
Effect of titanium surface topography on macrophage activation and secretion of proinflammatory cytokines and chemokines

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Abstract: The macrophage has a major role in normal wound healing and the reparative process around implants. Murine macrophage-like cells RAW 264.7 were used to investigate the effect of titanium surfaces on macrophage activation and secretion of proinflammatory cytokines [interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α] and chemokines (monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 α). Four topographies were used: those produced by mechanically polishing, coarse sand blasting, acid etching, and sandblasting and acid etching (SLA). Macrophages were plated on the four titanium surfaces at a population density of 5×10^5 cells/mL/well. Tissue culture plastic and tissue culture plastic plus lipopolysaccharide (LPS) served as negative and positive control, respectively. In addition, all surfaces were tested for their effects on macrophages in the presence of LPS. Supernatants were collected for assays after 6, 24, and 48 h and the numbers of macrophages attached to the surfaces were quantified using the DAPI (4,6-di-amidino-2-phenylindole) assay. Cytokine and chemokine levels were measured with sandwich enzyme-linked immunosorbent assays. Statistical comparison between the surfaces and the controls was determined by using the two-way analysis of variance including interaction effect (two tailed and $p \leq 0.05$). Unstimulated macrophages increased their secretion of the proinflammatory cytokine (TNF- α) when attached to rough surfaces (acid

etching and SLA, $p \leq 0.05$). In macrophages stimulated with LPS, the roughest surface SLA produced higher levels of IL-1 β , IL-6, and TNF- α at 24 and 48 h than all other surfaces ($p \leq 0.05$). Surface topography also modulated the secretion of the chemokines monocyte chemoattractant protein-1 and macrophage inflammatory protein-1 α by macrophages. Unstimulated macrophages attached to the SLA surface down-regulated their production of chemokines ($p \leq 0.05$) whereas LPS-stimulated macrophages attached to the SLA surface up-regulated their production ($p \leq 0.05$). Moreover, the SLA surface was found to act synergistically with LPS as well as the combination of blasting and etching features of the SLA surface resulted in significant release of proinflammatory cytokines and chemokines by stimulated macrophages at 24 and 48 h ($p \leq 0.05$). This *in vitro* study has demonstrated that surface topography, in particular the SLA surface, modulated expression of proinflammatory cytokines and chemokines by macrophages in a time-dependent manner. © 2004 Wiley Periodicals, Inc. *J Biomed Mater Res* 70A: 194–205, 2004

Key words: surface topography; sandblasted large-grits acid-etched (SLA) surface; macrophages; lipopolysaccharide (LPS); proinflammatory cytokines [interleukin (IL)-1 β , IL-6, tumor necrosis factor (TNF)- α] and chemokines [monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1 α]

INTRODUCTION

The insertion of an artificial implant into the human body evokes a sequence of healing processes and host

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responses including a range of vascular, humoral, and cellular responses, known collectively as inflammation.^{1,2} The use of biocompatible materials in implants generally results in a progression of the inflammatory response from a high intensity, acute phase to a low-activity, quasi-equilibrium state, called the foreign body reaction.³ The macrophage is central to direct host inflammatory and immune processes; thus, its response to biomaterials is extremely important in understanding material-mediated host response.⁴ Furthermore, the macrophage has a pivotal role in modulating the repair process, mediating phagocytosis,

and producing a myriad of cytokines for control of wound healing and cell recruitment as well as proliferation.⁵⁻⁷

The success of dental implants is dependent on the material properties of the implant material including mechanical properties, surface chemistry, and surface topography.⁸ The main goal of implanting any material is to obtain an appropriate host tissue response for the particular application.⁹⁻¹¹ Surface topography is known as one of the major determinants of implant performance *in vivo* and influences cell behavior in myriad ways including cell adhesion, cell selection, mechanical interlocking, cell orientation and topographic (contact) guidance, tissue organization, cell shape, production of local microenvironments,^{8,12} and production of growth factors and cytokines.¹¹ The response of surface topography (roughness) to cells including fibroblasts, epithelium, and osteoblasts has been extensively studied.¹¹⁻¹⁵ For example, many studies have investigated the effect of surface topography on the ability of cells to attach, proliferate, differentiate, and secrete extracellular matrix and proteins [i.e., transforming growth factor (TGF)- β 1 and bone morphogenetic protein-2].^{11,16,17} Moreover, increasing the surface roughness of titanium (Ti) implants generally results in increased resistance to compressive, tensile, and shear forces as well as increase in bone formation at the implant surface.¹⁸⁻²¹

Several dental implant systems with rough surfaces are available for clinical use; one example is the SLA (sandblasted-large grits-acid etched) Ti surface developed by ITI (International Team for Implantology).²² The SLA surface is created by first sandblasting with large-grit particles of Al_2O_3 , followed by acid etching with $\text{HCl}/\text{H}_2\text{SO}_4$ at elevated temperatures for 5 min.²³ SLA has compared favorably in histometric and biomechanical testing with some other implant surfaces such as TPS (Ti plasma-sprayed).²⁴ SLA has been successfully used clinically²⁵ and has produced higher removal torque values than the machined and acid-etched surfaces by 75–125% at each healing period.²⁴

The potential role of macrophages in the performance and longevity of an implanted material is not well understood. The macrophage is one of the first cells to arrive at the tissue–implant interface.^{26,27} Macrophage interaction with implanted materials is thought to involve adhesion, activation, and secretion of cytokines at the implant site.^{28,29} Typically, macrophages prefer rough surfaces to smooth ones (a phenomenon called rugophilia)^{30,31} and elongate significantly on grooved substrata.³²

It is acknowledged that macrophages are a major source of cytokines, chemokines, and growth factors.³³⁻³⁵ Cytokines and chemokines are involved in both the destructive and reparative stages of early wound healing after insertion of the implant. Proinflammatory cytokines, such as interleukin (IL)-1 β ,

IL-6, and tumor necrosis factor (TNF)- α and chemokines such as monocyte chemoattractant protein (MCP)-1 and macrophage inflammatory protein (MIP)-1 α are widely expressed at the early stages of implantation. It would appear likely their presence would direct host responses such as cellular recruitment and possibly bone formation. In this study, the topography of Ti surfaces, in particular the SLA surface, was found to effect macrophage activation and increase production of the proinflammatory cytokines and chemokines in the presence of lipopolysaccharide (LPS).

MATERIALS AND METHODS

Preparation of replica surfaces

Ti disks (15 mm in diameter and 1-mm thickness) of four surface topographies: mechanically polished (PO), coarsely blasted (CB), acid etched (AE), and SLA were provided by Institute Straumann (Waldenburg, Switzerland). Impressions of the four surfaces were made with vinyl polysiloxane impression material (PROVIL Light; Dormagen, HK, Germany). Vinyl polysiloxane negative replicas were used to cast epoxy-resin (EPO-TEK 302-3; Epoxy Technology, Bellerica, MA) positive replicas of these surfaces, then left in a fume hood for 24 h for initial polymerization. For polymerization completion, positive replicas were baked at 58°C for 3 days. The epoxy replicas were then cleaned by ultrasonication in a detergent (7X) (ICN Biomedicals, Inc., Costa Mesa, CA) and sputter-coated (Randex 3140 Sputtering System, Palo Alto, CA) with 50 nm of Ti. Finally, replicas were treated for 3 min in an argon-gas glow-discharge chamber and placed into 24-well culture plates (Falcon; Becton Dickinson Labware, Franklin Lakes, NJ).

Surface characterization

Briefly, X-ray photoelectron spectroscopy was used to determine the chemical composition of the oxide layer of the Ti-coated epoxy-resin replica surfaces. Surface topographies of replicas prepared in this manner have been characterized using a noncontact laser profilometer (LPM) (UBM; UBM Messtechnik GmbH, Ettlingen, Germany) equipped with a microfocus sensor based on an autofocus system, as well as a scanning electron microscope (Cambridge Stereoscan 260, Cambridge, UK) and the stereo-scanning electron microscopy (SEM) application. LPM and stereo-SEM were used for the quantitative topographic comparisons of the original surfaces and their replicas and the replicas have been found to faithfully model the original surfaces. For examples, values calculated from stereo-SEM showed that the integral roughness parameters (i.e., R_a , R_q , R_t) of the SLA original surface were 2.69 μm (R_a), 3.21 μm (R_q), and 13.76 μm (R_t); for the SLA replicas, the integral roughness parameters were 2.59 μm (R_a), 2.93 μm (R_q), and 12.03 μm (R_t),

respectively. Values calculated from noncontact LPM also demonstrated close similarity between the SLA original surface and its replica. Roughness parameters were $4.39 \mu\text{m}$ (R_a), $5.56 \mu\text{m}$ (R_q), and $35.46 \mu\text{m}$ (R_t) for the SLA original surface and the roughness parameters were $4.33 \mu\text{m}$ (R_a), $5.46 \mu\text{m}$ (R_q), and $33.39 \mu\text{m}$ (R_t) for the SLA replica. The roughness parameters were almost identical for the AE original surface and its replica using noncontact LPM.¹⁵

Cell culture

Murine macrophage-like cells RAW 264.7 cell line (ATCC, Manassas, VA) were cultured on tissue culture plastic (TCP) (75 cm² flask) (Falcon; Becton Dickinson Labware) in RPMI 1640 medium (Stemcell, Vancouver, BC, Canada) supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, Inc., Rockville, MD), antibiotics, and were incubated at 37°C, 5% CO₂ in a humidified atmosphere 95% air. The antibiotic mixture contained 0.5 mg penicillin G (Sigma, St. Louis, MO), 5 mL gentamicin sulfate (Sigma), and 60 mL of amphotericin B (Fungizone) (Gibco, Grand Island, NY). Cells were routinely passaged by scraping with a split ratio of 1:5 for assays. RAW cells were then plated onto the Ti-coated epoxy-resin replica surfaces at a population density of 5×10^5 cells/mL/well. TCP wells and TCP wells plus LPS (*Escherichia coli*; Sigma) served as negative and positive controls, respectively. Supernatants were harvested for assays after 6, 24, and 48 h and stored at -80°C .

LPS-stimulated and unstimulated macrophages

In the first part of the study, all experimental wells were seeded with unstimulated macrophages except some TCP wells containing LPS that constituted the positive controls. In the second part of the study, all the wells except the negative controls (TCP) were seeded with macrophages stimulated with a suboptimal dose of LPS (6.4 ng/mL). The purpose of using this low dose of LPS was to determine whether surface topography acted synergistically with LPS. An additional reason for testing the effect of surface topography in the presence of LPS is that it is likely that all implanted materials would be exposed to LPS *in vivo*, because there is evidence that LPS are present to some degree even under aseptic conditions. LPS are thought to derive from the systemic circulation after transient bacteremia due to normal activities such as eating and tooth brushing. It is, however, very difficult to identify the sources of LPS because it is so prevalent and surfaces can be easily contaminated.³⁶

Quantification of macrophages using 4,6-diamidino-2-phenylindole (DAPI) assay

After harvesting supernatants, the macrophage monolayer attached to the surfaces was covered with 2 mL of a Tris buffer and sonicated using an ultrasonic processor with

microtip attachment (Branson, Sonifier 250; VWR Scientific, Danbury, CT). Macrophage disruption was checked using phase-contrast microscopy. DNA content was assessed by adding DAPI (Sigma) to each well to a final concentration of 0.5 $\mu\text{g}/\text{mL}$ and the content of the wells transferred to a cuvette and read in a fluorescence spectrofluorometer at 365 nm excitation and 460 nm emission. The concentration of DNA was calculated from a standard curve obtained using known concentrations of salmon DNA.³⁷ Finally, all the results obtained from the assays were standardized accordingly (ng/ μg DNA/mL).

Cytokine and chemokine determinations

Cytokine (TNF- α , IL-1 β , and IL-6) and chemokine (MCP-1 and MIP-1 α) levels were measured with sandwich enzyme-linked immunosorbent assays (ELISAs) as prescribed by the manufacturer (QuantikineTM; R&D Systems, Minneapolis, MN). Briefly, 50 μL of standard, control, or sample were added per well. The tested samples were incubated for 2 h at room temperature. After washing unbound substances, 100 μL of an enzyme-linked polyclonal antibody were added per well and then incubated for 2 h at room temperature. After a wash to remove any unbound antibody-enzyme reagent, 100 μL of substrate solution was added to each well and then incubated and protected from light for 30 min. The optical density of each well was then determined using a microplate reader (Titertek Multiskan; Flow Laboratories, Mississauga, ON) set to 450 nm and corrected at 570 nm. The sample values were determined by comparison with a standard curve. All samples were assayed in triplicate.

Statistics

Data were analyzed using the software program SPSS (version 10 for Windows XP; SPSS Inc., Chicago, IL). Statistical comparison between the surfaces and the controls was determined by using the two-way analysis of variance (ANOVA) including interaction effect (two tailed and $p \leq 0.05$) and *post hoc* Tukey multiple comparison testing.

RESULTS

Scanning electron micrographs of unstimulated macrophages cultured on the Ti surfaces used in this study (PO, CB, AE, and SLA) are presented in Figure 1. Macrophages attached and proliferated more on the SLA surface compared with other Ti surfaces.

Cytokine release

The surface topography on which macrophages were cultured influenced the release of cytokines from unstimulated and stimulated macrophages.

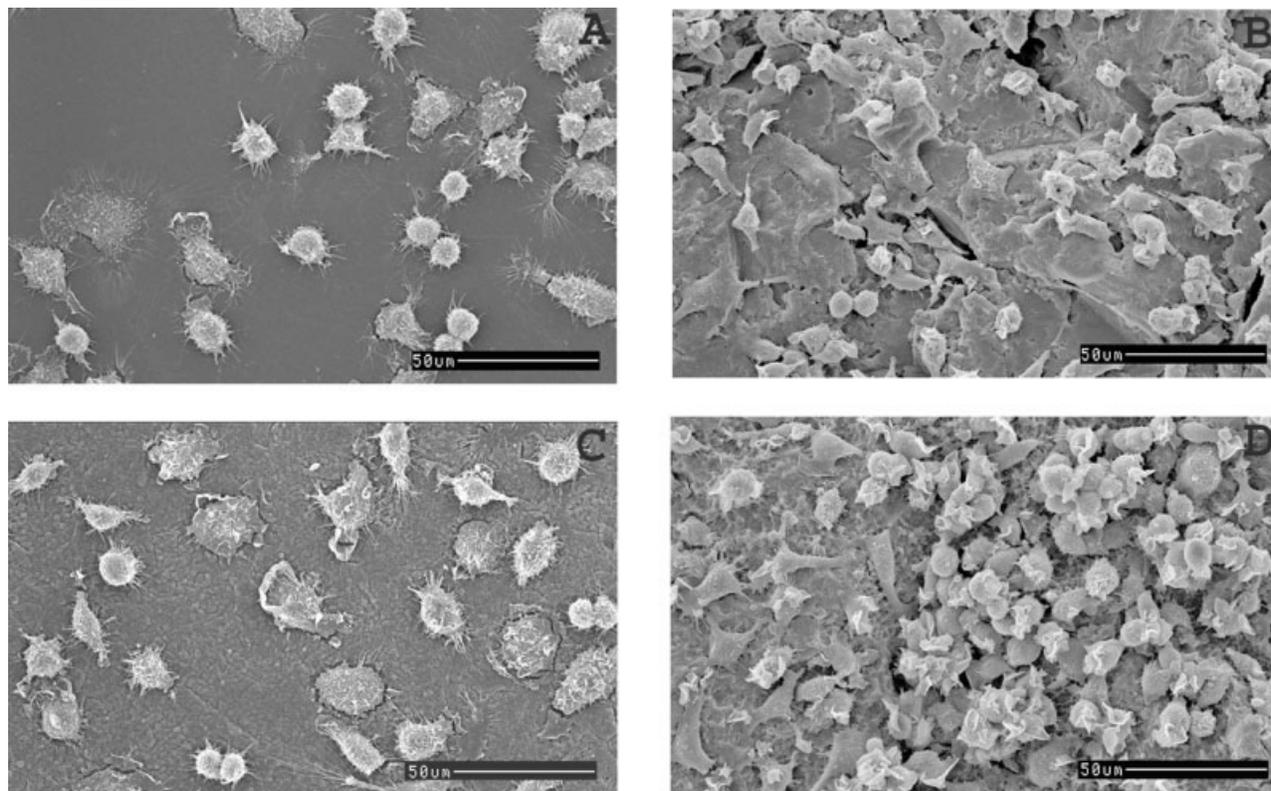


Figure 1. Scanning electron micrographs of unstimulated macrophages cultured on the Ti surfaces used in this study. Macrophages cultured on Ti surfaces for 48 h. Bar 50 μm ; original magnification, 500 \times . (A) Mechanically polished surface, (B) coarsely blasted surface, (C) acid-etched surface, and (D) SLA surface.

TNF- α

Unstimulated macrophages [Fig. 2(a)]

At 6 h, TNF- α secreted by unstimulated macrophages cultured on the AE surface increased significantly compared with TCP, PO, CB, and SLA Ti surfaces ($p \leq 0.05$). At 24 h, TNF- α secreted by the macrophages cultured on SLA and AE surfaces increased significantly compared with all other Ti surfaces: the response to AE was greater than that to SLA ($p \leq 0.05$). At 48 h, TNF- α secreted by the macrophages cultured on the AE surface increased significantly compared with all other Ti surfaces ($p \leq 0.05$).

Stimulated macrophages [Fig. 2(b)]

After 6 h of incubation, there was no difference in TNF- α secretion when LPS-stimulated macrophages were cultured on all Ti surfaces, the levels being significantly increased only when compared with TCP [Fig. 2(a), $p \leq 0.05$]. After 24 h and 48 h, the macrophages cultured on SLA surfaces secreted 9- and 14-fold more TNF- α , respectively, when compared with unstimulated macrophages cultured on TCP [Fig.

2(a)]. This higher production of TNF- α by these macrophages was also statistically significant when compared with all other surfaces including the positive control (TCP + LPS) ($p \leq 0.05$). TNF- α levels did not differ significantly among other Ti surfaces including PO, CB, and AE.

IL-1 β

Unstimulated macrophages

IL-1 β was undetectable in unstimulated macrophage cultures on all surfaces at all times tested.

Stimulated macrophages (Fig. 3)

Treatment of macrophages with LPS resulted in significant IL-1 β production. For example, the IL-1 β level increased approximately 6-fold at 24 h and 16-fold at 48 h by stimulated macrophages cultured on SLA surfaces compared with unstimulated macrophages cultured on TCP (data not shown). At 6 h, IL-1 β production by the macrophages cultured on the

PO surface increased significantly compared with SLA surfaces. At 6 h also, IL-1 β production by the macrophages cultured on the positive control (TCP plus LPS) increased significantly compared with all other surfaces ($p \leq 0.05$). At 24 h, IL-1 β secretion by the macrophages cultured on the SLA surface increased significantly compared with PO and AE surfaces ($p \leq$

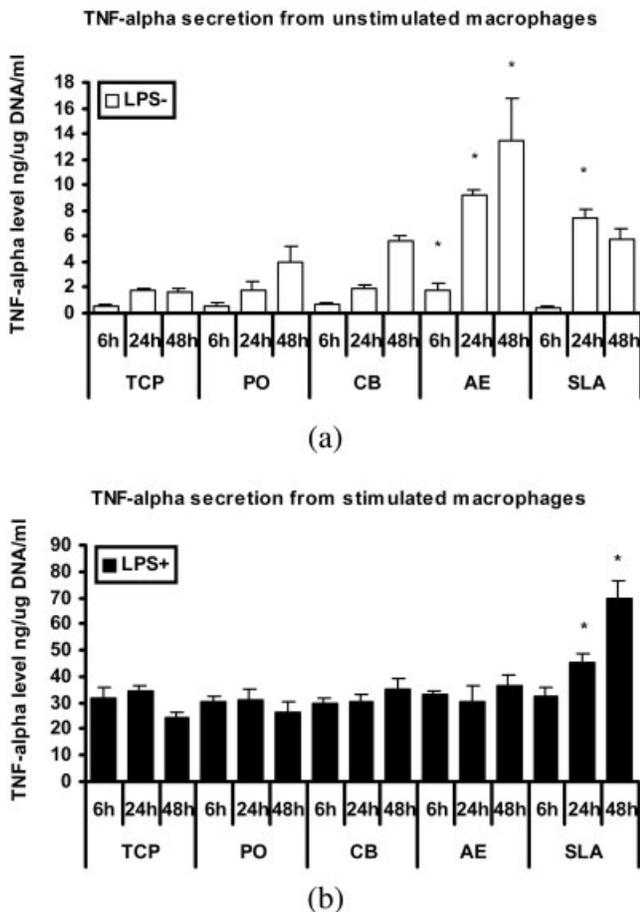


Figure 2. (a) TNF- α production by unstimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. The TNF- α content was measured by ELISA and quantified by DAPI. Data are presented as means \pm standard error of the mean (SEM) and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP at each time tested. At 24 h, TNF- α secreted by the macrophages cultured on SLA and AE surfaces increased significantly compared with all other Ti surfaces ($p \leq 0.05$). (b) TNF- α production by LPS-stimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. Macrophages were stimulated with LPS (6.4 ng/mL). The TNF- α content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP plus LPS at each time tested. The macrophages cultured on SLA surfaces secreted significant amounts of TNF- α compared with all other tested surfaces at 24 and 48 h ($p \leq 0.05$).

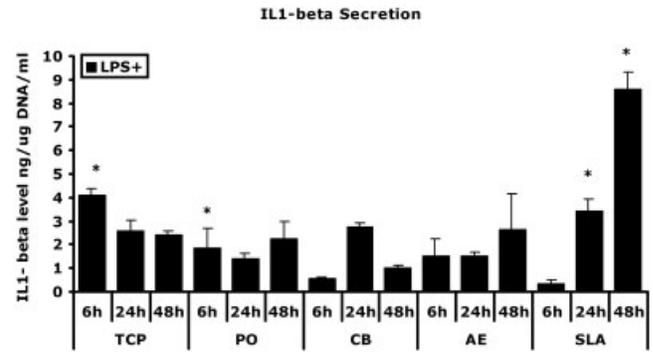


Figure 3. IL-1 β production by LPS-stimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. Macrophages were stimulated with LPS (6.4 ng/mL). The IL-1 β content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP plus LPS at each time tested. Treatment of macrophages with LPS resulted in significant IL-1 β production. IL-1 β was not detected in unstimulated cultures.

0.05). At 48 h, the IL-1 β level produced by the macrophages increased on the SLA surface compared with all other surfaces including positive control (TCP plus LPS) ($p \leq 0.05$).

IL-6

Unstimulated macrophages

Similar to IL-1 β , IL-6 also was undetectable in unstimulated macrophage cultures on all surfaces at all times tested.

Stimulated macrophages (Fig. 4)

Similar to IL-1 β , IL-6 production was significantly greater in the presence of LPS. For example, the IL-6 level increased approximately 7-fold at 24 h and 13-fold at 48 h by stimulated macrophages cultured onto SLA surfaces compared with unstimulated macrophages cultured onto TCP (data not shown). At 6 h, IL-6 production by the macrophages cultured on SLA surfaces increased significantly compared with TCP ($p \leq 0.05$). At 24 h, the IL-6 level produced by the macrophages cultured on the SLA surface was greater than that observed on TCP, TCP plus LPS, PO, and AE surfaces ($p \leq 0.05$). At 48 h, the IL-6 level produced by the macrophages was greater on the SLA and AE surfaces and differed significantly compared with all other surfaces including the positive control (TCP plus LPS) ($p \leq 0.05$).

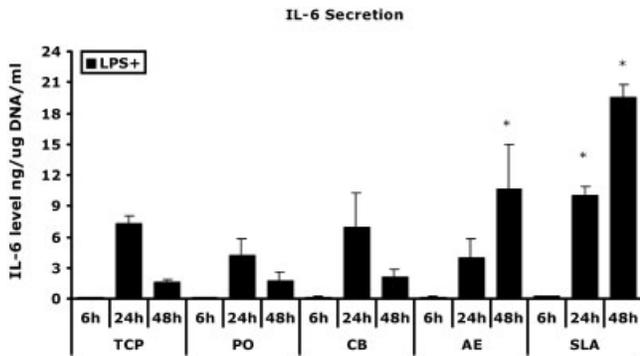


Figure 4. IL-6 production by LPS-stimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. Macrophages were stimulated with LPS (6.4 ng/mL). The IL-6 content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP plus LPS at all times tested. IL-6 production was not detected in unstimulated macrophages.

Chemokine release

The surface topography on which macrophages were cultured also influenced the release of chemokines from unstimulated and stimulated macrophages.

MCP-1

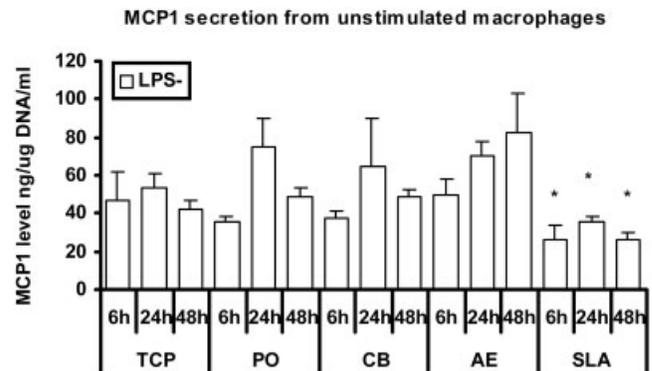
Unstimulated macrophages [Fig. 5(a)]

Generally, the SLA surface was found to down-regulate MCP-1 secretion in unstimulated macrophages at 6, 24, and 48 h, respectively, in comparison with other surfaces. This decrease in secretion of MCP-1 was statistically significant when compared with the AE surface at 6 h, the PO surface at 24 h, and the AE surface at 48 h ($p \leq 0.05$).

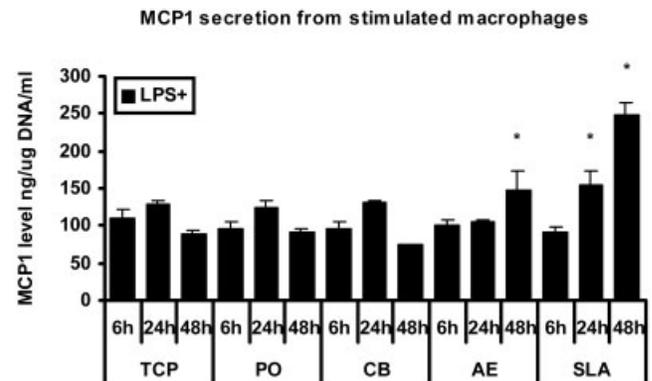
Stimulated macrophages [Fig. 5(b)]

LPS treatment resulted in an increased secretion of MCP-1 approximately 4-, 8-, and 14-fold by macrophages cultured onto the SLA surface compared with unstimulated macrophages cultured onto the SLA surface at 6, 24, and 48 h, respectively [Fig. 5(a)]. At 6 h, MCP-1 production by the macrophages cultured on all Ti surfaces including positive control (TCP plus LPS) was significantly greater than that observed on TCP [Fig. 5(a), $p \leq 0.05$]. At 24 h, the MCP-1 level produced by the macrophages cultured on the SLA surface increased significantly compared with all other surfaces

except CB and TCP plus LPS ($p \leq 0.05$). At 48 h, the MCP-1 produced by the macrophages cultured on SLA and AE surfaces was significantly greater than that observed in cultures cultured on all other surfaces including TCP plus LPS ($p \leq 0.05$).



(a)



(b)

Figure 5. (a) MCP-1 production by unstimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. The MCP-1 content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP at each time tested. The SLA surface down-regulated MCP-1 production in unstimulated macrophages at 6, 24, and 48 h, respectively, in comparison with other surfaces ($p \leq 0.05$). (b) MCP-1 production by LPS-stimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. Macrophages were stimulated with LPS (6.4 ng/mL). The MCP-1 content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP plus LPS at each time tested. LPS treatment resulted in an increased secretion of MCP-1 approximately 4-, 8-, and 14-fold by macrophages cultured onto the SLA surface compared with unstimulated macrophages cultured onto the SLA surface at 6, 24, and 48 h, respectively [$p \leq 0.05$, (a)].

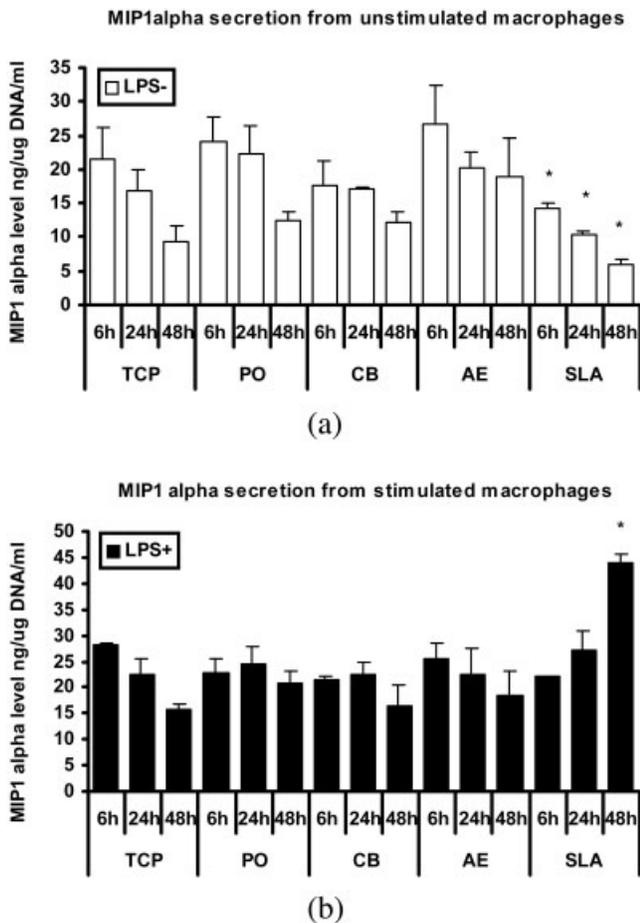


Figure 6. (a) MIP-1 α production by unstimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. The MIP-1 α content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP at each time tested. Similar to MCP-1, the SLA surface down-regulated MIP-1 α secretion in unstimulated macrophages at 6, 24, and 48 h, respectively, when compared with other surfaces ($p \leq 0.05$). (b) MIP-1 α production by LPS-stimulated macrophages cultured on TCP and Ti surfaces of varying roughness after 6, 24, and 48 h. Macrophages were stimulated with LPS (6.4 ng/mL). The MIP-1 α content was measured by ELISA and quantified by DAPI. Data are presented as means \pm SEM and were performed in triplicate. Two-way ANOVA and Tukey *post hoc* were performed to test significance ($p \leq 0.05$). * $p \leq 0.05$ compared all Ti surfaces and TCP plus LPS at each time tested. MIP-1 α production up-regulated nearly 1.5-, 3-, and 8-fold by macrophages cultured onto the SLA surface compared with unstimulated macrophages cultured onto the SLA surface at 6, 24, and 48 h, respectively [$p \leq 0.05$, (a)].

MIP-1 α

Unstimulated macrophages [Fig. 6(a)]

Similar to MCP-1, the SLA surface was also found to down-regulate MIP-1 α secretion in unstimulated mac-

rophages at 6, 24, and 48 h, respectively, when compared with other surfaces. The reduction of MIP-1 α secretion by the macrophages cultured onto the SLA surface was statistically significant with the AE surface at 6 h, all other Ti surfaces at 24 h, and AE surface at 48 h ($p \leq 0.05$).

Stimulated macrophages [Fig. 6(b)]

In contrast, MIP-1 α production up-regulated nearly 1.5-, 3-, and 8-fold by macrophages cultured onto the SLA surface compared with unstimulated macrophages cultured onto the SLA surface at 6, 24, and 48 h, respectively [Fig. 6(a)]. At 6 and 24 h, MIP-1 α secretion did not differ significantly among cultures on all the Ti surfaces and the positive control (TCP plus LPS). At 48 h, the MIP-1 α level increased significantly on SLA surfaces compared with all other surfaces and the positive control (TCP plus LPS) ($p \leq 0.05$).

Synergism: Surface topography and LPS

Surface topography was found to act synergistically with the LPS. This synergistic effect was time dependent and was statistically significant at 24 and 48 h. Generally, when macrophages were stimulated with LPS, all the Ti surfaces increased their production of the proinflammatory cytokines and chemokines. Interestingly, the stimulated macrophages cultured on SLA surfaces secreted significantly more of the proinflammatory cytokines and chemokines compared with all other Ti surfaces and the positive controls (TCP + LPS) at 48 h (Fig. 7).

Interaction between blasted and AE components of the SLA surface

The data obtained from our study were analyzed using the two-way ANOVA, which allows testing for interaction between the components of the surfaces on cytokine and chemokine secretion. In brief; the surfaces used in the study can be considered as two factors, acid etching at two levels (present and absent) and grit blasting also at two levels (present and absent). Thus, for example, the SLA surface has both the acid-etching and grit-blasting produced features present whereas the PO surface has neither. The combination of blasting and etching features found on the SLA surface resulted in stimulated macrophages releasing greater amounts of proinflammatory cytokines and chemokines than that predicted by summing the amounts produced by blasting and acid etching. This

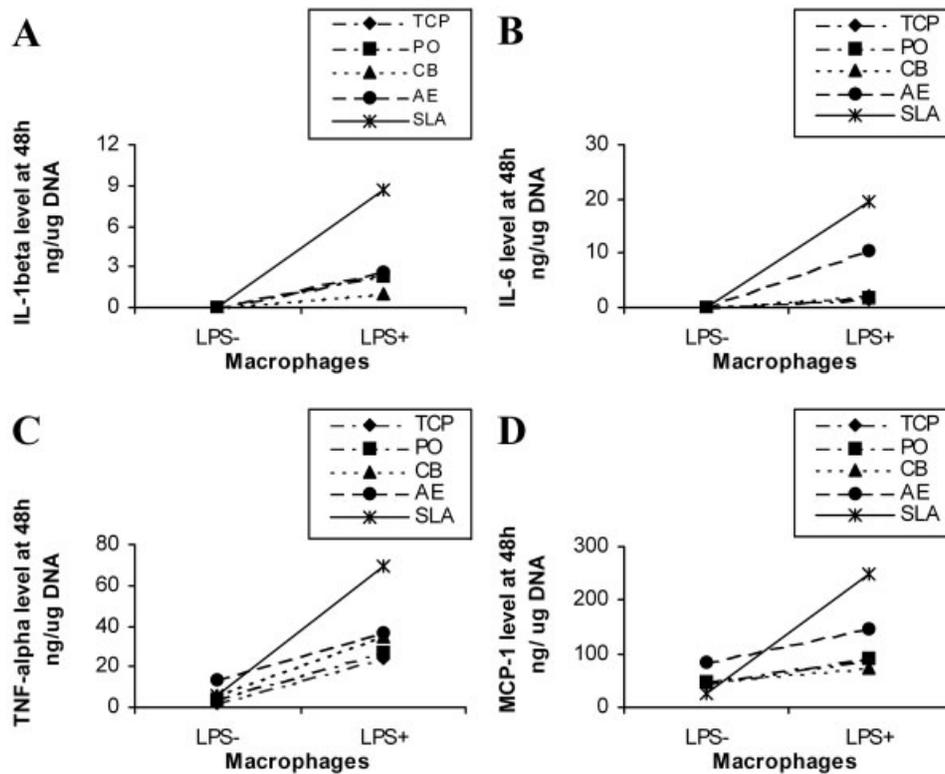


Figure 7. Interaction between topography and LPS stimulation at 48 h. Cytokine and chemokine profiles of macrophages (stimulated vs unstimulated) cultured on all tested surfaces. After 48 h, stimulated macrophages cultured on the SLA surface released significantly more cytokines and chemokines than TCP positive controls ($p \leq 0.05$). (A) IL-1 β , (B) IL-6, (C) TNF- α , and (D) MCP-1.

interaction was statistically significant for TNF- α and MCP-1 at 24 h and on all cytokines and chemokines tested at 48 h ($p \leq 0.05$, Fig. 8).

DISCUSSION

The purpose of this study was to investigate the effect of the topography of Ti surfaces on macrophage activation and their secretory profile. Unstimulated macrophages increased their secretion of the proinflammatory cytokine (TNF- α) when attached to rough surfaces (AE and SLA). In macrophages stimulated with LPS, the roughest surface SLA produced higher levels of IL-1 β , IL-6, and TNF- α at 24 and 48 h than all other surfaces. Surface topography also modulated the secretion of the chemokines MCP-1 and MIP-1 α by macrophages. Unstimulated macrophages attached to the SLA surface down-regulated their production whereas LPS-stimulated macrophages attached to the SLA surface up-regulated their production.

The early stages after implant insertion are important because the early events form the foundation of wound healing around the implant.³⁸ For example, the placement of endosseous implants leads to fibrin clot formation, which serves as a scaffold for red blood

cells, platelets, and peripheral monocytes that eventually become macrophages.³⁹ Park and Davies⁴⁰ and Davies⁴¹ have suggested that implant surface topography increases physical retention of the fibrin clot on the implant surface and could activate the platelets within the fibrin clot. Moreover, Cooper³⁹ proposed that cells resident in the fibrin clot might contribute to inductive and chemotactic signals that support osseointegration. It has been postulated that activation of the macrophages in the context of the wound microenvironment results in enhanced release of chemokines (MCP-1 and MIP-1 α) and cytokines (IL-1 β , IL-6, and TNF- α), that can act as paracrine, autocrine, and potentially, endocrine mediators of host response.⁴²

The present study demonstrates that the SLA surface modulated expression of proinflammatory cytokines and chemokines by stimulated macrophages. The secretion of these factors was time and topography dependent. Stimulated macrophages on SLA surfaces in particular, produced significant levels of both cytokines and chemokines when compared with positive controls (TCP plus LPS). Our work confirms in part the previous work in which the increase in TNF- α secretion from LPS-stimulated macrophages was associated with surface roughness¹⁰ and has extended the finding to several other cytokines and chemokines

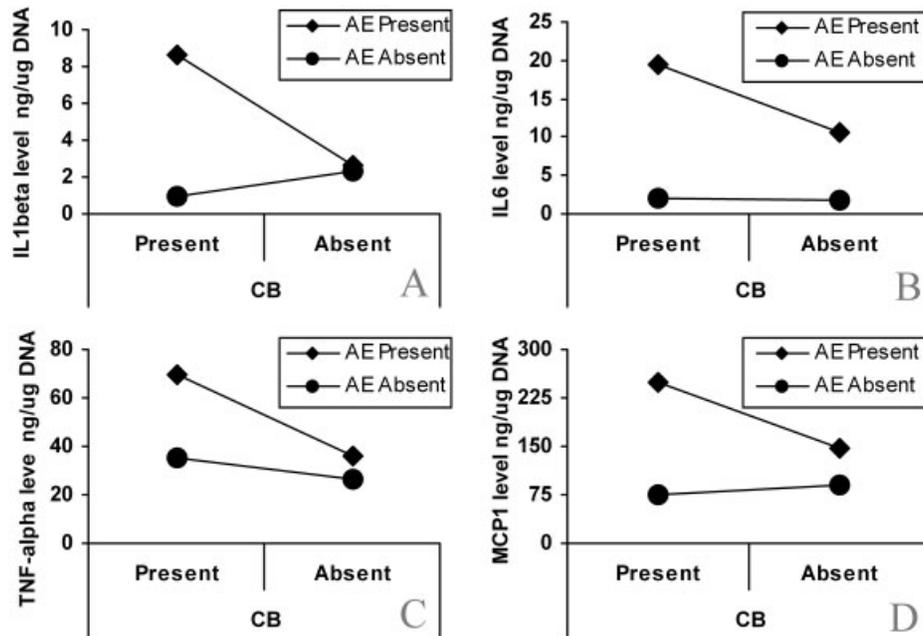


Figure 8. Interaction effects between the features produced by blasting and etching on cytokine and chemokine secretion by stimulated macrophages at 48 h. The presence of both CB and AE features is characteristic of the SLA surface whereas the mechanically polished surface (PO) has neither. Two-way ANOVA was used to test for significance and the interaction effects between the two components of the SLA surface. The combination of both CB and AE resulted in significant production of all cytokines and chemokines tested in this study at 48 h ($p \leq 0.05$). (A) IL-1 β , (B) IL-6, (C) TNF- α , and (D) MCP-1.

(IL-1 β , IL-6, MCP-1, and MIP-1 α). Of particular interest is TNF- α , which has been implicated indirectly in angiogenesis during wound healing,⁴² stimulation of osteoclastic activity,⁴³ and regulation of osteoblast proliferation⁴⁴; and is thus of potential importance in implant osseointegration.

Our study also found that the IL-1 β level increased by LPS-stimulated macrophages cultured on SLA surfaces. This cytokine has been shown to participate in neovascularization (angiogenesis) during wound healing⁴² and it stimulates fibroblast proliferation and granulocyte replication.^{45,46} Both TNF- α and IL-1 β are key mediators of the inflammatory process and they contribute to the reparative phase either directly by influencing endothelial and fibroblast functions or indirectly, by inducing additional cytokines and growth factors.⁴² The significance of increased IL-6 levels in the culture media from LPS-stimulated macrophages may relate to their role in wound healing and bone remodeling. IL-6 has been shown to be crucial to epithelialization and influences granulation tissue formation during wound healing.⁴⁷ IL-6 also induces osteoclast formation and consequently is involved in both bone resorption and bone formation in the remodeling stage.^{48,49}

Interestingly, MCP-1 and MIP-1 α were secreted by both stimulated and unstimulated macrophages cultured on all the Ti surfaces. Both MCP-1 and MIP-1 α have monocyte chemotactic activity and have key roles in immunoregulatory and inflammatory processes.⁵⁰

MCP-1 and MIP-1 α also have a critical role in macrophage recruitment into wounds, and appropriate tissue repair is dependent on this recruitment.^{50,51} It has been found that MCP-1 knockout mice exhibit a remarkable delay in wound healing.⁵²

There are at least four possible mechanisms whereby surface topography may affect macrophage activation and secretion. First, the surface roughness itself has been found to affect the spreading, proliferation, and differentiation of cells *in vitro*. For example, Martin et al.,¹⁷ found that surface roughness decreased osteoblast proliferation, increased differentiation, and increased protein synthesis and matrix production. Surface roughness seemed to affect not only protein synthesis but also gene expression. Furthermore, surface roughness also alters osteoblast thickness and morphology, but not cell volume.¹⁵ This alteration on cell shape due to surface roughness might also affect gene expression and growth factor secretion. Second, the increase of surface area characteristic of a rough surface has been found to affect cytokine production. For example, macrophages secreted more IL-1 when exposed to Ti particles at higher surface area ratios.⁵³ Third, the interaction between a Ti surface and conditioned medium results in a thin layer of adsorbed macromolecules that modify cell behavior. It has been speculated that the rough surface may have absorbed more fibronectin than other surfaces⁵⁴ and, consequently, increased macrophage attachment through their RGD-integrin recep-

tor domains. This organization on Ti surfaces of different roughness between macrophages and extracellular proteins might also affect the macrophages' secretory profile.

The fourth possible mechanism is quorum sensing—a phenomenon whereby gene expression responds to population density.⁵⁵ This mechanism might explain the effect of SLA surfaces on MCP-1 and MIP-1 α secretion where these chemokines were down-regulated on SLA surfaces but not stimulated relative to control values when LPS was present. It was evident that unstimulated macrophages proliferated more on SLA surfaces compared with stimulated macrophages (Fig. 1). We speculate that under the condition of our high cell population density in the pits of the SLA surfaces, the unstimulated macrophages sense that additional macrophages are not required and decrease chemokine production. In contrast, when macrophages were stimulated with LPS and cell number decreased, MCP-1 and MIP-1 α secretion were elevated to recruit more macrophages to the surfaces.

To date, three pathways have been identified in macrophage activation with three distinct biological functions. The first is the classically activated macrophage, which involves the Th1 cellular immune responses. The second is the alternative activated macrophage pathway that has been implicated in immunosuppression and tissue repair. The third pathway is type 2-activated macrophage, which inhibits inflammation and induces Th2-type humoral immune responses.⁵⁶ These three types of macrophage activation may form their own regulatory network to prevent a well-intentioned immune response from progressing to immunopathology.⁵⁶ Although in the past, the presence of macrophages around implants has been considered to be a threat to osseointegration,^{30,31} more recent studies have indicated that macrophages might instead have a beneficial effect.^{9,16,57} For example, Takebe et al.¹⁶ showed that Ti surface topography modulates osteoinductive (bone morphogenetic protein-2) and osteogenic cytokine (TGF- β 1) expression in a macrophage cell line.

The effect of material surfaces on macrophage activation and cytokine secretion has been predominately studied *in vitro*. It has been found that macrophages secreted IL-1, IL-6, IL-10, and TNF- α after stimulation by different material surfaces.² The secretion of such cytokines was generally low without exogenous stimulus. For example, addition of LPS showed a marked increase of proinflammatory cytokines by macrophages attached to Ti alloy particles and Ti discs. Gretzer et al.⁵⁸ reported that low levels of TNF- α were detected when monocytes were unstimulated whereas TNF- α levels were increased about fourfold by LPS stimulation of cells on Ti discs. It has also been found that unstimulated macrophages did not induce IL-1 β

and TNF- α production when macrophages were challenged with Ti alloy particles whereas IL-1 β and TNF- α production increased when the LPS was added to the medium.⁵⁹

Only a few *in vivo* studies in soft tissues have reported the expression of TNF- α , IL-1 β , and TGF- β around implant materials.^{60–62} Thomsen and Gretzer² in a thorough review of the macrophage and material surfaces interactions concluded that the precise modulatory effects of cytokines on the inflammatory and reparative processes around implants are not known. Two general problems that make clear interpretation difficult are redundancy and pleiotropism. Redundancy exists because most functions of cytokines can be performed by many different types of cytokines. For example, many different cytokines can act to stimulate cell division in activated T cells. Pleiotropism exists because a single cytokine has many different functional effects, not only on different cell types but even on the same cell type.⁶³ In view of this complexity, additional studies using model systems, such as the one described in this study, are still required.

CONCLUSIONS

This *in vitro* study demonstrates that surface topography, in particular the SLA surface, modulates production of cytokines and chemokines including TNF- α , IL-1 β , IL6, MCP-1, and MIP-1 α from a macrophage cell line in a time-dependent manner. It is possible that these effects on macrophages form part of the basis for the successful and rapid development of osseointegration in implants with SLA surfaces. The model system used in this study whereby surfaces produced by each of the processes (AE, CB) involved in SLA surface fabrication can be assessed singly and in combination is helpful in determining how surface topography exerts its complex effects. In particular, the SLA surface demonstrated synergism in its effects on cytokine and chemokine secretion by LPS-stimulated macrophages. Additional studies are needed to determine whether the responses produced by these Ti surfaces also occur *in vivo*.

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