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3-dimensional laser structured scaffolds improve macrophage adherence and antigen-specific response

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Abstract

Biomaterial surface properties are determined by the synergistic effect of the morphology and chemistry. Specific combinations of chemical and topographical cues, similar to those experienced by cells during *in vivo* development, have been shown to control cellular adhesion, migration and function. The aim of the study was to investigate whether 3D micro and submicron laser textured transplantable Si scaffolds with tunable morphology and chemistry could support macrophage adherence, antigen presentation and specific antibody production. The 3D scaffolds could provide useful materials for *in vitro* and *in vivo* applications.

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Keywords: scaffold;macrophages;antigen presentation;adherence;antibody production.

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1. Introduction

The development of an immune response lies on the successful antigen presentation by antigen presenting cells (APCs) and recognition by CD4+ T helper (T_H) cells. The traditional view of antigen presentation states that extracellular antigens are presented by professional APCs as a complex with MHC class II molecules to T_H cells. The generated antigenic peptides are loaded onto MHC class II molecules and transported to the cell surface for presentation to CD4+ T_H cells [1]. Activation of T_H cells represents one of the most important activities of the immune system since it leads to humoral or cellular immunity as well as tolerance. Thus, T_H will stimulate B cells for specific antibody production or Tcyt for target cell killing, while the expression of negative surface markers will suppress both types of immunity to ensure homeostasis of the immune system.

1.1. Antigen presentation

Activation of both the humoral and cell-mediated counterparts of the immune system requires cytokines produced by T_H cells. It is essential for T_H activation to be carefully regulated, since an inappropriate T-cell response to self-components can display fatal autoimmune disorders. To this extend, T_H cells can only recognize antigen as a complex with class MHC II molecules on the surface of antigen-presenting cells [2, 3]. The specialized APCs, which include mainly macrophages, B lymphocytes and dendritic cells, are characterized by two essential properties: expression of surface class II MHC molecules and ability to deliver co-stimulatory signals necessary to T_H-cell activation. Antigen-presenting cells first absorb antigen, either by phagocytosis or endocytosis, and then display a part of that antigen (antigenic epitope) on their membrane bound to a class II MHC molecule. T_H cells recognize the MHCII-antigen complex through a specific T-cell receptor (TCR) and upon activation will trigger maturation of B cells and production of antigen-specific antibody. As a result of this process, each mature B synthesizes and displays antibodies with a single specificity, corresponding to a distinct specificity for a specific antigenic epitope. The mature B lymphocyte is therefore said to be antigen-committed.

1.2. Cytokines and antibody production

Cytokines bind to specific receptors on the membrane of target cells, triggering signal-transduction pathways that ultimately alter gene expression in the target cells. Cytokines were found to regulate proliferation, differentiation and antigen response. Although a variety of cells can secrete cytokines, the two principal producers are T cells and macrophages. The cytokines that are produced from T cells are known as interleukins. Specifically, interleukin-2 (IL-2), which is considered to belong to inflammatory cytokines, regulates the activity of T lymphocytes that are responsible for immunity. It is necessary for growth, proliferation and differentiation towards the 'effector' state of T cells. IL-2 is normally produced by T cells during an immune response. Antigen binding to the TCR stimulates the secretion of IL-2 and the expression of IL-2 receptors (IL-2R). The IL-2/IL-2R interaction then stimulates the growth, differentiation and survival of antigen-specific CD4+ T cells. Interleykin-4 (IL-4), which belongs to the anti-inflammatory cytokines, activates B and T cell proliferation, while differentiating B cells into plasma cells. Antibodies are major components of the immune system. IgG is the predominant isotype found in blood and extracellular fluid controlling infection of body tissues. IgG antibodies are generated following class switch and maturation of the antibody response and thus participate predominantly in the secondary immune response.

1.3. Silicon scaffolds

The transplantable scaffolds to be used herein consist of 3D micro and submicron texturing of biomaterials surfaces produced by ultrafast lasers [4-8]. These are preferred over 2D surfaces since they offer more realistic micro- and local-environment for cell activity. Indeed, 2D surfaces have been shown to alter cell metabolism and gene expression patterns [9]. In addition, it has been shown that 3D micro/nano topography and surface chemistry influence the differentiation and migration of macrophages [10, 11]. The proposed ultrafast laser structuring

presents distinct advantages [12] as it (a) is versatile and material independent (b) is rapid, easily adaptable and scalable through parallel processing and (c) allows the unique possibility for controllable, high resolution features at both the micro- and nano- length scales. The latter is due to the limited size of the affected volume - very close to the diffraction-limited volume. Additional advantages of laser structuring include high fabrication rate, non-contact interaction, applicability to many types of materials and reproducibility. Furthermore, lasers can be easily incorporated to computer-assisted fabrication systems for complex and customized 3D matrix structure design and manufacture. Such systems gave rise to a versatile class of scaffold production techniques which are laser-based solid-free-form (SFF) fabrication techniques. The SFF is essentially a rapid prototyping technique which allows control over macroscopic properties, such as scaffold shape and microscopic internal architecture.

In the present study we ensure optimal macrophage adherence and function on micro-textured 3D Si scaffolds with gradient roughness and ionic forces and define the conditions for antigen presentation and activation of T cells. Seeding the system of macrophage-coated Si scaffolds with T and B cells, we examine their ability for activation and specific antibody production. Such approach could be envisaged as a future transplantable personalized vaccination process, which would also eliminate all side effects due to the non-specific stimulation of adjuvants [13-18].

2. Materials and Methods

2.1. Spleen cell isolation and cell cultures

Upon spleen isolation under sterile conditions, the tissue was single cell suspended in HBSS medium (Gibco BRL, Grand Island, NY). After successive centrifugations at 1200rpm and red cells lysis, the cell pellet was resuspended in RPMI culture medium (Gibco) supplemented with 10% FBS (Gibco) and cultured in 12-well plates (Sarstedt, Numbrecht, Germany) at a concentration of 10,000,000 cells / ml at a final volume of 2 mls in the presence or not of Si scaffold substrates, placed at the center of the well. After 24h of incubation, scaffolds were thoroughly washed and were either subjected to scanning electron microscopy (Scanning Electron Microscopy-SEM) analysis or placed into new plates to exclude cells that had adhered to the plastic and continue the culture for antigen pulsing. In this case, the antigen (human serum albumin, HSA, 20mg/ml, Sigma-Aldrich Co., MO, USA) was added to the cultures and after 24h of incubation T and B lymphocytes were added to the cultures ($6.5x10^6$ cells/ml). T and B lymphocytes were isolated from whole spleen cells upon removal of macrophages with magnetic beads bound with anti-CD11 antibody (EuroBioSciences GmbH, Friesoythe, Germany). After 4 or 7 days of incubation the scaffolds were either submitted to SEM or confocal microscopy analysis. Culture supernatants were tested for the presence of cytokines and antigen-specific antibody. All cells were grown in an incubator Forma Scientific at 37 º C in the presence of 5% CO2.

2.2. Silicon scaffolds

The micro-structure of the planar Si surfaces was irradiated using a femtosecond (fs) laser under pressure in the presence of reactive gas (SF_6). The different topologies of the surface were obtained by varying the density of laser radiation. Thus, silicon crystals were subjected to laser radiation in a vacuum chamber at a pressure 10-1 mbar using a rotary pump, which was then filled with active gas (SF6). The laser beam was focused through a quartz lens to the silicon surface, in a direction perpendicular to the sample. The sample was positioned in the chamber on XY moving plates, which allowed movement of the sample in 1mm step. The laser radiation source emitted 150 fs pulses at frequency 1 kHz. Three different power densities laser (0.34 J/cm2 - 1.69 J/cm2) were tested using an attenuator. The samples were moving with high precision at the X-Y translational level, normal to the instantaneous laser beam. After the irradiation, the samples were cleaned in ultrasonic baths of trichlorethylene, acetone and methanol, followed by washing in 10% HF aqueous solution to remove the acid developed on the surface. Upon irradiation, the laser structured surfaces were morphologically characterized by contact angle measures and SEM analysis.

2.3. Enzyme-Linked ImmunoSorbent Assay, ELISA

ELISA experiments were performed as previously described [19]. Briefly, for cytokine detection, culture supernatants were coated to 96-well flat-bottom plates and reacted anti-IL-2 or anti-IL-4 primary antibodies (1/100 dilution; Immunotools). The reaction was developed using an anti-mouse IgG coupled to horse radish peroxidase secondary antibody (Santa Cruz, CA, USA). For the detection of HSA-specific antibodies, ELISA plates were initially coated with HSA and thereafter reacted with culture supernatants, followed by addition of the secondary anti-mouse IgG coupled to horse radish peroxidase antibody. Qnatification was performed by comparing the obtained values to standard curves, produced with known amounts of commercial cytokines and IgG antibodies (Immunotools).

2.4. SEM

After culture termination, scaffolds were washed with 0.1 M sodium cacodylate buffer (SCB) and then incubated in the same solution for 15 min. After repeating this step twice, scaffolds were fixed using a 2% glutaraldehyde, 2% formaldehyde in 1% SCB fixative buffer for 1 h at 4 C. All surfaces were then washed twice (from 15 min each time) with 1% SCB at 4°C, dehydrated using serially graded ethanol immersions (from 50% to 100%) and incubated for 15 min in dry 100% ethanol. Prior to electron microscopy examination the samples were sputter coated with a 10 nm gold layer. SEM analysis used a JEOL 7000 field emission scanning electron microscope with an acceleration voltage of 15 kV.

2.5. Immunofluorescence

Upon culture termination, scaffolds were fixed with 4% paraformaldehyde, washed and reacted with mouse anti-mouse CD90-FITC coupled antibody (Serotec, Oxford, UK) and CSFR polyclonal antibody (Antibody, Assay biotech, Sunnyvale, CA), developed using an anti-rabbit coupled to PE secondary antibody (Serotec). Confocal microscopy analysis was performed using a 'Zeiss AxiosKop 2 plus' laser scanning confocal microscope. .

3. Results

3.1. Macrophage adhesion and morphology upon culture on 3D Si scaffolds

Using oxidized or non-oxidized Si scaffolds (oxidation increases scaffold hydrophilicity) irradiated with three consecutive energy intensities (0.68 J/cm^2, 0.96 J/cm^2 and 1.5 J/cm^2), the ability of macrophage adherence and the effect of substrate roughness to cell morphology was estimated. The results showed that macrophages preferred either non oxidized low energy substrates or oxidized high energy structures, as evaluated by cell number counting (Fig. 1A).

However, cell morphology was also affected by scaffold roughness. Macrophages are adherent cells, a property that enables them to environmental stability, functionality and effective engagement in the processes of antigen presentation. Scanning microscopy showed that the substrates displaying greater adhesion points, facilitated adherence and cells displayed a healthy morphology similar to that expected in a natural environment. At low roughness (0.68 J/cm^2), macrophages exhibited excellent morphology, showing rounded cell bodies cytoplasmic extensions spread in a three-dimensional environment (Fig. 1B, 1C).

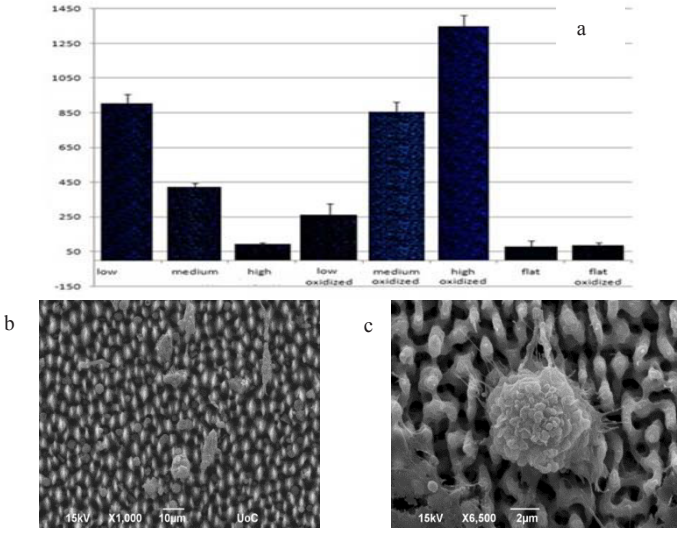


Figure.1 (a) Number of cells/unit area; (b) cell morphology in low roughness and (c) in high roughness non-oxidized surfaces.

3.2. Functionality control of macrophages for antigen presentation and immune response development

The functionality of macrophages was tested through their ability to activate an antigen-specific response. For this reason, scaffolds of single roughness (0.68 J/cm^2 and 1.5 J/cm^2) oxidized or not, were used. Upon adherence, macrophages were pulsed for 24h with HSA and after removal of the antigen, virgin T-and B-lymphocytes (6.5x10^{6}cells/culture) were added in the culture and incubated for 4 or 7 days.

3.2.1. Morphology and interactions of macrophages and T-cells on scaffolds

After the 7-day culture, the non-oxidized surfaces exhibited better supported cell growth, as assessed by SEM analysis. In the presence of T-lymphocytes, macrophages showed significant activation and vigorous secretory vesicle production from both types of cells was observed (Fig. 2).

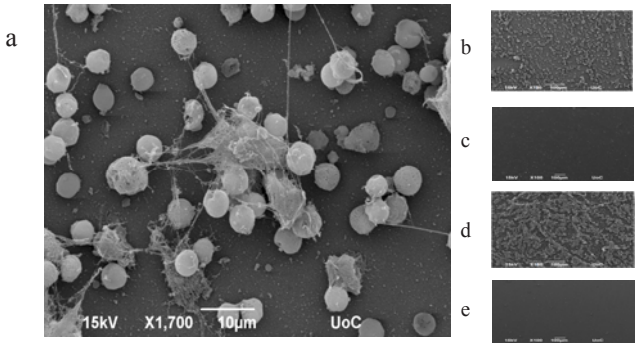


Figure2. (a) Illustration of the immune response on the silicon scaffolds ;(b, c, d, e) Adhesion of macrophages and T lymphocytes during the immune response in the substrates of all asperities, oxidized or not, after 7 days of culture

3.2.2. Identification of cell populations attached to the surface of the scaffolds

The functionality of macrophages was tested through their ability to activate an antigen-specific response. Interaction of T-cells with macrophages was initially visualized by double fluorescence staining with CSFR

(colony stimulating-1 receptor specifically expressed on macrophages) and CD90 (specifically characterizing T cells) specific antibodies. Confocal microscopy analysis identified macrophages (labeled with green fluorescent substance) to interact with T cells (labeled with red fluorescent substance), where T cells were either shown to surround macrophages or to be deposited on their surface (Fig. 3). Functional analysis of the developed system was assessed by cytokine and specific antibody production by ELISA. The cytokines tested in here were interleukin 2 and interleukin 4. These cytokines are considered to be important indicators of T lymphocyte activation in the course of a humoral immune reaction. Therefore, their detection in the culture supernatant could be a proof for a successful immune response development, demonstrating the effectiveness of the system and the prospect of using a three dimensional environment in cell growth and function. As depicted in Fig. 4, IL-2 production was higher after 7 days of culture using the high roughness oxidized or non-oxidized scaffolds, while IL-4 production was predominately activated by the non-oxidized low roughness scaffolds equally well after 4 or 7 days of culture. Antigen-specific antibody production, which is the most valuable evidence for immune response development was detected in all culture supernatants collected from low or high roughness substrates after 4 or 7 days of culture (Fig. 5). As expected higher amounts of specific antibody production was obtained after 7 days of culture.

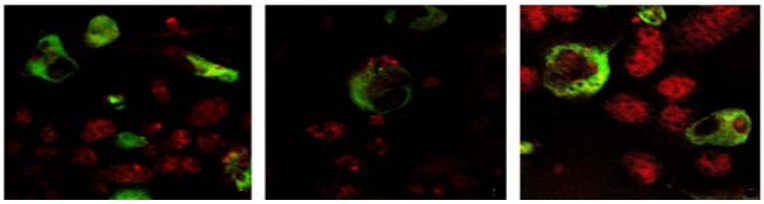
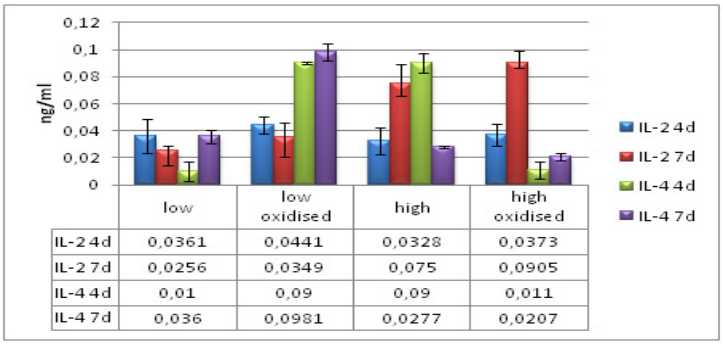
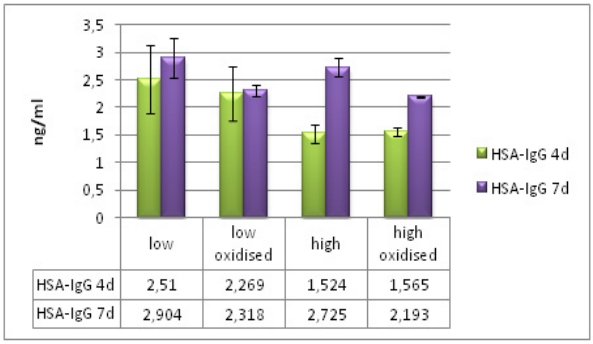


Figure.3 Confocal microscopy analysis. Macrophages are shown in green and T lymphocytes in red fluorescence.



	low	low oxidised	high	high oxidised
IL-2 4d	0,0361	0,0441	0,0328	0,0373
IL-2 7d	0,0256	0,0349	0,075	0,0905
IL-4 4d	0,01	0,09	0,09	0,011
IL-4 7d	0,036	0,0981	0,0277	0,0207

Figure 4 Quantification of interleukins detected in the culture supernatants.



	low	low oxidised	high	high oxidised
HSA-IgG 4d	2,51	2,269	1,524	1,565
HSA-IgG 7d	2,904	2,318	2,725	2,193

Figure5. Quantification of anti-HSA antibody detected in the culture supernatants.

4. Conclusions

Since the mechanisms of cell adhesion have been studied, the role of tissue spatial architecture in cell function was confirmed. In addition, material properties, such as surface chemistry, energy and morphology were recognized as key factors in controlling the assembly of cells in three-dimensional features. Therefore, the ability to adjust morphology and surface chemistry is considered to be very useful in the study of surface models including three-dimensional micro-nano structures on cell behavior and growth. In this perspective, the present study concentrated on the role of hierarchically structured silicon surfaces fabricated using fs laser pulses on the development of an immune response. The intensity modulation of the laser causes significant changes in the morphological characteristics of micro-cones such as height and roughness. Therefore, the produced substrates provided three-dimensional spaces providing culture conditions closer to those of the natural environment facilitating thus cell adhesion and proliferation. Moreover, the possibility of controlled shaping of surface wettability properties allowed modification of the surface chemistry, supporting in a better way cell survival and function. It has been shown that the growth of fibroblasts was promoted in hydrophilic low roughness substrates, allowing the cells to take advantage of this contact surface acquiring force, which benefits adhesion and spreading. However, adhesion was shown to be almost impossible onto ultra-hydrophobic or high surface roughness substrates [19]. It has been shown that after oxidation the hydrophobic high roughness substrate is converted to hydrophilic, allowing proliferation and viability of nerve cells (unpublished observations). In experiments using neural cell lines, the cells tend to generate attachments in areas where there a balance of forces necessary for the cytoskeleton can be generated. Therefore, the wettability of the surface is critical as it describes to what extent the surface is exposed to the culture medium and thus the protein absorption. Decrease in absorbance, resulting in reduced protein coating on the surface may adversely affect the ability of cells to create attachments. In any case it is generally accepted that surface energy affects the contact area of the cell membrane to the substrate, while the profile of the membrane could also change the wettability of the adjacent solid [20-22]. The structures of silicon treated with laser to form micro-and nano-cones, with ability to control the roughness and surface chemistry can serve as a new approach for studying the dimensional interactions with biomaterials *in vivo*. It has been shown that using such techniques, substrate wettability can be modified from hydrophobic to hydrophilic by independent control wettability and chemistry. Studies on fibroblasts showed that the fundamental parameters certifying cell adhesion to 3D structures include cooperation of roughness and surface chemistry, both needed to determine wettability and surface energy of the substrate. The design of Si scaffolds is crucial as it affects cell adhesion and provides tools to micro and nano scale, exploring thus the complex interactions of anchored cells within the *in vitro* environment. The hierarchical roughness should provide opportunities for orientation and proliferation of cells, while the holes between the interim cones should provide space for remodeling tissue structures.

The present work focused on the adhesion and function of immune cells involved in antigen presentation, namely macrophages and T and B lymphocytes. The ultimate goal of this work was to find the appropriate conditions of macrophage adhesion and development of an antigen-specific immune response on 3D Si scaffolds. Low and high roughness 3D Si substrates in an oxidized or non-oxidized form were tested for their ability to support macrophages adherence and growth. The results showed that macrophages prefer low roughness, non-oxidized surfaces or high roughness, oxidized surfaces. The fact that untreated flat scaffolds failed to support cell growth led to the conclusion that laser-decorated 3D surfaces create a favorable milieu for adhesion and cell growth. Antigen pulsing and addition of T and B lymphocytes to 3D-scaffolf absorbed macrophages activated cytokine and specific antibody production, demonstrating the development of a direct immune response. The results showed that the non-oxidized, low roughness (0.68 J/cm^2) surfaces were the best structures for IL-2, IL-4 and antigen-specific antibody production, the levels of which reached the concentration of 4.8 ng /ml.

Scanning electron microscopy analysis allowed mapping the detailed interaction of T lymphocytes with macrophages, where T lymphocytes were shown to debouch projections interacting with the surface of macrophages, probably during the process of antigen presentation. Such technology could be useful in generating a transplantable *in vivo* immune response. Preliminary results show that Si scaffolds are able to induce immunostimulation when transplanted in mice, indicating that the present work could open new areas of research in vaccine technology. The activation of an antigen-specific immune response without the need of adjuvants will

provide the possibility for natural selection of the antigenic epitope *ex vivo* and upon transplantation development of the specific immune response *in vivo*.

Acknowledgements

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