IMPLANT SURFACE TOPOGRAPHY MODULATES MACROPHAGE MORPHOLOGY AND SUBSEQUENT SIGNALING CASCADES

by

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ABSTRACT

Implant surface topography is a major determinant in the success of dental implants.

The objective of this thesis was to investigate macrophage responses to implant surface topography *in vitro* and *in vivo*. The *in vitro* studies investigated the effects of surface topography on the RAW 264.7 macrophage cell line's morphology and signaling over time. Cells plated on surfaces of varying degrees of roughness responded with specific topography-directed time-dependent changes in cell morphology. These changes were accompanied by alterations in F-actin and vinculin organization and localization. Activation of focal adhesion kinase (FAK), proto-oncogenic tyrosine kinase (Src), and extracellular signal-regulated kinases1/2 (ERK 1/2) signaling molecules were also found to be both surface topography and time dependent. Phosphorylation of FAK and ERK1/2 appeared to be integrin-mediated as the Src inhibitor pyrazolopyrimidine (PP1) blocked their activation. Surface topography also affected RAW 264.7 cell number.

The *in vivo* section comprised two parts. First, an immunostaining technique was developed that allowed recognition of the lysosomal marker ED1 on macrophages embedded in L.R-White embedding medium. We then used this technique to identify recruited ED1-positive macrophages at the implant-tissue interface in cryosections and in sections of L.R-White-embedded polished and rough implants placed in rats.

We conclude that RAW 264.7 cells are highly sensitive to micron-scale features of surface topography; the features affected cell morphology as well as the organization and localization of F-actin and vinculin. For the first time ever we report that topographies used in clinical implants differentially regulate activation of FAK, Src and ERK1/2 signaling molecules. As

these pathways lead to production of inflammatory cytokines, surface topography may be important in deciding host responses to/and eventual outcomes of implantation. The results point to the possibility of designing implants with specific surface features to control host responses and thereby improve clinical outcomes.

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LIST OF ABBREVIATIONS

Ac/P	: Calcium phosphate.
AE	: Acid-etched surface.
Al	: Aluminum.
AI_2O_3	: Aluminum oxide.
ANOVA	: Analysis of variance.
ATCC	: American Type Culture Collection.
avb3-integrin	:Vitronectin receptor.
В	: Sandblasted surface.
BD%	: Percentage of bone density.
bFGF	: Basic fibroblast growth factor.
BIC%	: Percentage of implant-bone contact.
BMPs	: Bone morphogenetic proteins.
BSA	: Bovine serum albumin.
C°	: Degree centigrade.
CCL2	: Expression and cellular localization.
CD14	: Cluster of differentiation-14.
CD16	: Cluster of differentiation-16.
CD163	: Cluster differentiation 163.
CD68	: Cluster differentiation 68.
CpTi	: Commercially pure titanium.
CS	: Cvtoskeletal Stabilization buffer.
CsK	: C-terminal Src kinase.
C-C	: Cvsteine-cvsetine.
CXC	: Cvsteine Amino acid Cvsteine.
DAB	: 3.3-diaminobenzidine-tetrahydrochloride.
DFP	: Diisopropyl fluorophosphates.
DMEM	: Dulbecco's modified Eagle's medium.
EBC%	: Percentage of expected bone contact.
ECM	: Extracellular matrix.
ED1	: Lysosomal rat macrophage ED1 antigen.
ED2	: Surface membrane rat macrophage ED2 antigen.
EDTA	: Ethylenediaminetetraacetic acid.
EGF	: Epidermal growth factor.
EGTA	: Ethyleneglycoltetraacetic acid.
ERK1/2	: Extracellular signal-regulated kinases1/2.
EthD-1	: Ethidium homodimer-1.
F4/80	: Macrophage receptor.
FAK	: Focal adhesion kinase.
FAs	: Focal adhesions.
F-actin	: Filamentous actin.
FBGC	: Foreign body giant cells.
FCS	: Fetal calf serum.
Fc-γ	: Fc-gamma receptor.
FGF	: Fibroblast growth factor.
GAPDH	: Glyceraldehydes-3-phosphate-dehydrogenase (housekeeping gene).
G-CSF	: Granulocyte-colony stimulating factor.

GEMM-CFU	: Granulocyte-erythrocyte-megakaryocyte-macrophage colony forming unit.
GLM	: Generalized Linear Model.
GM-CFU	: Granulocyte-macrophage colony-forming unit.
GPCR	: G-protein coupled receptor.
Grb2	: Growth-factor receptor bound protein 2.
Grb7	: Growth-factor receptor bound protein 7.
GTP	: Guanosine triphosphate.
GTPases	: Hydrolase enzymes.
H_2SO_4	: Sulfuric acid.
H_3PO_4	: Phosphoric acid.
HA	: Hydroxyapatite.
Hcl	: Hydrochloric acid.
IFN-γ	: Interferon-y.
IL-1	: Interleukin-1
IL-10	: Interleukin-10.
IL-12	: Interleukin-12.
II -1α	· Interleukin-1alpha
IL_1®	: Interleukin-1heta
IL-1p	: Interleukin 23
IL-2J	: Interleukin 3
IL-3 II ⊿	: Interleukin 4
IL-4	· Interleukin 6
IL-0	. Interneukin-o.
Juli	. N-lettillidi Nildse.
	. Polassium chionue.
KDa	
LPS	
LSE	: Laser surface engineering.
M1	: Classically activated macrophages.
M2	: Alternatively activated macrophages.
MAPK	: Mitogen-activated protein kinase.
MARCO	: Macrophage receptor with collagenous structure.
MBL	: Marginal bone loss.
MCP-1	: Monocyte chemotactic protein-1.
M-CSF	: Macrophage-colony stimulating factor.
MDM	: Monocyte-derived macrophage.
MIP-1 α	: Macrophage inflammatory protein 1 alpha.
ml	: Milliliter.
MLC	: Myosin light chains.
MLCK	: Myosin light chains kinase.
mm	: Millimeter.
mМ	: Millimoler.
MMP-1	: Matrix metallopeptidase 1.
MMP-2	: Matrix metallopeptidase 2.
MMP-3	: Matrix metallopeptidase 3.
MDM	: Monocyte-derived macrophage
MPS	: Mononuclear phagocyte system.
$Na_3C_6H_5O_7$:Trisodium citrate.
ΝF-Κβ	: Nuclear factor-kappa beta.
nm	: Nanometer.
NO	: Nitric oxide.

OsO ₄ : Osmium tetroxide.	
P : Polished surface.	
P130Cas : p130 Crk-associated substrate.	
P388D1 : Macrophage cell line.	
PBS : Phosphate buffer saline.	
PDGF : Platelet derived growth factor.	
PEG : Polvethylene glycol.	
pERK1/2 Phospho-ERK1/2	
nFAK · Phospho-FAK	
nH · A measure of the degree of the acidity or the alk	alinity of a solution
PIP-3 : Phoenbatidylinositol 3-kinase	
PIPES : Piperazine-N N-bis (2-ethanesulfonic acid)	
$PI C_a 1$: Phoenbolingeo C_a 1	
DIE : Periodontal ligament enithelial cells	
PMSE : Phonylmothanosulfonyl fluorido	
PD1 : A amino 5 (4 mothylinbonyl) 7 (t butyl) pyrazolaľ	A dl nurimidino
PP1 . 4-animo-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3	2,4-uj-pyrimiume.
PP2 . 4-amino-5-(4-chiorophenyi)-7-(t-butyi)pyrazoio(3	,4-uj-pyrimiume.
PP5 . 4-animo-7-prienyipyrazoi[5,4-u]-pyrimiume.	
PSR . Phosphalluyi serine receptor.	
psic . Priospino-sic.	
PTKS Protein tyrosine kinases.	
p-Tyr : Phosphotyrosine.	
PVD : Physical vapor deposition technique.	
PVDF : Polyvinylidene fluoride.	
R _a : Average roughness value.	
RCOs : Rat calvarian osteoblasts.	
RES : Reticulo-endothelial system.	
RGDC : Peptide sequence Arg-Gly-Asp-Cys.	
rhBMP-2 : Recombinant human bone morphogenic protein	-2.
RIPA : RadioImmuno Precipitation Assay.	
RPM : Revolutions per minute.	
R-Ras : Small hydrolase enzyme.	
RSV : Ruos sarcoma virus.	
SAS : Statistical Analysis Software.	
SDS/PAGE : Sodium dodecyl sulfate polyacrylamide gel elect	trophoresis.
SEM : Scanning electron microscope.	
SH1 : Homology domain 1.	
SH2 : Homology domain 2.	
SH3 : Homology domain 3.	
SH4 : Homology domain 4.	
SLA : Sand-blasted large-grit acid-etched.	
SOCS : Suppressor of cytokine signaling.	
SOS : Son of seveless protein.	
SR-A : Scavenger receptor family.	
Src : Proto-oncogenic tyrosine kinase.	
Sre : Serine residue.	
Srs : Sarcoma virus.	
STAT3 : Signal transducer and activator of transcription3	
TBS Tris-buffer saline	

TGF-α	: Transforming growth factor alpha.
TGF-β1	: Transforming growth factor-beta1.
Th1	: T helper cell 1.
Th2	: T helper cell 2.
Thr	: Threonine residue.
Ti	: Titanium.
Ti-6AL-4V	: Grade 5 titanium alloy.
TiO ₂	: Titanium oxide.
TLRs	: Toll-like receptors.
TNF-α	: Tumor necrosis factor alpha.
TNF-α:	: Tumor necrosis alpha.
TPS	: Titanium plasma sprayed.
TTBS	: Tween-20 Tris-buffer saline.
Tyr	: Tyrosine residue.
V	: Vanadium.
VEGF	: Vascular endothelial growth factor.
v-Src	: Rous sarcoma virus-Src.
XPS	: X-ray photoelectron spectrometry.
β2-GPIR	: Beta2-glycoprotein I receptor.
μΙ	: Microliter.
μ m	: Micrometer.

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DEDICATION

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COAUTHORSHIP STATEMENT

The chapters presented in this thesis have been prepared by the candidate with guidance from the graduate supervisors, Dr. Douglas Waterfield and Dr. Don Brunette. The data in this thesis was acquired and prepared by the candidate.

CHAPTER 1: Introduction

1.1 General introduction

Endosseous dental implants are currently the most sophisticated, innovative and exciting treatment modality for replacing missing teeth. They are also being widely used for a variety of other applications such as anchoring in orthodontics. In general the various techniques and applications in use are based on good evidence and are highly predictable.

Brånemark introduced endosseous implants and demonstrated that with the control of different factors during implant insertion, osseointegration can be reliably achieved. These factors include the surgical technique, the monitoring of healing and loading times of the implant, general health and the bone quality of the recipient site, as well as the biocompatibility of the implanted material (commercially pure titanium and titanium alloys) being the most widely used materials.

Researchers have endeavored to improve implant success by incremental modification of these factors. A great number of advances have occurred and led to the widespread use of implants in a larger selection of patients, so that now implants demonstrate faster healing times and shorter loading times, even in recipients with limited or poor bone quantity.

Many commercially produced implant systems have aimed to improve on the original Brånemark approach. Some have achieved improved cellular/clinical responses by alteration of implant surface topography thereby demonstrating the importance of this property. Since the introduction of micro-patterning and micro-machining methods by Brunette and coworkers in the early 1980s, many different aspects of implant surface topography and cell behavior have been studied. Numerous effects of surface topography on cell behavior *ex vivo* in terms of their adhesion, migration, cell shape, cell selection and cell differentiation are well established (1-5). However, these effects are not limited to the whole cell level. Recent data show that surface topography modulates cellular gene expression, which in turn, further affects patterns of cytokine secretion (6-9).

Implant surface topography plays a crucial role in the wound healing responses in situ. Topographical effects include the retention of the blood clot and the activation of its hematopoietic cellular contents, the recruitment and activation of different cell populations including peripheral monocytes that give rise to macrophages, and finally bone formation and bone remodeling at the implant interface. Macrophages are one cell type that is prominent among cell populations at the wound site within 24 hours. Macrophages clear bacteria deposited as a result of the wounding and implantation procedure. The macrophage is also a major source of cytokine production at the wound site. The production of cytokines in turn affects the recruitment and differentiation of other cell populations. Moreover, macrophages are "plastic cells" and can exhibit different phenotypes such as pro-inflammatory, anti-inflammatory, angiogenic, and an osteoinductive effects. In this thesis we have investigated the role of surface topography on RAW 264.7 cell shape, the patterning of the cytoskeletal proteins, cell viability/number and activation of intracellular signaling molecules. We also determined whether surface topography would differentially affect accumulation of macrophages on implanted material in situ.

1.2 Biocompatibility and biofunctionality of implanted devices

Biocompatibility is concerned with the interplay between an implanted material (biomaterial) and the surrounding body tissues and the outcomes of these interactions. As such, biocompatibility is the most important criteria in selecting materials intended for clinical applications. Biofunctionality refers to the ability of a biomaterial to partially or completely replace and/or restore the function of a body tissue when it is exposed to and/or in contact with body fluids continuously or intermittently (10). The first and foremost characteristic of a biomaterial intended for use within the interior of the body is that it be non-toxic. Moreover, it should neither elicit an adverse response from the body nor be carcinogenic (11).

1.3 Biocompatible material

A biocompatible material is defined by its ability to perform with "an appropriate host response in a specific application" (12). In other words it should produce the optimal expected outcome in terms of biological response and the intended clinical application (13). Ceramics, either in single crystals or composites, and metals are examples of biocompatible materials used in implanted devices. For example a single crystal Aluminum oxide (Al₂O₃) characterized by a high strength has been used in dental implants. Glass-ceramic composite has been widely used in vertebrae replacement as it binds strongly to bone. However, these materials have limitations in load-bearing applications because their fracture toughness values are low compared to bone (14).

Metallic biomaterials such as stainless steel and titanium (Ti) are superior candidates in load bearing situations. Titanium is available naturally and is produced by the Kroll process where titanium chloride is reduced by its chemical reaction with magnesium (15). Among the materials available, Ti and its alloys have become the materials of choice in the field of oral implantology and orthopedic applications (16-18).

1.4 The properties of titanium and titanium alloys

The use of titanium and its alloys in medicine has been comprehensively reviewed (19). Commercially pure titanium (cpTi) has four grades based on the degree of purity (graded 1 to 4). This purity is characterized by oxygen, carbon and iron content. CpTi is not a strong enough material for use in high load-bearing conditions. The addition of aluminum (AI) and vanadium (V) produces a significantly stronger titanium alloy TI-6AL-4V (grade 5 titanium alloy). In addition, this treatment improves the biocompatibility and reduces the elastic modulus of the titanium alloy while increasing the yield strength and fatigue properties relative to pure titanium (20). Titanium is highly reactive and has a high affinity for oxygen. After contact with air or with an aqueous electrolyte environment a passive surface titanium oxide film forms spontaneously (21-23). This protective titanium oxide layer is responsible for the superior corrosion resistance and offers a potent barrier against dissolution and metal ion release of Ti alloys (24). Other properties of Ti alloys include a high tensile strength, low specific weight, poor heat conductivity, and little or no adverse tissue reaction.

Titanium and titanium alloys have been described as naturally osteoinductive and osteoconductive materials that permit bone growth on their surfaces (25). Nevertheless,

there have been several studies aimed at improving the surface biological and chemical properties of Ti devices by the application of different coatings and chemical treatment methods (26). The use of osteoinductive substances as biomimetic coatings on the surface of implants holds great promise for controlling and optimizing the cascade of biological events that result in the bone formation appropriate for anchoring implanted devices to meet functional criteria. For example, coating with hydroxyapatite (27-29), calcium phosphate (30-32), and the peptide sequence Arg-Gly-Asp-Cys (RGDC) leads to considerably greater cell attachment and proliferation and a significant increase in bone thickness when compared to non-coated implants (33, 34). In addition, studies utilizing a recombinant human bone morphogenic protein-2 (rhBMP-2) as a coating or delivered into the preparations followed by implant placement have shown enhanced initial integration of dental implants in animal models (35-38).

Coatings, though, face the long-term clinical problems of possible degradation and delamination that could compromise their intended biological effect during initial stages of wound healing and bone formation, leading to possible failure at the implant-coating interface during implant function (39). For example, in a case report on two human hydroxyapatite (HA)-coated dental implants retrieved after 14 years of functional loading, there was a 46% resorption of the HA coating. Nevertheless, no gaps or connective fibrous tissue were found at the implant-bone interface (40).

In order to surmount these limitations and to improve implant longevity, investigators have reconsidered implant design, and developed microtextured implant surfaces (3, 41). Currently surface topography (surface texture) is an important area of research due to its powerful and persistent effect on cell behavior such as shape, orientation, and

adhesion. (42-45). Such surface directed alteration of cell shape is postulated to account for differential gene expression (46-48) that leads to changes in cell growth (49), cytoskeletal organization (50), collagen synthesis, extracellular matrix metabolism (51), differentiation and secretory profile (52-54).

1.5 Commercially available dental implant designs

Among the commercially available implants most frequently described in the dental literature and used clinically, two basic types of surface can be identified: (1) roughened surfaces obtained by subtractive processes such as those achieved by sand blasting, acid etching, or a combination of the two processes; electropolishing, mechanical polishing and (2) those with roughened surfaces achieved by additive processes such as coating with titanium plasma-spray (TPS) or hydroxyapatite as well as ion deposition. Surfaces are also subdivided into isotropic surfaces that have no orientation of their features and anisotropic surfaces that contain orienting features such as the fine lines or grooves produced by turning on a lather (machining).

Moreover, the surfaces are integrated into a larger structure with visible, so called macro geometric properties, such as screw threads and vents, which are designed to facilitate mechanical stability. In reviewing the approaches used to modify implant surfaces, we have selected some illustrative examples from several implant manufacturers. No attempt has been made to be comprehensive because of the large number of implant systems available and the numerous, sometimes subtle, variations in production methods. The emphasis is on those implants that are widely used or illustrate particular principles of surface modification.

1.5.1 Polished (smooth) surface

Polished surfaces are prepared by several different techniques, such as mechanopolishing and electropolishing. The simplest way, however, is smoothing the surface by means of grit-papers and/or diamond cloths. The finest particles produce surfaces with the highest levels of polishing (mirrored/polished surface) with 0.1 μ m roughness. On the other hand, grinding with larger particles can produce a surface with a roughness of 1 μ m (55, 56). Smoothing does not produce a significant increase in the geometrical surface area of the treated surface (57).

1.5.2 Blasted surface

The TioblastTM (Astratech AB, Molandal, Sweden) surface is produced by blasting the titanium implant surface with hard ceramic particles such as titanium oxide (TiO₂) at a high speed by means of compressed air. TiO₂ particles of approximately 25 μ m produce a moderately rough surface in the range of 1 to 2 μ m. A second technique used for blasting titanium implant surfaces uses Al₂O₃ beads (average particle size: 250 μ m). Results from an *in vivo* study show that in, the size range studied, the optimal size of blasting particles is 75 μ m (58) and the surface roughness value produced is within the range of 1.0 to 2.0 μ m (59). Embedding of the blasting material into the surface is a noticeable problem as these particles have been released in the surrounding tissues and interfered with osseointegration (60). A third way of roughening titanium implant surfaces is by using biocompatible and osteoconductive blasting materials such as hydroxyapatite, beta-tricalcium phosphate, or a mixture of both. The surface roughness

of titanium implants depends on the size of the ceramic particles. The blasting procedure results in a surface with an increased area of 29%.

1.5.3 Acid-etched surface

The roughed surface of the OsseotiteTM implant produced by acid etching is an example of this implant type (Implant innovations, Palm Beach Gardens, FL). The technique involved in producing this surface involves a two-step etching procedure where titanium implants are immersed in an over 100°C hot solution of strong acids such as sulfuric acid/ hydrochloric acid (H₂SO₄/HCl). This etching produces a micro-rough surface characterized by micro-pits with a diameter in the range of 0.5 to 2 μ m (61). High frequency irregularities and an increased surface area of 20% characterize this surface. The specific surface produced by dual acid etching is hypothesized to promote the adhesion of the osteogenic cells, and thus promote bone apposition at the implant interface (62).

1.5.4 Sand-blasted Large-grit Acid-etched (SLA) surface

One of the most widely used commercial treatment processes for titanium implant surface roughening is a combination of sand blasting and acid etching (63, 64). The SLATM (Institute Straumann AG, Waldenburg, Switzerland) is such a surface produced by the combination of blasting and etching. The surface is first blasted with large size AI_2O_3 beads under industrial particle-blasting conditions (average particle size: 250 µm). The blasted surface is then etched in a hot solution of H_2SO_4 /HCl. The SLA technique combines the advantages of both sand blasting and acid-etching methods to produce

implant surface characterized by both macro-roughness and micro-pits with an average roughness value (R_a) of 1.5 μ m (65). Furthermore, the micro-pits and the sharp peaks produced by acid etching on the SLA surface increased the surface area by 33% and enhanced osteoblast proliferation and bone formation (63, 64). The acid-etching procedure also cleans the titanium implant surface of the remaining air-abrasive blasting particles. The SLA surface properties of macro-roughness, the micro-pits, and the cleanness of the implant surface are crucial factors for the osseointegration (58, 66).

1.5.5 Coated surfaces

An example of coated surfaces is the Steri-Oss TM family of implants that is available with HA or TPS coatings. The HA coating obviously alters the surface chemistry as well as the topography. This dual alteration has the potential at least to combine beneficial effects synergistically. *In vitro* experiments comparing bone formation on microfabricated grooves of different depths with surfaces consisting of titanium or coated with HA found that HA coated surfaces produced consistently more bone nodules at all depths with a statistically significant interaction term indicating synergy between the effects of coating and topography. The Steri-Oss HA coated implants had an average height deviation of 1.68 μ m, an average wavelength of 13.74 μ m, and an increased surface area of 55% (59). The Steri-Oss TPS surface was rougher than its HA coated counterpart, with an average height deviation of 3.86 μ m, an average wavelength of 19.55 μ m, and an increased surface area of 134 %. No long-term follow up studies have been published (59).

1.5.6 Oxidized surface

The TiUniteTM surface (Nobel Biocare AB, Götenborg, Sweden) is an example of oxidized titanium implant surfaces. Treatment of titanium in strong acids such as sulfuric acid (H_2SO_4) and phosphoric acid (H_3PO_4) at a high current density increases the thickness of the titanium oxide layer to more than 1000 nm. The subsequent dissolution of the oxide layer creates micro- or nano-pores on the titanium surface (67) and produces microstructure and crystallinity modifications on the titanium surface (68), which increases the surface area 37%. Anodized surfaces demonstrated more bone apposition and higher values for biomechanical and histomorphometric tests when compared to machined surfaces (69, 70).

1.5.7 Turned (machined) surface

Machining can produce implant surfaces with threads, steps, holes, and microgrooves. For example, the MK IIITM surface (Nobel Biocare AB, Götenborg, Sweden) produced by machining is characterized by cutting marks that resulted in a surface with oriented features and an increased surface area of 19%. Turning also can modify surface properties. Such changes include an increased surface hardness and an average roughness of 0.3 to 0.6 μ m is produced on the surface.

More titanium surface roughening methods have recently been introduced. One such approach involves chemical treatment of the surface with fluoride solution. This treatment creates surface roughness and additionally incorporates fluoride into the

titanium surface. It may also enhance osteoblastic differentiation and promote osseointegration of treated dental implants compared to non-treated controls (71, 72).

In the 1990's, Laser Surface Engineering (LSE) was introduced (73-75). LSE produced surface micotopography depends on the wavelength of the source. For example features of around 64 nm requires vacuum ultraviolet (157 nm) wavelength. Whereas features of 20 nm require Extreme ultraviolet (13 nm) wavelength source (76). Karacs et al. reported in vivo experiments where the torque removal force was higher for sand blasted and laser treated implants compared to smooth implants (77). Another use of the LSE is material surface coating called laser-assisted coating. Hydroxyapatite and zirconia are typically coated on substrates such as Ti alloys aiming to induce the formation of new chemical species that could improve the interaction between the biological environment and the surface of the implanted material. (78). The claimed advantages of the LSR method are that laser processing can be rapidly conducted in air with high precision and reproducibility (73).

The above described techniques and methods of implant surface preparation were designed for implant surface roughening according to the belief that roughening provides a greater surface area for cell attachment and eventually more bone apposition with subsequent mechanical stabilization of the implanted devices (4, 62, 79). In certain unfavorable clinical situations such as poor quality and/or volume of the host bone, using an implant with rough surface appears to be highly indicated. Furthermore, short implants with rough surfaces may be the implant of choice in recipients with anatomical abnormalities and insufficient bone quantity. These implants demonstrate superior clinical outcome compared to smooth surfaced implants (80, 81).

1.6 Clinical performance of some commercial implant systems

Many types of roughened surface have proved successful in implants regardless of the procedures used to produce them. The clinically and commercially important question arises, what implant system is best? The answer to this question is complicated because of the complexities involved in clinical trials of dental implants.

First, many implant systems have very high success rates for certain patient populations. Thus distinguishing small relative effects in a clinical trial requires large samples.

Secondly, there is heterogeneity among patients with respect to medical conditions, compliance with oral hygiene, and habits such as smoking or clenching. When not dealt with by exclusion from the subject pool or methods to distribute their effects, these factors will cause increased variance in the data.

Thirdly, sites chosen for implant placement can vary markedly in quality of bone within the same patient.

Fourthly, implant systems are developed at a faster rate than the time required to obtain long-term clinical data.

Fifthly, implant placement is technique sensitive so the skill of the surgical team contributes to their overall success rate. Usually studies do not take into account the experience of the surgeons used to place the implants.

Sixthly, investigators are frequently associated with and/or sponsored by manufactures of particular implant systems. When such investigators carry out comparative studies the implant system with which they are most familiar appear to have an advantage; these investigators would encounter fewer technical problems than they would face with unfamiliar systems.

Seventhly, There is ample evidence from pharmaceutical research that systematic bias exists that favors products made by the company that sponsors the research. Explanations for the above include the selection of inappropriate comparison products or conditions as well as publication bias (studies which show the company's product to be inferior are simply not published) (82). In the dental setting implants cannot be placed "blind" (in the experimental design sense of the investigator not knowing which implant system he is using). If different teams associated with different manufacturers get contradictory results, meta-analysis of the studies will be hard pressed to find differences.

Considering these problems it is not surprising that the most recent Cochrane systematic review, Esposito et al. (2007) concluded there was no evidence showing that any particular type of dental implant has superior long-term success (83). The authors noted

that their conclusions were based on a few RCTs, often at high risk of bias, with few participants and relatively short follow-up periods.

It should be noted that the development of new implant surface topographies has largely been approached through trial and error optimization with little consideration of the mechanisms whereby surfaces produce biological responses. In large part, however, detailed information on specific mechanisms is not available. This thesis attempts to rectify this deficiency by examining the effects of topography on FAK-SRC complex signaling in macrophages. Macrophages were chosen as the cell of interest because they are central to the wound healing that occurs around implants and they are often associated with implanted rough surfaces *in vivo*. The FAK-SRC complex signaling pathway was chosen because it is intimately involved with cell adhesion as well as cytokine secretions that are important in establishing a healing milieu.

1.7 Surface topography and cell behavior

Although this thesis is restricted to the effects of surface topography on the RAW 264.7 macrophage cell line It has been reported that surface topography affects a wide variety of cell behavior (84). Among the most relevant cell behaviors influencing implant performance are cell adhesion, contact guidance, cell selection, cell differentiation and cell signaling.

1.7.1 Cell adhesion

In general cellular adhesion, spreading, and migration are phenomena mediated by the cytoskeleton, including filamentous actin (F-actin) structures. Strong cell attachment to a substratum is associated with the assembly of thick bundles of F- actin, known as stress fibers, which connect to focal adhesions (FAs). FAs are large macromolecular assemblies on the cell membrane that connect the cell to its substratum. Vinculin, an integrin-associated protein complex, is a component of FAs (45, 85) that is frequently used to visualize them. Specialized attachment proteins in the FAs are called integrins. They are a family of transmembrane glycoprotein receptors composed of α and β subunits that are encoded by 18 α and 8 β integrin genes in mammals that interact with extracellular matrix molecules, opposing cells or substrata (86). Integrins are also involved in intracellular events such as regulation of gene expression, cell proliferation, differentiation, migration, and cell death (87, 88). Cellular adhesion molecules are called receptors and the molecules they recognize are termed "ligands". Surface topography is known to modulate and affect the organization of these adhesion molecules and FAs act as a biochemical-signaling hub (4, 6).

Studies on eukaryotic cells indicated that surface roughness could affect cell adhesion. Osteoblasts showed higher levels of adhesion on surfaces produced by electro-erosion, sandblasting or acid etching compared to that formed on surfaces produced by polishing and machining (89) with a roughness value of ($R_a \approx 0.15 \mu m$) being optimal for adhesion and spreading (90, 91). Similarly, epithelial cells cultured on smooth surfaces demonstrated stronger adhesion as determined by area of FAs compared to cells on rough surfaces (92).
Adhesion of macrophages to biomaterials is a complex process. Several different types of adhesive structures have been described in macrophages cultured in vitro. One such structure is called a focal contact and is visualized as a rectangular structure that colocalizes with vinculin, talin, and F-actin (93). Another means of macrophage adhesion to biomaterials is through podosomes that appeared as distinct, circular structures of colocalized vinculin and talin surrounding an F-actin core as described by DeFife et al. 1999. Although DeFife et al. claim that macrophages and foreign body giant cells (FBGC) adhere to surfaces mainly through podosomal structures and not focal contacts, their experiments used only coveslips as substrata for macrophage adherence (94). Podosomes are similar to FAs but they are short living substructures and undergo cycles of rapid assembly/disassembly with a life span of 2-4 minutes (95). When macrophages adhere to biomaterials they most likely recognize surface adsorbed proteins and then adhere onto the surface via integrin-mediated adhesive interactions (96). Macrophages, through their integrin receptors can recognize extracellular matrix molecules such as fibronectin and vitronectin as well as adsorbed blood proteins (97). Components of the complement cascade can also bind to biomaterial surfaces and the complement cascade can potentially be activated by blood-contacting biomaterials. Binding could occur through recognition of C3b by receptors on macrophages. Surface topography has also been reported to have an effect on macrophage adhesion. In a study using the macrophage cell line P388D1, 44-nm deep grooves increased cell adhesion compared to smooth surfaces. The authors claimed that cell adherence was both groove depth and pitch dependent with more cells found on substrata with small pitches (98).

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1.7.2 Contact guidance

Contact or topographic guidance occurs when surface features such as grooves and ridges of micromachined substrata direct cell alignment and locomotion (99, 100). This tendency of topographic influence on cell morphology, spreading, and locomotion is called "contact guidance" (Weiss 1959, Brunette 1999). Reports from *in vitro* studies have demonstrated that fibroblasts align themselves parallel to grooves on the substratum (3, 52). This cell orientation in turn affects orientation of ECM deposition (101). Cell orientation as determined by surface topography guides ECM alignment and deposition, and can also alter gene expression (8, 9).

Osteoblasts also exhibit contact guidance. On grooved substrata the cells migrate parallel to the grooves. On the other hand, the cells migrate randomly on a rough topography (SLA). Time-lapse movies showed that osteoblasts enter the pits of the SLA surface and are trapped in them at least for short periods before they migrate in another direction (102).

Macrophages also demonstrated contact guidance in that they were oriented by multiple grooves, showed spreading and migration along the groove/ridge edges. These cells also exhibited significantly faster movement and traveled longer distance on grooved substrata than on plain ones. However, restricted movement and shorter travel distance was demonstrated on 5 μ m deep grooves compared to 0.5 μ m shallow grooved substrata (103).

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Although topographic guidance was one of the first cell behavior observed *in vitro*, major advances were made possible by the introduction of micromachined and nanofabrication methods (3, 104). Surfaces produced using this methodology made it possible to investigate the effect of precisely defined surface topographies on cell behavior in both *in vitro* and *in vivo* experimental models.

1.7.3 Cell selection

Variations in cellular adhesion to topography indicate that some cell types prefer to attach to and accumulate on certain topographies and shun others. For example, osteoblasts and macrophages favor rough surfaces, a behavior termed (rugophilia) (105, 106). In contrast, fibroblasts and epithelial cells prefer smooth surfaces (107).

Cell selection can be modulated by the size of the topographical feature. For instance certain topographies can exclude cells above certain dimension(s) (108). Fabrication methods developed for the microelectronics industry provide micro-machined topographies with precisely defined features, a variety of sizes of pits or pores that can be employed in cell selection (3, 42). Similarly, cell traps produced by micropatterning can be utilized for cell selection where these traps direct cells to a location from which they can't escape (109).

1.7.4 Cell differentiation

Topographic features can affect cell phenotype of the attached cells most likely by altering cell shape leading to changes in gene expression and cell differentiation (46, 47). Osteoblasts are critical for implant osseointegration because they synthesize and produce ECM and control its mineralization. Surface topography can modulate such osteoblast activity through a change in cell shape (110). Osteoblasts exhibited increased matrix secretion and mineralization when cultured on rougher surfaces ($R_a \approx 4 \mu m$) (2). Similarly when monocytes and macrophages were challenged with titanium particles 1-3 μm in diameter, translocation of the transcription factor nuclear factor kappa-beta (NF-k β) and secretion of pro-inflammatory cytokines were increased (9, 111). In addition, studies utilizing fixed surfaces, the SLA surfaces increased LPS-stimulated macrophage's production of pro-inflammatory cytokines when compared to a P surface (7). How cell size and shape alterations exhibited by macrophages exposed to different surface topographies alter signaling pathways that are involved in the macrophage's phenotypic secretory functions has not been determined.

1.7.5 Cell types reacting to surface topography

There are many cell types used in the *in vitro* investigation of cell responses to surface topography. Table 1.1 presents some of these cell types.

Cell type	Reference
Chondrocytes	(103, 112).
Endothelial cells	(113)
Epithelia	(4, 42, 104, 114, 115).
Eukaryotic cells	(116).
Fibroblasts	(52, 115, 117).
Keratinocytes	(54, 118).
Keratocytes	(119).
Macrophage	(7, 53, 98).
Mesenchymal stem cells	(120).
Mesenchymal progenitors	(121).
Monocytes	(122)
Myoblasts	(123).
Neurons	(124).
Neutrophils	(125, 126).
Osteoblasts	(4, 110, 127, 128).
Osteocytes	(129).
Smooth muscle cells	(130).
Platelets	(131).

Table 1.1: Cell types reacting to surface topography.

1.8 The macrophage

1.8.1 Origin and subpopulations

Macrophages are considered a mature component of the mononuclear phagocyte system (MPS) as described in the nomenclature classification established by Van Furth in 1972 (132, 133). The precursors of macrophages originate from the bone marrow. Differentiation induced by interleukins-1 (IL-1), IL-3, and/or IL-6 in bone marrow stem cells give rise to a new stem cell and a pluripotent myeloid cell known as a granulocyteerythrocyte-megakaryocyte-macrophage colony forming unit (GEMM-CFU). These cells in the presence of IL-1 and/or IL-3 then differentiate into progenitor cells of both macrophages and granulocytes called granulocyte-macrophage colony-forming unit, (GM-CFU) (134, 135). Macrophage colony-stimulating factor (M-CSF) induces the proliferation and differentiation of these progenitors into monoblast cells that further develop to promonocytes (136). Bone marrow monocytes differentiate from the promonocytes and enter the blood circulation (137, 138). They remain in the circulation for some time before they settle into their targeted body tissue or organ where they fully differentiate to resident macrophages in the absence of an endogenous inflammatory stimulus (139). As they migrate to different tissues they acquire different characteristic morphological and functional diversification according to their location and the microenvironment (140, 141). Macrophage populations in different body tissues are known by different names. For example those resident in lungs are called alveolar macrophages. Those associated with nervous system and nerve tissues are called microglia while those found in the liver are called kupffer cells (140). Macrophages can be thought of as divided into three main subpopulations: resident macrophages (socalled histiocytes), exudate macrophages, and cells that differentiate into dendritic cells (142). This unique proliferation, differentiation and tissue localization of macrophages is modulated by different growth factors and cytokines such as macrophage- colony stimulating factor (M-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), tumor necrosis factor- α (TNF- α), and the chemokines (CCL 14, 15,16, and 19) (137, 143-145). A diagrammatic illustration of these stages and factors involved is shown in figure 1.1.

In vivo studies showed that the newly recruited monocytes are of two subsets. One is strongly positive for the cluster of differentiation14 (CD14) cell surface molecule and negative for CD16 and is designated the CD14++ CD16- monocyte. These cells have a short life span and are the monocytes recruited to inflamed tissues. The second set of cells, designated CD14+ CD16+, have a slow turnover and are the monocytes recruited to non-inflamed tissues (146-148). The pattern of surface antigens seen in tissue macrophages in many respects resembles those of the tissue monocytes (149).



Figure 1.1: Differentiation of stem cells to monocytes/macrophages.

Growth factors involved in each stage are indicated with arrows. Oblique arrows indicate the points where derivations to other lineages are generated. The name of each derived lineage is also indicated. (Modified from (137)).

1.8.2 Macrophage markers and identification

Macrophages are identified by the expression of unique cell surface and cytoplasmic molecules recognized by specific antibodies. These molecules differ from species to species. Antigens among macrophages of the same species also differ according to the type of tissue where they reside and the microenvironments surrounding them. For example, the monoclonal antibody F4/80 is specific for the F4/80 membrane mouse macrophage antigen. The majority of macrophages (but not all) express this antigen (150). Other general macrophage markers include the functional pattern recognition receptors. Examples of these are the scavenger receptor family (SR-A) that consists of MARCO and CD36 receptors (151). These receptors are used by macrophages to recognize different bacterial species. A macrophage receptor called the mannose receptor is involved in the clearance of inflammatory agents and molecules (152). The other common pattern recognition receptors found on macrophage are the Toll-like receptors (TLRs). The ten human TLRs are membrane and intra-cellular receptors. Each of the membrane receptors is consisting of an extracellular ligand-binding region and an intracellular signaling domain (153).

In addition to the above receptors there are certain receptors involved in phagocytosis namely vitronectin receptor ($\alpha v\beta$ 3-integrin), phosphatidyl serine receptor (PSR) (154, 155), and β 2-glycoprotein I receptor (β 2-GPIR) (156). Human macrophages also express other antigens such as CD68 and CD163 (157). The rat macrophage expresses antigens such as complement receptor-3, sialoadhesin, ED1, and ED2. ED1 and ED2 utilized in this thesis for *in situ* localization are the rat homologue of the human CD68 and CD163, respectively (158). ED1 antigen is a heavily N-glucosylated protein of 110

kDa located on lysosomal membranes. It is identified by the ED1 monoclonal antibody and gives a patchy granular cytoplasmic appearance upon staining (159, 160). ED2 monoclonal antibody is specific for ED2 antigen, which is a 175kDa cell surface membrane antigen (160). The functions of ED1 and ED2 antigens are still unknown. However, it has been suggested that ED1 may have a role in phagocytosis (159). During inflammation, ED1 is expressed by newly recruited macrophages; whereas ED2 is expressed by mature resident macrophages (161). The expression of these antigens is assumed to be related to both the stage of resident macrophage differentiation and the surrounding microenvironment (159, 160, 162).

1.8.3 Macrophage activation, regulation and biological functions

Upon stimulation, macrophages display diverse activities not restricted to a specific single set of functions. Rather they display a progression of functional changes in response to changes in their microenvironment (163). Macrophages have a remarkable plasticity to adapt to the changes in their microenvironment and to display unique tissue-specific or response-specific functional profiles. Macrophages thus exhibit specific phenotypes for each specific homing situation (164). Macrophage phenotypes can be diametrically opposed such as pro-inflammatory versus anti-inflammatory activities and tissue destructive versus tissue reparative functions (165, 166). Resident macrophages, when activated, are involved in the process of defense against injury and invading microorganisms (140). In this concept of macrophage activation, macrophages secrete pro-inflammatory mediators as well as eliminate phagocytosed pathogens. This form of activation, dependent on two signals, was among the first discovered, and is thus termed "classical activation". The first signal is the cytokine interferon-γ (IFN-γ) produced by Th1

and natural killer cells. IFN-y does not activate macrophages but it primes them for activation by the second signal cytokine TNF- α or LPS (167). Classically activated M1 murine macrophages have a major role in the immune response by releasing proinflammatory cytokines such as IL-1, IL-6, and TNF- α , which then activate other cells involved in the innate and adaptive immune responses (168). These macrophages also produce select chemotactic proteins of the C-C chemokine subfamily and the CXC chemokine family such as expression and cellular localization (CCL2) (Monocyte chemotactic protein-1 (MCP-1)) and CCL3 macrophage inhibitor protein $1-\alpha$ (MIP1- α)), which facilitate recruitment of macrophages to areas of inflammation. (169) A second type of macrophage activation is termed "alternative activation" of macrophages. The cytokines responsible for this activation are interleukin-4 (IL-4) and/or IL-13, which are produced by Th2 cells. The alternative activation phenotype, (M2a and M2b cells) has diverse biological roles that differ from the classically activated macrophages and entails producing anti-inflammatory cytokines (170). Furthermore, alternatively activated macrophages have the ability to mediate wound healing, angiogenesis, and ECM deposition (171). Thus, it is speculated that alternatively activated macrophages apparently have the potential to serve a regulatory and recovery function rather than a killing function that is characteristic of classically activated macrophages.

1.8.4 Macrophage activation signals

Macrophages can respond to signals originating from other cells or from the surrounding environment. Typically there are at least six different receptor systems on the cell's membrane that can be activated (172). Cells can sense electromagnetic signals (such as light), signals from biological mediators such as growth factors and hormones, and

mechanical signals such as touch. Most of the signal transduction pathways (the processes that involve a series of steps by which a signal on a cell's surface is converted into a specific cellular response) are initiated due to interactions between a ligand and receptor. Cell signaling involves three stages. The first, reception, is the target cell's detection of a signal coming from outside the cell by a specific receptor. Most signal receptors are plasma membrane proteins. They are divided into three major types: G-protein-linked receptors, tyrosine-kinase receptors, and ion-channel receptors. The second stage, transduction, requires a sequence of changes in a series of different molecules, (a so called signal transduction pathway). Signal amplification occurs in such pathways, whereby some signal molecules in the pathway transmit the signal to multiple molecules in the next component of the series so that a large number of molecules can be activated at the end of the pathway. In these signaling pathways protein phosphorylation plays a major role in the mechanism of signal transduction (51). The final stage, *response*, is the endpoint or the "output response" of the process whereby the transduced signal finally triggers a specific cellular response. It could be any type of cellular activity in the cytoplasm such as regulation of enzyme activity, a change in the cell's metabolism or rearrangement of the cytoskeleton, or secretion of pro-inflammatory cytokines. The final activated molecule in a signaling pathway may function as a transcription factor that turns a gene on or off. Often a transcription factor regulates many different genes.

Cellular signal transduction from external stimulation is coordinated and modulated by protein tyrosine kinases (PTK)'s. The outcomes of these interactions are biochemical changes, which are processed and delivered to the nucleus to modulate gene expression. (173).

It has also been suggested that mechanical forces from cellular binding of surface topography can lead to activation of intracellular signals by integrin clustering at the focal adhesions and as such interaction with cytoskeletal and catalytic proteins (174). The topography of a titanium surface alters cell attachment patterns and shape. Cell shape changes may in turn affect cell membrane receptors and trigger signaling pathways involved in the activation of transcriptional factors and gene expression leading to macrophage activation. This hypothesis has not been investigated to date.

A number of signaling pathways are involved in macrophage activation; some proteins of which will be discussed below:

1.8.4.1 Focal adhesion kinase (FAK)

FAK is a 120kDa non-receptor tyrosine kinase. It was first identified as a protein phosphorylated in response to Src transformation in v-Src-transformed fibroblasts (175). FAK localizes in focal adhesions, which are known as sites of cellular interaction with the ECM through the focal-adhesion-targeting sequence (176). During FA assembly, tyrosine phosphorylation is one of the key signaling events. Recruitment of FAs proteins such as FAK, talin, vinculin, and paxillin to FAs is an essential step preceding FAK tyrosine phosphorylation (177). FAK, talin, vinculin, and paxillin link the integrin receptors and the cytoplasmic actin cytoskeleton (178). FAK is implicated in signaling cascades involved in cell motility, migration, proliferation, and apoptosis (179). It is suggested that integrins could directly interact with FAK during FAK phosphorylation. In this instance FAK phosphorylation is initiated by integrin clustering at the focal adhesions upon engagement of their extracellular matrix protein ligands during formation of focal

adhesions and cell spreading (180). Transmembrane permutations that induce clustering enhance adhesion and FAK phosphorylation (181), a process known as integrinmediated FAK phosphorylation. The second mechanism of FAK activation is mediated by integrin through G-protein coupled receptors (GPCR) and depends on Src family kinase members (182). In a variation of this theme, the N-terminal domain of FAK contains a putative binding site stimulated by integrin activation. This FAK N-terminal domain could bind to peptides mimicking beta integrins cytoplasmic domains that in turn, activate G- proteins intracellularly (183).

FAK tyrosine phosphorylation is postulated to play a role in signal transduction through the focal adhesion complex, which, as mentioned above, is triggered by diverse extracellular signals (184). The major autophosphorylation site Tyr³⁹⁷ of FAK is located in the kinase domain of the N-terminal of the FAK molecule (176). Activation of FAK at this site creates a specific docking binding site for Src family kinases through Src homology domain 2 (SH2)-mediated interactions (185). Fyn (186), Phospholipase C-y1 (PLC- g1), growth-factor-receptor bound protein 7 (Grb7), suppressor of cytokine signaling (SOCS). and Src-homology containing proteins (187-190) can all bind to this site. Autophosphorylation of the Tyr³⁹⁷ site on FAK recruits more Src and Src-like kinases through their SH2 domains (185). The binding between FAK and Src is called FAK-Src complex formation. Binding of the SH3 domain of Src and stabilization of FAK-Src complex is facilitated by a proline-rich sequence upstream of Tyr³⁹⁷ site (189, 191). Upon binding to FAK, Src phosphorylates additional tyrosine sites on FAK namely, Tyr⁵⁷⁶, Tyr⁵⁷⁷, and Tyr⁹²⁵ (188). Maximum activation of FAK is observed with phosphorylation of Tyr⁵⁷⁶ and Tyr⁵⁷⁷ residues located in the activation loop of FAK, while activation of FAK phosphorylation at Tyr⁹²⁵ residue creates binding sites for integrin associated proteins

such as talin and paxillin, and the SH2 domain of Grb2 and small GTPases such as R-Ras. This latter binding links FAK to mitogen-activated protein kinase (MAPK), inducing activation of the Ras-ERK1/2 signaling pathway (192, 193). Furthermore, the ERK/MAPK signaling pathway plays an important role in regulating focal adhesion dynamics and cell motility (194). In this instance, ERK is recruited to FAs in response to several stimuli. One such stimulus is v-Src activation that is primarily activated by FAK phosphorylation (195). Cell migration induced by PDGF and EGF depends on localized cell process de-adhesion from the surface that requires ERK/MAPK signaling at FAs (196). FAK-Src binding complex and activation sites are illustrated in figure 1.2.



Figure 1.2: FAK-Src complex formation and phosphorylation sites.

1: Inactive FAK during FAs assembly. 2: Maturation of FAs, integrin clustering activates autophosphorylation of FAK at Tyr 397. 3: Integrin mediated FAK phosphorylation initiates Src recruitment and phosphorylation at Tyr 416, then it binds to FAK. 4: FAK-Src complex formation leads to further phosphorylation of FAK at Tyr 567, Tyr 577, Tyr 862, and Tyr 925 phosphorylation sites were it links it to ERK/MAPK signaling pathway. 5: FAs dis-assembly, cell de-adhesion, and cell migration. (Modified from (197)).

Overall, FAK is involved in the regulation of focal adhesion turnover and cell survival. Inhibition of FAK signaling leads to cell apoptosis (198), while over-expression reduces cell apoptosis (199). A diagrammatic illustration of FAK structure with three main domains and phosphorylation sites is shown in figure 1.3.



Figure 1.3: Domain structure and phosphorylation sites (P) of FAK molecule.

Focal adhesion kinase (FAK) contains a FERM domain, a kinase domain and a focal adhesion-targeting (FAT) domain. The FERM domain mediates interactions of FAK with the epidermal growth factor (EGF) receptor; platelet-derived growth factor (PDGF) receptor. The Ezrin and the FERM domain can be conjugated to SUMO (small ubiguitinrelated modifier) at Lys152. The FAT domain recruits FAK to focal contacts by associating with integrin-associated proteins such as talin and paxillin. It also links FAK to the activation of Rho GTPases by binding to guanine nucleotide-exchange factors (GEFs) such as p190 RhoGEF. FAK contains three proline-rich regions (PRR1-3), which bind Src-homology-3 (SH3) domain-containing proteins such as p130Cas, the GTPase regulator associated with FAK (GRAF) and the Arf-GTPase-activating protein ASAP1. FAK is phosphorylated (P) on several tyrosine residues, including Tyr397, 576, 577, 861 and 925. Tyrosine phosphorylation on Tyr397 creates Src-homology-2 (SH2) binding site for Src, phospholipase C gamma (PLC γ), suppressor of cytokine signaling (SOCS), growth-factor-receptor-bound protein 7 (GRB7), the Shc adaptor protein, p120 RasGAP and the p85 subunit. FAK phosphorylation at Tyr925 creates a binding site for GRB2. (Reproduced from (181) with permission).

1.8.4.2 Src family kinases

There are a number of (52-62 kDa) proteins identified as members of the Src family kinases: Fyn, Yes, Blk, Fgr, Hck, Lck, Lyn, and the Frk subfamily proteins Frk/Rak and lyk/Bsk. These proteins have similar amino acid sequence homology to Src (200, 201). Src is a non-receptor protein kinase first discovered in Rous sarcoma virus (RSV), a chicken tumor virus where it is known as v-Src (a viral protein) (202). Generally Src is bound to endosomes, perinuclear membranes, secretory vesicles, and the cytoplasmic face of the plasma membrane where it can be activated with a wide range of growth factors and integrin receptors(203). The Src protein molecule includes six distinct functional domains as illustrated in figure 1.4: Src homology domain 4 (SH4); a unique domain at the N-terminal segment; SH3 domain; SH2 domain; a catalytic domain Src homology domain 1 (SH1); and a short C-terminal negative regulatory tail (200, 204, 205). In the dormant form of the enzyme, the tyrosine 527 (Tyr^{527}) binds intra-molecularly with the Src SH2 domain (205). Upon Src activation, the pTyr⁵²⁷ residue, located six residues from the C-terminus, is the primary site of tyrosine phosphorylation and in vivo up to 95% of Src is phosphorylated at this site (206). Kinase activity is also promoted by Src autophosphorylation at the Tyr⁴¹⁶ site; which is present at the activation loop (205). Activation of other Src kinases such as the cytoplasmic protein tyrosine kinase Cterminal Src kinase (CsK), involves the phosphorylation of the regulatory C-terminal tyrosine of Src (207).

Src kinases are activated through receptor protein kinases, integrin receptors, G-proteincoupled receptors, cytokine receptors, and steroid hormone receptors (203). In the case of platelet derived growth factor (PDGF) receptor protein-tyrosine kinase mediates other Src phosphorylation at residues Tyr²¹³ in the SH2 domain and Tyr¹³⁸ in the SH3 domain (208). In addition to tyrosine residues, Src is activated and phosphorylated at serine and threonine residues (Ser¹², Ser¹⁷, Ser⁴⁸, & Ser⁷²) and (Thr³⁴ & Thr⁴⁶) respectively (209, 210). Src signals to different downstream effectors including p85 (the regulatory subunit of phosphatidylinositol 3-kinase- (PIP-3), RasGAP, phospholipase-Cγ, some integrin signaling proteins (tenesin, vinculin, paxillin), as well as interacting back to FAK (200). Furthermore, Src is involved in the activation and phosphorylation of the signal transducer and activator of transcription3 (STAT3) tyrosine in a JAK-independent manner and further activates the DNA binding and transcriptional gene expression (211).

Src family kinases are involved in cell cytoskeletal alteration, differentiation, motility, proliferation, and survival (200). Hck, Lyn, and Fgr Src family tyrosine kinases are implicated in the biological modulation of the TLR signaling pathway in LPS-activated macrophages responses and production of pro-inflammatory cytokines. Monocytes treated with the Src tyrosine kinase inhibitor, pyrazolopyrimidine (PP1), exhibited reduced LPS-induced biological responses (212). In addition, Src knockout macrophages exhibited suppressed secretion of the pro-inflammatory cytokines IL-1 and TNF- α (213). As mentioned previously a signaling complex can form between activated Src family members and FAK that further stimulates FAK activity on downstream substrates such as the small G protein Ras, and then ERK1/2. The FAK–Src complex mediates the phosphorylation of paxillin and p130 Crk-associated substrate (CAS). Paxillin and p130CAS can recruit other molecules to adhesions and regulate the organization of the actin cytoskeleton and control cell spreading and migration (214, 215). This activation of p130CAS also phosphorylates the MAPK/ERK that is involved in the phosphorylation of downstream substrates myosin light chain kinase (MLCK) and

myosin light chain (MLC), which in turn mediates FA formation and adhesion disassembly (216). Thus FAK, Src, p130CAS, paxillin, ERK and MLCK are required for FA function (194).

Implant surface topography is involved in the formation of such binding complexes and the phosphorylation of downstream signaling substrates. From the forgoing it can be appreciated that many signaling pathways are either originated or modulated by events at cell attachment sites, in particular focal adhesions. Surface topography can modulate the number and surface distribution of FA.



Figure 1.4: Domain structure and phosphorylation sites (P) of Src kinase molecule. The Src protein molecule includes six distinct functional domains: Src homology domain 4 (SH4); a unique domain at the N-terminal segment; SH3 domain; SH2 domain; a catalytic domain Src homology domain 1 (SH1); and a short C-terminal negative regulatory tail. Tyr 527 site is located at the C-regulatory tail. The SH1 contains the binding site of Tyr 416 that autophosphorylates upon binding to FAK. The SH2 and SH3 domains contain respectively the Tyr 213 and Tyr 138 that subsequently are phosphorylated in association with PDGF receptor. At the unique domain, Src phosphorylates at threonine sites (T34, T46, and T72) by a cyclin-dependent kinase 1 (CDK1) and also phosphorylates at serine site 12 (S12) during protein kinase C activation. Furthermore, Src phosphorylation occurs at S17 through protein kinase A (PKA). (Modified and reproduced from (204, 205) with permission).

1.8.4.3 Extracellular regulated signal protein kinase (ERK1/2)

Mitogen-Activated Protein Kinases (MAPKs) were members of the first signaling cascade studied and are probably the most thoroughly investigated. There are three major groups of MAPKs which constitute more than twelve proteins: the p38 family with four members; (p38a, p38b, p38γ, and p38δ) share similar structural homology (217), the c-Jun NH2-terminal kinase family (JNK1, JNK2, and JNK3) (218, 219), and ERK1 and ERK2 that have similar structures and functions (219, 220).

All the above mentioned signaling components operate in concert, upon activation of certain membrane receptors, and thus form a complex network of interacting proteins that regulate cellular processes. Generally, all MAPKs depend on dual phosphorylation sites for their activation by different stimuli, for example, the threonine (Thr) and Tyrosine (Tyr) residues located on selected motifs for each individual kinase group such as Thr-Glu-Tyr for ERK1/2 (217, 221). In the inactive form ERK1/2 localizes to the cytoplasm. This localization is mediated and stabilized by its association with MEK1/2.

There are a variety of stimuli that activate MAPK such as growth factors, inflammatory cytokines, and cellular stress (222, 223). MAPK members can signal independently from each other manifesting specific and distinct physiologic responses (224). The activation of each of the MAPK cascades is initiated by small GTP binding proteins, or by adaptor proteins that transmit the signal to protein kinases. These are commonly referred to as MAPK kinase kinases (225). In each kinase-signaling cascade a downstream kinase serves as a substrate for the upstream activator. This direct enzyme-substrate interaction modulates signal transmission and substrate specificity.

The signaling Raf-MEK-ERK pathway was the first to be mapped from the upstream kinase at the cell membrane to the far end substrate in the nucleus (226). The small G protein Ras is the prime initiator for Raf activation and recruitment from the cytoplasm to the cell membrane. Upon activation Raf phosphorylates and activates MEK (227), which in turn phosphorylates ERK. MEK1/2 are the only known activators of ERK1/2 and this activation is directly regulated by the dual phosphorylation of Thr¹⁸³ and Tyr¹⁸⁵ (228). Activated ERK1/2 translocates to the nucleus, where it affects gene expression and phosphorylates transcription factors Ets, Elk, Myc, and SRF (229).

Human macrophages challenged with titanium particles exhibited higher levels of tyrosine phosphorylation and MAPK activation that is associated with increased release of TNF- α , IL-1, and IL-6 (230). Evidence from previous studies using several adherent cells showed that mechanical forces could activate these kinases By altering the pattern of cell adhesion, surface topography may influence these mechanical forces experienced by macrophage at the implant surface, and in turn the phosphorylation and activation of the ERK1/2 signaling cascade (231, 232).

In summary, FAs are suggested to be involved in cell adhesion, proliferation and cell migration. Integrin clustering in these FAs will lead to autophosphorylation of FAK. Src phosphorylation and recruitment towards phosphorylated FAK will occur and a FAK-Src binding complex will be formed. Association between FAK and Src leads to phosphorylation of FAK on additional tyrosine residues and recruitment of additional structural and signaling molecules that contribute to the assembly of focal complexes. Furthermore, this association and further phosphorylation of more sites on FAK activate downstream substrates such as Shc and Ras, which are known components of the

ERK1/2-MAPK signaling pathway. The hypothesized cross-talk between FAK, Src, FAK-Src complex formation and the activation of ERK1/2-MAPK signaling cascade presented as FAK-mediated signaling is illustrated in figure 1.5.



Figure 1.5: Diagrammatic illustration of FAK-mediated signaling pathways.

Integrin clustering at focal adhesions initiates FAK autophosphorylation at Tyr 397, subsequently FAK phosphorylation provides a binding site for the Src SH2 domain, this binding leads to FAK-Src signaling complex molecule formation and FAK phosphorylation in additional sites such as Tyr576, Tyr577, and Tyr925. FAK phosphorylation at Tyr925 is suggested to link integrin-mediated FAK phosphorylation to downstream ERK-MAPK substrates.

The significance of signaling through membrane-associated integrins and FAK lies in the possibility that the topography of implant surface could modulate cell adhesion and alter cell shape. This in turn could affect integrin clustering at FAs/podosomes, which would trigger signaling pathways involved in the activation of transcriptional factors and gene expression leading to macrophage activation and functional specialization.

1.8.5 Macrophage responses to biomaterials

Monocyte-derived macrophages (MDMs) are able to respond to most biomaterial implants in *vitro* (53, 233, 234). This response is complicated by two factors, differentiation of macrophages from monocytes and subsequent macrophage response to the biomaterial. Few studies have been carried out on the effects of implant material on differentiation of macrophages. One study of note has reported that differentiation can be affected by a material surface. The authors reported that the material surface affected the morphology, phenotype, and degradative capacity of differentiating monocytes (235). The second factor, macrophage function can be influenced by the size of the material, chemical composition, and topography.

Macrophages incubated with titanium, polystyrene, polyethylene and poly (methyl methacrylate) particles responded by production of inflammatory cytokines and increased bone resorption. This response was size, composition and dose dependent (236-238). Such wear particles have been implicated in osteolysis around orthopedic implants. The proposed mechanism is the phagocytosis of these wear particles by macrophages leading to the production of bone resorbing cytokines (239).

It has also been shown that biomaterial surface chemistry has important ramifications on cellular and host responses. However, the underlying molecular mechanisms are poorly understood. Numerous studies systematically investigated cellular responses to surface chemistry. Implant surface chemical composition of the biomaterial affects hydrophilicity. Highly hydrophilic surfaces showed desirable interactions with biological fluids, cells and tissues compared to hydrophobic ones (63, 240, 241). A good example is a study showing that the complement component C3 increases the density of macrophages adhered to surface implant material (242). Furthermore, implants treated with an intact peptide growth factor, TGF- β , which is one of the major growth factors produced by macrophages increases the amount of bone deposition compared to untreated controls (243). Similarly, in a study investigating the effect of titanium, chromium and cobalt ions on the production of cytokines IL-1, IL-6, TNF- α , and transforming growth factor-beta1 (TGF- β 1) by LPS- stimulated macrophages, titanium increased production of IL-6 and TNF- α (compared to the other two materials) (244). Chemically altered high density polyethylenes also demonstrated differential responses in the production of proinflammatory cytokines (245). That changes in chemical composition has a clinically relevant effect is demonstrated by the finding that calcium phosphate coatings accelerate early healing and they bond to bone ((246).

Implant surface topography is a persistent factor that remains as long as the implant is *in situ*. Rough surface topography can affect cell behavior. Macrophages, for example, prefer rough surfaces to smooth ones; a phenomenon called Rugophilia (105). *In vitro* studies in our laboratory (7) carried out on the RAW 264.7 macrophage cell line attached to rough and smooth surfaces for 12, 24, and 48 hours showed the macrophages responded differently. Rough surfaces increased secretion of the proinflammatory

cytokine TNF- α compared to smooth ones. (7). Thus it is clear that surface topography plays a role in the macrophage response to biomaterials. One caveat in studying the effect of surface topography on macrophage function is that roughening procedures also alter the surface composition and surface energy of the produced topographies (66). It is difficult to produce substrata that vary in topography by common industrial processes without altering the substrate chemistry.

We can conclude that the surface chemical properties, as well as surface topographical features can modulate macrophage response and subsequent events involved in wound healing and bone formation at the implant-tissue interface.

1.9 Wound healing around implants and the role of macrophages

Immediately following implant insertion the surface of the implant will be occupied by a blood clot. It is postulated that rough surface topography provides a mechanical retention to this clot influencing the osteoconductive behavior of the cells in the implant vicinity, (247). The red blood cell and platelet recruitment increase with increased roughness (248). Platelets in the fibrin clot are enriched with osteogenic cytokines but lack osteoinductive cytokines. Degranulation of the adhered platelets appeared to produce high levels of PDGF (249), which is considered to be the initial chemoattractant mediator for leukocytes that are responsible for the secretion of pro-inflammatory cytokines. As mentioned above macrophages actively respond to almost all biomaterial implants *in vivo* such as metals, ceramics and cements, and polymers as they produce cytokines and growth factors (233, 250). Published data demonstrate that during post-biomaterial implantation, macrophages are the dominant infiltrating cells in soft and hard

tissues (250, 251). TGF- β produced by the implant-adherent macrophages increase the recruitment of mesenchymal cells that differentiate into osteoblasts (252). The influence of rough surface topography, the availability of high levels of osteoinductive cytokines produced by macrophages, and the presence of osteoblasts are factors influencing bone formation around the rough surfaced implant. A diagrammatic illustration of stages of wound healing is presented in figure 1.6.



Figure 1.6: Diagrammatic illustration of stages of wound healing. Adapted from (www.worldwidewound.com).

1.9.1 Role of macrophages in osseointegration

The term osseointegration was introduced and defined by Brånemark in the seventies (253). Osseointegration refers to the firm anchoring of a surgical implant by the direct growth and contact of bone around it without intervening fibrous tissue formation at the interface as observed in the light microscope (254). Clinically, osseointegration suggests ankylosis of the implant-bone interface. This condition is achieved in various implant systems by the formation of an interlock between the implant surface features (threaded, porous, or textured) and the bone tissue. A general biomechanical definition of osseointegration has been accepted, "It is a process whereby clinically asymptomatic rigid fixation of implanted materials is achieved, and maintained, in bone during functional loading"(255, 256). The direct contact between the implant metallic surface and the bone with no existing space suggests the possibility of direct chemical bonding between tissue and the implant. In any case the close apposition of bone to implant allows the efficient transfer of force from implant to bone. If chemical bonding has occurred the displacement forces would be restricted to atomic distances within the chemical bonds (257).

Bone healing and/or new bone formation depends on cells of the osteoblastic lineage, which differentiate from pluripotent mesenchymal stromal cells. This differentiation to a bone-forming cell lineage is called osteoinduction (255, 258). Urist introduced the term osteoinduction when describing his experiments involving demineralized bone as an osteoinductive agent. The differentiation process of pluripotent mesenchymal stromal cells is modulated by osteoinductive signals that are provided by agents that are members of the TGF- β family of growth factors, such as bone morphogenetic proteins (BMPs) (259, 260).

The macrophage is an important cell in the osteogenic process. *In vitro* studies demonstrate that human and murine macrophages secrete the pro-inflammatory cytokines IL-1, IL-6 and TNF- α that stimulate osteoclastic bone resorption prior to bone remodeling (261). Macrophages also secrete BMP-2, BMP-6 and TGF- β (261, 262). Osteoblastic differentiation is stimulated by the anti-inflammatory cytokine TGF- β (262). There is also a large body of evidence to suggest that implant topography has a profound effect on osseointegration by affecting both bone formation and fixation strength (254, 263). An osteoconductive surface is one that permits bone growth on its surface or down into pores, channels, and pits (255). The role of macrophages and the signaling molecules involved in osteoinduction, osteoconduction, and osseointegration demonstrated by certain implant surface topographies is not fully understood.

1.10 In vitro and in vivo experimental studies

Most experimental evaluation of surface topography is initially carried out *in vitro* followed by *in vivo* studies using animal models. A rat model will be used in this project to investigate the functions of macrophages at the implant surface.

The selection of a species for study is based on many factors. The rat is a model that is used extensively in the study of human disease. For example a rat model has been used to study the role of macrophages in infectious diseases such as acute meningitis and pneumonitis (264, 265). The model is also used in the study of some autoimmune diseases like anterior uveitis (Endotoxin Induced Uveitis: EIU) (266). In addition to infectious and autoimmune diseases, rats are used in various models to understand the role of macrophages in organ transplantation such as lung, liver, heart and cornea (267, 268). Furthermore, the use of rats has increased our knowledge of the role that macrophages play in the immune response to tumors (269).

Immunological analysis in the rat has been facilitated by the availability of specific antibodies to many leukocyte populations as well as cell depletion technologies. In the case of macrophage dependent responses there are a number of antibodies that specifically identify macrophage subpopulations. As mentioned previously the rat macrophage expresses many different antigens. During inflammation, ED1 is expressed by newly recruited macrophages, whereas ED2 is expressed by mature resident macrophages (161) allowing workers to dissect the role of specific macrophage phenotypes in inflammatory responses. A second technology has seen the development of methods of selective macrophage depletion by administration of clodronate-containing liposomes. Both these technologies have contributed to the increased insight in the role of macrophage in various disease models (160).

Surgically the rat has many advantages over the mouse; the primary one being its size that makes the performance of microsurgical manipulations easier. For these reasons the rat was used for our *in situ* studies

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1.11 Statement of the problem

1.11.1 Background

The success or failure of a biomaterial implant depends in part on the cellular interactions that occur at the bone-implant interface. Host reactions to an implant include surgical injury, acute inflammation, chronic inflammation (foreign body response), and possible fibrous capsule development. These processes are highly regulated and involve a complex interplay between many different cell types. At the later stages of chronic inflammation and the early stages of endosseous wound healing, macrophages play a pivotal role in the clinical sequelae through production of cytokines and growth factors that initiate tissue destruction and/or reparative responses.

1.11.2 Hypothesis

The central hypothesis of this thesis is that surface topography of commonly used dental implants can modulate macrophage morphology, cytoskeletal patterning and subsequent activation of signaling cascades. The following sub-hypotheses are addressed in their respective chapters.

- 1. In Chapter 2 we hypothesized that surface topography would alter cell shape, the patterning of the cytoskeletal proteins and the cell number. Our objectives were:
- To investigate topographically activated macrophages in terms of changes to their morphology over time.

- To investigate topographically activated macrophages in terms of the changes to the appearance of their filamentous actin and the patterns of focal adhesions by vinculin staining over time.
- To investigate the effect of surface topography on macrophage cell number and a possible association with FAs/podosome disassembly and cell death.
- In Chapter 3 we hypothesize that surface topography would affect intracellular signaling molecules. Our objectives were:
- To investigate topographically activated macrophages in terms of the phosphorylation of tyrosine residues in general, as well as phosphorylation of the specific FAK, Src, and ERK1/2 signaling molecules.
- To investigate the linkage between the above three signaling molecules (induced by different surface topographies) by using the Src protein kinase inhibitors PP1 and PP2.
- 3. In Chapter 4 we hypothesize that surface topography would differentially affect accumulation of macrophages on implanted material *in situ*. Our objectives were:
- To develop an immunostaining technique for staining the ED1 and ED2 macrophage markers using a rat cell line.
- To demonstrate the presence of these macrophages at the tissue-implant interface in a rat model.
1.11.3 Rationale for the study

Since surface topography has the capacity to influence cell behavior, it is important to understand the responses of macrophages to different topographical cues *in vitro*. Such *in vitro* tests would enable the screening of topographies for their effects on macrophages before the surfaces were tested in animal models or clinical applications. Moreover, the in vitro approach provides an efficient method for designing topographies to produce desired responses.

1.12 References

- 1. Bigerelle, M., and Anselme, K. (2005) Statistical correlation between cell adhesion and proliferation on biocompatible metallic materials. *J Biomed Mater Res A* 72, 36-46
- 2. Boyan, B. D., Bonewald, L. F., Paschalis, E. P., Lohmann, C. H., Rosser, J., Cochran, D. L., Dean, D. D., Schwartz, Z., and Boskey, A. L. (2002) Osteoblastmediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int* 71, 519-529
- 3. Brunette, D. M., Kenner, G. S., and Gould, T. R. (1983) Grooved titanium surfaces orient growth and migration of cells from human gingival explants. *J Dent Res* 62, 1045-1048
- 4. Hamilton, D. W., Chehroudi, B., and Brunette, D. M. (2007) Comparative response of epithelial cells and osteoblasts to microfabricated tapered pit topographies in vitro and in vivo. *Biomaterials* 28, 2281-2293
- 5. Schuler, M., Owen, G. R., Hamilton, D. W., de Wild, M., Textor, M., Brunette, D. M., and Tosatti, S. G. (2006) Biomimetic modification of titanium dental implant model surfaces using the RGDSP-peptide sequence: a cell morphology study. *Biomaterials* 27, 4003-4015
- Dalby, M. J., Yarwood, S. J., Johnstone, H. J., Affrossman, S., and Riehle, M. O. (2002) Fibroblast signaling events in response to nanotopography: a gene array study. *IEEE Trans Nanobioscience* 1, 12-17
- 7. Refai, A. K., Textor, M., Brunette, D. M., and Waterfield, J. D. (2004) Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. *J Biomed Mater Res A* 70, 194-205
- Scotchford, C. A., Ball, M., Winkelmann, M., Voros, J., Csucs, C., Brunette, D. M., Danuser, G., and Textor, M. (2003) Chemically patterned, metal-oxide-based surfaces produced by photolithographic techniques for studying protein- and cellinteractions. II: Protein adsorption and early cell interactions. *Biomaterials* 24, 1147-1158
- Wong, N., Trindade, M. C., Patel, R., Yaszay, B., Goodman, S. B., and Smith, R. L. (2004) Effects of interleukin-10 on titanium particle-induced macrophage transcription factor activation and cytokine expression in vitro. *J Biomed Mater Res A* 69, 40-46
- 10. Agrawal, C. M. (1998) Reconstruction of the human body using biomaterials. *J. O. M.*, 31-35
- 11. Williams, D. F. (1981) Implants in dental and maxillofacial surgery. *Biomaterials* 2, 133-146

- 12. Williams, D. F. (1989) Biocompatibility considerations in the use of orthopaedic implant alloys. *Curr. Presp. on Implanta. Devi.* 1
- 13. Williams, D. F. (1987) Definitions in biomaterials, in progress in Biomedical Engineering.
- 14. Kokubo, T., Kim, H. M., Kawashita, M., and Nakamura, T. (2004) Bioactive metals: preparation and properties. *J Mater Sci Mater Med* 15, 99-107
- 15. Boyer, R., Welsch, G., and Collings, E. W. (1994) Materials properties handbook: titanium alloys; ASM international metals park, Ohio.
- 16. Goto, T., Yoshinari, M., Kobayashi, S., and Tanaka, T. (2004) The initial attachment and subsequent behavior of osteoblastic cells and oral epithelial cells on titanium. *Biomed Mater Eng* 14, 537-544
- 17. Meffert, R. M., Langer, B., and Fritz, M. E. (1992) Dental implants: a review. *J Periodontol* 63, 859-870
- 18. Ryan, G., Pandit, A., and Apatsidis, D. P. (2006) Fabrication methods of porous metals for use in orthopaedic applications. *Biomaterials* 27, 2651-2670
- 19. Brunette, D. M., Tengvall, P., Textor, M., and Thomsen, P. (2001) Titanium in medicine. *Springer, Berlin.*
- 20. Steinemann, S. G. (1998) Titanium--the material of choice? *Periodontol 2000* 17, 7-21
- Johansson, C., Lausmaa, J., Ask, M., Hansson, H. A., and Albrektsson, T. (1989) Ultrastructural differences of the interface zone between bone and Ti 6AI 4V or commercially pure titanium. *J Biomed Eng* 11, 3-8
- 22. Lausmaa, J. (1996) Surface spectroscopic chracterization of titanium implant materials. *J. Electron. Spectrosco. Relat. Phenom.* 81, 343-361
- 23. Sittig, C., Textor, M., Spencer, N. D., Wieland, M., and Vallotton, P. H. (1999) Surface characterization of implant materials c.p. Ti, Ti-6AI-7Nb and Ti-6AI-4V with different pretreatments. *J Mater Sci Mater Med* 10, 35-46
- 24. Tengvall, P., and Lundstrom, I. (1992) Physico-chemical considerations of titanium as a biomaterial. *Clin Mater* 9, 115-134
- 25. Fujibayashi, S., Neo, M., Kim, H. M., Kokubo, T., and Nakamura, T. (2004) Osteoinduction of porous bioactive titanium metal. *Biomaterials* 25, 443-450
- 26. Hanawa, T. (1999) *In vivo* metallic biomaterials and surface modification. *Mater. Sci. Eng.* A276, 260-266
- Costa Cde, A., Sena, L. A., Pinto, M., Muller, C. A., Cavalcanti, J. H., and Soares Gde, A. (2005) In vivo characterization of titanium implants coated with synthetic hydroxyapatite by electrophoresis. *Braz Dent J* 16, 75-81

- 28. Perizzolo, D., Lacefield, W. R., and Brunette, D. M. (2001) Interaction between topography and coating in the formation of bone nodules in culture for hydroxyapatite- and titanium-coated micromachined surfaces. *J Biomed Mater Res* 56, 494-503
- 29. Rouahi, M., Gallet, O., Champion, E., Dentzer, J., Hardouin, P., and Anselme, K. (2006) Influence of hydroxyapatite microstructure on human bone cell response. *J Biomed Mater Res A* 78, 222-235
- 30. Ban, S., and Hasegawa, J. (2002) Morphological regulation and crystal growth of hydrothermal-electrochemically deposited apatite. *Biomaterials* 23, 2965-2972
- 31. Kawashita, M., Itoh, S., Miyamoto, K., and Takaoka, G. H. (2008) Apatite formation on titanium substrates by electrochemical deposition in metastable calcium phosphate solution. *J Mater Sci Mater Med* 19, 137-142
- 32. Lopez-Heredia, M. A., Weiss, P., and Layrolle, P. (2007) An electrodeposition method of calcium phosphate coatings on titanium alloy. *J Mater Sci Mater Med* 18, 381-390
- Davis, D. H., Giannoulis, C. S., Johnson, R. W., and Desai, T. A. (2002) Immobilization of RGD to < 1 1 1 > silicon surfaces for enhanced cell adhesion and proliferation. *Biomaterials* 23, 4019-4027
- Ferris, D. M., Moodie, G. D., Dimond, P. M., Gioranni, C. W., Ehrlich, M. G., and Valentini, R. F. (1999) RGD-coated titanium implants stimulate increased bone formation in vivo. *Biomaterials* 20, 2323-2331
- Bessho, K., Carnes, D. L., Cavin, R., Chen, H. Y., and Ong, J. L. (1999) BMP stimulation of bone response adjacent to titanium implants in vivo. *Clin Oral Implants Res* 10, 212-218
- Boyne, P., and Jones, S. D. (2004) Demonstration of the osseoinductive effect of bone morphogenetic protein within endosseous dental implants. *Implant Dent* 13, 180-184
- Liu, Y., de Groot, K., and Hunziker, E. B. (2005) BMP-2 liberated from biomimetic implant coatings induces and sustains direct ossification in an ectopic rat model. *Bone* 36, 745-757
- Wikesjo, U. M., Sorensen, R. G., Kinoshita, A., and Wozney, J. M. (2002) RhBMP-2/alphaBSM induces significant vertical alveolar ridge augmentation and dental implant osseointegration. *Clin Implant Dent Relat Res* 4, 174-182
- Giavaresi, G., Fini, M., Cigada, A., Chiesa, R., Rondelli, G., Rimondini, L., Torricelli, P., Aldini, N. N., and Giardino, R. (2003) Mechanical and histomorphometric evaluations of titanium implants with different surface treatments inserted in sheep cortical bone. *Biomaterials* 24, 1583-1594

- 40. lezzi, G., Scarano, A., Petrone, G., and Piattelli, A. (2007) Two human hydroxyapatite-coated dental implants retrieved after a 14-year loading period: a histologic and histomorphometric case report. *J Periodontol* 78, 940-947
- 41. Schroeder, A., and Buser, D. A. (1988) ITI-system. Basic and clinical procedures. *Shikaku-Odontol.* 77, 1267-1288
- 42. Brunette, D. M. (1986) Spreading and orientation of epithelial cells on grooved substrata. *Exp Cell Res* 167, 203-217
- 43. Curtis, A. S. G., and Clark, P. (1990) The effects of topographic and mechanical properties of materials on cell behaviour. *Crit. Rev. Biocompat.* 5, 343-362
- 44. Gronowicz, G., and McCarthy, M. B. (1996) Response of human osteoblasts to implant materials: integrin-mediated adhesion. *J Orthop Res* 14, 878-887
- 45. Oakley, C., and Brunette, D. M. (1993) The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* 106 (Pt 1), 343-354
- Akiyama, S. K. (1996) Integrins in cell adhesion and signaling. *Hum Cell* 9, 181-186
- 47. Chicurel, M. E., Singer, R. H., Meyer, C. J., and Ingber, D. E. (1998) Integrin binding and mechanical tension induce movement of mRNA and ribosomes to focal adhesions. *Nature* 392, 730-733
- 48. Krause, A., Cowles, E. A., and Gronowicz, G. (2000) Integrin-mediated signaling in osteoblasts on titanium implant materials. *J Biomed Mater Res* 52, 738-747
- 49. Folkman, J., and Moscona, A. (1978) Role of cell shape in growth control. *Nature* 273, 345-349
- 50. Ben-Ze' ev, A. (1991) Animal cell shape changes and gene expression. *BioAssaya* 13, 207-212
- McCubrey, J. A., May, W. S., Duronio, V., and Mufson, A. (2000) Serine/threonine phosphorylation in cytokine signal transduction. *Leukemia* 14, 9-21
- 52. Brunette, D. M. (1986) Fibroblasts on micromachined substrata orient hierarchically to grooves of different dimensions. *Exp Cell Res* 164, 11-26
- 53. Takebe, J., Champagne, C. M., Offenbacher, S., Ishibashi, K., and Cooper, L. F. (2003) Titanium surface topography alters cell shape and modulates bone morphogenetic protein 2 expression in the J774A.1 macrophage cell line. *J Biomed Mater Res A* 64, 207-216
- 54. Watt, F. M., Jordan, P. W., and O'Neill, C. H. (1988) Cell shape controls terminal differentiation of human epidermal keratinocytes. *Proc Natl Acad Sci U S A* 85, 5576-5580

- 55. Bagno, A., and Di Bello, C. (2004) Surface treatments and roughness properties of Ti-based biomaterials. *J Mater Sci Mater Med* 15, 935-949
- Taborelli, M., Jobin, M., Francois, P., Vaudaux, P., Tonetti, M., Szmukler-Moncler, S., Simpson, J. P., and Descouts, P. (1997) Influence of surface treatments developed for oral implants on the physical and biological properties of titanium. (I) Surface characterization. *Clin Oral Implants Res* 8, 208-216
- 57. Francois, P., Vaudaux, P., Taborelli, M., Tonetti, M., Lew, D. P., and Descouts, P. (1997) Influence of surface treatments developed for oral implants on the physical and biological properties of titanium. (II) Adsorption isotherms and biological activity of immobilized fibronectin. *Clin Oral Implants Res* 8, 217-225
- Wennerberg, A., Hallgren, C., Johansson, C., and Danelli, S. (1998) A histomorphometric evaluation of screw-shaped implants each prepared with two surface roughnesses. *Clin Oral Implants Res* 9, 11-19
- 59. Wennerberg, A., and Albrektsson, T. (2006) Implant surfaces beyond micron roughness. Experimental and clinical knwledge of surface topography and surface chemistry. *App. Osseointegration Res.* 5, 40-44
- 60. Aparicio, C., Gil, F. J., Fonseca, C., Barbosa, M., and Planell, J. A. (2003) Corrosion behaviour of commercially pure titanium shot blasted with different materials and sizes of shot particles for dental implant applications. *Biomaterials* 24, 263-273
- 61. Zinger, O., Anselme, K., Denzer, A., Habersetzer, P., Wieland, M., Jeanfils, J., Hardouin, P., and Landolt, D. (2004) Time-dependent morphology and adhesion of osteoblastic cells on titanium model surfaces featuring scale-resolved topography. *Biomaterials* 25, 2695-2711
- 62. Trisi, P., Lazzara, R., Rao, W., and Rebaudi, A. (2002) Bone-implant contact and bone quality: evaluation of expected and actual bone contact on machined and osseotite implant surfaces. *Int J Periodontics Restorative Dent* 22, 535-545
- 63. Buser, D., Broggini, N., Wieland, M., Schenk, R. K., Denzer, A. J., Cochran, D. L., Hoffmann, B., Lussi, A., and Steinemann, S. G. (2004) Enhanced bone apposition to a chemically modified SLA titanium surface. *J Dent Res* 83, 529-533
- 64. Li, D., Ferguson, S. J., Beutler, T., Cochran, D. L., Sittig, C., Hirt, H. P., and Buser, D. (2002) Biomechanical comparison of the sandblasted and acid-etched and the machined and acid-etched titanium surface for dental implants. *J Biomed Mater Res* 60, 325-332
- Szmukler-Moncler, S., Perrin, D., Ahossi, V., Magnin, G., and Bernard, J. P. (2004) Biological properties of acid etched titanium implants: effect of sandblasting on bone anchorage. *J Biomed Mater Res B Appl Biomater* 68, 149-159

- 66. Wieland, M., Textor, M., Spencer, N. D., and Brunette, D. M. (2001) Wavelengthdependent roughness: a quantitative approach to characterizing the topography of rough titanium surfaces. *Int J Oral Maxillofac Implants* 16, 163-181
- 67. Sul, Y. T., Johansson, C., Wennerberg, A., Cho, L. R., Chang, B. S., and Albrektsson, T. (2005) Optimum surface properties of oxidized implants for reinforcement of osseointegration: surface chemistry, oxide thickness, porosity, roughness, and crystal structure. *Int J Oral Maxillofac Implants* 20, 349-359
- Sul, Y. T., Johansson, C. B., Jeong, Y., Wennerberg, A., and Albrektsson, T. (2002) Resonance frequency and removal torque analysis of implants with turned and anodized surface oxides. *Clin Oral Implants Res* 13, 252-259
- 69. Rocci, A., Martignoni, M., and Gottlow, J. (2003) Immediate loading of Branemark System TiUnite and machined-surface implants in the posterior mandible: a randomized open-ended clinical trial. *Clin Implant Dent Relat Res* 5 Suppl 1, 57-63
- 70. Sul, Y. T., Johansson, C. B., Roser, K., and Albrektsson, T. (2002) Qualitative and quantitative observations of bone tissue reactions to anodised implants. *Biomaterials* 23, 1809-1817
- 71. Cooper, L. F., Zhou, Y., Takebe, J., Guo, J., Abron, A., Holmen, A., and Ellingsen, J. E. (2006) Fluoride modification effects on osteoblast behavior and bone formation at TiO2 grit-blasted c.p. titanium endosseous implants. *Biomaterials* 27, 926-936
- 72. Ellingsen, J. E., Johansson, C. B., Wennerberg, A., and Holmen, A. (2004) Improved retention and bone-toImplant contact with fluoride-modified titanium implants. *Int J Oral Maxillofac Implants* 19, 659-666
- 73. Duncan, A. C., Weisbuch, F., Rouais, F., Lazare, S., and Baquey, C. (2002) Laser microfabricated model surfaces for controlled cell growth. *Biosens Bioelectron* 17, 413-426
- 74. Hao, L., and Lawrence, J. (2003) Effects of CO₂ Laser erradiation on the wettability and human skin fibroblast cell response of magnesia partially stabilized zirconia. *Mater. Sci. Eng.* C, 627-239
- 75. Nakayama, Y., and Matsuda, T. (1995) Surface microarchitectural design in biomedical applications: preparation of microporous polymer surfaces by an excimer laser ablation technique. *J Biomed Mater Res* 29, 1295-1301
- 76. Kurella, A., and Dahotre, N. B. (2005) Review paper: surface modification for bioimplants: the role of laser surface engineering. *J Biomater Appl* 20, 5-50
- 77. Karacs, A., Fancsaly, A. J., Divinyi, T., PetImsgge, G., and Kovach, G. (2003) Morphological and animal study of titanium dental implant surface induced by blasting and high intensity pulsedNd-glass laser. *Mater. Sci. Eng. C.* 23, 398-410

- 78. D'Alessio, L., Teghil, R., Zaccagnino, M., Zaccardo, I., Ferro, D., and Marotta, V. (1999) Pulsed laser ablation and deposition of bioactive glass as coating material for biomedical applications. *Appl. Surf. Sci.* 138-139, 527-532
- 79. Hallgren, C., Reimers, H., Gold, J., and Wennerberg, A. (2001) The importance of surface texture for bone integration of screw shaped implants: an in vivo study of implants patterned by photolithography. *J Biomed Mater Res* 57, 485-496
- 80. Conner, K. A., Sabatini, R., Mealey, B. L., Takacs, V. J., Mills, M. P., and Cochran, D. L. (2003) Guided bone regeneration around titanium plasmasprayed, acid-etched, and hydroxyapatite-coated implants in the canine model. *J Periodontol* 74, 658-668
- 81. Testori, T., Wiseman, L., Woolfe, S., and Porter, S. S. (2001) A prospective multicenter clinical study of the Osseotite implant: four-year interim report. *Int J Oral Maxillofac Implants* 16, 193-200
- 82. Lexchin, J., Bero, L. A., Djulbegovic, B., and Clark, O. (2003) Pharmaceutical industry sponsorship and research outcome and quality: systematic review. *Bmj* 326, 1167-1170
- Esposito, M., Murray-Curtis, L., Grusovin, M. G., Coulthard, P., and Worthington, H. V. (2007) Interventions for replacing missing teeth: different types of dental implants. *Cochrane Database Syst Rev*, CD003815
- 84. Harrison, R. G. (1914) The reaction of emberyonic cells to solid structures. *J. Exp. Zool.* 17, 521
- 85. Owen, G. R., Meredith, D. O., ap Gwynn, I., and Richards, R. G. (2005) Focal adhesion quantification a new assay of material biocompatibility? Review. *Eur Cell Mater* 9, 85-96; discussion 85-96
- 86. Hynes, R. O. (2002) Integrins: bidirectional, allosteric signaling machines. *Cell* 110, 673-687
- Humphries, M. J., Travis, M. A., Clark, K., and Mould, A. P. (2004) Mechanisms of integration of cells and extracellular matrices by integrins. *Biochem Soc Trans* 32, 822-825
- Maqueda, A., Moyano, J. V., Gutierrez-Lopez, M. D., Ovalle, S., Rodriguez-Frade, J. M., Cabanas, C., and Garcia-Pardo, A. (2006) Activation pathways of alpha4beta1 integrin leading to distinct T-cell cytoskeleton reorganization, Rac1 regulation and Pyk2 phosphorylation. *J Cell Physiol* 207, 746-756
- 89. Bowers, K. T., Keller, J. C., Randolph, B. A., Wick, D. G., and Michaels, C. M. (1992) Optimization of surface micromorphology for enhanced osteoblast responses in vitro. *Int J Oral Maxillofac Implants* 7, 302-310
- 90. Anselme, K., and Bigerelle, M. (2005) Topography effects of pure titanium substrates on human osteoblast long-term adhesion. *Acta. Biomater.* 1, 211-222

- 91. Huang, H. H., Ho, C. T., Lee, T. H., Lee, T. L., Liao, K. K., and Chen, F. L. (2004) Effect of surface roughness of ground titanium on initial cell adhesion. *Biomol Eng* 21, 93-97
- 92. Baharloo, B., Textor, M., and Brunette, D. M. (2005) Substratum roughness alters the growth, area, and focal adhesions of epithelial cells, and their proximity to titanium surfaces. *J Biomed Mater Res A* 74, 12-22
- 93. Ono, M., Murakami, T., Tomita, M., and Ishikawa, H. (1993) Association of the actin cytoskeleton with glass-adherent proteins in mouse peritoneal macrophages. *Biol Cell* 77, 219-230
- 94. DeFife, K. M., Jenney, C. R., Colton, E., and Anderson, J. M. (1999) Cytoskeletal and adhesive structural polarizations accompany IL-13-induced human macrophage fusion. *J Histochem Cytochem* 47, 65-74
- Destaing, O., Saltel, F., Geminard, J. C., Jurdic, P., and Bard, F. (2003) Podosomes display actin turnover and dynamic self-organization in osteoclasts expressing actin-green fluorescent protein. *Mol Biol Cell* 14, 407-416
- 96. Phillips, J. M., and Kao, W. J. (2005) Macrophage adhesion on gelatin-based interpenetrating networks grafted with PEGylated RGD. *Tissue Eng* 11, 964-973
- 97. Labat-Robert, J., Bihari-Varga, M., and Robert, L. (1990) Extracellular matrix. *FEBS Lett* 268, 386-393
- Wojciak-Stothard, B., Curtis, A., Monaghan, W., MacDonald, K., and Wilkinson, C. (1996) Guidance and activation of murine macrophages by nanometric scale topography. *Exp Cell Res* 223, 426-435
- 99. Clark, P., Connolly, P., and Moores, G. R. (1992) Cell guidance by micropatterned adhesiveness in vitro. *J Cell Sci* 103 (Pt 1), 287-292
- 100. Weiss, P. (1959) Cellular dynamics. Rev. Modern. Phys., 11-20
- 101. Abiko, H., and Brunette, D. M. (1993) Immunohistochemical investigation of tracks left by the migration of fibroblasts on titanium surfaces. *Celles Mater.*, 161
- 102. Brunette, D. M., Khakbaznejad, A., Takekawa, M., Shimonishi, M., Murkami, H., Wieland, M., Chehroudi, B., Glass-Burdzinski, J., and Textor, M. (2003) Improving the bio-implant interface by controlling cell behaviour using surface topography. in bio-implant interface: improving biomaterials & tissue reactions. *Ellingsen JE and Lyngstadaas SP. Eds. Lavoisier.*, 114-115
- Wojciak-Stothard, B., Madeja, Z., Korohoda, W., Curtis, A., and Wilkinson, C. (1995) Activation of macrophage-like cells by multiple grooved substrata. Topographical control of cell behaviour. *Cell Biol Int* 19, 485-490
- 104. Clark, P., Connolly, P., Curtis, A. S., Dow, J. A., and Wilkinson, C. D. (1991) Cell guidance by ultrafine topography in vitro. *J Cell Sci* 99 (Pt 1), 73-77

- 105. Rich, A., and Harris, A. K. (1981) Anomalous preferences of cultured macrophages for hydrophobic and roughened substrata. *J Cell Sci* 50, 1-7
- Soskolne, W. A., Cohen, S., Sennerby, L., Wennerberg, A., and Shapira, L. (2002) The effect of titanium surface roughness on the adhesion of monocytes and their secretion of TNF-alpha and PGE2. *Clin Oral Implants Res* 13, 86-93
- 107. Mustafa, K., Oden, A., Wennerberg, A., Hultenby, K., and Arvidson, K. (2005) The influence of surface topography of ceramic abutments on the attachment and proliferation of human oral fibroblasts. *Biomaterials* 26, 373-381
- 108. Chvapil, M., Holusa, R., Kliment, K., and Stoll, M. (1969) Some chemical and biological characteristics of a new collagen-polymer compound material. *J Biomed Mater Res* 3, 315-332
- Dow, J. A., Clark, P., Connolly, P., Curtis, A. S., and Wilkinson, C. D. (1987) Novel methods for the guidance and monitoring of single cells and simple networks in culture. *J Cell Sci Suppl* 8, 55-79
- 110. Brunette, D. M., and Chehroudi, B. (1999) The effects of the surface topography of micromachined titanium substrata on cell behavior in vitro and in vivo. *J Biomech Eng* 121, 49-57
- 111. Pollice, P. F., Hsu, J., Hicks, D. G., Bukata, S., Rosier, R. N., Reynolds, P. R., Puzas, J. E., and O'Keefe, R. J. (1998) Interleukin-10 inhibits cytokine synthesis in monocytes stimulated by titanium particles: evidence of an anti-inflammatory regulatory pathway. *J Orthop Res* 16, 697-704
- 112. Hamilton, D. W., Riehle, M. O., Monaghan, W., and Curtis, A. S. (2005) Articular chondrocyte passage number: influence on adhesion, migration, cytoskeletal organisation and phenotype in response to nano- and micro-metric topography. *Cell Biol Int* 29, 408-421
- 113. Dalby, M. J., Riehle, M. O., Johnstone, H., Affrossman, S., and Curtis, A. S. (2002) In vitro reaction of endothelial cells to polymer demixed nanotopography. *Biomaterials* 23, 2945-2954
- 114. Chehroudi, B., Gould, T. R., and Brunette, D. M. (1989) Effects of a grooved titanium-coated implant surface on epithelial cell behavior in vitro and in vivo. *J Biomed Mater Res* 23, 1067-1085
- 115. Hamilton, D. W., and Brunette, D. M. (2005) "Gap guidance" of fibroblasts and epithelial cells by discontinuous edged surfaces. *Exp Cell Res* 309, 429-437
- 116. Lydon, M. J., and Clay, C. S. (1985) Substratum topography and cell traction on sulphuric acid treated bacteriological-grade plastic. *Cell Biol Int Rep* 9, 911-921
- 117. Curtis, A. S., and Varde, M. (1964) Control of Cell Behavior: Topological Factors. *J Natl Cancer Inst* 33, 15-26

- 118. Lauer, G., Wiedmann-Al-Ahmad, M., Otten, J. E., Hubner, U., Schmelzeisen, R., and Schilli, W. (2001) The titanium surface texture effects adherence and growth of human gingival keratinocytes and human maxillar osteoblast-like cells in vitro. *Biomaterials* 22, 2799-2809
- 119. Teixeira, A. I., Nealey, P. F., and Murphy, C. J. (2004) Responses of human keratocytes to micro- and nanostructured substrates. *J Biomed Mater Res A* 71, 369-376
- 120. Albelda, S. M., and Buck, C. A. (1990) Integrins and other cell adhesion molecules. *Faseb J* 4, 2868-2880
- Wood, A. (1988) Contact guidance on microfabricated substrata: the response of teleost fin mesenchyme cells to repeating topographical patterns. *J Cell Sci* 90 (Pt 4), 667-681
- 122. Ernsting, M. J., Labow, R. S., and Santerre, J. P. (2007) Human monocyte adhesion onto RGD and PHSRN peptides delivered to the surface of a polycarbonate polyurethane using bioactive fluorinated surface modifiers. *J Biomed Mater Res A* 83, 759-769
- 123. Williamson, M. R., and Coombes, A. G. (2004) Gravity spinning of polycaprolactone fibres for applications in tissue engineering. *Biomaterials* 25, 459-465
- 124. Rajnicek, A., and McCaig, C. (1997) Guidance of CNS growth cones by substratum grooves and ridges: effects of inhibitors of the cytoskeleton, calcium channels and signal transduction pathways. *J Cell Sci* 110 (Pt 23), 2915-2924
- 125. Chang, S., Popowich, Y., Greco, R. S., and Haimovich, B. (2003) Neutrophil survival on biomaterials is determined by surface topography. *J Vasc Surg* 37, 1082-1090
- 126. Meyle, J., Gultig, K., and Nisch, W. (1995) Variation in contact guidance by human cells on a microstructured surface. *J Biomed Mater Res* 29, 81-88
- 127. Chehroudi, B., Ratkay, J., and Brunette, D. M. (1993) Topographical influence of micromachined surfaces on mineralization *in vitro*. *J. Dent. Res.* 72, 390
- 128. Schwartz, Z., Lomann, C. H., Offinger, J., Bonewald, L. F., Dean, D. D., and Boyan, B. D. (1999) Implant surface characteristics modulate differentiation behaviour of cells in the osteoblast linage. *Adv. Den. Res.* 13
- 129. Vesely, P., Boyde, A., and Jones, S. J. (1992) Behaviour of osteoclasts in vitro: contact behaviour of osteoclasts with osteoblast-like cells and networking of osteoclasts for 3D orientation. *J Anat* 181 (Pt 2), 277-291
- Webb, A., Clark, P., Skepper, J., Compston, A., and Wood, A. (1995) Guidance of oligodendrocytes and their progenitors by substratum topography. *J Cell Sci* 108 (Pt 8), 2747-2760

- 131. Kikuchi, L., Park, J. Y., Victor, C., and Davies, J. E. (2005) Platelet interactions with calcium-phosphate-coated surfaces. *Biomaterials* 26, 5285-5295
- 132. Gordon, S. (2007) The macrophage: past, present and future. *Eur J Immunol* 37 Suppl 1, S9-17
- 133. van Furth, R., Cohn, Z. A., Hirsch, J. G., Humphrey, J. H., Spector, W. G., and Langevoort, H. L. (1972) The mononuclear phagocyte system: a new classification of macrophages, monocytes, and their precursor cells. *Bull World Health Organ* 46, 845-852
- 134. Metcalf, D. (1971) Transformation of granulocytes to macrophages in bone marrow colonies in vitro. *J Cell Physiol* 77, 277-280
- Ogawa, M., Porter, P. N., and Nakahata, T. (1983) Renewal and commitment to differentiation of hemopoietic stem cells (an interpretive review). *Blood* 61, 823-829
- 136. Goud, T. J., and van Furth, R. (1975) Proliferative characteristics of monoblasts grown in vitro. *J Exp Med* 142, 1200-1217
- Valledor, A. F., Borras, F. E., Cullell-Young, M., and Celada, A. (1998) Transcription factors that regulate monocyte/macrophage differentiation. J Leukoc Biol 63, 405-417
- 138. van Furth, R. (1982) Current view on the mononuclear phagocyte system. *Immunobiology* 161, 178-185
- 139. David, A., Hume, I. L., Ross, S., Roy, H. R., Tedjo, S., Christine, A. W., and Ravasi, T. (2002) The mononuclear phagocyte system revisited. *J. Leukoc. Biol.* 72, 621-627
- 140. Gordon, S., Keshav, S., and Chung, L. P. (1988) Mononuclear phagocytes: tissue distribution and functional heterogeneity. *Curr Opin Immunol* 1, 26-35
- 141. Morrissette, N., Gold, E., and Aderem, A. (1999) The macrophage--a cell for all seasons. *Trends Cell Biol* 9, 199-201
- 142. Yamate, J., Yoshida, H., Tsukamoto, Y., Ide, M., Kuwamura, M., Ohashi, F., Miyamoto, T., Kotani, T., Sakuma, S., and Takeya, M. (2000) Distribution of cells immunopositive for AM-3K, a novel monoclonal antibody recognizing human macrophages, in normal and diseased tissues of dogs, cats, horses, cattle, pigs, and rabbits. *Vet Pathol* 37, 168-176
- 143. Mantovani, A., Sica, A., and Locati, M. (2007) New vistas on macrophage differentiation amd activation. *Eur. J. Immunol.* 37, 14-16
- 144. Proost, P., Wuyts, A., and Van Damme, J. (1996) Human monocyte chemotactic proteins-2 and -3: structural and functional comparison with MCP-1. *J Leukoc Biol* 59, 67-74

- 145. Riches, D. W., Chan, E. D., and Winston, B. W. (1996) TNF-alpha-induced regulation and signalling in macrophages. *Immunobiology* 195, 477-490
- 146. Fogg, D. K., Sibon, C., Miled, C., Jung, S., Aucouturier, P., Littman, D. R., Cumano, A., and Geissmann, F. (2006) A clonogenic bone marrow progenitor specific for macrophages and dendritic cells. *Science* 311, 83-87
- 147. Geissmann, F., Jung, S., and Littman, D. R. (2003) Blood monocytes consist of two principal subsets with distinct migratory properties. *Immunity* 19, 71-82
- 148. Ziegler-Heitbrock, L. (2007) The CD14+ CD16+ blood monocytes: their role in infection and inflammation. *J Leukoc Biol* 81, 584-592
- 149. Passlick, B., Flieger, D., and Ziegler-Heitbrock, H. W. (1989) Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood* 74, 2527-2534
- 150. Austyn, J. M., and Gordon, S. (1981) F4/80, a monoclonal antibody directed specifically against the mouse macrophage. *Eur J Immunol* 11, 805-815
- 151. Peiser, L., Mukhopadhyay, S., and Gordon, S. (2002) Scavenger receptors in innate immunity. *Curr Opin Immunol* 14, 123-128
- 152. Zamze, S., Martinez-Pomares, L., Jones, H., Taylor, P. R., Stillion, R. J., Gordon, S., and Wong, S. Y. (2002) Recognition of bacterial capsular polysaccharides and lipopolysaccharides by the macrophage mannose receptor. *J Biol Chem* 277, 41613-41623
- 153. Norris, J. L., and Manley, J. L. (1996) Functional interactions between the pelle kinase, Toll receptor, and tube suggest a mechanism for activation of dorsal. *Genes Dev* 10, 862-872
- 154. Fadok, V. A., Voelker, D. R., Campbell, P. A., Cohen, J. J., Bratton, D. L., and Henson, P. M. (1992) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol* 148, 2207-2216
- 155. Savill, J., Dransfield, I., Gregory, C., and Haslett, C. (2002) A blast from the past: clearance of apoptotic cells regulates immune responses. *Nat Rev Immunol* 2, 965-975
- Balasubramanian, K., and Schroit, A. J. (1998) Characterization of phosphatidylserine-dependent beta2-glycoprotein I macrophage interactions. Implications for apoptotic cell clearance by phagocytes. *J Biol Chem* 273, 29272-29277
- 157. Hogger, P., Dreier, J., Droste, A., Buck, F., and Sorg, C. (1998) Identification of the integral membrane protein RM3/1 on human monocytes as a glucocorticoid-inducible member of the scavenger receptor cysteine-rich family (CD163). *J Immunol* 161, 1883-1890

- 158. van den Berg, T. K., Dopp, E. A., and Dijkstra, C. D. (2001) Rat macrophages: membrane glycoproteins in differentiation and function. *Immunol Rev* 184, 45-57
- Damoiseaux, J. G., Dopp, E. A., Calame, W., Chao, D., MacPherson, G. G., and Dijkstra, C. D. (1994) Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* 83, 140-147
- 160. Dijkstra, C. D., Dopp, E. A., Joling, P., and Kraal, G. (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Adv Exp Med Biol* 186, 409-419
- Rosengren, A., Danielsen, N., and Bjursten, L. M. (1997) Inflammatory reaction dependence on implant localization in rat soft tissue models. *Biomaterials* 18, 979-987
- 162. Barbe, E., Damoiseaux, J. G., Dopp, E. A., and Dijkstra, C. D. (1990) Characterization and expression of the antigen present on resident rat macrophages recognized by monoclonal antibody ED2. *Immunobiology* 182, 88-99
- 163. Stout, R. D., and Suttles, J. (2004) Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol* 76, 509-513
- 164. Laskin, D. L., Weinberger, B., and Laskin, J. D. (2001) Functional heterogeneity in liver and lung macrophages. *J Leukoc Biol* 70, 163-170
- 165. Gordon, S. (2003) Alternative activation of macrophages. *Nat Rev Immunol* 3, 23-35
- 166. Gordon, S., Fraser, I., Nath, D., Hughes, D., and Clarke, S. (1992) Macrophages in tissues and in vitro. *Curr Opin Immunol* 4, 25-32
- 167. Nathan, C. (1991) Mechanisms and modulation of macrophage activation. *Behring Inst Mitt*, 200-207
- 168. Verreck, F. A., de Boer, T., Langenberg, D. M., Hoeve, M. A., Kramer, M., Vaisberg, E., Kastelein, R., Kolk, A., de Waal-Malefyt, R., and Ottenhoff, T. H. (2004) Human IL-23-producing type 1 macrophages promote but IL-10-producing type 2 macrophages subvert immunity to (myco)bacteria. *Proc Natl Acad Sci U S* A 101, 4560-4565
- 169. Gibran, N. S., Ferguson, M., Heimbach, D. M., and Isik, F. F. (1997) Monocyte chemoattractant protein-1 mRNA expression in the human burn wound. *J Surg Res* 70, 1-6
- Denholm, E. M., and Rollins, S. M. (1993) Expression and secretion of transforming growth factor-beta by bleomycin-stimulated rat alveolar macrophages. *Am J Physiol* 264, L36-42

- 171. Gratchev, A., Schledzewski, K., Guillot, P., and Goerdt, S. (2001) Alternatively activated antigen-presenting cells: molecular repertoire, immune regulation, and healing. *Skin Pharmacol Appl Skin Physiol* 14, 272-279
- 172. Zagris, N. (2001) Extracellular matrix in development of the early embryo. *Micron* 32, 427-438
- 173. Geng, Y., Zhang, B., and Lotz, M. (1993) Protein tyrosine kinase activation is required for lipopolysaccharide induction of cytokines in human blood monocytes. *J Immunol* 151, 6692-6700
- 174. Clark, E. A., and Brugge, J. S. (1995) Integrins and signal transduction pathways: the road taken. *Science* 268, 233-239
- 175. Kanner, S. B., Reynolds, A. B., Vines, R. R., and Parsons, J. T. (1990) Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc Natl Acad Sci U S A* 87, 3328-3332
- 176. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1993) Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J Cell Biol* 123, 993-1005
- 177. Kirchner, J., Kam, Z., Tzur, G., Bershadsky, A. D., and Geiger, B. (2003) Live-cell monitoring of tyrosine phosphorylation in focal adhesions following microtubule disruption. *J Cell Sci* 116, 975-986
- 178. Aplin, A. E. (2003) Cell adhesion molecule regualetion of nucleocytoplasmic trafficking. *FEBS Lett* 543, 11-14
- 179. Parsons, J. T. (2003) Focal adhesion kinase: the first ten years. *J Cell Sci* 116, 1409-1416
- Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat Rev Mol Cell Biol* 2, 793-805
- 181. Mitra, S. K., Hanson, D. A., and Schlaepfer, D. D. (2005) Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol* 6, 56-68
- 182. Salazar, E. P., and Rozengurt, E. (2001) Src family kinases are required for integrin-mediated but not for G protein-coupled receptor stimulation of focal adhesion kinase autophosphorylation at Tyr-397. *J Biol Chem* 276, 17788-17795
- Schaller, M. D., Otey, C. A., Hildebrand, J. D., and Parsons, J. T. (1995) Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J Cell Biol* 130, 1181-1187
- 184. Abedi, H., and Zachary, I. (1997) Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* 272, 15442-15451

- 185. Schaller, M. D., Hildebrand, J. D., Shannon, J. D., Fox, J. W., Vines, R. R., and Parsons, J. T. (1994) Autophosphorylation of the focal adhesion kinase, pp125FAK, directs SH2-dependent binding of pp60src. *Mol Cell Biol* 14, 1680-1688
- Polte, T. R., and Hanks, S. K. (1995) Interaction between focal adhesion kinase and Crk-associated tyrosine kinase substrate p130Cas. *Proc Natl Acad Sci U S* A 92, 10678-10682
- 187. Han, D. C., and Guan, J. L. (1999) Association of focal adhesion kinase with Grb7 and its role in cell migration. *J Biol Chem* 274, 24425-24430
- Schlaepfer, D. D., and Hunter, T. (1997) Focal adhesion kinase overexpression enhances ras-dependent integrin signaling to ERK2/mitogen-activated protein kinase through interactions with and activation of c-Src. *J Biol Chem* 272, 13189-13195
- 189. Thomas, J. W., Ellis, B., Boerner, R. J., Knight, W. B., White, G. C., 2nd, and Schaller, M. D. (1998) SH2- and SH3-mediated interactions between focal adhesion kinase and Src. *J Biol Chem* 273, 577-583
- 190. Zhang, X., Chattopadhyay, A., Ji, Q. S., Owen, J. D., Ruest, P. J., Carpenter, G., and Hanks, S. K. (1999) Focal adhesion kinase promotes phospholipase Cgamma1 activity. *Proc Natl Acad Sci U S A* 96, 9021-9026
- 191. Fincham, V. J., James, M., Frame, M. C., and Winder, S. J. (2000) Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *Embo J* 19, 2911-2923
- 192. Furuhjelm, J., and Peranen, J. (2003) The C-terminal end of R-Ras contains a focal adhesion targeting signal. *J Cell Sci* 116, 3729-3738
- 193. Schlaepfer, D. D., and Hunter, T. (1996) Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol* 16, 5623-5633
- 194. Webb, D. J., Donais, K., Whitmore, L. A., Thomas, S. M., Turner, C. E., Parsons, J. T., and Horwitz, A. F. (2004) FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol* 6, 154-161
- 195. Fincham, V. J., Brunton, V. G., and Frame, M. C. (2000) The SH3 domain directs acto-myosin-dependent targeting of v-Src to focal adhesions via phosphatidylinositol 3-kinase. *Mol Cell Biol* 20, 6518-6536
- 196. Hauck, C. R., Hsia, D. A., and Schlaepfer, D. D. (2000) Focal adhesion kinase facilitates platelet-derived growth factor-BB-stimulated ERK2 activation required for chemotaxis migration of vascular smooth muscle cells. *J Biol Chem* 275, 41092-41099

- 197. Hanks, S. K., Ryzhova, L., Shin, N. Y., and Brabek, J. (2003) Focal adhesion kinase signaling activities and their implications in the control of cell survival and motility. *Front Biosci* 8, d982-996
- Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996) Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J Cell Biol 135, 1383-1390
- 199. Sonoda, Y., Matsumoto, Y., Funakoshi, M., Yamamoto, D., Hanks, S. K., and Kasahara, T. (2000) Anti-apoptotic role of focal adhesion kinase (FAK). Induction of inhibitor-of-apoptosis proteins and apoptosis suppression by the overexpression of FAK in a human leukemic cell line, HL-60. *J Biol Chem* 275, 16309-16315
- Brown, M. T., and Cooper, J. A. (1996) Regulation, substrates and functions of src. *Biochim Biophys Acta* 1287, 121-149
- Cance, W. G., Craven, R. J., Bergman, M., Xu, L., Alitalo, K., and Liu, E. T. (1994) Rak, a novel nuclear tyrosine kinase expressed in epithelial cells. *Cell Growth Differ* 5, 1347-1355
- 202. Purchio, A. F., Erikson, E., Brugge, J. S., and Erikson, R. L. (1978) Identification of a polypeptide encoded by the avian sarcoma virus src gene. *Proc Natl Acad Sci U S A* 75, 1567-1571
- 203. Thomas, S. M., and Brugge, J. S. (1997) Cellular functions regulated by Src family kinases. *Annu Rev Cell Dev Biol* 13, 513-609
- 204. Tatosyan, A. G., and Mizenina, O. A. (2000) Kinases of the Src family: structure and functions. *Biochemistry (Mosc)* 65, 49-58
- 205. Roskoski, R., Jr. (2004) Src protein-tyrosine kinase structure and regulation. *Biochem Biophys Res Commun* 324, 1155-1164
- 206. Zheng, X. M., Resnick, R. J., and Shalloway, D. (2000) A phosphotyrosine displacement mechanism for activation of Src by PTPalpha. *Embo J* 19, 964-978
- 207. Okada, M., and Nakagawa, H. (1989) A protein tyrosine kinase involved in regulation of pp60c-src function. *J Biol Chem* 264, 20886-20893
- Broome, M. A., and Hunter, T. (1997) The PDGF receptor phosphorylates Tyr 138 in the c-Src SH3 domain in vivo reducing peptide ligand binding. *Oncogene* 14, 17-34
- Gould, K. L., and Hunter, T. (1988) Platelet-derived growth factor induces multisite phosphorylation of pp60c-src and increases its protein-tyrosine kinase activity. *Mol Cell Biol* 8, 3345-3356
- 210. Stover, D. R., Liebetanz, J., and Lydon, N. B. (1994) Cdc2-mediated modulation of pp60c-src activity. *J Biol Chem* 269, 26885-26889

- Cao, X., Tay, A., Guy, G. R., and Tan, Y. H. (1996) Activation and association of Stat3 with Src in v-Src-transformed cell lines. *Mol Cell Biol* 16, 1595-1603
- Novogrodsky, A., Vanichkin, A., Patya, M., Gazit, A., Osherov, N., and Levitzki, A. (1994) Prevention of lipopolysaccharide-induced lethal toxicity by tyrosine kinase inhibitors. *Science* 264, 1319-1322
- Aki, D., Mashima, R., Saeki, K., Minoda, Y., Yamauchi, M., and Yoshimura, A. (2005) Modulation of TLR signalling by the C-terminal Src kinase (Csk) in macrophages. *Genes Cells* 10, 357-368
- 214. Hagel, M., George, E. L., Kim, A., Tamimi, R., Opitz, S. L., Turner, C. E., Imamoto, A., and Thomas, S. M. (2002) The adaptor protein paxillin is essential for normal development in the mouse and is a critical transducer of fibronectin signaling. *Mol Cell Biol* 22, 901-915
- 215. Honda, H., Nakamoto, T., Sakai, R., and Hirai, H. (1999) p130(Cas), an assembling molecule of actin filaments, promotes cell movement, cell migration, and cell spreading in fibroblasts. *Biochem Biophys Res Commun* 262, 25-30
- 216. Schlaepfer, D. D., Hauck, C. R., and Sieg, D. J. (1999) Signaling through focal adhesion kinase. *Prog Biophys Mol Biol* 71, 435-478
- 217. Schaeffer, H. J., and Weber, M. J. (1999) Mitogen-activated protein kinases: specific messages from ubiquitous messengers. *Mol Cell Biol* 19, 2435-2444
- 218. Davis, R. J. (2000) Signal transduction by the JNK group of MAP kinases. *Cell* 103, 239-252
- 219. English, J., Pearson, G., Wilsbacher, J., Swantek, J., Karandikar, M., Xu, S., and Cobb, M. H. (1999) New insights into the control of MAP kinase pathways. *Exp Cell Res* 253, 255-270
- 220. Lai, C. F., Chaudhary, L., Fausto, A., Halstead, L. R., Ory, D. S., Avioli, L. V., and Cheng, S. L. (2001) Erk is essential for growth, differentiation, integrin expression, and cell function in human osteoblastic cells. *J Biol Chem* 276, 14443-14450
- 221. Hazzalin, C. A., and Mahadevan, L. C. (2002) MAPK-regulated transcription: a continuously variable gene switch? *Nat Rev Mol Cell Biol* 3, 30-40
- 222. Kharbanda, S., Pandey, P., Ren, R., Mayer, B., Zon, L., and Kufe, D. (1995) c-Abl activation regulates induction of the SEK1/stress-activated protein kinase pathway in the cellular response to 1-beta-D-arabinofuranosylcytosine. *J Biol Chem* 270, 30278-30281
- Winston, B. W., Chan, E. D., Johnson, G. L., and Riches, D. W. (1997) Activation of p38mapk, MKK3, and MKK4 by TNF-alpha in mouse bone marrow-derived macrophages. *J Immunol* 159, 4491-4497

- Madhani, H. D., Styles, C. A., and Fink, G. R. (1997) MAP kinases with distinct inhibitory functions impart signaling specificity during yeast differentiation. *Cell* 91, 673-684
- 225. Uhlik, M. T., Abell, A. N., Cuevas, B. D., Nakamura, K., and Johnson, G. L. (2004) Wiring diagrams of MAPK regulation by MEKK1, 2, and 3. *Biochem Cell Biol* 82, 658-663
- Wellbrock, C., Ogilvie, L., Hedley, D., Karasarides, M., Martin, J., Niculescu-Duvaz, D., Springer, C. J., and Marais, R. (2004) V599EB-RAF is an oncogene in melanocytes. *Cancer Res* 64, 2338-2342
- 227. Papin, C., Eychene, A., Brunet, A., Pages, G., Pouyssegur, J., Calothy, G., and Barnier, J. V. (1995) B-Raf protein isoforms interact with and phosphorylate Mek-1 on serine residues 218 and 222. *Oncogene* 10, 1647-1651
- Zhang, J., Zhang, F., Ebert, D., Cobb, M. H., and Goldsmith, E. J. (1995) Activity of the MAP kinase ERK2 is controlled by a flexible surface loop. *Structure* 3, 299-307
- Davie, J. R., and Spencer, V. A. (2001) Signal transduction pathways and the modification of chromatin structure. *Prog Nucleic Acid Res. Mol. Biol.* 65, 299-340
- 230. Nakashima, Y., Sun, D. H., Trindade, M. C., Maloney, W. J., Goodman, S. B., Schurman, D. J., and Smith, R. L. (1999) Signaling pathways for tumor necrosis factor-alpha and interleukin-6 expression in human macrophages exposed to titanium-alloy particulate debris in vitro. *J Bone Joint Surg Am* 81, 603-615
- 231. Boutahar, N., Guignandon, A., Vico, L., and Lafage-Proust, M. H. (2004) Mechanical strain on osteoblasts activates autophosphorylation of focal adhesion kinase and proline-rich tyrosine kinase 2 tyrosine sites involved in ERK activation. J Biol Chem 279, 30588-30599
- Fanning, P. J., Emkey, G., Smith, R. J., Grodzinsky, A. J., Szasz, N., and Trippel, S. B. (2003) Mechanical regulation of mitogen-activated protein kinase signaling in articular cartilage. *J Biol Chem* 278, 50940-50948
- Labow, R. S., Sa, D., Matheson, L. A., and Santerre, J. P. (2005) Polycarbonateurethane hard segment type influences esterase substrate specificity for humanmacrophage-mediated biodegradation. *J Biomater Sci Polym Ed* 16, 1167-1177
- 234. Lu, J., Descamps, M., Dejou, J., Koubi, G., Hardouin, P., Lemaitre, J., and Proust, J. P. (2002) The biodegradation mechanism of calcium phosphate biomaterials in bone. *J Biomed Mater Res* 63, 408-412
- 235. Dinnes, D. L., Santerre, J. P., and Labow, R. S. (2008) Influence of biodegradable and non-biodegradable material surfaces on the differentiation of human monocyte-derived macrophages. *Differentiation* 76, 232-244

- 236. Bhimaraj, P., Buris, D., Sawyer, W., Toney, C., Siegel, R., and Schadler, L. (2008) Tribological investigation of the effects of particle size, loading and crystallinity on poly(ethylene) terephthalate nanocomposites
- . Wear 264, 632-637
- 237. Rader, C. P., Sterner, T., Jakob, F., Schutze, N., and Eulert, J. (1999) Cytokine response of human macrophage-like cells after contact with polyethylene and pure titanium particles. *J Arthroplasty* 14, 840-848
- 238. Shanbhag, A. S., Jacobs, J. J., Black, J., Galante, J. O., and Glant, T. T. (1994) Macrophage/particle interactions: effect of size, composition and surface area. *J Biomed Mater Res* 28, 81-90
- Lapcikova, M., Slouf, M., Dybal, J., Zolotaarevova, E., Entllcher, G., Pokorny, D., Gallo, J., and Sosna, A. (2008) Nanometer size wear debris generated from ultra high molecular weight polyethylene in vivo. *Wear* 266, 349-355
- Sawase, T., Jimbo, R., Baba, K., Shibata, Y., Ikeda, T., and Atsuta, M. (2008) Photo-induced hydrophilicity enhances initial cell behavior and early bone apposition. *Clin Oral Implants Res* 19, 491-496
- Zhao, G., Schwartz, Z., Wieland, M., Rupp, F., Geis-Gerstorfer, J., Cochran, D. L., and Boyan, B. D. (2005) High surface energy enhances cell response to titanium substrate microstructure. *J Biomed Mater Res A* 74, 49-58
- 242. Kao, W. J., Sapatnekar, S., Hiltner, A., and Anderson, J. M. (1996) Complementmediated leukocyte adhesion on poly(etherurethane ureas) under shear stress in vitro. *J Biomed Mater Res* 32, 99-109
- 243. Sumner, D. R., Turner, T. M., Purchio, A. F., Gombotz, W. R., Urban, R. M., and Galante, J. O. (1995) Enhancement of bone ingrowth by transforming growth factor-beta. *J Bone Joint Surg Am* 77, 1135-1147
- 244. Wang, J. Y., Wicklund, B. H., Gustilo, R. B., and Tsukayama, D. T. (1996) Titanium, chromium and cobalt ions modulate the release of bone-associated cytokines by human monocytes/macrophages in vitro. *Biomaterials* 17, 2233-2240
- 245. Boynton, E. L., Waddell, J., Meek, E., Labow, R. S., Edwards, V., and Santerre, J. P. (2000) The effect of polyethylene particle chemistry on human monocytemacrophage function in vitro. *J Biomed Mater Res* 52, 239-245
- 246. Daculsi, G., Malard, O., and Goyenvalle, E. (2005) Efficacy and performance of bone substitute for bone reconstruction in place of allograft and autograft *ITBM-RBM* 26, 218-222
- 247. Davies, J. E. (1998) Mechanisms of endosseous integration. *Int J Prosthodont* 11, 391-401
- 248. Park, J. Y., and Davies, J. E. (2000) Red blood cell and platelet interactions with titanium implant surfaces. *Clin Oral Implants Res* 11, 530-539

- 249. Park, J. Y., Gemmell, C. H., and Davies, J. E. (2001) Platelet interactions with titanium: modulation of platelet activity by surface topography. *Biomaterials* 22, 2671-2682
- 250. Solheim, E., Sudmann, B., Bang, G., and Sudmann, E. (2000) Biocompatibility and effect on osteogenesis of poly(ortho ester) compared to poly(DL-lactic acid). *J Biomed Mater Res* 49, 257-263
- 251. Xia, Z., Ye, H., Choong, C., Ferguson, D. J., Platt, N., Cui, Z., and Triffitt, J. T. (2004) Macrophagic response to human mesenchymal stem cell and poly(epsilon-caprolactone) implantation in nonobese diabetic/severe combined immunodeficient mice. *J Biomed Mater Res A* 71, 538-548
- 252. Bolander, M. E. (1992) Regulation of fracture repair by growth factors. *Proc Soc Exp Biol Med* 200, 165-170
- 253. Branemark, P. I., Hansson, B. O., Adell, R., Breine, U., Lindstrom, J., Hallen, O., and Ohman, A. (1977) Osseointegrated implants in the treatment of the edentulous jaw. Experience from a 10-year period. *Scand J Plast Reconstr Surg Suppl* 16, 1-132
- 254. Albrektsson, T., Branemark, P. I., Hansson, H. A., and Lindstrom, J. (1981) Osseointegrated titanium implants. Requirements for ensuring a long-lasting, direct bone-to-implant anchorage in man. *Acta Orthop Scand* 52, 155-170
- 255. Albrektsson, T., and Johansson, C. (2001) Osteoinduction, osteoconduction and osseointegration. *Eur Spine J* 10 Suppl 2, S96-101
- 256. Zarb, G., and Albrektsson, T. (1991) Osseointegration a requiem for the peoriodontal ligament? An editorial. *Int. J. Periodont. Rest. Dentistry.* 11, 88-91
- 257. Listgarten, M. A., Buser, D., Steinemann, S. G., Donath, K., Lang, N. P., and Weber, H. P. (1992) Light and transmission electron microscopy of the intact interfaces between non-submerged titanium-coated epoxy resin implants and bone or gingiva. *J Dent Res* 71, 364-371
- 258. Urist, M. R. (1965) Bone: formation by autoinduction. Science 150, 893-899
- 259. Schmitt, J. M., Hwang, K., Winn, S. R., and Hollinger, J. O. (1999) Bone morphogenetic proteins: an update on basic biology and clinical relevance. *J Orthop Res* 17, 269-278
- 260. Urist, M. R., Mikulski, A., and Lietze, A. (1979) Solubilized and insolubilized bone morphogenetic protein. *Proc Natl Acad Sci U S A* 76, 1828-1832
- 261. Mundy, G. R. (1993) Role of cytokines in bone resorption. *J Cell Biochem* 53, 296-300
- Champagne, C. M., Takebe, J., Offenbacher, S., and Cooper, L. F. (2002) Macrophage cell lines produce osteoinductive signals that include bone morphogenetic protein-2. *Bone* 30, 26-31

- Ivanoff, C. J., Hallgren, C., Widmark, G., Sennerby, L., and Wennerberg, A. (2001) Histologic evaluation of the bone integration of TiO(2) blasted and turned titanium microimplants in humans. *Clin Oral Implants Res* 12, 128-134
- 264. Hashimoto, S., Pittet, J. F., Hong, K., Folkesson, H., Bagby, G., Kobzik, L., Frevert, C., Watanabe, K., Tsurufuji, S., and Wiener-Kronish, J. (1996) Depletion of alveolar macrophages decreases neutrophil chemotaxis to Pseudomonas airspace infections. *Am J Physiol* 270, L819-828
- 265. Polfliet, M. M., Zwijnenburg, P. J., van Furth, A. M., van der Poll, T., Dopp, E. A., Renardel de Lavalette, C., van Kesteren-Hendrikx, E. M., van Rooijen, N., Dijkstra, C. D., and van den Berg, T. K. (2001) Meningeal and perivascular macrophages of the central nervous system play a protective role during bacterial meningitis. *J Immunol* 167, 4644-4650
- Rosenbaum, J. T., McDevitt, H. O., Guss, R. B., and Egbert, P. R. (1980) Endotoxin-induced uveitis in rats as a model for human disease. *Nature* 286, 611-613
- 267. Sekine, Y., Bowen, L. K., Heidler, K. M., Van Rooijen, N., Brown, J. W., Cummings, O. W., and Wilkes, D. S. (1997) Role of passenger leukocytes in allograft rejection: effect of depletion of donor alveolar macrophages on the local production of TNF-alpha, T helper 1/T helper 2 cytokines, IgG subclasses, and pathology in a rat model of lung transplantation. *J Immunol* 159, 4084-4093
- 268. van Overdam, K. A., Verbakel, C. A., Kouwenhoven, E. A., van Rooijen, N., de Bruin, R. W., JN, I. J., and Marquet, R. L. (2000) Negligible role for NK cells and macrophages in delayed xenograft rejection. *Transpl Int* 13 Suppl 1, S504-507
- Heuff, G., van der Ende, M. B., Boutkan, H., Prevoo, W., Bayon, L. G., Fleuren, G. J., Beelen, R. H., Meijer, S., and Dijkstra, C. D. (1993) Macrophage populations in different stages of induced hepatic metastases in rats: an immunohistochemical analysis. *Scand J Immunol* 38, 10-16

CHAPTER 2:

Modulation of Macrophage Morphology, Patterning of Cytoskeletal

Organization, and Cell Viability by Surface Topography¹

¹ A version of this chapter will be submitted for publication. Ghrebi, S., Hamilton, D., Waterfield, D., Brunette, D. Modulation of Macrophage Morphology, Patterning of Cytoskeletal Organization, and Cell Viability by Surface Topography.

2.1 Introduction

It is widely accepted that alterations in the surface topography of titanium implant devices exert a significant influence on cell behavior in vitro and in vivo (1-3). This interaction is known as " topographic control of cell behavior" reviewed by Brunette and others (4, 5). In the case of placement of implanted devices the host reactions follow a generalized set pattern: injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction, and fibrosis/fibrous capsule formation (6, 7). Cells of the monocytic lineage are involved in the progression of these host responses. This requires the early recruitment of monocytes to the implantation site by chemoattractants released by platelets following blood-material interactions (8). As these monocytes populate the wound site they differentiate and produce further chemoattractant signals that attract more mononuclear cells to the implant site (8). The recruited monocyte-derived macrophages (MDM) aid in the further recruitment and activation of other cell types such as lymphocytes, fibroblasts and osteoblasts, as well as being involved in extra-cellular matrix turnover, angiogenesis, and stimulation of bone formation and bone resorption (9). Furthermore, activated macrophages produce cytokines, and growth factors that are crucially involved in guiding tissue repair and wound healing (3, 10-12).

Monocytes/macrophages can be modulated by the topography of Ti surfaces used in dental implants (3, 13). In such cases the size of the material they encounter is an important element in determining their response. As macrophages can only phagocytize materials of a small size (up to roughly 10 μ m in diameter), macrophages and macrophage-derived foreign body giant cells attach to the surface of bulky medical

devices, such as dental implants, and may stay on the surface for the lifetime of the implant (6, 14).

In many cell types it is well established that alteration of cell size, shape, and spreading can be controlled by implant surface topography (15-17). Generally, the first effect initiated by surface topography on cells, as they adapt to the surface is their shape through the organization of cytoskeletal elements and structures such focal adhesions (FAs), and podosomes (2, 18-20). In this report we determined whether surface topography of commonly used dental implants modulate macrophage morphology, patterning of the cytoskeletal organization and cell number.

2.2 Materials and methods

2.2.1 Fabrication and characterization of implant surface replicas

Ti-discs with a diameter of 10 mm and 1mm in thickness were kindly provided by Straumann AG, Waldenburg, Switzerland. Following the procedures previously developed in our laboratory (21, 22), negative impressions of polished (P), acid-etched (AE), sand-blasted (B), and sand-blasted large-grit and acid-etched (SLA) topographies were made using vinyl polysiloxane impression material (PROVIL[®] novo Light; Heraeus kulzer, Domrmagen, Germany). The negative impressions were then used to cast replica surfaces in epoxy resin (Epoxy Technology, Billerica, MA). The epoxy was mixed following manufacturer's guidelines. The negative impressions were filled with the epoxy, left to settle overnight and then baked in a 59°C oven for four days. After hardening, the replica surfaces were trimmed, cleaned, and left overnight to dry. The surfaces were

sputter-coated with 60 nm of titanium dioxide using the physical vapor deposition technique (PVD), reactive magnetron sputtering at the Paul Scherer Institute, Villingen, Switzerland. The chemical composition of the epoxy Ti-coated replicas produced following the above-described method was examined using X-ray photoelectron spectroscopy (XPS). The reported results determined that Ti, oxygen, carbon, and nitrogen were present on all Ti-coated epoxy replicas and the Ti oxide-layer thickness was found to be 4.5 to 5.5 nm and was dominated by TiO₂. In the same study, original Ti surfaces and their replicas were characterized using a non-contact laser profilometer (LPM) and a stereo scanning electron microscope (stereo-SEM). LPM and stereo-SEM characterization indicated that there was no significant difference in the integral roughness parameters found between the original Ti surfaces and their Ti-coated epoxy replicas (22). SEM images of the four different Ti-coated epoxy replica surfaces used in the study were obtained by stereo-SEM (Cambridge Stereoscan 260, Cambridge, UK) and are presented in figure 2.1.



Figure 2.1: Representative SEM images of Ti-coated epoxy surfaces used in the study. Surfaces were examined using Cambridge Sterioscan-260 SEM at accelerating voltage of 13 kV. All pictures were adjusted to the same size with Adobe Photoshop program. Ra'= arithmetic average roughness parameter (surface average roughness). SLA= Sand-blasted Large-grit Acid-etched.

2.2.2 Cell culture

The mouse RAW 264.7 macrophage cell line was purchased from ATCC, Manassas, VA. The cells were cultured in 75cm³ tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) and maintained in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc, Vancouver, B.C.) supplemented with antibiotics: penicillin G 100 mg/ml, gentamycin 50 mg /ml (Sigma-Aldrich, St, Louis, MO), amphoteracin B (Gibco, Grand Island, NY) 3 mg /ml, and 15% heat-inactivated fetal calf serum (FCS) (HyClone AdvancSTEM[™], Logan, UT). The cultured cells were incubated at 37 °C in a humidified 95% air and 5% CO₂ environment in a ThermoForma incubator (Thermo Electron Corporation, Waltham, MA). They were fed with fresh complete medium every 3 days, and sub-cultured just before reaching confluence.

2.2.3 Experimental design

The surfaces were randomly allocated to each experimental condition for both early and late times. Three experiments were carried out on each sample at each time. A split-plot analysis of variance was used to test hypotheses about treatment means and their interaction with time. In the split-plot model, surface represent treatments in the main plots. While the time steps constitute the sub-plots the interaction of surface and time was also considered.

The early times were selected to assess early events of macrophage responses to P and rough surface topographies and to determine any form of transient macrophage activation after macrophage implant contact. The later times were chosen to study macrophage responses to implant surface topography at the inflammatory and granulation tissue formation phases of wound healing where macrophages are known to participate in those events as outlined above.

2.2.4 Macrophage morphology and cell spreading

Polished, B, AE, and SLA surfaces were glow-discharged (21, 23) using a Plasma Cleaner/Sterilizer PDC-32G (Harrick Scientific, Ossining, NY,) and placed in 24 well plates. For short time points (0.5 h, 2 h, and 6 h), only P and SLA substrata were used and cells were plated at 2x10⁵ cells/sample. For longer time points the four different surface topographies were used and the cells were plated as follows: 1x10⁵ cells for an experiment lasting for one day, 4×10^4 for three days, and 2×10^4 for five days. The plating population densities were selected to ensure sufficient available area for macrophage spreading during the incubation period. For SEM processing, samples were washed in PBS twice for two minutes each. Each sample was then fixed in 4% paraformaldehyde (Fisher Scientific, Nepean, ON) in phosphate buffer saline (PBS) (Scientific, Nepean, ON) for 0.5 h at room temperature and washed in PBS 3 times for five minutes each. This was followed by fixation in 2.5 % glutaraldehyde (Sigma-Aldrich, St, Louis, MO) in PBS for one hour at 4°C then washing 3 times in PBS for five minutes each. The double fixation technique has been found to better preserve cellular structure. (24). Samples were further fixed in 1% OsO₄ (Sigma-Aldrich, St, Louis, MO) in PBS for one hour. The fixative was removed and samples washed 3 times in distilled water for two minutes each. The samples were then incubated in 1% tannic acid (Sigma-Aldrich, St, Louis, MO) for 20 minutes at room temperature, washed 3 times in distilled water two minutes each, fixed again in OsO₄ for 0.5 h, and finally washed 3 times in distilled water for two minutes each. Sample dehydration was carried out in steps using the following ethanol concentrations 50%, 70%, 90% 95%, and 100% (each concentration used twice for five minutes each). The samples were then critical point dried using the semi-automatic critical point dryer Samdri-795 (Tousimi Research Corporation, MD) according to the manufacturer's instructions. Finally the samples were mounted on aluminum stubs, and DC-sputtered with 15 to 20 nm gold in a Anatech Hummer-VI sputtering system coating unit (Surplus Process Equipment Corp. Santa Clara, CA). Samples were viewed using a Cambridge 260 stereo SEM (Leica Instruments, Wetzlar, Germany) at 10 to 13 KV accelerating voltage. Pictures were captured from fields of less cell-populated areas using Orion 1.0 software (E.L.I. Microscopy, Eghezee, Begium). The Adobe[®] Photoshop[®] CS2 version 9.0.2 program was used to adjust all the pictures to similar size. Using the cell tracing function of image J 1.36b software from NIH, the area of 20 individual cells was obtained. The mean cell area of the traced 20 cells from each sample was calculated. Data obtained were presented as means of early (0.5, 2, and 6 h.) and late (day one, three, and five) time points.

2.2.5 Filamentous actin (F-actin) and vinculin staining

Polished, B, AE, and SLA surfaces were glow-discharged using a Plasma Cleaner/Sterilizer PDC-32G (Harrick Scientific, Ossining, NY,) and placed in 24 well plates (Falcon, Becton Dickinson Labware, Franklin Lakes NJ). P and SLA surfaces were randomly allocated for the experimental group and the control group (primary antibody delete). The time points evaluated were 0.5 h, 2 hr, 6 h, one, two, and three days. Sub-confluent macrophages were scraped from the flasks with a cell scraper (Fisher Scientific, Nepean, Ontario, Canada) and adjusted to 1 x 10⁵ cells/ml. One ml of

cells was placed on each of the test surfaces in the 24 well plates. At each time point, samples were processed for immunostaining as previously described (2, 25) with minor modifications. In brief, samples were washed in PBS twice for two minutes each, followed by 2 washes in 37 °C warm CS buffer (prepared according to standard laboratory methods) for two minutes each. The samples were then fixed in 4% formaldehyde (Fisher Scientific, Nepean, Ontario, Canada) in PBS for 0.5 h, and rinsed in CS buffer (3 times for 10 minutes each). Following the wash, cells were permeabilized in 0.2% Triton X-100 (Fisher Scientific, Nepean, ON), for two minutes as described previously (26) and then rinsed in CS buffer (2 times for two minutes each). Non-specific antibody binding was blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich, St, Louis, MO) and 5% goat serum (Sigma-Aldrich, St, Louis, MO) in PBS at 37 °C for 0.5 h. For F-actin staining, samples were incubated with 50 µl Oregon Green[®] 514 phalloidin (Molecular Probes, Burlington, ON) at 1:200 in the blocking solution, washed (2 times for three minutes each), and then counter- stained with 30 μ l of Hoechst 33342 (Molecular Probes, Burlington, ON) for five minutes. Finally samples were washed (5 times for two minutes each in PBS).

Two samples were placed on one glass slide (Fisher Scientific, Nepean, ON), and a glass cover slip mounted with 30 μ l of fluoromount-G (Southern Biotech, Birmingham, AL). An indirect immunofluorescent labeling technique was carried out for vinculin staining. The primary antibody delete (negative control) samples were left in the blocking solution, whereas the test samples were incubated with 50 μ l of primary monoclonal mouse anti-vinculin (Chemicon International, Temecula, CA) at 1:50 dilution in the blocking solution. Samples were then rinsed in 1% BSA in PBS (3 times for five minutes each), blocked again for 20 minutes and incubated with the secondary antibody Alexa

fluor 546 goat anti-mouse (Molecular Probes, Burlington, ON) for 1 hour at room temperature. The samples were then washed in PBS (2 times for two minutes each), and counter-stained with 30 μ l of Hoechst stain solution (Sigma-Aldrich, St, Louis, MO) for five minutes. Finally samples were washed (5 times for two minutes each in PBS) and mounted as described above.

An indirect double immunostaining technique was performed when two cellular components needed to be visualized on the same sample. In the double staining technique the F-actin was done first followed by vinculin. Samples were observed for individual staining and for co-localization of the double staining using a Nikon Confocal Microscope C1. All pictures in each experiment were captured under similar parameter settings such as the gain and pin hole size. The 488 nm argon laser was used for green fluorescence, the 408 ultraviolet laser for blue fluorescence, and the 543 helium neon laser for red fluorescence.

Staining of F-actin and vinculin in cells on the various surfaces were compared to negative control (primary antibody omitted) samples. The Adobe[®] Photoshop[®] CS2 version 9.0.2 program was used to adjust all the pictures to similar size. In order to determine whether there was any difference between the different surfaces, staining was evaluated visually in terms of staining form and localization in randomly selected fields at each time point and for each experimental condition. Pictures of random fields from each surface sample at each time point and for each experimental condition.

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2.2.6 Live/dead viability staining assay

The LIVE/DEAD[®] Viability/Cytotoxicity Assay Kit (Molecular Probes, Burlington, Ontario, Canada) was used as described by the manufacturer. The number of dead cells in a total of 100 cells from each sample was determined by fluorescence observation under the confocal fluorescence microscope. Green fluorescence (calcein AM) in live cells was observed by using excitation/emission 495nm/515 nm filters. 495 nm/635 nm filters were used for observing the red fluorescence (ethidium homodimer-1 (EthD-1)) in dead cells.

2.2.7 Quantification and statistics

For each experiment, three tests were carried out under each condition and for each time point. The mean and the standard deviation of the readings (variable of interest) were calculated. Analysis of variance (ANOVA) was used to assess the statistical significance of comparisons among the four surfaces and the four conditions at the different time points as well as their interactions. The Generalized Linear Model (GLM) procedure in SAS version 9.13 (SAS Institute) was used for data analysis.

The analysis model was used for experiments in which different surfaces were observed over time (equation). The Bonferroni (Dunn) multiple t-tests were used for comparing means (main effects). In cases where the full model (equation) indicated a linear or quadratic treatment interaction over time, the analysis was repeated for each time separately.

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The model used in this analysis is as follows:

Model 1:

$\mathbf{y}_{ijk} = \boldsymbol{\mu} + \boldsymbol{\alpha}_i + \boldsymbol{\varepsilon}_{ir} + \boldsymbol{\theta}_j + (\boldsymbol{\alpha}\boldsymbol{\theta})_{ij} + \boldsymbol{\delta}_{ijk}$

Where:

y ijk	= response for replicate k on surface i at time j;
μ	= overall mean;
α_i	= effect of the ith surface;
ϵ_{ir}	= random experimental error for replications within surfaces;
θj	= effect of the jth time;
(αθ) _{ij}	= interaction between the surface and time;
δ_{ijk}	= random experimental error for replicates within surface and time.

2.3 Results

2.3.1 Topographic effects on macrophage morphology and spreading

In order to investigate the effect of surface topography on macrophage morphology, P, B, AE, and SLA surfaces were studied for up to 3 days. Observations were categorized by surface type and grouped by early times (0.5 h., 2 hr. and 6 h.) and late times (1, 3, and 5 days).

SEM observation and spreading area obtained by image J cell tracing indicated that macrophages spread faster on P surfaces at 0.5 h post-culturing compared to macrophages on SLA surfaces (figure 2.2.). The cells appeared morphologically round and significantly larger (p< 0.05, figure 2.4) with numerous microspikes attached to the P sample surfaces compared to cells on SLA surfaces that exhibited a smaller rounded morphology with fewer microspikes. At 2 hours, SEM images showed cell morphology was in transition on both surfaces. However, the macrophages on P surface were still significantly larger than those on SLA surfaces (p< 0.05, figure 2.4).

At 6 h the shapes of macrophages on SLA surfaces and P surfaces were reversed from those seen at 0.5 h. Significantly larger (p< 0.05, figure 2.3 and 2.4) spreading polygonal cells with numerous podosome-like structures and microspikes attached to the peaks and deeper pore features were formed on the SLA topography. In contrast, cells were smaller and rounded with fewer podosome-like structure and microspikes on the P topography.

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Figure 2.2: SEM images of macrophage morphology on P and SLA surfaces at 0.5 h. Cells on P surfaces showed more spreading and numerous thin micro-spike extensions compared to SLA surfaces where the cells appeared round with fewer micro-spikes.


Figure 2.3: SEM images of macrophage morphology on P and SLA surfaces at 6 h. Cells on SLA surfaces showed more spreading with more podosome-like extensions compared to P surfaces where the cells appeared smaller with fewer podosome-like structures.



Figure 2.4: Topographic effects on macrophage spreading on P and SLA surfaces. The means of cell areas of 20 cells on each surface obtained by image J analysis were plotted. Comparisons were made between the two surfaces at each time point. Means with different letters indicate significant differences (p< 0.05). At 0.5 h the mean cell area of macrophages on P surfaces was significantly higher than that of macrophages plated on SLA surfaces. At 2 and 6 h the macrophage spreading profiles changed and the SLA surfaces significantly increased macrophage spreading compared to P surfaces (p< 0.05) Data presented as means and SD (n=3). On day one macrophages on P and B surfaces were elongated with more micro-spike extensions being demonstrated on P surfaces (figure 2.5 A). Macrophages cultured on SLA surfaces showed more spreading compared to macrophages on P surfaces (figure 2.5 B). The cells on SLA surfaces were significantly larger than cells on B, AE, and P surfaces (p< 0.05, figure 2.8) and showed numerous longer podosome-like structures and microspikes with thick long lamellipodia that were stretched and attached to rough surface peaks and deep surface pores (figure 2.6 A & B). Macrophages on the rough B and SLA surfaces were also significantly larger than macrophages on the P and AE surfaces (p< 0.05, figure 2.8).

Figure 2.5 A and B: Representative SEM micrographs illustrate the effects of surface topography on macrophage morphology and cell spreading at day one.

(A) Morphology of macrophages on P and B surfaces. Macrophages on both surfaces appeared elongated with podosome-like structures. More microspikes were shown by macrophages on P surfaces compared to B surfaces. (B) Macrophages on AE surfaces were smaller with fewer microspikes. On the SLA macrophages were more spread, more elongated and showed more podosome-like structures and microspikes that stretched and attached to rough surface peaks and deep pores.



Figure 2.5 A: SEM images of macrophage morphology on P and B surfaces at day one.



Figure 2.5 B: SEM images of macrophage morphology on AE and SLA surfaces at day one.

On day three, macrophages on the P surfaces exhibited the largest polygonal-shaped morphology with distinct microspikes and thicker lamellipodia extensions with podosome-like structures compared to macrophages on the other three surfaces (figures 2.6 A & B) These differences in cell area were found to be statistically significant (p< 0.05, figure 2.8). The morphology of macrophages on AE and SLA surfaces shifted from the polygonal and elongated shape, respectively seen at day one towards a mixture of elongated and round-shape cells (figures 2.6 B). Interestingly, the morphology of macrophages cultured on SLA and AE surfaces were significantly the smallest (p< 0.05, figure 2.8) and differed from those cultured on P, and B surfaces in terms of shape, cellular extensions and number of podosome-like structures (Figures 2.6 A & B).

Figure 2.6 A and B: Representative SEM micrographs illustrate the effects of surface topography on macrophage morphology and cell spreading at day three.

(A) Macrophages on P surfaces appeared larger than macrophages on B surfaces as well as than macrophages on AE and SLA surfaces as shown in (figure 2.6 A & B). More podosome-like structures and microspikes were present on macrophages on P surfaces compared to macrophages on B surfaces. (B) Macrophages on AE and SLA surfaces were smaller and rounder than macrophages on P surfaces. Macrophages on SLA surfaces had lost some of their microspikes as compared to macrophages on other surfaces.



Figure 2.6 A: SEM images of macrophage morphology on P and B surfaces at day three.



Figure 2.6 B: SEM images of macrophage morphology on AE and SLA surfaces at day three.

On day five, macrophage spreading was further reduced and the cells adopted a spherical morphology with no lamellipodia extensions and very limited number of microspikes (Figures 2.7 A & B). Macrophages on SLA, AE, and B rough surfaces showed a significantly smaller morphology compared to macrophages on P surfaces (p< 0.05, figure 2.8). Nevertheless macrophages on B and AE surfaces, although significantly smaller than those on the P surfaces, still demonstrated numerous microspike extensions compared to macrophages on the SLA surfaces (Figures 2.7 A & B). In contrast, macrophages on P surfaces exhibited a polygonal wide-shaped morphology with a large cell body, long lamellipodia and numerous well-formed podosome-like structures and microspikes compared to macrophages on rough SLA, B, and AE surfaces (figures 2.7 A & B).

Figure 2.7 A and B: Representative SEM micrographs illustrate the effects of surface topography on macrophage morphology and cell spreading at day five.

(A) Macrophages on P surfaces appeared larger than macrophages on B surfaces as well as than macrophages on AE and SLA surfaces as shown in (figure 2.6 A & B). More podosome-like structures and microspikes were present on macrophages on P surfaces compared to macrophages on B surfaces. (B) Macrophages on AE and SLA surfaces were smaller and rounder than macrophages on P surfaces. Macrophages on SLA surfaces had lost some of their microspikes as compared to macrophages on other surfaces.



Figure 2.7 A: SEM images of macrophage morphology on P and B surfaces at day five.



Figure 2. 7 B: SEM images of macrophage morphology AE and SLA surfaces at day five.



Figure 2.8: Representative graph showed topographic effects on cell area of macrophages cultured on polished, blasted, acid-etched, and SLA surfaces at days one, three, and five. The means of cell areas of 20 cells on each surface obtained by image J analysis were plotted. Comparisons were made between the four surfaces at each time point. Means with the same letter are not significantly different. Means with different letters differ significantly (p< 0.05). SLA surfaces increased macrophage spreading compared to polished at day one. On days three and five polished surface increased macrophage spreading compared to acid-etched, blasted, and SLA surfaces. Data presented as means and SD (n= 3).

Generally, macrophages attached and spread on the sample surfaces as individual cells, not clusters. The adaptation of macrophages to the SLA surface features was marked; they were capable of stretching into the deep pores underneath the elevated peaks of the surface roughness. The peaks and the deeper pores on SLA surfaces appeared to provide attractive attachment points where macrophages anchored their fine microspikes. This behavior was exhibited primarily by macrophages cultured on SLA topography and to a lesser extent by macrophages cultured on the B and AE surfaces. A common finding at all time points for all surface topographies was that macrophages did not all exhibit a uniform morphology. However, there was a trend towards a common macrophage response to each surface, such as large elongated or small and rounded cells.

Based on those variations in macrophage spreading patterns and morphology determined by different topographies at different times, we hypothesized that those variations may have effects on the cellular formation and organization of F-actin at focal adhesions and podosome-like structures and we investigated that possibility.

2.3.2 F-actin staining and organization in respect to surface topography

F-actin staining was performed only on the P and SLA surface topographies to study the difference in the effect between the smooth and the roughest surface. At 0.5 h macrophages cultured on the P and SLA surfaces generally exhibited a patchy F-actin staining within central areas of spreading cells. More plaques that localized at the cell periphery were evident in macrophages cultured on P surfaces compared to macrophages cultured on SLA surfaces where the staining patterns appeared as circular bundles around the nucleus of rounded and less spread cells (figure 2.9). The negative controls showed no staining (figure 2.17).



Figure 2.9: Fluorescence images of F-actin staining on P and SLA surfaces at 0.5 h. Macrophage cytoskeletal organization is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for 0.5 h and stained with Oregon green phalloidin for F-actin (green). On both surfaces where cellular extensions are evident F-actin appeared as straight bundles in the extensions (white arrow heads). Whereas in round cell, the F-actin appeared as punctuate dots around the nucleus (red arrow heads). Nuclei stained with Hoechst 33342.

At 6 h, F-actin staining on SLA surfaces appeared as both large well formed dot-like patterns that were located around the nucleus and dense actin edge-bundles in cellular extensions that are likely the FAs and podosome-like structures that were attached to surface topographic features. On the P surfaces actin stained as a dark cloudy appearance around the nucleus of rounded cells and as a patchy appearance in spreading cells with few dense actin edge-bundles in cellular extensions (figure 2.10). The negative control samples showed no staining (figure 2.17).



Figure 2.10: Fluorescence images of F-actin staining on P and SLA surfaces at 6 h. Macrophage cytoskeletal organization is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for 6 h and stained with Oregon green phalloidin for F-actin (green). On both surfaces where cellular extensions are evident F-actin appeared as straight bundles in the extensions (white arrow heads). Whereas in round cell, the F-actin appeared as punctuate dots around the nucleus (red arrow heads). F-actin appeared as dense actin edge-bundles in cellular extensions located at the FAs and podosome-like structures that attached to surface topographic features. Nuclei stained with Hoechst 33342.

At later times (days one and three), F-actin localized differently in round-shaped and in spread cells as seen in figures 2.11 & 2.12). On day one macrophages cultured on SLA surfaces exhibited a large dense dot-like F-actin staining pattern localized not only at the protrusions of the spreading cells but also within central areas of the cell. The protrusions appeared extended and attached to the SLA surface features distant from the cell. Macrophages cultured on the P surfaces exhibited dense circular bundles of F-actin surrounding the nucleus of rounded cells, whereas in spreading cells the F-actin demonstrated a patchy appearance (figure 2.11).

On day three, F-actin staining on the P surfaces appeared as straight dense bundles at the cell protrusions (podosome-like structures) where the cells are attached to the surface. Generally F-actin staining of macrophages cultured on the SLA surface exhibited a patchy staining around the nucleus and in the periphery of round-shaped cells. This contrasted with the patterns seen at the early times (6 h and one day) where the actin staining appeared straight and located at the cellular extensions associated with FAs and podosomes (figures 2.10, 2.11 and 2.12). The negative control samples showed no staining (figure 2.17).

Generally differences in F-actin staining reflect a change in FAs and podosomes on the two surfaces over time.



Figure 2.11: Fluorescence images of F-actin staining on P and SLA surface at day one. Macrophage cytoskeletal organization is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for one day and stained with Oregon green phalloidin for F-actin (green). On both surfaces where cellular extensions are evident F-actin appeared as straight bundles in the extensions (white arrow heads). Whereas in round cells, the F-actin appeared as punctuate dots around the nucleus (red arrow heads). On SLA surface (bottom image), F-actin localized within central areas of the cell and at the protrusions of the spreading cells where the cells are attached to the SLA surface features distant from the cell. Nuclei stained with Hoechst 33342.



Figure 2.12: Fluorescence images of F-actin staining on P and SLA surfaces at day three. Macrophage cytoskeletal organization is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for three days and stained with Oregon green phalloidin for F-actin (green). On both surfaces where cellular extensions are evident F-actin appeared as straight dense bundles at the cell protrusions (FAs and podosome-like structures) where the cells are attached to the surface (white arrow heads). Whereas in round cells, the F-actin appeared as punctuate dots around the nucleus (red arrow heads). Nuclei stained with Hoechst 33342.

As discussed above the appearance and localization of the F-actin in the attachment sites of macrophages to their substratum differed between the P and the SLA surfaces. This pattern may indicate differences in the proteins such as FAK, vinculin, and paxillin recruited to those attachment sites. The following section will present the results obtained by immunostaining of vinculin, a component of FAs and podosomes.

2.3.3 Vinculin staining in respect to surface topography

Vinculin staining at the early times (0.5 and 6 h) can be seen in figures 2.13 & 2.14 respectively. At 0.5 h macrophages cultured on the P surfaces exhibited a dot-like staining pattern that differed from macrophages on SLA surfaces where the staining appeared patchy with few dots around the nucleus (figure 2.13). However, by 6 h the staining on the P surfaces was patchy, whereas macrophages on SLA demonstrated a well-formed dot-like appearance localized at the cellular extensions. This later dot-like appearance of vinculin was maintained and localized at both the cell periphery and cellular processes (figure 2.14). The negative primary antibody omitted controls showed no staining (figure 2.17).



Figure 2.13: Fluorescence images of vinculin staining on P and SLA surfaces at 0.5 h. Vinculin appearance and localization in cultured macrophage is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for 0.5 and stained for vinculin (red) with monoclonal mouse anti-vinculin. On both surfaces where cellular extensions are evident vinculin appeared as dense dots in the extensions (white arrow heads). Whereas in round cells, vinculin staining exhibited a patchy appearance with few dots located around the nucleus (green arrow heads). Nuclei stained with Hoechst 33342.



Figure 2.14: Fluorescence images of vinculin staining on P and SLA surfaces at 6 h. Vinculin appearance and localization in cultured macrophage is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for 6 h and stained for vinculin (red) with monoclonal mouse anti-vinculin. On both surfaces where cellular extensions are evident vinculin appeared as dense dots in the extensions (white arrow heads). Whereas in round cells, vinculin staining exhibited a patchy appearance with few dots located around the nucleus (green arrow heads). Nuclei stained with Hoechst 33342.

On day one vinculin staining in macrophages cultured on SLA surfaces demonstrated a dot-like appearance within the central areas of the cells and formed aggregates of wellformed and dense dots that were seen at the cell extensions (figure 2.15). These vinculin aggregates coincided with the cellular processes and extensions (focal contacts) that attached onto the surface features. When compared to the SLA surfaces, macrophages on the P surfaces demonstrated vinculin in the form of dense circular bundles in round-shaped cells with a slightly reduced dot-sized appearance. In addition, vinculin staining in these macrophages was diffuse with no preferential localization (figure 2.15). By day three the vinculin aggregate in macrophages cultured on SLA surfaces was reduced compared to those exhibited by macrophages cultured on SLA surfaces at day one. Furthermore, those vinculin features were reduced in numbers compared to those shown by macrophages cultured on the P surfaces at day three. Macrophages on P surfaces exhibited vinculin staining with well-developed vinculin plaques that were thicker and localized in the more prominent and more numerous cellular extensions (figure 2.16). The negative control samples (primary antibody delete) showed no staining (figure 2.17).

Generally differences in vinculin staining reflect a change in FAs and podosomes on the two surfaces over time.



Figure 2.15: Fluorescence images of vinculin staining on P and SLA surfaces at day one. Vinculin appearance and localization in cultured macrophage is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for one day and stained for vinculin (red) with monoclonal mouse anti-vinculin. On both surfaces where cellular extensions are evident vinculin appeared as dense dots in the extensions (white arrow heads). Whereas in round cells, vinculin staining exhibited a patchy appearance with few dots located around the nucleus (green arrow heads). The SLA surface features (peaks and deep pores) provided multiple attachment points where vinculin formed aggregates of short, thick and dense dots that were associated with the cellular processes and extensions (focal contacts and podosomes). Nuclei stained with Hoechst 33342.



Figure 2.16: Fluorescence images of vinculin staining on P and SLA surfaces at day three. Vinculin appearance and localization in cultured macrophage is dependent on surface topography. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for three days and stained for vinculin (red) with monoclonal mouse anti-vinculin. On both surfaces where cellular extensions are evident vinculin appeared as dense dots in the extensions (white arrow heads). Whereas in round cells, vinculin staining exhibited a patchy appearance with few dots located around the nucleus (green arrow heads). Vinculin aggregates in macrophages cultured on SLA surface were few compared to those on P surface. Nuclei stained with Hoechst 33342.



Figure 2.17: Fluorescence images of F-actin & vinculin (-) ve control (Primary antibody omitted). Representative images of macrophages cultured on P and SLA surfaces for 6 h and three days showed no staining of F-actin or vinculin. Nuclei stained with Hoechst 33342.

2.3.4 F-actin & vinculin double staining

F-actin and vinculin double staining in macrophages indicated co-localization of the two proteins. At 0.5 h, macrophages on the P surfaces showed F-actin and vinculin co-localized in the cellular extensions and at the cell periphery. In contrast, the two proteins were co-localized around the nuclei of macrophages on the SLA surfaces (figure 2.18). By 6 h, co-localization in macrophages on SLA surfaces appeared both in the cytoplasm as well as in the cellular extensions that attached to the topographic surface features. Co-localization on the P surfaces was reduced in the cellular processes and started to appear at the cellular periphery around the nucleus (figure 2.19).

Figure 2.18: Fluorescence images of co-localization of F-actin and vinculin staining on P and SLA at 0.5 h. F-actin and vinculin co-localization and appearance in cultured macrophage is a topography and time dependent. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for 0.5 h and stained with Oregon green phalloidin for F-actin (green) and with monoclonal mouse anti-vinculin for vinculin (red). On P (left column image) surfaces showed F-actin and vinculin co-localized in the cellular extensions and periphery of cells (white arrow heads). On the SLA surfaces (right column image), the two proteins were co-localized around the nuclei of macrophages (red arrow heads). A= F-actin, B= vinculin, and C= composite image of A & B. Nuclei stained with Hoechst 33342 (blue).



Figure 2.18: Fluorescence images of co-localization of F-actin and vinculin on P and SLA at 0.5 h.

Figure 2.19: Fluorescence images of co-localization of F-actin and vinculin staining on P and SLA at 6 h. F-actin and vinculin co-localization and appearance in cultured macrophages is topography and time dependent. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for 6 h and stained with Oregon green phalloidin for F-actin (green) and with monoclonal mouse anti-vinculin for vinculin (red). On P surfaces (left column image) co-localization F-actin and vinculin staining was reduced in the cellular processes and appeared more at the cellular periphery around the nucleus (red arrow heads). On SLA surfaces (right column image) co-localization appeared both in the cytoplasm as well as in cellular extensions that attached to the topographic surface features (white arrow heads). A= F-actin, B= vinculin, and C= composite image of A & B. Nuclei stained with Hoechst 33342 (blue).



Figure 2.19: Fluorescence images of co-localization of F-actin and vinculin on P and SLA at 6 h.

On day one, macrophages on SLA surfaces maintained co-localization of F-actin and vinculin staining compared to those on P surfaces. Localization of the two components can be seen at the cellular protrusions of macrophages on the SLA surface where the cells are attached to the surface topographic features. In contrast, the co-localization was reduced in macrophages on P surfaces as a result of the reduction in the staining appearance of the two proteins (figure 2.20). By day three, the co-localization of F-actin and vinculin in macrophages on SLA surfaces shifted from cellular FAs to be concentrated at the cell periphery. In contrast, the staining in macrophages on the P surfaces was now co-localized at the cellular extensions that attached firmly to the surface (figure 2.21).

In summary, this study determined that FA/podosome sub-structural contents such as F-actin and vinculin localization patterns varied with both time and topography.
Figure 2.20: Fluorescence images of co-localization of F-actin and vinculin staining on P and SLA at day one. F-actin and vinculin co-localization and appearance in cultured macrophages is topography and time dependent. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for one day and stained with Oregon green phalloidin for F-actin (green) and with monoclonal mouse anti-vinculin for vinculin (red). On P surfaces (left column picture) co-localization of F-actin and vinculin appeared around the nucleus (red arrow heads). On SLA surfaces (right column picture) co-localization of the two components can be seen more evident at the cellular extensions (FAs and podosomes) of macrophages where they are attached to the surface topographic features (white arrow heads). A= F-actin, B= vinculin, and C= composite image of A & B. Nuclei stained with Hoechst 33342 (blue).



Figure 2.20: Fluorescence images of co-localization of F-actin and vinculin on P and SLA at day one.

Figure 2.21: Fluorescence images of co-localization of F-actin and vinculin staining on P and SLA at day three. F-actin and vinculin co-localization and appearance in cultured macrophages is topography and time dependent. Representative fluorescence images of RAW 264.7 mouse macrophages differentiated for three days and stained with Oregon green phalloidin for F-actin (green) and with monoclonal mouse anti-vinculin for vinculin (red). On P surfaces (left column image) F-actin and vinculin staining was co-localized at the cellular protrusions of macrophages where they are attached to the surface topography (white arrow heads). In contrast, on SLA surfaces (right column image) F-actin and vinculin staining followed the cell shape patterns and was co-localized at the cellular periphery (red arrow heads). A= F-actin, B= vinculin, and C= composite image of A & B. Nuclei stained with Hoechst 33342 (blue).



Figure 2.21: Fluorescence images of co-localization of F-actin and vinculin on P and SLA at day three.

2.3.5 Effect of surface topography on macrophage cell viability over time

Cell viability was determined by a live/dead cell-staining assay in which the live cells fluoresce green and the dead ones fluoresce red. The immunofluorescence images of macrophages cultured on P and SLA surfaces at days one, three and five are shown in figure 2.22. At each time point the number of dead cells in 100 cells was counted on P and SLA surfaces and the representative graphs are illustrated in figure 2.23.

On day one there were significantly more dead cells on the P surfaces compared to the SLA surfaces (p< 0.05). On days three and five the number of dead cells significantly increased on the SLA surfaces compared to the P surfaces (p< 0.05).



Figure 2.22: Representative fluorescence images of topographic effect on macrophage viability (dead cells stained in red) at days, one, three, and five on P and SLA surfaces. Live/dead staining of macrophages indicated that the P surfaces prompted the rate of macrophage cell death at day one compared to SLA surfaces. Comparison at days, three and five revealed higher numbers of dead cells on the SLA surfaces compared to the P surfaces.



Figure 2.23: The effect of surface topography on macrophage cell viability at days one, three, and five. One hundred cells from randomly selected fields were counted and the percent of red stained dead cells for each surface at each time point was calculated. Comparisons were made between the two surfaces at each time point. Means with different letters indicate significant differences (p< 0.05). At day one the number of dead cells was significantly higher on P surfaces compared to SLA surfaces. By days three and five the numbers of dead cells on the SLA surfaces were significantly increased compared to P surfaces (p< 0.05). Data presents as means and SD (n= 3).

2.4 Discussion

Dental implant insertion induces a host inflammatory response that follows a prescribed sequence of events over time including blood material interaction and clot formation, provisional matrix production, acute inflammation, chronic inflammation, granulation tissue development, foreign body reaction, and possible fibrosis/fibrous capsule formation (7, 27, 28). A pivotal cell modulating the host response is the monocyte-derived macrophage (29). At the implant interface, the macrophage and it's morphological variant the foreign body giant cell (FBGC) appear rapidly after implantation and can remain on the surface throughout the wound healing process (6, 30, 31).

In this paper we investigated the topographic effects of dental implant surfaces on the macrophage *in vitro*. Although we recognize it is very difficult to separate the effects of surface chemistry/charge and surface topography we have tried to control this by using surfaces of different topographies sputter-coated with 60 nm of titanium dioxide. We evaluated the topographic effects on RAW 264.7 cell morphology and on F-actin, and vinculin organization followed by determination of cell number on the tested surfaces at days one, three and five post culturing.

In our study of topographical effects using RAW 264.7 cells, macrophage morphology between 0.5 h and 6hr changed from a predominantly spread pattern on the P surface to a predominantly spread pattern on the SLA surface. Macrophage spreading on P, B, AE, and SLA surfaces at latter times was also investigated. At day 1 spreading further increased on the SLA surface when compared to the P and AE surfaces. B surfaces also

significantly increased macrophage spreading compared to P and AE surfaces. By days three and five this pattern changed. The SLA, B, and AE surfaces significantly reduced macrophage spreading compared to the P surface. Our findings on rough surfaces complement those of Wójciak-Stothard *et al* (1996) who studied contact guidance of P388D1 murine macrophage cells on micro-grooved and smooth fused silica substrata and reported that after 15 minutes the macrophages aligned along the microgrooves but adopted a round shape on smooth surfaces (20).

The pattern of spreading of macrophages on the surfaces used in these experiments can be provisionally explained as a result of macrophage activation through three mechanisms that can interact. One mechanism appears to be operative immediately following cell plating and is controlled by the culture environment, in particular the proteins adsorbed to the culture surface. The second mechanism occurs later and progresses over time and is concerned with cell signaling in attachment structures. The third mechanism involves response to cytokines and chemokines. The first postulated mechanism is summarized as follows.

As a general rule cells do not adhere directly to the original TiO₂ surface but rather to a surface layer of adsorbed proteins (32). Three characteristics of a biomaterial affect protein absorption; surface chemistry, roughness and wetability. Absorbed proteins are cellular glycoproteins from the surrounding environment including sera-derived proteins *in vitro* as well as other body fluids *in vivo* (33, 34). These adsorbed proteins are responsible for the initial cellular adhesion to material surfaces. However, maintaining adhesion and further cell spreading and subsequent interaction with the surface depends also on the proteins produced and released to the ECM by the adherent cells

themselves. Activated macrophage populations are known to produce numerous enzymes and effector protein molecules (35) as well as fibronectin and the ECM protein betaIG-H3 (36). If the cells are not able to synthesize and deposit sufficient ECM molecules or obtain them from the surrounding environment, the cells undergo apoptosis (37, 38). In order for cells to adhere and spread they require a minimum number of ligands on the material surface to support focal contact formation. This minimum number of ligands required differs between cell types and is called the 'Hubbell limit' (39). The composition, type, amount, and conformation of the adsorbed proteins further influence cellular activities (40, 41) and are dependent on substratum surface properties such as chemical composition and surface roughness (42, 43).

In our *in vitro* model, the substratum and the surrounding medium environment activated the macrophages without interference from other stimuli that may be present *in vivo*. The proteins found in fetal calf serum (FCS) adsorbed onto the substratum give the macrophages a means of attaching via their surface integrin receptors. Integrins also act as mechanosensors transducing the structural cues in the extracellular environment to biochemical signals in the cytosol those subsequently direct cellular contractile forces through rearrangement of the cytoskeleton. As a consequence integrin signaling controls such cellular functions as adhesion, spreading, and migration (44).

Types, amounts, and properties of surface adsorbed proteins are time and surface topography dependent. Therefore, the effects of these proteins on cell-surface adhesion and spreading would be accordingly different. For example, after 10 minutes incubation, the total amount of adsorbed proteins is more pronounced on rough surfaces produced by polishing with 800-grit metallographic paper compared to smooth surfaces produced by polishing with 1200-grit metallographic paper. However, individual protein adsorption was differential and surface-dependent. There was more albumin protein adsorbed onto smooth surfaces compared to rough surfaces because Ti surface roughening increases surface hydrophilicity leading to a lower albumin adsorption (45). In contrast there was more fibronectin protein adsorbed onto rough surfaces compared to smooth surfaces (46). Furthermore, rough surfaces produced by sandblasting followed by acid etching (SLA) adsorbed 50% less fibronectin than smooth surfaces (47).

Consequently, the morphologies shown by macrophages on P surfaces at early may be due to different amounts or types of FCS-derived proteins adsorbed on the surface that promoted faster cell spreading.

A second mechanism may also modulate macrophage spreading on both P and SLA surfaces at later times. The previously mentioned integrin/protein substratum interactions are involved in regulating macrophage responses including rearrangement of the actin cytoskeleton, gene transcription, and cell proliferation and survival (44, 48). By day one the highest mean spread area was shown by macrophages cultured on SLA surfaces followed by those cultured on B surfaces compared to cells cultured on P and AE surfaces. This change in macrophage spreading on SLA and B surfaces can be provisionally explained as follows. Macrophage recognition of the surface adsorbed proteins initiates integrin-mediated intracellular signal transduction pathways affecting podosome formation, in which the FAK and Src mediated pathways are thought to play a pivotal role (49, 50). We hypothesize that phosphorylation of FAK and Src signaling molecules may be linked to macrophage spreading on Ti surfaces. Should these molecules be less phosphorylated (activated) in macrophages on P surfaces at day one,

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cell spreading would be reduced.

On day three and day five the highest cell spreading was shown by macrophages cultured on P surfaces followed by those cultured on B surfaces compared to cells cultured on SLA and AE surfaces. This later increase in cell size on P surfaces could be due to increased phosphorylation of FAK and Src molecules that in turn increased macrophage spreading. In support of this interpretation is a study demonstrating that osteoclasts of Src^{-/-} mice showed reduced F-actin formation and impaired migratory functions confirming a link between Src function and podosome assembly/disassembly (51).

The third postulated mechanism involves the role played by cytokines acting on cell receptors during cell adhesion and spreading. For example, IL-1 β , granulocyte-macrophage colony stimulating factor (GM-CSF), macrophage colony stimulating factor (M-CSF), and TNF- α regulate the ligation of macrophage integrin receptors with a variety of ECM proteins (52-55). De Nichilo *et al* showed differential time-dependent effects of M-CSF and GM-CSF on integrin expression in cultured human macrophages. This differential integrin expression caused differences in macrophage spreading at 24 hr but not at 4 days post-culturing. At 24 hr macrophage treated with M-CSF displayed $\alpha\nu\beta5$ integrins but not $\alpha\nu\beta3$ and the cells appeared flattened. On the other hand GM-CSF induced the expression of $\alpha\nu\beta3$ but not $\alpha\nu\beta5$ in macrophages still displaying only $\alpha\nu\beta5$ integrins and cells remained flattened. In contrast, GM-CSF-treated macrophages induced the expression of $\alpha\nu\beta3$ and $\alpha\nu\beta5$ and the cells adopted a well spread flattened morphology. Thereby changes in the integrin family members involved in attachment

caused significant differences in macrophage spreading (53). In a previous study from our laboratory, LPS-stimulated macrophages demonstrated an increased production of IL-1 β , IL-6, and TNF- α at 24 and 48 hrs with the highest levels being produced by macrophages attached to the roughest surface (SLA) compared to P surfaces (13). Therefore, in our system differences in cytokine production by macrophages cultured on P and SLA surfaces may affect integrin expression. Such expressed integrins may in turn direct cell spreading. It should be noted that macrophages on P and B surfaces may produced cytokines such as interleukin-4 and interleukin-13 that are known to induce macrophage spreading and FBGC formation (56, 57).

It is known that cell size varies as a cell progresses through the cell cycle. It should be noted that this size variation is a limitation of interpretations of cell area comparing populations with different proliferation rates. Nevertheless, we attempted to minimize this problem by measuring only well isolated cells that would not have divided recently (i.e. after mitosis). The recently divided cells are generally closely adhered to each other. Moreover, data obtained by measuring isolated cells was more reproducible than data obtained by measuring cells in clusters, as it is easier to exactly determine their outline. In any case the 2fold difference in area after only 0.5 h in culture cannot be explained by differences in cell proliferation rates, as the doubling time of the cells is around 18 h.

Macrophage spreading on P and rough surfaces necessarily involves cytoskeletal rearrangement. We used confocal scanning microscopy to examine formation and organization of F-actin and vinculin. Vinculin is known as one of the constituents of focal adhesion complexes and podosomes (58, 59). Our results showed that the F-actin appearance and localization corresponded with the morphological patterns induced by

surface topography. On both SLA and P surfaces, if the cells were well spread, the Factin was localized and concentrated in the cellular extensions that attached to the surface. On the other hand F-actin was concentrated close to the cell margin of macrophages having a rounded or limited spreading morphology. Similarly, vinculin was localized at cellular extensions (FAs and podosomes) of spread cells and at the periphery of round cells.

In summary we have demonstrated that at 0.5 h macrophages spread more on P surfaces and showed numerous extensions where their F-actin and vinculin were colocalized whereas in round macrophages on SLA the F-actin and vinculin co-localized at the cellular periphery. At day one macrophages on SLA showed co-localization of Factin and vinculin in FAs and podosomes but round macrophages on P surfaces showed bundles of F-actin and vinculin at the cell periphery. At days three and five, macrophages were well spread on P surfaces with well formed FAs and podosomes where the F-actin and vinculin appeared co-localized in plaques. In contrast, macrophages on SLA surfaces at days three and five were small and F-actin and vinculin co-localized at the periphery. These results indicated that the smoother the surface topography the more macrophages developed FAs and podosomes with time, and the more F-actin bundles formed and co-localized with vinculin in the FAs/podosomes.

2.5 References

- 1. Chou, L., Firth, J. D., Uitto, V. J., and Brunette, D. M. (1995) Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J Cell Sci* 108 (Pt 4), 1563-1573
- 2. Oakley, C., and Brunette, D. M. (1993) The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* 106 (Pt 1), 343-354
- 3. Takebe, J., Champagne, C. M., Offenbacher, S., Ishibashi, K., and Cooper, L. F. (2003) Titanium surface topography alters cell shape and modulates bone morphogenetic protein 2 expression in the J774A.1 macrophage cell line. *J Biomed Mater Res A* 64, 207-216
- 4. Brunette, D. M. (1988) The effects of implant surface topography on the behavior of cells. *Int J Oral Maxillofac Implants* 3, 231-246
- 5. Curtis, A., and Wilkinson, C. (1997) Topographical control of cells. *Biomaterials* 18, 1573-1583
- 6. Anderson, J. M. (2000) Multinucleated giant cells. *Curr. Opin. Hematol.* 7, 40-47
- 7. Gretzer, C., Emanuelsson, L., Liljensten, E., and Thomsen, P. (2006) The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials. *J Biomater Sci Polym Ed* 17, 669-687
- 8. Broughton, G., 2nd, Janis, J. E., and Attinger, C. E. (2006) The basic science of wound healing. *Plast Reconstr Surg* 117, 12S-34S
- 9. Garrigues, G. E., Cho, D. R., Rubash, H. E., Goldring, S. R., Herndon, J. H., and Shanbhag, A. S. (2005) Gene expression clustering using self-organizing maps: analysis of the macrophage response to particulate biomaterials. *Biomaterials* 26, 2933-2945
- 10. Bosca, L., Zeini, M., Traves, P. G., and Hortelano, S. (2005) Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *Toxicology* 208, 249-258
- 11. Hunt, T. K., Burke, J., Barbul, A., and Gimbel, M. L. (1999) Wound healing. *Science* 284, 1775
- 12. Tsirogianni, A. K., Moutsopoulos, N. M., and Moutsopoulos, H. M. (2006) Wound healing: immunological aspects. *Injury* 37 Suppl 1, S5-12

- 13. Refai, A. K., Textor, M., Brunette, D. M., and Waterfield, J. D. (2004) Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. *J Biomed Mater Res A* 70, 194-205
- 14. Xia, Z., and Triffitt, J. T. (2006) A review on macrophage responses to biomaterials. *Biomed Mater* 1, R1-9
- 15. Boyan, B. D., Bonewald, L. F., Paschalis, E. P., Lohmann, C. H., Rosser, J., Cochran, D. L., Dean, D. D., Schwartz, Z., and Boskey, A. L. (2002) Osteoblastmediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int* 71, 519-529
- 16. Brunette, D. M., Kenner, G. S., and Gould, T. R. (1983) Grooved titanium surfaces orient growth and migration of cells from human gingival explants. *J Dent Res* 62, 1045-1048
- 17. Hamilton, D. W., Chehroudi, B., and Brunette, D. M. (2007) Comparative response of epithelial cells and osteoblasts to microfabricated tapered pit topographies in vitro and in vivo. *Biomaterials* 28, 2281-2293
- 18. DeFife, K. M., Jenney, C. R., Colton, E., and Anderson, J. M. (1999) Cytoskeletal and adhesive structural polarizations accompany IL-13-induced human macrophage fusion. *J Histochem Cytochem* 47, 65-74
- Jiang, Takayama, S., Qian, X., Ostuni, E., Wu, H., Bowden, N., LeDuc, P., Ingber, D., and Whitesides, G. (2002) Controlling Mammalian Cell Spreading and Cytoskeletal Arrangement with Conveniently Fabricated Continuous Wavy Features on Poly(dimethylsiloxane)
- . American Cemical Society
- Wojciak-Stothard, B., Curtis, A., Monaghan, W., MacDonald, K., and Wilkinson, C. (1996) Guidance and activation of murine macrophages by nanometric scale topography. *Exp Cell Res* 223, 426-435
- 21. Chehroudi, B., Gould, T. R., and Brunette, D. M. (1988) Effects of a grooved epoxy substratum on epithelial cell behavior in vitro and in vivo. *J Biomed Mater Res* 22, 459-473
- 22. Wieland, M., Chehroudi, B., Textor, M., and Brunette, D. M. (2002) Use of Ticoated replicas to investigate the effects on fibroblast shape of surfaces with varying roughness and constant chemical composition. *J Biomed Mater Res* 60, 434-444
- 23. Rowland, S. A., Shalaby, S. W., Latour, R. A., Jr., and von Recum, A. F. (1995) Effectiveness of cleaning surgical implants: quantitative analysis of contaminant removal. *J Appl Biomater* 6, 1-7
- 24. Hayat, M. A. (1986) Glutaraldehyde: role in electron microscopy. *Micron Microsc. Acta.* 17, 115-135

- 25. Baharloo, B., Textor, M., and Brunette, D. M. (2005) Substratum roughness alters the growth, area, and focal adhesions of epithelial cells, and their proximity to titanium surfaces. *J Biomed Mater Res A* 74, 12-22
- 26. Ghrebi, S. S., Owen, G. R., and Brunette, D. M. (2007) Triton X-100 pretreatment of LR-white thin sections improves immunofluorescence specificity and intensity. *Microsc Res Tech* 70, 555-562
- 27. Anderson, J. M. (2001) Biological responses to biomaterials. *Annu. Rev. Mater. Res.* 31, 81-110
- 28. Anderson, J. M. (1993) Mechanismes of inflammation and infection with implanted devices. *Cardiovasc. Pathol.* 2, 33S-41S
- 29. Gordon, S. (2002) Pattern recognition receptors: doubling up for the innate immune responses. *Cell* 111, 927-930
- Brodbeck, W. G., Nakayama, Y., Matsuda, T., Colton, E., Ziats, N. P., and Anderson, J. M. (2002) Biomaterial surface chemistry dictates adherent monocyte/macrophage cytokine expression in vitro. *Cytokine* 18, 311-319
- 31. Xia, Z. D., Zhu, T. B., Du, J. Y., Zheng, Q. X., and Wang, L. (1994) Macrophages in degradation of calcium phosphate compound artificial bone: an in vitro study. *J Tongji Med Univ* 14, 137-141
- 32. ter Brugge, P. J., Dieudonne, S., and Jansen, J. A. (2002) Initial interaction of U2OS cells with noncoated and calcium phosphate coated titanium substrates. *J Biomed Mater Res* 61, 399-407
- 33. Kowalczyska, H. M., Nowak-Wyrzykowska, M., Dobkowski, J., Kolos, R., kaminski, J., Makowska-Cynka, A., and Marciniak, E. (2002) Adsorption characteristics of human plasma fibronectin in relationship to cell adhesion. *J. Biomed. Mater. Res. Part B; Appl. Biomater.* 61, 260-269
- 34. Wilson, C. J., Clegg, R. E., Leavesley, D. I., and Pearcy, M. J. (2005) Mediation of biomaterial-cell interactions by adsorbed proteins: a review. *Tissue Eng* 11, 1-18
- 35. Tsukamoto, Y., Helsel, W. E., and Wahl, S. M. (1981) Macrophage production of fibronectin, a chemoattractant for fibroblasts. *J Immunol* 127, 673-678
- 36. Gratchev, A., Guillot, P., Hakiy, N., Politz, O., Orfanos, C. E., Schledzewski, K., and Goerdt, S. (2001) Alternatively activated macrophages differentially express fibronectin and its splice variants and the extracellular matrix protein betaIG-H3. *Scand J Immunol* 53, 386-392
- 37. Garcia, A. J., Vega, M. D., and Boettiger, D. (1999) Modulation of cell proliferation and differentiation through substrate-dependent changes in fibronectin conformation. *Mol Biol Cell* 10, 785-798

- 38. Moiseeva, E. P. (2001) Adhesion receptors of vascular smooth muscle cells and their functions. *Cardiovasc Res* 52, 372-386
- Massia, S. P., and Hubbell, J. A. (1991) An RGD spacing of 440 nm is sufficient for integrin alpha V beta 3-mediated fibroblast spreading and 140 nm for focal contact and stress fiber formation. *J Cell Biol* 114, 1089-1100
- 40. Baujard-Lamotte, L., Noinville, S., Goubard, F., Marque, P., and Pauthe, E. (2008) Kinetics of conformational changes of fibronectin adsorbed onto model surfaces. *Colloids Surf B Biointerfaces* 63, 129-137
- 41. Iuliano, D. J., Saavedra, S. S., and Truskey, G. A. (1993) Effect of the conformation and orientation of adsorbed fibronectin on endothelial cell spreading and the strength of adhesion. *J Biomed Mater Res* 27, 1103-1113
- 42. Heitz, J., Svorcik, V., Bacakova, L., Rockova, K., Ratajova, E., Gumpenberger, T., Bauerle, D., Dvorankova, B., Kahr, H., Graz, I., and Romanin, C. (2003) Cell adhesion on polytetrafluoroethylene modified by UV-irradiation in an ammonia atmosphere. *J Biomed Mater Res A* 67, 130-137
- 43. Walachova, K., Svorcik, V., Bacakova, L., and Hnatowicz, V. (2002) Colonization of ion-modified polyethylene with vascular smooth muscle cells in vitro. *Biomaterials* 23, 2989-2996
- 44. Delon, I., and Brown, N. H. (2007) Integrins and the actin cytoskeleton. *Curr Opin Cell Biol* 19, 43-50
- 45. Valagao Amadeu do Serro, A. P., Fernandes, A. C., de Jesus Vieira Saramago, B., and Norde, W. (1999) Bovine serum albumin adsorption on titania surfaces and its relation to wettability aspects. *J Biomed Mater Res* 46, 376-381
- 46. Deligianni, D. D., Katsala, N., Ladas, S., Sotiropoulou, D., Amedee, J., and Missirlis, Y. F. (2001) Effect of surface roughness of the titanium alloy Ti-6AI-4V on human bone marrow cell response and on protein adsorption. *Biomaterials* 22, 1241-1251
- 47. Francosi, P., Vaudaux, P., Tabotelli, M., Tonetti, M., Lew, D. P., and Descouts, P. (1997) Influence of surface treatments developed for oral implants on the physical and biological properties of titanium. (II) Adsorption isotherms and biological activity of inmobilized fibronrctin. . *Clin. Oral Implants Res.* 8, 217-225
- 48. Mocsai, A., Abram, C. L., Jakus, Z., Hu, Y., Lanier, L. L., and Lowell, C. A. (2006) Integrin signaling in neutrophils and macrophages uses adaptors containing immunoreceptor tyrosine-based activation motifs. *Nat Immunol* 7, 1326-1333
- 49. Rose, D. M., Alon, R., and Ginsberg, M. H. (2007) Integrin modulation and signaling in leukocyte adhesion and migration. *Immunol Rev* 218, 126-134
- 50. Schaller, M. D., and Parsons, J. T. (1994) Focal adhesion kinase and associated proteins. *Curr Opin Cell Biol* 6, 705-710

- 51. Sanjay, A., Houghton, A., Neff, L., DiDomenico, E., Bardelay, C., Antoine, E., Levy, J., Gailit, J., Bowtell, D., Horne, W. C., and Baron, R. (2001) Cbl associates with Pyk2 and Src to regulate Src kinase activity, alpha(v)beta(3) integrinmediated signaling, cell adhesion, and osteoclast motility. *J Cell Biol* 152, 181-195
- 52. Akagawa, K. S. (2002) Functional heterogeneity of colony-stimulating factorinduced human monocyte-derived macrophages. *Int J Hematol* 76, 27-34
- 53. De Nichilo, M. O., and Burns, G. F. (1993) Granulocyte-macrophage and macrophage colony-stimulating factors differentially regulate alpha v integrin expression on cultured human macrophages. *Proc Natl Acad Sci U S A* 90, 2517-2521
- 54. Liote, F., Boval-Boizard, B., Weill, D., Kuntz, D., and Wautier, J. L. (1996) Blood monocyte activation in rheumatoid arthritis: increased monocyte adhesiveness, integrin expression, and cytokine release. *Clin Exp Immunol* 106, 13-19
- 55. Xie, B., Laouar, A., and Huberman, E. (1998) Autocrine regulation of macrophage differentiation and 92-kDa gelatinase production by tumor necrosis factor-alpha via alpha5 beta1 integrin in HL-60 cells. *J Biol Chem* 273, 11583-11588
- 56. Christenson, E. M., Dadsetan, M., and Hiltner, A. (2005) Biostability and macrophage-mediated foreign body reaction of silicone-modified polyurethanes. *J Biomed Mater Res A* 74, 141-155
- 57. DeFife, K. M., Jenney, C. R., McNally, A. K., Colton, E., and Anderson, J. M. (1997) Interleukin-13 induces human monocyte/macrophage fusion and macrophage mannose receptor expression. *J Immunol* 158, 3385-3390
- 58. Buccione, R., Orth, J. D., and McNiven, M. A. (2004) Foot and mouth: podosomes, invadopodia and circular dorsal ruffles. *Nat Rev Mol Cell Biol* 5, 647-657
- 59. Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat Rev Mol Cell Biol* 2, 793-805

CHAPTER 3:

Modulation of Macrophage Signaling Cascades by Surface Topography

and the Effect of Src Protein Kinase Inhibitors on Cell Signaling,

Morphology and Number²

 ² A version of this chapter will be submitted for publication. Ghrebi, S., Hamilton, D.,
Waterfield, D., Brunette, D. Modulation of Macrophage Signaling Cascades by Surface
Topography and the Effect of Src Protein Kinase Inhibitors on Cell Signaling,
Morphology and Number.

3.1 Introduction

It is widely accepted that alterations in the topography of titanium surfaces exert a significant influence on cell behavior *in vitro* and *in vivo*.(1-3). In the case of titanium dental implants the host reactions follow a set pattern: injury, blood-material interactions, provisional matrix formation, acute inflammation, chronic inflammation, granulation tissue formation, foreign body reaction, and fibrosis/fibrous capsule formation (4, 5). Cells of the monoctyic lineage are involved in the progression of these host responses. This requires the early recruitment of monocytes to the implantation site by chemoattractants released by platelets following blood-material interactions(6). At the site, the monocytes differentiate into macrophages, which become involved in extracellular matrix turnover, angiogenesis, and stimulation of bone formation and bone resorption (7). These activated macrophages exert their effects by producing cytokines, and growth factors that are crucially involved in guiding tissue repair and wound healing (3, 8-10).

Upon activation the macrophages differentiate into a select functional phenotype. This is determined according to the time and type of the stimulus. During inflammation induced by lipopolysaccharide (LPS), macrophages exhibit a functional phenotype termed macrophage 1 (M1), which is a classically activated proinflammatory phenotype (11, 12). The M2 macrophages refer to various subtypes of non-classically activated macrophages. The production of anti-inflammatory cytokines is a common feature of M2 macrophages (13, 14).

The production and control of an array of inflammatory substances by M1 macrophages is complex and involves the activation and phosphorylation of several signaling pathways. One such pathway involves a kinase associated with focal adhesions (FAs) known as focal adhesion kinase (FAK). FAK is a non-receptor protein kinase implicated in signaling cascades involved in cell motility, migration, proliferation, and apoptosis (15). FAK tyrosine phosphorylation plays a crucial role in signal transduction through the focal adhesion complex triggered by diverse extracellular signals (16). Another signaling molecule is proto-oncogenic tyrosine kinase (Src), which is important in the expression of pro-inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor- α (TNF- α); Src ^{-/-} macrophages secrete less IL-1 and TNF- α (17). Furthermore, extracellular signalregulated kinase 1/2 (ERK1/2) of the mitogen-activated protein kinase (MAPK) family is activated in both LPS and integrin stimulated macrophages and also contributes to proinflammatory cytokine TNF- α production (18).

Human macrophages challenged with titanium particles exhibit high levels of tyrosine phosphorylation and MAPK activation that is associated with increased release of TNF- α , IL-1, and IL-6 (19). In our laboratory we investigated topographic RAW 264.7 murine macrophage activation in the presence of suboptimal doses of LPS. We reported that rough surface topography increased secretion of pro-inflammatory cytokines (IL-1, IL-6, and TNF- α) by LPS-stimulated macrophages. We also noted that the surfaces themselves were capable of stimulating production of TNF- α (20). In a similar study Takebe *et al.* showed that LPS-stimulated macrophages that attached to substratum *in vitro* produced bone morphogenetic protein-2 (BMP-2) (3). These two findings are supported by those of Nora *et al.* who showed that surface topography influenced macrophage activation resulting in a specific cytokine and gene expression pattern

different from that elicited by LPS indicating that topography does not activate macrophages by a similar mechanism to LPS (21). However, as yet the signaling molecules that are involved in topographically activated macrophages have not been fully investigated.

In an article submitted for publication we reported that the surface topography of commonly used dental implants modulate macrophage morphology, viability and patterning of the cytoskeleton and attachment structures including focal adhesions (FA) (Ghrebi et al, submitted). As integrin-mediated signaling molecules such as FAK are associated with FA, this finding suggests the possibility that FAK and FAK-related signaling molecules may show differential signaling patterns that vary with time and surface. In this paper we investigate topographical activation of RAW 264.7 macrophages to test the hypothesis that different surface topographies will differentially modulate signaling molecules. Phosphorylated tyrosine residues were first evaluated. Subsequently we measured phosphorylation of FAK, Src, and ERK1/2 signaling molecules. Src protein kinase inhibitor, 4-amino-5- (4-methylphenyl)-7-(t-butyl) pyrazol [3,4-d]-pyrimidine (PP1) and 4-amino-5- (4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d]pyrimidine (PP2) were used to determine the crosstalk between Src and FAK signaling molecules and on the ERK1/2 MAPK signaling pathway. Finally, we report on the effect of the Src protein kinase inhibitor PP1 on macrophage morphology and cell number when plated on our test surfaces.

3.2 Materials and methods

3.2.1 Fabrication and characterization of implant surface replicas

Ti-discs with a diameter of 10 mm and 1mm in thickness were kindly provided by Dr. M Wieland (Straumann AG, Waldenburg, Switzerland). Following the procedures previously developed in our laboratory (22, 23), negative impressions of polished (P), acid-etched (AE), sand-blasted (B), and B and acid-etched (SLA) topographies were made using vinyl polysiloxane impression material (PROVIL[®] novo Light; Heraeus kulzer, Domrmagen, Germany). The negative impressions were then used to cast replica surfaces in epoxy resin (Epoxy Technology, Billerica, MA). The epoxy was mixed following manufacturer's guidelines. The negative impressions were filled with the epoxy, left to settle overnight and then baked in a 59°C oven for four days. After hardening the replica surfaces were trimmed, cleaned, and left overnight to dry. The surfaces were sputter-coated with 60 nm of titanium dioxide using the physical vapor deposition technique (PVD), reactive magnetron sputtering at the Paul Scherer Institute, Villingen, Switzerland. SEM images of the four surfaces used in the study are presented in figure 3.1. The chemical composition and the oxide layer of epoxy Ti coated replicas produced following the above-described method were examined using X-ray photoelectron spectroscopy (XPS). The reported results determined that that Ti, oxygen, carbon, and nitrogen were present on all Ti-coated epoxy replicas and the Ti oxide-layer thickness was found to be 4.5 to 5.5 nm and was dominated by TiO₂. In the same study, original Ti surfaces and their replicas were characterized using a non-contact laser profilometer (LPM) and a stereo scanning electron microscope (stereo-SEM). LPM and stereo-SEM characterization indicated that there was no significant difference in the integral roughness parameters was found between the original Ti surfaces and their Ti-coated epoxy replicas (23).

3.2.2 Cell culture

The mouse RAW 264.7 macrophage cell line was purchased from ATCC, Manassas, VA. These cells were chosen for their ability to secrete pro-inflammatory cytokines upon stimulation with LPS similar to murine peritoneal exudates cells (Dr. A. Refai personal communication). The cells were cultured in 75cm³ tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) in Dulbecco's modified Eagle's medium (DMEM) (StemCell Technologies Inc, Vancouver, B.C.) supplemented with antibiotics: penicillin G 100 mg/ml, gentamycin 50 mg /ml (Sigma-Aldrich, St, Louis, MO), amphoteracin B (Gibco, Grand Island, NY) 3 mg /ml, and 15% heat-inactivated fetal calf serum (FCS) (HyClone AdvancSTEM[™], Logan, UT). The cultured cells were incubated at 37°C in a humidified 95% air and 5% CO₂ environment in a ThermoForma incubator (Thermo Electron Corporation, Waltham, MA). They were fed with fresh complete medium every 3 days, and sub- cultured just before reaching confluence.

3.2.3 Experimental design

The surfaces were randomly allocated to each treatment condition for both early and late times. Three experiments were carried out on each sample at each time. A split-plot analysis of variance was used to test hypotheses about treatment means and their interaction with time. In the split-plot model, surface and condition represent treatments in the main plots while the time steps constitute the sub-plots. Two types of interactions were examined, one consisted of surface and time, and the second consisted of surface, condition, and time.

The early times were selected to assess early events of macrophage responses to P and rough surface topographies and to determine any form of transient macrophage activation after macrophage implant interaction. The later times were chosen to study macrophage responses to implant surface topography at the inflammatory and granulation tissue formation phases of wound healing where macrophages are known to participate in those events as outlined above.

3.2.3.1 Phosphotyrosine, pFAK, pSrc, and pERK1/2 staining

For immunofluorescence staining of phosphotyrosine, pFAK, pSrc, total ERK1/2 and phospho-ERK1/2 (pERK1/2) the samples were processed as follows. Polished, B, AE, and SLA surfaces were glow-discharged and placed in 24 well plates (Falcon, Becton Dickinson Labware, Franklin Lakes NJ). Macrophages were harvested from sub-confluent cultures and adjusted to 1 x 10⁵ cells/ml. One ml of cells was placed on each test surface. At the various time points, samples were processed for immunostaining as previously described (2, 24) with minor modifications. In brief, samples were washed in PBS twice for two minutes each, followed by 2 washes in 37 °C warm PBS buffer for two minutes each. The samples were then fixed in 4% formaldehyde (Fisher Scientific, Nepean, Ontario, Canada) in PBS for 0.5 h, and rinsed in PBS buffer (3 times for 10 minutes each), permeabilized in 0.2% Triton X-100 (Fisher Scientific, Nepean, ON) for two minutes as described previously (25) and then rinsed in PBS buffer (2 times for two minutes each). Non-specific antibody binding was blocked with 3% bovine serum albumin (BSA) (Sigma-Aldrich, St, Louis, MO) and 5% goat serum (Sigma-Aldrich, St,

Louis, MO) in PBS at 37 °C for 0.5 h. An indirect immunofluorescent labeling technique was carried out for the pTyrosine (Chemicon International, Temecula, CA), pFAK (Upstate Cell Signaling Solution, Charlottesville VA), pSrc (Upstate Cell Signaling Solution, Charlottesville VA) and pERK1/2 (Chemicon International, Temecula, CA) staining. The primary antibody delete (negative control) samples were left in the blocking solution, whereas the test samples were incubated with 50 µl of the relevant monoclonal antibodies at a 1:200 dilution in the blocking solution. Samples were then rinsed in 1% BSA in PBS (3 times for five minutes each), blocked again for 20 minutes and incubated with a 1/600 dilution of the secondary antibody Alexa fluor 546 goat anti-mouse (Molecular Probes, Burlington, ON) for 1 hour at room temperature. The samples were then washed in PBS (2 times for two minutes each), and counter-stained with 30 µl of Hoechst stain solution (Sigma-Aldrich, St, Louis, MO) for five minutes. Finally, samples were washed (5 times for two minutes each in PBS) and mounted as described above.

In some experiments cells were treated with the Src kinase inhibitors 4-amino-5- (4methylphenyl)-7-(t-butyl) pyrazolo [3,4-d]-pyrimidine (PP1) (Biomol International L.P. Plymouth Meeting, PA) at a concentration of 0.45 μ M PP1 in 1 ml of medium, 4-amino-5-(4-chlorophenyl)-7-(t-butyl) pyrazolo [3,4-d]-pyrimidine (PP2) at a concentration of 6 μ M of PP2 in 1 ml of medium or 4-amino-7-phenylpyrazol[3,4-d]-pyrimidine (PP3) as a negative control (Calbiochem[®], Gibbstown, NJ) at a concentration of 6 μ M of PP3 in 1 ml of medium (26-28) before plating cells on the surfaces. PP3 is structurally related to PP1 and PP2 but does not inhibit Src family members (negative control).

For fluorescence detection, the samples were observed with Nikon Confocal Microscope C1 and EZ-C1 3.50 software Ver. 1.0 (Nikon Instruments Inc. Melville, NY) and images

were captured with RETIGA 2000R camera (Q Imaging, Burnaby, BC, Canada). All pictures were captured under similar parameter settings such as the gain and pin hole size. The 488 nm argon laser was used for green fluorescence, the 408 ultraviolet laser for blue fluorescence, and the 543 helium neon laser for red fluorescence. Photoshop software was used to adjust all the pictures to a similar size.

3.2.3.2 Staining analysis and quantification

Staining of pFAK, pSrc, and pERK1/2 in cells on the various surfaces were compared to negative control (primary antibody deleted) samples. The Adobe[®] Photoshop[®] CS2 version 9.0.2 program was used to adjust all the pictures in similar size. In order to determine whether there was any difference between the different surfaces, staining was qualitatively evaluated visually in terms of staining form and localization in randomly selected fields at each time point and for each experimental condition. The pERK1/2-staining pattern was quantified in terms of nuclear translocation; pictures of random fields from each surface sample at each time point and for each experimental condition were captured. 200 cells were counted for each condition and the percentage of cells exhibiting complete nuclear ERK1/2 translocation were enumerated.

3.2.4 Western blotting

3.2.4.1 Protein isolation

Cells were seeded at 3x10⁵ cells /sample on 12 surfaces of each of the four surface topographies. At each time point, samples were washed for two minutes (twice with

PBS) then the cells were harvested and lysed with 0.37 μ l/ml of Diisopropyl fluorophosphates (DFP) (Toronto research Chemicals Inc., North York, ON, Canada) and 10 μ l/ml of protease inhibitor cocktail containing AEBSF 104mM, aprotinin 0.08 mM, leupeptin 2 mM, bestatin 4mM, pepstatin A 1.5mM, and E-64 1.4 mM (Sigma, Saint Louis, MO), 5 μ l/ml of PMSF (Sigma, Saint Louis, MO), and 10 μ l/ml of 100 mM EDTA (Sigma, Saint Louis, MO) in RIPA buffer (Sigma, Saint Louis, MO). Total protein concentration in each sample was measured using the BCA Protein Assay kit (Pierce Biotechnology, Inc. Rockford, IL).

3.2.4.2 Electrophoresis, membrane staining and gel quantification

Ten µl of the molecular weight marker Precision Plus Protein[™] all blue standards (Bio-Rad Laboratories, Hercules, CA) were loaded in the first left lane of the gel. An equal concentration (30 µg) of protein in loading buffer from each sample was loaded on the gel. Protein samples were resolved by SDS/PAGE 6% gel for pFAK, 14% gel for pSrc, and 12% gel for total and pERK1/2 and transferred to a polyvinylidene fluoride (PVDF) membrane (Sigma, Saint Louis, MO) for western blotting using PowerPac Basic[™] system (Bio-Rad Laboratories, Hercules, CA). The samples on the membrane were blocked (5% fat free dry milk in 0.05% TTBS containing 1% BSA for two hours) to prevent non-specific binding. After blocking, membranes were washed twice in TTBS for 10 minutes and incubated in the primary antibody at 1:1000 dilutions in TTBS overnight at 4°C. The same antibodies used for immunocytochemistry were used in the western blotting. The membranes were then washed twice in TTBS for 10 minutes and incubated for two hours with the secondary biotinylated goat anti-rabbit antibody (Bio-Rad Laboratories, Hercules, CA) at 1:3000 dilution in TTBS at room temperature. Following the incubation the membranes were washed in TTBS (twice for 10 minutes each) and incubated for a further two hours at room temperature in streptavidin-biotinylated alkaline phosphatase complex (Bio-Rad Laboratories, Hercules, CA). The membranes were then washed five times in TTBS (10 minutes each) and incubated in the AP color development buffer (Bio-Rad Laboratories, Hercules, CA) to develop the enhanced alkaline phosphatase into chromogenic color. All membranes used to detect the same signaling molecule (i.e Src) were developed in the color complex for the same length of time. Finally the membranes were washed in distilled water, dried overnight, and scanned with Epson Perfection V700 Photo Scanner (Digital ICE technologies). Densitometric quantification analysis of band area and gray value (integrated density) was carried out on a Macintosh computer using the image J analysis software (developed at the U. S. National Institutes of Health and available on the Internet at http://rsbweb.nih.gov/ij/download.html). For a protein loading control, total ERK1/2 was stained in the same experiment that was run to stain for pERK1/2. A similar volume and concentration was loaded and processed for staining the housekeeping proteins GAPDH (Chemicon International, Temecula, CA) and β -actin (Santa Cruz biotechnology[®], inc., Santa Cruz, CA) as controls for protein loading. These were run simultaneously during FAK and Src analysis.

3.2.5 Determination of macrophage cell number

After glow-discharge, surfaces were placed in 24 well plates. Cells were plated at 2.5x10⁴ cells/sample, fed and incubated as described above. PP1 and PP2 Src inhibitors and control PP3 (at concentrations described above) were incorporated into the cell culture. At each time point; one, two, three, four and five days samples were washed in

PBS twice for two minutes each and incubated in 0.5 ml of 0.25% trypsin for 10 minutes. One half ml of complete medium was used to counteract the effect of trypsin. Immediately afterwards the cells were removed from the surface and centrifuged for five minutes at 25,000 RPM. The cells were suspended in 1 ml of medium and the cell number of each sample was calculated. Three parallel samples were used in each experiment and the experiments were repeated three times.

3.2.5.1 Effect of Src inhibitors on macrophage morphology and spreading

Polished, B, AE, and SLA surfaces were glow-discharged using a Plasma Cleaner/Sterilizer PDC-32G (Harrick Scientific, Ossining, NY,) and placed in 24 well plates. For short time points (0.5, 2, and 6 h), only P and SLA substrate were used and cells were plated at 2x10⁵ cells/sample. For longer time points the four different surface topographies were used and the cells were plated as follows: 1x10⁵ cells for an experiment lasting for one day, $4x10^4$ for three days, and $2x10^4$ for five days. The plating population densities were selected to ensure sufficient available area for macrophage spreading during the incubation period. PP1 Src inhibitor and control PP3 (at concentrations described above) were incorporated into the cell culture. For processing, samples were washed in PBS twice for two minutes each. Each sample was then fixed in 4% formaldehyde (Fisher Scientific, Nepean, ON) in phosphate buffer saline (PBS) (Scientific, Nepean, ON) for 0.5 h at room temperature and washed in PBS 3 times for five minutes each. This was followed by fixation in 2.5 % glutaraldehyde (Sigma-Aldrich, St, Louis, MO) in PBS for one hour at 4°C then washing 3 times in PBS for five minutes each. The double fixation technique has been found to better preserve cellular structure. The rationale is that formaldehyde does not cross-link proteins but penetrates quickly. Thus, it serves to preserve the structure intact for the protein cross-linking agent glutaraldehyde (29). Samples were further fixed in 1% OsO₄ (Sigma-Aldrich, St, Louis, MO) in PBS for one hour. The fixative was removed and samples washed 3 times in distilled water for two minutes each. The samples were then incubated in 1% tannic acid (Sigma-Aldrich, St, Louis, MO) for 20 minutes at room temperature, washed 3 times in distilled water two minutes each, fixed again in OsO_4 for 0.5 h, and finally washed 3 times in distilled water for two minutes each. Sample dehydration was carried out in steps using the following ethanol concentrations 50%, 70%, 90% 95%, and 100% (each concentration used twice for five minutes each). The samples were then critical dried using the semi-automatic critical point dryer Samdri-795 (Tousimi Research Corporation, MD) according to the manufacturer's instructions. Finally the samples were mounted on aluminum stubs, and DC-sputtered with 15 to 20 nm gold in a Anatech Hummer-VI sputtering system coating unit (Surplus Process Equipment Corp. Santa Clara, CA). Samples were viewed using a Cambridge 260 stereo SEM (Leica Instruments, Wetzlar, Germany) at 10 to 13 KV accelerating voltage. Pictures were captured from random fields of less cell-populated areas using Orion 1.0 software (E.L.I. Microscopy, Eghezee, Begium). The Adobe[®] Photoshop[®] CS2 version 9.0.2 program was used to adjust all the pictures to similar size. Using the cell tracing function of image J 1.36b software from NIH, the area of 20 individual cells was obtained. The mean cell area of the traced 20 cells from each sample was calculated. Data obtained was presented as means of early (0.5, 2, and 6 h.) and late (day one, day three, and day 5) times.

3.2.6 Quantification and statistics

For each experiment, three tests were carried out under each condition and for each time point. The mean and the standard deviation of the readings (variable of interest) were calculated. Analysis of variance (ANOVA) was used to assess the statistical significance of comparisons among the four surfaces and the four conditions at the different time points as well as their interactions. The Generalized Linear Model (GLM) procedure in SAS version 9.13 (SAS Institute) was used for data analysis.

We used two models. One model was used for experiments in which different surfaces were observed over time (equation 1). The second model was used for experiments where the effect of both surfaces and conditions were studied over time (equation 2). The Bonferroni (Dunn) multiple t-tests were used for comparing means (main effects). In cases where the full model (equation 1 or 2) indicated a linear or quadratic treatment interaction over time, the analysis was repeated for each time separately.

The two models used in this analysis are as follows:

Model 1:

$y_{ijk} = \mu + \alpha_i + \varepsilon_{ir} + \theta_j + (\alpha \theta)_{ij} + \delta_{ijk}$

Where:

y ijk	= response for replicate k on surface i at time j;
μ	= overall mean;
α_i	= effect of the ith surface;
8 _{ir}	= random experimental error for replications within surfaces;
θj	= effect of the jth time;
(αθ) _{ij}	= interaction between the surface and time;

 δ_{ijk} = random experimental error for replicates within surface and time.

Model 2:

$$\mathbf{y}_{ijk} = \boldsymbol{\mu} + \boldsymbol{\alpha}_i + \boldsymbol{\beta}_j + (\boldsymbol{\alpha}\boldsymbol{\beta})_{ij} + \boldsymbol{\varepsilon}_{ijr} + \boldsymbol{\theta}_1 + (\boldsymbol{\alpha}\boldsymbol{\theta})_{il} + (\boldsymbol{\beta}\boldsymbol{\theta})_{jl} + (\boldsymbol{\alpha}\boldsymbol{\beta}\boldsymbol{\theta})_{ijl} + \boldsymbol{\theta}_{ijlk}$$

Where:

 $\begin{array}{lll} y_{ijlk} & = \mbox{response for replicate k on ith surface and jth condition at time l;} \\ \mu & = \mbox{overall mean;} \\ \alpha_i & = \mbox{effect of the ith surface;} \\ \beta_j & = \mbox{effect of the jth condition;} \\ (\alpha\beta)_{ij} & = \mbox{interaction between surface and condition;} \\ \hline \epsilon_{ijr} & = \mbox{random experimental error for replications within surfaces and conditions;} \\ \theta_1 & = \mbox{effect of time;} \end{array}$

 $(\alpha \theta)_{ii}$ = interaction between surface and time;

 $(\beta \theta)_{jl}$ = interaction between condition and time;

 $(\alpha\beta\theta)_{ijl}$ = interaction between surface, condition and time;

 δ_{ijlk} = random experimental error for replicates within surface and condition over time.

3.3 Results

3.3.1 Phosphotyrosine immunostaining and immunoblotting

3.3.1.1 Immunostaining of phosphorylated tyrosine residues

The major indication for activation of signaling molecules is the presence of phosphorylated tyrosine. Phosphorylation signal was localized at both the FAs and podosomes and the area around the nuclear envelope. Immunostaining of phosphorylated tyrosine residues of macrophages cultured on P and SLA surfaces at early times is shown in figure 3.1. The phosphorylation of the tyrosine residues observed in macrophages cultured on the P and SLA surfaces at 0.5 h showed a dot-like appearance. However, a more intense dot-like punctuate staining was seen on P surfaces compared to SLA. These dots were located more at the cell periphery. At 6 h tyrosine phosphorylation in macrophages cultured on SLA surfaces appeared as denser dot-like localized around the cell nucleus while the phosphorylation in cells on P surfaces was patchy. Negative controls (primary antibody omitted) showed no phosphotyrosine staining.



Figure 3.1: Representative immunofluorescence images of RAW 264.7 mouse macrophages cultured on P and SLA surfaces for (0.5 h & 6 h) and stained with rabbit anti-phosphotyrosine antibody for tyrosine phosphorylation (red). Tyrosine phosphorylation on P surfaces showed a dot-like appearance localized around the nucleus, whereas the staining on SLA surfaces appeared generally patchy allover the cytoplasm. By 6 h, tyrosine phosphorylation in macrophages on SLA surfaces showed a dot-like appearance whereas tyrosine phosphorylation in macrophages on P surfaces appeared patchy. Nuclei stained with Hoechst 33342 (blue).
At the later times (days one and three) the tyrosine phosphorylation patterns of the macrophages changed. On day one macrophages cultured on rough surfaces exhibited tyrosine phosphorylation that appeared as well formed dots located at the cellular extensions that attached to the surface features compared to macrophages on P surfaces that showed some dot-like staining located in the cytoplasm (figure 3.2). By day three, macrophages on all surfaces showed tyrosine staining that localized at the cellular extensions. The SLA surfaces also induced diffuse tyrosine phosphorylation that localized in the cytoplasm and at the cell periphery (figure 3.3). Negative controls (primary antibody omitted) showed no staining.



Figure 3.2: Images of tyrosine phosphorylation on P, B, AE, and SLA surfaces at day one. Representative immunofluorescence images of RAW 264.7 mouse macrophages cultured for one day and stained with rabbit anti-phosphotyrosine antibody for tyrosine phosphorylation (red). Tyrosine phosphorylation appeared as prominent dots-like in macrophages cultured on rough surfaces. The staining was localized at the cytoplasm and cellular extensions attached to topographic features. On P surfaces the staining appeared as diffused smaller dots that localized in the cytoplasm. Nuclei stained with Hoechst 33342 (blue).



Figure 3.3: Images of tyrosine phosphorylation on P, B, AE, and SLA surfaces at day three. Representative immunofluorescence images of RAW 264.7 mouse macrophages cultured on P, B, AE, and SLA for one day and stained with rabbit anti-phosphotyrosine antibody for tyrosine phosphorylation (red). Tyrosine phosphorylation on all the surfaces appeared as dots-like present both around the nucleus and in the cytoplasm. Nuclei stained with Hoechst 33342 (blue).

3.3.1.2 Immunoblotting of phosphorylated tyrosine residues

In addition to immunocytochemical staining of phosphotyrosine (days one, two and three), the phosphotyrosine residues in topographically activated macrophages were immunoblotted-using SDS/PAGE and stained for protein bands. The results indicated that the tyrosine phosphorylation increased in macrophages cultured on all surfaces over time. Both the number and appearance of bands demonstrated this finding (figure 3.4). On day one, macrophages on rough surfaces showed an increase in tyrosine phosphorylation compared to those on P surfaces as seen on most of the bands. Also there were some bands on rough surfaces that were missing on the P surfaces (mostly band number 7, which was hardly visible on P surfaces). On day two, the tyrosine phosphorylation in macrophages cultured on the P and B surfaces increased compared to macrophages on the AE and SLA surfaces as seen in some bands (mostly band number 10), which can be seen on P and B surfaces but barely seen on AE and SLA surfaces. By day three, there were more bands of tyrosine phosphorylation in macrophages cultured on SLA surfaces compared to other surfaces as represented by bands number 6 and 9 that were not seen on P, B, and AE surfaces.

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Figure 3.4: Representative images of immunoblotting of general tyrosine

phosphorylation. The western blots of phosphotyrosine from lysates of RAW 264.7 cells plated on P, B, AE, and SLA surfaces for 1, 2, and 3 days using rabbit antiphosphotyrosine antibody following polyacylamide gel electrophoresis (PAGE) of 30 µg. On day one it is evident that rough surfaces (AE, B, and SLA) increased the amount of phosphorylation compared to P surfaces. In addition, band number 7 was seen on the rough surfaces and absent on P surface. On day two, AE and SLA increased the amount of phosphotyrosine in macrophages compared to P and B surfaces. By day three, macrophages on SLA surfaces showed different tyrosine phosphorylation patterns as band number three was not seen compared to P, AE, and B surfaces. The qualitative evaluation was based on visual observation only.



Figure 3.4: Images of immunoblotting analysis of general tyrosine phosphorylation.

3.3.2 Immunocytochemistry and immunoblotting staining of FAK signaling molecules

3.3.2.1 Focal adhesion kinase activation and localization

Surface topography modulates macrophage expression of pro-inflammatory cytokines and chemokines. The production of pro-inflammatory cytokines in response to different biological stimuli is a complex process involving a number of different signaling pathways connected to a plethora of distinct cell surface receptors (15, 16). As noted above tyrosine residues were differentially phosphorylated on the various surfaces over time suggesting activation of signaling pathway(s). Therefore, we decided to investigate activation of the ERK1/2 family of MAP kinase pathways, as it is one of pathways primarily involved in production of proinflammatory cytokines. It was our hypothesis that integrins (transmembrane adhesion proteins) were involved in differential signaling on the various surface topographies. As mentioned in the introduction the ERK1/2 MAP kinase pathway is linked to the integrin-mediated FAK-Tyr397- Src binding in focal adhesion through the binding of Grb2 signaling molecule to FAK. Thereby the first target protein investigated was phosphorylated FAK.

On day one immunocytochemical staining of pFAK indicated that FAK phosphorylation in non-treated cultured macrophages on all surfaces appeared as small punctuate dots. However, the localization of the staining followed the macrophage's spreading patterns. The cells cultured on rough surfaces showed FAK staining in the cytoplasm and cellular extensions that comprised the FAs and podosomes. On P surfaces, FAK staining appeared more circular being found both at cell periphery as well as close to the nucleus. The Src kinase inhibitor PP1 reduced the levels of FAK phosphorylation in treated macrophages on both the rough and P surfaces, the reduction appearing more significant on the rough surfaces (figure 3.5 A). The Src kinase inhibitor PP2 also reduced FAK phosphorylation as well. In the PP3-treated macrophages (negative control), FAK phosphorylation was not reduced compared to non-treated samples (figure 3.5 B). No FAK staining was observed in negative control samples (primary antibody delete) as presented in figure 3. 12.

Figure 3.5 A & B: Representative immunofluorescence images of RAW 264.7

macrophages cultured on P, B, AE, and SLA surfaces cultured for one day and stained with anti-FAK (pTyr 397) for FAK phosphorylation (red). (A) Images of FAK phosphorylation in non-treated and PP1-treated macrophages. Macrophages cultured on rough surfaces showed FAK staining in the cytoplasm as well as in cellular extensions that comprised the FAs and podosomes. On P surfaces, FAK staining was found both at the cell periphery and close to the nucleus. The Src kinase inhibitor PP1 reduced the levels of FAK phosphorylation in treated macrophages cultured on all surfaces. (B) Images of FAK phosphorylation in PP2 and PP3 treated macrophages .The Src kinase inhibitor PP2 reduced FAK phosphorylation. However, in PP3-treated macrophages (negative control), FAK phosphorylation was not reduced compared to non-treated samples. Nuclei stained with Hoechst 33342 (blue).



Figure 3.5 A: Images of pFAK in non-treated and PP1-treated macrophages at day one.



Figure 3.5 B: Images of pFAK in PP2 and PP3-treated macrophages at day one.

By day three, FAK phosphorylation appeared as large well-formed dots in macrophages cultured on P surfaces and was uniformly distributed and localized to cellular extensions and around the nucleus. In macrophages cultured on AE, B, and SLA surfaces the staining appeared in a patchy diffuse pattern. Again, PP1 as well as PP2 reduced FAK phosphorylation as seen in figures 3.6 A and 3.6 B respectively. PP3 (negative control) showed no effect on FAK phosphorylation compared to non-treated samples (figure 3.6 B). No FAK staining was observed in negative control samples (primary antibody delete) as presented in figure 3. 12.

Figure 3.6: A & B: Representative immunofluorescence images of RAW 264.7 macrophages cultured on P, B, AE, and SLA surfaces for three days and stained with anti-FAK (pTyr 397) for FAK phosphorylation (red). (A) Images of FAK phosphorylation in non-treated and PP1-treated macrophages. FAK phosphorylation appeared as well-formed dots in macrophages cultured on P surfaces that uniformly distributed and localized to the cellular extensions, where as on rough surfaces the dots appeared smaller and fewer. PP1 reduced FAK phosphorylation in macrophages cultured on all surfaces. (B) Images of FAK phosphorylation in PP2 and PP3-treated macrophages, PP2 reduced FAK phosphorylation, however, in PP3-treated macrophages (negative control), FAK phosphorylation was not reduced compared to non-treated samples. Nuclei stained with Hoechst 33342 (blue).



Figure 3.6 A: Images of pFAK in non-treated and PP1-treated macrophages at day three.



Figure 3.6 B: Images of pFAK in PP2-treated and PP3-treated macrophages at day three.

3.3.2.2 Immunoblotting of topographically phosphorylated FAK

The pFAK 125kDa protein bands developed by immunoblotting from macrophage lysates at days one, two and three are presented in figure 3.7. The bands are divided into four experimental groups designated as non-treated (macrophages plated on the surfaces without exposure to any Src protein kinase inhibitors), PP1, PP2, and PP3-treated (macrophages were exposed to each of the Src protein kinase inhibitors immediately before plating). Each group consisted of the four surface topographies P, B, AE, and SLA. The house- keeping protein β -actin was immunoblotted as a gel loading control at day one and day two as seen in figure 3.7. The bands represent the topographic effects on FAK phosphorylation and were quantified with Image J and the means obtained were plotted as an area against time in days. The graphs are presented in figure 3.8. The effects of Src protein kinase inhibitors were also quantified with image J and the results were presented in figure 3.9. As a control for gel loading, the house keeping protein β -actin from lysates of non-treated macrophages cultured on all the surfaces at days one and two was immunoblotted. The phosphorylated levels of β -actin protein bands were equal on both day one and day two as visually observed (figure 3.7).

On day one the level of FAK phosphorylation in non-treated macrophages cultured on P, AE, and SLA surfaces was similar. However, the level of FAK phosphorylation on the three surfaces was significantly higher than that phosphorylated by macrophages cultured on B surfaces (p< 0.05) as seen in figure 3.8. On day two, phosphorylated FAK in non-treated macrophages cultured on P and AE surfaces was significantly higher than the phosphorylated FAK in macrophages cultured on SLA and B (p< 0.05). There were no significant differences in the phosphorylated FAK between P and AE or between SLA

and B surfaces of the non-treated macrophages at day two (p < 0.05) as seen in figure 3.8. In general, the levels of phosphorylated FAK on all surfaces were decreased at day two compared to day one.

By day three, the levels of phosphorylated FAK in non-treated macrophages cultured on P and B surfaces were higher than the levels of phosphorylated FAK in macrophages cultured on SLA and AE surfaces. Also these levels on P and B surfaces were higher at day three when compared to the levels at days one and two. In contrast, these levels on SLA and AE surfaces were lower at day three when compared to the levels at days one and two. In contrast, these levels on and two. Statistically, the level of phosphorylated FAK in macrophages cultured on P surfaces was higher than the levels of FAK phosphorylation on B, AE and SLA surfaces (p < 0.05). The second highest phosphorylated FAK level was seen in macrophages cultured on AE and SLA surfaces were not statistically significantly different (p > 0.05) as seen in figure 3.8.

PP1 and PP2 reduced the levels of phosphorylated FAK in macrophages that were cultured on all the surfaces; the reduced levels were statistically significant when compared to the levels of phosphorylated FAK within non-treated macrophages cultured on the same surface (p< 0.05). The levels of phosphorylated FAK demonstrated by non-treated and PP3-treated (negative control) macrophages were not statistically different (p> 0.05) as seen in figure 3.9. As expected the levels of phosphorylated FAK shown by non-treated and PP3-treated (negative control) macrophages were not statistically different (p> 0.05) as seen in figure 3.9. As expected the levels of phosphorylated FAK shown by non-treated and PP3-treated (negative control) macrophages were not statistically different (p> 0.05) as seen in figure 3.9. As expected the levels of phosphorylated FAK shown by non-treated and PP3-treated (negative control) macrophages were not statistically different (p> 0.05) as seen in figure 3.9.

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P= polished, B= blasted, E= acid-etched, and S=sand blasted & acid-etched

Figure 3.7: The presented blots are representative of the amounts of phosphorylated FAK from lysates prepared from RAW 264.7 macrophages plated on P, B, AE, and SLA surfaces for 1, 2, and 3 days and determined by western blotting analysis using anti-FAK (Tyr397) antibody following polyacylamide gel electrophoresis (PAGE). The pFAK blots are presented in three rows indicated as (day one, day two, and day three) and according to the experimental condition; each row has four columns (Non-treated, PP1-treated, PP2-treated, and PP3-treated). The last row in the figure represents the β -actin blots at days one and two.



Figure 3.8: Quantification by image J analysis of pFAK immunoblots shown in (figure 3.7) from non-treated macrophages cultured on polished, blasted, acid-etches, and SLA surfaces for days one, two, and three. Comparisons were made between the four surfaces at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences. Means labeled with two letters do not differ significantly from means labeled with either letters (i.e. A B is not significantly different from either A or B) (p< 0.05). On day one, FAK phosphorylation in macrophages cultured on blasted surfaces was significantly less than FAK phosphorylation in macrophages cultured on polished surfaces but not different from macrophages cultured on blasted surfaces. On day two, phosphorylated FAK was significantly reduced in macrophages cultured on blasted and SLA surfaces compared to macrophages cultured on polished surfaces but was not different from macrophages cultured on polished surfaces but was not different from macrophages cultured on polished surfaces. By day three, The SLA and acid-etched surfaces significantly reduced FAK phosphorylation in cultured RAW 264.7 cells compared to polished and blasted surfaces. Data presented as means and SD (n=3).



Figure 3.9: Effects of Src protein kinase inhibitors (PP1, PP2, and PP3) on

phosphorylated FAK. Quantification by image J analysis of pFAK immunoblots shown in (figure 3.7). Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences. Means labeled with two letters do not differ significantly from means labeled with either letters (i.e. A B is not significantly different from either A or B). PP1 and PP2 significantly reduced phosphorylated FAK in macrophages cultured on all surfaces at all time points compared to non-treated samples, where as PP3-treated macrophages (negative control) showed no significant effect on topographically phosphorylated FAK compared to non-treated macrophages (p < 0.05). Data presented as means and SD (n=3).

FAK autophosphorylation at Tyr 397 is driven by integrin clustering at FAs. This autophosphorylation of FAK creates binding sites for SH2-domain-containing proteins such as Src. We then investigated the effect of surface topography on the phosphorylation of Src.

3.3.3 Immunocytochemistry and immunoblotting of Src signaling molecule

3.3.3.1 Src protein kinase activation and localization

Immunocytochemical staining of pSrc on day one in non-treated macrophages on SLA and B surfaces demonstrated a well-formed dot-like appearance, localized both around the nucleus and in the extensions. Phosphorylated Src in macrophages cultured on P and AE surfaces appeared diffuse throughout the cell area. The Src kinase inhibitor PP1 reduced the levels of phosphorylated Src in treated macrophages on all surfaces (figure 3.10 A). The PP2 Src kinase inhibitor also reduced Src phosphorylation (figure 3.10 B). However, in PP3-treated macrophages (negative control), Src phosphorylation levels were not reduced compared to non-treated samples (figure 3.10 A & B). Src phosphorylation on negative controls (primary antibody omitted) was not observed (figure 3.12). Figure 3.10 A & B: Representative immunofluorescence images of RAW 264.7 mouse macrophages cultured on P, B, AE, and SLA surfaces for one day and stained with antipSrc (pTyr 416) for Src phosphorylation (red). (A) Images of Src phosphorylation in nontreated and PP1-treated macrophages cultured on P, B, AE, and SLA surfaces. Src phosphorylation on B and SLA surfaces showed a well-formed dot-like appearance, localized around the nucleus. Phosphorylated Src in macrophages cultured on P and AE surfaces appeared diffused in the cytoplasm. The Src kinase inhibitor PP1 reduced the levels of phosphorylated Src in treated macrophages on all surfaces. (B) Images of Src phosphorylation in PP2-treated and PP3-treated macrophages cultured on P, B, AE, and SLA surfaces. The PP2 Src kinase inhibitor also reduced Src phosphorylation. However, in PP3-treated macrophages (negative control), Src phosphorylation levels were not reduced compared to non-treated samples. Nuclei stained with Hoechst 33342 (blue).



Figure 3.10 A: Images of pSrc in non- treated and PP1-treated macrophages at day one



Figure 3.10 B: Images of pSrc in PP2 and PP3-treated macrophages at day one.

By day three, Src phosphorylation on P surfaces was seen as well formed large dots and small short plaques localized at cellular extensions that mostly associated with FAs and podosome-like structure attachments (figure 3.11 A). The staining pattern on B surfaces gave a diffuse dot-like patchy appearance. On AE and SLA surfaces Src phosphorylation demonstrated a patchy appearance that was localized in the narrow cytoplasmic area around the nucleus. PP1and PP2-treated macrophages showed a decreased Src phosphorylation compared to non-treated cells (figure 3.11 B). PP3 showed no effect on Src phosphorylation levels in treated macrophages compared to non-treated ones (figures 3.11 A & B). Src phosphorylation on negative control samples (primary antibody delete) was not observed (figure 3.12). Figure 3.11 A & B: Representative immunofluorescence images of RAW 264.7 mouse macrophages cultured on P, B, AE, and SLA surfaces for three days and stained with anti-pSrc (pTyr 416) for Src phosphorylation (red). (A) Images of Src phosphorylation in non-treated and PP1-treated macrophages cultured on P, B, AE, and SLA surfaces. Src phosphorylation on P surfaces appeared as large dots and small short plaques localized at cellular extensions. The staining pattern on AE, B and SLA surfaces showed a diffuse dot-like patchy appearance that localized in the narrow cytoplasmic area around the nucleus. PP1-treated macrophages showed a decreased Src phosphorylation compared to non-treated cells (B) Images of Src phosphorylation in PP2-treated macrophages showed a decreased Src phosphorylation compared showed a decreased Src phosphorylation compared to non-treated cells. PP3 showed no effect on Src phosphorylation levels in treated macrophages compared to non-treated compared to non-treated cells. Nuclei stained with Hoechst 33342 (blue).



Figure 3.11 A: Images of pSrc in non- treated and PP-treated macrophages at day three.



Figure 3.11 B: Images of pSrc in PP2 and PP3-treated macrophages at day three.



Figure 3.12: Fluorescence images of FAK & Src (-) ve control (Primary antibody omitted). Representative images of macrophages cultured on P and SLA surfaces for one and three days showed no staining of FAK or Src. Nuclei stained with Hoechst 33342 (blue).

3.3.3.2 Immunoblotting evaluation of topographically phosphorylated Src

The pSrc 60kDa protein bands developed by immunoblotting from macrophage lysates at days 1,2 and 3 are presented in figure 3.13. The bands were divided to represent the four experimental groups designated as non-treated (macrophages plated on the surfaces without exposure to any Src protein kinase inhibitors), PP1, PP2, and PP3-tretaed (macrophages were exposed to each of the Src protein kinase inhibitors immediately before plating). Each group consisted of the four surface topographies P, B, AE, and SLA. The house keeping protein GAPDH was immunoblotted as a gel loading control at days one and two as seen in figure 3.13. The bands represent the topographic effects on Src phosphorylation and were quantified with Image J and the means obtained were plotted as an arbitrary area against time in days. The graphs are presented in figure 3.14.

On day one and as shown in figure 3.14 the highest level of pSrc was demonstrated by non-treated macrophages cultured on the SLA surface compared to P, B, and AE surfaces. This difference was found to be statistically significant (p< 0.05). Phosphorylated Src levels in non-treated macrophages cultured on B surfaces were significantly higher than pSrc levels in macrophages cultured on P and AE surfaces (p< 0.05). In PP1 and PP2-treated macrophages Src phosphorylated levels were significantly reduced. PP3, however, did not reduce phosphorylation levels compared to non-treated macrophages as shown in figure 3.14 and figure 3.15.

On day two, Src phosphorylation generally decreased compared to day one. Phosphorylated Src was significantly higher in non-treated macrophages cultured on P

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surfaces when compared to macrophages cultured on AE and SLA surfaces (p< 0.05) figure 3.14. The lowest level of phosphorylated Src was seen in macrophages cultured on SLA surfaces. These were found to be significantly different compared to the level of Src phosphorylation in macrophages cultured on P and B surfaces (p< 0.05) but not when compared to macrophages cultured on AE surfaces (p> 0.05) figure 3.14.

By day three the highest levels of pSrc were seen in macrophages cultured on P and B surfaces. These levels were significantly higher than pSrc in macrophages cultured on AE and SLA surfaces (p< 0.05). The lowest level of pSrc was seen in macrophages cultured on SLA surfaces. Again this level of Src phosphorylation was significantly different from pSrc seen in macrophages cultured on AE, P and B surfaces (p< 0.05).

Topographically induced Src phosphorylation was generally reduced in Macrophages exposed to Src inhibitors. PP1 as well as PP2 significantly reduced pSrc compared to non-treated macrophages. As expected, PP3 has no effect on Src phosphorylation. There was no statistical difference between pSrc in PP3-treated and non-treated macrophages cultured on all surfaces used in the study at all time points investigated (p> 0.05) figure 3.15.

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P= polished, B= blasted, E= acid-etched, and S=sand blasted & acid-etched

Figure 3.13: The presented western blots are representative of the amounts of pSrc in in lysates prepared from RAW 254.7 macrophages plated on P, B, AE, and SLA surfaces for 1, 2, and 3 days using anti-pSrc (Tyr416) antibody following polyacylamide gel electrophoresis (PAGE). The pSrc blots are presented in three rows indicated as (day one, day two, and day three) and according to the experimental condition; each row has four columns (Non-treated, PP1-treated, PP2-treated, and PP3-treated). The last row in the figure represents the GAPDH blots at days one and two.



Figure 3.14: Graph showing image J analysis of pSrc immunoblots shown in figure 3.13 from non-treated macrophages cultured on P, B, AE, and SLA surfaces for days one, two, and three. Comparisons were made between the four surfaces at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences. Means labeled with two letters do not differ significantly from means labeled with either letters (i.e. A B is not significantly different from either A or B) (p< 0.05). On day one, pSrc in RAW 264.7 cells cultured on SLA and blasted surfaces were significantly higher than pSrc in cells cultured on polished and acid-etched surfaces. On day two, pSrc was significantly reduced in cells cultured on SLA surfaces compared to macrophages cultured on polished, blasted, and acid-etched surfaces. By day three, The SLA and acid-etched surfaces significantly reduced Src phosphorylation in cultured RAW 264.7 cells compared to polished and blasted surfaces. Data presented as means and SD (n=3).



Figure 3.15: Effects of Src protein kinase inhibitors (PP1, PP2, and PP3) on pSrc.

Quantification by image J analysis of immunoblots shown in (figure 3.14). Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences. Means labeled with two letters do not differ significantly from means labeled with either letters (i.e. A B is not significantly different from either A or B). PP1 and PP2 significantly reduced pSrc in RAW 264.7 cells cultured on all surfaces at all time points except on blasted and acid-etched surfaces at day three. PP3-treated cells (negative control) showed no effect on topographically phosphorylated Src compared to non-treated RAW 264.7 cells. Data presented as means and SD (n=3).

Integrin-induced signaling leads to FAK autophosphorylation, which then initiates Src recruitment and Src phosphorylation as shown above. The FAK-Src complex formation activates FAK at the Tyr⁹²⁵ phosphorylation site creating binding sites for the SH2 domain of Grb2 and small GTPases such as R-Ras, linking FAK to the MAPK, Ras-ERK1/2 signaling pathway. We tested activation of EFK1/2 in response to our different surface topographies. Src protein kinase inhibitors were also used to test pathway interactions.

3.3.4 Immunocytochemical and immunoblotting of the ERK1/2 signaling molecule

Phosphorylation and nuclear translocation of ERK1/2 was studied by immunocytochemical staining at day one, day two, and day three. The percentages of ERK1/2 nuclear translocation in 100 cells of non-treated, PP1, and PP3-treated macrophages was determined. Moreover, immunoblotting of pERK1/2 from non-treated, PP1, PP2, and PP3-treated macrophage lysates at days one, two, and three and the gel loading control, total ERK1/2 at days one and two was conducted.

3.3.4.1 Immunostaining evaluation of pERK 1/2 phosphorylation and nuclear translocation

On day one, the number of cells displaying pERK1/2 nuclear translocation in non-treated macrophages cultured on SLA and AE surfaces was significantly higher than those displaying pERK1/2 nuclear translocation when cultured on P and B surfaces (p< 0.05) as seen in Figure 3.16 A & figure 3.17. On day two, the number of cells displaying pERK1/2 nuclear translocation in non-treated macrophages cultured on P surfaces was significantly higher than those displaying pERK1/2 nuclear translocation in non-treated macrophages cultured on P surfaces was significantly higher than those displaying pERK1/2 nuclear translocation when cultured on B, AE, and SLA surfaces (p< 0.05) as seen in figure 3.17. The pERK1/2 nuclear

translocation in non-treated macrophages cultured on SLA surface was the lowest and found to be statistically significant compared to pERK1/2 nuclear translocation in non-treated macrophages cultured on P and B surfaces (p< 0.05) as seen in figure 3.17. By day three the number of cells displaying of pERK1/2 nuclear translocation in non-treated macrophages cultured on P and B surfaces was significantly higher than those cultured on AE, and SLA surfaces (p< 0.05) as seen in figure 3.17. pERK1/2 nuclear translocation in non-treated macrophages cultured macrophages cultured on SLA surfaces was significantly higher than those cultured on AE, and SLA surfaces (p< 0.05) as seen in figure 3.17. pERK1/2 nuclear translocation in non-treated macrophages cultured on SLA surfaces was significantly lower than pERK1/2 nuclear translocation in the cells cultured on P, B, and AE surfaces (p< 0.05) as seen in figure 3.16 B & figure 3.17.

pERK1/2 nuclear translocation in PP1 and PP2-treated macrophages was reduced significantly compared to non-treated macrophages on the test surfaces (p< 0.05) (figures 3.16 A & B and 3.18. As expected, there was no difference in pERK1/2 nuclear translocation in PP3-treated macrophages and non-treated macrophages (p> 0.05) on as seen in figure 3.16 A, B and in figure 3.18).
Figure 3.16 A & B: Representative immunofluorescent images of RAW 264.7 macrophages cultured on P, B, AE, and SLA surfaces for one and three days and stained with polyclonal rabbit anti-pERK1/2 (Thr 202, Tyr 204) for pERK1/2 nuclear translocation (purple). Nuclei were stained with Hoechst. (A) Immunofluorescent images on day one of pERK1/2 in RAW 264.7 cells plated on P, B, AE, and SLA surfaces. There were less RAW 264.7 macrophages exhibiting pERK1/2 nuclear translocation on P and B surfaces compared to AE and SLA surfaces in the non-treated RAW 264.7 macrophages. PP1 Src inhibitor reduced pERK1/2 translocation in the cells when cultured on all the surfaces, PP3 showed no effect on pERK1/2 nuclear translocation. Negative control samples (primary antibody omitted) presented in the bottom row showed no pERK1/2 nuclear translocation (B) Immunofluorescent images of pERK1/2 phosphorylation on P, B, AE, and SLA surfaces at day three. P surfaces induced more pERK1/2 nuclear translocation compared to AE, B, and SLA surfaces. Again PP1 reduced pERK1/2 translocation and PP3 had no effect. Negative control samples (primary antibody omitted) are presented in the bottom row showed no pERK1/2 nuclear translocation.



Figure 3.16 A: pERK1/2 staining and nuclear translocation at day one (white arrows).



Figure 3.16 B: pERK1/2 staining and nuclear translocation at day three (white arrows).





macrophages cultured on polished, blasted, acid-etched, and SLA surfaces for one, two, and three days. Representative images shown in figure 3.16 A & B. Comparisons were made between the four surfaces at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). On day one the percentage of RAW 264.7 cells showing ERK1/2 nuclear translocation on acid-etched and SLA surfaces was significantly higher than ERK1/2 translocation seen in macrophages cultured on polished and blasted surfaces (p< 0.05). On day three the polished surfaces increased ERK1/2 nuclear translocation compared to acid-etched, blasted, and SLA surfaces. Data presented as means and SD (n=3).



Figure 3.18; Quantification of the effects of Src inhibitors (PP1, PP2, and PP3) on topographic pERK1/2 phosphorylation and nuclear translocation in macrophages Cultured on polished, blasted, acid-etched, and SLA surfaces for one, two, and three days. Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences. PP1as well as PP2 significantly reduced pERK1/2 nuclear translocation in macrophages cultured on all the surfaces at all time points (p< 0.05) whereas PP3 had no effect on pERK1/2 nuclear translocation compared to non-treated samples. Data presented as means and SD (n=3).

3.3.4.2 Immunoblotting evaluation of topographically phosphorylated ERK1/2

Bands of pERK1/2 at day one, day two, and day three and bands of total ERK1/2 at day one and day two obtained by western blotting analysis are shown in figure 3.19. The bands obtained for non-treated samples were quantified with Image J and the means obtained were plotted as an arbitrary area against time in days (figure 3.20).

On day one the highest level of ERK1/2 phosphorylation was seen in non-treated macrophages cultured on SLA surfaces. This was significantly higher when compared to non-treated macrophages cultured on P, B and AE surfaces (p< 0.05). Among all the surfaces, the lowest level of ERK1/2 phosphorylation was seen in non-treated macrophages cultured on the P surfaces (figure 3.20). On day two the levels of ERK1/2 were still higher in non-treated macrophages cultured on P and AE surfaces (p< 0.05). By day three, the highest level of ERK1/2 phosphorylation was seen in non-treated macrophages cultured on P and AE surfaces (p< 0.05). By day three, the highest level of ERK1/2 phosphorylation was seen in non-treated macrophages cultured on P and AE surfaces (p< 0.05). By day three, the highest level of ERK1/2 phosphorylation was seen in non-treated macrophages cultured on B, AE, and SLA surfaces. This was statistically significant (p< 0.05). Non-treated macrophages cultured on SLA surfaces demonstrated significantly lowest level of ERK1/2 phosphorylation compared to non-treated macrophages cultured on the other surfaces (p< 0.05) as seen in figure 3.20.

PP1-treated as well as PP2-treated macrophages generally showed significantly lower level of ERK1/2 phosphorylation compared to non-treated macrophages on all surfaces at all time pointes (p< 0.05). However, there was no effect of PP1 on ERK1/2 phosphorylation on AE surfaces at day two and there was no effect of PP2 on ERK1/2 phosphorylation on P surfaces at days one and two and on AE at day two and on SLA

surfaces at days two and three. As expected there was no statistical difference between phosphorylated ERK1/2 in PP3-treated macrophages and phosphorylated ERK1/2 in non-treated macrophages (figure 3.21).



Figure 3.19: The presented western blots are representative of the amounts of pERK1/2 in RAW 264.7 in lysates prepared from RAW 264.7 macrophages plated on P, B, AE, and SLA surfaces for 1, 2, and 3 days using anti-pERK1/2 (Thr 202, Tyr 204) antibody following polyacylamide gel electrophoresis (PAGE). The pERK1/2 blots are presented in three rows indicated as (day one, day two, and day three) and according to the experimental condition; each row has four columns (Non-treated, PP1-treated, PP2-treated, and PP3-treated). The last row in the figure represents the total ERK1/2 blots at days one and two.



Figure 3.20: Quantification by image J analysis of pERK1/2 immunoblots shown in figure 3.19 for non-treated macrophages cultured on polished, blasted, acid-etches, and SLA surfaces for days one, two, and three. Comparisons were made between the four surfaces at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). On day one, pERK1/2 in RAW 264.7 cells cultured on SLA, acid-etched, and blasted surfaces were significantly higher than pSrc in cells cultured on polished surfaces. On day two, pERK1/2 was significantly reduced in cells cultured on acid-etched and polished surfaces compared to macrophages cultured on blasted, and SLA surfaces. By day three, The SLA, blasted, and acid-etched surfaces significantly reduced ERK1/2 phosphorylation in cultured RAW 264.7 cells compared to polished surfaces (p< 0.05). Data presented as means and SD (n=3).



Figure 3.21; Effects of Src protein kinase inhibitors (PP1, PP2, and PP3) on pERK1/2. Quantification by image J analysis of pERK1/2 immunoblots shown in (figure 3.19). Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences. Means labeled with two letters do not differ significantly from means labeled with either letters (i.e. A B is not significantly different from either A or B) (p< 0.05). PP1 significantly reduced pERK1/2 in RAW 264.7 cells cultured on all surfaces at all time points except on acid-etched at day two compared to non-treated samples. PP2 significantly reduced pERK1/2 in macrophages cultured on all surfaces the reduction was not significant at day three. On the SLA surfaces there was a significant reduction on day one only. PP3-treated cells (negative control) showed no effect on topographically pERK1/2 when compared to non-treated RAW 264.7 cells. Data presented as means and SD (n=3).

Effect of Src protein kinase inhibitors on macrophage morphology plated on the test surfaces.

In this section we evaluated the effects of Src protein kinase inhibitor PP1 on macrophage morphology in PP1-treated samples in early times (0.5, 2, and 6 h) and at later times (days one, three, and five). SEM observation of PP1-treated macrophage morphology cultured on P and SLA surfaces at 0.5, 2, and 6 h indicated that the macrophages exhibited a rounded morphology with almost very few short microspikes compared to non-treated samples (figure 3.22). The Src protein kinase inhibitor PP1 significantly reduced macrophage spreading compared to the morphology showed by macrophages of non-treated samples (p< 0.05, figure 3.23).

Figure 3.22: Representative SEM images have non-treated and PP1-treated RAW 264.7 mouse macrophages cultured on P and SLA surfaces for 0.5, 2, and 6h. Macrophages treated with PP1 demonstrated rounded morphology compared to non-treated samples. Furthermore, PP1-treated macrophages showed limited or no microspike extensions compared to non-treated.



Figure 3.23: SEM images showed the effect of PP1 on macrophage morphology at 0.5, 2, and 6 h.

Figure 3.23: The effects of Src kinase inhibitor PP1 on topographically induce

macrophage spreading cultured on P and SLA surfaces for 0.5, 2, and 6 h. The means of cell areas of 20 cells on P (top graph) and SLA (bottom graph) surfaces obtained from image J analysis were plotted against 05, 2 and 6 h time points. Comparisons were made between the treated and PP1-treated macrophages plated on polished and SLA surfaces at 0.5, 2, and 6 h. Means with the same letter are not significantly different and means with different letters indicate significant differences. The mean cell area of PP1-treated macrophages on polished and SLA surfaces were significantly lower than that of non-macrophages plated on polished and SLA surfaces (p< 0.05). Data presented as means and SD (n=3).



Figure 3.23: The effects of PP1 on macrophage spreading at early times.

At late times (days one, three, and five) macrophages treated with PP1 and cultured on all the surfaces were generally significantly smaller (p< 0.05) when compared to the morphology exhibited by macrophages of non-treated samples.

Of specific note, macrophages on PP1-treated SLA samples on day one showed a more spherical morphology with the cells exhibiting more microspikes compared to the morphology of macrophages of PP1-treated and cultured on P, B, and AE samples (figure 3.24 A). By day three this pattern had changed slightly. PP1 treated macrophages on P surfaces were still spherical but larger in size. Moreover, they exhibited more microspikes compared to macrophages on B, AE, and SLA surfaces (figure 3.24 B). By day five PP1-treated macrophages on P surfaces were still spherical but larger in size. Moreover, they exhibited more microspikes compared to macrophages on P surfaces were larger and had multiple microspikes. On the other surfaces the cells were smaller and the microspikes decreased; the macrophages on the SLA surface being the smallest cells with the fewest microspikes (figure 3.24 C). The morphology of macrophages of PP3-treated samples (negative control) was similar to that of cells of the non-treated samples at all days (figures 3.24 A, B, & C). The PP1 induced reduction in macrophage spreading was significant compared to macrophage spreading in non-treated samples, whereas PP3-treated macrophages showed no statistical difference in spreading compared to macrophage spreading of non-treated samples (p> 0.05) as seen in figure 3.24 D.

Figure 3.24 A, B, C, and D: Representative SEM images of non-treated, PP1-treated and PP3-treated RAW 264.7 mouse macrophages cultured on P, B, AE, and SLA surfaces for one, two, and three days. (A) SEM images of non-treated, PP1-treated, and PP3-treated macrophages cultured for one day. The PP1-treated macrophages demonstrated rounded morphology compared to non-treated samples. Furthermore, PP1-treated macrophages showed few microspike extensions compared to non-treated. (B) SEM images of non-treated, PP1-treated, and PP3-treated macrophages cultured for three days. The PP1-treated macrophages on P surfaces were smaller compared to non-treated with more microspikes compared to macrophages on B, AE, and SLA surfaces. (C) SEM images of non-treated, PP1-treated, and PP3-treated macrophages treated cultured for five days. PP1-treated macrophages on P surfaces were larger and had multiple microspikes. On the other surfaces the PP1-treated-cells were smaller and the microspikes decreased; the macrophages on the SLA surface being the smallest cells with the fewest microspikes. The morphology of macrophages of PP3-treated samples (negative control) was similar to that of cells of the non-treated samples at all days (figures 3.25 A, B, & C). (D) Graphs show the effects of PP1 and PP3 Src kinase inhibitors on cell area of macrophages cultured on polished, blasted, acid-etched, and SLA surfaces. The means of cell area of 20 cells from each treatment condition obtained from image J analysis were plotted against time points. Comparisons were made between the three treatment conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). The mean cell area of PP1-treated macrophages on all surfaces was significantly lower than that of non-treated macrophages. No significance difference in spread area was found between PP3-treated and non-treated macrophages at all time points (p > 0.05). Data presented as means and SD (n=3).



Figure 3.24 A: SEM images show the effects PP1 and PP3 Src kinase inhibitor on macrophage morphology at day one.



Figure 3.24 B: SEM images show the effects of PP1 and PP3 Src kinase inhibitors on macrophage morphology at day three.



Figure 3.24 C: SEM images show the effects of PP1 and PP3 Src kinase inhibitors on macrophage morphology at day five.



Figure 3.24 D: Graphs show the effects of PP1 and PP3 Src kinase inhibitors on cell area of macrophages cultured on polished, blasted, acid-etched, and SLA surfaces for one, three, and five days. Comparisons were made between the three treatment conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). Data presented as means and SD (n=3).

Effect of surface topography on macrophage cell number over time in the presence of Src kinase inhibitors

Although macrophages *in situ* are terminally differentiated non-proliferating cells the RAW 264.7 cell line is capable of proliferation. We therefore tested the effects of surface topography on macrophage cell number and the influence of Src protein kinase inhibitors PP1, PP2, and PP3 on the cell number (figures 3.25 & 3.26).

On day one the macrophage cell number was higher on rough surfaces than on P surface. The highest macrophage number was found on SLA surface (p< 0.05), probably reflecting different levels of cell attachment. On day two the number of macrophages on SLA and B surfaces was significantly higher than the number of macrophages attached to AE and P surfaces (p< 0.05). Among all the surfaces the number of cells was the lowest on the P surface. On day three the number of macrophages in non-treated samples was again higher on the SLA and B surfaces compared to AE and P surfaces (p< 0.05) as seen in figure 3.25.

On day four the pattern changed slightly. Macrophage numbers in non-treated samples were significantly higher on AE and B surfaces compared to SLA and P surfaces. The lowest cell number was found on the P surfaces (p < 0.05). By day five the macrophage number on P and B surfaces was significantly higher compared to the number on AE, and SLA surfaces in non-treated samples (p < 0.05). The lowest cell number was seen on SLA non-treated samples (p < 0.05) as seen in figure 3.25.

In PP1-treated samples at days one, three, and five the numbers were significantly lower than the numbers on non-treated samples (p < 0.05). In PP1 and PP2-treated

cells, macrophage numbers were reduced significantly compared to non-treated samples (p < 0.05). There was no statistical difference between macrophage numbers attached to all surfaces of PP3-treated and non-treated samples (figure 3.26).



Figure 3.25 : The effect of surface topography on cell number of non-treated RAW 264.7 macrophages cultured on polished, blasted, acid-etched, and SLA surfaces. Macrophage numbers were calculated with electronic cell counter at days one, two, three, four, and five. Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). On day one the highest cell numbers were found on SLA surfaces followed by acid-etched surfaces compared to polished and blasted surfaces (p< 0.05). On days two and three the highest cell numbers were associated with SLA and blasted surfaces, whereas the cell numbers on acid-etched and polished surfaces were the lowest and the difference was statistically significant (p< 0.05). By days three and five SLA and acid-etched showed significant reduction in macrophage cell numbers compared to polished and blasted surfaces (p< 0.05). Data presented as means and SD (n=3).



Figure 3.26: Graphs show the effects PP1, PP2, and PP3 Src kinase inhibitors on cell number of macrophages cultured on polished, blasted, acid-etched, and SLA surfaces. Macrophage numbers were calculated with electronic cell counter at days one, three, and five. Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). The presence of macrophages was reduced in all PP1-treated samples compared to macrophage number on non-treated samples and the reduction was statistically significant (p< 0.05). PP2 had no effect on macrophage numbers on all surfaces at day three. PP3-treated macrophages showed no numerical differences from those seen on non-treated samples. Data presented as means and SD (n=3).

3.4 Discussion

Upon implant placement, recruited monocytes mature to terminally differentiated macrophages under the stimulus of both the chemical composition and surface topography of the implant. These cells are intimately involved in the biological responses that will determine the success or failure of the implant (30).

It is well established that alteration of cell size, shape, and spreading can be controlled by implant surface topography (31-36). In an earlier article (Ghrebi et al, to be submitted) on topography and patterning of the cytoskeleton it was found that the smoother the surface topography the more the RAW 264.7 cells developed FAs and podosomes with time and the more F-actin bundles formed and co-localized with vinculin in these adhesive structures. Such changes require activation of a variety of signaling pathways and downstream substrates. The activity of such signaling pathways is regulated in part by phosphorylation of tyrosine amino acids in signaling molecules. In this paper we first analyzed the effects of surface topography on tyrosine phosphorylation, and phosphorylation of FAK-Src and ERK1/2- MAPK signaling molecules.

Our results from immunocytochemistry indicated that the intensity and localization of phosphotyrosine (p-Tyr) corresponded to macrophage shape. Cells anchor themselves to a given substratum through FAs, these cellular sites are known to modulate translocation and phosphorylation of signal transduction molecules (37-40). Cross-linking of integrins by ECM molecules at FAs leads to rearrangement of the actin cytoskeleton and cell spreading (41). Our immunocytochemical results from the four

surface topographies indicated that the intensity and localization of phosphotyrosine corresponded to the spreading of the RAW 264.7 macrophages.

The differential tyrosine phosphorylation patterns of RAW 264.7 macrophages cultured on our surfaces over time can be provisionally explained as follows. Where macrophages demonstrated a large spread area they developed more and larger FAs and podosomes. As a result these well-formed adhesion structures induced high levels of integrin clustering and recruited higher amounts of actin associated proteins such as paxillin, tensin, and vinculin (42) that lead to autophosphorylation of tyrosine sites on FAK, which initiated FAK-Src signaling complex formation that directed phosphorylation of further tyrosine sites on Src and FAK and subsequently phosphorylation of other downstream tyrosine sites on ERK1/2-MAPK. In contrast, tyrosine phosphorylation was accordingly reduced in macrophages demonstrating a round morphology and smaller attachment structures that apparently lacked the ability to induce integrin clustering and vinculin recruitment.

During cell adhesion integrin clustering is associated with FA/podosome attachment to the ECM and results in adhesion dependent tyrosine phosphorylation (43, 44). Thus integrin clusters function not only as adhesion molecules but also as mechanotransducers (45, 46) that link ECM ligands and cytoskeletal structures and thereby orchestrate intracellular events (47, 48). Integrins, however, do not themselves possess enzymatic activity. In order for them to signal they must trigger downstream events including phosphorylation of FAK. FAK autophosphorylates when it localizes to focal adhesions (as a result of integrin clustering) via its C-terminal FAT (focal adhesion

targeting) domain (49). As topography influenced tyrosine phosphorylation patterns, we determined how surface topography affected FAK phosphorylation,

Localization of pFAK staining again followed the RAW264.7 macrophage's spreading patterns. The SLA surface down-regulated pFAK with time, whereas P surfaces up-regulated pFAK with time as shown both by immunostaining and immunoblotting.

Activation of FAK has long reaching consequences. FAK activation from integrins, when integrated with signals derived from growth factor/cytokine receptors, is instrumental in organization of the cytoskeleton, induction of cellular proliferation/ migration, rescue from apoptosis and production of inflammatory cytokines. These processes occur through interaction(s) with a number of interconnected signaling pathways.

One molecule that is capable of feeding several pathways is Src (a non-receptor tyrosine kinase) (50-52). Src localizes to endosomes, perinuclear membranes, secretory vesicles, and the cytoplasmic face of the plasma membrane. It is well placed to interact with a wide range of growth factors and integrin receptors (53). Our results showed that Src phosphorylation was associated with FAK phosphorylation and was surface and time dependent. We speculate that Src phosphorylation is initiated by FAK. Subsequent Src phosphorylation may be attributed to secondary stimuli such as cytokines produced by macrophages themselves (3, 20).

The high affinity binding of autophosphorylated FAK to Src also permits binding of other signaling molecules such as Grb2 to FAK. This binding is involved in initiating

other downstream signaling events in particular the ERK1/2-MAPK pathway. Immunocytochemical and western blot investigation of the pSrc and pERK1/2 translocation/pERK1/2 indicated that phosphorylation of these signaling intermediates again followed the same general pattern as cell spreading. When macrophages were spread with well-formed extensions, more FAK and Src phosphorylation was seen as well as the ERK1/2 phosphorylation and nuclear translocation. In contrast ERK1/2 together with FAK and Src phosphorylation was reduced in macrophages exhibiting a round morphology.

In order to test the crosstalk between ERK1/2 and FAK-Src complexes we treated macrophages with Src protein kinase inhibitors. The Src inhibitors PP1 and PP2 significantly reduced the levels of phosphorylated FAK on all the surfaces suggesting Src can influence phosphorylation of FAK. Furthermore, these Src inhibitors (particularly PP1) reduced ERK1/2 phosphorylation and pERK1/2 translocation on all surfaces indicating the dependence of ERK1/2 activation on Src phosphorylation. However, as the reduction mentioned above was not complete it is possible that ERK1/2 activation might also occur through other mechanisms such as secondary signaling stimuli resulting from growth factors in the medium.

Furthermore, effects of surface stresses may be involved in cellular activation of ERK1/2. Plotkin *et al.* 2005 tested the effect of mechanical stretching on ERK1/2 phosphorylation in osteocytes. They reported that mechanical forces transduced by integrins and a signalsome comprising actin filaments, microtubules, FAK, and Src kinase resulted in phosphorylation of the ERK1/2-MAPK signaling pathway (54).

Similarly Hamilton *et al.* 2007 using epithelial cells demonstrated higher levels of ERK1/2 nuclear translocation on topographically complex surfaces (grooves120 µm depth and 280 µm pitch) compared to P surfaces (33). ERK1/2 activation will then phosphorylates downstream transcription factors that are involved in many cellular functions (55, 56). That mechanical strain can affect monocytes pro-inflammatory function (acid phosphatase activity and IL-6 production) has been reported by Matheson *et al.* (57). Our findings on the differential effects of cells topography on pro-inflammatory cytokine and chemokine secretion can also been interpreted on the basis of strain related activation of ERK1/2. (20).

We conclude that the FAK, Src and ERK1/2 signaling molecules play an important role in regulating focal adhesion dynamics and cell spreading. Therefore we would expect to see a significant effect of Src inhibitors on RAW 264.7 morphology. This is what we found. Treatment of macrophages with PP1 prior to plating significantly reduced macrophage spreading on all surfaces both at early and late times compared to nontreated macrophages.

Results obtained from evaluation of macrophage morphology, F-actin and vinculin (Ghrebi *et a*l, to be submitted) distribution as well as of our reported FAK, Src, and ERK1/2 activation indicated differential cell responses to the smooth (P) and rough (B, AE, and SLA) surface topographies. As ERK1/2 is a major participant in cell growth and is differentially phosphorylated by our surfaces it is possible that the RAW 264.7 macrophages might exhibit different numbers of cells on the different surfaces. Upon investigation we found that on day one SLA surfaces demonstrated the highest number of macrophages followed by the B and AE rough surfaces. The lowest macrophage

number was found on P surfaces. On days two and three a greater number of macrophages attached to SLA and B surfaces compared to those attached to P and AE surfaces. However, by day five the numbers were found on SLA surfaces were significantly lower than those attached to B and P surfaces. At this point we can't conclude that the differential effects on the cell number reflect the ability of the surfaces to induce cellular proliferation. Cell number on a surface is a complex function of the number of cells that attach to a surface as well as the subsequent rates of cell proliferation and cell death. Our findings are supported by an *in vitro* study where the authors reported higher monocyte numbers attached to rough surfaces compared to the P surfaces after one day of culture (58). The authors similarly reported a 30% decrease in cell number attached to rough surfaces during the course of one week. More work is required to completely differentiate the roles of attachment, proliferation and cell death in producing these patterns. It would be interesting to determine whether the rougher topographies induced more cell death than the smooth surfaces in the long term; thereby providing another mechanism for immunoregulation.

In summary, we have demonstrated that surfaces of differing roughness topography demonstrated a differential effect on activation of intracellular signaling molecules. Thus it may be possible to use these findings to investigate cellular signaling pathways by differentially activating them by culturing the cells on substrata with specific roughness and feature shape.

3.5 References

- 1. Chou, L., Firth, J. D., Uitto, V. J., and Brunette, D. M. (1995) Substratum surface topography alters cell shape and regulates fibronectin mRNA level, mRNA stability, secretion and assembly in human fibroblasts. *J Cell Sci* 108 (Pt 4), 1563-1573
- 2. Oakley, C., and Brunette, D. M. (1993) The sequence of alignment of microtubules, focal contacts and actin filaments in fibroblasts spreading on smooth and grooved titanium substrata. *J Cell Sci* 106 (Pt 1), 343-354
- Takebe, J., Champagne, C. M., Offenbacher, S., Ishibashi, K., and Cooper, L. F. (2003) Titanium surface topography alters cell shape and modulates bone morphogenetic protein 2 expression in the J774A.1 macrophage cell line. J Biomed Mater Res A 64, 207-216
- 4. Anderson, J. M. (2000) Multinucleated giant cells. *Curr. Opin. Hematol.* 7, 40-47
- 5. Gretzer, C., Emanuelsson, L., Liljensten, E., and Thomsen, P. (2006) The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials. *J Biomater Sci Polym Ed* 17, 669-687
- 6. Broughton, G., 2nd, Janis, J. E., and Attinger, C. E. (2006) The basic science of wound healing. *Plast Reconstr Surg* 117, 12S-34S
- 7. Garrigues, G. E., Cho, D. R., Rubash, H. E., Goldring, S. R., Herndon, J. H., and Shanbhag, A. S. (2005) Gene expression clustering using self-organizing maps: analysis of the macrophage response to particulate biomaterials. *Biomaterials* 26, 2933-2945
- 8. Tsirogianni, A. K., Moutsopoulos, N. M., and Moutsopoulos, H. M. (2006) Wound healing: immunological aspects. *Injury* 37 Suppl 1, S5-12
- 9. Bosca, L., Zeini, M., Traves, P. G., and Hortelano, S. (2005) Nitric oxide and cell viability in inflammatory cells: a role for NO in macrophage function and fate. *Toxicology* 208, 249-258
- 10. Hunt, T. K., Burke, J., Barbul, A., and Gimbel, M. L. (1999) Wound healing. *Science* 284, 1775
- 11. Mantovani, A., Sica, A., and Locati, M. (2007) New vistas on macrophage differentiation and activation. *Eur J Immunol* 37, 14-16
- 12. Stout, R. D., and Suttles, J. (2004) Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol* 76, 509-513
- 13. Gerber, J. S., and Mosser, D. M. (2001) Reversing lipopolysaccharide toxicity by ligating the macrophage Fc gamma receptors. *J Immunol* 166, 6861-6868

- Mantovani, A., Sica, A., Sozzani, S., Allavena, P., Vecchi, A., and Locati, M. (2004) The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* 25, 677-686
- 15. Parsons, J. T. (2003) Focal adhesion kinase: the first ten years. *J Cell Sci* 116, 1409-1416
- 16. Abedi, H., and Zachary, I. (1997) Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J Biol Chem* 272, 15442-15451
- Aki, D., Mashima, R., Saeki, K., Minoda, Y., Yamauchi, M., and Yoshimura, A. (2005) Modulation of TLR signalling by the C-terminal Src kinase (Csk) in macrophages. *Genes Cells* 10, 357-368
- 18. Roach, S. K., and Schorey, J. S. (2002) Differential regulation of the mitogenactivated protein kinases by pathogenic and nonpathogenic mycobacteria. *Infect Immun* 70, 3040-3052
- 19. Boutahar, N., Guignandon, A., Vico, L., and Lafage-Proust, M. H. (2004) Mechanical strain on osteoblasts activates autophosphorylation of focal adhesion kinase and proline-rich tyrosine kinase 2 tyrosine sites involved in ERK activation. *J Biol Chem* 279, 30588-30599
- 20. Refai, A. K., Textor, M., Brunette, D. M., and Waterfield, J. D. (2004) Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. *J Biomed Mater Res A* 70, 194-205
- 21. Paul, N. E., Skazik, C., Harwardt, M., Bartneck, M., Denecke, B., Klee, D., Salber, J., and Zwadlo-Klarwasser, G. (2008) Topographical control of human macrophages by a regularly microstructured polyvinylidene fluoride surface. *Biomaterials* 29, 4056-4064
- 22. Chehroudi, B., Gould, T. R., and Brunette, D. M. (1988) Effects of a grooved epoxy substratum on epithelial cell behavior in vitro and in vivo. *J Biomed Mater Res* 22, 459-473
- 23. Wieland, M., Chehroudi, B., Textor, M., and Brunette, D. M. (2002) Use of Ticoated replicas to investigate the effects on fibroblast shape of surfaces with varying roughness and constant chemical composition. *J Biomed Mater Res* 60, 434-444
- 24. Baharloo, B., Textor, M., and Brunette, D. M. (2005) Substratum roughness alters the growth, area, and focal adhesions of epithelial cells, and their proximity to titanium surfaces. *J Biomed Mater Res A* 74, 12-22
- 25. Ghrebi, S. S., Owen, G. R., and Brunette, D. M. (2007) Triton X-100 pretreatment of LR-white thin sections improves immunofluorescence specificity and intensity. *Microsc Res Tech* 70, 555-562

- 26. Bain, J., McLauchlan, H., Elliott, M., and Cohen, P. (2003) The specificities of protein kinase inhibitors: an update. *Biochem J* 371, 199-204
- 27. Hanke, J. H., Gardner, J. P., Dow, R. L., Changelian, P. S., Brissette, W. H., Weringer, E. J., Pollok, B. A., and Connelly, P. A. (1996) Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynTdependent T cell activation. *J Biol Chem* 271, 695-701
- 28. Tatton, L., Morley, G. M., Chopra, R., and Khwaja, A. (2003) The Src-selective kinase inhibitor PP1 also inhibits Kit and Bcr-Abl tyrosine kinases. *J Biol Chem* 278, 4847-4853
- 29. Hayat, M. A. (1986) Glutaraldehyde: role in electron microscopy. *Micron Microsc. Acta.* 17, 115-135
- Geiger, B., Bershadsky, A., Pankov, R., and Yamada, K. M. (2001) Transmembrane crosstalk between the extracellular matrix--cytoskeleton crosstalk. *Nat Rev Mol Cell Biol* 2, 793-805
- Boyan, B. D., Bonewald, L. F., Paschalis, E. P., Lohmann, C. H., Rosser, J., Cochran, D. L., Dean, D. D., Schwartz, Z., and Boskey, A. L. (2002) Osteoblastmediated mineral deposition in culture is dependent on surface microtopography. *Calcif Tissue Int* 71, 519-529
- 32. Brunette, D. M., Kenner, G. S., and Gould, T. R. (1983) Grooved titanium surfaces orient growth and migration of cells from human gingival explants. *J Dent Res* 62, 1045-1048
- 33. Hamilton, D. W., Chehroudi, B., and Brunette, D. M. (2007) Comparative response of epithelial cells and osteoblasts to microfabricated tapered pit topographies in vitro and in vivo. *Biomaterials* 28, 2281-2293
- 34. DeFife, K. M., Jenney, C. R., Colton, E., and Anderson, J. M. (1999) Cytoskeletal and adhesive structural polarizations accompany IL-13-induced human macrophage fusion. *J Histochem Cytochem* 47, 65-74
- 35. Jiang, Takayama, S., Qian, X., Ostuni, E., Wu, H., Bowden, N., LeDuc, P., Ingber, D., and Whitesides, G. (2002) Controlling Mammalian Cell Spreading and Cytoskeletal Arrangement with Conveniently Fabricated Continuous Wavy Features on Poly(dimethylsiloxane)

. American Cemical Society

- Wojciak-Stothard, B., Curtis, A., Monaghan, W., MacDonald, K., and Wilkinson, C. (1996) Guidance and activation of murine macrophages by nanometric scale topography. *Exp Cell Res* 223, 426-435
- 37. Ingber, D. E. (1997) Tensegrity: the architectural basis of cellular mechanotransduction. *Annu Rev Physiol* 59, 575-599
- 38. Wozniak, M. A., Modzelewska, K., Kwong, L., and Keely, P. J. (2004) Focal adhesion regulation of cell behavior. *Biochim Biophys Acta* 1692, 103-119

- 39. De Nichilo, M. O., and Yamada, K. M. (1996) Integrin alpha v beta 5-dependent serine phosphorylation of paxillin in cultured human macrophages adherent to vitronectin. *J Biol Chem* 271, 11016-11022
- 40. Weber, C., Alon, R., Moser, B., and Springer, T. A. (1996) Sequential regulation of alpha 4 beta 1 and alpha 5 beta 1 integrin avidity by CC chemokines in monocytes: implications for transendothelial chemotaxis. *J Cell Biol* 134, 1063-1073
- 41. Yamada, K. M., and Geiger, B. (1997) Molecular interactions in cell adhesion complexes. *Curr Opin Cell Biol* 9, 76-85
- 42. Parsons, J. T. (1996) Integrin-mediated signalling: regulation by protein tyrosine kinases and small GTP-binding proteins. *Curr Opin Cell Biol* 8, 146-152
- 43. Kornberg, L., Earp, H. S., Parsons, J. T., Schaller, M., and Juliano, R. L. (1992) Cell adhesion or integrin clustering increases phosphorylation of a focal adhesion-associated tyrosine kinase. *J Biol Chem* 267, 23439-23442
- 44. Lipfert, L., Haimovich, B., Schaller, M. D., Cobb, B. S., Parsons, J. T., and Brugge, J. S. (1992) Integrin-dependent phosphorylation and activation of the protein tyrosine kinase pp125FAK in platelets. *J Cell Biol* 119, 905-912
- 45. Ingber, D. (1991) Integrins as mechanochemical transducers. *Curr Opin Cell Biol* 3, 841-848
- 46. Orazizadeh, M., Cartlidge, C., Wright, M. O., Millward-Sadler, S. J., Nieman, J., Halliday, B. P., Lee, H. S., and Salter, D. M. (2006) Mechanical responses and integrin associated protein expression by human ankle chondrocytes. *Biorheology* 43, 249-258
- 47. Giancotti, F. G., and Ruoslahti, E. (1999) Integrin signaling. *Science* 285, 1028-1032
- 48. Larsen, M., Artym, V. V., Green, J. A., and Yamada, K. M. (2006) The matrix reorganized: extracellular matrix remodeling and integrin signaling. *Curr Opin Cell Biol* 18, 463-471
- 49. Hildebrand, J. D., Schaller, M. D., and Parsons, J. T. (1993) Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J Cell Biol* 123, 993-1005
- 50. Alper, O., and Bowden, E. T. (2005) Novel insights into c-Src. *Curr Pharm Des* 11, 1119-1130
- 51. Frame, M. C. (2004) Newest findings on the oldest oncogene; how activated src does it. *J Cell Sci* 117, 989-998
- 52. Playford, M. P., and Schaller, M. D. (2004) The interplay between Src and integrins in normal and tumor biology. *Oncogene* 23, 7928-7946

- 53. Brown, M. T., and Cooper, J. A. (1996) Regulation, substrates and functions of src. *Biochim Biophys Acta* 1287, 121-149
- 54. Plotkin, L. I., Mathov, I., Aguirre, J. I., Parfitt, A. M., Manolagas, S. C., and Bellido, T. (2005) Mechanical stimulation prevents osteocyte apoptosis: requirement of integrins, Src kinases, and ERKs. *Am J Physiol Cell Physiol* 289, C633-643
- 55. Juliano, R. L., Reddig, P., Alahari, S., Edin, M., Howe, A., and Aplin, A. (2004) Integrin regulation of cell signalling and motility. *Biochem Soc Trans* 32, 443-446
- 56. Parsons, J. T., Martin, K. H., Slack, J. K., Taylor, J. M., and Weed, S. A. (2000) Focal adhesion kinase: a regulator of focal adhesion dynamics and cell movement. *Oncogene* 19, 5606-5613
- 57. Matheson, L. A., Maksym, G. N., Santerre, J. P., and Labow, R. S. (2006) The functional response of U937 macrophage-like cells is modulated by extracellular matrix proteins and mechanical strain. *Biochem Cell Biol* 84, 763-773
- 58. Soskolne, W. A., Cohen, S., Sennerby, L., Wennerberg, A., and Shapira, L. (2002) The effect of titanium surface roughness on the adhesion of monocytes and their secretion of TNF-alpha and PGE2. *Clin Oral Implants Res* 13, 86-93
CHAPTER: 4

The Effects of Surface Substratum Surface Topography on Macrophage

Behavior in vivo³

³ A version of this chapter has been published. Ghrebi, S. S., Owen, G. R., and Brunette, D. M. (2007) Triton X-100 pretreatment of LR-white thin sections improves immunofluorescence specificity and intensity. *Microsc Res Tech* 70, 555-562.

4.1 Introduction

The implantation of a medical device initiates a cascade of events that occur in response to the presence of a foreign body as well as to the injury of the surrounding tissue during surgery (1). During the chronic inflammatory response of wound healing macrophages are the predominant cell types present. The macrophages involved in the early stages of chronic inflammation are termed recruited macrophages and are thought to be important in the wound healing response to implants (2). Macrophages are essential in the wound healing process since they secrete active proteins that exhibit chemotactic, mitogenic and angiogenic properties, (3). In the rat, recruited macrophages can be distinguished from resident macrophages by the presence of a cytoplasmic marker named ED1 (4). ED1 is a heavily glycosylated protein of 90,000-110,000 MW, located in the lysosomal plasma membrane (5), and thought to be involved in the process of phagocytosis (5, 6). The ED1 antigen has been found to present a patchy granular appearance in the cytoplasm of the macrophage (4, 5). Immunoperoxidase techniques for light microscopy have previously been used to identify ED1 macrophages. The peroxidase activity was observed by incubation of samples with a solution of 3,3-diaminobenzidinetetrahydrochloride (DAB) (4, 5, 7) DAB, however, is difficult to observe on sections without amplification with silver developer (8, 9). The immunofluorescence technique in this chapter uses one of the Alexa fluor dyes (Molecular probes) as secondary antibodies. These have advantages over other long-wavelength dyes (10). The fluorescence of this dye could be observed without amplification and moreover it does not detect endogenous peroxidase activity that can be problematic for the immunoperoxidase method.

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Pre-embedding techniques have been used with success to localize surface antigens. Physically sectioning embedded tissue followed by the post-embedding labeling procedure is the most feasible means of intracellular antigen identification such as ED1. Peroxidase and gold light scattering (peroxidase-DABI) techniques for post-embedding immunohistochemistry have been used previously to identify macrophages expressing the ED1 marker in tissue sections. (11-16). Fluorescent probes have the advantages of convenience and versatility, lending themselves for example, to double labeling procedures as a variety of probes varying in excitation and emission wavelength have been developed.

In the first study my goal was to develop a technique that could specifically identify recruited macrophages in tissue sections using immunofluorescent histochemistry. Our main refinement was to use a technique that would preserve and protect the antigen after tissue processing. The second study applied the developed immunofluorescent technique in the *in vivo* rat model to test whether "SLA (rough) surface topography would selectively enhance both macrophage attachment/proliferation at the implant-tissue interface".

4.2 Materials & methods

In order to test the second hypothesis that "SLA (rough) surface topography would selectively enhance both macrophage attachment/proliferation *in vivo*" it was necessary to develop a technique to identify macrophages at the implant-tissue interface. My first experiments involved developing an immunostaining technique to identify macrophage

phenotype ED1 using a rat cell line. This technique was then applied to identify macrophages at the tissue-implant interface.

4.2.1 Identification of ED1 macrophage marker in vitro

4.2.1.1 Identification of ED1 in macrophages cultured on Ti-coated glass coverslips

Our approach was to first optimize the staining procedure on cultured macrophages. Rat alveolar NR8383 macrophages (ATCC, Manassas, VA) were cultured in 75cm³ tissue culture flasks (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ) in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY) supplemented with antibiotics: penicillin G 100 mg/ml, gentamycin 50 mg /ml (Sigma-Aldrich, St, Louis, MO), amphoteracin B (Gibco, Grand Island, NY) 3 mg /ml, and 10% of heat-inactivated FCS (Cansera, Rexdale, ON,). The cultured cells were incubated at 37 ^oC in 95% air and 5% CO₂. When the cells were at sub-confluence, they were scraped from the flasks with a cell scraper (Fisher Scientific, Nepean, ON). The cells were plated at a population density of 2x 10⁵ cells/ml/well on 50 nm titanium-coated (Randex 3140 sputtering System, Palo, Alto) glass cover slips (Fisher Scientific, Nepean, ON) placed in a 24 well culture tissue plate (Falcon, Becton Dickinson Labware, Franklin Lakes NJ). Each group contained experimental positive samples and others as negative controls. All cells were fed and incubated for 24 hours and then processed for indirect immunofluorescent labeling. Samples were washed in 0.1M PBS, pH 7.3, twice for two minutes each. Afterward, the group one (not Triton X-100-treated) samples (controls) were immediately fixed in a mixture of 4% formaldehyde (Fisher Scientific, Nepean, ON) and 0.2% glutaraldehyde (Sigma-Aldrich, St, Louis, MO) in PBS (17) for 15 minutes and washed 3 times for 10 minutes each in PBS. The group two (Triton X-100-treated samples) were fixed and washed identically to the controls then incubated for two minutes in 0.2% Triton X-100 (Fisher Scientific, Nepean, ON). The third group (Triton X-100-treated samples) was first incubated for two minutes in 0.2% Triton X-100 (18), fixed with the same fixative for 15 minutes and similarly washed in PBS as well. After fixation and washing, all samples were incubated for 20 minutes in 3% BSA (Sigma-Aldrich, St, Louis, MO), 0.1% glycine (Electron Microscopy Sciences, Hornby, ON), 0.2% Tween 20 (Fisher Scientific, Nepean, ON), and 5% normal goat serum (Sigma-Aldrich, St, Louis, MO) for blocking of non-specific binding. The (primary antibody omitted) negative control for each group was left in the blocking solution, whereas the putatively positive samples were incubated at 1:50 dilution of primary mAB ED1 (Serotec, Ltd. Raleigh, NC) in the blocking solution for 0.5 h, washed 3 times for 10 minutes each in PBS and blocked again for 20 minutes. All samples were then incubated with Alexa fluor 546-goat antimouse (Molecular Probes, Burlington, ON) secondary antibody for 0.5 h. Finally samples were washed 5 times for two minutes each in PBS and mounted on glass cover slips with Prolong anti-fade mounting medium (Molecular Probes, Burlington, ON). For fluorescence detection, the samples were observed with an AXIO Skop 2 epifluorescence microscope (Zeiss, Oberkochen, Germany) using a rhodamine, 540 nm wavelength excitation filters and a barrier filter for the desired emitted wavelength (red light). Pictures were captured with a low light-intensity CCD camera (PentaMax 12 bit CCD; Princeton Instrument Inc. Trenton, NJ) using Northern Eclipse 6.0 software.

4.2.1.2 Identification of ED1on sections of LR-White-embedded macrophage pellets

The cell line and the antibodies used were the same as those used in the first study. All processing steps were carried out at room temperature unless otherwise indicated. The attached macrophages were removed by scraping and were collected in 15 ml centrifuge tubes (Falcon, Becton Dickinson Labware, Farnklin Lakes NJ). The cell suspensions were centrifuged at 500,000 rpm for five minutes then each pellet was re-suspended with 5 ml of 0.1M PBS and centrifuged again for five minutes. After the supernatant was discarded, the samples were washed in 0.1M PBS twice for two minutes each, fixed with a mixture of 4% formaldehyde and 0.2% glutaraldehyde for 15 minutes, and then washed with PBS three times for five minutes each. Partial dehydration of samples with 30%, 50%, and 70% ethanol for five minutes each was performed. After dehydration, gradual infiltration of the samples on a rotary device were carried out with hard grade LR-White resin (Canemco Inc., St., Laurent, Quebec, ON) at 2:1 LR-White and 70% ethanol for 1 hour and then at 100% LR-White twice for 1 hour each. Following infiltration, the manufacturer's accelerator was mixed at the proportion of 1.5 ml per 1 ml of 0° C pre-cooled LR-White resin. The samples were placed in "0"-gauge gelatin capsules (Canemco Inc., St., Laurent, Quebec, ON) and filled with the LR-White mixture. The capsules were capped air tight and placed in a solid aluminum block with drilled holes of corresponding size for a tight fit. The solid aluminum block was pre-cooled to 0°C and served to absorb the heat energy released by polymerization. Afterwards the whole block was placed in a refrigerator and left to polymerize for three hours at 0° C.

Two mm thick sections were cut using a Sorvall MT2 ultra-microtome and transferred to poly-L- lysine pre-coated glass slides (Electron Microscopy Sciences, Hornby, ON) and

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left overnight to dry. Slides were divided into two groups, each containing experimental and control samples. Sections were then washed in PBS twice for two minutes each. The first group samples processed directly without pre-treatment with Triton X-100 (Fisher Scientific, Nepean, ON). Samples of the second group were incubated in 0.2% Triton-X-100 for two minutes, and then both groups were processed equally. Blocking for non- specific binding was performed by incubation of sections with 5% normal goat serum, 3% BSA (Sigma-Aldrich, St, Louis, MO), and 0.2% Tween 20 (Fisher Scientific, Nepean, ON) in PBS for 20 minutes. Experimental samples were then incubated with 1:50 proportions of ED1 and the blocking solution for 1 hour. Then samples were washed in PBS 3 times for five minutes each, blocked again for 20 minutes, and incubated with Alexa fluor 546 in blocking solution at 1:50 dilution for 1 hour. Afterwards, the samples were washed in PBS 5 times for two minutes each and mounted on glass cover slips with Prolong anti-fade mounting medium (Molecular Probes, Burlington, ON). Samples were observed using AXIO Skop2 epifluorescence microscope (Zeiss, Oberkochen, Germany) and photographed.

4.2.1.2.1 Effect of Triton X-100 on LR-White sections

4.2.1.2.1.1 Fluorescent microscopic evaluation

Both the Triton exposure time and Triton concentration were investigated. Two samples were used for each condition. Samples of the first group were considered as the positive controls and were exposed to 0.2% of the detergent for two minutes. The second group was exposed to 0.2% for 10 minutes. The third group was exposed to 2% for two minutes and the fourth group was exposed to 2% of the detergent for 10 minutes. We

carried out an experiment with the same technique and incubated the experimental samples in 0.4% Pepsin pH 0.2 for 15 minutes at 37^oC. The control samples were not exposed to Pepsin treatment.

4.2.1.2.1.2 SEM evaluation

The effect of Triton X-100 concentration and incubation time on sections of macrophage pellet embedded in LR-White resin was also evaluated by SEM. Sections of similar thickness to those used in immunostaining were cut and transferred to Thermanox tissue culture treated plastic cover slips (Nalgen Nunc International, Rochester, NY), left overnight to dry and then washed in PBS twice two minutes each. Samples for negative controls were washed twice in distilled water for two minutes each and left to dry in a tissue culture laminar-flow hood (Canadian Cabinet Co Ltd., Ottawa, ON). After exposure to the detergent as described, samples of each experimental group were washed in distilled water and allowed to dry. Then all samples were mounted on SEM aluminum stubs (Canemco Inc., St., Laurent, Quebec, ON), gold sputter coated (Edwards Coating unit, Gibco, Grand Island, NY), and observed with SEM (Cambridge Stereoscan 260, Cambridge, UK). To investigate the effect of Triton X-100, sections were cut and prepared as described above, washed with distilled water, and incubated in 0.2% osmium tetroxide (Canemco Inc., St., Laurent, Quebec, ON) for one hour. Then samples were washed 5 times in distilled water for five minutes each, each sample group was incubated with its specific Triton X-100 concentration for the intended duration, washed in distilled water, and left to dry. SEM observation was carried out as described above and images were captured using Orion 1.0 software.

4.2.2 Identification of ED1 macrophage marker in vivo

4.2.2.1 Fabrication and characterization of implants

Following the same procedure described previously in our laboratory (19, 20), vinyl polysiloxane impressions of SLA and P topographies were cut and assembled to fabricate a negative form of the designed implant shape, which was used to cast master implants in epoxy resin (EPO-TEK 302-3; Epoxy Technology). Negative impressions of master implants were made, placed in 5 ml sample vials (VWR. inter. Mississauga, Ontario, Canada) and cast in L. R. White[®] resin. The obtained implants were sputter-coated with 50 nm of titanium (Randex 3140 Sputtering System, Palo, Alto, CA) and stored in Teflon jars containing sterile degassed distilled water until use

4.2.2.2 Animal model and implantation procedure

Sprague Dawley rats were used in the *in vivo* experimental studies. The animal care committee, UBC, approved all protocols. The implants were placed subcutaneously as described by Chehroudi (19, 21). In brief, the rat was anesthetized, the fur above the parietal area shaved, and the skin scrubbed with Betadine (Purdue Frederich Inc. Toronto, Canada) followed by 70% ethanol. An access incision at the middle of the cranium was made, the periostium was elevated, the implant was rested firmly on the bone, and the skin sutured back by 4-0 polypropylene sutures (Ethico). This location was chosen as the site provides good surgery access and is less subject to movement. A prophylactic intramuscular injection of antibiotics (Pen-Di-Strep, Roger/STP. London, Ontario, Canada) was given to each animal. Dr. Chehroudi supervised all procedures

and the animals were monitored postoperatively on a daily basis. The number of rats found to be sufficient for statistical analysis was 6 rats for each time point.

4.2.2.3 Implant retrieval, fixation, and embedding

At each time point (three days, one week, two, three, four, five, six, seven, and eight weeks) animals were sacrificed with an overdose of sodium pentobarbital (MTC. Pharmaceutical, Mississauga, ON). Before heart collapse a five min. flush of warm heparinated saline into the circulation followed by perfusion with 150 ml of 4% formaldehyde (Sigma-Aldrich) in PBS was carried out through the left ventricle. The implant with the surrounding tissues was gently removed and placed overnight in the same fixative (22). Afterwards, the protruding portions were separated and processed at room temperature unless otherwise stated. Samples were washed in 0.1M PBS, pH 7.3 and partial dehydration with 30%, 50%, and 70% ethanol was carried out. This method preserves the antigenicity of the delicately fixed tissues (23-25). After dehydration, gradual infiltration with L. R-White[®] resin was performed in 2:1 L. R-White[®] and 70% ethanol for two hrs, followed by 100% L. R-White[®] overnight. Finally the samples were placed in fresh 100% LR-White for two hrs. Following infiltration, each implant portion was placed in a separate labeled "000"-gauge gelatin capsule and covered with a mixture of L. R-White[®] accelerator and 0°C pre-cooled L.R-White[®] plastic at1.5 µl to 1 ml proportions. The capsules were capped tightly and held in close contact in 0°C precooled solid aluminum block, the whole block returned to 0°C and the resin left for cold polymerization for three hours.

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4.2.2.4 Sectioning and immunohistochemistry

Groups of 20 serial 2 μ m thick sections were cut from throughout the implant. Five sections were transferred to each poly-L- lysine pre-coated glass slides (Electron Microscopy Sciences). Four slides from each time point were obtained and left overnight to dry. One slide was randomly allocated for negative control (Primary antibody omitted) with three slides left for ED1 staining. Sections were washed in PBS twice for 2 min, incubated in 0.2% Triton-X-100 (Fisher Scientific) for 2 min, and washed once with PBS. They were then incubated with 5% normal goat serum, 3% BSA (Sigma-Aldrich), and 0.2% Tween 20 (Fisher Scientific) in PBS for 20 min for blocking of non-specific binding. For ED1, sections were incubated with 1:50 dilution in the blocking solution for 1 hour. The sections were then washed in 0.1% BSA 3x 5 min, blocked again for 20 min, and incubated with Alexa fluor 546 in blocking solution at 1:50 dilution for 1 hour. Finally, samples were washed in PBS 5 times for 5 min each, and mounted with Fluoromount-G (Southern Biotech). From frozen samples, serial 4 to 6 µm sections of the frozen tissues were cut, collected on pre-coated slides and stored in -80°C until processed (26). Short immunofluorescence staining protocol was performed on cryosections for ED1macrophages (27, 28). Samples from both L. R-White[®] -embedded and frozen implants were analyzed for fluorescent staining using AXIO Skop2 microscope (Zeiss). Pictures were captured by Pentamax camera (Princeton) using Northern Eclipse 6.0 program.

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4.3 Results

Results of the experiments involved in developing the immunostaining technique to identify macrophage phenotype ED1 using a rat cell line are presented in the following section. This technique was then applied to identify macrophages at the tissue-implant interface (please refer to appendix A).

4.3.1 Identification of ED1 in macrophages in vitro

4.3.1.1 Immunohistochemistry of cultured macrophages

In developing the staining method, the first approach was to use cultured macrophages on cover slips as these provided a convenient source of cells with ED1 antigen that could be stained without the problems induced by embedding and sectioning. Previous studies using peroxidase staining have described ED1 as an intracellular antigen located on the lysosomal membranes and having a cytoplasmic patchy granular appearance (4, 5). We observed the same pattern of distribution of the ED1 antigen around the nucleus using the fluorescence technique. Our preliminary results, however, from cultured macrophages without any detergent treatment showed some non-specific staining (Figure 4.1 A). Cells permeabilized with Triton X-100 after fixation showed increased specific staining compared to untreated samples (Figure 4.1 B). However, cells incubated with the detergent for the same duration before fixation exhibited the best staining in terms of specificity and intensity (Figure 4.1 C). One negative control involved exposing the macrophages only to the secondary antibody (i.e. not to the ED1 antibody) and staining was not observed. In addition, we examined cell specificity by applying the technique to cells known not to express ED1, which included epithelial cells, fibroblasts, and osteoblasts and these cells failed to exhibit staining of ED1.



Figure 4.1: Representative images of alveolar macrophages plated on titanium coated glass cover slips and labeled with an immunofluorescent antibody to ED1 (arrow). (A) Cells untreated with Triton X-100. (B) Cells treated with 0.2% for 2 min after fixation. (C) Cells treated with 0.2% Triton X-100 for 2 min before fixation. D, E, and F (primary antibody omitted). Macrophages treated with 0.2% Triton X-100 for 2 min before fixation X-100 for 2 min before fixation showed specific staining with high intensity of granular appearance. (Reproduced with permission).

4.3.1.2 Immunostaining of sections of LR-White-embedded macrophage pellets

After optimization of the technique on whole cultured macrophages, we applied the method to sections of macrophage pellets. Improvement in staining specificity and intensity was observed when sections were incubated with 0.2% Triton X-100 for two minutes (Figure 4.2 A) in comparison to those samples processed in the same way but not exposed to Triton X-100 (Figure 4.2 B). Sections used for negative controls that were not incubated with ED1 antibody showed no staining.



Figure 4.2: Representative images of sections of LR-White embedded macrophage cell pellet labeled with an immunofluorescent antibody for ED1 (arrows) post-embedding. (A) Sections treated with 0.2% Triton X-100 for 2 min. (B) Sections untreated with Triton X-100. (C) Sections Treated with 0.2% Triton X-100 for 10 min. D) Sections treated with 2% Triton X-100 for 2 min. Improved staining specificity was seen after sections were incubated with 0.2% Triton X-100 for 2 min. Limited staining can be seen in sections exposed to higher concentrations of detergent or longer incubation times. (Reproduced with permission).

4.3.1.2.1 Effect of Triton X-100 on LR-White sections

4.3.1.2.1.1 Fluorescent microscopic evaluation

As Triton X-100 treatment improved staining, we examined the time and concentration dependence of this treatment. The standard group, which was exposed to 0.2% concentration and two minutes duration, showed the same pattern and intensity of ED1 staining as seen above, (Figure 4.2 A). Samples of the second group that was exposed to a 0.2% concentration of the detergent but for 10 minutes duration showed no staining and the outline form of the macrophages was affected (Figure 4.2 C). Prolonging incubation time results in loss of the cell integrity due to the extraction effect of Triton X-100. The same pattern could be readily seen on samples exposed to 2% Triton for 10 minutes (Figure 4.2 D). However, a limited ED1 staining could be detected in samples exposed to 2% concentration of the detergent for two minutes. Samples exposed to Pepsin showed no improvement of ED1 staining compared to non-treated samples.

4.3.1.2.1.2 SEM Evaluation

We examined the effect of Triton X-100 concentration and incubation time on sections of macrophage cell pellet embedded in LR-White resin using SEM. On sections exposed to 0.2% detergent concentration for two minutes the nuclei were prominently visible and surrounded with cytoplasmic structures (Figure 4.3 A). On the control sections that did not receive detergent treatment the nuclei of the macrophage cells were not clearly observed and the surface topography of the sections appeared unaffected (Figure 4.3 B). In sections that were exposed to the same concentration (0.2%) but for 10 minutes, the nuclei were obvious, but the cytoplasmic structures surrounding them were deformed and had partially lost their shape (Figure 4.3 C). These cytoplasmic structures could not be distinguished at all in samples of the third experimental group, which was exposed to 2% detergent concentration for two minutes and the nuclei also became less distinct (Figure 4.3 D). As expected, a greater extraction effect was seen after incubation with 2% detergent concentration or when sections were exposed to 0.2 % for a longer period (10 minutes). These effects were not found on sections that were incubated with Triton X-100 after Osmium treatment. All tissue-containing areas appeared similar with no loss of the cytoplasmic structures regardless of Triton X-100 concentrations or exposure times (Figure 4.4 A-C).



Figure 4.3: Representative SEM images of sections of LR-White-embedded macrophage cell pellet. (A) Sections treated with 0.2%Triton X-100 for 2 min. Nuclei (N) were prominent and cell outline (arrow) can be seen. (B) Sections untreated with Triton X-100. The nuclei (N) are less obvious but the cell outline (arrow) is partially visible. (C) Sections treated with 2%Triton X-100 for 10 min. Nuclei (N) became less distinct and the cell outline could not be distinguished. (D) Sections treated with 2%Triton X-100 for 2 min. Nuclei and cell outline are less obvious at this high concentration. (Reproduced with permission).



Figure 4.4: Representative SEM images of sections of macrophage cell pellet embedded in LR-White (R). Resin sections treated with 0.2% Osmium tetroxide before exposure to Triton X-100. (A) Sections treated with 0.2% Triton X-100 for 2 min. (B) Sections treated with 0.2% Triton X-100 for 10 min. C) sections treated with 2% Triton X-100 for 10 min. All tissue containing areas on the sections appeared identical with no loss of nuclei or cell outline, regardless of detergent concentration or exposure times. (Reproduced with permission).

4.4 Discussion

Different tissue preparation techniques can effect the distribution of intracellular protein antigen. For example, the distribution of calmodulin is affected by whether cell permeabilization is carried out before or after cell fixation (29). Exposure of cells to mild formaldehyde fixation followed by detergent extraction leads to the stabilization of both soluble and insoluble proteins in their native localizations (30). Macrophages treated in this way showed no staining which could be due to less membrane porosity caused by the fixation that affected antibody accessibility. Optimal staining was seen in cells fixed after gentle detergent treatment. This procedure removes some soluble proteins from the unfixed cells leaving insoluble structures that can be observed by immunostaining (18). This might explain the extensive and clear ED1 staining of this group.

Successful post embedding immunostaining is principally dependent on two parameters: antigen preservation and antibody accessibility to the antigen. These two parameters depend on physical interactions that can be influenced by tissue fixation, dehydration and the embedding material. During this study we were successful in post-embedding immunofluorescence labeling of the macrophage marker, ED1. Interestingly, pretreatment of the section with the detergent Triton X-100 improved the staining intensity. Triton X-100 treatment is a method normally associated with permeabilizing samples prepared for pre-embedding immunostaining but from our study it was apparent that Triton X-100 could be used to facilitate the penetration of antibodies into LR-White resin sections. Using Triton X-100 on LR-White resin sections to improve staining specificity and intensity has not been reported previously.

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In addition to ultra-structural preservation, the main consideration for tissue fixation in immunocytochemistry is the preservation of the antigens. Antigenicity is maintained by using fixatives that cross-link proteins but do not adversely affect the amino acid sequences that bind to the antibody. Mild fixation is possible with aldehydes, they crosslink proteins but are known not to fix lipid. Nevertheless, aldehydes preserve the protein portion of the phospholipid cell membrane (31).

For post-embedding labeling, the sample must be dehydrated in preparation for resin embedding. Partial dehydration (up to 70% ethanol) was applied to reduce the extraction of hydrophobic molecules because full dehydration can be detrimental to antigens (9, 24, 32). An embedding media, particularly suited for antigen preservation for immunostaining, was used. LR-White resin is an acrylic resin, with a very low viscosity (10cps) that facilitates tissue penetration. The hydrophilic nature of LR-White makes it possible to embed partially dehydrated samples with up to 12% volume of water. After polymerization, the hydrophilic characteristic of the resin is maintained and is advantageous for immunolabeling since the sections are permeable to aqueous solutions.

Another means for maintaining antigenicity is the use of low temperature embedding with chemical polymerisation. In this study a chemical accelerator was used to crosslink the monomer rather than using heat. When using chemical polymerisation it is possible to carefully monitor the extent of cross-linking by modifying the volume of accelerator used (33). A low cross-linked resin was used in this protocol since it has been demonstrated that low cross-linked resins are associated with higher immunostaining (34). One consequence of under polymerization is the swelling of sections in aqueous solutions. However, although such swelling can distort spatial relationships, it is advantageous for immunolabeling because sections are permeable to antibodies.

The topography on the resin surface is also thought to be important in exposing the antigen. LR-white section relief is rough, increasing the surface area of the section and consequently antibody accessibility to the antigen (25, 33, 35).

One of the most commonly used detergents in immunostaining is Triton X-100. Triton is a synthetic non-ionic, non-denaturing detergent (36, 37). The high solubilization of lipids by Triton is the result of its hydrophobic character (38). Non-ionic detergents are also used in immunolabeling to increase antibody specificity by reducing hydrophobic and charge interactions on the sample surface (39). Triton X-100 has also been used in the pre-treatment of cryo-sections to facilitate immunolabeling (40). We hypothesized that this step may improve specific labeling of ED1 by extracting lipids present in partially dehydrated LR white sections. Various Triton X-100 concentration and treatment duration showed that ED1 labeling was optimal at a concentration of 0.2% for two minutes.

Amphiphilic membrane proteins (such as ED1) are fixed by aldehydes but if they are still bound to lipid the proteins are vulnerable for detergent extraction (41). A 0.2 % concentration of Triton X-100 for 10 minutes incubation resulted in diminished labeling intensity and relocalisation of the antigen, indicating that extraction of the ED1 antigen was occurring. This observation agrees with previously published results, when the researchers exposed the samples to a concentration of 0.05% Triton X-100 for 20 minutes (41, 42). To investigate the physical effect of Triton X-100 on the sections, we imaged the resin section surface with SEM. LR-White resin sections treated by Triton X-

100 showed differences in surface topography only in the area occupied by tissue, changes in the resin itself were not detected. The surface of non-treated sections remained somewhat smooth while the surface of the optimum Triton X-100 procedure (0.2% for two minutes) was rougher.

The two most likely explanations for the increased staining of ED1 after Triton extraction of lipid membranes are: 1) The ED1 protein persisted in the sample after 70% ethanol dehydration or 2) the ED1 antigenic sites were exposed by breaking bonds that resulted from over fixation of the tissue by aldehyde cross-linking. To test the first possibility we carried out post-fixation of the sections with Osmium fixation (known to preserve unsaturated lipids (43) prior to Triton X-100 treatment. To test the second possibility, we pre-treated sections with pepsin that is known to break aldehyde bonds (24, 44). Treatment with osmium tetraoxide, however, mitigated the effect of Triton X-100 treatment. Treatment with pepsin did not significantly improve the labeling intensity (data not shown). Thus lipid extraction is the most likely action of Triton on the L. R. White tissue section. This result supports our hypothesis that Triton X-100 extracts unfixed lipid components (such as membranes) from the tissue areas in LR-White sections.

Macrophages are known to persist at the implant-tissue interface being preferentially found on rough surfaces (45). *In vitro* studies demonstrated that macrophages when activated by surface topography released proinflammatory cytokines and growth factors (46) and bone morphogenetic proteins (47). Using several surface topographic as well as LPS stimulation Refai *et al.* as well as Soskolne *et al.* studies demonstrated that macrophages adhered, proliferated and functioned on rough surfaces (46, 48). As ED1 macrophages were associated with rough subcutaneous implants, we speculate that

these cells will be activated early to produce pro-inflammatory cytokines. These studies have yet to be performed.

Our results showed that it is possible to identify ED1 macrophage in tissues attached to implants by employing immunohistochemistry techniques. Macrophages were attached to the SLA surface at the implant interface (please refer to appendix A). These macrophages were not seen on P surfaces. Gretzer *et al* 2006 showed that the highest numbers of ED1 macrophage were seen around implants in the period of 1-7 days post-implantation (49). Although our results from *in vitro* proliferation experiments showed more macrophages on P surfaces at day five compared to SLA surfaces, the cells seen in tissue may represent different macrophage subpopulations with different phenotypes. The extensively spread macrophages with their multiple long extensions we observed on P surfaces using SEM may indicate the potential of these surfaces to induce FBGC around these implants. In contrast, macrophages spreading on SLA surfaces were smaller with fewer extensions that may indicate transition from active inflammatory macrophages to another phenotype such as anti-inflammatory macrophages.

In conclusion, the accessibility of the antibody to ED1 antigen on alveolar macrophages in LR-White sections was improved by the extraction effected by Triton X-100. The most intense and clear specific fluorescent staining was observed when sections were pretreated with 0.2% Triton X-100 for 2 min. Longer exposure of sections to 0.2% Triton or 2 min exposure to 2% Triton lead to reduced ED1 labeling. SEM observations indicated that the detergent affected the cells and not the resin. This novel technique could be applied in many research areas where post-embedding fluorescent immunolabeling with higher labeling intensity is desired for recognition of ED1 on other macrophage subsets. In an *in vivo* study we noted the presence of recruited ED1 macrophages at the implanttissue interface using this technique. Once macrophages are identified we will be able to study more aspects of macrophage behavior at the implant vicinity such as macrophage attachment/number and gene expression.

4.5 References

- 1. Anderson, J. M. (1993) Mechanisms of inflammation and infection with implanted devices. *CArdiovasc. Pathol.* 2, S33-S41
- Rosengren, A., Bjursten, L. M., Danielsen, N., Persson, H., and Kober, M. (1999) Tissue reactions to polyethylene implants with different surface topography. J Mater Sci Mater Med 10, 75-82
- 3. Anderson, J. M. (2000) The cellular cascades of wound healing. In bone engineering, . *ed. JE Davies EM squared incorporated Toronto.*, 81-94
- 4. Dijkstra, C. D., Dopp, E. A., Joling, P., and Kraal, G. (1985) The heterogeneity of mononuclear phagocytes in lymphoid organs: distinct macrophage subpopulations in the rat recognized by monoclonal antibodies ED1, ED2 and ED3. *Immunology* 54, 589-599
- Damoiseaux, J. G., Dopp, E. A., Calame, W., Chao, D., MacPherson, G. G., and Dijkstra, C. D. (1994) Rat macrophage lysosomal membrane antigen recognized by monoclonal antibody ED1. *Immunology* 83, 140-147
- Yamate, J., Maeda, M., Benn, S. J., Laithwaite, J. E., Allan, A., Ide, M., Kuwamura, M., Kotani, T., Sakuma, S., and LaMarre, J. (2001) Effects of lipopolysaccharide on a macrophage-like cell line (HS-P) from a rat histiocytic sarcoma. *J Comp Pathol* 125, 15-24
- 7. Beelen, R. H. J., Eestermans, I. L., A., D. E., and Dijkstra, C. D. (1987) Monoclonal antibodies ED1, ED2, and ED3 againest rat macrophages: Expression of recognized antigens in different satges of differentiation. *Transplant. Proc.* 19, 3166-3170
- 8. Newman, G. R., and Jasani, B. (1998) Silver development in microscopy and bioanalysis: a new versatile formulation for modern needs. *Histochem J* 30, 635-645
- 9. Newman, G. R., Jasani, B., and Williams, E. D. (1983) A simple post-embedding system for the rapid demonstration of tissue antigens under the electron microscope. *Histochem J* 15, 543-555
- Berlier, J. E., Rothe, A., Buller, G., Bradford, J., Gray, D. R., Filanoski, B. J., Telford, W. G., Yue, S., Liu, J., Cheung, C. Y., Chang, W., Hirsch, J. D., Beechem, J. M., Haugland, R. P., and Haugland, R. P. (2003) Quantitative comparison of long-wavelength Alexa Fluor dyes to Cy dyes: fluorescence of the dyes and their bioconjugates. *J Histochem Cytochem* 51, 1699-1712
- Campoccia, D., Hunt, J. A., Doherty, P. J., Zhong, S. P., O'Regan, M., Benedetti, L., and Williams, D. F. (1996) Quantitative assessment of the tissue response to films of hyaluronan derivatives. *Biomaterials* 17, 963-975

- 12. Hunt, J. A., Vince, D. G., and Williams, D. F. (1993) Image analysis in the evaluation of biomaterials. *J Biomed Eng* 15, 39-45
- Maldonado, I. R., Ferreira, M. L., Camargos, E. R., Chiari, E., and Machado, C. R. (2004) Skeletal muscle regeneration and Trypanosoma cruzi-induced myositis in rats. *Histol Histopathol* 19, 85-93
- 14. McLennan, I. S. (1993) Resident macrophages (ED2- and ED3-positive) do not phagocytose degenerating rat skeletal muscle fibres. *Cell Tissue Res* 272, 193-196
- 15. Mohanty, M., Hunt, J. A., Doherty, P. J., Annis, D., and Williams, D. F. (1992) Evaluation of soft tissue response to a poly(urethane urea). *Biomaterials* 13, 651-656
- 16. Rosengren, A., Danielsen, N., and Bjursten, L. M. (1997) Inflammatory reaction dependence on implant localization in rat soft tissue models. *Biomaterials* 18, 979-987
- 17. Kraehenbuhl, J. P., Racine, L., and Jamieson, J. D. (1977) Immunocytochemical localization of secretory proteins in bovine pancreatic exocrine cells. *J Cell Biol* 72, 406-423
- 18. Osborn, M., and Weber, K. (1982) Immunofluorescence and immunocytochemical procedures with affinity purified antibodies: tubulin-containing structures. *Methods Cell Biol* 24, 97-132
- 19. Chehroudi, B., Gould, T. R., and Brunette, D. M. (1988) Effects of a grooved epoxy substratum on epithelial cell behavior in vitro and in vivo. *J Biomed Mater Res* 22, 459-473
- 20. Wieland, M., Chehroudi, B., Textor, M., and Brunette, D. M. (2002) Use of Ticoated replicas to investigate the effects on fibroblast shape of surfaces with varying roughness and constant chemical composition. *J Biomed Mater Res* 60, 434-444
- 21. Chehroudi, B., Gould, T. R., and Brunette, D. M. (1992) The role of connective tissue in inhibiting epithelial downgrowth on titanium-coated percutaneous implants. *J Biomed Mater Res* 26, 493-515
- 22. Chehroudi, B., McDonnell, D., and Brunette, D. M. (1997) The effects of micromachined surfaces on formation of bonelike tissue on subcutaneous implants as assessed by radiography and computer image processing. *J Biomed Mater Res* 34, 279-290
- 23. Hayat, M. A. (1986) Glutaraldehyde: role in electron microscopy. *Micron. Microsc. Acta.* 17, 115-135
- 24. Herken, R., Fussek, M., Barth, S., and Gotz, W. (1988) LR-White and LR-Gold resins for postembedding immunofluorescence staining of laminin in mouse kidney. *Histochem J* 20, 427-432

- 25. Hobot, J. A., and Newman, G. R. (1991) Strategies for improving the cytochemical and immunocytochemical sensitivity of ultrastructurally well-preserved, resin embedded biological tissue for light and electron microscopy. *Scanning Microsc Suppl* 5, S27-40; discussion S40-21
- Pedersen, T. X., Leethanakul, C., Patel, V., Mitola, D., Lund, L. R., Dano, K., Johnsen, M., Gutkind, J. S., and Bugge, T. H. (2003) Laser capture microdissection-based in vivo genomic profiling of wound keratinocytes identifies similarities and differences to squamous cell carcinoma. *Oncogene* 22, 3964-3976
- 27. Fink, L., Kinfe, T., Seeger, W., Ermert, L., Kummer, W., and Bohle, R. M. (2000) Immunostaining for cell picking and real-time mRNA quantitation. *Am J Pathol* 157, 1459-1466
- Goldsworthy, S. M., Stockton, P. S., Trempus, C. S., Foley, J. F., and Maronpot, R. R. (1999) Effects of fixation on RNA extraction and amplification from laser capture microdissected tissue. *Mol Carcinog* 25, 86-91
- 29. Nielsen, T. B., Field, J. B., and Dedman, J. R. (1987) Association of calmodulin with lysosomes. *J Cell Sci* 87 (Pt 2), 327-336
- Heggeness, M. H., Wang, K., and Singer, S. J. (1977) Intracellular distributions of mechanochemical proteins in cultured fibroblasts. *Proc Natl Acad Sci U S A* 74, 3883-3887
- 31. Jones, D. (1969) The reaction of formaldehyde with unsaturated fatty acids during histological fixation. *Histochem J* 1, 459-491
- 32. Craig, S., and Goodchild, D. J. (1982) Post-embedding immunolabeling. Some effects of tissue preparation on the antigenicity of plant proteins. *Eur J Cell Biol* 28, 251-256
- 33. Newman, G. R., and Hobot, J. A. (1987) Modern acrylics for post-embedding immunostaining techniques. *J Histochem Cytochem* 35, 971-981
- 34. Newman, G. R. (1987) Use and abuse of LR White. *Histochem J* 19, 118-120
- 35. Newman, G. R., and Hobot, J. A. (1989) Role of tissue processing in colloidal gold methods. *In: Hayat, M.A. (ed) Colloidal gold; Principles, Methods, and applications Academic press San Diego.* 2, 35-45
- 36. Helenius, A., and Simons, K. (1975) Solubilization of membranes by detergents. *Biochim Biophys Acta* 415, 29-79
- 37. Jones, M. N. (1999) Surfactants in membrane solubilisation. *Int J Pharm* 177, 137-159
- Prete, P. S., Malheiros, S. V., Meirelles, N. C., and de Paula, E. (2002) Quantitative assessment of human erythrocyte membrane solubilization by Triton X-100. *Biophys Chem* 97, 1-5

- 39. Grube, D. (1980) Immunoreactivities of gastrin (G-) cells. II. Non-specific binding of immunoglobulins to G-cells by ionic interactions. *Histochemistry* 66, 149-167
- 40. Larsson, L. I. (1981) Peptide immunocytochemistry. *Prog Histochem Cytochem* 13, 1-85
- 41. Goldenthal, K. L., Hedman, K., Chen, J. W., August, J. T., and Willingham, M. C. (1985) Postfixation detergent treatment for immunofluorescence suppresses localization of some integral membrane proteins. *J Histochem Cytochem* 33, 813-820
- 42. Melan, M. A., and Sluder, G. (1992) Redistribution and differential extraction of soluble proteins in permeabilized cultured cells. Implications for immunofluorescence microscopy. *J Cell Sci* 101 (Pt 4), 731-743
- 43. Behrman, E. J. (1984) The chemistry of osmium tetroxide fixation. In scanning electron microscopy. *p.i SEM. Inc. AMF O'Hare, Chicago*
- 44. Barsky, S. H., Rao, N. C., Restrepo, C., and Liotta, L. A. (1984) Immunocytochemical enhancement of basement membrane antigens by pepsin: applications in diagnostic pathology. *Am J Clin Pathol* 82, 191-194
- 45. Salthouse, T. N. (1984) Some spects of macrophage behavior at the implant interface. *J. Biomed. Mater. Res.* 18, 395-401
- 46. Refai, A. K., Textor, M., Brunette, D. M., and Waterfield, J. D. (2004) Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. *J Biomed Mater Res A* 70, 194-205
- 47. Takebe, J., Champagne, C. M., Offenbacher, S., Ishibashi, K., and Cooper, L. F. (2003) Titanium surface topography alters cell shape and modulates bone morphogenetic protein 2 expression in the J774A.1 macrophage cell line. *J Biomed Mater Res A* 64, 207-216
- 48. Soskolne, W. A., Cohen, S., Sennerby, L., Wennerberg, A., and Shapira, L. (2002) The effect of titanium surface roughness on the adhesion of monocytes and their secretion of TNF-alpha and PGE2. *Clin Oral Implants Res* 13, 86-93
- 49. Gretzer, C., Emanuelsson, L., Liljensten, E., and Thomsen, P. (2006) The inflammatory cell influx and cytokines changes during transition from acute inflammation to fibrous repair around implanted materials. *J Biomater Sci Polym Ed* 17, 669-687

CHAPTER: 5

General Discussion and Future Directions

5.1 General discussion

The success or failure of a dental implant depends in part on the cellular interactions that occur at the bone-implant interface. Host reactions to an implant include surgical injury, fluid-material interactions, acute inflammation, chronic inflammation (foreign body response), and possible fibrous capsule development. The characteristics of implants that can be recognized by the immune system (particularly macrophages) include surface chemistry/charge and surface topography. Recognition of these features is highly regulated and involves a complex interplay between many different cell types. At the later stages of chronic inflammation and the early stages of endosseous wound healing, macrophages play a pivotal role in the clinical sequelae through production of cytokines and growth factors that initiate tissue destruction and/or reparative responses.

The research presented in this thesis expands our knowledge of the roles of surface topography in controlling cell behavior. More specifically I addressed the effects of Ti surface topographies on the behavior of the transformed macrophage cell line RAW 264.7. I determined that these macrophages were responsive to their substrata, showing different responses to substrata of different surface roughness over time.

The most significant findings of my research can be summarized as follows:

5.1.1 Effect of surface topography on cellular morphology and cytoskeleton

The topographic features of the substrata can markedly influence cell shape (1-3). Wójciak-Stothard et al. 1996 reported the orientation and the alignment of

macrophages along grooved substrata (4). I demonstrated that macrophage morphology differed among surface roughness and changed with time of culture. As cells spread they form more numerous strong focal adhesions and podosomes to attach to the substrata, a process that is associated with vinculin recruitment to these attachment sites (5). The immunostaining of F-actin and vinculin enabled me to observe the localization of these FA's and podosomes. I found an association between a decrease in these two cellular structures and reduced cell spreading suggesting that surfaces that induced poor assembly of focal contacts and podosomes also supported less cell spreading.

5.1.2 Effect of surface topography on generalized tyrosine phosphorylation, and activation of FAK, Src and ERK 1/2-MAPK signaling cascades

High levels of cellular tyrosine phosphorylation are often associated with vinculin-rich focal adhesions (6). I demonstrated that high levels of tyrosine phosphorylation coincided with the surfaces and time points where macrophages exhibited a well-formed dot-like vinculin appearance together with expression of cellular FAs and podosomes. Examples of tyrosine molecules that are phosphorylated in integrin-mediated signaling downstream of FAs and podosome attachments include FAK, Src, and the ERK1/2 signaling cascade (7, 8). In this study I found that topographic activation of FAK, Src, and ERK1/2-MAPK signaling molecules were again both surface and time dependent (Figure 5.1). Generally, the same trend of FAK, Src, and ERK/2 activations was found. This same pattern was confirmed in studies of the nuclear translocation of ERK1/2, a process downstream from FAK, Src phosphorylation.

The amount of activation of these intracellular mediators is associated with the level of cellular spreading on the various substrata; the more spreading the more phosphorylation of Src and ERK 1/2. PP1 inhibition of Src phosphorylation reduced both FAK and ERK1/2-MAPK phosphorylation suggesting an integrin-mediated FAK-Src and a subsequent ERK1/2-MAPK dependent phosphorylation. These data on the activation of one signaling cascade explains a possible mechanism for reported observations (by our group and others) in which surface topography modulated expression of macrophage pro-inflammatory cytokines and chemokines (9-11).

Figure 5.1: Summary of topographic effects on amount of activated (phosphorylated) signaling signaling molecules (FAK, Src and ERK1/2) on days one and three. (A) There was no significant difference in FAK phosphorylation levels in macrophages cultured on tested surfaces for one day. However, by day three rough surfaces significantly reduced FAK phosphorylation levels compared to P surfaces. (B) On day one, there was a significant increase in Src phosphorylation levels in macrophages cultured on B, and SLA surfaces compared to AE and P surfaces. By day three rough surfaces significantly reduced Src phosphorylation levels compared to P surfaces with the highest reduction levels seen on AE and SLA. (C) On day one, there was a significant increase in ERK1/2 phosphorylation levels in macrophages cultured on AE, B, and SLA surfaces compared to P surfaces. By day three rough surfaces compared to P surfaces. A star from the same bar color indicates a significant difference (p< 0.05).



Figure 5.1: The effect of surface topography on amount of activated (phosphorylated) signaling molecules FAK, Src and ERK1/2 at days one and three. Comparisons were made between the four conditions on each surface at each time point. Means with the same letter are not significantly different and means with different letters indicate significant differences (p< 0.05). Data presented as means and SD (n=3).
5.1.3 Effect of surface topography on cellular proliferation/viability

The numbers of macrophages differed between the test surfaces at each time point. Cell number in our test system is a complex function of number of cells initially attached as well as rates of cell proliferation and death. I found that the cell number was higher on the rougher surfaces on day one. By days three and five cell numbers on rough surfaces were lower than those observed on the P surfaces. There are many studies that claim that FAK protects cells from a known form of apoptosis called anoikis, which is induced by cell detachment from the ECM (12). In contrast, inhibition of FAK induces apoptosis (13). The role FAK plays in the mechanisms of cell survival is not clear. However, it is suggested that FAK-Src association is an essential event in FAK-inhibited cell apoptosis (14). I showed that the SLA surfaces reduced FAK and Src phosphorylation. Cell viability assays indicated that the rough surfaces may be attributed to reduced FAK and Src phosphorylation at FAs and podosomes that subsequently lead to cell detachment.

5.1.4 Identification of macrophages on implant-tissue interface *in vivo*

I developed a new immunohistological staining technique to identify macrophage populations *in vitro* (15) and *in vivo*. Using this technique we were able to identify ED1 positive macrophages on SLA surfaces transplanted into rat craniums.

5.2 The "Big Picture"

It is useful at this point to envisage what we feel is happening after cells are placed on implant surfaces *in vitro*. First, the cells adhere to the adsorbed surface proteins through integrin-ligand interactions. As a result of this cellular adhesion the cells respond to the mechanical stress (stretching and pulling) being applied by the ligand bound integrins and the cytoskeleton. These responses are demonstrated morphologically by cell spreading and an increase in number and size of both the FAs and podosomes. In our study well formed F-actin arrays and vinculin plaques co-localized at FAs and podosomes. Surfaces of differing roughness topography demonstrated a differential effect on cell spreading and FA's/podosomes as they probably produce different opportunities for attachment and that may lead to different patterns of mechanical stresses.

This influence of surface topography also translated into differential activation of intracellular signaling molecules; the signals being initiated by the above mentioned integrin/ligand interactions. Integrin clustering in the FAs and/or podosomes activates FAK at the sites of cellular attachment. This in turn opens docking sites in Src family kinases and subsequent phosphorylation of Tyr925. Furthermore, our PP1 inhibitor study suggested that FAK phosphorylation at Tyr397 and Tyr925 creates binding sites for Grb2-SOS complex, which is known to activate the ERK1/2-MAPK signaling pathway (Fig. 5.2). ERK1/2-MAPK is a transcription factor intimately involved in the production of pro-inflammatory cytokines. Thus, events occurring at the Ti surface-cell interface can affect the signaling pathway and eventual production of pro-inflammatory cytokines.

I investigated the presence of ED1-positive macrophages on implants with different topographie. After one week *in situ*, macrophages were found attached almost exclusively to the rough SLA surface. The presence of ED1 on these cells indicates a macrophage population that has been recruited to the site of the implant. A complete inventory of macrophage phenotype(s) on the surface and how they change with time is currently not available. After one week we see only a snapshot of events influenced by regional cytokine production as well as the surface chemistry and topography of the implant. As macrophages are highly plastic cells it is entirely possible that surface topography could differentially activate different phenotypes of macrophages *in situ* at different times, with these phenotypes being selected according to the stimuli exerted on them from their microenvironment. Such a change in environment could possibly reflect a change in the community of macrophages as well and there is even the possibility that these macrophages adopt a healing and/or immunosuppressive phenotype.

In summary, we have demonstrated that surfaces of differing roughness topography demonstrated a differential effect on activation of intracellular signaling molecules. Thus, it may be possible to use these *in vitro* techniques to screen different surface topographies for their ability to induce specific macrophage phenotypes.

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Figure 5.2: Diagrammatic illustration of FAK-mediated signaling pathways and the proposed effect of Src kinase inhibitor PP1 on (FAK, Src and ERK1/2) signaling molecules.

5.3 Future directions

I feel a number of studies should be carried out to expand on the findings of this thesis. The cell-surface topography interaction should be investigated in greater detail. More specifically we need:

- to determine which of the integrin family members interact with the surface topography as this may have downstream effects on the intracellular signaling pathways activated.
- 2. to investigate the role of the unique podosome structures in cell signaling (as opposed to FAs). This could entail an investigation into the localization of vinculin, FAK, and Src signaling molecules in the transiently expressed podosome. An immunogold staining detection technique using back-scattered electron imaging with a scanning electron microscope could be employed.
- to Investigate the early membrane requirements to signal transduction. More specifically we need to determine the effect of cholesterol depletion from membrane-associated rafts (using Methyl-β-cyclodextrin) on topographic activation and phosphorylation of FAK, Src, and ERK1/2.
- 4. to determine the macrophage phenotype on our test surfaces at various time points This could be accomplished by staining the macrophage with 27E10 antibody directed against M1 and with CD163 that is a specific surface marker

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expressed by M2 phenotype macrophages. It could also be addressed by investigation of transcriptional patterns for each phenotype (see below).

- 5. to determine the effects of topography on the transcriptome using microarray analysis.
- to investigate other signaling pathways that could by activated by surface topography (such as the JUK signaling pathway) by applying the techniques used in this study.
- 7. to carrying out *in situ* hybridization in order to identify whether the ED1 positive macrophages have a transcription pattern that identifies them as having a healing/immunosuppressive phenotype. Alternatively, we could use guided laser micro-desection and qRT-PCR to identify these transcription factors.
- to use guided laser micro-dissection of macrophages around the implants to carry out transcriptom analysis using cytokine arrays.
- 9. to modify the test sample to enable easier handling and develop a freezefracture technique for our *in vivo* model. I suggest that a disk-shaped implant would improve sample handling for cryosectioning the tissues attached to L R-White made implants.

5.4 References

- 1. Brunette, D. M. (1988) The effects of implant surface topography on the behavior of cells. *Int J Oral Maxillofac Implants* 3, 231-246
- 2. Curtis, A. S., and Wilkinson, C. D. (1998) Reactions of cells to topography. *J Biomater Sci Polym Ed* 9, 1313-1329
- 3. Hamilton, D. W., Chehroudi, B., and Brunette, D. M. (2007) Comparative response of epithelial cells and osteoblasts to microfabricated tapered pit topographies in vitro and in vivo. *Biomaterials* 28, 2281-2293
- Wojciak-Stothard, B., Curtis, A., Monaghan, W., MacDonald, K., and Wilkinson, C. (1996) Guidance and activation of murine macrophages by nanometric scale topography. *Exp Cell Res* 223, 426-435
- 5. Zaidel-Bar, R., Ballestrem, C., Kam, Z., and Geiger, B. (2003) Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci* 116, 4605-4613
- 6. Zamir, E., and Geiger, B. (2001) Molecular complexity and dynamics of cellmatrix adhesions. *J Cell Sci* 114, 3583-3590
- Marzia, M., Sims, N. A., Voit, S., Migliaccio, S., Taranta, A., Bernardini, S., Faraggiana, T., Yoneda, T., Mundy, G. R., Boyce, B. F., Baron, R., and Teti, A. (2000) Decreased c-Src expression enhances osteoblast differentiation and bone formation. *J Cell Biol* 151, 311-320
- Xing, Z., Chen, H. C., Nowlen, J. K., Taylor, S. J., Shalloway, D., and Guan, J. L. (1994) Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Mol Biol Cell* 5, 413-421
- 9. Refai, A. K., Textor, M., Brunette, D. M., and Waterfield, J. D. (2004) Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines. *J Biomed Mater Res A* 70, 194-205
- 10. Soskolne, W. A., Cohen, S., Sennerby, L., Wennerberg, A., and Shapira, L. (2002) The effect of titanium surface roughness on the adhesion of monocytes and their secretion of TNF-alpha and PGE2. *Clin Oral Implants Res* 13, 86-93
- 11. Tan, K. S., Qian, L., Rosado, R., Flood, P. M., and Cooper, L. F. (2006) The role of titanium surface topography on J774A.1 macrophage inflammatory cytokines and nitric oxide production. *Biomaterials* 27, 5170-5177
- 12. Frisch, S. M., and Screaton, R. A. (2001) Anoikis mechanisms. *Curr Opin Cell Biol* 13, 555-562

- Hungerford, J. E., Compton, M. T., Matter, M. L., Hoffstrom, B. G., and Otey, C. A. (1996) Inhibition of pp125FAK in cultured fibroblasts results in apoptosis. J Cell Biol 135, 1383-1390
- 14. Frisch, S. M., Vuori, K., Ruoslahti, E., and Chan-Hui, P. Y. (1996) Control of adhesion-dependent cell survival by focal adhesion kinase. *J Cell Biol* 134, 793-799
- 15. Ghrebi, S. S., Owen, G. R., and Brunette, D. M. (2007) Triton X-100 pretreatment of LR-white thin sections improves immunofluorescence specificity and intensity. *Microsc Res Tech* 70, 555-562

APPENDIX: A

Identification of ED1 in Macrophages at Implant-tissue Interface in vivo⁴

⁴ The results presented in Appendix A accepted for publication as part of manuscript by BabaK Chehroudi, Salem Ghrebi, Hiroshi Murakami, John Douglas Waterfield, Gethin Owen, Donald Maxwell Brunette. Bone formation on rough, but not smooth, subcutaneously implanted Ti surfaces is preceded by macrophage accumulation. Biomed. Mater. Res., in press.

Sections taken from LR-White and frozen samples and processed for immunostainingcontained cells with intense positive staining for ED1 at the implant tissue interface of SLA implants on day seven after implantation. ED1 stained cells observed on sections from smooth implants were seen within the tissues but not at the tissue implantinterface. ED1 stained cells were not observed on sections of the negative controls (Primary antibody omitted).



Photomicrographs of ED1 positive staining macrophages (white arrows) at the interface of SLA surface at one week. a; on sections from LR-White embedded blocks, b: from frozen tissues, c: positive staining was seen within the tissue but not at the interface of the implants with smooth surfaces.

APPENDIX: B

UBC Research Ethics Board Certificate

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THE UNIVERSITY OF BRITISH COLUMBIA

ANIMAL CARE CERTIFICATE

Application Number: A05-1677	
Investigator or Course Director: Donald M. Brunette	
Department: Dentistry	
Animals:	
	Rats Sprague Dawley 60
Start Date:	April 1, 2004 Approval December 10, 2008
Funding Sources:	
Funding Agency:	Canadian Institutes of Health Research (CIHR)
Funding Title:	Mesenchymal stem cells and biomaterials in bone regeneration: a team approach
Unfunded title:	N/A

The Animal Care Committee has examined and approved the use of animals for the above experimental project.

This certificate is valid for one year from the above start or approval date (whichever is later) provided there is no change in the experimental procedures. Annual review is required by the CCAC and some granting agencies.

A copy of this certificate must be displayed in your animal facility.