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Titanium Surface Characteristics, Including Topography and Wettability, Alter Macrophage Activation

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Abstract

Biomaterial surface properties including chemistry, topography, and wettability regulate cell response. Previous studies have shown that increasing surface roughness of metallic orthopaedic and dental implants improved bone formation around the implant. Little is known about how implant surface properties can affect immune cells that generate a wound healing microenvironment. The aim of our study was to examine the effect of surface modifications on macrophage activation and cytokine production. Macrophages were cultured on seven surfaces: tissue culture polystyrene (TCPS) control; hydrophobic and hydrophilic smooth Ti (PT and oxygen-plasma-treated (plasma) PT); hydrophobic and hydrophilic microrough Ti (SLA and plasma SLA), and hydrophobic and hydrophilic nano-and micro-rough Ti (aged modSLA and modSLA). Smooth Ti induced inflammatory macrophage (M1-like) activation, as indicated by increased levels of interleukins IL-1 β , IL-6, and TNF α . In contrast, hydrophilic rough titanium induced macrophage activation similar to the anti-inflammatory M2-like state, increasing levels of interleukins IL-4 and IL-10. These results demonstrate that macrophages cultured on high surface wettability materials produce an anti-inflammatory microenvironment, and this property may be used to improve the healing response to biomaterials.

Graphical abstract

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Keywords

Macrophages; M1 activation; M2 activation; Surface roughness; Wettability; Immune response; Titanium

1. Introduction

The goal of many dental and orthopaedic implants is complete integration of the implant with host bone with limited adverse effects in the surrounding tissue [1]. After implantation into the body, the cascade of events initiated by immune cells after interacting with the material surface determines the fate of a biomaterial [1–3]. Many studies have focused on how material surface modifications can promote stem cell differentiation toward osteoblasts [4, 5]. However, before osteoblasts can arrive and begin forming bone, the inflammatory response must be resolved. Activation of the immune system controls the initial response to the implanted material and affects its long-term survival and integration. Immune cells release factors in response to their interaction with the biomaterial surface to condition the microenvironment surrounding the implant controlling the immune response. Continued immune system activation can lead to chronic inflammation that can result in the breakdown of healthy tissue surrounding the implant [1]. However, a lack of inflammatory response will leave the debris from implantation to remain and affect the integration of the material and generation of new tissue [6, 7].

Neutrophils, platelets, and macrophages migrate from blood vessels to the wound site. There, they produce growth factors, chemokines, and cytokines that recruit additional immune cells to the site, and in a normal wound healing response, induce phagocytosis of the damaged cells/tissue and stimulate the wound healing process [1]. Macrophages are responsible for the initial immune response, inflammation, and maintaining tissue homeostasis [3, 8, 9]. The ability of a material surface to control the reaction of these cells will influence the host's initial response to the device, and ultimately decide the integration of the material.

Two macrophage phenotypes have been established: the classical pro-inflammatory M1 and the alternative anti-inflammatory, wound healing M2 [4, 9–11]. Classical M1 polarization is generated by activation of the naïve macrophage by interferon gamma (INF γ) and lipopolysaccharide (LPS). The alternative M2 activation results from cell stimulation by interleukin (IL)-4 and IL-13 released from the cells of the adaptive immune system [9, 12]. Macrophage activation is characterized by the profile of cytokines and growth factors

released by the cells into their microenvironment. The M1 activation is a pro-inflammatory response responsible for rapid immune activation in the presence of microbiological or other acute threats [13], characterized by high levels of interleukin IL-1 β , IL-6, and TNF α [9, 10, 13]. M2 activation is a wound healing and tissue remodeling response marked by anti-inflammatory cytokines IL-10, IL-4, IL-13, and TGF- β [9, 11]. A balance between these macrophage activations is required for the proper healing of an injury and biomaterial integration [1, 14]. The aim of our study was to examine the effect of surface microstructure and wettability on macrophage activation, polarization, and cytokine production in response to Ti substrates.

2. Materials and Methods

2.1 Disk Preparation

Ti disks were provided by Institut Straumann AG (Basel, Switzerland), with SLA and modSLA corresponding to the commercially available SLA® and SLActive® respectively. Each disk was created by a 15 mm punch from 1 mm thick sheets of grade 2 unalloyed Ti. The disks were sized to fit securely in a 24 well plate. The sample disks were prepared as previously described [4]. Disks were cleaned and degreased by acetone bath, and then processed in a 2% ammonium fluoride/2% hydrofluoric acid/10% nitric acid solution at 55°C for 30 seconds to produce the pretreatment (PT) surfaces. SLA surfaces were created by coarse-grit blasting PT disks with 0.25–0.50 mm corundum followed by acid etching in a mixture of HCl and H₂SO₄ in order to create surface structures and roughness at the macro and micro scale. PT and SLA disks were rinsed in deionized water and dried after processing. modSLA disks were created using the same procedure as SLA but were rinsed under nitrogen protection to prevent air exposure and stored in an isotonic NaCl solution in sealed glass tubes until use. This process results in a hydrophilic surface with roughness at the micron-, submicron-, and nano-scale. Disks were sterilized by γ -irradiation.

A set of hydrophilic, PT and SLA disks were created by oxygen plasma cleaning (plasmaPT, plasmaSLA) as established in prior experiments [15, 16]. Disks were treated in an oxygen plasma cleaner (PDC-32G, Harrick Plasma, NY) at medium radio frequency for 2 minutes per side. The hydrophobic aged modSLA surface was created by sonicating modSLA surfaces in ultrapure water for 10 minutes two times to remove the residual saline solution and aged by exposure to air for two weeks under sterile conditions.

2.2 Surface Characterization

The roughness generated under each surface condition was determined by laser scanning confocal microscopy (LSCM, Zeiss LSM 710, Carl Zeiss). Measurements were taken with a scan size of 600 μm $\times$ 600 μm with a 20-x lens. Roughness values (average roughness over area: S_a , skewness: S_{sk} , kurtosis: S_{ku} , and developed interfacial area ratio: S_{dr}) were calculated with a 100 μm threshold. Measurements were taken at three different points on each disk.

Qualitative assessments of macro-, micro-, and nanostructure of each surface were acquired by scanning electron microscopy (SEM, Zeiss Auriga, Carl Zeiss, Jena, Germany) at 1k $\times$ and 100k $\times$ magnifications. Disks were analyzed without the addition of a conductive coating

with a secondary electron detector at 5kV, under vacuum and a distance of approximately 2mm.

The wettability of each surface type was indirectly measured by sessile drop contact angle (ramé-hart contact angle goniometer 250, model 100-25a, ramé-hart instrument co., Succasunna, NJ). Measurements using 1µL drops of deionized water were taken at three locations of six disks per surface condition. A contact angle of 0° considered hydrophilic and greater than 80° considered hydrophobic.

The chemical composition of the surface was determined by X-ray photoelectron spectroscopy (XPS, ESCALAB 250, Thermo Scientific, Waltham MA) under ultra-high vacuum (10^{-9} Torr or below) with a microfocused monochromatic AlK α x-ray source. The focus of the XPS was to assess differences in carbon levels; therefore, disks were secured to the mount with stainless steel clips in order to remove potential carbon readings from the adhesive tape. Prior to analysis clips and mount were sonicated in acetone. Survey scans were completed at each region, followed by high-resolution scans for C1s, Ti2p, O1s, Na1s, and Cl2p. Scans were aligned to the binding energy of the C1s peak at 284.8 eV. Thermo Advantage software was used to evaluate spectrum results. Each surface characterization procedure was performed on three regions of six disks per surface condition. modSLA surfaces were rinsed in ultrapure water prior to each surface characterization procedure.

2.3 Cell Culture

Primary murine macrophages were isolated from femurs of 6–8 week-old male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) under VCU IACUC approval using previously described methods [17]. Briefly, bone marrow cells were flushed from the femurs using Dulbecco's phosphate-buffered saline (Life Technologies, Carlsbad, CA). Red blood cells were lysed from the bone marrow extract with ACK Lysing Buffer (Quality Biological, Inc., Gaithersburg, MD). Cells were counted (TC20™ Automated Cell Counter, Bio-Rad Laboratories, Hercules, CA) and plated in a 75 cm² flask at a density of 500,000 cells/mL in 10mL RPMI 1640 media (Life Technologies) supplemented with 10% fetal bovine serum (Life Technologies), 50U/mL penicillin-50 µg/mL streptomycin (Life Technologies), and 30ng/mL macrophage colony-stimulating factor (M-CSF, PeproTech, Rocky Hill, NJ). Cells were cultured at 37°C, 5% CO₂, and 100% humidity. Fresh media supplemented with M-CSF was added after four days. Seven days after plating, macrophages were passaged and seeded onto Ti surfaces for experiments. modSLA surfaces were removed from saline solution and rinsed with ultrapure water prior to cell seeding.

2.4 Cell Staining

Differentiated macrophages were plated on surfaces at a density of 20,000 cells/cm² in RPMI with 10% fetal bovine serum (Life Technologies), 50U/mL penicillin-50 µg/mL streptomycin (Life Technologies) without M-CSF and cultured at 37°C, 100% humidity, and 5% CO₂ for 24 hours. Cells were fixed with a 4% paraformaldehyde solution overnight. Cell membranes were permeabilized in 0.1% Triton X-100 (Sigma-Aldrich). The cytoskeleton was stained using a 1:40 dilution of Alexa Fluor 488 conjugated phalloidin in PBS and nuclei stained using a 3:2000 dilution of Hoechst 34580 (Life Technologies). Cells were

imaged at three randomly selected regions on six independent disks using LSCM at 40-x magnification.

2.5 Protein Quantification

Differentiated, un-activated macrophages were plated on Ti surfaces in RPMI 1640 culture medium without M-CSF. Cells plated on tissue culture polystyrene (TCPS) served as a control of naïve macrophages. Conditioned media were harvested from cell cultures 24 and 72 hours after plating. Medium was changed 24 hours prior to harvest. Levels of IL-1 β , IL-4, IL-6, IL-10, and TNF α in the conditioned media were measured by enzyme-linked immunosorbent assays (ELISA, PeproTech) following the manufacturer's protocol. Immunoassay results are presented as normalized to dsDNA content in cell lysates. Cell monolayers were washed in PBS, lysed in 0.05% Triton X-100, and homogenized by sonication. DNA levels were quantified in cell lysates using the Quant-iT^{TM} PicoGreen dsDNA Assay Kit (Invitrogen, Life Technologies) as per manufacturer protocol.

2.6 Statistical Analysis

Surface characteristic experiments were conducted on a minimum of three regions of six separate disks per surface condition. Each cell study experiment was conducted with six independent cultures per surface. Experiments were performed at least twice to ensure consistent results; presented data are from one experiment. A one-factor, equal-variance analysis of variance (ANOVA) was used to test the null hypothesis that the group means are equal, against an alternative hypothesis that at least two of the group means are different, at the $\alpha=0.05$ significance level. Upon determination of a p -value less than 0.05 from the overall ANOVA model, multiple comparisons between the group means were made using the Tukey-HSD method. All statistical analysis was completed using JMP pro11 software.

3. Results

3.1 Surface Characterization

The surface roughness (Figure 1) was characterized using parameters of average roughness (S_a), skewness (S_{sk}), kurtosis (S_{ku}) and developed interfacial area ratios (S_{dr}). The application of wettability modifications did not change the surface roughness of the disks in any of the three parameters measured. The average roughness of PT was found to be $0.59 \mu\text{m} \pm 0.019 \mu\text{m}$ and the average roughness of plasma treated PT was found to be $0.59 \mu\text{m} \pm 0.023 \mu\text{m}$. Both were significantly less rough than microstructured surfaces. The roughness values of the four micro-rough surfaces were not significantly different (Figure 1A). The average roughness of SLA was $3.58 \mu\text{m} \pm 0.042 \mu\text{m}$ and plasmaSLA was $3.61 \mu\text{m} \pm 0.047 \mu\text{m}$, modSLA was $3.64 \mu\text{m} \pm 0.029 \mu\text{m}$ and aged modSLA was $3.49 \mu\text{m} \pm 0.029 \mu\text{m}$. Skewness values are a ratio between peaks and valleys present throughout the surface microstructure, which represents the symmetry between peaks and valleys. A negative value is indicative of more distinct valleys and positive is more distinct peaks about the average plane. Skewness measurements were -1.59 ± 0.114 on PT and -1.44 ± 0.053 on plasmaPT for smooth and -0.19 ± 0.048 SLA, -0.19 ± 0.024 plasmaSLA, -0.20 ± 0.014 aged modSLA, and -0.19 ± 0.042 on modSLA (Figure 1B). The kurtosis value assigns a number to the relative steepness of each peak. Kurtosis values were 6.52 ± 0.218 for PT, $5.99 \pm$

0.231 for plasmaPT and 3.30 ± 0.125 on SLA, 3.19 ± 0.067 plasmaSLA, 3.39 ± 0.088 aged modSLA, and 3.19 ± 0.086 modSLA indicating smaller but sharper peaks present on smooth surfaces in comparison to the rough (Figure 1C). The developed interfacial area ratios (Figure 1D) were similar between smooth PT and plasmaPT were similar ($41\% \pm 2.2\%$ and $39\% \pm 2.3\%$), between the two SLA and plasmaSLA ($53\% \pm 0.6\%$ and $51\% \pm 1\%$) and finally aged modSLA and modSLA ($62\% \pm 1.3\%$ and $60\% \pm 1.5\%$).

SEM images and the 3D topographical view from LSCM of PT (Figure 2A, 2G, 2M) and plasmaPT (Figure 2B, 2H, 2N) show smooth surfaces at the micron (2A, 2B) and submicron (2G, 2H) scale. At the micron scale SLA (Figure 2E), plasmaSLA (Figure 2G), aged modSLA (Figure 2I) and modSLA (Figure 2K) have similar topography. A similar microtopography is also evident in the 3D topographical reconstruction in Figure 2O–R. However, viewed under 100kx magnification, nanostructures are visible on the aged modSLA and modSLA surfaces (Figure 2K and L) that are lacking on the SLA and plasma treated SLA surfaces (Figure 2I and J).

The wettability was altered successfully by our chosen treatments (Figure 3). plasmaPT, plasmaSLA, and modSLA were hydrophilic with contact angles of 0° while PT (93.6°), SLA (120.9°), and aged modSLA (110.4°) surfaces were hydrophobic (Figure 3).

XPS analysis revealed more carbon was present on hydrophobic surfaces in comparison to hydrophilic surfaces (Figure 4). Additional trace elements of nitrogen were present on some disks but are not shown in the figure. More oxygen was present on the plasma-treated and modified surfaces compared to untreated surfaces. Similar levels of carbon and oxygen were measured on smooth and rough surfaces with similar surface wettability. Analyses of binding state analysis are listed in Table 1. Plasma treated PT and SLA surfaces had the highest percent of binding at 284.8 eV, which is representative of C-C or C-H binding. These surfaces only displayed this peak and did not show C-O (286 eV) or C=O (288 eV) binding. Untreated PT and SLA surfaces had similar levels of each form of carbon binding. Both modSLA and aged modSLA surfaces showed the highest amount of C=O binding compared to the other surfaces. No differences were measured between Ti2p measurements on each surface at the TiO₂ (peaks at 458.5 eV and 465 eV). Levels of Ti metal were not detected on the aged modSLA surface. The greatest portion of O1s binding was present in the TiO_x oxide layer of each of the surfaces.

3.2 Cell Staining

Macrophages were attached on each surface condition tested in this study. Cells appeared to attach and spread more on the smooth PT and plasmaPT surfaces in comparison to all microrough surfaces (SLA, plasma SLA, modSLA, and aged modSLA) (Figure 5). Macrophages appeared similar in number and morphology on smooth Ti surfaces and the glass substrate control. Cells on rough surfaces had a less elongated morphology. No multinucleated cells were detected under visual assessment of cells attached to the surfaces.

3.3 24-Hour Microenvironment

After 24 hours, greater levels of pro-inflammatory factors were present in comparison to anti-inflammatory factors. A reduced amount of IL-1 β was released by cells on all titanium

surfaces compared to TCPS (Figure 6B). IL-1 β was significantly lower on rough hydrophilic surfaces (plasmaSLA and modSLA) compared to rough hydrophobic SLA and aged modSLA, with no difference on smooth (PT and plasmaPT). IL-6 was reduced on Ti, with the exclusion of SLA, in comparison to the TCPS control (Figure 6C), and levels were similar between PT, plasmaPT, plasmaSLA, and modSLA surfaces. Hydrophobic microrough surfaces (SLA and aged modSLA) produced increased IL-6 levels. TNF α was reduced on smooth surfaces (PT and plasmaPT) and hydrophilic microrough surfaces (plasmaSLA and modSLA) compared to TCPS (Figure 6D), and greater on hydrophobic rough surfaces than hydrophilic.

Levels of anti-inflammatory IL-4 and IL-10, characteristic of an M2 phenotype, were upregulated on rough hydrophilic plasmaSLA and modSLA in comparison to both smooth and rough hydrophobic surfaces. IL-4 was significantly reduced on PT, plasmaPT, SLA, and aged modSLA as compared to the TCPS control (Figure 6E) and was significantly higher in cells on plasmaPT compared to PT. An increase in the level of both IL-4 and IL-10 was measured on both plasmaSLA and modSLA compared to the matching hydrophobic surfaces (SLA and aged modSLA, respectively). IL-10 levels were significantly higher on hydrophilic Ti surfaces in comparison to the TCPS control (Figure 6F). Moreover, levels of IL-10 were higher in cells on hydrophilic Ti surfaces than those on their hydrophobic counterparts.

3.4 72-Hour Microenvironment

After 72 hours, there was approximately twice the amount of protein present in media collected from each of the test surfaces per μ g DNA in comparison to the 24-hour time point.

There was no significant difference in IL-1 β secreted by cells on PT or plasmaPT compared to TCPS (Figure 7B). Secretion of pro-inflammatory IL-1 β , IL-6, and TNF α was lower in cells cultured on rough hydrophilic surfaces (plasmaSLA and modSLA) in comparison to rough hydrophobic surfaces (SLA and aged modSLA) (Figure 7B–D). Reduced levels of IL-6 were measured in cultures on plasmaPT compared to PT. No difference was detected between the plasmaSLA and modSLA surfaces or the SLA and aged modSLA surfaces in the three pro-inflammatory factors measured, suggesting no effect of the presence of nanostructures.

Secretion of anti-inflammatory IL-4 and IL-10 were similar on the PT and plasmaPT surfaces (Figure 7C, 7F). Levels increased in cells on hydrophilic plasmaSLA and modSLA in comparison to hydrophobic SLA and aged modSLA. However, there was no difference between levels on the two rough hydrophilic surfaces (plasmaSLA and modSLA) or the rough hydrophobic (SLA and aged modSLA)

3.5 DNA Quantification

Differences in comparison to the TCPS control and all test surfaces, excluding plasmaPT, were detected in DNA levels at the 24-hour time point. A significant reduction in DNA measured on each surface in comparison to PT. By the 72-hour time point, there was no

significant difference detected between the test surfaces and TCPS. DNA content was lower on Ti surfaces at 72 hours in comparison to 24 hours. (Figure 6A, 7A)

4. Discussion

In this study, we examined the effect of surface roughness and wettability on activation of naïve macrophages as characterized by the production of cytokines released by the cells. The surface roughness and wettability were varied independently of each other to determine the effect of each property on macrophage activation. We found that increased surface wettability had a stronger immunomodulatory effect than increases in roughness.

Disks modified by sandblasting and acid etching created a surface with a more similar topography to osteoclast conditioned bone than the smooth PT surfaces [18]. A qualitatively similar microstructured surface was seen in SEM images of the rough surfaces (SLA, plasmaSLA, modSLA, aged modSLA). We selected this imaging technique due to the high micron-scale roughness of the disk topography. While atomic force microscopy would allow for the assessment of nanostructures on a smooth surface, it may not accurately measure the large-scale roughness [19]. The tapping mechanism utilized for surface roughness assessment in AFM prevents the accurate perception of steep peaks and valleys, which may be generated because of sandblasting treatment. In addition, a sample height of greater than 20 μm and scan area of less than 150 $\mu\text{m} \times 150 \mu\text{m}$ is necessary to utilize this technique [20]. LSCM was selected as the optimal method for surface roughness measurements due to these limitations. This characterization method can select a larger scan area to give a better overall description of disk roughness. Near positive skewness values (S_{sk}) on each microrough surface indicated a greater number of peaks present on sandblasted surfaces compared to smooth. Similarities in developed interfacial area ratio (S_{dr}) between disks pre- and post-wettability modification demonstrate our ability to maintain matching surface topography. This ratio represents the amount of textured area on the surface. The highest percent of textured area was quantified on surfaces with both micro- and nanostructure (aged modSLA and modSLA). The hydrophilic surfaces used in this study had less carbon contamination and more oxygen, consistent with studies comparing SLA and modSLA by our group and others [18, 21, 22]. The application of an oxygen plasma treatment successfully modified the oxide layer of each hydrophobic disk by the removal of hydrocarbon contamination, thus generating a hydrophilic surface. Reduced levels of carbon present in the XPS scans were due to clean sample preparation and the use of metallic clips instead of carbon adhesive tape. These reduced levels resulted in only minor differences between the surfaces. Surfaces treated with oxygen plasma did not show any carbon bound to oxygen, suggesting the successful removal of hydrocarbons bound to the oxide layer. This surface modification may have altered surface chemistry in addition to the surface energy. These changes in surface features may have altered protein adsorption to the surfaces [23], which can change the cell response to a material.

Previous studies have shown an improved implant success rate in correlation with the increase in surface roughness and wettability. An increase in the differentiation of MSCs and their production of osteogenic and angiogenic microenvironment has been shown to occur with increased roughness and wettability of a Ti surface [24]. High success rates of

bone substitute materials such as beta tri-calcium phosphate with microstructure have also been studied [25, 26]. However, the resorption of these materials makes it difficult to determine if the success is due to activation of macrophages or osteoclasts in the remodeling of material and bone following implantation. Macrophages interact with the biomaterial surface before progenitor cells initiate contact with it. The immune system interacts with a biomaterial in different ways, which may be either harmful or beneficial. Macrophages are phagocytic cells, responsible for clearing away pieces of broken cells and tissue to allow for the generation of new tissue. The importance of the interaction of immune cells and biomaterial surface has been previously established [27]. Successful implant integration relies on a balance of classically activated (M1) macrophages to clear the wound site coupled with anti-inflammatory (M2) activated macrophages to promote wound healing and regeneration. A consistently high M1 response will recruit additional immune cells to the site, and this chronic inflammation can lead to fibrous encapsulation instead of successful tissue integration. The ability to control the ratio of M1 and M2 macrophages at the host-biomaterial interface will allow damaged tissue to be removed without a prolonged immune response that can lead to the creation of foreign body giant cells and inhibition of healing and integration.

The increased surface wettability resulted in a greater anti-inflammatory cell response in comparison to hydrophobic surfaces with matching roughness characteristics. DNA content on each of the surfaces was similar to levels on TCPS, showing that macrophages can interact with Ti surfaces. The reduced level of cells at 72 hours on all surfaces may correlate with the fast acting and short lifespan of macrophages [28]. The reduced cell number also suggests that the cause of chronic immune responses may be due to recruitment of new immune cells to the injury site and not because of a single constant inflammatory cell population [28].

Surface topography and wettability may control the attachment of cells in different ways. Smooth surfaces may allow the cells to attach and spread more than on rough surfaces. The configuration of the cells may alter the factors produced [29]. Increased levels of DNA were present on smooth PT surfaces in this study. A surface similar to that present in natural tissue, such as those with microroughness and hydrophilicity, may be able to stimulate the cells to switch activation and prevent a chronic immune response. A switch in cell activation can lead to the resolution of a previously chronic immune microenvironment. Interestingly, most of the research performed on macrophage activation has been done on TCPS or glass substrates that have lack biological relevance or similarity with any tissue in our body. The wettability of a material surface will control the proteins able to adsorb to the surface and the formation of a blood clot and fibrin network [30]. Cells such as macrophages will then interact with the adsorbed proteins through integrin complexes. These interactions may activate different pathways and allow various factors to be produced by the cell.

The results of this study show that while wettability has the strongest effect on interleukin release, it may not be solely responsible for the increase in anti-inflammatory factors. The combination of hydrophilicity and the increase of both pro- and anti-inflammatory protein production due to increased surface roughness resulted in M2-activated macrophages. The reduction in pro-inflammatory cytokines here is consistent with observations from other

studies, which have shown a decrease of gene expression of pro-inflammatory markers on hydrophilic rough Ti surfaces at 24 hours in comparison to rough hydrophobic surfaces [4, 21, 31]. Our study used primary bone-marrow-derived macrophages, whereas others have focused on the RAW 264.7 macrophage-like cell line [21]. Experiments conducted with both cell types have shown similar results, with an increase in pro-inflammatory factors secretion by macrophages cultured on hydrophobic surfaces. Studies focused on the macrophage cell line have quantified the effect of surface parameters on mRNA expression [21]. This study considers the next step in the actual production of proteins by the cells in contact with the surfaces and the microenvironment generated that will alter additional cell responses. Our results demonstrate the ability of rough, hydrophilic surfaces to produce initial levels of pro-inflammatory cytokines and ultimately produce the highest concentrations of anti-inflammatory and immunomodulatory factors. Additional studies have measured TGF- β as a marker for M2 activation. However, TGF- β is known to exist in three highly conserved isoforms in mammals, 1, 2 and 3, each having slightly different effects on surrounding tissue. Adverse effects of the different isoforms of TGF- β have been recorded, with increased levels being present at times of fibrous encapsulation and cancer [32]. Due to these negative potential outcomes and difficulty in distinguishing between isoforms, this cytokine was not included in the model of pro- and anti-inflammatory activation in this study.

The specific removal of macrophages through chemical or transgenic modification has shown to alter bone formation following tibia fracture in mice [33, 34]. Studies have demonstrated the importance of tissue resident macrophages (termed osteomacs) macrophages on fracture repair [35, 36] and have highlighted the actions of macrophages in the deposition of collagen matrix and the formation of new bone. Both circulating and tissue resident macrophages can be activated along the gradient from M1 to M2 and have differing effects on the wound healing cascade. The increased level of anti-inflammatory factors produced on rough, hydrophilic surfaces in this study may allow for faster resolution of inflammation and initiation of tissue regeneration.

5. Conclusion

Increased wettability, both inherent to the surface due to the manufacturing process or generated by exposure to an oxygen plasma treatment, increases anti-inflammatory macrophage activation more than surface roughness. Also, increased wettability of a micro-rough surface stimulates more anti-inflammatory cytokine release by macrophages than cells on hydrophilic smooth surfaces. The combination of increased surface roughness and hydrophilicity may interact synergistically to yield a microenvironment suitable for reduced healing times and increased osseointegration, which may lead to a higher level of implant success.

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Figure 1. Characterization of surface topography. Roughness parameters A) average roughness, B) skewness, and C) kurtosis D) Surface Area Ratio of the test samples were quantified by laser confocal microscopy. * $p < 0.05$ vs. PT, # vs. plasmaPT, \$ vs. SLA, % vs. plasmaSLA, & vs. aged modSLA.

Figure 2. Qualitative scanning electron images of surface topography at 1kx magnification A) PT, B) plasmaPT, C) SLA, D) plasmaSLA, E) aged modSLA, F) modSLA, and at 100kx G) PT, H) plasmaPT, I) SLA, J) plasmaSLA, K) aged modSLA, L) modSLA and 3D topographical images from LCSM M) PT, N) plasmaPT, O) SLA, P) plasmaSLA, Q) aged modSLA, and R) modSLA

Figure 3. Sessile drop contact angle (1 μ m DI water) on A) PT, B) SLA, C) aged modSLA, D) plasmaPT, E) plasmaSLA, F) modSLA.

Figure 4.
Elements present at material surface analyzed by x-ray photo spectroscopy.

Figure 5. Primary macrophages cultured on A) glass B) PT C) plasmaPT D) SLA E) plasmaSLA F) aged modSLA and G) modSLA for 24 hours, then fixed in 4% paraformaldehyde and stained cytoskeleton stained with phalloidin (green) and nucleus stained with Hoechst 34580(blue). Images were taken at 40x magnification (scale bar = 50 μm).

Figure 6.

DNA and protein quantification of media harvested from primary macrophages cultured on Ti surfaces for 24 hours. A) DNA B) IL-1β, C) IL-6, D) TNFα, E) IL-4, F) IL-10 *p<0.05 vs. TCPS, # vs. PT, \$ vs. plasmaPT, % vs. SLA, & vs. plasmaSLA, @ vs. aged modSLA.

Figure 7.

DNA and protein quantification of media harvested from primary macrophages cultured on Ti surfaces for 72 hours. A) DNA B) IL-1β, C) IL-6, D) TNFα, E) IL-4, F) IL-10 *p<0.05 vs. TCPS, # vs. PT, \$ vs. plasmaPT, % vs. SLA, & vs. plasmaSLA, @ vs. aged modSLA.

Binding energies determined from high resolution XPS scans of C1s, Ti2p, and O1s. Values are mean $\pm$ standard error of three regions of six disks per surface.

Table 1

Surface	C1s			Ti2p			O1s	
	C-C or C-H	C-O	C=O	TiO ₂	Ti(M)	C-O or OH	TiOx	
PT	67 $\pm$ 2.8	10 $\pm$ 2.2	8 $\pm$ 1.1	88 $\pm$ 0.1	5 $\pm$ 0.5	37 $\pm$ 0.7	63 $\pm$ 0.7	
pPT	100 $\pm$ 0.0	0 $\pm$ 0.0	0 $\pm$ 0.0	88 $\pm$ 0.5	5 $\pm$ 0.6	31 $\pm$ 1.4	69 $\pm$ 1.4	
SLA	67 $\pm$ 4.9	20 $\pm$ 6.9	17 $\pm$ 2.0	87 $\pm$ 0.2	5 $\pm$ 0.2	39 $\pm$ 0.6	61 $\pm$ 0.6	
pSLA	100 $\pm$ 0.0	0 $\pm$ 0.0	0 $\pm$ 0.0	89 $\pm$ 0.3	3 $\pm$ 0.1	29 $\pm$ 1.0	71 $\pm$ 1.0	
aged modSLA	75 $\pm$ 0.9	1 $\pm$ 0.5	24 $\pm$ 0.5	93 $\pm$ 0.4	ND	30 $\pm$ 4.3	68 $\pm$ 3.6	
modSLA	76 $\pm$ 1.2	1 $\pm$ 0.7	23 $\pm$ 1.2	88 $\pm$ 0.9	3 $\pm$ 0.5	27 $\pm$ 1.0	73 $\pm$ 1.0	