. The surfaces were then rinsed with sterile phosphate buffered saline (PBS), and put in sterile PBS until use. The subcutaneous insertions of the implants in the mice were performed within 2 hours (see technique below).

Characterization of the Implants

The implants were characterized by contact angle measurements, after washing and sterilization with absolute ethanol, by the sessile drop method using deionized water with conductivity not greater than 1μS/cm. The measuring system was an Optical Contact Angle Device OCA 15, with an electronic syringe (Dataphysics), and was connected to a video camera. The software SCA20 was used for image analysis and for the calculation of the contact angles. The measurements were performed at 25°C, in a thermostat-controlled environmental chamber saturated with water. The samples were dried in a vacuum oven for 1 hour before the contact angles were determined. Eight measurements on each surface were recorded.

Murine Subcutaneous Air Pouch

In each set of experiments, 7 male BALB/c mice (Charles River, Spain) were used at 8 weeks of age. An air-pouch was induced in each mouse and it was generated according to the method of Sedgwick et al. [13], as adapted before by one of us [17]. Ether-anaesthetized mice were injected subcutaneously in the dorsal area with 5 mL of sterile air that caused the formation of an air pouch. A second subcutaneous injection of 3 mL of sterile air into the air pouch was performed 5 days later. This two-injection variant of the air-pouch model favors the formation of lining cells, resembling a sinovial membrane, that increase the reactivity of the air-pouch cavity [18]. Each animal received a single implant. The procedures involved in the murine air-pouch model were submitted and evaluated by the in-house ethics committee and were approved before the experiments were performed.

Implantation of the Biomaterial

One day after the second subcutaneous injection, the mice were anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4,0-8,0 mg/Kg of weight) and xilazine (Rompum, Bayer Co., Portugal; 0,8-1,6 mg/Kg) and the skin covering the air pouch area was shaved and cleaned with betadine. A surgical incision was made, the methyl-coated implant was placed inside the air pouch and the incision was sutured. Control animals received a gold-coated implant or were sham-operated.

Inflammatory Exudates

The exudates were recovered from the mouse air-pouches 24, 48 and 72 hours after the implantation. The mice were ether-anaesthetized and sacrificed. Harvesting of inflammatory exudates was done by washing the air pouch cavities with 2 mL of PBS supplemented with 3% of saccharose (Panreac, Spain), followed by recovery of the lavage fluid. The total number of cells collected from the air pouches was determined with an automatic cell counter (Seac Cell Counter S2013, Metronik). For quantification of inflammatory cells, the exudates were centrifuged onto microscope slides (Cytospin3, Shandon), fixed in formol/ethanol and stained by the Giemsa method. The cells were counted by light microscopy at a magnification of 1000x, and the percentage of the different cell types was determined.

Implants

Explantation occurred immediately after the recovery of the inflammatory exudates. The sutures were cut, and the wound edges separated; the implants were

then carefully removed from the pouches and fixed for scanning electron microscopy, as described below.

Scanning Electron Microscopy

The materials were removed from the pouches and fixed in 1,25% glutaraldehyde, dehydrated in a graded series of ethanol and subjected to critical-point drying from 100% ethanol. The samples were sputter-coated with gold and observed with a JEOL JSM-6301F scanning electron microscope. The acceleration voltage used was 15KeV.

Statistical Analysis

Data from the experiments are expressed as mean values and standard deviation. The significance of differences between group averages was assessed by Mann-Whitney test. Significance was defined at $p<0.05$.

RESULTS

Contact Angle

The CH_3-terminated SAM provided a very hydrophobic substrate, with a contact angle of 108.0º ± 0.7. The gold surface (control) was also hydrophobic, with a contact angle of 75.8º ± 1.9. These data are in accordance with those cited in previously published studies [19-21].

Inflammatory exudates

We have studied the cellular kinetics of the acute inflammatory response caused by CH_3-coated implants in mice. Implants with this chemically defined surface were inserted in an air-pouch cavity of the mouse, and the ensuing inflammatory exudates were evaluated 24, 48 and 72 hours after implantation. Low numbers of inflammatory cells were seen in the air pouches of sham-operated animals; no significant differences in cell numbers were seen in these mice during the 3-day course of the study. For air pouches that had received gold-coated implants, the number of recovered inflammatory cells was higher, in comparison with the sham-operated animals, only at the first day, with no significant differences at 48 and 72 hours. The presence of CH_3 on the implant surface caused a significant increase in

the total number of leukocytes collected from the air pouches all along the 3-day course of the study (Fig.1).

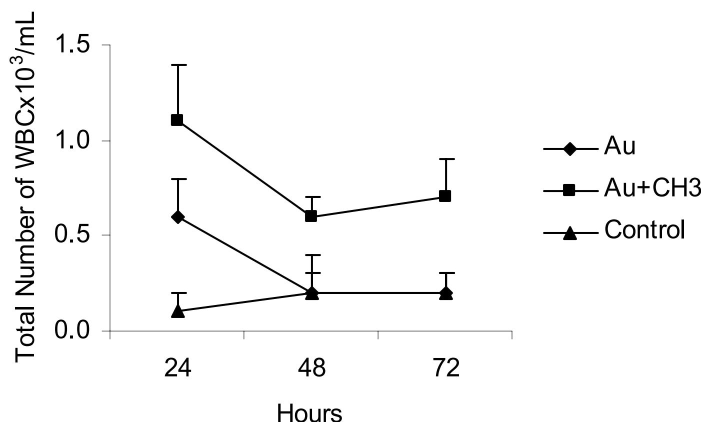


Figure 1
Total number of white blood cells (WBC) present in the inflammatory exudates recovered from the air pouches 24, 48 and 72 hours after the insertion of the implants with CH_3 or pure gold surfaces (and sham-operated mice, as controls of surgery). The differences obtained between the three groups of mice (CH_3-coated SAMs, gold-surfaces and sham-operated) are statistically significant ($p<0.05$), with the exception between animals receiving gold-surfaces and sham-operated mice at 48 and 72 hours.

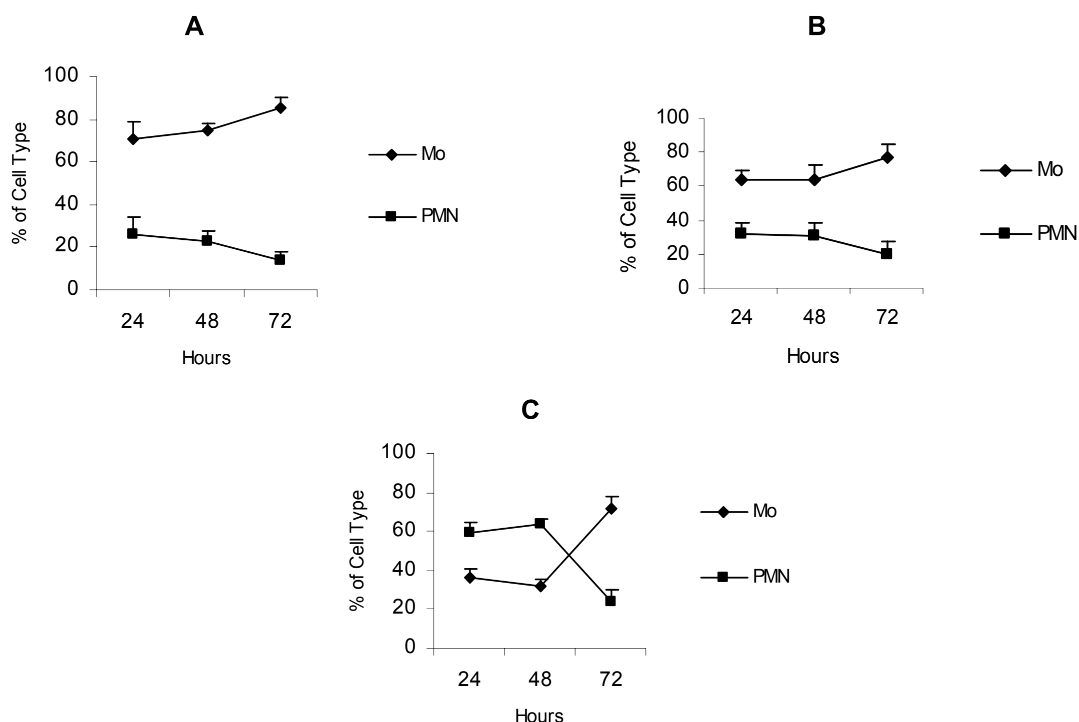
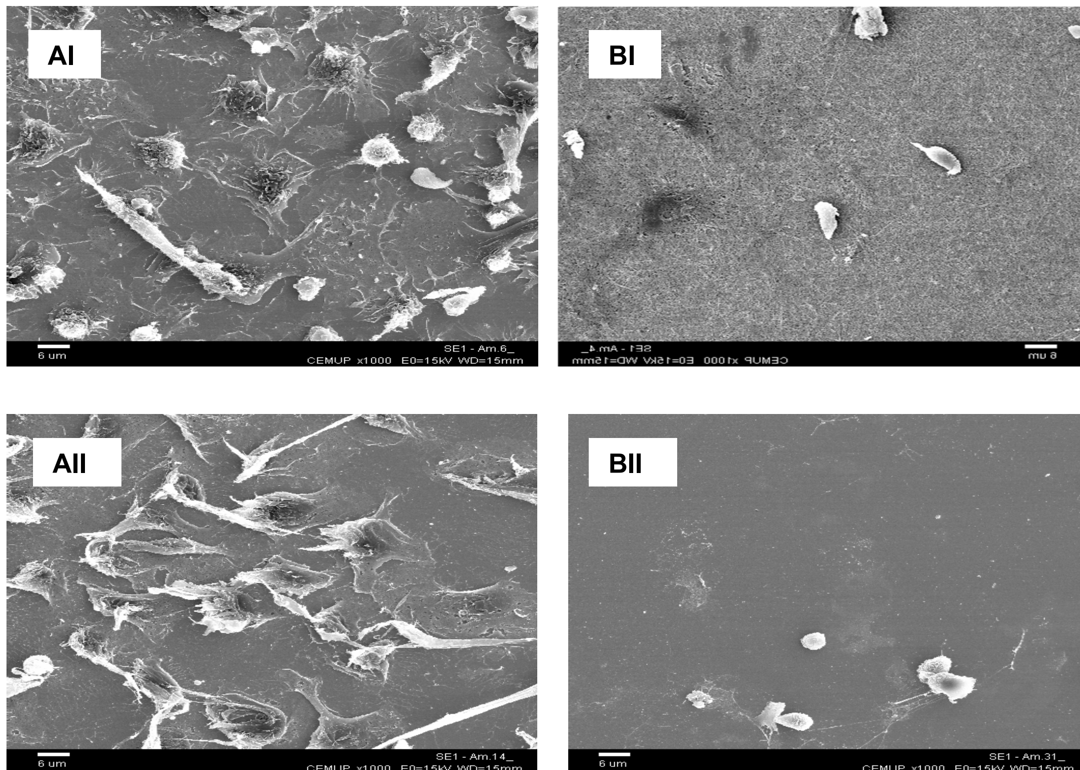


Figure 2
Percentage of mononuclear (Mo) and polymorphonuclear (PMN) cells present in the inflammatory exudates 24, 48 and 72 hours after implantation. (A) Sham-operated animals; (B) Gold-covered surfaces; and (C) CH_3-coated implants.

The different proportion of mononuclear (Mo) and polymorphonuclear (PMN) cells present in the exudates is illustrated in figure 2. Mo was more numerous than PMN in the inflammatory exudates recovered from the air pouches of sham-operated animals or of mice with gold-coated implants (Fig 2A and 2B). For the CH_3-coated implants, PMN were predominant over Mo cells at 24 and 48 hours after implantation; an inversion of this proportion was observed at 72 hours post-implantation (Fig. 2C).

Cell Adhesion

We have quantified the number of adherent inflammatory cells seen on CH_3-coated surfaces and on gold-coated surfaces by the use of SEM observations at a magnification of 1000x (Fig. 3). We found that the number of adherent inflammatory cells was low on CH_3-covered surfaces, during the whole 3-day course of the study. This was in contrast with the higher densities of cells seen on the gold-covered surfaces during the whole course of the investigation (Fig. 4).



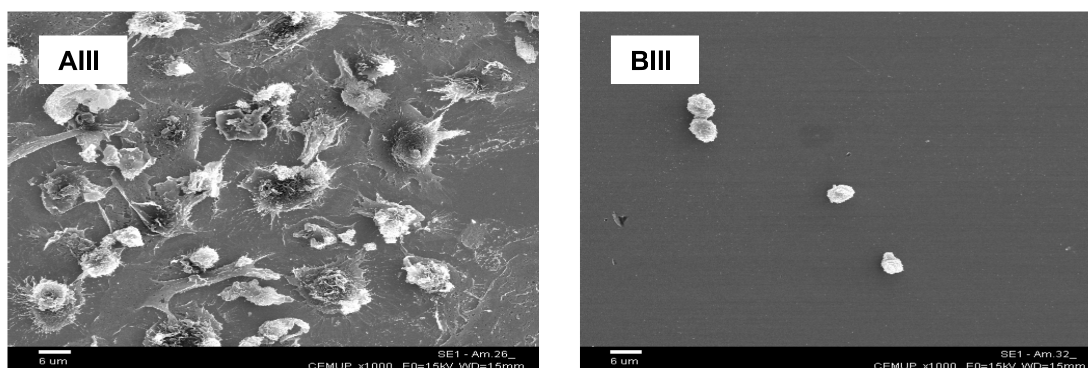


Figure 3
Scanning electron microscopy micrographs of cells adherent to the implanted surfaces, with a magnification of 1000x. (A) Gold and (B) CH_3-coated surfaces (**I**. 24 hours; **II**. 48 hours; **III**. 72 hours).

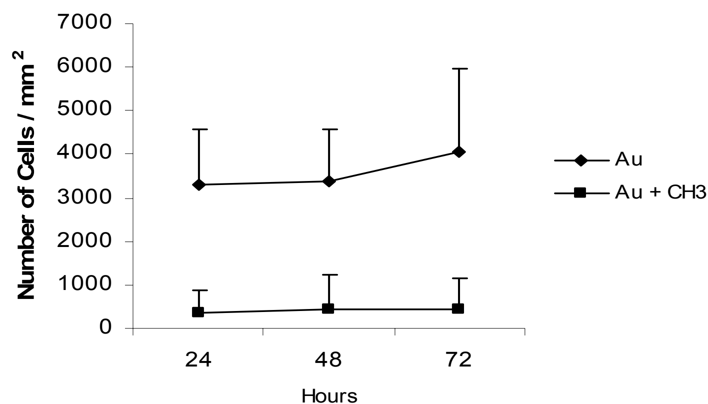


Figure 4
Adhesion of inflammatory cells to the surfaces implanted in the subcutaneous air pouches and removed 24, 48 and 72 hours after implantation. The differences observed between the gold and CH_3-coated surfaces are statistically significant ($p<0.05$) during the 3-day course of the study.

DISCUSSION

Implanted biomaterials are known to trigger acute inflammatory responses of different severity and this difference has been associated namely with the chemical composition of the surface of the implant. The mechanisms involved in such modulation of the acute inflammatory response can be divided into phagocyte transmigration, chemotaxis and adhesion to implant surfaces [22].

In this study, we have used SAMs to study the kinetics of inflammatory cell recruitment and adhesion induced by methyl terminal functional groups during acute

inflammatory reaction; for that, we have used a rodent air-pouch model of inflammation. The air pouch model of inflammation involves the formation of a sterile subcutaneous cavity where the biomaterial is inserted. This model has been employed in the *in vivo* study of the inflammatory response to several biomaterials. It has been shown to be a model that is sensitive to differences in implant composition [23, 24].

We document here that the implantation of a model surface with CH_3 terminal functional groups, in comparison with gold implants, induces a significant increase in the number of leukocytes recruited to the inflammatory cavity. This phenomenon was observed during the whole course of the acute inflammation herein studied (from 1 to 3 days). Also, we have observed that PMN were predominant over Mo cells during the first 2 days after implantation, in contrast with the observations regarding gold-covered surfaces.

In spite of methylated surfaces inducing the migration of larger numbers of leukocytes into the inflammatory cavity, very few of these cells were seen attached to the surface of the implant. This phenomenon was observed during the 3-day course of the study. This finding extends previous observations by us [12] and also by Källtorp et al. [5] and Lindblad et al. [25]. The results described by Tang et al. [26], although revealing low numbers of cells adhering to gold-covered surfaces do not include CH_3-covered surfaces, which renders difficult a comparison with our studies. In addition they have used an explant-associated enzyme activity to estimate the number of adherent cells, instead of direct observation of the surfaces by SEM as we have done.

It is likely that the phlogistic action of the methyl surface is related to its modulation of host proteins that adhere to the surface of the implant. In fact, after implantation, it is known that biomaterials acquire a layer of host proteins well before the arrival of inflammatory cells. Phagocytes will interact with this protein layer rather than with the material itself [27]. This protein layer influences, or even dictates, further responses to implanted materials [28].

Källtorp et al. [5] have investigated the adsorption of some plasma proteins to SAMs. Their results indicate that the surface chemistry influences the protein adsorption pattern. For instances, complement fragment C3 and IgG were detected on gold-coated surfaces but they were adsorbed in very low amounts to CH_3-covered surfaces. The herein described adherence of PMN and Mo to the implant surface is probably related with cell membrane receptors for proteins such as IgG and complement activated fragment C3b present in these cells. The receptor-mediated binding of the inflammatory cells to this adsorbed proteins results in cell attachment

and activation [29, 30]. Fibrinogen is also important in mediating short-term accumulation of inflammatory cells on implanted biomaterials. Fibrinogen adsorbs spontaneously and denaturates on implant surface and becomes proinflammatory, mainly by causing an increased recruitment and adhesion of leukocytes in inflammation and in tissue repair at implant surfaces [31, 32].

Several authors have studied the adsorption of fibrinogen to SAMs. The concentration of the adsorbed protein is higher for gold-coated surfaces than for the other types of SAMs investigated, but it is also relatively high for the CH_3-covered surfaces [5, 19, 25]. These results may seem conflicting if one considers the low number of cells adherent to the methyl-terminated surfaces. Lindblat et al. [25] offered an explanation by postulating that it is possible that fibrinogen binds to the hydrophobic surface and undergoes degradation before the arrival of the recruited cells, thus offering a lower number of accessible cell binding sites.

Taken together these results may explain the herein data on the low number of cells adherent to the methyl-terminated SAMs in comparison with the gold-covered surfaces during the 3 days of this investigation.

The herein-reported use of SAMs offers an elective model for the *in vivo* and *in vitro* study of the attachment of inflammatory cells to surfaces bearing a precise and homogeneous chemical nature. Because the samples can be easily processed for observation by different microscopy methods, we propose that these coated samples surfaces will be useful for the *in situ* definition of subpopulations of attached leukocytes, by the use of immunocytochemistry.

CONCLUSIONS

Implants with methylated surfaces induced an early recruitment of high numbers of inflammatory cells, but this phenomenon was associated with low density of leukocytes seen attached to the implant surface. Thus, our study illustrates that a high phlogistic action of a biomaterial of well-defined chemistry is dissociated from its capacity to induce the attachment of inflammatory cells.

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CHAPTER V

The Attraction of Mac-1$^+$ Phagocytes During Acute Inflammation by Methyl-Coated Self-Assembled Monolayers

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ABSTRACT

We have used self-assembled monolayers (SAMs) of alkanethiolates on gold to study the contribution of methyl terminal functional groups in implant-triggered inflammation. The CH_3-coated biomaterials were inserted in an air-pouch cavity of the BALB/c mouse and the *in situ* inflammatory response was monitored 4, 24, 48 and 72 hours later. Flow cytometry was applied to define surface expression of the adhesion receptor Mac-1 (CD11b/CD18), a marker of activated leukocytes, and also of CD3 and B220 antigens (T and B lymphocytes). The CH_3-coated surfaces caused a significant enhancement in the number of Mac-1$^+$ cells in the implant. The only significant change in T and B lymphocytes was a transient increase in T cells detected 48 hours after the implantation. Peak numbers of Mac-1$^+$ phagocytes were observed 24 hours after implantation. We conclude that if CH_3 is present at the surface of implants, this chemical group will trigger a significant enhancement of

activated phagocytes involved in the inflammatory reaction, and this phenomenon may extend the local phlogistic event.

INTRODUCTION

The increasing usage of implanted devices in medical practice demands detailed knowledge of inflammatory events occurring at the host/implant interface. Biomaterials may trigger an array of iatrogenic effects that usually include inflammation, but may also encompass fibrosis, coagulation, and infection [1]. Implantation of any medical device, whether into the blood stream or into the soft or hard tissue, causes interaction of inflammatory cells with the material surface to differing extents [2]. Within a few hours after implantation, most biomaterials cause acute inflammatory responses, reflected by an accumulation of leukocytes on implant surfaces [3]. Different leukocytes, such as polymorphonuclear leukocytes (PMN), monocytes, macrophages and lymphocytes participate in host inflammatory and in immune processes; therefore, leukocyte response to biomaterials is important to understand material-mediated host reaction [4, 5].

Integrins participate in many aspects of inflammation, namely those involving cell migration, adherence and activation [6]. Circulating leukocytes express adhesion molecule receptors, which are up-regulated in many inflammatory states, thus allowing leukocyte binding to endothelial adhesion molecules. Ligands such as the integrin Mac-1 (CD11b/CD18) that binds to intercellular adhesion molecule-1 (ICAM-1) on the endothelial surface are exposed at the surface of phagocytes. Expression of these ligands may be taken as a measure of phagocyte up-regulation in inflammatory reactions [7]. Up-regulation of Mac-1 expression by phagocytes has been described after contact with surfaces of biopolymeric materials, which leads to increased adhesiveness to the polymer surface; this activation occurs at different extents, after implantation of any medical device and this is due to an interaction between inflammatory cells and the surface of the biomaterial [8, 9].

It is thus important to evaluate the potential inflammatory response that is produced by an implant, namely with regards to the specific chemical groups expressed on the surface of the biomaterial. For that, we have used here self-assembled monolayers (SAMs) that are a class of organic surfaces that are well suited to study interactions of chemically defined surfaces with cells and tissues. SAMs of alkanethiolates on gold can be prepared by immersing a clean film of gold into a solution of terminally substituted alkanethiols [10-12]. SAMs are structurally the

best-ordered interfaces currently available for studying the interactions of cells and proteins with substrates of different surface chemistries [13-15]. We have adopted this model to investigate by flow cytometry the changes in inflammatory cells (activated phagocytes, T and B lymphocytes) that can be ascribed to the methyl terminal functional group when it is present at the surface of a biomaterial.

MATERIALS AND METHODS

Preparation of the Implants

Gold substrates were prepared with an automated load locked ion beam deposition system (Nordiko N3000). A 5 nm chromium adhesion layer and a 25 nm gold layer were deposited by ion beam sputtering from chromium and gold targets (99,9% purity) on silicon wafers (AUREL, Gmbh). Deposition rates used were 0,050 nm/s for chromium and 0,033 nm/s for gold. Deposition pressure was $3,5x10^{-5}$ Torr. The silicon substrates are coated with gold on one side. The gold -coated surfaces act as a gold plus silicon control. The wafers were coated with 1,5 µm of photoresist (PFR7790EG, JSR Electronics), soluble in acetone, to protect the film surface. The wafers were then cut into 0,5 x 0,5 cm squares using a DISCO DAD 321 automated saw.

The gold substrates were pre-cleaned with acetone and immersed in a solution of 7:3 volume ratio of concentrated H_2SO_4 and 30% H_2O_2 for 10 minutes. The substrates were then washed with absolute ethanol, water and absolute ethanol again. The gold-coated substrates were immersed for 24 hours in a 1 mM ethanolic solution [16] of 1-hexadecanethiol ($HS(CH_2)_{15}CH_3$) (92% purity) from Aldrich. Upon the removal from the thiol solutions, the monolayers were rinsed in absolute ethanol, ultrasonically cleaned in absolute ethanol for 2 minutes and rinsed again in absolute ethanol. The surfaces were then rinsed with sterile phosphate buffered saline (PBS), and put in sterile PBS until use. The subcutaneous insertions of the implants in the mice were performed within 2 hours (see technique below).

Characterization of the Implants

The implants were characterized by contact angle measurements, after washing and sterilization with absolute ethanol, by the sessile drop method using deionized water with conductivity not greater than 1μS/cm. The measuring system was an Optical Contact Angle Device OCA 15, with an electronic syringe (Dataphysics), and was connected to a video camera. The software SCA20 was

used for image analysis and for the calculation of the contact angles. The measurements were performed at 25°C, in a thermostat-controlled environmental chamber saturated with water. The samples were dried in a vacuum oven for 1 hour before the contact angles were determined. Eight measurements on each surface were recorded.

Murine Subcutaneous Air Pouch

In each set of experiments, 5 male BALB/c mice (Charles River, Spain) were used at 8 weeks of age. An air-pouch was induced in each mouse and it was generated according to the method of Sedgwick et al. [17], as adapted before by one of us [18]. Ether-anaesthetized mice were injected subcutaneously in the dorsal area with 5 mL of sterile air that caused the formation of an air pouch. A second subcutaneous injection of 3 mL of sterile air into the air pouch was performed 5 days later. This two-injection variant of the air-pouch model favors the formation of lining cells, resembling a synovial membrane, that increase the reactivity of the air-pouch cavity [19]. Each animal received a single implant.

Implantation of the Biomaterial

One day after the second subcutaneous injection, the mice were anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4,0-8,0 mg/Kg of weight) and xilazine (Rompum, Bayer Co., Portugal; 0,8-1,6 mg/Kg) and the skin covering the air pouch area was shaved and cleaned with betadine. A surgical incision was made, the methyl-coated implant was placed inside the air pouch and the incision was sutured. Control animals received a gold-coated implant or were sham-operated.

Inflammatory Exudates

The exudates were recovered from the mouse air-pouches 4, 24, 48 and 72 hours after implantation. The mice were ether-anaesthetized and sacrificed. Harvesting of inflammatory exudates was done by washing the air pouch cavities with 3 mL of BSS (balanced salt solution) supplemented with 10% of fetal calf serum (Gibco Biocult, UK), followed by the recovery of the lavage fluid.

Flow Cytometry Analysis

Flow cytometric analysis was carried out on the inflammatory exudates recovered from the air pouches. Before the flow analysis, 10 μL of the exudate was

withdrawn and the number of cells per ml was counted by light microscopy (after Tück staining).

Cells were single and double stained with anti-mouse monoclonal antibodies (MoAb) conjugated with flourescein isothiocianate (FITC) or phycoerythrin (PE). Briefly, cell concentration was adjusted to $2x10^7$ cells/ml and $0.5\text{-}1x10^6$ cells were incubated with optimal concentrations of the appropriate antibodies for 20 minutes on ice. After staining the cells were washed twice and suspended in FACS (flow analysis by cell sorting) medium containing propidium iodide (PI, Sigma). Data were collected on cells selected by forward ligh scattered (FSC) and PI gating in a FacScan analyser (Becton Dickinson) with the CellQuest software. Cell size was defined by FSC analysis. Monoclonal antibodies with the following specificities were used: anti-CD3 (for T cells; BD, Lexington, USA), anti-B220 (for B cells; Pharmingen, San Diego, USA) and anti-Mac-1 (CD11b/CD18, for activated phagocytes; Pharmingen, San Diego, USA).

Statistical Analysis

Data from the experiments are expressed as mean values and standard deviation. The significance of differences between group averages was assessed by Mann-Whitney test. Significance was defined at $p<0.05$.

RESULTS

Contact Angle

The methyl-terminated surface (CH_3) provided a very hydrophobic substrate, with a contact angle of 108.0º ± 0.7. The gold surface (control) was also hydrophobic, presenting a contact angle of 75.8º ± 1.9. These data are in accordance with those cited in previously published studies [20-22].

Flow Cytometry

We have studied the cellular kinetics of the acute inflammatory response caused by the implantation of CH_3-coated SAMs. These implants were inserted in an air pouch cavity of the mouse and the inflammatory exudates were collected and evaluated by light microscopy and flow cytometry at 4, 24, 48 and 72 hours after the implants were inserted. A low number of inflammatory cells were seen in air pouches of animals with no implant material and, also there were no significant variations

during the 3-day course of the study. The CH_3-coated implant caused a significant increase in the total number of cells collected from the inflammatory cavity. The maximum number of inflammatory cells was observed 24 hours after the introduction of the material (both for CH_3-coated SAMs and gold surfaces) (Fig. 1). This enhancement was derived mostly from phagocytes, namely from macrophages and granulocytes.

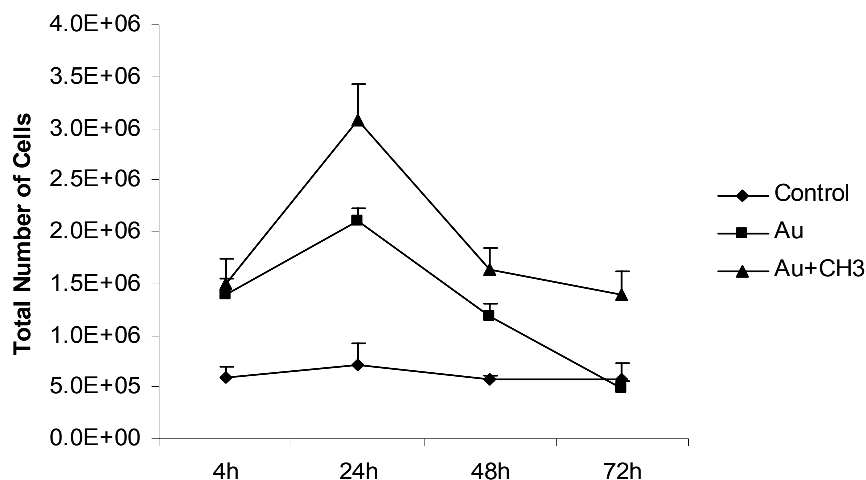

Figure 1
Total number of white blood cells (WBC) present in the inflammatory exudates collected from the air pouches 4, 24, 48 and 72 hours after the insertion of the methyl-coated implant (n=5). The differences observed between mice with methyl-coated implant and controls (gold-coated implant or sham-operated animals) are statistically significant ($p<0.05$), except between gold and CH_3-coated surfaces at 4h and between the two control groups at 72h.

Detection of the Mac-1 antigen was used to evaluate the number of activated phagocytes by flow cytometry (Fig. 2). The analytical gate shown in figure 2 was selected in order to ensure that all of the leukocytes were considered for the analysis. All of the cells marked with antibodies for Mac-1, CD3 and B220 were present in this gate. The gate was also designed to include only viable cells by the use of propidium iodide staining (data not shown). We show here that the number of cells expressing Mac-1 was increased by the presence of the CH_3 chemical group on the implant surface. In fact, this marker showed that activated phagocytes were at least half of the cells present in the air pouches containing CH_3-coated implants. Values for Mac-Values for Mac-1^+ cells were almost always significantly higher in mice with CH_3-coated implants than in mice with control (gold) implants or with no implants at all (Fig. 3). After 48 hours of inflammation no differences were seen between mice with gold implants and animals without implants in the air pouches (Fig. 3).

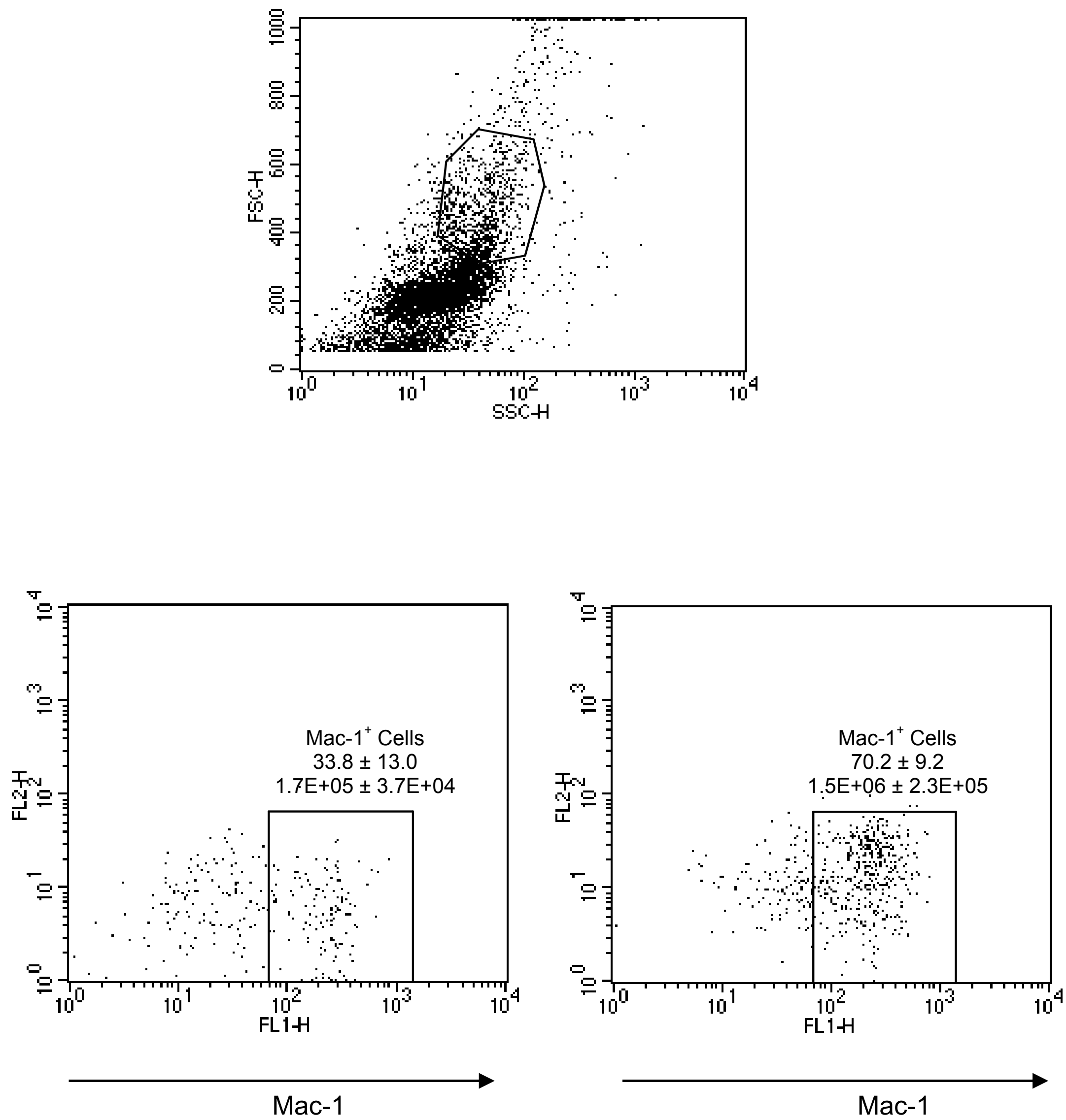

Figure 2
Cytofluorimetric analysis of the inflammatory exudates recovered from the air pouches 24 hours after implantation of the materials (n=5). (A) Dot plot of one representative experiment correlating the FSC-H (size) / SSC-H (granulation) of total cells recovered from the air pouches. (B) Dot plot of Mac-1+ cells of a sample from control animals. (C) Dot plot of Mac-1+ cells of a sample recovered from an air pouch with CH_3-coated implant. The numbers inside the dot plots are mean values of percentage and total number of cells with the standard deviation.

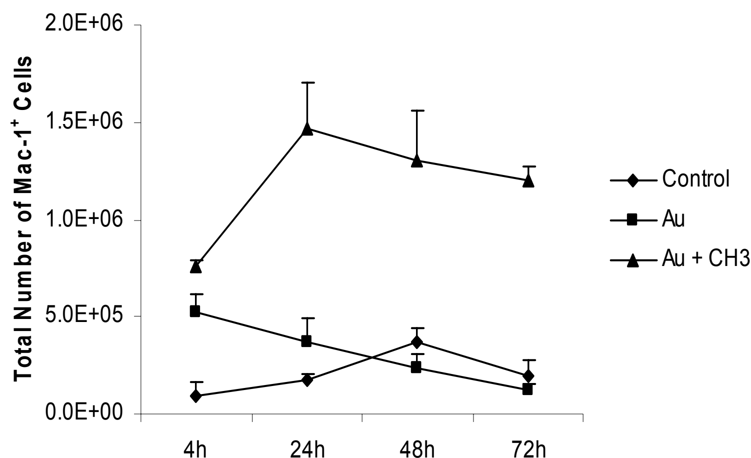

Figure 3
Quantitative kinetics of Mac-1+ phagocytes present in the inflammatory exudates recovered from the air pouches for 3 days after the implantation of materials (n=5). The differences obtained between the three groups of mice (CH_3-coated implant, gold implant and sham-operated) are statistically significant ($p<0.05$), with the exception between animals receiving gold and CH_3-coated implants at 4h, and between sham-operated mice and animals receiving gold-coated implants at 48 and 72h.

In contrast to phagocytes, both T and B lymphocytes were present in low number. B cell numbers were not altered during the acute inflammatory response to CH_3-coated surfaces; an elevation in T cells was observed 48 and 72 hours after implantation of the CH_3-coated surfaces (Figs. 4 and 5).

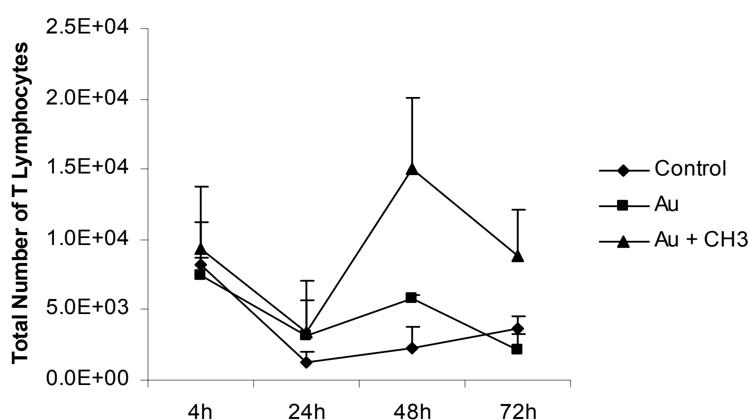

Figure 4
Number of T lymphocytes present in the exudates recovered from the inflammatory air pouches 4, 24, 48 and 72 hours after the implantation of the different materials (n=5). The differences obtained are not statistically significant, with the exception between control and CH_3-coated implants at 48h and between gold-coated and CH_3-coated implants at 48 and 72h.

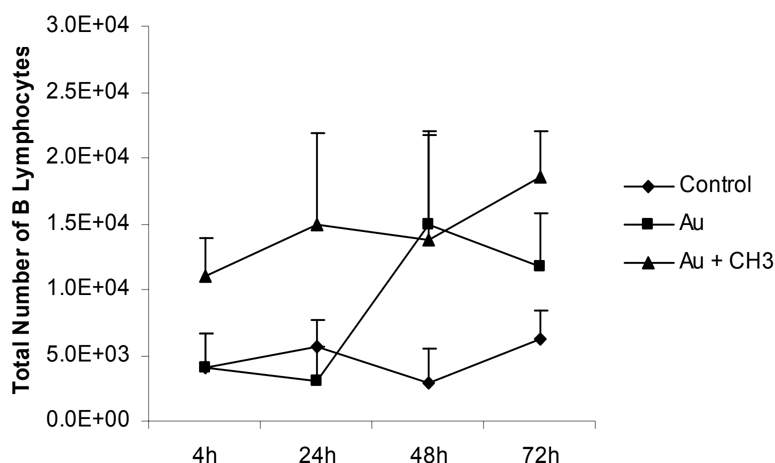

Figure 5
Number of B lymphocytes present in the inflammatory exudates recovered from the air pouches 4, 24, 48 and 72 hours after the implantation of the self-assembled monolayers (n=5). The differences observed are not statistically significant, except between control and CH_3-coated implants at 4 and 48h and between gold and CH_3-coated implants at 4h.

DISCUSSION

On implantation, biomaterials, like any foreign body, trigger acute inflammatory response that is reflected by an *in situ* accumulation of leukocytes [23]. This accumulation is often considered to be the most important feature of the acute inflammatory reaction, namely with regards to inflammatory lesions mediated by adhesion of leukocytes. Increased leukocyte adhesion in inflammation involves cell activation and specific interactions between complementary adhesion molecules present on the leukocyte and endothelial surfaces [24].

We show here that CH_3 terminal functional groups trigger an acute phlogistic action from the host when this chemical group is exposed at the surface of an implanted biomaterial. In fact, we observed that the implantation of CH_3-coated SAMs in a subcutaneous air-pouch of the mouse was capable of increasing the number of leukocytes recruited to this inflammatory cavity. The total number of cells harvested from the air pouch was higher when CH_3-coated SAMs were implanted. This phenomenon was detected during the whole course of acute inflammation herein studied (from 4 to 72h).

Flow cytometry revealed that CH_3-coated SAMs induced a significant recruitment of phagocytes expressing the integrin Mac-1 (also referred as CD11b/CD18). Because the surface expression of Mac-1 is known to reveal

activation of phagocytes, our finding suggests that the CH_3 terminal functional group, by itself, will foster an ongoing inflammatory reaction.

The predominant cell type present in the inflammatory response varies with the age of injury, and lymphocytes are in general present in chronic inflammation whereas phagocytes are predominant during the first days following injury, the acute inflammation [25]. As expected, at the early phase of acute inflammation neither T nor B lymphocytes were significantly attracted to the cavity where the implants were located. Almost no significant differences in the number of lymphocytes attracted to the air pouches were found between the different materials and the control group of this study. The only significant change was an increase in the number of T cells that occurred 48 hours after the implantation of either of the two types of surfaces, and persisted at 72 hours in mice with CH_3-coated implants. The kinetics of these T cell values suggests that this is a transient phenomenon.

The herein reported expression of Mac-1 on the surface of leukocytes is known to be associated with increased adherence of inflammatory cells to the endothelium. This is an important parameter to determine the degree of the inflammatory response. In fact, Mac-1 is an integrin molecule that has a key role in leukocyte adhesion which has been demonstrated in an autosomal recessive disorder called leukocyte-adhesion deficiency [26]. Mac-1 is expressed at the surface of activated monocytes, macrophages, granulocytes and natural killer cells. This receptor can bind to the intercellular adhesion molecule ICAM-1, the complement factor iC3b, fibrinogen and coagulation factor X [26]. Increased surface expression of Mac-1 has been associated not only with enhanced adherence but also with chemotaxis, opsonization and aggregation of inflammatory cells [27]. Leukocytes from sites of active inflammation have been shown to increase surface density of Mac-1 compared with leukocytes from non-inflammed tissue [7]. Therefore, the comparative expression of Mac-1 on the surface of leukocytes has been proposed as a reliable parameter for estimating the inflammatory risk of different implanted biomaterials [28].

The expression of the integrin Mac-1 at the surface of leukocytes after contact with the implants has been monitored before in several studies assessing inflammatory responses to biomaterials. Schmidt et al. [2] have shown that polyurethane, polymethylmetacrylate and poly-D,L-lactide strongly induced Mac-1 up-regulation of human neutrophils, whereas polyethylene, polyisoprene and silicone only induced a weak response. Sundaram et al. [29] studied polyurethanes with different ionic groups and reported that the expression of Mac-1 was higher in adherent cells recovered from the surface of negatively charged polyurethane.

Gourlay et al. [30] have investigated the expression of Mac-1 on neutrophils that have been in contact with PVC with different concentrations of plasticizer, and reported that the up-regulation of this integrin may be significantly reduced by employing a methanol-washing technique to the plasticized PVC to decrease the amount of plasticizer, and also by coating the surface with heparin [31]. Risbud et al. [32] have studied the biocompatibility of a polytetrafluoroethylene/wollastonite composite and used the expression of Mac-1 as an activation marker of the immunological response.

The herein investigation addresses the acute inflammatory reaction caused by CH_3 chemical group. This endeavor was made possible by the use of the SAMs. In fact, the development of SAMs has offered the possibility of testing the biological activity of implant surfaces covered with coatings of well-defined surface chemistry. However, only some reports about *in vivo* biological reactions to SAMs implants can be found. Källtorp et al. [34] and Lindblad et al. [35] reported that the *in vivo* cell recruitment and distribution was greatly influenced by the different surfaces functionalities. The methylated surfaces had the lowest amount of cells associated with the surface. However, in the fluid space around the methylated implant, a higher number of inflammatory cells were found than around the hydroxylated or gold implants. Källtorp et al. [33] and Tang et al. [36] evaluated the influence of implant surface chemistry on *in vitro* complement activation and on *in vivo* inflammatory cell recruitment, and they report that the tendency of some surfaces to activate the complement *in vitro* predict the *in vivo* inflammatory responses to implants of the same material, although other factors than complement activation may stimulate cell recruitment to implant surfaces.

The rapidly growing area of material surface chemical functionalization is likely to result in future implants engineered to fulfill specific demands. An understanding of the biological response to materials surface is a key biomaterial research area [37]. Ideally, implanted devices would forego the foreign body reaction and lead into “normal” wound healing and to provide for integration of the implant with the body [38]. Hydrophylic surfaces cause a decrease in the rates of leukocyte adhesion and macrophage fusion; also, cytokine production is significantly decreased by cells adherent to hydrophilic substrates [38, 39]. In contrast to these studies, there is solid clinical evidence that hydrophobic biomaterials (such as PTFE) are suitable to be used as implants in human medicine [40, 41]. Until now, the majority of the results have been obtained *in vitro* and important information is still missing on the role of material surface properties for inflammation and tissue repair *in vivo*.

The surface of CH_3-coated gold disks work as an electric insulator whereas the surface of pure Au is an electrical conducting material. This difference in the electrochemical behavior deserves to be addressed. Our previous investigation using both SAM-coated implants (CH_3, COOH and OH-coated disks) and Au implants indicated that the chemistry of the implant surface was a greater modulator of the attraction of inflammatory cells than differences in the electrochemical behavior [42]. It is therefore plausible to interpret the herein reported differences in phlogistic activity between CH_3-coated SAMs and Au surfaces as deriving mostly from the different chemistry of the two surfaces, rather than from distinct electrochemical behavior.

We have shown before that SAMs coated with CH_3 terminal functional groups attract a large numbers of cells to the cavities where the implants are introduced, and we also found that adherence of inflammatory cells to these implants was low [42]. The present data offer further information on this inflammatory response by documenting that attracted phagocytes contain a large population of activated cells revealed by the Mac-1 labelling.

CONCLUSIONS

The implantation of CH_3-coated SAMs in a subcutaneous air-pouch of the mouse increased the number of leukocytes recruited to this inflammatory cavity. This phenomenon was observed during the whole course of the study. These CH_3-covered surfaces also induced a significant recruitment of phagocytes expressing the integrin Mac-1. Because the surface expression of Mac-1 is known to correspond to activation of phagocytes, our finding suggests that the CH_3 terminal functional group will extend the phlogistic reaction.

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CHAPTER VI

The Influence of Functional Groups of Self-Assembled Monolayers on Fibrous Capsule Formation and Cell Recruitment

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ABSTRACT

The contribution of the surface chemistry of an implant to the thickness of the fibrous capsule formed after implantation was herein investigated. For that, self-assembled monolayers (SAMs) of alkanethiols on gold with different terminal functional groups (COOH, OH and CH_3) were produced. These surfaces were implanted in subcutaneous air pouches of BALB/c mice and the ensuing fibrous capsules were evaluated and compared with the initial inflammatory response caused by the implant. The thickness of the fibrous capsules that are under organization around the implant was measured 1 week after implantation by histology. Inflammatory exudates were collected from the air pouches 24 hours after the implantation of SAMs and were analysed by flow cytometry. A significant increase in the thickness of fibrous capsules was seen around implants coated with CH_3 groups, and also in gold surfaces, in comparison with the air pouch wall of sham-operated mice and of COOH- and OH-covered SAMs. The CH_3-coated implants also recruited higher numbers of inflammatory cells; this enhancement

involved a significant number of Mac-1^{+} cells. Our data indicate that implant surfaces coated with CH_3 induce thick fibrous capsules and this may be the result of the stronger phlogistic effect of CH_3 in comparison with COOH or OH chemical groups.

INTRODUCTION

The implantation of a biomaterial leads to a response to injury that activates mechanisms of healing of the damaged tissues. A sequence of events is initiated starting with an acute inflammatory response and involving granulation tissue development, a foreign body reaction and fibrous capsule development [1]. The size, shape, and chemical and physical properties of the biomaterial may be responsible for variations in the intensity and time duration of the inflammatory and wound healing processes [2].

The first phase of wound healing - acute inflammation - follows as neutrophils and monocytes migrate to the locus of the inflammatory stimulus. Persistent inflammatory stimuli lead to chronic inflammation which is characterized by the presence of macrophages, monocytes and particularly lymphocytes, with the proliferation of blood vessels and connective tissue. The healing response of inflammation, granulation tissue, is initiated by the action of macrophages and is characterized by the proliferation of fibroblasts, synthesis of collagen and proteoglycans and angiogenesis. The foreign body reaction is comprised of foreign body giant cells apposed to the biomaterial surface, surrounded by granulation tissue and fibrous encapsulation of the implant [3, 4].

We have investigated here the size of the fibrous capsule that is under organization around implants with different surface chemistry and compared it with the early inflammatory response to the same implant. We have used self-assembled monolayers (SAMs) with the terminal functionalities of COOH, OH and CH_3. SAMs are a class of organic surfaces well suited to study interactions of chemically defined surfaces with cells and tissues. SAMs of alkanethiolates on gold can be prepared by immersing a clean film of gold into a solution of terminally substituted alkanethiols [5-7].

MATERIALS AND METHODS

Substrate Preparation

The gold substrates were prepared with an automated load locked ion beam deposition system (Nordiko N3000). The 5 nm chromium adhesion layer and the 25 nm gold layer were deposited by ion beam sputtering from gold and chromium targets (99,9% purity) on silicon wafers (AUREL, Gmbh). Deposition rates used were 0,050 nm/s for chromium and 0,033 nm/s for gold. Deposition pressure was $3{,}5 \times 10^{-5}$ Torr. The silicon substrates are coated with gold on one side. The gold -coated surfaces act as a gold plus silicon control. The wafers were coated with 1,5 μm of photoresist (PFR7790EG, JSR Electronics), which is soluble in acetone, to protect the film surface. The wafers were then cut into 0.5 x 0.5 cm squares using a DISCO DAD 321 automated saw.

SAM Preparation

Before being used the gold substrates were cleaned with acetone and immersed in “piranha solution” (7:3 volume ratio of concentrated H_2SO_4 and 30% H_2O_2) for 10 minutes. The substrates were then washed with absolute ethanol, water and absolute ethanol again. The gold-coated substrates were immersed for 24 hours in a 1 mM ethanolic solution [8] of 11-mercapto-1-undecanol ($HS(CH_2)_{11}OH$) (97% purity), 1-hexadecanethiol ($HS(CH_2)_{15}CH_3$) (92%) and 16-mercaptohexadecanoic acid ($HS(CH_2)_{15}COOH$) (90%) all from Aldrich. Upon the removal from the different thiol solutions, the monolayers were rinsed in absolute ethanol, ultrasonically cleaned in absolute ethanol for 2 minutes, rinsed again in absolute ethanol. The surfaces were then rinsed in three baths of sterile phosphate buffered saline (PBS), and put in sterile PBS until use. The subcutaneous insertions of the implants in the mice were performed within 2 hours.

Characterization of the Implants

The implants were characterized by contact angle measurements, after washing and sterilization with absolute ethanol, by the sessile drop method using deionized water with conductivity not greater than 1μS/cm. The measuring system was an Optical Contact Angle Device OCA 15, with an electronic syringe (Dataphysics), and was connected to a video camera. The software SCA20 was used for image analysis and for the calculation of the contact angles. The measurements were performed at 25°C, in a thermostat-controlled environmental chamber saturated with water. The samples were dried in a vacuum oven for 1 hour

before the contact angles were determined. Eight measurements on each surface were recorded.

Mouse Animal Model

In each set of experiments, 5 male BALB/c mice (Charles River, Spain) were used at 8 weeks of age. Air-pouches were generated according to the method of Sedgwick et al. [9], as adapted before by one of us [10]. Ether-anaesthetized mice were injected subcutaneously in the dorsal area with 5 mL of sterile air that caused the formation of an air pouch. A second subcutaneous injection of 3 mL of sterile air into the air pouch was performed 5 days later. This two-injection variant of the air-pouch model favors the formation of lining cells, resembling a sinovial membrane, that increase the reactivity of the air-pouch cavity [11]. Each animal received a single implant.

Insertion of the Implant Materials

One day after the second subcutaneous injection, the mice were anaesthetized by intramuscular injection of ketamine (Ketalar, Parke-Davis Co., Spain; 4,0-8,0 mg/Kg of weight) and xilazine (Rompum, Bayer Co., Portugal; 0,8-1,6 mg/Kg) and the skin covering the air pouch area was shaved and cleaned with betadine. A surgical incision was made, the materials were placed inside the air pouch, and the incision was sutured. The same technique was performed for control animals, except that no material was implanted inside the air pouches (sham-operated mice).

Histology of the Air Pouch

Mice were sacrificed one week after the implantation of the materials in the air pouches. Due to the nature of the implant material, it had to be removed prior to sectioning. After extraction of the implant the air pouches were injected with black drawing ink (made up of charcoal microparticles) in order to visualize the inner surface of the subcutaneous cavity. The samples were surgically removed from the skin deep into the muscle layer, fixed in formol, processed and embedded in paraffin wax and cut in thin sections. The sections were stained using standard hematoxylin and eosin. Color images of the histology slides were taken on a light microscope. The magnification used for all images was 100x. Pouch membrane thickness was determined at several points on each section, and the mean values were calculated.

Inflammatory Exudates

The exudates were recovered from the mouse air-pouches 24 hours after the implantation. The mice were ether-anaesthetized and sacrificed. Harvesting of inflammatory exudates was done by washing the air pouch cavities with 3 mL of BSS (balanced salt solution) supplemented with 10% of foetal calf serum (Gibco Biocult, UK), followed by the recovery of the lavage fluid.

Flow Cytometry Analysis

Flow cytometric analysis was carried out on the inflammatory exudates recovered from the air pouches. Before the flow analysis, 10 µL of the exudate was withdrawn and the number of cells per ml was counted by light microscopy (after Tück staining).

Cells were single and double stained with anti-mouse monoclonal antibodies (MoAb) conjugated with flourescein isothiocyanate (FITC) or phycoerythrin (PE). Briefly, cell concentration was adjusted to $2x10^7$ cells/ml and $0.5-1x10^6$ cells were incubated with optimal concentrations of the appropriate antibodies for 20 minutes on ice. After staining the cells were washed twice and suspended in FACS (fluorescence activated cell sorter) medium containing propidium iodide (PI, Sigma). Data were collected on cells selected by forward light scattered (FSC) and PI gating in a FacScan analyser (Becton Dickinson) with the CellQuest software. Cell size was defined by FSC analysis. Monoclonal antibodies with the following specificities were used: anti-CD3 (for T cells; BD, Lexington, USA), anti-B220 (for B cells; Pharmingen, San Diego, USA) and anti-Mac-1 (CD11b/CD18, for activated phagocytes; Pharmingen, San Diego, USA).

Statistical Analysis

Data from the experiments are expressed as mean values and standard deviation. The significance of differences between group averages was assessed by Mann-Whitney test. Significance was defined at $p<0.05$.

RESULTS

Contact Angle

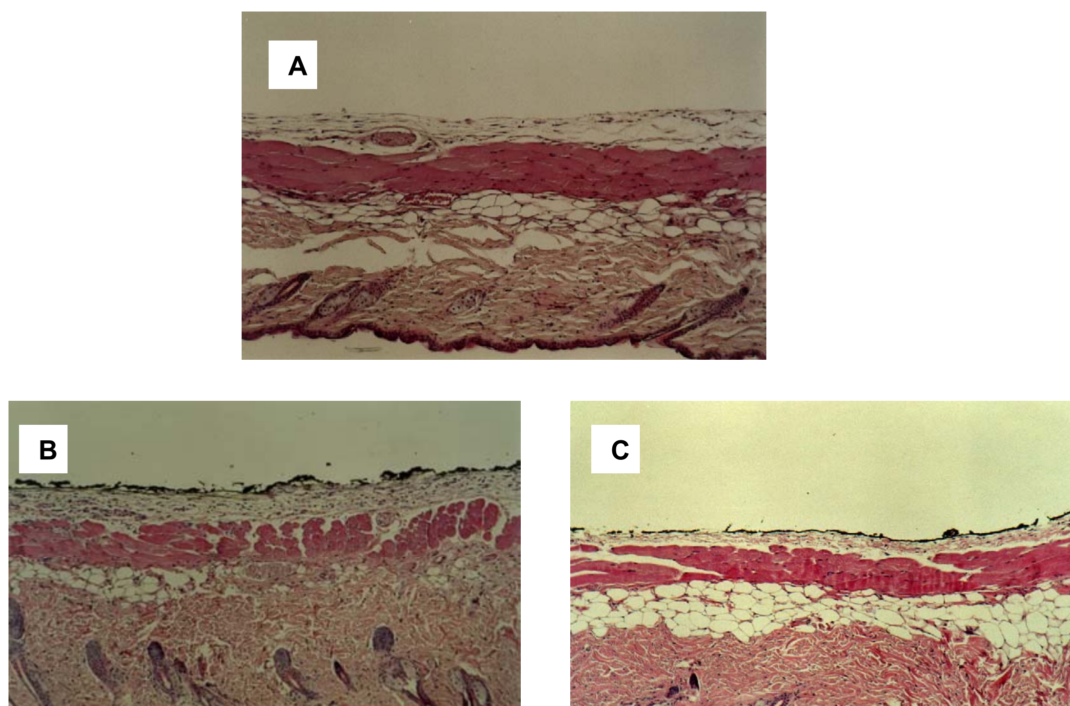
The methyl-terminated surface (CH_3) provided an extremely hydrophobic substrate, with a contact angle of 108.0º ± 0.7. The hydroxyl (OH) and the carboxyl

(COOH) terminated surfaces provided hydrophilic substrates (contact angles of 31.9º ± 3.6 and 47.7º ± 2.4, respectively). The gold surface (control) is hydrophobic, presenting a contact angle of 75.8º ± 1.9. These data are in accordance with those cited in previously published studies [12, 13].

Histology of the Air Pouch

The subcutaneous cavity (air pouch) induced by the repeated injection of sterile air resembled a synovial cavity. In fact, the gap induced by the air between the subcutaneous tissues was filled up with edema and the wall of this liquid space was lined by flat cells. This is illustrated in figure 1 where the air pouch was injected with a particle marker (black drawing ink) in order to offer a precise identification of the luminal surface of the air pouch.

In order to compare the changes in the thickness of the wall of the air pouches after implants were inserted, we have measured the width of organized layers of the air-pouch lining observed in sections of the samples. This quantitative evaluation revealed that the CH_3-coated surfaces induced a significantly enhancement in thickness of the fibrous capsule of the implants, in comparison with surfaces coated with COOH or OH (Fig. 2). In comparison with gold surfaces, the CH_3-coated SAMs were not significantly different in the thickness of fibrous capsule.



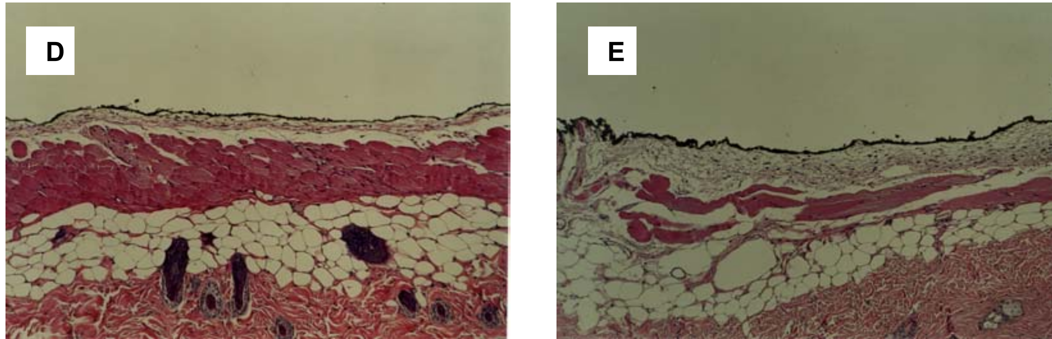


Figure 1
Light micrographs of hematoxylin and eosin stained sections of the wall of the subcutaneous air pouch of BALB/c mice 1 week after the implantation of the different self-assembled monolayers. (A) Sham operated animals; (B) Gold-covered surfaces; (C) COOH-covered SAMs; (D) OH-covered SAMs; (E) CH_3-terminated SAMs.

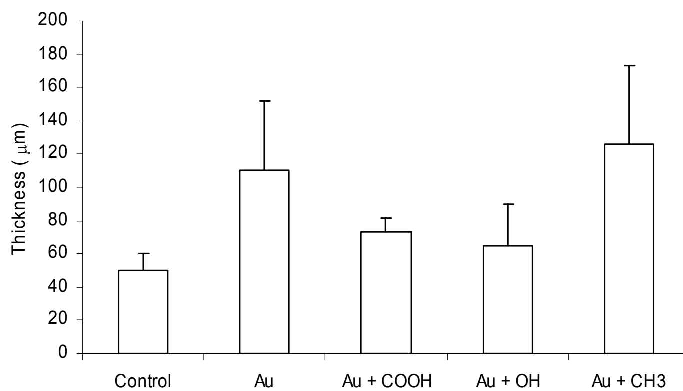


Figure 2
Thickness of the fibrous capsule formed around implants 1 week after their insertion in the mouse air pouch. The differences between gold and CH_3-covered surfaces, between COOH- and OH-covered surfaces and between sham-operated animals and OH-covered surfaces are not statistically significant; all other values are significantly different ($p<0.05$) from each other.

Flow Cytometry

We have studied the cellular recruitment to the subcutaneous air pouches 24 hours after the implantation of the different SAMs. The inflammatory exudates were collected and evaluated both by light microscopy and flow cytometry. A low number of inflammatory cells were collected from the air pouches of animals with no implant. The OH- and CH_3-covered surfaces caused the highest recruitment of inflammatory cells to the air pouches (Figure 3).

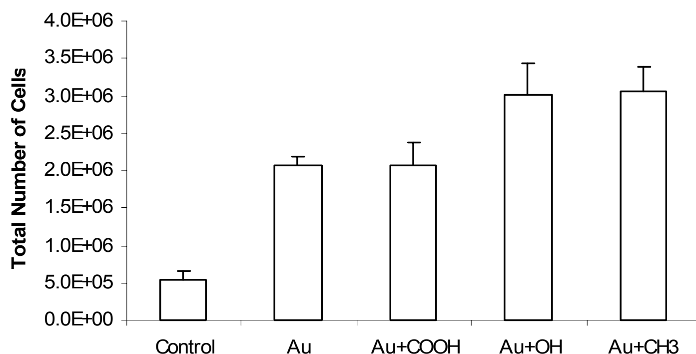


Figure 3
Total number of white blood cells (WBC) present in the inflammatory exudates recovered from the mouse air pouches 24 hours after the implantation of the different self-assembled monolayers. The differences between gold and COOH-covered surfaces and between OH- and CH_3-covered surfaces are not statistically significant; all other values are significantly different ($p<0.05$) from each other.

The evaluation of the Mac-1 adhesion receptor was used to assess the number of activated phagocytes by flow cytometry (Figure 4). In control animals (with no implanted material), the number of Mac-1$^+$ cells was low, and after the insertion of the different SAMs there was a significant increase in the number of these cells. The number of Mac-1$^+$ cells was significantly higher for implants coated with OH and CH_3. The methyl-coated SAMs induced the recruitment of the highest number of activated phagocytes. Both T and B lymphocytes were present in low numbers, with no statistically significant differences between the materials studied (Figures 5 and 6).

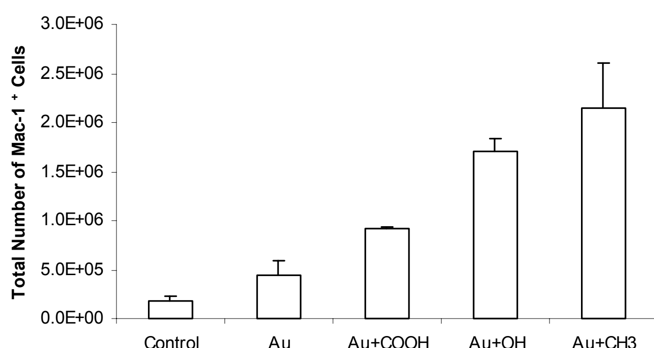


Figure 4
Number of Mac-1+ phagocytes recovered from the inflammatory exudates 24 hours after implantation of the materials. All the differences between the materials are statistically significant ($p<0.05$) from each other and from control.

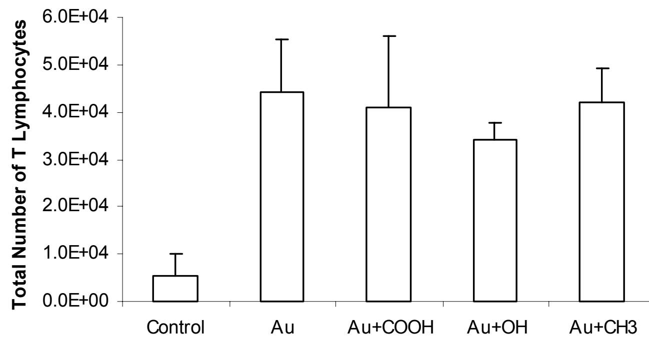


Figure 5
T lymphocytes present in the inflammatory exudates recovered from the air pouches 24 hours after the implantation of the different materials. Differences between materials are not statistically significant; differences between the control and the materials are statistically significant.

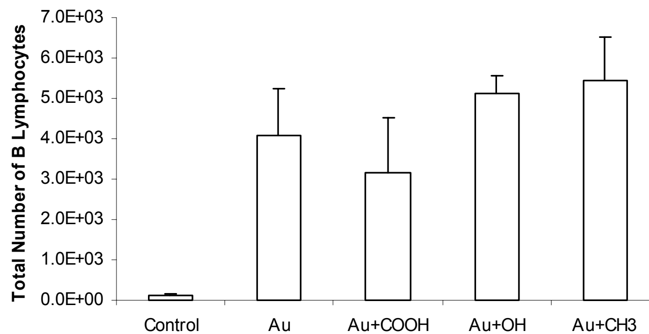


Figure 6
B lymphocytes present in the inflammatory exudates recovered from the air pouches 24 hours after the implantation of the different materials. Differences between the materials are not statistically significant; differences between the control and the materials are statistically significant.

DISCUSSION

The herein investigation shows that the chemical nature of the surface of a subcutaneous implant modulates the thickness of the fibrous capsule that is organized around the implant. The formation of this fibrous capsule is a secondary adaptative response of local tissues of the host to implantation. The structure of this fibrous capsule depends on the nature of the implant and on the inflammatory response caused by the biomaterial. The inflammation is a result of the inflicting surgical trauma and the presence of the implanted material. The inflammatory process is closely linked to the subsequent repair/regeneration of tissues. The

chemical composition of the surface of the biomaterial may modify the local inflammatory response, the secretion of cytokines/fibrogenic factors and development of the fibrous capsule [14].

To obtain well-defined chemical surfaces, we have used SAMs with different terminal functional groups (COOH, OH and CH_3). SAMs offer an elective system to obtain chemically homogeneous surfaces, as it has been documented before in a number of studies [15-17]. SAMs were inserted in subcutaneous air-pouches of BALB/c mice. The local acute inflammatory response to the implant was investigated by flow cytometry and compared with the thickness of the implant fibrous capsule measured in tissue sections of the walls of the implant cavity one week after the implantation of the different model surfaces.

We have used the rodent air pouch model of inflammation [9]. In this model a sterile subcutaneous cavity is formed, giving rise to an area where the biomaterial can be implanted, allowing the study of the inflammatory response caused by the implant. This model has been used as a reliable model for studies of inflammation by several authors. In fact, Yang et al. [18] have used this model to study the effect of particle shape of ultra-high molecular weight polyethylene (UHMWPE) in adverse inflammatory reactions to debris, and they conclude that elongated particles generated more active inflammatory cavities and with enhanced thickness of fibrous capsule than globular particles. Sieving et al. [19] investigated the shape and surface texture of UHMWPE wear debris and their data suggest that both shape and texture influence the severity of the inflammatory response; also, the rough debris surface texture was found to exert a marked effect on adverse tissue responses when combined with particles that have a sharp elongated shape. Wooley et al. [20] investigated the inflammatory response to polymer particles of UHMWPE and polymethylmethacrylate (PMMA) and metal particles of cobalt-chrome (Co-Cr) and titanium alloy (Ti-6Al-4V) and also combinations of the different biomaterials with UHMWPE, they observed that all particulate biomaterials caused significant increases in membrane thickening in comparison with controls, with the highest reaction seen in response to Ti-6Al-4V particles. They also observed a synergistic increase in wall thickness of the air pouch when PMMA was combined with UHMWPE.

We found that surfaces coated with CH_3 induced thicker fibrous capsules around the implanted material than COOH- and OH-coated SAMs did. The latter surfaces trigger the formation of fibrous capsules with thicknesses that were comparable to those observed in sham-operated animals. Interestingly, CH_3-covered surfaces induced the recruitment of the highest number of Mac-1$^+$ phagocytes. The

OH-covered surfaces although inducing the recruitment of high numbers of inflammatory cells and also high numbers of Mac-1$^+$ phagocytes, generate the formation of thinner fibrous capsules. Mac-1 is an integrin molecule (also known as Cd11b/CD18) that binds to intercellular adhesion molecule-1 (ICAM-1) present on the endothelial surface. The expression of Mac-1 at the surface of leukocytes is associated with enhanced adherence, chemotaxis, opsonization and aggregation of inflammatory cells [21]. Leukocytes from sites of active inflammation have been shown to increase surface density of Mac-1 compared with leukocytes from non-inflamed tissues [22].

B and T lymphocytes were present in low numbers, with no statistically significant differences between the different types of SAMs herein studied. This is an expected result since significant enhancement in the number lymphocytes occurs only in chronic inflammation, being phagocytes the predominant cell type usually found during acute inflammation.

Our data on the initial inflammatory response to the three types of chemical surfaces suggest that the increase in the thickness of the fibrous capsule is associated with severity of the early inflammatory response caused by the implant material. In our previous investigations using SAMs we have found that CH_3-coated implants induce a sustained number of activated phagocytes in the inflammatory cavity of the implant [23]. This phenomenon is, however, not associated with a high degree of *in vivo* adhesion of leukocytes to the surface of the implant [24]. In contrast, gold-coated surfaces which also exhibit thick fibrous capsules, although not inducing the recruitment of high numbers of inflammatory cells, are associated with large numbers of leukocytes adherent to the implant surface [24]. The OH-covered surfaces that induce the recruitment of high numbers of inflammatory cells and high numbers of activated phagocytes, and are associated with high numbers of adherent inflammatory cells [24] presented thinner fibrous capsules.

In this work we did not investigate whether the thiol groups of the SAMs were present on the surface of the implanted material for the whole period (1 week) of the study. It is plausible to consider that the oxidants secreted by inflammatory phagocytes may damage the thiol groups of the SAMs. However, our previous reports on the acute inflammation to SAMs [23, 24] documented that the chemical nature of SAMs changed the inflammatory response. This finding is not consistent with a fast damage and removal of the thiol groups from the surface. The herein differences in capsule thickness add a further argument in favor of the persistence of the thiol groups in the surface of the implants.

Biomaterial-mediated inflammatory responses have been related to the subsequent fibrotic responses, and it has been postulated that surface functionality may affect the degrees of inflammatory responses to biomaterial implants, which means that surface functionalities might influence the extent of implant mediated fibrotic reactions [23]. Although the correlation between extent of inflammation and fibrosis is poor, the results obtained by Tang et al. [23] do not exclude the possibility that the adherent inflammatory cells may influence the formation of fibrotic tissue associated with biomaterial implants.

In conclusion, our results indicate the possibility that recruited and/or adherent inflammatory cells may influence the formation of fibrotic tissue associated with these type of surface. We propose here that fibrous capsules increase their thickness around implants when chemical groups, such as CH_3, capable of inducing acute inflammatory reactions with large numbers of activated phagocytes are present at the surface of the implanted material.

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CHAPTER VII

1. General Discussion

The aim of this thesis was to study inflammatory responses to biomaterials and how these reactions are altered by the chemical composition of the surface of the implant. For that purpose, model surfaces with well-defined chemistry were used to investigate the relationship between inflammatory cells and biomaterial surface chemical properties. Self-assembled monolayers (SAMs) of alkanethiols on gold with the terminal functionalities of OH, COOH and CH_3 were used to assess the *in vitro* adhesion of human leukocytes, and the *in vivo* recruitment, activation and adhesion of inflammatory cells of the mouse. The healing response was also investigated through the microscopic analysis of the formation of the fibrous capsule.

Contemporary medicine has seen an increasing usage of implanted devices; this advancement in medical practice requires a detailed knowledge of inflammatory events that occur at the host/implant interface. This is because biomaterials may potentially trigger an array of iatrogenic effects that always include inflammation, but may also encompass fibrosis, coagulation and infection [1].

In general, inflammation can be seen as a phenomenon that protects the body from invasion by microorganisms or foreign bodies. However, inflammation can also produce unwanted effects that may impair therapeutical goals of improving human health. In fact, because implants work as foreign bodies, the implantation of a biomaterial may cause inflammatory reactions of such severity that they can go as far as to inactivate the implant [2]. The surfaces of the implants are rapidly covered by leukocytes and this event is often related to the inflammatory response seen after implantation of biomaterials [3]. Leukocytes are both the cells of host inflammatory reactions and the cells of the ensuing immune response; thus, the reaction of leukocytes to biomaterials is central in the understanding of material-host interactions [4]. It must, nevertheless, be kept in mind that leukocyte adhesion to biomaterials may occur during a number of different situations involved in blood-biomaterial interactions such as haemodialysis, hemofiltration, cardiopulmonary bypass, and implantation of heart-assisting devices. Furthermore, it must be considered that events associated with leukocyte-biomaterial interactions encompass formation of microthrombi due to the aggregation of platelets, detachment of thrombi after

activation of proteases from white blood cells, and secretion by leukocytes of active molecules that modify local and systemic vascular reactions [5].

Usually, within a few hours after implantation, most biomaterials cause acute inflammatory responses that are reflected by accumulation of leukocytes on implant surfaces. These leukocytes involve distinct cell types such as, polymorphonuclear leukocytes (PMN), monocytes, macrophages and lymphocytes[6].

To address the adhesion of leukocytes to solid surfaces, a number of different factors have to be considered, namely those related with surface chemistry, charge or hydrophilicity and protein adsorption to the surfaces. In order to investigate the role of surface chemistry of biomaterials, adequate models have to be selected and employed. To reach this goal, we have identified self-assembled monolayers (SAMs) as a class of organic surfaces that can be easily prepared by immersing a clean film of gold into a solution of terminally substituted alkanethiols. SAMs have been considered to be structurally the best ordered interfaces available to investigate the interactions of cells and proteins with substrates of different surface chemistries [7]. This model system allowed us to produce monolayers with three different terminal functionalities: COOH, OH and CH_3. This was obtained by treatment of gold surfaces with 16-mercaptohexadecanoic acid, 11-mercapto-1-undecanol and 1-hexadecanethiol, respectively.

In our first investigation, we have used isolated human leukocytes to study their reaction *in vitro* to SAMs. For the isolation of the leukocytes from the peripheral blood we have used a method of centrifugation of whole blood cells through a density gradient made with Histopaque (Sigma). This approach is considered to be the most efficient one to obtain a rich cell band after a single centrifugation. Our results confirmed that this method is quite appropriate to obtain both PMN and mononuclear cells from whole blood and also to keep their physiological features intact for *in vitro* studies. To activate the isolated human leukocytes *in vitro* we have employed the lectins PHA and PMA. This is because lectins can agglutinate cells, and this phenomenon triggers cell activation as it has been demonstrated before, namely in studies that have researched the response of activated blood cells to biomaterials [8, 9].

Regarding these initial experiments, our data indicate that human leukocytes, both mononuclear and PMN leukocytes show adhesion in greater numbers to CH_3–covered surfaces than to OH and COOH-covered substrates. Also, we demonstrated that pre-activation of leukocytes resulted in a general increase in the number of attached cells seen on the model surfaces. This enhancement was higher for PHA-

activated than for PMA-stimulated mononuclear leukocytes, whereas PMA treatment of PMN leukocytes resulted in a higher adhesion of these cells than PHA stimulation. Because the use of PMA and PHA was found to increase the number of leukocytes that were attached to the surface of SAMs, we conclude that the adherence of leukocytes to the SAMs is greater when the white blood cells are activated, i.e., the same physiological state of leukocytes that are present in inflammatory reactions. Our results also confirmed previous evidence that PMA is an elective activator of neutrophils, whereas PHA is more efficient in the activation of mononuclear cells.

Why does leukocyte activation by lectins result in greater degree of cell adhesion to implant surfaces? The event is due to enhancement in the expression of molecules that mediate both intercellular aggregation and adhesion to vascular or other surfaces. In fact, it is well known that PMA activates protein kinase C (PKC) [10] and this leads to an increased expression of adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) and intercellular cell adhesion molecules-1 (ICAM-1) [11]; and also of a sulfatide receptor [12]. PHA, on the other hand, increases the density of a number of surface receptors (e.g. CD18, CD11a, CD54, CD58, CD44, CD49d, and CD25) that are also involved in activation and adhesion [13].

This investigation offers a specific example that the chemical nature of the surface of the biomaterial modulates the degree of adhesion of leukocytes *in vitro*, and confirms and extends previous data obtained by Tegoulia and Cooper [14] who have investigated neutrophil adhesion to SAMs. In addition, we also report that the degree of activation of the human white blood cells influences their adhesion to the materials surfaces.

Several previous investigations using SAMs deserve to be mentioned. Tidwell et al. [15] used SAMs to study the different growth of endothelial cells. They reported that COOH terminated SAMs were the best surface to obtain endothelial cell growth. The same terminal group was also associated with adhesion of platelets [16], human corneal epithelial cells [17] and human osteoblast-like cells [18]. According to our data, human leukocytes behave differently: these cells adhered in higher numbers to CH_3 than to COOH coated SAMs

The *in vivo* inflammatory response was investigated using a rodent air-pouch model of inflammation [19]. This model involves the formation of a sterile subcutaneous cavity in the dorsal area of mice that can be used to insert the implant and to study the inflammatory reaction caused by the biomaterial [20, 21]. This *in vivo* model has been used in the evaluation of the biological response to several

biomaterials [22-24], and has proved to be sensitive to differences in implant composition [22, 25]. The major advantage of this model is that it offers a system that allows the insertion of an implant in a sterile cavity from where inflammatory leukocytes can be easily harvested by washing the air pouch with a physiological solution.

A large number of phagocytes, specifically PMN and macrophages/monocytes are often observed in the inflammatory reaction caused by implantation of biomaterials. Phagocytes may modify the severity and extension of the inflammatory response and thus may alter biomaterial biostability and biocompatibility [26]. The degree of surgical trauma and the chemical nature of the surface of the biomedical material are known to modify the number and cell type of phagocytes that are detected near or on the surface of implants [27]. The participation of phagocytes in the initial inflammatory response to a biomaterial may also determine the how the healing process will evolve. Phagocyte interaction with biomaterials depends, namely on the chemical nature, surface free energy and charge, as well as on the porosity and roughness of the implant [28].

Our *in vivo* studies were initiated by defining the acute inflammatory reaction and cell adhesion to SAMs with the same three terminal functionalities (OH, COOH and CH_3). After 24 hours of implantation of the different SAMs, the inflammatory exudates and the implants were removed and analyzed. It was found that the *in vivo* cell recruitment to the air pouches was influenced by the different surface functionalities of the SAMs. Methyl-covered SAMs attracted the highest number of leukocytes to the inflammatory cavity of the mice. However, higher numbers of cells adhered to the OH-coated and gold surfaces in comparison with what was found regarding COOH and with CH_3-coated ones. The number of adherent cells was in fact quite low for the methyl-terminated SAMs. Therefore, it was concluded that methylated surfaces induced the migration of larger numbers of inflammatory cells into the air pouches but this phenomenon was not associated with high number of adherent cells detected on the surface of the implant. This findings extended previous observations by Källtorp et al. [29] and Lindblad et al. [30], that had subcutaneously implanted similar surfaces in the back of the rat. Our data indicate that hydrophobic surfaces (such as CH_3-coated ones) induce a more intense acute inflammatory response and a lower cell adhesion than hydrophilic surfaces (such as COOH and OH-coated surfaces). We conclude that the chemical nature of the

surface of the implant modifies both local acute inflammatory reaction and cell adhesion, and that the two events are not necessarily correlated.

The *in vivo* behavior of the methyl-terminated surfaces, inducing the recruitment of high numbers of inflammatory cells but demonstrating very low adhesion of cells, lead us to investigate this phenomenon for a longer period of time. CH_3-coated model surfaces were implanted and the inflammatory exudates and implants were retrieved and examined 24, 48 and 72 hours after implantation. Again, methyl-covered surfaces were associated with the recruitment of high numbers of inflammatory cells and with low numbers of adherent cells. This phenomenon was observed during the whole course of the acute inflammation studied, thus strengthening our initial evidence of lack of correlation between the intensity of the inflammatory response and the density of cells attached to the implant surface.

It is known that immediately after implantation proteins are adsorbed on biomaterials surfaces. In seconds to minutes a monolayer of proteins usually adsorbs to most surfaces. The protein adsorption event occurs well before inflammatory cells arrive at the surface. Therefore, inflammatory cells see firstly a protein layer, rather than the actual surface of the biomaterial [31-34]. Among proteins that most readily adsorb to biomedical implants fibrinogen, Immunoglobulin G (IgG) and complement factor 3, usually predominate and have an important role in the inflammatory process [6, 35].

Fibrinogen is important in mediating the short-term accumulation of inflammatory cells on implanted materials [6]. Fibrinogen adsorbs spontaneously and denaturates on implant surfaces and thus becomes pro-inflammatory, mainly by causing an increased recruitment and adhesion of leukocytes during inflammation and also during tissue repair at implant surfaces [27, 36, 37]. Several authors have studied the adsorption of fibrinogen to SAMs. The concentration of the adsorbed protein is higher for gold surfaces than for the other types of SAMs, but is also relatively high for the CH_3-coated surfaces [14, 29, 30]. It was postulated that fibrinogen possibly binds to the hydrophobic surface and undergoes degradation before the arrival of the recruited cells, thus offering a lower number of accessible cell-binding sites [30]. This phenomenon may explain the low number of cells that were found in our experiments to be adherent to the methyl-terminated model surfaces.

It has been shown that surfaces that present low adsorption of IgG generally are more biocompatible than those displaying a high adsorption of this protein, because immobilized IgG activates the complement system and promotes the adhesion of monocytes to the surface of biomaterials [38, 39]. Biomaterials activate the complement by the alternative pathway through the binding of C3b to the biomaterial surface [40-42]. The OH-covered surfaces were shown to deposit C3 and IgG that are present in very low or non-measurable amounts in the methyl-terminated surface [38, 43, 44]. This previous finding offers a putative explanation for the higher number of cells that in our experiments were found to be adherent to the OH-covered surfaces in comparison with CH_3-covered ones. Our findings may be due to the activation of the complement system as observed through the surface binding of C3 and/or IgG.

Flow cytometry was then applied to investigate the surface expression of the adhesion receptor Mac-1 (CD11b/CD18), a marker of activated leukocytes, in the retrieved inflammatory exudates. This is because increased surface expression of the integrin molecule Mac-1 has been associated not only with enhanced adherence but also with chemotaxis, opsonization and aggregation of inflammatory cells [45]. In addition, leukocytes from sites of active inflammation have been shown to increase surface density of Mac-1 compared with non-inflamed tissues [46]. Integrins are cell surface molecules that are central in the physiology of inflammation, involved in cell migration, adherence and activation of leukocytes [47]. Adhesion molecule receptors are present on the surface of circulating leukocytes. These receptors are up-regulated in many inflammatory states, and this change is necessary for leukocyte binding to adhesion molecules located on the surface of endothelial cells. The integrin Mac-1 is one of those ligands on the leukocyte surface that binds to intercellular adhesion molecule-1 (ICAM-1) on the endothelial surface and this attachment makes the leukocyte to move from circulation into inflammatory tissues [46]. Expression of Mac-1 on leukocyte surface may be taken as a measure of its up-regulation that usually occurs in inflammatory reactions. Thus, the high density of Mac-1 antigen on the surface of leukocytes is currently considered to be a reliable parameter to evaluate the pro-inflammatory risk of implanted biomaterials [48].

After 24 hours of implantation, in sham-operated animals the number of Mac-1^+ cells was low, and after the insertion of the different SAMs there was a significant increase in the number of these cells. The implants with methyl-terminated surfaces induced a further significant increase in the number of activated leukocytes. In addition, the inflammatory exudates induced by methyl-terminated SAMs were

monitored 4, 24, 48 and 72 hours after implantation. Flow cytometry revealed that methyl-terminated surfaces induced a significant enhancement in the recruitment of phagocytes expressing Mac-1. This phenomenon was detected during the whole course of the study. Since the surface expression of this adhesion molecule reveals activation of phagocytes, our results suggest that the methyl group will promote stimulation of white blood cells with consequent enhancement of the inflammatory reaction. Because the surface expression of Mac-1 is known to reveal activation of phagocytes, this finding can be related with our initial experiments using PMA or PHA activated human leukocytes *in vitro*.

Flow cytometry also allowed concluding that, at the early phase of acute inflammation neither T nor B lymphocytes were present in significant numbers in cavity where the implants were located. This is not surprising taking into consideration that lymphocytes are known to take just a minor role during acute inflammation.

The wound healing that is observed after implantation of a biomaterial starts after acute inflammation is over. However, if inflammatory stimuli continue to be induced by the implant this will lead to chronic inflammation with the presence of macrophages, monocytes and particularly lymphocytes, and with the proliferation of blood vessels and accumulation of fibers and cells of the connective tissue. The healing process of the implant cavity is characterized by the proliferation of fibroblasts, synthesis of collagen and proteoglycans and also by local angiogenesis. The foreign body reaction caused by the implant leads to the detection of giant cells apposed to the biomaterial surface that becomes surrounded by granulation tissue and is followed by fibrous encapsulation of the implant [32, 49].

The end stage of the wound healing response to biomaterial implantation is usually fibrosis or fibrous encapsulation of the inserted body. In the last set of experiments of this thesis, we have investigated the influence of the surface chemistry of implants on the thickness of the fibrous capsule that is under organization around the material. The organization of this fibrous capsule depends on the biomaterial and on the inflammatory response that is initiated by its implantation. The degree of severity of the inflammatory process is closely linked to the features of subsequent repair/regeneration of tissues. This is a pertinent issue since the chemical composition of the surface of the biomaterial may change the local inflammatory response, the secretion of cytokines/fibrogenic factors, and finally, the development of the fibrous capsule [27]. The thickness of fibrous capsules was measured by histology one week after implantation. A significant increase in the

thickness of the fibrous capsule was seen around implants coated with methyl groups, and also in gold surfaces, in comparison with sham-operated mice and COOH- and OH-covered surfaces. It should be stressed that our data showed that the increase in the capsule thickness was not associated with a high degree of *in vivo* adhesion of leukocytes to the surface of the implant. Thus, it appears that fibrous capsule formation depends more from the severity of the local inflammatory reaction than on the adhesion of leukocytes to the implant. These results indicate that surfaces coated with CH_3 groups induce thick fibrous capsules and, considering the above cited data, it can be proposed that the CH_3 groups trigger stronger phlogistic effects than COOH or OH chemical groups.

Taken together the data obtained in the different studies performed in this thesis, it is pertinent to consider that CH_3-covered surfaces are rather pro-inflammatory implants. In fact, in the *in vitro* studies, the methyl-terminated surfaces induced the adhesion of the highest number of human leukocytes, both mononuclear and polymorphonuclear cells. In the *in vivo* studies that were performed, the CH_3-covered SAMs although presenting very low numbers of adherent cells at the surface, have demonstrated to have a strong phlogistic effect. These surfaces are accountable for the recruitment of high numbers of inflammatory cells during several days in the acute inflammatory response, and a large percentage of these cells are activated ones, as revealed by the flow cytometric studies using Mac-1 labeling as a marker of cell activation. Also, the methyl-covered surfaces induce the formation of thick fibrous capsules around the implants which may be the result of this strong pro-inflammatory effect in comparison with that observed regarding the other chemical groups investigated (COOH and OH).

2. Future Work

Several issues still need to be addressed in future studies in order to advance our understanding of the influence of the biomaterial surface chemistry in the inflammatory response:

- The use of self-assembled monolayers with other terminal functional groups, such as amine (NH_2) which is positively charged at physiological pH, or

polyethylene glycol (PEG), a non-fouling material, which means that it resists protein and cell adhesion.

- The kinetics of the *in vitro* adhesion of human leukocytes to the different self-assembled monolayers.

- The quantification of apoptosis among adherent inflammatory cells since apoptosis is a mechanism by which cells undergo programmed cell death and the appearance of apoptotic cells represents a potential method of removing adherent cells from a biomaterial surface with a limited inflammatory response.

- The role of the adsorbed protein layer needs to be addressed in more detail. The role of fibrinogen and its pro-inflammatory action needs to be further investigated, namely the changes in the conformation of this protein after adsorption to the biomaterials surfaces.

- The *in vivo* stability of the self-assembled monolayers should be investigated, to determine the limit time that the model surfaces can be implanted.

- The application of other techniques to better characterize the inflammatory response, namely immunocytochemistry for the *in situ* definition of subpopulations of attached leukocytes.

- The study of the attached leukocytes by flow cytometry, through the recovery of the adherent cells from the surface of implants.

- Self-assembled monolayers are model surfaces very useful for fundamental studies. Based on the results obtained using SAMs it would be important now to prepare polymers with adequate compositions and to study their behavior towards the activation of the immunological response. These studies would likely provide information to be applied in the development of biomedical devices.

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