Research Article

Attenuating Immune Response of Macrophage by Enhancing Hydrophilicity of Ti Surface

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Immune responses can determine the *in vivo* fate of implanted materials. The strategy for developing implants has shifted towards using materials with immunomodulatory activity. However, the immunoregulatory effect of hydrophilicity of titanium surface on the macrophage behavior and its underlying mechanism remain poorly understood. Here, the Ti surface hydrophilicity-dependent behavior of murine RAW264.7 macrophages was investigated *in vitro*. Two laboratory models with significantly different surface hydrophilicity and similar roughness were established with Ti-polished and Ti-H_2O_2 surfaces. The results of cell morphology observation showed that the Ti-H_2O_2 surface yielded enhanced cell adhesion and less multinucleated cell formation. CCK-8 assay indicated that the growth rate of macrophage on Ti-H_2O_2 surface is higher than that of Ti-polished. ELISA assay result revealed lower level of proinflammatory factor TNF-α and higher level of anti-inflammatory factor IL-10 on the Ti-H_2O_2 surface compared to Ti-polished. Subsequently, immunofluorescence and western blotting analysis showed that activation of the NF-κB-TNF-α pathway might be involved in the modulation of the immune response by surface hydrophilicity. Together, these results suggested that relative high hydrophilic Ti surface might attenuate the immune response of macrophage by activating NF-κB signaling. These findings could provide new insights into designing implant devices for orthopedic applications.

1. Introduction

Because of excellent mechanical and corrosion resistance properties, titanium- (Ti-) based materials have been widely used in making dental and orthopedic implants [1, 2]. However, immune responses mediated by inflammatory cells have been found to significantly impact the biocompatibility and function of implanted medical devices or prostheses and can ultimately precipitate their clinical failure [3, 4]. The paradigm for developing an ideal implant material has been shifted using from inert to immunomodulatory materials, emphasizing the importance of immune cells in the evaluation of a candidate material [5]. Macrophages, the major effector cells in the immune response to implants,

have been reported to determine the long-term immune response and outcome of an inflammatory reaction [6, 7]. Modulation of adhesion and activation of macrophages with anti-inflammatory drugs can reduce immune responses [8]. However, in the clinic the long-term systematic use of drugs can compromise host defenses, leading to impaired immune responses and an increased potential for infection [9]. To avoid this problem, appropriate surface modifications of implants have been considered as an alternative approach to inhibit or attenuate inflammation reaction temporarily for achieving high success rates [10–12].

Recently, several types of surface modification methods have been introduced to slow macrophage-mediated immune

responses triggered by Ti-based implants, including coatings, sand blasting, and acid etching [13–15]. However, delamination of the coating was found to sometimes occur during implant placement or in load bearing situations, which was probably caused by poor stability of the coating bound to the substrate [13]. Additionally, normal bone mineralization can be inhibited by the release of constituents, such as Al_2O_3 or Ta_2O_5, from the material when they were modified by sand blasting [14]. The etched implant surface has been shown to be capable of promoting osseointegration because of hydrophilicity [15]. Furthermore, surface hydrophilicity of Ti-based implants has been established to modulate osteogenic cell adhesion, proliferation, and differentiation [16]. Therefore, it can be hypothesized that incorporating surface hydrophilicity might be a promising alternative for regulating macrophage behavior. Nevertheless, little attention has been paid to the immunoregulatory effect of surface hydrophilicity of titanium on the macrophage behavior and data about this topic has been needed.

Herein, the purpose of this work is to investigate the effects of the hydrophilicity of Ti surface on macrophage behavior *in vitro*. Two kinds of Ti surfaces with different hydrophilicity including polished Ti surface (Ti-polished) and etched Ti surface (Ti-H_2O_2) were used in present study. The morphology, roughness, and wettability of the Ti surfaces were firstly characterized. The morphology of macrophage RAW264.7 cells on the Ti surfaces was observed by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Cell proliferation was assessed by CCK-8 assay. The activation status of macrophages was quantified by measuring TNF-α and IL-10 secretion by ELISA assay. Subsequently, the underlying mechanism of modulation of surface hydrophilicity on the immune response was explored by both immunofluorescence and western blotting analysis.

2. Materials and Methods

2.1. Samples Preparation. Ti disks with 20 mm in diameter and 2 mm thick were used to prepare two kinds of different Ti surfaces including hydrophilic (Ti-H_2O_2) and polished (Ti-polished). Ti-polished surfaces were prepared by polishing with sand paper from 400 to 1500 grit. Ti-H_2O_2 surfaces were obtained via immersing the Ti disks in 10% H_2O_2 solution for 24 h.

2.2. Sample Characterization. The surfaces were imaged by scanning electron microscope (SEM, Hitachi S-4800, Tokyo, Japan) at a 15 kV accelerating voltage. Atomic force microscopy (AFM, SPA-300HV, Seiko, Tokyo, Japan) was performed to measure the surface roughness parameter Ra over a $10 \times 10\,\mu m$ area. X-ray photoelectron spectroscopy (XPS, Thermo V6 Escalab 250, West Sussex, UK) was carried out to analyze element composition of these Ti surfaces. Surface water contact angles of Ti materials were measured using a drop shape analysis system (JY-82A, China).

2.3. Cell Culture. Murine macrophage-like RAW264.7 cells were obtained from Cell Resource Center, IBMS,

CAMS/PUMC, and cultured in DMEM medium with 10% FBS (Gbico, Carlsbad, CA, USA). After two passages, cells were plated at 2.0×10^5 cells per well in 24-well plates.

2.4. Cell Morphology Observation

2.4.1. Scanning Electron Microscopy (SEM). Cells were incubated on Ti disks for 1, 3, and 7 days. After incubation, cells were fixed with 2.5% glutaraldehyde and incubated with 0.18 mol/L sucrose solution. Samples were dehydrated through a graded ethanol series (30–100%) and air-dried.

2.4.2. Confocal Laser Scanning Microscopy (CLSM). Cells were cultured on Ti disks for 7 days. Cells were then fixed with 4.0% paraformaldehyde, treated with Alexa Fluor 546-phalloidin (50 μg/mL) for 1 h at room temperature, and then stained with DAPI for 10 min according to the manufacturer's directions. Images of the stained cells were acquired using CLSM (Zeiss, LSM 780, Oberkochen, Germany).

2.5. Cell Proliferation Assay. To assess macrophage activity on Ti disks, cell counting kit-8 (CCK-8) was performed on days 1, 3, 5, and 7. According to the manufacturer's directions, CCK-8 solution was added to each well of the plate at a ratio of 1 : 10 and then was incubated for 2 h in an incubator. After incubation, absorbance was measured using a microplate reader (Bio-Rad, Hercules, CA, USA).

2.6. Cell Cytokine Secretion Examination. To analyze cytokine secretion, cells were cocultured with Ti disks and supernatants were collected at days 1, 3, 5, and 7. The levels of TNF-α and IL-10 in the supernatants were quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's directions.

2.7. Assessment of NF-κB. In accordance with the manufacturer's directions, cells grown on Ti disks for 3 days were fixed, blocked, and incubated with anti-NF-κB p65 primary antibody at 4°C overnight. Then samples were treated with an appropriate Cy3-conjugated secondary antibody for 1 h and stained with DAPI for 5 min at room temperature. A confocal microscopy (Zeiss, LSM 780, Oberkochen, Germany) was used to observe NF-κB activation.

2.8. Statistical Analysis. Data were analyzed using an independent Student's t-test and are presented as means ± standard deviation (S.D.). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results and Discussion

Given the importance of macrophages in bone dynamics, some studies have investigated interactions between bone substitute biomaterials (e.g., bioceramics, polymers, or titanium) and macrophages [11, 17, 18]. However, in the field of implant materials research, the immunoregulatory effect of surface hydrophilicity of titanium on the macrophage
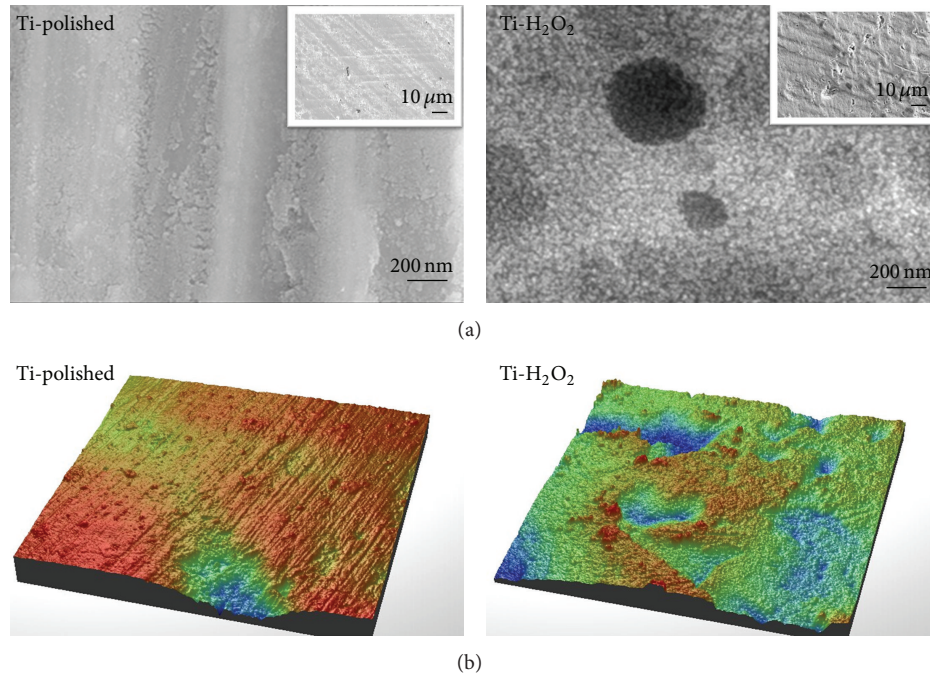
(a)

(b)

FIGURE 1: Scanning electron microscopy (a) and atomic force microscopy (b) images of the Ti-polished and Ti-H_2O_2 surfaces.

behavior and its underlying mechanism still remain largely unknown. To devise a strategy for Ti surface design for clinical regenerative therapies, additional data regarding the effects of surface hydrophilicity on immune responses and the underlying mechanisms involved will be required. The results in present study demonstrated that a more hydrophilic Ti surface might more favorably attenuate macrophage immune responses via NF-κB-TNF-α signaling.

The SEM observation clearly indicated that Ti-H_2O_2 possessed a nanostructure with a uniformly distributed nanoparticle compared to Ti-polished surface (Figure 1(a)). As shown in Table 1, AFM images showed that the two kinds of Ti surfaces shared similar roughness that Ra of Ti-polished and Ti-H_2O_2 were 23.25 ± 5.89 nm and 87.13 ± 23.72 nm. While Ti-H_2O_2 surface ($35.21 \pm 3.08°$) was found to be more hydrophilic than the Ti-polished surface ($64.46 \pm 2.384°$). The quantified element composition given by XPS indicated a little increment in the O1s peak for the hydrophilic Ti-H_2O_2 surface compared to hydrophobic Ti-polished surface, supported by atomic ratios of oxygen (Figure 2 and Table 2). The results were consistent with previous report that O_2^- and –OH group would be generated on surface when treated with H_2O_2 solution [19]. Thus, the two kinds of Ti surface models used in our work presented significantly different surface hydrophilicity and similar roughness.

The proliferation rate of cells on the Ti surfaces partially reflects biocompatibility of the materials, which is essential for implant survival. Mouse macrophage RAW264.7 cells viability on different Ti surfaces was measured by CCK-8 assay for 1, 3, 5, or 7 days (Figure 3). Overall, relative high hydrophilic Ti-H_2O_2 surface resulted in enhanced cell proliferation. Although cell viabilities on different surfaces

TABLE 1: Water contact angle and surface roughness of the Ti samples.

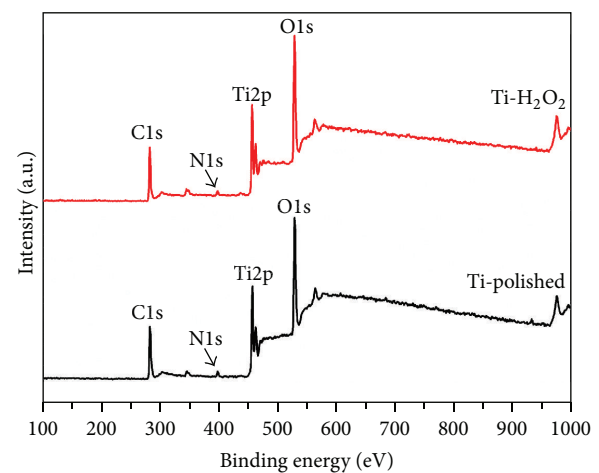| Sample	Contact angle (°)	Ra (nm)
Ti-H_2O_2	35.21 ± 3.08	87.13 ± 23.72
Ti-polished	64.46 ± 2.38	23.25 ± 5.89

FIGURE 2: X-ray photoelectron spectroscopy analysis of the Ti-polished and Ti-H_2O_2 surfaces.

were similar after 1 day of culture, significantly higher cell viabilities were found on hydrophilic Ti-H_2O_2 surfaces compared to hydrophobic Ti-polished surfaces after culture for 3 days. Additionally, this trend continued to be maintained

TABLE 2: Element distribution recorded by XPS analysis.

	Ti-H_2O_2	Ti-polished
C1s	42.31%	44.71%
Ti2p	13.35%	13.15%
N1s	2.58%	2.72%
O1s	41.77%	39.44%
F1s	0	0
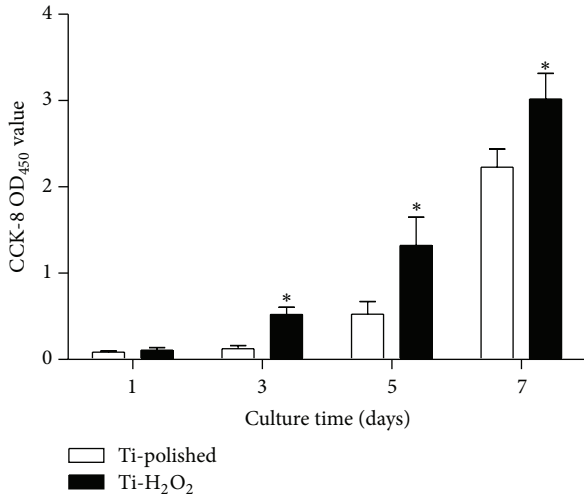
FIGURE 3: The effect of surface hydrophilicity on RAW264.7 cells viability measured by CCK-8 ($^*P < 0.05$).

at days 5 and 7. These data demonstrate that hydrophilic Ti-H_2O_2 surfaces are more biocompatible than hydrophobic Ti-polished surfaces. The excellent biocompatibility of Ti-H_2O_2 surfaces for macrophage may be benefit for wound healing. Previous studies also indicated that improved wound healing was associated with enhanced macrophage recruitment [20].

The influence of materials on macrophage morphology played an important role in the assessment of materials immunoregulatory property. Cell shape changes have been reported to be related to different functional states of cells, such as proliferation, nuclear organization, and differentiation [21–23]. Additionally, cell shape was found to modulate the phenotypic polarization of macrophages. In this study, we clearly observed different initial cell adhesion behaviors of macrophages on two types of Ti surfaces after 1 day of culture (Figure 4(a)). More filopodia were observed on hydrophobic Ti-polished surfaces than on those of hydrophilic Ti-H_2O_2 surfaces. Filopodia are specialized macrophage adhesion structures that can form in the early stages of cell adhesion and consist of punctuate F-actin on plasma membrane extensions [24], which facilitate attachment and migration [25, 26]. It has been proposed that a round shape might indicate a resting nonactivated macrophage, whereas the presence of multiple filopodia represents an activated macrophage [26]. Therefore, the difference in filopodia numbers clearly suggested that macrophages would be more prone to become activated on the hydrophobic Ti-H_2O_2 surface than on the hydrophilic Ti-polished.

After 7 days of culture, SEM observation showed that several suspected FBGCs formed by cell fusion appeared on Ti-polished surfaces, whereas only elongated cells with numerous cellular projections were present on Ti-H_2O_2 surfaces (Figure 4(a)). Furthermore, immunofluorescence staining of cytoskeleton organization and CLSM analysis of nuclei confirmed that more FBGCs formed on hydrophobic Ti-polished surfaces than on hydrophilic Ti-H_2O_2 surfaces (Figure 4(b)). This phenomenon was consistent with previous work of Collier et al. who reported that hydrophilic surfaces exert a robust inhibitory effect on FBGCs formation [25]. The formation of FBGCs in response to implanted biomaterials or medical devices, also known as a foreign body reaction, has been implicated in the degradation and cracking of implanted material and in initiating the formation of fibrous capsules that surround the implanted biomaterials [27, 28]. FBGCs with multiple nuclei might release profibrotic factors, including transforming growth factor beta (TGF-β) and platelet-derived growth factor (PDGF), which could in turn activate fibroblasts and endothelial cells to facilitate the subsequent fibrotic encapsulation and inflammatory response [21, 29]. Thus, increased FBGCs formation on hydrophobic Ti-polished surfaces might imply a more serious inflammatory response of macrophages, and hydrophilic Ti-H_2O_2 surfaces might be more beneficial for inhibiting inflammation.

Notably, the two types of Ti surfaces also yielded macrophages with significantly different cell morphologies. More macrophages with spindles appeared on Ti-H_2O_2 surface, whereas almost all macrophages exhibited a round cell appearance with multiple filopodia on Ti-polished surfaces (Figure 4(b)). These results suggested that Ti-H_2O_2 surfaces might promote the polarization of macrophages towards a prohealing macrophage phenotype, implying that they can exert anti-inflammatory activities and improve wound healing responses. Our results were also consistent with previous report that prohealing cells exhibited a more elongated shape compared to proinflammatory cells [30]. By contrast, the round cell morphology on Ti-polished surfaces might be a phenotypic marker of proinflammatory macrophages, leading to fibrotic responses and inflammation around any materials.

It has been well established that the immune system is closely related to skeletal development and that they share many common cytokines [31]. Those cytokines can mediate macrophage-dependent inflammatory cascades that are triggered by innate immune activation and act to influence implant integration and osteogenesis [32–35]. Proinflammatory cytokines can enhance osteoclast differentiation and resorbing activity and inhibit osteoblast activity and bone formation, whereas anti-inflammatory cytokines exert opposite effects [33–35]. TNF-α, a key proinflammatory regulator that is mainly released by stimulated macrophages, contributes to fibrosis via TNF receptor 2, which then acts through extracellular signal-regulated kinase 1/2 (ERK1/2) to stimulate cell proliferation and inhibit collagen degradation [33]. TNF-α was also found to activate the ERK-specific mitogen-activated protein kinase (MAPK) pathway, leading to increased TGF-β1 production, which can facilitate fibrosis
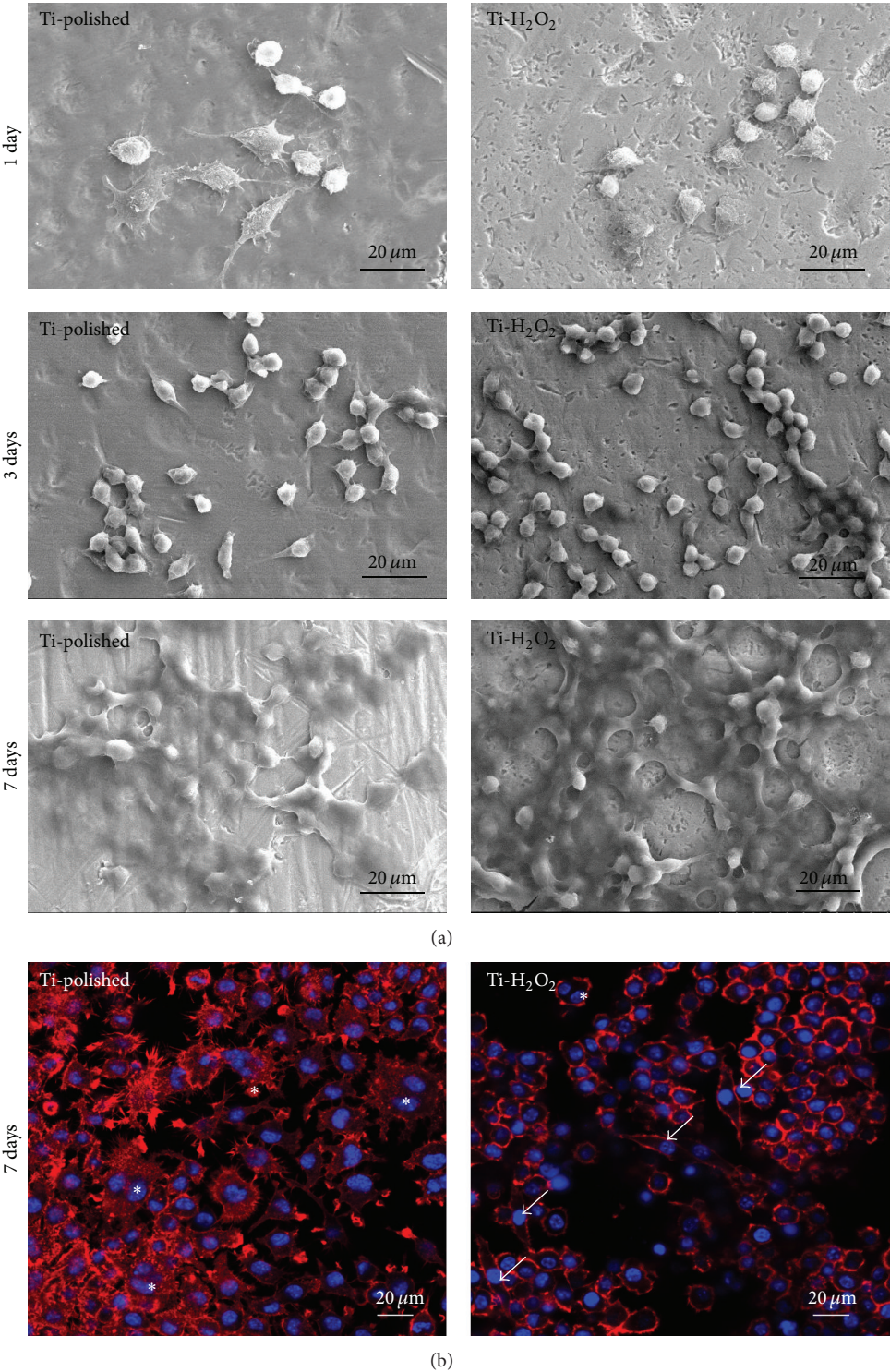
(a)

(b)

FIGURE 4: The influence of surface hydrophilicity on cell morphology and FBGCs formation. (a) Scanning electron microscopy images of macrophages cultured on Ti-polished or Ti-H_2O_2 surfaces for 1, 3, and 7 days. (b) The cytoskeleton organization of macrophages cultured on Ti-polished and Ti-H_2O_2 surfaces for 7 days. White asterisks indicate FBGCs. White arrow indicates the spindle cells. F-actin and cell nuclei were immunostained with Alexa Fluor 546-phalloidin (red) and DAPI (blue), respectively.
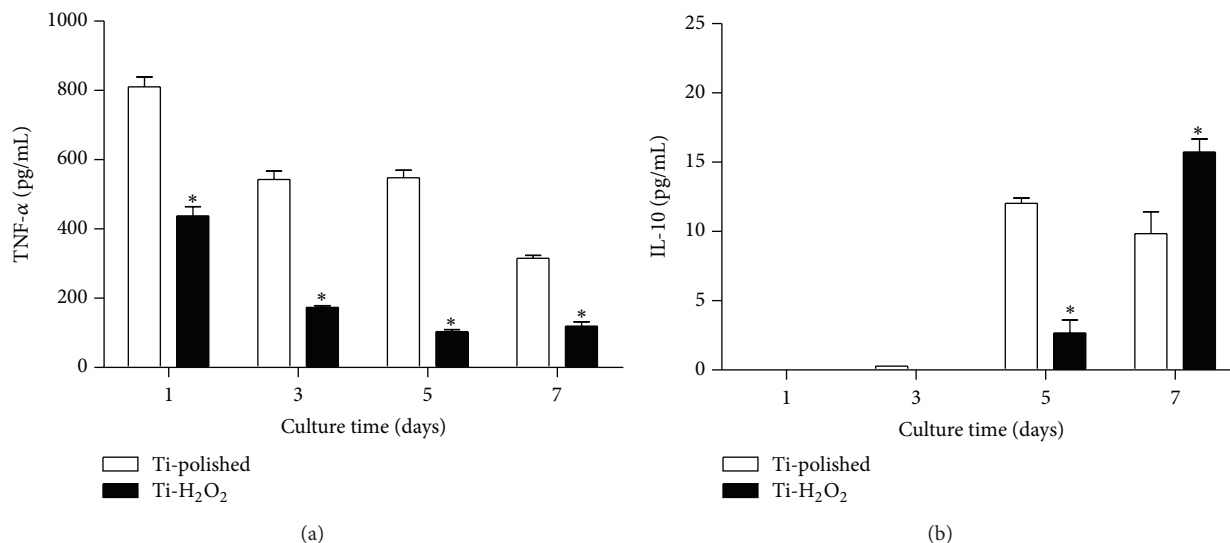
FIGURE 5: The levels of secreted TNF-α (a) and IL-10 (b) in the supernatants of macrophages cultured on different surfaces for 1, 3, 5, and 7 days ($^{*}P < 0.05$).

[36]. The ELISA quantitative analysis showed that TNF-α secretion was significantly increased on hydrophobic Ti-polished surfaces compared to hydrophilic Ti-H$_2$O$_2$ surfaces at all time points (Figure 5(a)). Production of the proinflammatory cytokine TNF-α peaked on both Ti surfaces after 1 day of culture and then decreased with the time, suggesting that a robust inflammatory response occurred at an early stage of culture. IL-10 is a typical anti-inflammatory cytokine that plays a critical role in suppressing proinflammatory cytokines [35]. In contrast to the pattern of proinflammatory cytokine secretion, production of the anti-inflammatory cytokine IL-10 increased in a time-dependent manner on both Ti surfaces (Figure 5(b)). These results indicated that the surface wettability of Ti implants has a profound effect on the immune response of macrophages. Our results are also in line with previous studies. Lee et al. reported that hydrophilic titanium elicited fewer inflammation reactions [37]. Tsang et al. demonstrated that hydrophilic carbon nanofibers (CNFs) caused a weaker macrophage-dependent inflammatory response than hydrophobic CNFs [38]. Taken together, hydrophilic Ti-H$_2$O$_2$ surfaces may potentially be better osteogenesis-promoting materials, due to the ability to inhibit the production of proinflammatory cytokines and promote anti-inflammatory molecule secretion.

The confirmation that macrophage behavior can be affected by different hydrophilic Ti surfaces prompted us to further explore the possible mechanism underlying the hydrophilicity-dependent inflammatory response to implanted materials. Activation of the NF-κB pathway, a key intracellular regulator of inflammatory signaling, has been known to promote the secretion of proinflammatory cytokines, including TNF-α and IL-1β [30, 31]. In our work, the significantly different levels of TNF-α production on these two Ti surfaces implied that the NF-κB pathway might be critical in the hydrophilicity-dependent immune

response. The NF-κB family consists of five members—p50, p52, p65 (RelA), c-Rel, and RelB—that share an N-terminal Rel homology domain (RHD) [32]. Most of these proteins can form different NF-κB homodimers and heterodimers. Among the heterodimers, p50/p65 heterodimers are considered to be key mediators of gene expression [33]. Therefore, p65 was chosen in present research as the mark for NF-κB expression. It can be seen from immunofluorescence images and western blotting results in our work that more NF-κB p65 was present in macrophages cultured on Ti-polished surfaces than in those cultured on Ti-H$_2$O$_2$ surfaces (Figure 6). Combined with inflammatory cytokines secretion results, we can conclude that NF-κB signaling may be involved in the regulation effects of hydrophilic Ti surfaces on the acute phase inflammation response of macrophage. Conclusively, these findings could provide an approach to optimize the Ti implant materials for desired bone integration and orthopedic applications.

4. Conclusions

In this study, Ti surface hydrophilicity-dependent immune response and underlying mechanism were explored. The results showed that more hydrophilic Ti surface (Ti-H$_2$O$_2$) yielded enhanced cell adhesion, increased proliferation, and less multinucleated cells formation. The hydrophilic Ti-H$_2$O$_2$ surfaces that exhibited the inhibition in the production of pro-inflammatory cytokines and the promotion in the secretion of anti-inflammatory molecules may be more beneficial to serve as potentially osteogenesis-promoting materials. Moreover, it was revealed that hydrophilic Ti surface might be more favorable in attenuating macrophage immune response via NF-κB signaling. These results may provide new insight in surface-designing of novel implant devices.
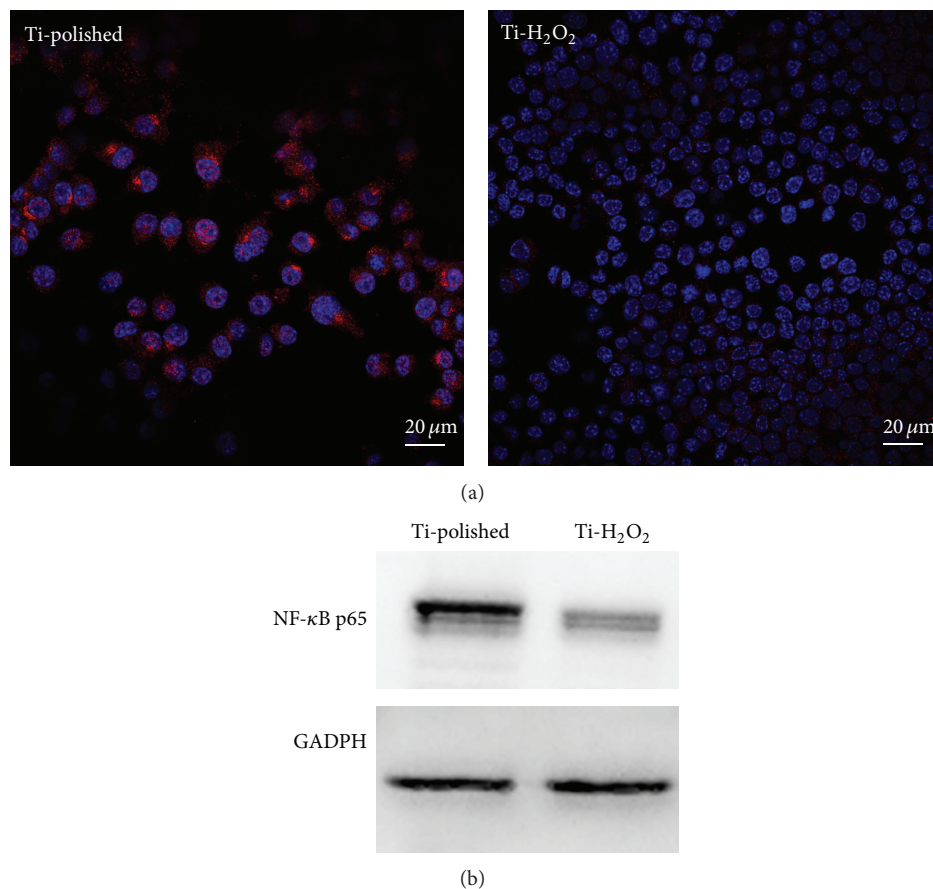
(a)

(b)

FIGURE 6: Immunofluorescence images (a) and western blotting images (b) of NF-κB p65 in macrophage nuclei. NF-κB p65 and cell nuclei were immunostained by Cy3 (red) and DAPI (blue), respectively.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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