The effect of PMMA stimulated Complement-Macrophage cascade on Osteogenesis of Preosteoblast-like MC3T3-E1 cells on PMMA surface

DISSERTATION

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Abstract

A model of the initial complement mediated inflammatory response to tissue engineering (TE) scaffolds is developed in this study. TE has been given increasing attention in cases of large defects caused by severe trauma or disease based on the concept that the scaffold degrades and is replaced by tissue regeneration. Numerous attempts have been made to modify biomaterials chemical compositions, mechanical properties, and biomolecular incorporation to improve biocompatibility. The surface properties have been shown to affect host inflammatory responses and the ultimate fibrous capsule formation through foreign body reaction around implanted biomaterials. Although extensive research has been conducted regarding immune responses, such as macrophage activity, upon biomaterial particles and to a lesser extent on biomaterials surface, the inflammatory cascade and its effect on differentiation of preosteoblasts to osteoblasts is not completely understood. Biomaterials are known to adsorb serum proteins and activate the complement system followed by subsequent inflammatory cascade. Complement activation through either direct deposition or interaction with adsorbed protein layer on biomaterial surface is believed to be the initiation process of inflammatory responses to implanted biomaterials. By setting the goal of constructing a tissue engineering model, the present study utilizes complement, macrophages, and preosteoblast-like cells to investigate the initial step of the inflammatory response and, furthermore, their effect on osteogenesis on biomaterial surface, poly(methylmethacrylate).
PMMA polymer surfaces with different tacticities effects on MC3T3-E1 cell proliferation and differentiation were tested with cell proliferation and viability assay (MTS assay). In addition, their effect on MC3T3-E1 cell differentiation was assessed with MTS assay and osteogenic activity markers quantifications. No significant differences between different tacticity PMMA and tissue culture polystyrene were found.

The effect of complement on macrophage, RAW264.7, activation on biomaterial surface was investigated in present study. Three cytokines, TNF-α, IL-6, and IL-1β, expression were quantified with ELISA to evaluate macrophage activation on PMMA surfaces and particles. Cytokine expressions on PMMA surface were not significantly different from control group. At 6 hour, 1-10 μm PMMA beads induced significantly increased TNF-α expression. Mouse complement pretreatment resulted in increased expression of IL-6 and decreased expression of TNF-α while IL-1β was barely detectable in present experimental conditions.

Co-culture system is constructed by means of transferring RAW264.7 cell culture media to preosteoblast culture with corresponding material surfaces or particulates. The mRNA expression of Runx2, ON, and Collagen Type I are quantified with real time PCR to evaluate osteogenic activity of MC3T3-E1 cells under osteogenic condition. MC3T3-E1 cells cultured with osteogenic media supplemented with RAW264.7 cell culture media with LPS showed elongated morphology under light microscope. Real time PCR study for mRNA expression shows significantly lower Runx2 and SPARC expression in LPS
stimulated co-culture system. Complement involvement in co-culture system generally decreased all three mRNA expressions.

Conclusion: within the limitations of this study, complement treatment decreases TNF-α and increases IL-6 expression of RAW cells interacting with PMMA surfaces and particles. Culture media from LPS activated macrophage induced MC3T3-E1 cells differentiating toward fibroblasts even with osteogenic media. Complement-macrophage system inhibits osteogenic activity of MC3T3-E1 cells on PMMA surfaces and particles.
Dedication

This document is dedicated to my wife Yun, daughter Anji, and my parents.
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Introduction

Tissue engineering (TE) has been given increasing attention in order to repair critical tissue loss due to traumas or diseases in which the natural healing process fails to allow complete recovery. TE is based on the concept that degradable biomaterial induced tissue regeneration eventually replaces the original defect site while the TE scaffold provides framework for the regenerating processes. Since TE scaffolds are designed to degrade, a precisely controlled degradation rate and biocompatibility of the scaffold material are major criteria for their clinical application. However, biomaterials are capable of inducing an inflammatory response in both bulk and debris phases. Hence, if TE scaffolds create an environment that inhibits regeneration then healing may not occur before the scaffold absorbs.

The host immune system constantly interrogates implanted materials to break down and isolate or eliminate through encapsulation or phagocytosis in accordance with particulate size. The inflammatory response to biomaterials is a complicated process involving dynamic interactions between complement system, monocytes/macrophage and other immune cells, and tissue regenerating stem cells and precursor cells. Biomaterials are known to activate the complement system through interaction between adsorbed proteins and complement system. Despite the known macrophage stimulation by activated
complement system in response to pathogens, the role of the complement system that is activated by biomaterials surfaces in macrophage activation remains unclear.

The initial inflammatory response, which is thought to be the same process as the response to conventional biomaterials, is prone to create a non-permissive environment for tissue regeneration. This could lead to ultimate failure of the TE therapy. Because TE scaffolds are degradable, the effect of the initial inflammatory response to the TE scaffold materials surface on proliferation and differentiation of regenerative cells is not often considered.

The overall objective of the present study is to construct a co-culture system to elucidate the interaction between biomaterials, complement, and macrophage and their effect on the osteogenic activity of preosteoblasts on a biomaterial surface.
Chapter 1 Literature Review

1.1 The interaction of complement system and biomaterial surface

The complement system plays a major role in the immune response through opsonization and recognition of foreign materials (Passirani, Barratt et al. 1998; Mosqueira, Legrand et al. 2001). Hence, complement activation has been thought to be responsible for triggering an inflammatory response to biomaterials. This is based on the fact that certain biomaterial surfaces induce complement activation and activated complement components are known to mediate granulocyte aggregation, chemotaxis, and adhesiveness. The chemistry and protein adsorption were demonstrated to affect the complement response to a biomaterial surface (Tang, Liu et al. 1998; Pokidysheva, Maklakova et al. 2001; Andersson, Ekdahl et al. 2005; Nilsson, Ekdahl et al. 2007; Arima, Toda et al. 2008; Toda, Kitazawa et al. 2008).

Many studies have demonstrated that protein adsorption, also known as Vroman effect, is a primary process of the host response to biomaterial implantation. The biological response towards artificial materials is initiated by the adsorption of plasma proteins. Hence, the initial protein adsorption largely determines biocompatibility and blood compatibility of medical devices (Pokidysheva, Maklakova et al. 2001; Arima, Toda et al. 2008). Upon placement of biomaterials in a host, the surface was immediately covered
with a plasma protein film of approximately 8 nm (Andersson, Ekdahl et al. 2005). Human Serum Albumin (HSA), Immunoglobulin G (IgG), and fibrinogen have all been reported as the most abundantly bound to biomaterial surfaces. The non-specific adsorption and conformational changes of serum proteins has been believed to be closely related to complement activation on biomaterials (Nilsson, Ekdahl et al. 2007). The surface properties of biomaterials have shown to play important role in interaction between biomaterials and serum proteins.

Surface chemistry plays an important role in determining the immobilizing and activation of complement. Surface hydroxyl groups and oxidation products have been demonstrated to activate the complement system and immobilize C3 convertase (Arima, Toda et al. 2008). In contrast, it appears that surface groups with amine functionalities cause relatively little complement activation and inflammatory response. The nascent C3b molecule is able to bind specifically to proteins and carbohydrates via free hydroxyl or amino groups, forming a covalent ester or amide bonds, respectively. The amide bond is stable while the ester bond is more labile because of the esterolytic activity of C3b (Andersson, Ekdahl et al. 2005; Nilsson, Ekdahl et al. 2007). Carboxylic acid-bearing surfaces and certain material such as gold (Au) prompt neither complement activation nor inflammatory responses.

The proteolytic cleavage of C3 into C3b and C3a is considered to be the main process in the activation of complement (Andersson, Ekdahl et al. 2005; Nilsson, Ekdahl et al.
Activation is achieved by two enzyme complexes, the C3 convertases (C4b,2a and C3bBb, respectively), which are assembled by three different activation pathways, classical pathway (CP), mannose-binding lectin pathway (LP), and alternative pathway (AP) (Andersson, Ekdahl et al. 2005; Nilsson, Ekdahl et al. 2007; Arima, Toda et al. 2008).

Some studies propose that certain types of biomaterial polymers activate complement system through AP while other studies suggest that biomaterials trigger a rapid activation by the classical pathway (CP) and/or mannose-binding lectin pathway (LP) (C4-dependent), followed later by alternative pathway (AP) activation (Nilsson, Ekdahl et al. 2007). Even though the CP deposits only minute amounts of C3b on the surface, the deposition is rapid and sufficient to instantaneously trigger the AP amplification loop. The proposed mechanism for this phenomenon is that C3b is conformationally changed, exposing a highly reactive thioester group which reacts with nucleophilic groups, such as hydroxyl groups, on biomaterials to form a covalent linkage and subsequently complex with factors B and D (Toda, Kitazawa et al. 2008). On the other hand, C3 in the initially adsorbed protein layer is able to generate an initiating C3 convertase (Andersson, Ekdahl et al. 2002). The explanation in this circumstance is that the adsorbed C3 is conformationally changed into a configuration that mimics that of bound C3b. This initial convertase can then trigger further activation and amplification via AP (Pokidysheva, Maklakova et al. 2001; Nilsson, Ekdahl et al. 2007). The combined sequential activation
of the two pathways results in the total deposition of C3b molecules on the surface (Andersson, Ekdahl et al. 2005).

### 1.2 Complement system inhibition

Because of complement’s pivotal role as an inflammation mediator, inhibition of the complement cascade appeared to be a promising approach for the reduction of the inflammatory reaction induced by biomaterials (Schmidt, Haase et al. 2003). Heat inactivation of serum has been introduced decades ago and appeared to be a promising practice to eliminate complement system from serum. However, different components of the complement cascade served as a target for complement inhibition in other applications. The application of complement system inhibitors may reduce or remove the potential interrogation of host to TE scaffolds prior to their degradation and enhance tissue regeneration.

One approach was the inhibition of complement enzymes of which the majority are classified as serine proteases (Schmidt, Haase et al. 2003). However, because of their high substrate specificity and their inaccessibility by universal protease inhibitors, this approach was not successful.

Other compounds are able to modulate the action of complement, including heparin, the well-known anticoagulant, which has also been shown to inhibit complement activation
at different stages (Passirani, Barratt et al. 1998). In order to improve the compatibility of biomaterials with blood by reducing activation of complement system, heparin coatings have been extensively used (Nilsson, Ekdahl et al. 2007). Nanoparticles made from heparin-PMMA copolymers retain on their surface at least some of the complement-inhibiting properties of soluble heparin (Passirani, Barratt et al. 1998).

A soluble form of the complement receptor type 1 has also been seen to inhibit complement as well as cell activation (Schmidt, Haase et al. 2003). However, in comparison, Compstatin showed a higher efficiency in reduction of cell activation (Nilsson, Larsson et al. 1998). Compstatin is thought to be the most effective and reliable substance in this context (Schmidt, Haase et al. 2003). Compstatin, a C3-binding protein, inhibits the complement via binding to C3 and thus prevents the adsorption of C3 on polymer surfaces, the generation of C3a and sC5b-9, as well as cell activation during extracorporal circulation; this has been demonstrated using two in vitro models (Schmidt, Haase et al. 2003). However, recent studies on compstatin showed unpromising inhibitory effect on mouse complement system due to species-specific binding (Sahu, Morikis et al. 2003).

1.3 Macrophage adhesion and stimulation on biomaterials surface

The precise mechanism by which macrophages, the primary initiators of inflammatory response, become activated by biomaterials remains unclear. The current consensus is
that macrophages phagocytose various biomaterials particles through membrane receptor binding and are subsequently activated (Laing, Dillon et al. 2008). However, other studies with macrophage culture on material surfaces state that monocyte/macrophage can be activated and produce pro-inflammatory cytokines by direct interaction with a material surface, without phagocytosis of particulates. Once activated, a cascade of proinflammatory events occurs, involving other cellular participants including osteoblasts, fibroblasts and osteoclasts, stimulated by cytokines such as TNF-α, PGE-2, IL-1 and IL-6, chemokines such as MCP-1, and metalloproteinases (Laing, Dillon et al. 2008). All these studies were conducted with macrophages cultured on biomaterial surfaces with cell culture media without the involvement of the complement system.

In vitro studies regarding material-dependent nature of cytokine and chemokine proteomic profiles with time have shown that the macrophage activation on biomaterials to be combination of classical and alternative pathway (Jones, Chang et al. 2007). Biomaterial adhered monocyte/macrophages showed a phenotypic switch from an activation state similar to classically activated macrophages to one similar to alternatively activated macrophages (Jones, Chang et al. 2007). IL-10 seems to play an important role in this phenotypic switch. However, the protein adsorption and following complement adsorption and activation have to be taken into account that may explain the different phenotype of biomaterial-induced macrophage activation.
The process of recognition and phagocytosis involves multiple ligand-receptor interactions, and it is now clear that various opsonins, including immunoglobulins, collectins and complement components, guide the cellular activities required for pathogen internalization. A good example is the cross-talk between Fc- and complement-receptors in mediating efficient phagocytosis of immune complexes (van Lookeren Campagne, Wiesmann et al. 2007). There are now three recognized gene superfamilies of complement receptors (CR): the short consensus repeat (SCR) modules that code for CR1 and CR2, the β-2 integrin family members CR3 and CR4, and the immunoglobulin Ig-superfamily member CRIg (van Lookeren Campagne, Wiesmann et al. 2007). Through specific antibodies blocking test, Leukocyte β2 integrins Mac-1 and p150,95 have been proved to be promiscuous cell-surface receptors that recognize and mediate cell adhesion to a variety of adsorbed and denatured proteins (Brevig, Holst et al. 2005).

The central role of complement as a mediator to recruit and activate leukocytes has been known for decades. Complement activation generates fragments (C3b, iC3b, and C3dg) and soluble peptides (C3a and C5a) that bind to biomaterial surfaces. Although they are not covalently bound, the anaphylatoxins C3a and C5a may be passively adsorbed to certain charged surfaces. All these fragments are ligands for receptors on leukocytes (PMNs, monocytes and mast cells) that can trigger inflammation and release pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and the highly potent chemokine IL-8 (Passirani, Barratt et al. 1998; Nilsson, Ekdahl et al. 2007). Another effect of complement activation is the up-regulation of receptors on leukocytes (e.g.,
CD11b/CD18 and CD35), which together with down-regulation of L-selectin makes the cells very adhesive and prone to interact with platelets and endothelial cells in the vascular walls (Nilsson, Larsson et al. 1998). Furthermore, iC3b is an important ligand for CD11b/CD18 (CR3); through their interaction, activated leukocytes are attached to surface-bound iC3b (Nilsson, Ekdahl et al. 2007).

1.4 Cytokines released by stimulated macrophages and their effects on osteoblast behavior

Biomaterials induce monocyte/macrophage activation and secretion of pro-inflammatory cytokines such as IL-1β, TNF-α, IL-6 and IL-8 via up-regulation of transcription factor NFκβ, and activation of inflammasome danger signaling in macrophages (Trindade, Lind et al. 2001). The activation of local (and systemic) inflammation result in decreased osteoblast function and increased osteoclast activity (Iwamoto, Kurachi et al. 2005; Yamamoto, Kurachi et al. 2007; Hallab and Jacobs 2009).

It’s been reported that a PMMA particle challenge of macrophages leads to the induction of TNF-α through transactivation of the TNF-promoter-CAT reporter (Chua, Chua et al. 2002; Taki, Tatro et al. 2007). Tumor necrosis factor α (TNF-α), a pro-inflammatory cytokine secreted by monocytes, plays a key role in abnormal bone remodeling and inflammatory bone diseases, especially in apoptosis of osteoblasts (Chua, Chua et al. 2002). TNF-α induces apoptosis by interacting with its receptors (TNF-R1 and TNF-R2)
and death receptors such as FAS (Chua, Chua et al. 2002). TNF-α induces apoptosis in murine osteoblastic MC3T3-E1 cells by upregulating FAS expression and by activating caspase-1, -3, -7, -11, and -12. TGF-β1 can decrease the apoptotic process by attenuating the expression of these apoptotic proteins (Chua, Chua et al. 2002). In addition, IL-1 and TNF-α have been reported to indirectly induce osteoclast formation by stimulating osteoblasts (Hikiji, Shin et al. 2000; Le, Laflamme et al. 2009).

TNF-α and IL-1β lead simultaneous generation of both NO and O$_2^-$ to form ONOO$^-$ which inhibits the osteoblast-like cells differentiation and ALPase activity (Hikiji, Shin et al. 2000). In addition, these two cytokines have synergistic interaction on increased NO and O$_2^-$ production by MC3T3-E1 (Hikiji, Shin et al. 2000). In agreement with gene expression, it has been reported that TNF-α and IL-1β had led two-to threefold inhibition of osteocalcin (OC) synthesis in osteoblasts (Hikiji, Shin et al. 2000). In addition, TNF-alpha and IL-1beta are known to stimulate osteoblasts to produce IL-6 which in turn promote a shift in bone homeostasis towards bone resorption (Patil, Zhu et al. 2004).

IL-6 has long been considered as a centrally important cytokine for osteoclastogenesis and thus bone resorption (Patil, Zhu et al. 2004). Since its discovery three decades ago, interleukin IL-6 has been recognized as pleiotropic cytokine influencing many biological events in several organs including the bone marrow, the central nervous system (CNS) or the immune system (Kamimura, Ishihara et al. 2003). IL-6 has been reported to indirectly stimulate osteoclastogenesis through the production of RANKL by osteoblasts (Duplomb,
Baud’huin et al. 2008). However, more recently, IL-6 has been demonstrated to supports osteoblast generation through the gp-130-STAT1/3 pathway (Sims, Jenkins et al. 2004). The IL-6/sIL-6R complex enhances alkaline phosphatase (AP) activity or the number of AP-positive (AP+) cells or colonies. This indicates that IL-6 in the presence of sIL-6R might influence osteoblast differentiation (Franchimont, Wertz et al. 2005). IL-6 in the presence of sIL-6R was shown to stimulate insulin-like growth factor (IGF)-I and bone morphogenic protein-6 (BMP-6) transcripts in osteoblasts obtained from rat calvariae (Franchimont, Wertz et al. 2005). IL-6 and TGF-β1 were able to inhibit TNF-α-induced apoptosis in murine osteoblastic MC3T3-E1 cells (Chua, Chua et al. 2002).

Conversely, not only can macrophages produce and secret IL-6, other types of cells such as osteoblasts, under stimulation, produce and secret, in agreement between mRNA level and supernatant content, IL-6 to interact with monocytes/macrophages and osteoclasts (Patil, Zhu et al. 2004; Le, Laflamme et al. 2009).

1.5 Biological activity markers for the study of interactions between osteoblasts and biomaterials

Osteogenic activity has been studied for decades and numbers of osteogenic markers were discovered at the level of proteins and RNA expressions. Among numbers of markers, Osteocalcin (OC) and Alkaline Phosphatase (ALP) have been given great attention.
Osteocalcin (OC), a γ-carboxylated protein synthesized primarily by osteoblasts during bone formation, is present in bone matrix and is known to be a differentiation marker at a later stage (Weinreb, Shinar et al. 1990). Not only does it incorporate into bone extracellular matrix (ECM), but also secreted into circulation, where it has served as marker of bone turnover in the clinic for decades (Pregizer, Baniwal et al. 2008).

Alkaline phosphatase (ALPase), also called basic phosphatase, is a membrane-bound enzyme that is abundant in many tissues. A high level of ALPase is found in preosteoblasts in bones. From its pattern of gene expression, ALPase is known to be an early differentiation marker during the formation of bone (Weinreb, Shinar et al. 1990).

COL1A1, Type I collagen α 1, encodes the major component of type I collagen which is main structural component of connective tissues, and in bone collagen type I is essential for matrix formation and mineralization (Pedraza, Marelli et al.).

Runx2, a key osteoblast transcription factor, is commonly used as marker of osteoblast differentiation. It regulates OC expression by recruiting and binding to OC promoter (Pregizer, Baniwal et al. 2008).

SPARC (secreted glycoprotein, acidic, rich in cysteine), also known as Osteonectin (ON), is a phosphorylated 32-kDa glycoprotein which serve as the major non-collagenous
protein of organic component of bone matrix (Renn, Schaedel et al. 2006). It is expressed in bone and undergoing active remodeling. ON binds to collagen and promotes crystal deposition through two high-affinity Ca$^{2+}$ binding sites and modulates matrix organization and mineralization (Ohsawa, Neo et al. 2001; Renn, Schaedel et al. 2006). The level of SPARC has been directly linked to the extent of bone mineralization. mRNA level of SPARC detection is preferred because of its transient detection in the extracellular environment caused by rapid proteolysis of SPARC by extracellular proteases (Motamed 1999).

1.6 Significance of this study

In depth understanding in biomaterial induced inflammation cascade provides multiple targets through which the inflammation will be controlled and biocompatibility and healing processes will be greatly improved. Biomaterials can be modified to control serum protein adherence and conformation consistency to reduce the interaction with complement system. Furthermore, subsequent cascade of inflammatory responses can be controlled by application of inhibitory molecules for multiple target points such as complement cascade, membrane receptors of macrophages, and cytokine production cascade of macrophages. Addition to reducing inflammatory responses caused by biomaterials, tissue regeneration promoting factors can be administered with biomaterial implantation. Ultimately, TE scaffolds can be designed and produced by combination of inflammation-regulation, tissue regeneration-promotion, and controlled biomaterial
degradation rate which matches with tissue regeneration. This proposed study constructs in vitro model for investigation of inflammatory responses to biomaterial surfaces, and furthermore, TE scaffold.

1.7 Hypothesis and specific aims

We hypothesized that biomaterial surface activated complement system provokes macrophage activation on the material surface and complement-macrophage cascade has inhibitory effect on the osteogenic activity of preosteoblasts on biomaterial surface.

1.3.1 Investigation of MC3T3-E1 cell viability and osteogenic differentiation on different copolymer PMMA

The null hypothesis was that preosteoblast-like MC3T3-E1 cells proliferate and differentiate similarly on PMMA polymers with different tacticities.

1.3.2 Study of the role of complement system on macrophage stimulation on atactic PMMA surface

By quantification of specific cytokines such as TNF-α, IL-6, and IL-1β, the null hypothesis was that no difference in cytokine expression between complement-
macrophage-PMMA system and macrophage-PMMA system. In addition, heat inactivated complement application has no difference with intact complement application in terms of activating macrophages on biomaterial surface.

1.3.3 Evaluation of the effect of complement-macrophage system on osteogenic activity of preosteoblast-like MC3T3-E1 cells on PMMA

The null hypothesis was no osteogenetic activity difference between osteoblasts with and without treatment with culture media transferred from complement-macrophage-PMMA system.
Chapter 2 Materials and Methods

2.1 Biomaterials PMMA (polymethylmethacrylate)

The isotactic, syndiotactic, and atactic PMMA polymers were synthesized in our lab and characterized with NMR. The tacticity of PMMA is originated from the chirality of β-carbon and forms isotactic, syndiotactic, and atactic PMMA polymer which has distinct arrangement of ester bonds on the polymer chains. As illustrated in [Fig.1], isotactic polymer chain has a regular arrangement of the ester group relative to one another while syndiotactic polymer chain has alternating arrangement of ester bonds. Atactic PMMA, however, has a random arrangement of ester groups. PMMA polymers with different tacticities were used to study preosteoblast behavior on PMMA surfaces based on the concept that distinct ester group arrangement affects cell behavior differently through discrete interaction with surrounding molecules and cells. Atactic PMMA was chosen for macrophage activation studies and co-culture system since no significant difference was found in the study of preosteoblast behavior on polymers with different tacticities as shown in section 3.2.
Figure 1. PMMA polymer chains with different tacticities. The tacticity is originated from the stereocenter $\beta$-carbon. Isotactic PMMA chain has a regular arrangement of the ester group relative to one another. Syndiotactic PMMA chain has alternating arrangement of ester groups. Atactic PMMA chain has a random arrangement of ester groups.
2.1.1 Smooth surface preparation.

Atactic, syndiotactic, and isotactic PMMA particles were dissolved in toluene to make 3 % w/w solution with shaking overnight. Three drops of PMMA solution were evenly distributed on the 15 mm glass coverslip which is fixed on sample holder of spin-coater (P-6000 SPIN COATER, Specialty Coating Systems, Inc. IN) with vacuum. 3,500 rpm with moderate acceleration rate was applied for spin-coating and the spin was held for 30 seconds. 15 mm PMMA coated glass discs were used in 24 well culture plates for cell culture.

35 mm diameter circular microscope cover glass (Erie Scientific Co, New Hampshire, USA) was purchased and trimmed off 0.5 mm evenly in order to fit into 6 well culture plates. 34 mm glass discs were then cleaned with freshly prepared piranha solution, 1:3 volume ratio of 30 % hydrogen peroxide and pure sulfuric acid, and rinsed with acetone and deionized water thrice followed by drying at oven. 5 % w/w atactic PMMA solution was applied to 34 mm glass disc covering entire area to assure complete coating. 5,000 rpm, due to larger coating area, with moderate acceleration rate was applied for 34 mm disc preparation and the spin coating was held for 30 seconds. These 34 mm diameter cover class discs coated with atactic PMMA were used in 6 well culture plates for cell cultures and RNA separation.
Glass discs that were spin-coated with atactic PMMA then were sterilized by dry heat method at 120 °C under vacuum in vacuum oven (Precision Scientific Inc., Chicago, IL) for 2 days. Overnight direct UV exposure in the biological safety cabinet was applied prior to cell seeding.

2.1.2 Rough surface preparation.

Cast PMMA rod was purchased from McMaster-Carr (Aurora, OH, USA) and machined (Maximat Super 11, EMCO, Austria) down to appropriate diameter for 6 well culture plate, 34.2 mm, and sliced to form PMMA discs with diamond saw (Vari/Cut™ VC-50, LECO co. St Joseph, MI, USA) to 1 mm thickness. The surfaces were roughened with 320 grit sandpaper with polishing rotor (Vari/Pol VP-50, LECO co. St Joseph, MI, USA). Counter-direction circular motion was applied to form randomly roughened surfaces. Roughened PMMA discs were cleaned with distilled water and methanol with ultrasonic bath 15 min each. Cleaned discs were then dried in vacuum oven at 100°C for overnight. Overnight direct UV exposure in biological safety cabinet was employed prior to cell culture.
2.1.3 Particulates, particle size and surface properties.

PMMA particles have been studied for decades and considered a primary trigger of inflammatory response in peri-implant site and particle size plays major role in macrophage/monocyte stimulation through phagocytosis. In addition, opsonization of particles by serum proteins especially complements components, promote the phagocytosis and consequently lead to inflammatory responses. A range of particle size from 1-10 μm to \( \frac{1}{16}" \) was tested in this study to elucidate the effect of size in phagocytosis and macrophage activation. Furthermore, mouse complement was employed to evaluate the effect of opsonization on initial phagocytosis and activation.

\( \frac{1}{16}" \) beads were purchased from McMaster-Carr (Aurora, OH, USA) and 1-10 μm and 200 μm beads were purchased from Polysciences (Polysciences, PA). Chemical compositions of particles (beads) were determined by FT-IR while thermo analysis was performed with Differential Scanning Calorimetry (DSC) as described in next section. 1-10 μm PMMA beads were washed thrice with 75 % ethanol and dried completely prior to characterization whereas 200 μm and \( \frac{1}{16}" \) beads were characterized in as received condition.
2.1.4 PMMA surface and thermal properties characterization.

2.1.4.1 Topographical characterization with Optical microscopy and Atomic Force Microscopy (AFM).

In present study, spin-coated and custom made rough PMMA surface topography were observed and evaluated with optical measuring microscopy and AFM for micrometer and nanometer scale resolution, respectively.

Since the spin-coated PMMA surface was too smooth to be appropriately imaged under optical measuring microscopy (Nikon measurescope, NIKON Co. Japan), the boundary of coated PMMA and glass coverslip was observed at a defect area of spin-coated surface which was resulted from insufficient amount of PMMA solution. Optical images were captured under the highest magnification of 40x (BD Plan 40, 0.65, 210/0) with Nikon COOLPIX 990 camera. For roughened PMMA disc surface, Nikon SC-112 and SC-111 measuring devices were applied to measure XY (horizontal) plane and Z value (vertical), respectively. Z value was measured with focus variation method i.e. set zero point as focusing at the beam surface and measured the movement in vertical axis when most inner surface was focused. Measurements were performed at 5 different area of one sample and 5 samples were analyzed. Data was reported as mean ± standard deviation.
According to measurescope result, area of 50 x 50 μm² was imaged with Dimension 3000 AFM (Veeco, Santa Barbara, CA) with contact mode at a rate of 0.3 Hz under ambient conditions to investigate the roughness of spin-coated and roughened PMMA surface. Roughness is assessed with parameters of Ra, Rq, and Rmax. Ra is the arithmetic average of absolute values of height of surface topography [Eq.1]. Rq is the root mean squared values of absolute roughness profile [Eq.2]. Rmax records maximum value of surface roughness.

\[
Ra = \frac{1}{n} \sum_{i=1}^{n} |y_i| \quad \text{--- Equation 1}
\]

\[
Rq = \sqrt{\frac{1}{n} \sum_{i=1}^{n} y_i^2} \quad \text{--- Equation 2}
\]

2.1.4.2 Contact angle.

Static water contact angles on PMMA disc surface were determined by the sessile drop method using a goniometer (ramé-hart, inc. Mountain Lakes, NJ, USA). A droplet of 6 μl of distilled and deionized water was placed on a PMMA surface and the contact angle was read three times during 1 min. This procedure was repeated five times at different places on the same surface. The contact angle of a surface was expressed as the mean value of five contact angle measurements. For PMMA discs sliced by diamond saw, contact angles were measured on parallel and perpendicular to the groves. After
roughening, contact angle was measured in random direction due to random distribution of grooves. Tissue culture polystyrene discs were obtained by cutting off the bottom of a 6 well culture plate and were rinsed with methanol and water and air dried prior to contact angle measurement.

2.1.4.3 FT-IR.

The chemical composition of spin-coated PMMA, bulk PMMA disc, and different particle size PMMA beads were evaluated with FT-IR (PerkinElmer Spectrum One FT-IR spectrometer). Spectra were collected under attenuated total reflectance (ATR) mode. Specimens were placed on Universal diamond ATR top-plate with 30-50 pressure gauge and scanned 256 cycles to minimize the background noise. Assurance of identical chemical composition was desired to avoid unknown variations caused by different compound.

2.1.4.4 Differential Scanning Calorimetry

The thermal properties of PMMA samples were investigated to provide guidance for the sterilization procedure of PMMA specimens. The glass transition temperature (Tg) of a non-crystalline material is the critical temperature at which the material undergoes phase transformation from a glassy to rubbery state. The glass transition temperature of different size PMMA particulates, 1-10 μm, 200 μm, and 1/16” (1.5875 mm) in diameter,
was studied with differential scanning caloremetry (DSC, DSCQ100 with DSC refrigerated cooling system, TA instrument, New Castle, DE). Spin-coated PMMA and roughened PMMA discs were not included in DSC study because it was impractical to prepare specimens. Standard heating was performed with ramp from 50 °C to 350 °C with a temperature rate increase of 10 °C/min under sample purge flow of N₂ 50 ml/min. For the reheating experiment, the heating and cooling cycle with a range of 50 °C – 200 °C was performed prior to data collection. A second heating procedure was then performed with same standard heating ramp mentioned above. Data was analyzed with Universal Analysis 2000 (TA instrument, New Castle, DE). The glass transition temperatures (Tg) was determined as the midpoint of the change in heat capacity (ΔCp/2) in the heating curve.

2.2 cell lines and cell culture

2.2.1 RAW 264.7 cell lines and culture

RAW 264.7 cells were generously provided by Dr. Quan’s laboratory and propagated with High Glucose Dulbecco's Modified Eagle Medium (DMEM, Gibco Invitrogen) with 10% Heat inactivated FBS (Gibco Invitrogen) in accordance with American Type Culture Collection (ATCC) instruction. Subculture was performed at 80-90 % confluence and splitting ratio was 1:4-6 by scrapping method. Cells were dislodged by cell scraper and
aspirated in 5 ml complete culture media and the appropriate volume of cell suspension was transferred to new culture flask. A total volume of 10 ml media was used with 75 cm² flasks. Cell subculture was performed every 3 days with cells reaching 80-90 % confluency. Cell culture was held under 100 % humidity with 5 % CO₂ at 37°C.

2.2.2 MC3T3-E1 cell line and culture

MC3T3-E1 cells, generously provided by Dr. Mooney laboratory, a mouse calvaria-derived preosteoblast cell line, were selected because of their ability to differentiate to osteoblast under osteogenic condition. Cells were plated in complete growth media. Subculture was performed with Trypsin (Invitrogen Corp.) i.e. incubated with 2 ml 0.25 % Trypsin for 15 min at 37 °C and 6 ml growth media was added to neutralize the trypsin for splitting cells into 1:4 ratio.

Growth media was prepared by supplementing 10%FBS, 1 % penicillin/streptomycin antibiotics, and 2 mM L-glutamine in Dulbecco's Modified Eagle Medium (DMEM). Osteogenic media was supplemented with 50µg/mL ascorbic acid and 10mM β-glycerophosphate in complete growth media. Cell culture was performed under 100 % humidity and 5 % CO₂ at 37°C.
2.3 Experimental design

2.3.1 MC3T3-E1 behavior on PMMA surface

2.3.1.1 MC3T3-E1 proliferation study on PMMA surface

Sterile 15mm glass coverslips (see 2.1.1) that were spin-coated with atactic, syndiotactic, and isotactic PMMA were carefully placed into 24-well culture plate. 15 coverslips were used for each copolymer PMMA, five time points and triplicate for each time point. MC3T3-E1 cells were counted and seeded to a density of 20,000 cells/well in accordance with group assignments. Cells were then cultured with growth media only for 28 days at 37 °C with 5 % CO2.

MTS assay was performed at each time point, 3, 7, 14, 21, and 28 days to compare cell proliferation and viability. CellTiter 96 aqueous one solution cell proliferation assay kit was purchased from Promega and the assay was performed as follows. In this assay, the formation of formazan from methyl tetrazolium salt (MTS) by mitochondrial NADH/NADPH dependent dehydrogenases in living cells is quantified by colorimetric measurement at 490 nm. 160 μl MTS solution was added to each well containing 800 μl fresh media. 800 μl fresh phenol free media supplemented with 160 μl MTS solution in
empty well served as reference. After incubating at 37°C for 3 hr, 160 μl homogenized media was transferred to 96 well plate through pipette and absorbance at 490 nm was measured with 96 well plate reader (Vmax kinetic microplate reader, Molecular Devices, PerkinElmer Inc.).

2.3.1.2 MC3T3-E1 osteogenic differentiation on PMMA surface

Cell culture conditions were identical to proliferation study except that growth media was substituted with osteogenic media (see section 2.2.2) once cells were confluent. Two control groups were assigned: positive control group with osteogenic culture media, negative control group with growth media only. Cells were cultured for 3, 7, 14, 21, and up to 28 days with changing media at every 4 and 3 days alternatively to assure that media collection at each time point is consistent in culture duration. Culture media were collected and cell components were acquired after lysing with 150 μl 1 x passive lysis buffer (Promega) incubating at room temperature for 15 min at each time point for future analyses. Samples were frozen at -20°C until further quantification. Prior to cell lysis, MTS assay was performed to assess cell proliferation and viability.

Osteocalcin expression in culture media was quantified with Mouse Osteocalcin EIA kit (catalog No. BT-470, Biomedical Technologies Inc., MA). Assay was performed with as follows: 1.56, 3.12, 6.25, 12.5, 25, and 50 ng/ml standards were obtained by diluting the stock standard (50 ng/ml). 25 μl of collected culture media, standards, and sample buffer
(blank) were pipetted into 96 well plate followed by 100 μl of osteocalcin antiserum in each well and incubated for 20 hr at 4°C. 100 μl streptavidin-horseradish peroxidase reagent was added to each well after complete wash with 300 μl/well phosphate-saline wash buffer and incubated for 30 min at room temperature. 100 μl equal volume mixture of TMB and hydrogen peroxide solution was added to each well after wash as described before and incubated for 15 min at room temperature in the dark. Reaction was stopped with 100 μl stop solution which contains hydrochloric acid and phosphoric acid. Absorbance was measured at 450 nm immediately (Vmax kinetic microplate reader, Molecular Devices, PerkinElmer Inc.). Unknown concentrations were obtained from standard calibration curve.

Alkaline phosphatase (ALP) activity quantification working solution was obtained by mixing equal parts in volume three different solutions: 1.5 mol/L 2-amino-2-methyl-1-propanol (Sigma 104-0, #221), 20 mM p-nitrophenylphosphate, disodium (prepared in water) (Sigma, # 104-100), and 1 mM MgCl₂. The mixture was maintained in a bottle that is protected from light by aluminum foil. Standards were prepared by serially diluting the mixture of 2 mL 0.02 N NaOH and 25 μl of pNPP standard in the bottles that were protected from the light. 50 μl lysed cell component samples and standards were transferred to 96 well plate followed by adding 50 μl of ALP working solution. The mixture was incubated for 30 min at 37°C in the dark. 100 μl 1 N NaOH was added to stop the reaction and read at 405 nm with plate reader (Vmax kinetic microplate reader, Molecular Devices, PerkinElmer Inc.).
Relative Alkaline phosphatase (ALP) and osteocalcin (OCN) activities were assessed by normalizing ALP and OCN expression to total protein that is quantified from lysed cell components using Pierce® BCA protein Assay Kit (Thermo Fisher Scientific, IL). The Thermo Scientific Pierce BCA Protein Assay is a detergent-compatible formulation based on bicinchoninic acid (BCA) for the colorimetric detection and quantitation of total protein. Reaction is based on the reduction of $\text{Cu}^{2+}$ to $\text{Cu}^{+}$ by protein in an alkaline medium and the highly sensitive and selective colorimetric detection of the cuprous cation ($\text{Cu}^{+}$) using a unique reagent containing bicinchoninic acid. Assay was performed as follows: Serial concentration standards were prepared and green working reagents were obtained by mixing 50:1 of reagent A and B. 25 $\mu l$ cell component aqueous solution and standards were added to 96 well plate followed by adding 200 $\mu l$ working reagent and mixed thoroughly. Plate was covered and incubated for 30 min at 37°C and the absorbance was measured at 550 nm after cooling down to room temperature.

Total DNA quantification was performed to evaluate cell numbers on PMMA polymer surfaces with different tacticities over time. To remaining lysate, equal volume of Caron’s lysis buffer was added and homogenized with sonication for 4-5 seconds (power=3). Serial DNA standards with capillary assay solution were prepared from stock DNA (10 mg/ml) (Gibco Invitrogen) for equipment calibration. 5 $\mu l$ lysate, 90 $\mu l$ 1xTNE, and 5$\mu l$ CAS were mixed for each sample and measured the fluorescence of the samples with Hoefer DyNA Quant 200 (Hoefer Inc., CA).
2.3.2 Complement-Macrophage system activation on material surface

2.3.2.1 Direct interaction between macrophage and PMMA surface

RAW 264.7 cells were cultured on spin-coated atactic PMMA surface in a 24-well plate. 1μg/ml LPS treated cells on tissue culture polystyrene (TCPS) served as positive control while cells cultured on TCPS with plain growth media served as control group. Cells were cultured at 37 °C and 5 % CO₂ for up to 48 hours and culture media were collected at 0, 3, 6, 24, and 48 hr time points. TNF-α, IL-1β, IL-6 quantification was carried out with BD OptEIA™ ELISA kits (BD Biosciences, Franklin Lakes, NJ) as follows: High binding 96 well plate (Corning Life Sciences) was coated with 100 μl capture antibody in coating buffer and incubated overnight at 4°C. 100 μl collected culture media and standards were added to the plate after complete washing and blocking with assay diluent and incubated for 2 hours at room temperature. Detection antibody and Sav-HRP reagents were added in accordance with manufacture instruction after washing with assay diluent to make final volume of 100 μl and incubated for 1 hour. After thorough washing, 100 μl of substrate solution composed of TMB and hydrogen peroxide was added and incubated for 30 min at room temperature in the dark. 50 μl stop solution, 1 M H₃PO₄, was then added prior to reading absorbance at 450 nm with reference wavelength at 550 nm (Vmax kinetic microplate reader, Molecular Devices, PerkinElmer Inc.).
2.3.2.2 The effect of complement pretreatment on activation of macrophage on PMMA surface

In 24-well plates, 15mm smooth PMMA surface was pretreated with mouse complement (Innovative Research, IL), either intact or heat inactivated, by incubating in DMEM 20% mouse complement on spin-coated PMMA surface at 37°C and 5% CO2. Macrophages were cultured without FBS, for preventing undesired stimulation of macrophages, for up to 24 hour due to limited growth condition of media without FBS in control groups. Culture media were collected at 3, 6, and 24 hr time points and cytokine concentration was quantified with ELISA kits as described above.

2.3.2.3 The effect of complement pretreatment on activation of macrophage by PMMA surfaces and particulates

Sterilized PMMA discs, smooth and rough, and different size beads were pretreated with 1.5 ml DMEM supplemented with 20% mouse complement (Innovative research, IL) for 1 hr at 37°C and 5%CO2. RAW264.7 cells were counted and seeded at a density of 10^6 cells/well with 1.5 DMEM to make final concentration of mouse complement of 10%. Positive control group was treated with 1μg/ml LPS in DMEM while control group and non-complement pretreatment groups were cultured with 3ml DMEM. No FBS was employed to avoid undesired variables since increasing cytokine expression with time was detected in previous study with 10% heat inactivated FBS.
2.3.3 Co-culture system

2.3.3.1 MC3T3-E1 osteogenic activity under influence of complement-macrophage system on PMMA

The co-culture system was established by cell culture media transferring. 0.5 ml/well 6 hour RAW 264.7 cell culture media were transferred to preosteoblast like MC3T3-E1 cell culture in correspondence with the condition of biomaterials surface or particulates. 0.5 ml/well fresh 2x osteogenic media or 2x growth media were added to MC3T3-E1 culture according to group assignment. Detailed group assignments were shown in [Table.1]. All cell culture in co-culture system was performed in 6 well culture plate. Media were changed every 3 days. At day 3, 6, and 12, media of co-culture system were collected for analysis of relevant molecules in the future and cells were lysed with Trizol to preserve RNA for quantitative analysis of osteogenic markers expression.
Table 1. Group assignments of the study of Complement-Macrophage system effect on osteogenic activity of MC3T3-E1 cells in co-culture system. Co-culture system was established by culture media transfer i.e. macrophage cell culture media (blue) were transferred to preosteoblast like MC3T3-E1 culture (red).

<table>
<thead>
<tr>
<th>Groups assignment</th>
<th>Osteogenic media</th>
<th>Growth media</th>
<th>RAW 264.7</th>
<th>Complement</th>
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<tr>
<td>Growth</td>
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<td>Co-culture growth</td>
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<td>Co-culture LPS</td>
<td>Y</td>
<td>Y with LPS</td>
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<td>Smooth w Comp</td>
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<td>Y</td>
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<td>Smooth</td>
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<td>Rough w Comp</td>
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<td>Rough</td>
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<td>1/16” w Comp</td>
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<td>1/16” w/o Comp</td>
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<td>200μm w Comp</td>
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<td>200μm w/o Comp</td>
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<td>200μm</td>
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<td>1-10μm w Comp</td>
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<td>1-10μm w/o Comp</td>
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<td>1-10μm</td>
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2.3.3.2 The evaluation of ostogenic activity of MC3T3-E1

Osteogenic markers COL1A1, Runx2, and SPARC mRNA expression were quantified at each time points by quantitative PCR.

RNA isolation was performed as follows: Cells were lysed by adding 1 ml of TRIZOL Reagent (catalog No.15596-018, Invitrogen) and homogenized by pipette. RNA is separated by means of phase separation with adding 0.2ml chloroform to homogenized samples. Precipitation and washing are performed with 0.5 ml pure isopropyl alcohol and 1 ml 75% ethanol, respectively. RNA is reconstituted in 20 μl RNase-free water and concentration and A260/280 ratio were measured with Nanodrop (Thermo Fisher Scientific Inc.) and the RNA concentration is adjusted to 100 ng/μl with RNase-free water for complementary DNA synthesis. Complementary DNA is synthesized with High capacity cDNA kit (Invitrogen) following the manufacture instruction. 1 μg RNA was reverse transcripted to complemetry DNA in total volume of 20 μl.

Real time PCR is performed to quantitatively evaluate mRNA expression on osteogenesis markers. Primers and probes for COL1A1, Runx2, SPARC (ON), and GAPDH (internal control) were purchased from Applied Biosystems (Taqman Gene Expression Assay). Quantitative PCR was performed with 7500 real time PCR system (Applied Biosystems, Thermo Fisher Scientific). GAPDH probe was labeled with VIC while the probes for
target genes were labeled with FAM. Taqman Universal PCR Master Mix (Applied Biosystems) was used for reagent mixing according to manufacturer’s protocol. The 20 μl mixture contained 10 μl master mix, 1 μl target gene probes and primers, 0.4 μl GAPDH forward primer, 0.4 μl reverse primer, 0.4 μl GAPDH probe, 6.8 μl RNase and DNase free water, and 1 μl cDNA sample. Total 40 thermo cycles were applied at 95ºC for 10 sec and 60ºC for 1 min as one cycle. Data was collected and analyzed with 7500 system v 1.2.3.f2.

2.4 Statistical analyses

Results were reported as mean ± standard deviation and ANOVA with Tucky’s test is employed for detecting the differences among groups. In cases of comparing two treatment groups, student t test was used to detect the statistical difference between two groups. Differences are considered statistically significant when p<0.05.
Chapter 3 Results and Discussions

3.1 Surface and thermal properties characterization of PMMA

The surface properties of PMMA were characterized because of the initial contact and interaction between biomaterials surfaces and host upon implantation. Surface roughness may affect non-specific protein adhesion and cell attachment through mechanical interlocking. Contact angle is essential indication of surface wetting properties which directly affect protein attachment and conformational switch and consequence interaction with cellular components. In addition, surface chemistry determines the interaction between surfaces and surrounding extracellular matrix proteins. Thermo properties could demonstrate the microstructure of material and provide temperature range for heat sterilization. By optical measuring microscopy, Atomic Force Microscopy (AFM), and contact angle measurement, the surface topography and wetting properties were determined while surface chemistry and thermal properties were assessed with FT-IR and DSC, respectively.
3.1.1 Characterization of topography

3.1.1.1 Optical Microscopy

Under optical measuring microscopy (Nikon measurescope, NIKON Co. Japan), as shown in [Fig.2], spin-coated PMMA was observed to have little contrast with the glass [Fig.2A] so that coating defect area, which is resulted from insufficient PMMA solution, is imaged to verify that PMMA is coated. On the contrary, the roughened surface provided superior optical contrast with complex microstructure [Fig.2B] for cell attachment through mechanical interlocking. By applying counter-directional rotation with sandpaper fixed on a spinning motor, randomly oriented scratches are made on PMMA disc surface to avoid pattern dependant cell attachment and growth. Average width of main beams on roughened surface was 10.12 ± 1.43μm and mean depth, measured with focus variation method, from the surface of main beams to the bottom, was 3.89 ± 0.69μm. The defect edge area of a spin-coated surface was chosen to elucidate the boundary of coated PMMA and glass coverslip since PMMA surface was too smooth to be appropriately imaged.
Figure 2. Optical microscopy image of spin-coated PMMA and roughened PMMA surface. A, defect area of spin-coating shows an optically smooth PMMA surface. B, PMMA disc roughened with 320 grit sandpaper, shows an intricate microstructure.
3.1.1.2 Atomic Force Microscopy

According to measurescope result, area of 50x50 μm² was imaged with Dimension 3000 AFM (Veeco, Santa Barbara, CA) with contact mode at a rate of 0.3 Hz under ambient conditions to investigate and quantify the roughness of spin-coated and roughened PMMA surface. AFM images [Fig.3] and data [Table.2] show that spin-coating produces an extremely smooth finish with mean Ra of 1.35±0.53nm. Conversely, roughened PMMA disc surface reached the Ra of 493.32±83.97. Rmax of roughened disc group, 5.10 μm, was much higher than that of spin coated group, 349.40 nm. Rmax of roughened group was in agreement with non-contacting 3D optical measuring microscope result. Variations may be caused by interference of cantilever in AFM and field of depth in optical microscopy.

Table 2. AFM roughness data of smooth and rough PMMA surface

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<th>Rough disc PMMA</th>
<th>Spin coated PMMA</th>
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<tr>
<td></td>
<td>mean</td>
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<tr>
<td>Ra</td>
<td>493.32 nm</td>
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</tr>
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<td>Rq</td>
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<td>Rmax</td>
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<td>0.27 μm</td>
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Figure 3. Atomic Force Microscopy image. Left: Spin-coated PMMA. Right: Roughened PMMA disc. Area: 50 x 50 μm². Mean Ra for spin coated PMMA surface: 1.35 nm, Mean Ra for rough surface: 493.32 nm.
3.1.2 Contact angle

Contact angle measurement provides essential information regarding hydrophilicity and hyrophobicity, as well as wetting ability and surface energy of a given surface. The lower contact angle, the better wettability the material exhibits during interaction with water. Wetting plays an important role on interaction between biomaterials and extracellular matrix proteins and cells. A clean glass surface served as reference and had a contact angle of $42.63\pm1.93^\circ$. Data is shown in [Table.4], while tissue culture polystyrene exhibits lowest contact angle of $55.09\pm2.49^\circ$, smooth PMMA surface showed more hydrophobic property with a $65.83\pm1.48^\circ$ contact angle. The roughened surface generally had higher contact angle than that of the smooth surface. Contact angles on sliced PMMA discs were examined both parallel and perpendicular with the grooves. Wetting anisotrophy was observed as described by Yang et al. 2009 i.e. different contact angle are observed on orientations parallel and perpendicular to cutting grooves. The contact angle observed from a direction that is perpendicular to grooves was statistically significantly higher than that of smooth surface ($p=0.006$). Random rough surface exhibited a CA that is in between that of parallel and perpendicular to regular grooves which can be explained by surface energy rearrangement. Roughened PMMA surfaces generally showed higher variance (standard deviation) which may be due to the variance in a given sample area tested which resulted from the intricate microstructure.
Table 3 Water contact angle on smooth and rough PMMA and TCPS surface

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<th></th>
<th>Glass</th>
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<th>TCPS</th>
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<td>69.31°</td>
<td>90.94°</td>
</tr>
<tr>
<td>Mean contact angle</td>
<td>42.63°</td>
<td>65.83°</td>
<td>94.06°</td>
</tr>
<tr>
<td>Standard Deviation</td>
<td>1.93°</td>
<td>1.48°</td>
<td>3.96°</td>
</tr>
<tr>
<td></td>
<td>4.55°</td>
<td>4.95°</td>
<td>2.49°</td>
</tr>
<tr>
<td></td>
<td>55.09°</td>
<td>90.94°</td>
<td>55.09°</td>
</tr>
</tbody>
</table>

However, surface wettability is not the only determinant factor for interaction between biomaterials and ECM. As shown in [Fig.12-14], cytokine expression in control group on PMMA was higher than that of TCPS. However, regarding the effect of complement on macrophage activation, no significant difference was found between the surfaces of two materials [Section 3.3].
3.1.3 FT-IR chemical composition study

The chemical composition of different size PMMA beads, PMMA discs acquired from acrylic rod, and spin-coated PMMA was investigated with FT-IR to assure that cells will interact with materials with of same composition. Spectra, as shown in [Fig.4], as expected, demonstrated identical chemical composition of different size particles, spin-coated film, and bulk roughened disc. Relatively low intensity was detected in smooth PMMA spectrum due to weak signal from the thin film. The baseline was affected by the signal from glass substrate.
3.1.4 Differential scanning calorimetry study of Tg

The thermal properties of PMMA samples were investigated to provide guidance for sterilization of PMMA specimens. The glass transition temperature (Tg) of a non-crystalline material is the temperature at which the phase transformation occurs.

DSC study of PMMA particles are shown in [Fig.5]. The glass transition temperatures are summarized in [Table.3]. Tg increased with decreasing particle size. The smallest particle 1-10 μm beads had highest Tg at 130.13 and 126.03°C in first and second heating
cycle, respectively. The largest “particle”, 1/16” PMMA beads, showed glass transition at 72.14 and 101.95°C in first and second thermo cycle, respectively. The intermediate size particle, 200 μm beads, on the other hand, revealed an increase of Tg to 110.74°C at second cycle from 106.66°C at first heating cycle. Commercially available low molecular weight PMMA particle has a Tg of 108°C (data not shown). Exothermic peaks were observed following Tg at initial heating process evidencing the release of strain from the surface of beads.

The exhibition of different Tg’s at the first and second heating cycle might be due to release of strain from the beads surface during first heating process. Smaller size had higher Tg possibly is due to much higher surface area where extra strain is concentrated during manufacturing to spherical beads. Hence, smaller beads needed more energy to release surface tension. All Tg were above 100°C so that the sterilization of spin-coated PMMA and roughened PMMA discs on vacuum oven at 100°C will not alter the surface microstructure.

Table 4. Tg of different size PMMA beads

<table>
<thead>
<tr>
<th>Particle size</th>
<th>Tg at first heating °C</th>
<th>Tg at reheating °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/16 inch</td>
<td>72.14</td>
<td>101.95</td>
</tr>
<tr>
<td>200 μm</td>
<td>106.66</td>
<td>110.74</td>
</tr>
<tr>
<td>1-10 μm</td>
<td>130.13</td>
<td>126.03</td>
</tr>
</tbody>
</table>
Figure 5. Differential Scanning Calorimetry. A: first heating cycle, an exothermic peak was observed after glass transition. B: second heating cycle, no exothermic peak was observed after glass transition. Tg of 200 µm and 1/16” PMMA beads were increased in the second heating cycle whereas that of 1-10 µm PMMA beads was decreased. Red: 1/16” bead, Maroon: 200 µm beads, Green: 1-10 µm beads.
3.1.5 Summary

Smooth and rough PMMA surfaces were obtained. Spin coating provided a surface with average roughness of 1.35 ± 0.53 nm while 320-grit sandpaper produced a rough surface on PMMA discs with an average roughness of 493.32 ± 83.97 nm. Generally, PMMA surface exhibited more hydrophobic property compared to tissue culture polystyrene (TCPS). Roughened PMMA showed more hydrophobicity compared with smooth PMMA surface. Rough surface with regular arrangement grooves exhibited anisotropic wetting property while randomly roughened surface showed isotropic wetting property. FT-IR revealed identical chemical composition of PMMA surfaces and different size particles. Sterilization with 100ºC in vacuum oven will not affect the PMMA microstructure because the Tg of different size particles, including atactic particle used for spin coating, was higher than 100ºC.

3.2 MC3T3-E1 cell behavior on surface of PMMA with different tacticity

The biological activity of a cell on a surface is based on molecular interactions. Hence, conformation of molecules and proximity between molecules play major role. The tacticity of PMMA is originated from the chirality of β-carbon and forms isotactic, syndiotactic, and atactic PMMA polymer which has distinct arrangement of ester bonds
on the polymer chains. So, it is reasonable to hypothesize that different tacticities may have distinct effects on the interaction between polymers and ECM and cells. As illustrated in [Fig.1], isotactic polymer chain has a regular arrangement of the ester group relative to one another while syndiotactic polymer chain has alternating arrangement of ester bonds. Atactic PMMA, however, has a random arrangement of ester groups. The purpose of this experiment was to assess the effect of PMMA polymer tacticities on proliferation and differentiation of preosteoblast like MC3T3-E1 cells that were cultured on the polymer surfaces with different tacticities.

3.2.1 The effect of different PMMA tacticity on preosteoblast-like MC3T3-E1 cell proliferation

Initially, MC3T3-E1 cells were cultured to determine if the tacticity of PMMA had any effect on proliferation. The proliferation study of MC3T3-E1 cell on different copolymers of PMMA showed no significant difference between groups at day 3 ($p=0.431$) and 14 ($p=0.295$), as shown in [Fig.6]. However, at day 7, cells cultured on all PMMA surfaces had lower viability compared to TCPS with statistical significance ($p=0.01$). MTS measurement at day 21 and 28 were out of range with plate reader (Bio-Tek Instruments) for all groups. No significant difference was found between groups on day 3 and day 14.

The tacticity of PMMA may not have effect on cell proliferation due to promising biocompatibility of PMMA surface since most inflammatory responses have been
reported in accordance with particulates that are generated from functioning bulky materials. However, the ester bond orientation varies in different copolymer PMMA as shown in [Fig.1]. The variability of orientation and arrangement of ester bond may lead different interaction between copolymer and ECM, furthermore, complement adhesion and activation. Conversely, even though the ester bond arrangement varies in atactic, syndiotactic, and isotactic polymer chains, the microstructure of bulk material might be very similar due entanglement and random organization of polymer chains which leads random exposure of ester bonds to the surface. More importantly the interactions between adjacent MA groups will lead alternate orientations with different span by rotation of γ-carbons. The size of preosteoblast determines that these cells will span a large area in comparison to single polymer chain or even crystalline phase of polymer which may lead similar ratio and amount exposure of ester bond to covering cells.

Even though cell viability and proliferation did not reveal the difference between PMMA polymer surfaces with different tacticities, cell differentiation may be distinct on different polymer surfaces.
3.2.2 Osteogenic study on PMMA with different tacticity.

Osteogenic activity of MC3T3-E1 cell was investigated by culturing cells on desired material surface with osteogenic media for up to 28 days. Cell viability and ALP, OCN activities were tested. Cell differentiation might be more sensitive to small changes in surface properties.

3.2.2.1 Cell viability
As shown in [Fig.7], TCPS growth group exhibited significantly higher cell viability at all time points ($p<0.05$). TCPS osteogenic group showed significantly higher ($p<0.05$) cell viability at day 3 and after day 21 compared to PMMA osteogenic groups. No statistically significant difference was found between PMMA osteogenic groups with different tacticities. The cell viability reached a plateau at day 14 for all groups except TCPS osteogenic group which showed increasing viability with time but still lower than TCPS growth group. Osteogenic condition showed an inhibitory effect on cell viability and proliferation since no differences were found under growth condition in previous study.

![Figure 7. MTS assay for determination of MC3T3-E1 cell viability on PMMA polymers with different tacticity under osteogenic condition](image-url)
However, total protein expression did not follow the trend observed in the cell viability study. As shown in [Fig.8], at day 28, TCPS growth group has significantly lower protein expression while no statistically significant differences were found between PMMA groups and TCPS osteogenic group. Nevertheless, protein expression on PMMA groups was slightly higher than that of TCPS osteogenic group.

The phenomenon that lower cell viability accompanied with higher protein expression could be resulted from excessive production of extracellular matrix protein from preosteoblast like cells upon interaction with PMMA surface under osteogenic condition.

![Total Protein quantification in the study of osteogenic activity of MC3T3-E1 cells on polymer PMMA with different tacticity](image)

Figure 8. Total protein quantification in osteogenic study of MC3T3-E1 cells on polymer PMMA with different tacticity
Total DNA is useful as a measurement of cell numbers. As shown in [Fig.9], TCPS osteogenic group showed higher total DNA i.e. higher number of cells than PMMA groups at all time points without statistically significant difference except that isotactic PMMA group showed significantly lower total DNA expression ($p=0.003$). No statistically significant differences were found between PMMA polymers with different tacticities at all time points. The initial difference in cell numbers could be caused by variations in the process of cell seeding or different proliferation rates on different surfaces.

Figure 9. Total DNA quantification in osteogenic study of MC3T3-E1 cells on PMMA polymer with different tacticity
Combining with cell viability study and total protein quantification, a conclusion can be made that PMMA polymers with different tacticities will not affect cell proliferation and differentiation compared to TCPS. Same number of cells with lower viability and higher extracellular matrix lead to the postulation that PMMA is capable of leading preosteoblast like cells differentiate toward fibroblastic path. Hence, the quantifications of osteogenic activity markers were performed.

3.2.2.2 Alkaline phosphatase activity

Alkaline phosphatase (ALP) has been identified to be a potent osteogenic marker (Weinreb, Shinar et al. 1990). ALP is quantified with ELISA kit and its activity is evaluated by normalizing ALP concentration to total protein and data is shown in [Fig.10]. TCPS growth group exhibited consistent and significantly low expression of ALP activity. Prominent activity of ALP was observed at day 21 in PMMA groups and TCPS osteogenic group. Cells cultured on PMMA surface exhibited higher ALP activity than TCPS osteogenic group at day 21 and 28 without a statistically significant difference.
3.2.2.3 Osteocalcin activity

Osteocalcin (OC) is quantified and its activity is evaluated after normalizing to total protein expression to avoid false positive or negative result caused by possible different cell number, since cells were cultured for a long period of time. As shown in [Fig.11], TCPS growth exhibited significantly low activity of OCN even though increasing activity was detected at day 21 and 28. At day 21 and 28, no significant difference was found between PMMA groups and TCPS osteogenic groups. Moreover, no statistically significant difference was found between PMMA polymers with different tacticities at
day 21 and 28. However, generally, cells cultured on PMMA surface with osteogenic media exhibited higher activity of ALP and OCN.

![OCN activity of MC3T3-E1 cells on PMMA surfaces under osteogenic condition](image)

Figure 11. OCN activity in osteogenic study of MC3T3-E1 cells on different copolymer PMMA surfaces

### 3.2.3 Summary

PMMA polymer surfaces with different tacticities exhibited no effect on cell proliferation and viability compared to tissue culture polystyrene under growth condition. In addition, the different tacticities failed to induce different osteogenic behavior of MC3T3-E1 cells in terms of ALP and OCN activity. Interestingly, osteogenic condition resulted in
decreased cell proliferation and viability in both PMMA polymer surfaces and tissue culture polystyrene surface. However, the relatively lower viability and lower cell numbers accompanied with same level of protein expression and higher ALP and OCN activation suggests that PMMA surface has promising biocompatibility regardless of tacticity. Nevertheless, there were no statistically significant differences between PMMA surfaces with different tacticities and TCPS surface.
3.3 Macrophage cells, RAW264.7, activation study

Since no significant difference has been detected in the cell proliferation and osteogenic activity study of MC3T3-E1 cell line, commercially available atactic PMMA was chosen for RAW cell activity study to construct a coculture system. In order to construct a co-culture system through culture media transfer, the cytokine profile upon activation of macrophages on PMMA surface had to be understood. Upon implantation of biomaterial into the host, macrophages encounter and interact with materials surface through the protein layer formed by non-specific adsorption. The initial adherence and activation of macrophages is a determinant signal for following inflammatory cascade. Hence, macrophage, RAW 264.7, activation on PMMA surfaces was studied in this section. Upon activation of macrophages, three proinflammatory cytokines, TNF-α, IL-1β, and IL-6 were quantified because not only were they important markers of macrophage activation but also they express regulatory effect on osteoblast as described in [Section 1.4].

3.3.1 Macrophage activation study performed on plain atactic PMMA surface

RAW 264.7 cells were cultured on spin-coated atactic PMMA surface in 24-well plate. 1μg/ml LPS treated cells on tissue culture polystyrene served as positive control group.
and assigned as “LPS stimulation” while cells cultured on TCPS with growth media served as negative control group. Cells were cultured for 48 hours and culture media were collected at 0, 3, 6, 24, 48 hr time points for target cytokine quantification. TNF-α, IL-1β, and Il-6 expression were quantified with BD OptEIA™ ELISA kits as described in [Section 2.3.2.1].

As shown in [Fig.12], TNF-α secretion gradually increased over time and, at 48 hours, reached 232±7 pg/ml and 280±23 pg/ml in TCPS control and PMMA groups, respectively, while positive control group (1 μg/ml LPS stimulation) produced peak amount of 1007±53 pg/ml within 6 hours. PMMA induced higher amount of TNF-α compare to TCPS and there is significant difference at 24 hour time points (p<0.05). No statistically significant difference was detected between TCPS control and PMMA surface groups at 48 hours.
Figure 12. TNF-α quantification in study of RAW 264.7 cell activation on PMMA surface. Positive control group (LPS stimulation) reached to the peak, 1007±53 pg/ml, at 6 hr. PMMA surface and TCPS surface induced gradual increase in TNF-α secretion and reached to a peak value at 48 hr, 280±23 and 232±7 pg/ml. PMMA surface induced significantly higher secretion at 24 hr (p<0.05). No significant differences have been found in other time points.
IL-6 secretion under LPS stimulation reached a peak value of 1500±13 pg/ml at 24 hours after treatment and slightly increased at 48 hours as shown in [Fig.13]. The TCPS Control group, however, secreted significantly lower IL-6 amounts at all time points. IL-6 levels increased with time and reached a peak at 24 hour and showed slight decrease at 48 hours. Cells that cultured on smooth PMMA surface expressed higher IL-6 than TCPS control group at all time points except 6 and 48 hour time points. However, no statistically significant differences were found between PMMA and TCPS control groups except at 6 hr time point at which TCPS control group expressed significantly higher level of IL-6 ($p=0.013$). At 24 h, PMMA group and TCPS control group expressed 7.89 pg/ml and 4.73 pg/ml, respectively. The reason that PMMA group at the 6 hr time point had a low IL-6 levels remains unknown.
Figure 13. IL-6 quantification in study of RAW 264.7 cell activation on PMMA surface. Positive control group (LPS stimulation) reached to the peak value 1500±13 pg/ml at 24 hr. PMMA and TCPS induced gradual secretion of IL-6 up to 24 hr reaching at 7.89±7.8 and 4.73±4.6 pg/ml, respectively. No statistically significant difference was found.
IL-1β expression, as shown in [Fig.14], followed the same trend of IL-6 expression for PMMA and TCPS control group i.e. gradually increase with time but recession at 6 h time points and slight decrease at 48 hr time point. However, no significant difference was found between PMMA group and TCPS control group at all time points. LPS stimulus did not result in significantly different IL-1β expression until 24 hours and its expression increased at 48 hours.
Figure 14. IL-1β quantification in study of RAW 264.7 cell activation on PMMA surface. Positive control group (LPS stimulation) reached to the peak at 48 hr. PMMA induced slightly higher expression compared to TCPS, but no statistical significance was found.
The proinflammatory cytokine profile in this study is in agreement with other studies in which the biomaterial surface did not induce the production of TNF-α, IL-6, and IL-1β without complement involvement (Lappegard, Bergseth et al. 2008). However, generally, PMMA surface induced higher expression of TNF-α, IL-6, and IL-1β without statistical significance. Other cytokines and chemokines such as IL-8, PGE2, and MCP1 are susceptible to change in profile since macrophage activation on biomaterial surface differs from classical and alternative pathway (Lappegard, Fung et al. 2004; Anderson, Rodriguez et al. 2008; Lappegard, Bergseth et al. 2008).

### 3.3.2 Complement effect on macrophage, RAW264.7, activation on PMMA surface.

Mouse complement (Innovative Research, IL) pretreatment was employed by incubating 20% mouse complement on spin-coated PMMA surface at 37°C and 5% CO2. Macrophages were cultured without FBS, for preventing undesired stimulation of macrophages, with intact or heat inactivated complement pretreatment, for 24 hours due to limited growth condition of media without serum. Culture media were collected at each time points and cytokine concentration is quantified with BD OptEIA™ ELISA kits as described in [Section 2.3.2.1]. Cells were lysed with 1x passive lysis buffer and collected for total protein quantification.
As shown in [Fig.15], at the 3 hr time point, the total protein expression of cells cultured on PMMA surface that were pretreated with mouse complement and heat inactivated mouse complement were significantly higher ($p=0.013$) than that of control groups on either TCPS or PMMA surfaces. At the 6 hr time point, total protein expression of PMMA-iComp group was significantly higher than all other groups followed by PMMA-Comp and positive control group. No significant differences have been found between other groups. After 24 hrs in cell culture, total protein expression of cells that were cultured on either PMMA or TCPS pretreated with mouse complement or heat inactivated mouse complement were significantly higher than that of 1 μg/ml LPS treated groups or control groups. In addition, cells that were cultured on PMMA surface expressed significantly higher level of protein than those on TCPS. No statistically significant differences have been found between 1 μg/ml LPS treated and growth media groups, either on PMMA or TCPS. Pretreatment with intact or heat inactivated mouse complement induced significantly higher ($p=0.013$) total protein indicates that macrophages were stimulated to produce more protein including cytokines, chemokines, and extracellular matrix proteins. Hence, it is reasonable to hypothesize that macrophages produce more TNF-α, IL-6, and IL-1β upon stimulation by surfaces that were pretreated with mouse complement.
Figure 15. Total protein quantification in the study of complement effect on RAW 264.7 cell activation on PMMA surface. iComplement refers to heat inactivated mouse complement.
TNF-α and IL-6 were quantified with BD OptEIA™ ELISA kit and shown in [Fig.16-17]. When ANOVA was performed for all groups, at all time points, IL-6 expression in positive control groups with PMMA or TCPS surface was significantly higher than all other groups. No statistical significant differences have been detected between other groups. However, once analyzed the groups except for the positive control group, differences between groups were revealed.

IL-6 quantification is shown in [Fig.16]. The significantly higher expression of IL-6 was detected in both PMMA and TCPS groups with complement pretreatment ($p<0.05$). Similar levels of IL-6 expression were detected in PMMA negative group and both TCPS and PMMA groups pretreated with complement. Macrophages that were cultured on TCPS expressed slightly higher IL-6 than the groups aforementioned with statistical significance. Even though a slight decrease in expression has been found in the groups with complement pretreatment, IL-6 expression in other groups was consistent over 24 hr.
The TNF-α expression pattern, however, was different from IL-6. TNF-α quantification data is shown in [Fig.17]. Similar to previous study, the expression of TNF-α increased over time in all treatment groups. Surprisingly, the groups that were pretreated with heat inactivated complement exhibited significantly higher TNF-α expression at all time points ($p<0.05$). On PMMA surface, complement pretreatment resulted in higher expression at the 6 hr time point but lower expression at the 24 hr time point. No significant difference was found between complement treated TCPS and TCPS control group.
Figure 17. TNF-α quantification in the study of complement effect on RAW 264.7 cell activation on PMMA surface

From this study, we could make conclusion that complement that activated by material surface could lead to increasing IL-6 and TNF-α secretion at 6 hr. Heat inactivation of mouse serum resulted in further increased TNF-α levels and decreased IL-6 expression.

We hypothesized that heat inactivation process forms a variety of proteins in the serum to go through conformation change and may express functional epitopes that has stimulatory effect on macrophages through interacting with membrane receptors. In addition, protein coagulation caused by heat inactivation may stimulate macrophages through phagocytosis. Other components in mouse serum such as antibodies along with
serum proteins that are altered in conformation may stimulate macrophages through unknown mechanisms.

Within the limitations of this study, part of second hypothesis is rejected. However, other studies evidenced that material surface could be friendly to macrophages and not to induce TNF-\(\alpha\), IL-6, and IL-1\(\beta\)(Lappegard, Fung et al. 2004; Lappegard, Bergseth et al. 2008). In addition, investigation of three cytokines is insufficient to determine whether macrophages are activated to proinflammatory stage. Other cytokines and chemokines and protein profile in depth study is required to draw safe conclusion. IL-8 has been reported to be synthesized in completely complement dependent manner(Lappegard, Bergseth et al. 2008).
3.3.3 The macrophage, RAW264.7, activation by PMMA surfaces and particulates under the influence of complement

Material surface roughness may play a role in macrophage activation. However, there is increasing controversy on the interaction between rough surface and macrophages (Refai, Textor et al. 2004; Tan, Qian et al. 2006; Fink, Fuhrmann et al. 2008). Conversely, PMMA particles are known to induce proinflammatory cytokine production of macrophage. Phagocytosis plays major role in activating macrophage/monocyte in sites of implantation (Bosetti, Zanardi et al. 2003; Anderson, Rodriguez et al. 2008; Hallab and Jacobs 2009).

Complement involvement in macrophage adhesion and activation were proposed. Hence, material surface roughness, different size beads, and complement were introduced into the study of complement effect on macrophage activation. Because of unclear effect of heat inactivated complement in previous study, the complement effect on macrophage activation is elucidated with and without complement treatment in the present phase.

In the study of macrophage, RAW264.7, activation on PMMA surfaces and particulates, as shown in [Fig.18], all groups with complement treatment showed significantly higher IL-6 expression at 6 hr time point ($p<0.05$). IL-6 secretion increased as smooth<rough<1/16”<200μm<1-10μm sequence and smallest particle induced significantly higher IL-6 production in 6 hr culture. However, this trend is disappeared
with complement treatment. Smooth surface with complement expressed highest production of IL-6 and different particle sizes did not show effect on IL-6 expression.

Figure 18. IL-6 quantification in the study of complement effect on RAW 264.7 cell activation on PMMA surfaces and particulates

Similar to the previous study, TNF-α expression in this experiment, as shown in [Fig.19], increased with time up to 20 hrs culture. At 20 hr time point, both 1-10 μm groups with and without complement treatment produced significantly higher TNF-α ($p<0.05$) while no significant difference has been found among other groups. Furthermore, with smooth and rough surface, 1/16” beads, and 200μm beads groups with complement treatment
expressed significantly lower (p<0.05) TNF-α level than corresponding groups without complement treatment which was in agreement with previous study.

IL-1β expression in this study was barely detectable (data not shown).
Figure 19. TNF-α quantification in the study of RAW 264.7 cell activation on PMMA surface and particulates with and without complement.
3.3.4 Summary

In this study, we evaluated the complement effect on macrophage activation on PMMA surface. We hypothesized that complement activation on biomaterial surface stimulates macrophages through complement receptors [section 1.3.2]. As important markers of proinflammatory cascade of macrophage, three cytokines, TNF-α, IL-6, and IL-1β were expected to increase upon complement involvement. In addition, heat inactivation of mouse complement was expected to have no effect on macrophage stimulation. In this study, the expressions of cytokines were toward more classical pathway when macrophages were cultured on PMMA surface by increasing TNF-α, IL-1β and IL-6 expression. Complement pretreatment, however, reduced the expression of TNF-α expression and promoted the expression of IL-6. Conversely, heat inactivated complement showed opposite effect on TNF-α and IL-6 expression compared to the effect of complement [Section 3.3.2]. IL-1β was barely detectable.

The present study regarding cytokine expression upon macrophage activation under complement influence was insufficient to draw a conclusion that complement activation by biomaterial surface has stimulatory effect on macrophage activation on biomaterial surfaces. However, there is evidence that complement has effect on cytokine profile of macrophages that were cultured on PMMA surfaces. Additional studies are required to
elucidate cytokine and chemokine profile upon macrophage activation on biomaterial surface under influence of surface activated complement system.

3.4 Complement-Macrophage effect on osteogenic activity of MC3T3-E1 cells cultured on corresponding PMMA surface or with particulate treatment.

Despite the fact that the complement effect on macrophage activation was not completely clear, co-culture system was established by culture media transfer because of two known facts from previous studies: first, PMMA surface behave similarly to preosteoblast proliferation and differentiation compared to TCPS; second, even though exact pathway is not clear, complement appeared to have certain extent effect on macrophage activation on PMMA surfaces and particulates. The purpose of co-culture study is to elucidate the effect of complement-macrophage system on preosteoblast differentiation on PMMA surfaces and particulates.

3.4.1 Morphology

Macrophages, RAW264.7, were cultured with and without mouse complement on PMMA surface or with PMMA beads in plain media for 6 hours and media were collected and transferred to MC3T3-E1 cell culture. In addition, identical volume of 2x
fresh osteogenic media or 2x growth media were added to MC3T3-E1 culture according to group assignment. All cell cultures were performed in 6-well plates and media were changed every 3 day interval. Media transfer is performed when MC3T3-E1 cells reached confluence which typically takes 3-5 days. At desired time points, 3, 6, and 12 days, media were collected for future study and the cells were lysed with Trizol to protect RNA from degradation. cDNA was synthesized from mRNA and realtime PCR was performed to investigate gene expression of osteogenic markers of MC3T3-E1 cell [Section 2.3.3.2].

As shown in [Fig.20], at 12 days co-culture, the preosteoblast like cells that were treated with 1 \( \mu \text{g/ml} \) LPS stimulated macrophage culture media showed spindle shaped morphology while cells maintained in osteogenic or growth media kept the morphology intact. Other groups that were treated with macrophage culture media had similar morphology which was intermediate between intact and elongated [Fig.20]. The morphological change typically indicates the differentiation pattern. We hypothesized that complement-macrophage system affects the differentiation and osteogenic activity of preosteoblast like MC3T3-E1 cell through cytokines that were produced by complement-macrophage system upon interaction with PMMA surfaces and particulates. The osteogenic activity markers were quantitatively determined by real time PCR [Section 2.3.3.2].
Figure 20. MC3T3-E1 cell optical microscopy image in co-culture system. A. osteogenic media; B. Growth media; C. LPS stimulated co-culture; D. co-culture system with 6 micron PMMA and complement; E. co-culture system with 6 micron PMMA; F. osteogenic media with 6 micron PMMA.
3.4.2 mRNA expression of osteogenic activity markers

3.4.2.1 Collagen type I α 1 (COL1A1)

Among types of collagen, collagen type I is the main structural base for bone formation. The COL1A1 gene produces a component of type I collagen, called the pro-alpha1 chain. This chain combines with another pro-alpha1 chain and also with a pro-alpha2 chain (produced by the COL1A2 gene) to make a molecule of type I procollagen. Hence, COL1A1 can be a potent marker for collagen type 1 production.

As shown in [Fig.21], at day 3, control group with osteogenic condition exhibited significantly high expression of COL1A1 mRNA. Groups with complement showed lower expression than groups without complement at all time points. The co-culture system on rough surface exhibited increasing expression at day 12 and it was significantly higher than control (p<0.05).

LPS stimulated co-culture system expressed interesting pattern over time regarding COL1A1 mRNA expression. At day 3, even though it was lower than control, it was significantly higher than most of other groups except co-culture system on smooth PMMA surface. However, at day 6, it decreased to the level lower than the group that was under growth condition only. Interestingly, at day 12, expression of COL1A1
increased to the level higher than groups under growth condition and co-culture groups with complement. Co-culture groups under growth condition showed the lowest expression from 6 day time points but without significant difference with MC3T3-E1 growth group.

However, it is not feasible to draw a conclusion regarding osteogenic activity through mRNA expression of COL1A1 solely. Collagen expression and secretion are closely related to not only osteogenic activity, but also extracellular matrix formation since collagen type 1 plays a role in many other biological activities. Runx2 and SPARC expression were investigated to evaluate osteogenic activity with a closer focus.

Figure 21. COL1A1 expression
3.4.2.2 Runx 2

Runx2 has long been recognized as important marker of osteogenic activity. Runx2, a key osteoblast transcription factor, is commonly used as marker of osteoblast differentiation. It regulates OC expression by recruiting and binding to OC promoter.(Pregizer, Baniwal et al. 2008). It has been reported that Runx2 gene expression decreases with time(Pregizer, Baniwal et al. 2008).

In this study, Runx2 expression followed the trend that was reported in other studies, increased expression at early stage and decreasing over time [Fig.22]. Nevertheless, the co-culture system on the rough surface showed increased Runx2 expression at day 12. Positive control group expressed significantly high level of Runx2 whereas co-culture with LPS stimulated macrophage group showed significantly low expression.

The co-culture system with mouse complement had a lower expression at day 3 [Fig.23] and 6 [Fig.24] than that without complement on PMMA surfaces. At day 12 [Fig.25], the opposite expression was detected. However, with different size beads, groups with complement expressed lower level of Runx2 at all time points. At day 12, 1-10µm group with complement expressed significantly low level of Runx2.
Figure 22. Runx2 expression

Figure 23. Runx2 expression in co-culture system at day 3
Figure 24. Runx2 expression in co-culture system at day 6

Figure 25. Runx2 expression in co-culture system at day 12
3.4.2.3 SPARC (ON)

SPARC (Osteonectin, ON) expression is shown in [Fig.26-29]. Positive control group expressed consistent level of osteonectin (ON) mRNA after reaching the peak at day 3 following media transduction. The positive control, under osteogenic condition, group exhibited significantly higher SPARC (ON) mRNA at all time points. SPARC (ON) expression slightly decreased over time but without significant difference except 1-10 μm beads group in which SPARC expression increased. SPARC (ON) expression in co-culture system with LPS stimulation was significantly lower than that of positive control group (osteogenic condition), control group (growth media), and control with RAW group (both cell lines in growth media) ($p<0.05$).

Co-culture system with complement, regardless of the surfaces or particle size, expressed significantly lower level of SPARC at all time points ($p<0.05$). At day 12, co-culture system with different size beads without complement expressed significantly high activity of osteonectin, SPARC mRNA expression, 2-3 fold change has been detected. MC3T3-E1 cells cultured with different size beads under osteogenic condition showed SPARC level in between co-culture systems that were with and without complement along with corresponding materials and it was comparable to that of positive control. At day 12, co-culture system on rough surface and beads showed increased SPARC expression.
Figure 26. SPARC expression

Figure 27. SPARC expression in co-culture system at day 3
Figure 28. SPARC expression in co-culture system at day 6

Figure 29. SPARC expression in co-culture system at day 12

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3.4.3 Summary

Co-culture system was constructed by means of media transfer. COL1A1, Runx2, and SPARC (ON) mRNA expression were quantified with real time PCR. LPS stimulated co-culture system revealed the lowest expressions of all three mRNA. It indicates that culture media from LPS stimulated macrophage culture significantly inhibits osteogenesis of preosteoblasts by reducing production of collagen type I, osteonectin, and indirectly osteocalcin by reducing Runx2 transcriptor expression. Spindle like transformation of preosteoblasts in LPS stimulated co-culture system indicates that not only did co-culture inhibit osteogenesis but also it promoted fibroblastic differentiation. The exact mechanism by which the co-culture system inhibited osteogenic differentiation remains unknown.

Complement involvement reduced all three mRNA at all groups, especially in the groups with different size particles. In addition, co-culture systems on surfaces generally showed reduced osteogenesis. Complement involvement further reduced the activity of osteogenesis on these surfaces.

However, it is possible that TNF-α, which is produced by LPS stimulated macrophages, plays important role (Chua, Chua et al. 2002). In addition, TNF-α and IL-1β inhibit osteocalcin (OC) synthesis in osteoblasts (Hikiji, Shin et al. 2000). Furthermore, TNF-
alpha and IL-1beta are known to stimulate osteoblasts to produce IL-6 which in turn promote a shift in bone homeostasis towards bone resorption (Patil, Zhu et al. 2004).

Nevertheless, the TNF-α expression in complement-macrophage system was reduced in comparison to control. Conversely, IL-6 expression was increased and IL-6 plays complex role in bone remodeling (Chua, Chua et al. 2002; Patil, Zhu et al. 2004; Duplomb, Baud'huiin et al. 2008). In addition, IL-1β was barely detectable in complement-macrophage system. Hence, we speculate that there might be other cytokines and/or chemokines are responsible for reduction of osteogenic activity in co-culture system.
Chapter 4 conclusion and prospective

4.1 Conclusions

The first hypothesis was rejected by present study. The PMMA polymer surfaces with different tacticities have no effect on proliferation and differentiation of preosteoblast like MC3T3-E1 cells.

Second hypothesis was partially rejected. Complement pretreatment resulted in increased IL-6 and reduced TNF-α on the surfaces and larger particles. However, 1-10μm PMMA particles, which can be phagocytosed, stimulate macrophages to produce greater amount of TNF-α with higher expression from complement pretreatment. Hence, complement plays a complex role in biomaterial induced macrophage stimulation. An extended study of cytokine profile is required to understand complete role of complement in initial interaction between biomaterials and macrophages.

Within the limitations of present study, third hypothesis was supported. Co-culture system is established by means of media transfer. The osteogenic activity of MC3T3-E1 cells was inhibited by cytokines secreted by macrophages that were stimulated with 1μg/ml LPS. Complement incorporation generally reduced osteogenesis by down
regulating COL1A1, Runx2, and SPARC (ON). The down regulation of COL1A1 by complement incorporation in co-culture system was comparable with that of LPS stimulation. Particulates, as described elsewhere, have inhibitory effect on osteogenic differentiation and activity of MC3T3-E1 cells. The complement incorporation intensified the inhibitory effect on all groups.

4.2 Prospectives

Only three cytokines, TNF-α, IL-1β, and IL-6 were studied in present. Our hypothesis regarding complement effect on macrophage activation on PMMA surface was partially rejected due to complex role that complement system plays. More detailed and in depth study of additional cytokines and chemokines are required in future studies. In addition, there are possible correlations between cytokine concentration and their effect on osteogenic activity of MC3T3-E1 i.e. promotes osteogenesis at lower concentration and inhibits at high concentration as seen present study. Along with cytokine profile, the individual cytokine effect on osteogenesis and its pathway has to be discovered.

Complement adhesion and activation study are needed for demonstrating correlation between the amount of activated complement and the rate of macrophage activation and cytokine production. Mouse complement purification is required for eliminating undesired side effect of mouse serum. However, purification could cause conformational change and result in false positive effect. In addition, mouse complement inhibitor is
needed in future study to specifically neutralize complement system. In terms of adhesion and conformational change, XRD might be useful.

Possible fibroblastic differentiation of MC3T3-E1 cell in co-culture system with stimulation of LPS leads to the study of fibroblastic markers detection under similar condition in the future.
Reference:


