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Implantable vaccine development using *in vitro* antigen-pulsed macrophages absorbed on laser micro-structured Si scaffolds

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ABSTRACT

To overcome the limiting antigenic repertoire of protein sub-units and the side effects of adjuvants applied in second generation vaccines, the present work combined *in vitro* and *in vivo* manipulations to develop biomaterials allowing natural antigen-loading and presentation *in vitro* and further activation of the immune response *in vivo*. 3-dimensional laser micro-textured implantable Si-scaffolds supported mouse macrophage adherence, allowed natural seeding with human serum albumin (antigen) and specific antibody and inflammatory cytokine production *in vitro*. Implantation of Si-scaffolds loaded with antigen-activated macrophages induced an inflammatory reaction along with antigen-specific antibody production *in vivo*, which could be detected even 30 days post implantation. Analysis of implant histology using scanning electron microscopy showed that Si-scaffolds could be stable for a 6-month period. Such technology leads to personalized implantable vaccines, opening novel areas of research and treatment.

1. Introduction

In order to avoid virulence and infectivity of vaccines, novel technologies have replaced bacterial or viral inactivation with sub-unit vaccines. Highly purified antigenic peptides or their corresponding DNA are usually emulsified into adjuvants and used for vaccination. Although such approaches eliminate infectivity of the pathogen itself, non-specific side effects of the adjuvants *cannot* be safely monitored yet. On the other hand, small antigenic peptides *cannot* be immunogenic by themselves and therefore the use of adjuvants is obligatory for the initiation of an inflammatory reaction that could increase chances for antigen presentation. Furthermore, small antigenic peptides have limited affinity for complexion with MHC class II antigens for presentation. Because of the extended polymorphism of MHC in the human race, only a limited number of MHC polymorphisms will display the right affinity for the provided antigenic epitopes to proceed to specific immune stimulation.

The vision towards an antigen-specific immune stimulation *in vivo* would be to find a way to trigger T-helper cells without the need of adjuvants. During a natural infection, because of the size and the complexity of the antigen (immunogenic), the organism develops a polyclonal immune response without the need of external manipulation. The idea developed in the present study was to allow antigen presentation to occur *in vitro* on implantable substrates, which upon implantation *in vivo* could stimulate specific immune response. The advantages of the *in vitro* antigen presentation process in case of a pathogen would be first, to allow natural epitope selection for loading onto self-MHC and second to avoid infectivity of the pathogen, since whole attenuated or dead organisms could be provided to the culture for a short period of time, necessary for antigen presentation and thereafter cleared out from the culture. Furthermore, the advantage of using an implantable biomaterial would be first, to provide a focal nest of antigen-loaded APCs capable to trigger a specific immune response and second, the material itself could cause a systemic inflammatory response [1] attracting immune cells to the implantation site.

The implantable scaffolds used herein consisted of 3-dimension (3D) micropatterned silicon (Si)-based substrates with tunable morphology and chemistry, which have been successfully used *in vitro* for fibroblast and nerve cell growth [2–4] and are preferred versus 2D surfaces because they provide more usable area and adhesion points for cell growth [5,6]. The choice of a so far

Abbreviation: HSA, human serum albumin; FCA, Freund complete adjuvant; SEM, scanning electron microscopy; WBC, white blood cells.

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considered hard, non-biodegradable material was essential in order to avoid any side effects of degradable bio-products. In the present work, 3D micro and submicron texturing of Si surfaces were produced by ultrafast lasers [2], which provide reproducible patterned areas with controlled hydrophilicity, while providing the opportunity for chemical modifications.

2. Materials and methods

2.1. Animals

BALB/c inbred mice were purchased from Charles River (Milan, Italy) and bred in the animal facility of the Department of Biology at the University of Crete (Crete, Greece). Males 4–8 weeks of age were handled according to the international and national bioethical rules and conformed to the bioethics regulations of the University of Crete, approved by the Animal Facility responsible officer of the Department of Biology. For serological and cellular analyses tail bleeding was performed. Mice were sacrificed by cervical dislocation and spleens were removed under antiseptic conditions.

2.2. Spleen cell cultures

Upon elimination of red cells, spleen cells were washed, resuspended in RPMI culture medium (Gibco BRL, Grand Island, NY) supplemented with 10% FBS (Gibco) and cultured in 12-well plates (Sarstedt, Numbrecht, Germany) at a concentration of 10^7 cells/ml at a final volume of 2 ml in the presence or not of Si scaffold substrates ($5 \times 5\,mm^2$), placed at the center of the well. After 24 h of incubation, scaffolds were thoroughly washed and either subjected to scanning electron microscopy (SEM) analysis or transferred to new plates to exclude adherent cells and continue the culture for antigen pulsing. In this case, human serum albumin (HSA, 20 μg/ml, Sigma-Aldrich Co., MO, USA) was added to the cultures for 24 h. After washing the antigen away, lymphocytes, isolated from whole spleen cells upon removal of macrophages with magnetic beads bound with anti-CD11 antibody (EuroBio-Sciences GmbH, Friesoythe, Germany) [7], were added to the cultures (6.5×10^6 cells/ml). 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.

2.3. Silicon scaffold preparation

Planar Si surfaces $5 \times 5\,mm^2$ were irradiated using a femtosecond (fs) laser under pressure in the presence of reactive gas (SF_6) as previously described [3]. The different surface topologies were obtained by varying the density of laser radiation including $0.68\,J/cm^2$ (low roughness), $0.96\,J/cm^2$ (medium roughness) and $1.5\,J/cm^2$ (high roughness). In some experiments, all three laser energies were included on the same $5 \times 5\,mm^2$ scaffold separated from each other with a narrow flat surface.

2.4. Scaffold implantation

Scaffolds loaded or not with antigen-activated macrophages were implanted to the left rear foot of anesthetized mice (Avertin, Sigma), sutured using 3-0 silk suture material (Deme TECH, USA). Tail bleeding was performed before and after implantation. In some experiments, implants were surgically excised 7, 14, 21 or 30-days later and animals were sacrificed 2 weeks after implant excision. Implanted animals were also followed up to 7 months and sacrificed thereafter. Implant histology was evaluated by SEM analysis. For classical immunization protocols, animals were injected intraperitoneally with 100 μg HSA emulsified into 100 μl Freund's complete adjuvant (FCA, Sigma), while control groups received only the emulsified FCA solution.

2.5. Enzyme-linked immunosorbent assay, ELISA

ELISA experiments were performed as previously described [7]. Briefly, for cytokine detection, culture supernatants (1:2 dilution) or serum (1:1000 dilution) were coated to 96-well flat-bottom plates and reacted anti-IL-2, anti-IL-4, anti-IL-10, anti-TNFα or anti-IFN-γ primary antibodies (1/100 dilution; Immunotools, Friesoythe; Germany). 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 antibody. Quantification was performed by comparing the obtained values to standard curves, produced with known amounts of commercial cytokines and IgG antibodies (Immunotools).

2.6. SEM analysis

Upon culture termination, scaffolds were washed with 0.1 M sodium cacodylate buffer (SCB) and then incubated in the same solution for 15 min, twice. Samples were fixed using a 2% glutaraldehyde, 2% formaldehyde in SCB fixative buffer for 1 h at 4 °C. All surfaces were washed twice (from 15 min per time) with SCB 0.1 M at 4 °C, dehydrated using serially graded ethanol immersions (from 30, 50, 70, 90 to 100%) and incubated for 15 min in dry 100% ethanol twice. The samples were critical point dried (Bal-Tec CPD 030), and mounted on appropriate stubs and sputter coated (Bal-Tec SCD 050) with a 15 nm gold layer prior to observation. SEM analysis was processed with a JEOL JSM 6390LV Scanning Electron Microscope operated at 15 kV and for high definition analysis a JEOL FESEM 7000F field emission scanning electron microscope with an acceleration voltage of 15 kV.

2.7. Immunofluorescence

Upon culture termination, scaffolds were fixed with 4% paraformaldehyde, washed and reacted with anti-mouse CD90-FITC coupled antibody (Serotec, Oxford, UK), colony stimulating factor-1 receptor (CSFR) polyclonal antibody (Antibody, Assay biotech, Sunnyvale, CA) or Mac-1 (Serotec) 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. In other experiments, samples were washed with PBS and mounted in coverslips with antifade reagent containing DAPI for the staining of the nuclei (ProLong® Gold reagent, Molecular Probes). Cell imaging was performed using an epifluorescence microscope coupled to a high resolution "CarlZeiss, Axiocam" color camera. Cell number quantification was assessed using an image processing algorithm (ImageJ; National Institutes of Health, Bethesda, MD).

2.8. Statistical analysis

Paired *t*-test was employed in order to compare the significance levels (p) between control and test values. Statistical analysis was performed using the Origin Pro 8 program.

3. Results and discussion

Vaccine development is still in the first line of research interests and is considered as a long and complex process to achieve. Difficulties lie on pathogen infectivity, sub-unit antigenicity, adjuvant safety and related side effects. To overcome these problems,