Inflammatory Response to a Titanium Surface with Potential Bioactive Properties: An In Vitro Study

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ABSTRACT

Background: The current hard tissue implants research aims to accelerate bone healing by designing surfaces that are bioactive. However, the role of the inflammatory response to these surfaces is so far incompletely described.

Purpose: The aim of the study was to evaluate early inflammatory response in vitro to a potentially bioactive surface—an anodized surface with Mg ions incorporated (anodized/Mg)—and to compare it to a turned, a blasted, and an anodized surface.

Materials and Methods: An interferometer was used for topographical characterizations. The disks were incubated with human mononuclear cells. Adherent cells were investigated with respect to number of cells, viability, differentiation, and cytokine production with and without lipopolysaccharide stimulation after 24 and 72 hours.

Results: The number of adhered mononuclear cells differed significantly between the different modified surfaces, with the highest number on the anodized surface. However, there were no significant differences in cytokine production and differentiation between the different modified surfaces. The amount of anti-inflammatory mediator interleukin-10 remained over time, while the number of cells and pro-inflammatory cytokine tumor necrosis factor-α decreased. The cells were viable on all surfaces, respectively.

Conclusion: The anodized surfaces with and without Mg ions showed an increased cell adherence, however, otherwise an inflammatory response similar to the turned and blasted surfaces. Furthermore, the potentially bioactive anodized/Mg surface showed a similar response to the TiUnite-like anodized surface despite the former having a surface roughness of a smoother character.

KEY WORDS: bioactivity, inflammation, in vitro, magnesium, surface modification, titanium oxide

Since osseointegration depends on biomechanical bonding, by means of bone ingrowth into micrometer-sized surface irregularities, the original turned surface has been by and large replaced by surfaces with moderate roughness, since those have demonstrated improved bone anchorage.[1–3]

One way of establishing moderate surface roughness is by increasing the native oxide layer of the titanium surface by different techniques resulting in increased bone response.[4–6]

The establishment of biochemical bonding is an attempt to further improve bone integration. Biochemical bonding would, at least theoretically, be possible with bioactive implants, where *bioactivity* is defined as "the characteristic of a material which allows it to form a bond with living tissue."[7]

The first materials claimed to be bioactive, solid hydroxyapatite, bioactive glasses, and glass ceramics were not suitable for dental applications because of poor mechanical properties. To part, this problem was solved by ceramic coatings on titanium implants. However, it has been demonstrated during the last 10 years that even commercially pure (CP) titanium can be made poten-

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tially bioactive by the anodization in electrolytes containing certain ions such as calcium and magnesium, microarch oxidation (MAO).[8] The earlier technical problems with ion beam-based modification techniques/line of sight limitations and the drawbacks of plasma-sprayed calcium phosphate coatings (hydroxyl apatite) such as delaminating and biodegradation during function have been eliminated.

In several studies, Sul and colleagues[9–11] have demonstrated excellent bone response in experimental in vivo studies by using this technique, particularly with incorporated Mg ions. Besides receiving a biomechanical bond, anodized/Mg surfaces may have a bioactive capacity.

However, the mechanisms behind these results are not fully explained and, in general, the mechanisms involved in bone formation around titanium implants are poorly understood. When an implant is inserted, proteins from body fluids immediately adsorb.[12] The surface characteristics have been shown to influence composition of the adsorbed protein and this may in turn be of importance for the regulation of the homeostatic systems, cellular events during early inflammation, etc.[13,14]

Since the inflammatory response is related to the bone response in this cascade of events, it is of importance to evaluate the underlying mechanisms of inflammation leading to bone formation.

Monocytes are useful for evaluating inflammatory response because several characteristics such as protein production (cytokines, growth factors), phagocytosis, and the formation of foreign body giant cells have been identified as critical events in the material host interaction.[15]

Since the host inflammatory reaction is a normal response to injury and to the presence of foreign agents and the precise magnitude and duration of the inflammatory response for optimal bone healing to an implant surface is unknown, the new experimental anodized/Mg was compared to a turned, a blasted, and an anodized surface—surfaces found on commercially available implant systems.

The aim of the study was to evaluate early inflammatory response in vitro to a potentially bioactive surface—the anodized/Mg surface.

MATERIALS AND METHODS

Disks and Surface Modification

The surfaces evaluated were the following: (1) turned, (2) blasted, (3) anodized, and (4) anodized/Mg.

A total of 216 CP titanium (grade 3) disks were prepared with a turned surface. Of those implants, 72 were kept turned, 72 were additionally blasted with 75 μm-sized Al_2O_3 particles, and further 72 underwent electrochemical oxidation and Mg ion incorporation by the MAO technique (anodized/Mg group). The electrochemical oxidation method employed in the present study has been described in previous studies.[8,16] In brief, all test disks were prepared using MAO methods at galvanostatic mode. Two platinum plates having a surface area of 32 cm^2 were used as cathodes at both sides of the titanium anode. Currents and voltages were continuously recorded at intervals of 1 s by a computer. The content of ripple was controlled to less than 0.1%. The disk was oxidized in a mixed electrolyte containing magnesium ions. In this study, the anodized/Mg group implants were deliberately manufactured with smooth surfaces.

Seventy-two implants were prepared with TiUnite-like surfaces and provided by Nobel Biocare AB, Göteborg, Sweden (anodized group).[17]

All surfaces were rinsed and cleaned ultrasonically in diluted Extran MA01® (Merck, Darmstadt, Germany) for 15 minutes, in distilled water for 10 minutes, and in absolute ethanol for 15 minutes, then dried at 60°C for 24 hours before sterilized by autoclaving.

The presence of endotoxins was evaluated with the Limulus amoebocyte lysate (LAL) assay (Sahlgrenska University Hospital, Sweden) and the surfaces showed no traces of contamination after the cleaning procedure.

The disks were placed in sterile 24-well polystyrene plates, in triplicate.

Surface Characterization

An interferometer was used for topographical characterization. The instrument has a vertical resolution of 0.05 nm and a horizontal resolution of 0.3 μm. Three disks of each surface modification were measured using 50× magnification objective and zoom factor of 0.62 resulting in a measuring area of 200 × 260 μm. A Gaussian filter sized 50 × 50 μm was used to remove shape and waviness before surface roughness was calculated.

Isolation and Culture of Monocytes

Buffy coats from six human blood doors were subjected to a two-step gradient centrifugation according to the method of Pertoft and colleagues.[18] In brief, 7 mL of undiluted blood was carefully layered over 4 mL of 60% v/v of polyvinylpyrrolidone-coated silica gel in conical centrifugation tubes and centrifuged at room temperature at 800 *g* for 30 minutes to separate the monocytes and lymphocytes from the remaining blood elements. The silica gel works to isolate mononuclear cells by exposing them to heterogenic nontoxic gel of particles of different sizes and shapes. This is known not to cause the activation of cells during centrifugation. The interface, containing mononuclear cells, was aspirated and transferred to a fresh tube and washed once with cold phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}). Cells were resuspended in cold (+4–8°C) PBS and layered over 3 mL of 50% v/v silica gel and centrifuged at 800 *g* for 60 minutes at +4. The middle layer containing mainly monocytes was harvested, washed, and counted. The cell viability was 99% as determined by trypan blue dye exclusion. The isolated mononuclear cells were resuspended in RPMI 1640 (Gibco-In Vitrogen™, Paisley, UK), 5% fetal calf serum, and 1% each of Penicillin-Streptomycin and fungizone at a concentration of 10^6 cells/mL. Cells were cultured at 10^6 cells per well in 24-well culturing plates for 24 and 72 hours.

Experimental Procedure

Half the number of disks with the different modified surfaces and adherent cells were immediately treated with lipopolysaccharide (LPS) 10 µg/mL to evaluate the effect of exogenous stimuli, while the other half were left without any stimuli and served as controls and background. Disks with cells were cultured in 37°C, 100% humidity, and 5% CO_2 for 24 hours or in parallel experiments for 72 hours, respectively.

In Vitro Evaluation Methods

Cell Viability After Cultivation. Cell viability was estimated by analyzing the content of lactate dehydrogenase (LDH) in the culture medium of the mononuclear cells after 24 and 72 hours.

LDH-mediated conversion of puruvic acid to lactid acid in the presence of reduced nicotinamide adenine dinucleotide and 2,4-dinitrophenylhydrazine was determined colorimetrically (Sahlgrenska University Hospital, Sweden).

Cytokine Assay: Tumor Necrosis Factor-α (TNF-α) and Interleukin-10 (IL-10) Content in the Supernatant. The supernatants from cells cultivated for 24 and 72 hours were carefully harvested, centrifuged free from cells, frozen and kept at –70°C, and then analyzed with respect to the amount of TNF-α and IL-10.

The TNF-α and IL-10 amounts were analyzed by the use of commercially available assays (Biotrak™, Amersham, Little Chalfont, Bucks, UK). The assay is based on quantitative "sandwich" enzyme immunoassay techniques, a solid-phase enzyme-linked immunosorbent assay (ELISA), which utilizes an antibody for TNF-α and IL-10 bound to the wells of a microtiter plate together with a biotinylated antibody to TNF-α and IL-10 and streptavidin-conjugated horseradish peroxidase.

The optical absorption was measured with an automatic plate reader (Vmax, Molecular Device, Crawley, UK). By comparing the optical density of the samples to the standard curve provided by the manufacturer, the concentration of TNF-α and IL-10 in unknown samples was determined. The sensitivity of the ELISA kits was <2 and <4 pg/mL, respectively.

Immunofluorescence: Cells and Differentiation. In parallel experiments, the monocytes were cultured on the differently treated titanium surfaces at a concentration of 10^6 cells/mL in 24-well culturing plates (Falcon®, Multiwell™, Becton Dickinson Labware, Franklin Lakes, NJ) for 24 and 72 hours to compare the number of cells and the cell differentiation. The disks with the adherent cells were fixed in absolute ethanol for 15 minutes at –20°C after the different culture periods. After washing with PBS, the cells were pre-incubated at room temperature with dilution buffer, consisting of PBS (pH 7.4), 5% fat-free milk, 0.1% gelatin, and 7.5% sucrose, followed by incubation for 20 minutes with Vectastains® normal blocking serum (Vector Lab., Burlingame, CA, USA) to reduce nonspecific binding. The cells were then incubated with monoclonal antibodies RM3/1 and 27E10 (Biogenesis, Poole, UK), respectively, for 30 minutes. Bound antibodies were detected by sequential incubation with biotinylated anti-mouse immunoglobulin G, and fluorescein isothiocyanate-conjugated avidin (Amersham) after incubation with Vectastain Elite® (Vector Lab.) for 30 minutes. Cells were rinsed with KCl to open up the cell membrane and were immediately immunolabeled and mounted with Vectashield® 4,6-diamidino-2-

phenylindole (Vector Lab.) (Sigma) and examined in Nikon Mikrophot FXA® epifluoroscence microscope (Nikon Eclips 600, Nikon Instruments, Inc., Melville, NY). The area of the positively stained cells was counted in five randomly selected areas ($302 \times 241\,\mu m^2$) on each disk and the mean value of the number of cells was calculated.

Statistics

The nonparametric Kruskal-Wallis test was used for statistical evaluation.

RESULTS

Surface Roughness

The different modified surfaces showed significant differences regarding surface roughness values for the S_a (arithmetic mean height deviation from mean plane [μm]) value and S_{dr} (developed interfacial area ratio [%]), where the anodized surface showed the roughest surface (1.13 μm + 0.04 and 52.60% + 6.77, respectively) followed by blasted (1.02 μm + 0.04 and 36.03% + 2.05, respectively), anodized/Mg (0.27 μm + 0.03 and 10.92% + 2.23, respectively), and turned (0.19 μm + 0.03 and 4.31% + 0.85, respectively) surfaces (Figure 1, A and B).

A spatial descriptive parameter, density of summits (Sds) demonstrated that the anodized surface having the lowest value $0.06/\mu m^2$ + 00.01, while the anodized/Mg showed a significant larger value $0.14/\mu m^2$ + 0.01 (Figure 1C).

Viability

The results showed, in general, low LDH values for all surfaces (within the range of 0.8–1.6 μkat/L), but were slightly increased after LPS stimulation (not significant) and after 72 hours ($p < .001$) (Figure 2, A and B).

Cell Number and Differentiation

The total number of cells differed significantly between the different modified surfaces ($p < .001$) with the highest number of cells on the anodized surface. There was a significant decrease of the total cell numbers from 24 to 72 hours ($p < .001$), but there were no significant differences between stimulated and un-stimulated wells.

In general, the percentages of differentiated cells were low and there were no significant differences between the tested surfaces.

Acute monocytic phenotype 27E10 marker dominated after 24 hours ($p < .001$), while the expression of

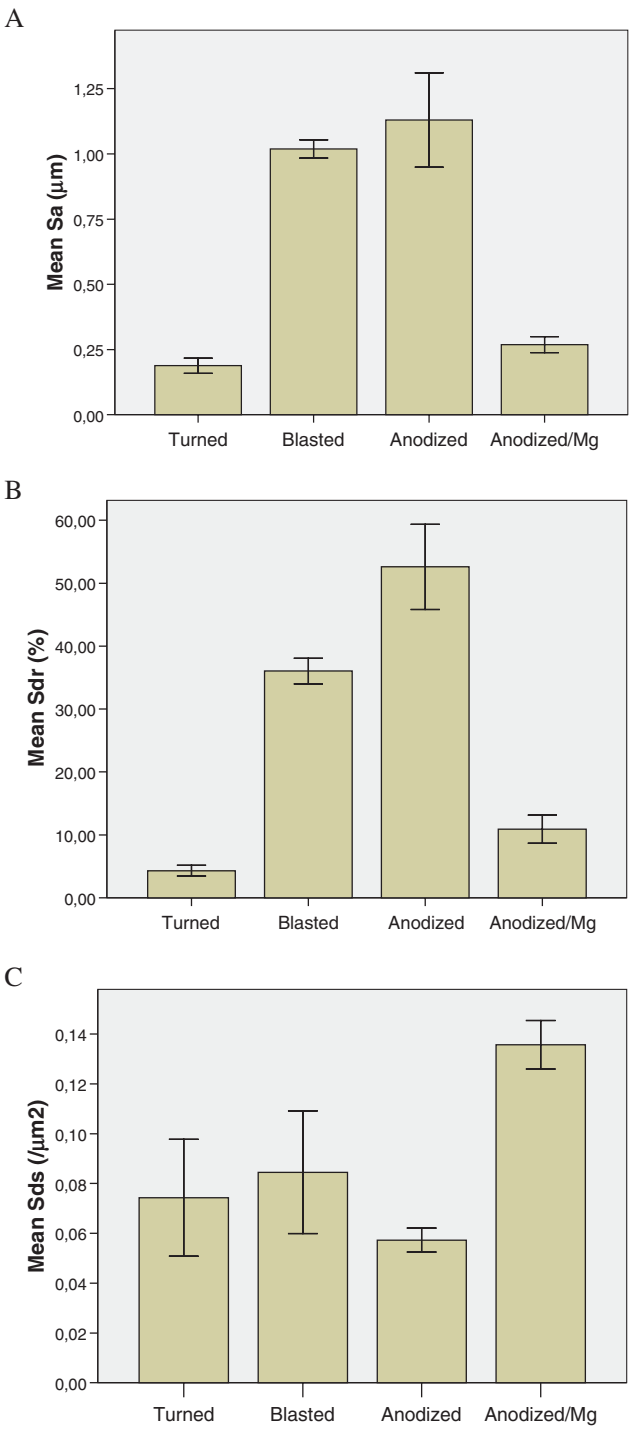

Figure 1 Three disks of each surface modification were analyzed and each disk measured at three areas. The mean value and the standard deviation of the nine measurements on every surface modification; (A) Sa, (B) Sdr, and (C) density of summits (Sds) values. Sa $p < .001$ for the blasted and anodized surface versus the turned and anodized/Mg surface. Sdr $p < .001$ for the turned versus the blasted versus the anodized versus the anodized/Mg surface. Sds $p < .001$ for the anodized/Mg versus the turned, the blasted, and the anodized surface.

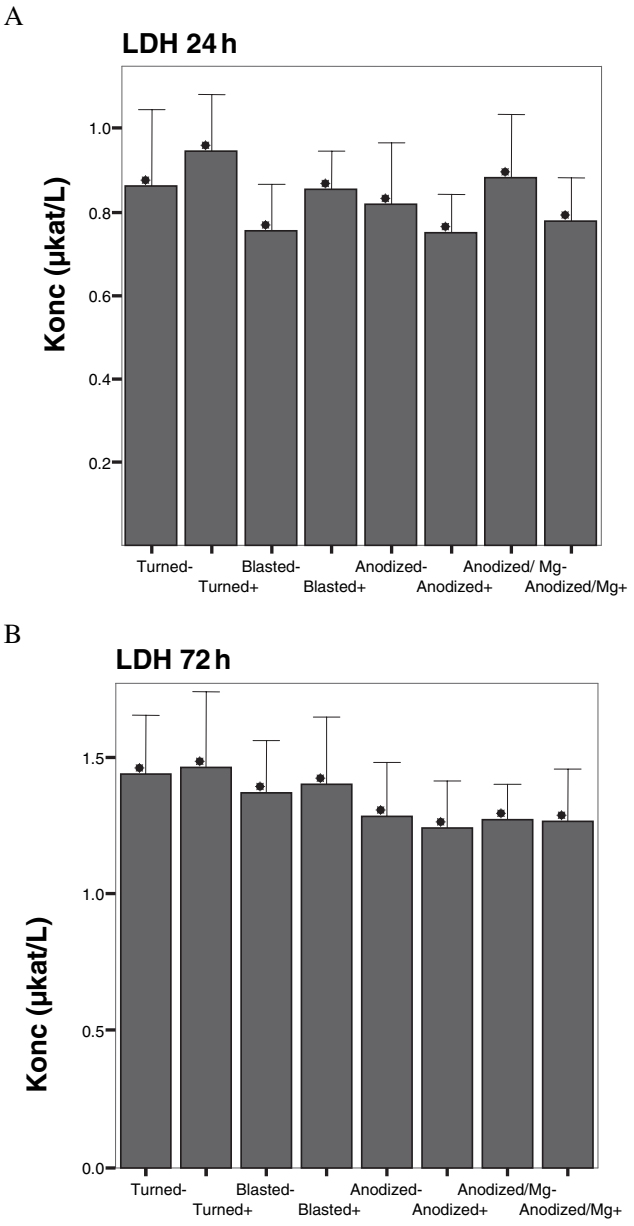

Figure 2 The mean value and the standard deviation of lactate dehydrogenase (LDH) content (µkat/L) in the culture medium around the disks after (A) 24 and (B) 72 hours. $p < .001$ for 24 versus 72 hours ($n = 144$).

the chronic RM3/1 dominated thereafter 72 hours ($p < .001$) (Figure 3).

Cytokine Release of TNF-α

There was no significant difference between the responses of the cultured cells on different modified surfaces in the TNF-α release. However, there were significantly higher concentrations after 24 hours than after 72 hours ($p < .001$). Additionally, the LPS-stimulated cells showed significantly higher values than the un-

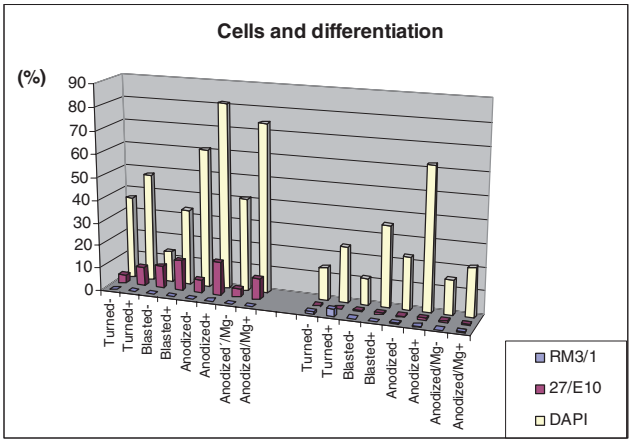

Figure 3 Mean area of total adherent and differentiated cells per measured area (%) on the disks with the different modified surfaces after 24 and 72 hours with (+) and without (–) lipopolysaccharide stimulation. $p < .001$ for total adhered cells; 24 versus 72 hours ($n = 72$), anodized versus turned, blasted, and anodized/Mg ($n = 36$), E27/10; 24 versus 72 hours ($n = 24$) and RM3/1; 24 versus 72 hours ($n = 24$).

stimulated cells on the different surfaces ($p < .001$) (Figure 4).

Cytokine Release of IL-10

There were no significant differences between the responses of the cultured cells on the different modified surfaces in IL-10 release or between the different evaluation times. The LPS-stimulated cells on all tested surfaces showed significantly higher values than the un-stimulated surface adherent cells ($p < .001$) (Figure 5).

DISCUSSION

The present study showed that there were surface-related differences in the cell accumulation, where the anodized surface expressed significantly larger number

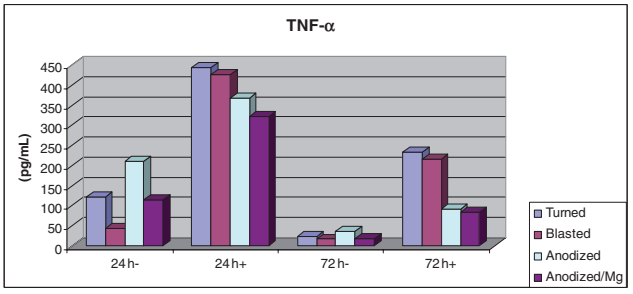

Figure 4 Mean tumor necrosis factor-α (TNF-α) concentrations (pg/mL) in the culture medium around the disks with the different modified surfaces after 24 and 72 hours with (+) and without (–) lipopolysaccharide (LPS) stimulation. $p < .001$ for 24 versus 72 hours ($n = 144$), with versus without LPS stimulation ($n = 144$).

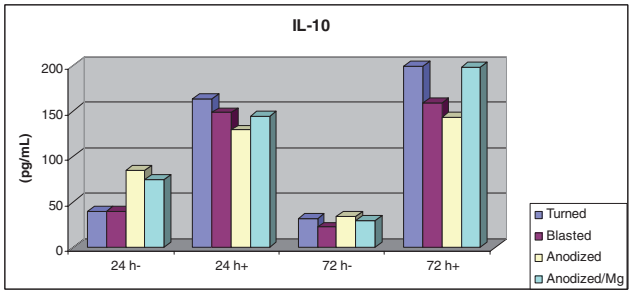

Figure 5 Mean interleukin-10 (IL-10) concentrations (pg/mL) in the culture medium around the disks with the different modified surfaces after 24 and 72 hours with (+) and without (−) lipopolysaccharide (LPS) stimulation. $p < .001$ for with versus without LPS stimulation ($n = 144$).

of adhered cells both at 24 and 72 hours. This is in agreement with other studies, where both macrophage and osteoblast cells have been observed to be "rugophilic" in contrast to epithelial cells and fibroblasts that prefer smoother surfaces.[19–23] Against this background, it is surprising that the blasted surface showed low cell adherence. However, comparisons with other studies have to be made with caution since other materials, cell types, evaluation methods, etc. have been used.[19–22] In yet other cases, it is difficult to compare results since surface characterization has been left out.[23]

Furthermore, the concomitant increase in porosities and change in physicochemical properties of the thickened oxide of the anodized surface may contribute as well.

This would explain the substantial cell adherence on the anodized/Mg surface after 24 hours despite its quite smooth surface (S_a). Osteoblasts seem to have a higher adherence to Mg ion-reinforced ceramic surfaces[24] and this may be valid for the cells in this study as well. An explanation may be the supply of Mg ions, since early adhesion to a biomaterial surface is mediated by integrins, and integrin function depends on divalent cations such as Mg ions.[25] Parise and Phillips[26] have demonstrated that platelets bound more readily to extracellular matrix with magnesium compared to calcium ions. Except for the change in physicochemical properties and increased porosity because of the thickened oxide, the surface expressed a high value for peaks per area unit, and this may be favorable for cell attachment. Since both of these anodized surfaces have performed well in vivo, one may speculate that the small number of inflammatory cells may not be the optimal for an increased bone response.

The decreased number of adhered cells for all surfaces over time is in accordance with results from Suska and colleagues[27,28] that showed that inflammatory response to titanium is transient compared to copper.

Many investigators have begun to study cell response to various experimental surfaces using markers of differentiation as indicators, rather than merely examining attachment and spreading. However, differentiation of monocytes related to titanium surface characteristics is so far poorly investigated in vitro. The markers used in the study suggest 27E10 expression denotes an "acute" phenotype of the macrophage and that RM3/1 expression indicates resident macrophages, hence a switch to a more mature cell.[29,30] This is in accordance with the present study where the acute monocytic phenotype 27E10 marker dominated after 24 hours, while the expression of the chronic RM3/1 dominated after 72 hours. When it comes to osteoblasts, differentiation has been shown to depend both on surface chemistry[31] and surface roughness.[32] In this study, there were no significant differences between the surfaces and, in general, the percentage of differentiated cells was low.

According to Zwadlo and colleagues[29,30], the surface antigen for 27E10 and RM/1 is found on 20% of peripheral blood monocytes and is increasingly expressed upon culture of monocytes, reaching a maximum between 48 and 72 hours.

A possible explanation may be that maximum expression of the two phenotypes occurred at a later time point not chosen for evaluation in this study, that the antigen was missing on the cells, or that the surfaces did not have the capacity to stimulate the cells to differentiate. Furthermore, LPS stimulation is supposed to decrease the expression of RM3/1 while 27E10 is not or is only weakly affected. In this study, LPS did not alter the kinetics of either 27E10 or RM3/1 expression compared to un-stimulated controls.

As for cell adherence, surface characteristics have shown to modulate macrophage mRNA expression and cytokine release with a stronger response to a rougher surface ($>1.0\,\mu m$) compared to a smoother one ($<0.6\,\mu m$).[22]

In the present study, the cytokine production did not reflect the cell accumulation and consequently there were no surface-related differences in the early cytokine release. However, all surfaces induced higher TNF-α at early time points and lower release at later time points. The transient nature of TNF-α secretion at the titanium

surfaces indicates potentially important down regulatory factors in the vicinity of these implants.[27]

One important factor is the IL-10 secretion which in vitro has been shown to promote degradation of pro-inflammatory cytokine mRNA decrease TNF-α receptor expression and down-regulate the secretion of macrophage-derived TNF-α.[33]

Another factor is that TNF-α promotes the spontaneous apoptosis of monocytes in vivo.[34]

Additionally, there were no surface-related differences in early cytokine release of IL-10 but unlike the pro-inflammatory mediator, the anti-inflammatory mediator did not decrease significantly at 72 hours.

LPS stimulation compared to no stimulation increased cytokine levels 10-fold for all surface adherent cells at both time points.

The precise mechanism of the LPS-stimulated cytokine release is not known. However, interactions between LPS and LPS binding sites on the macrophage surface and LPS binding proteins have been suggested.[35]

Furthermore, all surfaces showed viable cells with, in general, low LDH values for all time points even after LPS stimulation. Extracellular LDH levels are markers of plasma membrane injury and are increased if the monocytes, for example, are exposed antigens.[28]

CONCLUSION

The anodized surfaces with and without Mg ions showed an increased cell adherence, however an inflammatory response similar to the turned and blasted surfaces.

Furthermore, the potentially bioactive anodized/Mg surface showed a similar response to the TiUnite-like anodized surface despite the former having a surface roughness of a smoother character.

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