Front. Mater. Sci. 2017, 11(4): 318–327
https://doi.org/10.1007/s11706-017-0402-z

RESEARCH ARTICLE

Regulation of RAW 264.7 macrophages behavior on anodic TiO_2 nanotubular arrays

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ABSTRACT: Titanium (Ti) implants with TiO_2 nanotubular arrays on the surface could regulate cells adhesion, proliferation and differentiation to determine the bone integration. Additionally, the regulation of immune cells could improve osteogenesis or lead in appropriate immune reaction. Thus, we evaluate the behavior of RAW 264.7 macrophages on TiO_2 nanotubular arrays with a wide range diameter (from 20 to 120 nm) fabricated by an electrochemical anodization process. In this work, the proliferation, cell viability and cytokine/chemokine secretion were evaluated by CCK-8, live/dead staining and ELISA, respectively. SEM and confocal microscopy were used to observe the adhesion morphology. Results showed that the small size nanotube surface was benefit for the macrophages adhesion and proliferation, while larger size surface could reduce the inflammatory response. These findings contribute to the design of immune-regulating Ti implants surface that supports successful implantation.

KEYWORDS: RAW 264.7 macrophages; nanotopography; TiO_2 nanotubular arrays; inflammation

Contents

Received August 27, 2017; accepted September 25, 2017

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1 Introduction

Titanium (Ti) and its alloys, with favorable mechanical

properties and biocompatibility, were widely used to fabricate implantable devices such as artificial blood vessels, and orthopedic and dental implants [1–2]. To achieve better tissue acceptance or rapid osseointegration during the implantation, the surface topography of Ti implants was modified by various methods [3]. Among those common modified methods, electrochemical anodization known as anodization or anodic oxidation is a well-established surface modification approach for metals to produce bioactive and protective layers [4]. Diameters of nanotubes on the surface could be regulated from 10 to 5000 nm by adjusting the preparation parameters such as voltage, water content, pH of electrolyte, and the anodization time [1]. Studies have demonstrated that the diameter of the nanotubes on the Ti implants could serve as a topography cue to stimulate behavioral variations to both cells and tissues. For the surface energy and protein aggregation, as the main factors to determine cells adhesion behavior, could be regulated by the nanotube size [5–6]. Such as the TiO_2 nanotube surface with small size diameter ($\leqslant$50 nm) was benefit for the adhesion and proliferation of mesenchymal stem cells (MSCs), and the large size ($\geqslant$100 nm) nanotube surface could promote the MSCs differentiation toward an osteoblast lineage [7]. Meanwhile, a number of studies showed that the nanoscale surface topography largely affects bone cell differentiations involving osteoclastic activation and bone-forming activity, indicated that 15 nm is an optical geometric constant of surface-topography-supporting cell adhesion and differentiation [8]. So far, no universal conclusion has been drawn from previous studies on whether there is an optimum size of TiO_2 nanotubular arrays favorable for bioactivity.

While, the stem cells behavior on the implants surface is not the single condition we should consider. Once implanted, all surgical implants will encounter a complicated microenvironment, which may cause the failure of implantation. Frequent early implant failure most often due to the inflammation occurs even for Ti implants, which are the most widely used implant type in the clinic and the most recognized as ideal endosseous implantable devices [9–10]. Thus, to understanding the integration process between the bone tissue and implants is necessary for bone materials design and success of implantation. Generally, following the implantation, the medical devices cause the host body a universal response to the materials, which is an inflammatory response to the implant on the surface. And the intensity of this immediate inflammatory response directly impacts healing around and tissue integration of the implant [11–12]. For the inflammatory response, firstly various proteins from blood and interstitial fluids will adsorb to the devices surface within seconds to form a transient surface matrix. Then an acute inflammation is initiated, that the immune cells such as neutrophils, polymorphonuclear leukocytes and mast cells are active to release chemoattractants and activation cytokines in this phase. This will cause the monocyte recruit to the implant and differentiate into macrophage, which clear cell debris and begin to produce growth factors, chemokines and cytokines. Those released factors can regulate osteogenesis and influence the progression of subsequent both inflammatory and bone healing events. A favorable immune reaction creates an osteogenic microenvironment that can improve osteogenesis, whereas an inappropriate immune reaction may lead to the chronic inflammation and the formation of a fibrous capsule around the implant [13]. Thus, the new generation of bone biomaterials should be able to modulate the local immune environment such that it favors osteogenesis and the osseointegration of the implant.

During the formation of new bone between the materials and bone, the macrophages as one of the first cells to arrive at the tissue/implant interface play an important role in the inflammation and wound healing. For example, the tumour necrosis factor-alpha (TNF-α) secreted by the macrophages at low levels can promote wound healing by indirectly stimulating inflammation and increasing macrophage growth factors, while the high level of TNF-α has a detrimental effect on healing. Recent studies indicate that macrophages have remarkable functional plasticity, and can be regulated by the surface topography properties. Previous studies have revealed that variation of the surface roughness and topography of implants can mediate altered macrophage functions such as survival, adhesion, and secretion [14–16]. For example, the TiO_2 nanotube surface exhibited a significant reduction in pro-inflammatory activity of LPS stimulated RAW 264.7 macrophages with respect to cytokine/chemokine gene expression and protein secretion, and nitric oxide (NO) release, as compared with titanium flat surface [17]. Also, the diameter of the nanotube is a key topography cue influencing macrophage cells inflammatory response. Such as the diameters of TiO_2 nanotube ranging from 30 to 100 nm, the 70 nm showed the weakest inflammatory response [18–19]. While, small size of nanotube (~30 nm) causing less inflammation and inducing healing-associated M2 macrophages polarization *in vitro* was also reported [20].

Considering both the bone cells and immune cells are sensitive to the diameter of implant surfaces, we prepared TiO_2 nanotubes arrays with a wide range diameter from 20 to 120 nm to evaluate the macrophage cells adhesion, proliferation, cellular morphology and cytokine secretion. In this work, electrochemical anodization, the reactive parameter voltage and solution content were adjusted to fabricate TiO_2 nanotubular arrays with different tube diameter (20, 50 and 120 nm). Macrophage-like RAW 264.7 cells were cultured on the Ti-based discs coated with different diameter TiO_2 nanotubes on the surface with/without the LPS pro-inflammatory. The behavior of macrophage cells was evaluated to penetrate the influence of TiO_2 nanotubes diameter with a wide range on the host immune response.

2 Materials and methods

2.1 Fabrication and characterization of TiO_2 nanotubes

Pure Ti discs (purity > 99.7%, ϕ14.5 mm × 1 mm) were used substrates for anodization. All the sheets were polished with SiC papers (from 600 to 2000), and followed by sonication in acetone, ethanol and deionized water for 15 min respectively. Prior to anodization, all the sheets need to be acid-etched in a mixture solution (the volume ratio is $V(HF):V(HNO_3):V(H_2O)$ = 1:4:2) for 3 s, then rinsed with deionized water and dried.

Ti discs were anodized in a two-electrode configuration with Ti sheet as the anode, and Pt electrode as the cathode. For the control of nanotubes diameter, the electrolyte component, voltage and reaction time were regulated. The 20 and 50 nm nanotubular arrays were fabricated in electrolyte with 2 vol.% H_2O and 2.5 g/L NH_4F at 20 and 40 V for 2 h respectively. The nanotubular array with 120 nm diameter was formed in electrolyte with 10 wt.% H_2O and 0.5 wt.% NH_4F at 60 V for 1 h. Moreover, to ensure the diameter of nanotubes, two-step method was chosen for the 50 and 120 nm groups. Firstly, the Ti sheets were anodized for 1 h and 30 min at 40 and 60 V. After the first anodization, the samples were treated by a reverse voltage of 4 V in 0.5 mol/L H_2SO_4 and ultrasounded in distilled water to remove the grown oxide layer. Subsequently, the samples were anodized through the same condition as the first anodization to fabricate a nanotubular arrays on the surface. After that, all the anodized sheets were annealed at 450°C for 2 h.

The morphology of the anodized sheets was observed by field emission scanning electron microscopy (FE-SEM, FEI Nava 450) after being sputter-coated with a layer of Au.

2.2 Macrophage cell culture

Macrophage-like RAW 264.7 cells (China Infrastructure of Cell Line Resources), a murine leukemic monocyte cell line, were thawed and expanded in cell growth medium containing Dulbecco's minimal essential medium (DMEM, Hyclone), 10% fetal bovine serum (FBS, Gibco), and 1% penicillin/streptomycin (Invitrogen), at 37°C in a humidified 5% CO_2 atmosphere. And the cells were seeded at a density of 4000 cells/cm^2 on the Ti-based discs (ϕ14.5 mm × 1 mm) placed in 24-well tissue culture plates, except for the cell viability the cell density was 1×10^4 cells/cm^2. For the pro-inflammatory environment, 1 μg/mL lipopolysaccharide (LPS, Sigma) was added into the cell growth medium. Prior to macrophage seeding, the Ti-based discs coated with different diameter TiO_2 nanotubes on the surface were sterilized by soaking in 75% ethanol overnight. Then, the discs were rinsed three times for 30 min in sterile filtered Milli-Q water, air dried and exposed to ultraviolet light in a sterile 24-well tissue culture plate for 30 min.

2.3 Observation of the morphology

The macrophage cells adhesion morphology on the Ti-based discs with/without LPS pro-inflammatory were observed by SEM. The samples were fixed with 2.5% glutaraldehyde for 2 h, then dehydrated through graded concentrations of ethanol (30%, 50%, 60%, 70%, 80%, 90%, 95% and 100%), and finally critical point dried with CO_2. The cells cultured on the Ti-based discs were imaged with a field-emission SEM (Zeiss, Germany) after being sputter-coated with a layer of Au.

2.4 Cell viability and proliferation assays

Cell proliferation was quantified by Cell Counting Kit-8 (CCK-8, Dojin, Japan) at 2, 4 and 6 d post-seeding. Briefly, cells cultured on tissue culture plate (TCP) as the blank group were incubated with CCK-8 work solution (10% CCK-8 solution in serum free culture medium) for 2 h at 37°C. Afterward, the absorbance was measured at a wavelength of 450 nm and recorded using a multimodal plate reader (EnSpire, PerkinElmer).

The live/dead staining was used according to the manufacturer's instructions to image cellular viability on the tested substrates. After 2 d of culture, samples were washed with phosphate buffered saline (PBS), and then stained with a calcein acetoxymethyl ester (calcein AM): propidium iodide (PI) solution (2 mol/L:10 μmol/L, Dojin, Japan) for 10 min in the dark, at room temperature. Following incubation, the cell-populated discs were washed with PBS, and subsequently, visualized using an inverted fluorescence microscope (Leica, Germany). Images of representative microscopic fields were captured.

2.5 Cytoskeleton morphology study

Cells F-actin fluorescent staining was used to observed the macrophage morphology. After 4 d of cells culture, the cells on the surface of the discs were fixed with 4% formaldehyde at 4°C for 2 h, and washed three times with PBS, then permeabilized in a solution of 0.1% Triton-X-100 for 5 min. Then, after washed three times with PBS, the samples were blocked with 1% BSA in PBS at room temperature for 1 h. After removal of blocking reagent, the samples were stained with F-actin marker rhodamine-phalloidin (Dojin, Japan) at room temperature for 40 min, and then counterstained with 4', 6-diamidino-2-phenylindole (DAPI, Dojin, Japan) for 20 min. Representative images were acquired on a confocal laser scanning microscope (CLSM) (Zeiss LSM 710, Germany).

2.6 Cytokine/chemokine measurements by ELISA

After 4 d in culture, the supernatant was collected, centrifuged, and stored frozen at −80°C until analyses were performed. Commercially available enzyme-linked immunosorbent assay (ELISA) kits (Cusabio, China) were used to determine the concentration of tumor necrosis factor (TNF)-α and monocyte chemotactic protein (MCP)-1 according to package insert instructions. The optical density (OD) was read by a multimodal plate reader (EnSpire, PerkinElmer) and cytokine concentrations (expressed in pg/mL) were calculated for each cytokine according to its related standard curve.

2.7 Statistical analysis

Data are presented as means ± SD. Statistical comparisons were performed using ANVOA, and p-values < 0.05 are statistically significant.

3 Results

3.1 Surface characterization of TiO_2 nanotubular structures

As previous study showed, the morphology of TiO_2 nanotubular arrays could influence the behavior of cells, such as the morphology, viability/proliferation and differentiation. And the TiO_2 nanotubular array thin film on the surface of Ti substrate could be easily obtained by electrochemical anodization with a good control over the morphology. In this work, the parameters of voltage and fluoride concentration were tailored to fabricate various diameter nanotubes. Figures 1(b)–1(d) show SEM images of the 20, 50 and 120 nm TiO_2 nanotubular arrays, and the inserts are the representative reverse side of the nanotubular array film. The self-assembled nanotubular layers were generated by anodizing Ti sheets, and the surface diameter size is consistent to the back. The images show highly ordered, vertically aligned nanotubes with three different pore sizes between 20 and 120 nm. Flat substrates of Ti with a native TiO_2 oxide layer having a chemical composition analogous to that of the TiO_2 nanotube surface were used as control surfaces (shown in Fig. 1(a)).

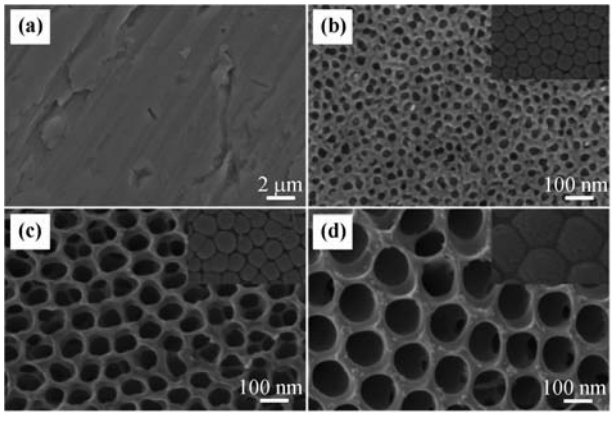

Fig. 1 SEM images of the various sample surfaces (the insert images are the bottom of the TiO_2 nanotubular arrays): **(a)** flat Ti with native TiO_2 oxide layer; **(b)** 20 nm, **(c)** 50 nm and **(d)** 120 nm TiO_2 nanotubular surface.

3.2 Macrophage adhesion morphological features and proliferation

Macrophage was seeded on the Ti-based discs with TiO_2 nanotubular film to investigate the direct interplay between the cells and the surface of the TiO_2 nanotube array. After 2 d cell culture, SEM was used to observe the cell

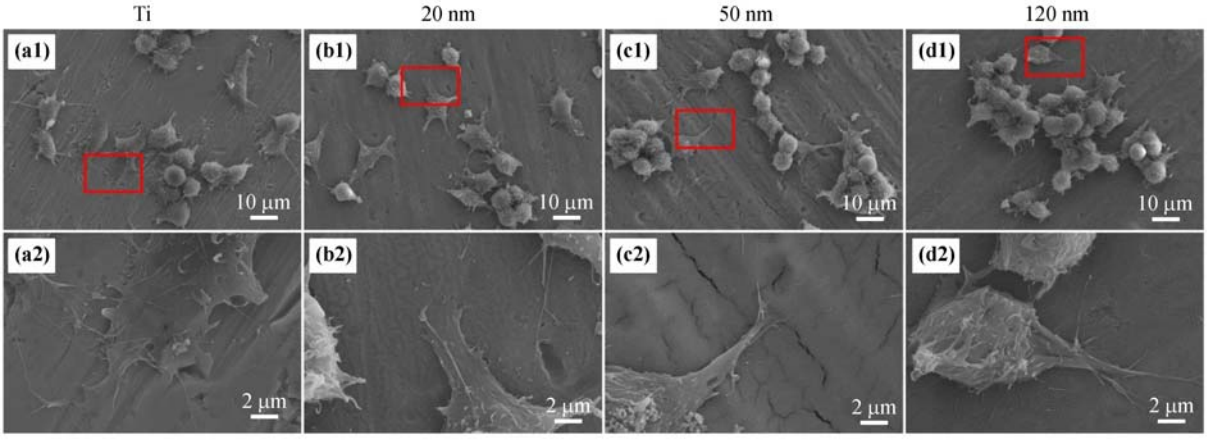

Fig. 2 Typical SEM morphologies of macrophage cells cultured on **(a1)(a2)** flat Ti and **(b1)(b2)(c1)(c2)(d1)(d2)** TiO_2 nanotubular surface with various sizes (20, 50 and 120 nm). The red block area in (a1)–(d1) are magnified in (a2)–(d2).

morphology and attachment on these samples. As shown in Figs. 2(a1) and 2(a2), the macrophage on the substrate of Ti with native TiO_2 oxide layer had a round morphology. Zooming in the edge of the cell, most of the macrophage cultured on the flat Ti discs had a broad and flat boundary. By contrast, the macrophage on the TiO_2 nanotubular film had a smaller size but were more stereoscopic, and with the increase of the TiO_2 nanotube diameter the cell adhesion morphology became more obvious (Figs. 2(b1), 2(b2), 2(c1), 2(c2), 2(d1) and 2(d2)). More importantly, the macrophage on the TiO_2 nanotubular film grew with more elongated pseudopodia, and decreased the adhesion area with the nanotube diameter. These results show that the macrophage exhibit different morphologies when they are cultured on TiO_2 nanotubular film with different surface topographies. Macrophage on a TiO_2 nanotubular film produced more elongated protrusions, and increased with the nanotube diameter.

The CCK-8 assay was further used to evaluate the cell proliferation on these samples quantitatively. As shown in Fig. 3, the macrophage on all the samples have an obvious proliferation tendency from 2 to 6 d, indicating the good biocompatibility of TiO_2 nanotubular film with different surface topographies. After culture for 2 d, there was no obvious difference between the native TiO_2 oxide layer and the TiO_2 nanotubular film for the adhered cells number. While, there are more cells on the native TiO_2 oxide layer than the TiO_2 nanotubular film at 4 and 6 d, which suggests that the nanotubular surface could not promote the self-renewal property of macrophage compared to the flat Ti with a native TiO_2 oxide layer. More importantly, after 6 d of cell culture, the cell number adhered on 20 nm nanotubular film obviously more than 50 and 120 nm,

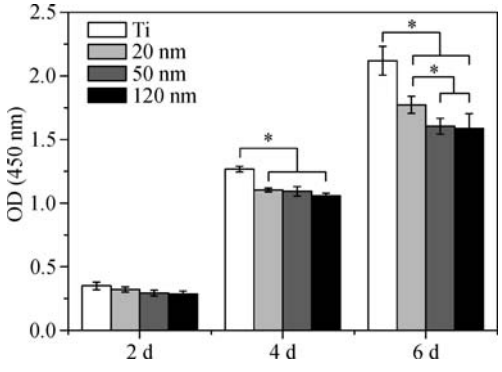

Fig. 3 Macrophage cells adhesion and proliferation on the various sample surfaces by CCK-8 assay. Error bars represent standard deviation for three specimens for each piece of data. * Statistical significance ($p < 0.05$).

which suggests the smaller nanotubular surface could promote the proliferation of macrophage compared to the larger size of TiO_2 nanotube. These results demonstrate that macrophage on the different size of TiO_2 nanotube surface exhibit different adhesion properties, cell morphologies, and self-renewal properties. Considering the interrelation between cell morphology, cell proliferation, and cell differentiation, we hypothesize that the macrophage will have different differentiation behaviors on these nanotubular surfaces.

3.3 Macrophage viability with/without LPS pro-inflammatory

Taking into account that the ability of different size of nanotubular arrays to support cellular survival of adherent macrophage may influence their inflammatory response, the viability of RAW 264.7 cells at 2 d post-seeding with/

without LPS pro-inflammatory were evaluated by live/dead staining. The fluorescence images were showed in Fig. 4, the live cells were labeled green due to ester hydrolysis of calcein AM to calcein, while the dead cells were red for DNA binding of ethidium homodimer in cells with compromised nuclear membranes. Without the LPS pro-inflammatory, the fluorescence images (Figs. 4(a1), 4(b1), 4(c1) and 4(d1)) revealed similar viabilities on analyzed samples, but much lower cell densities were observed on larger size of nanotube surfaces. Thus, microscopic observations are in line with the results of CCK-8 assay showing a decrease in the number of macrophages on the larger size of nanotube substrate. For the LPS pro-inflammatory culture condition, the macrophages show a lower cell density and a larger adhered area compared with the growth medium. Also the results show a lower cell density on the larger size of nanotube surface (Figs. 4(a2), 4(b2), 4(c2) and 4(d2)). For example, on the 50 and 120 nm nanotube surfaces, the adhered macrophages were obviously less than that of the flat Ti with native TiO_2 oxide layer. Notably, macrophages cultured on the 20 nm nanotube surface were similar to those of the control flat Ti discs. Those results proved that the size of the nanotube could affect the macrophage viability with/without LPS pro-inflammatory, and the large size of the TiO_2 nanotubular surface showed lower cell viability.

3.4 Macrophage morphological features with LPS pro-inflammatory

The macrophage morphology could not only reflect the cell adhesion property, but also show the behavior of differentiation. To assess the cellular morphology, fluorescence microscopic visualization of actin cytoskeleton was performed after 4 d of culture with/without LPS pro-inflammatory (Fig. 5). In standard culture condition, the macrophages on different samples showed similar actin morphology, and the cells density was decreased with the nanotube size that was in line with the proliferation results. While, with the stimulation of LPS, macrophages exhibited a markedly increased degree of spreading and extended numerous filopodia. Especially for the flat Ti group after exposure to LPS for 4 d, some macrophage cells became flat and achieved large spread area, and some of the cells became multinucleated as the arrow showed in Fig. 5(a2). While macrophages cultured on the TiO_2 nanotubular surface tended to be round and achieved smaller spread areas. Moreover, with the nanotube size larger than 50 nm, the filopodia was elongated (as shown by arrows), and the cells remained mononuclear without significant change in size compared with the untreated condition.

To further observe the cellular morphology change under the LPS stimulation, SEM was used after 1 and 6 d cell culture (Fig. 6). As researches reported, the morphological changes induced by surface topography are highly related to a cell's function and differentiation status. For the macrophages, the polarized or stretched morphology are considered activated, whereas static oval-like macrophages are considered inactivated [21]. With LPS pro-inflammatory for 1 d, the cell density of macrophages on the flat Ti surface was the highest, and some of the cells were

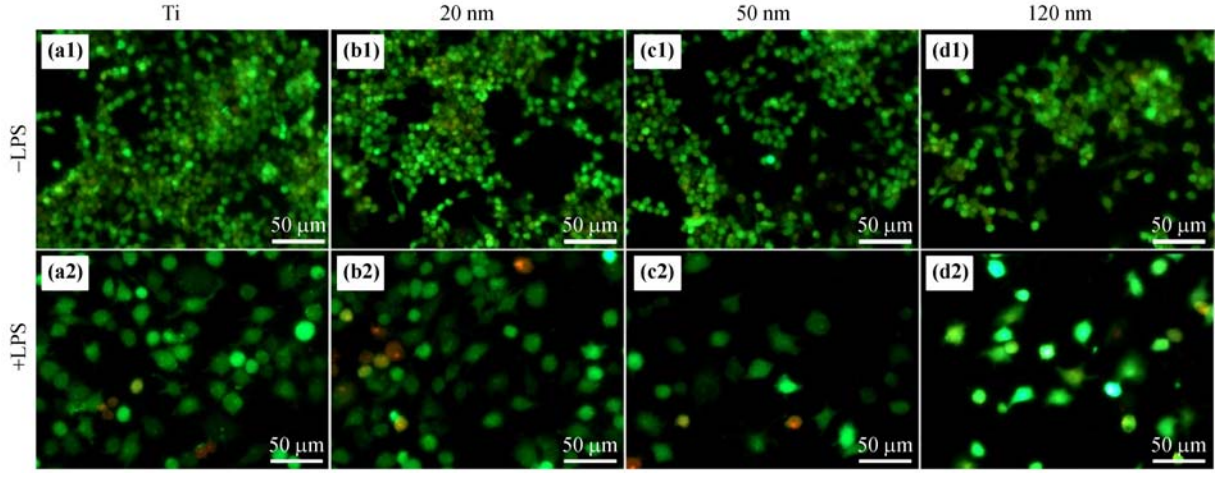

Fig. 4 Fluorescence images showing live/dead assays at 2 d post-seeding with/without the LPS pro-inflammatory. With the LPS stimulation (+ LPS), the live cell density on all the surface (**(a2)** Ti, **(b2)** 20 nm, **(c2)** 50 nm and **(d2)**120 nm) is lower than the standard culture condition (–LPS) on **(a1)** Ti and **(b1)(c1)(d1)** TiO_2 nanotubular surfaces (20, 50 and 120 nm). Green = live cells; Red = dead cells.

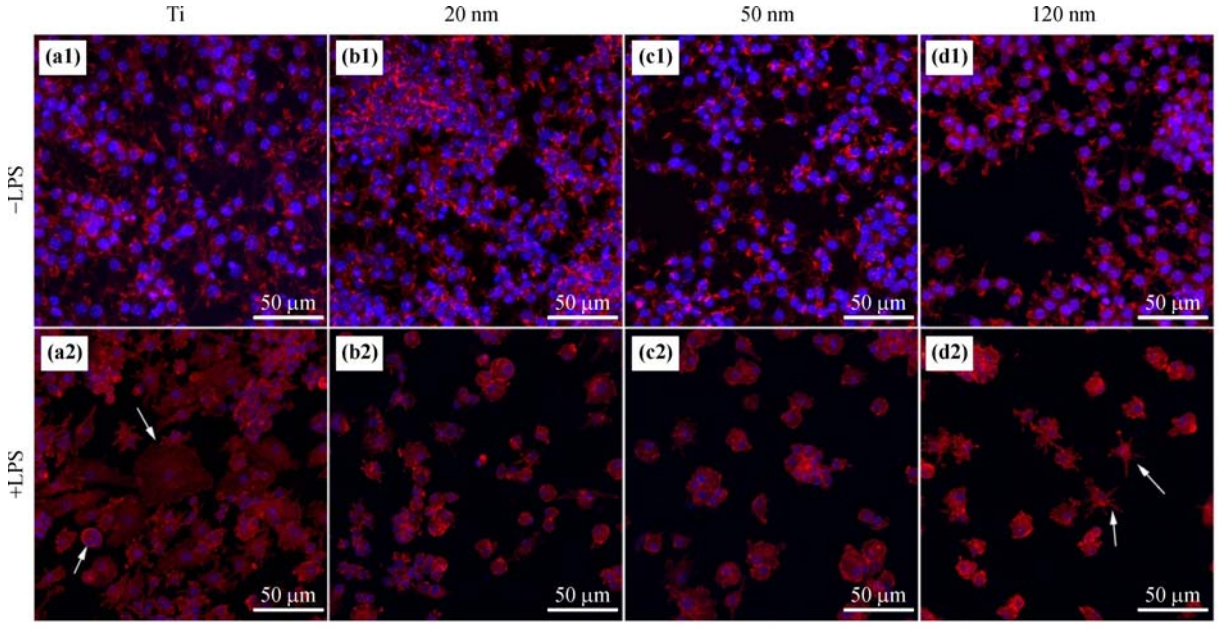

Fig. 5 Fluorescence micrographs of RAW 264.7 cells stained with rhodamine phalloidin (F-actin) and DAPI (nucleus) after 4 d of culture. Comparing to the standard culture condition (–LPS) on various surfaces (**(a1)** Ti, **(b1)** 20 nm, **(c1)** 50 nm and **(d1)** 120 nm), the stimulated cells (+ LPS) on various surfaces (**(a2)** Ti, **(b2)** 20 nm, **(c2)** 50 nm and **(d2)** 120 nm) showed larger spread area.

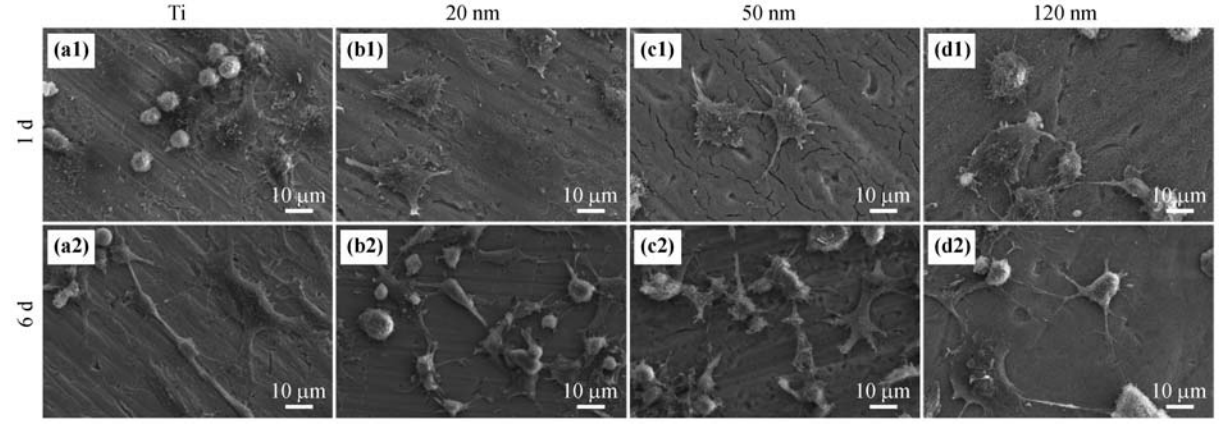

Fig. 6 Morphology of macrophage cells cultured on **(a1)(a2)** flat Ti and **(b1)(b2)(c1)(c2)(d1)(d2)** TiO_2 nanotubular surface with various sizes (20, 50 and 120 nm) with the LPS pro-inflammatory.

spherical and stereoscopic, while some cells were flat spread with large size. On the TiO_2 nanotubular surface, most of the macrophages showed flat spread, and with the nanotube size increase the pseudopodia of macrophage became thinner and stereoscopic. The SEM results showed the morphology of macrophage with the LPS stimulation 1 d were similar with the standard culture condition except the spread size, and did not show obvious differentiation status. After 6 d LPS stimulation, the macrophages cultured on the flat Ti surface stretched and their podosomes were mainly organized into a large belt-like structure at the cell periphery, and some of the adjacent cells fused to form foreign body giant cells (FBGC). On the TiO_2 nanotubular surface, the macrophage morphology showed oval shape without elongated stretch when the nanotube size more than 20 nm. For the 20 nm TiO_2 nanotube surface, there were still some cells stretched to spindle-like shape. The results indicated that the macrophage differentiation status could be affected by the surface structure in 6 d stimulation culture, and especially on the large size TiO_2 nanotube surface (50 and 120 nm) could obviously weaken the macrophage activation.

3.5 Cytokine/chemokine protein secretion

To test the inflammatory response of the macrophages on the TiO_2 nanotubular surface, ELISA analysis was used to test cytokine/chemokine protein (TNF-α and MCP-1) secretion. The cytokine production was normalized to cell number through CCK-8 value, and the levels of cytokines secretion after 4 d culture without the LPS pro-inflammatory were presented in Fig. 7. As shown in Fig. 7 (a), the levels of MCP-1 were lower in the culture media of macrophages cultured on all TiO_2 nanotubular surface than Ti. The amount of TNF-α expressed by macrophages grown on TiO_2 nanotubular surface also lower than the Ti surface, moreover the expression of 50 and 120 nm TiO_2 nanotubular surface were significantly lower than Ti surface. Thus, for the no pro-inflammatory culture condition at 4 d post-seeding, a tendency can be established, indicating that TiO_2 nanotubular surface will probably have a lower chemoattractant potential as compared to control surface, leading to a lower overall inflammatory response. Especially for the large size TiO_2 nanotubular surface (50 and 120 nm) could obviously weaken the inflammatory response, which was in line with the results of LPS pro-inflammatory culture condition shown in Section 3.4.

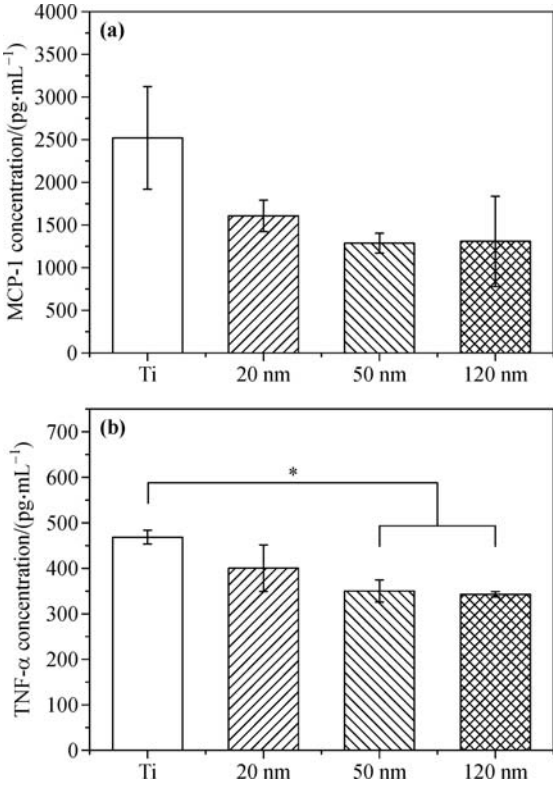

Fig. 7 Mean (± SD) levels of **(a)** MCP-1 and **(b)** TNF-α secreted into culture media after 4 d culture of RAW 264.7 macrophages without the LPS pro-inflammatory, as detected by ELISA. * Statistical significance ($p < 0.05$).

4 Discussion

Cells are able to sense the external material features and integrate physiochemical signals, which eventually result in altered cell behavior [22], e.g., the nanotopography of materials is believed to represent a promising management to precisely control seeded cell function and differentiation [23]. As one of the bone implants, Ti could be modified by anodization to form TiO_2 nanotubes with different diameters that can act as a new set of tools to study systematically the influence of nanoscale geometry on cell behaviors. Studies already have demonstrated that the TiO_2 nanotubular surface is able to mitigate the activation of macrophages by reducing the secretion of the pro-inflammatory mediators that is benefit for the osteogenesis. For TiO_2 nanotubes, the activation of macrophages was shown to depend on the nanotopography, which is the diameter of the nanotubes is the key morphological parameter influencing inflammatory responses. So we fabricated TiO_2 nanotubular arrays with a wide size range from 20 to120 nm to research the effect of nanotube diameter on macrophages behavior. The results showed that small size nanotube surface (20 nm) was benefit for the macrophages adhesion and proliferation, and the larger size surface (50–120 nm) affected macrophages to be less well spread and proliferation in both standard and LPS stimulation medium. All the size of nanotubes surface had lower macrophage activation and decreased levels of cytokine expression, especially for the larger size nanotubular surface had obvious effect in reducing the inflammatory response.

The cell adhesion and proliferation are mainly affected by the surface energy and adsorbed protein density. For the small size of TiO_2 nanotubular surface, nanoscale proteins more easily aggregate at top portion of the nanotube walls to reach a higher density of protein aggregate adsorption. The extracellular matrix (ECM) proteins are required for a cell to adhere to the surface and to be able to spread out, so the macrophages cultured on small size nanotubular surface could more easily attach to the surface for the high population of ECM proteins deposited across the entire surface. While, the large size nanotubular surface shows a lower density of ECM proteins, because there is much more inter proteins aggregate spacing due to the large

pore sizes in the nanostructure. And there was less proteins aggregates adsorption for the macrophages to establish initial contact on the large size nanotubular surface. As a result, the morphology of macrophages on different diameter TiO_2 nanotubular surface showed sharp distinction. The macrophages showed flat spread with large area on small size nanotubes surface, while displayed small size adhesion with large number of stereoscopic pseudopodia when the nanotubes diameter increased.

As studies reported, the morphology or the cell actin changes induced by the topography cue is highly related to a cell's functional and differentiation status [24–25]. For the macrophages, the polarized or stretched morphology are considered activated, whereas static oval-like macrophages are considered inactivated [21]. With the stimulation of LPS, macrophages on nanotubular surface showed less stretched morphology compared with the flat Ti, which demonstrated that the nanotubular surface could reduce the macrophages activation, and the cytokine/chemokine protein (TNF-α and MCP-1) secretion results also proved that the nanotubular surface led to lower inflammatory response. The diameter of nanotubes also affected macrophages activation and inflammatory response. Macrophages cultured on the larger size of nanotubular surface showed more inactivated morphology and less cytokine/chemokine protein secretion, and that was likely for the lower density of serum protein adsorption on the surface.

5 Conclusions

The present work demonstrated that a smaller number of macrophages adhered to TiO_2 nanotubular surface, and weak inflammatory response comparing to the flat Ti with native oxide layer. In the range of 20–120 nm, the small size nanotubular surface was benefit for the macrophages adhesion and proliferation; while, on the larger size surface, macrophages appeared to be less well spread in both standard and LPS stimulation medium. In general, it was proven that TiO_2 nanotubular surfaces had lower macrophage activation, and decreased levels of cytokine expression, especially for the larger size (50–120 nm) nanotubular surface had obvious effect in reducing the inflammatory response. This work proved that the role of nanotopography in dictating inflammatory cell responses and demonstrated that nanotopography can be utilized to control the inflammatory likelihood of medical implants.

Acknowledgements This work was in part supported by the China Postdoctoral Science Foundation (2016M591075) and the Fundamental Research Funds for the Central Universities (2302016FRF-TP-16-001A1).

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