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Laser micro-structured Si scaffold-implantable vaccines against *Salmonella Typhimurium*

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ABSTRACT

Salmonella Typhi is responsible for typhoid fever in humans. Despite the efforts, the development of long-lasting vaccines has failed and the available vaccines display only moderate activity, being considered as "international traveler's" vaccines. Taking advantage of the previously described implantable vaccine technology consisting on 3D laser-microstructured Si scaffolds loaded with antigen-seeded macrophages, the present study aimed to apply an antigenic stimulus of whole extracts of *S. Typhimurium*, which is the mouse analogue of the human Salmonella Typhi, and examine its ability to mount specific antibody response. After defining the experimental conditions for specific anti-*S. Typhimurium* IgG production *in vitro*, antigen-seeded macrophages loaded onto the 3D Si-scaffolds were implanted to mice, while parallel experiments used conventional Freund-complete-adjuvant vaccination protocols. The results showed that only the implantable vaccine protocol could mount a specific antibody response 14 days after implantation. The cytokine profile showed increase of IL-10 and IFN-γ in the case of implantable and conventional vaccination respectively, 7 days after implantation. Morphological studies on the excised scaffolds 14 days after implantation, showed the development of a well-structured adherent monolayer, establishing multiple contacts with lymphocytes in favor to immune response development. Based on the hypothesis that both stimulatory and suppressive components in the vaccination preparation, could affect the overall activity, peptidoglycan was applied as an antigen to the vaccination protocols. Surprisingly, peptidoglycan was shown to induce a mitogenic rather than specific immunogenic response. In this case, histological analysis of the excised scaffolds showed a restricted layer of adherent cells with cytoplasmic extensions, but hard to distinguish cell contacts with lymphocytes. Finally, the presented results showed a differential behavior of antigen presenting cells in accordance to the antigenic stimulus and consequently the activation state of the cells. Tailoring the micro/sub-micron 3D structures and chemistry of Si scaffolds, could control cell behavior according to the user's needs.

1. Introduction

Salmonella belongs in the family of Enterobacteriaceae and is a prototype Gram-negative intracellular bacterial pathogen, which lives as saprophyte or parasite within eukaryotic cells. Typically, *Salmonella* infects individuals through food consumption or drinking water and transmission uses the fecal-oral root, where it invades intestinal epithelial cells in the distal ileum. It infects many different animal hosts where the majority of diseases in animals and humans are caused by *Salmonella enterica* subspecies and range from local gastroenteritis to death [1].

Since typhoid fever, which is caused by serovar Typhi and Paratyphi is manifested in >20 million cases and accounts for more than 200,000 deaths per year [2], it has been a great interest in vaccine development. Attenuated whole cell vaccines have been replaced by an orally administered attenuated strain (Ty21a) and parenteral Vi capsular polysaccharide antigen which are mostly considered as traveler's vaccines due to their moderate efficacy and duration [3]. The need of in-depth study of host-pathogen interactions during Typhi infection turned scientists in defining animal experimental models. Although serovar Typhi only infects humans, *Salmonella typhimurium*-infected mice display many

Abbreviations: FCA, freund complete adjuvant; SEM, scanning electron microscopy; PG, peptidoglycan; IL-2, interleukin-2; IL-10, interleukin-10; IFN-γ, interferon-gamma; TNF-α, tumor necrosis factor-alpha; HSA, human serum albumin.

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similar characteristics to typhoid fever in humans in terms of lesions in internal organs and bacterial tissue distribution.

Although innate and acquired immunity to Salmonella infection, mainly based on the *Salmonella typhimurium* mouse models, have been extensively studied [4], it seems that innate protection overcomes immune specificity. After clearance of primary infection, mice although developing robust protective immunity to secondary challenge, this could not be transferred to naive mice by adoptive transfer of spleen cells, but required the addition of immune serum [5].

In most cases bacteria are removed by phagocytes, but *Salmonella typhimurium* can survive in phagocytes due to his type III secretion system [6–8]. In addition, it has been shown that peptidoglycan, which is the major morphogenetic element of the bacterial cell envelop, in intracellular residing *S. typhimurium* could trigger massive release of lipopolysaccharide to vesicles in the host-cell cytosol [9], which could favor mitogenic instead of antigen –specific response.

The need to overcome the side effects caused by adjuvants and the limited number of antigenic epitopes of the second-generation vaccines has prompted us to develop 3-dimensional (3D) laser micro-textured Si scaffolds, which allow natural antigen-loading, antigen presentation *in vitro* and further activation of the immune response *in vivo*. Previous studies have shown that these structures supported mouse macrophage adherence, allowed natural seeding with human serum albumin (HSA; antigen) and specific antibody and inflammatory cytokine production *in vitro* [10]. 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 [11]. The implantable scaffolds consist of 3D micro and submicron texturing of biomaterials surfaces produced by ultrafast lasers [12,13], and have been shown to influence the differentiation and migration of macrophages [14].

In the context of the 3D-Si scaffold technology, the present study focused on development of specific immune response to *S. typhimurium* and peptidoglycan. After defining antigenicity of the two agents *in vitro*, appropriately functionalized 3D-Si scaffolds were implanted to mice and tested as to the production of specific antibody and cytokines. The results demonstrated that the applied technology could indeed induce significantly higher and longer lasting response as compared to typical vaccination against *S. typhimurium*, while demonstrating a mitogenic effect of peptidoglycan.

2. Materials & methods

2.1. Animals

BALB/c 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, EL91-BIObr-09). Males 2–4 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 analyses tail bleeding was performed.

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 10^7cells/ml at a final volume of 2 ml in the presence of Si scaffold substrates (5×5 mm^2), placed at the center of the well. After 24 h of incubation, scaffolds were thoroughly washed and transferred to new plates to exclude adherent cells and continue the culture for antigen pulsing. In this case, *Salmonella typhimurium* fragments or peptidoglycane were added to the cultures. After 24 h of incubation T and B lymphocytes, isolated from the total spleen cell population, were added to the cultures (6.5×10^6 cells/ml). After 4 or 7 days of incubation the scaffolds were submitted to scanning electron microscopy (SEM) analysis and culture supernatants were tested for the presence of total or antigen-specific IgG antibody. All cells were grown in an incubator Forma Scientific at 37 °C in the presence of 5% CO_2.

2.3. Silicon scaffold preparation

Planar Si surfaces 5×5 mm^2 were irradiated using a femtosecond (fs) laser in the presence of reactive gas (SF6) as previously described [11]. The surface topology was obtained using a laser radiation density of 0.68 J/cm^2 (low roughness).

2.4. Salmonella Typhimurium

Salmonella enterica serovar *Typhimurium* (strain ATCC14028) were grown in medium L.B. (Lysogeny Broth) for 16 h at 37 °C. One ml of the resulting culture was centrifuged using a microcentrifuge at 1792 g for 4 min. The pellet was resuspended in 1 ml PBS and the optical density at 600 nm (OD_{600}) was calculated in order to obtain a value of 0.2 which corresponded to a bacterial concentration of 3.2×10^8 CFUs/ml. The preparation was further diluted in PBS to reach the concentration of 10^8 CFU/ml. Bacteria were heat shocked in a heating block (Bioblock Scientific, Fisher) for 10 min at 100 °C and stored at −20 °C. This preparation was added to the cultures at a concentration 3×10^6 CFUs/ml.

2.5. Peptidoglycan

Peptidoglycan (PG; 3 mg; SIGMA) from the Gram positive bacterium *Micrococcus luteus* was dissolved in 1 ml sterile dH_2O, and aliquots at the concentration of 3 mg/ml were stored at −20 °C. The protein was added to the culture at a final concentration of 1 μg/ml.

2.6. Scaffold implantation

Scaffolds loaded with antigen-activated macrophages were implanted to the left rear foot of anesthetized mice (Avertin, Sigma) and sutured using 3–0 silk suture material (Deme TECH, USA). Tail bleeding was performed before and after implantation. Implants were surgically excised 7 or 14 days later. Implant histology was evaluated by SEM analysis. For classical immunization protocols, animals were injected intraperitoneally with 3×10^6 CFUs/ml *S. Typhimurium* fragments or 1 μg/ml peptidoglycane emulsified into 100 μl Freund's complete adjuvant complete adjuvant (FCA, Sigma), while control groups received only the emulsified FCA solution.

2.7. Enzyme-linked immunosorbent assay, ELISA

ELISA experiments were performed as previously described [11]. Briefly, for the detection of specific antibodies, ELISA plates were initially coated with *S. Typhimurium* fragments or peptidoglycan and then reacted with culture supernatants, followed by addition of the secondary antibody.

2.8. 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 CPD030), 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 (SEM) operated at 15 kV.

2.9. Cell proliferation assays

Spleen cells were cultured in 96-well V-bottomed plates (Sarstedt, Numbrecht, Germany) at the concentration of 1×10^6 cells/well in DMEM culture medium (Biosera, Kansas City, USA) supplemented with 10% fetal bovine serum (FBS, Gibco) at a final volume of 200 μl with or without *Typhimurium* fragments or peptidoglycane (in various concentrations) and processed for ^{3}HTdR incorporation assays after 4 or 7 days of culture. The cultures were pulsed with 1 μCi of ^{3}HTdR (ICN, Costa Mesa, CA) 18 h prior to harvest. After transferring the cells on to cellulose filters, these were put in scintillation fluid (toluene-omnifluor; 1.38 g/l, NEN) and counted using a LKB beta-counter (Finland).

2.10. Statistical analysis

Data were analyzed with two-tailed Paired (*in vitro* experiments) or Unpaired (*in vivo* experiments) Student's T-test. P-values <0.05 were considered significant (*), values <0.01 were considered very significant (**), and values <0.001 and <0.0001 were considered highly significant (*** and ****). Statistics were performed using GraphPad Prism 6.01 (Graphpad Software, La Jolla, CA).

3. Results

The poor vaccination outcome against Salmonalla prompted us to apply the previously described implantable vaccine technology to a mouse model using the antigenic stimulus of *S. Typhimurium*, which displays symptoms similar to the human *S. Typhi*.

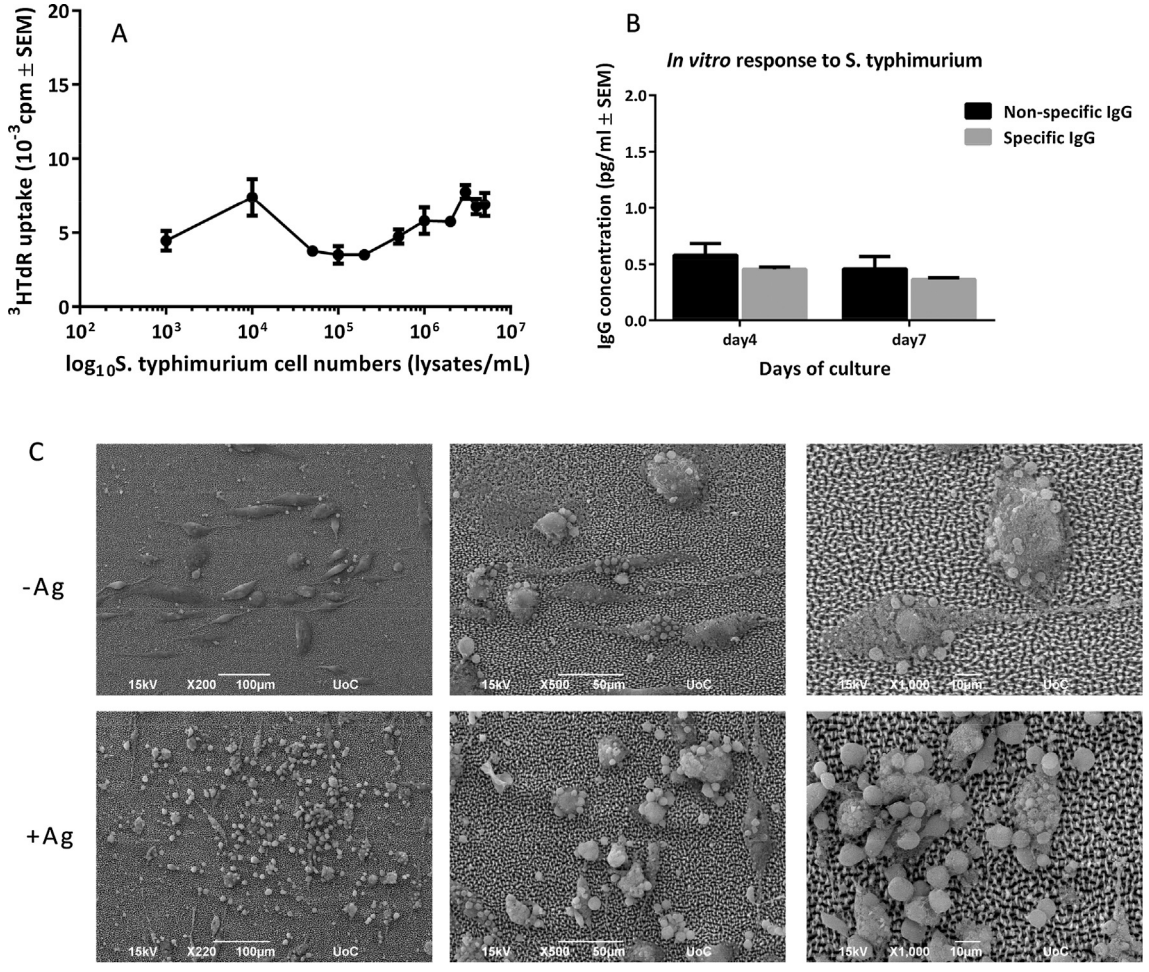

Fig. 1. In vitro immune response to *S. Typhimurium*. The most effective dose to be used was determined by total spleen cell proliferation assays as assessed by ^{3}HTdR uptake (A). The results represent the mean of 3 experiments and are expressed as cpm ± SEM. For evaluating the ability of *S. Typhimurium* to mount a specific immune response *in vitro*, spleen macrophages were cultured on laser patterned micro-structured Si-substrates for 24 h, transferred to new plates and seeded with 3×10^6 *S. Typhimurium* fragments/ml for 24 h and after antigen elimination lymphocytes (6.5×10^6 cells/ml) were added to the cultures and incubated for 4 or 7 days. Culture supernatants were tested for the presence of specific or non-specific IgG by ELISA (B). The results represent the mean of three experiments and are expressed as pg/ml ± SEM. Concentration was calculated according to an IgG standard curve. Upon culture termination, scaffolds were processed for SEM analysis (C). Panels show increasing magnification ranging from X100 to X1000.

3.1. Development of specific immune response against S. Typhimurium

In order not to exclude any antigenic epitopes, whole *S. Typhimurium* extracts were used as the antigenic stimuli. Following dose response experiments vis-à-vis spleen cell proliferation, the concentrations of 10^4 and 3×10^6 cell-lysates/ml showed the best ^{3}HTdR uptake (Fig. 1A). The concentration of 3×10^6 cell-fragments/ml was used in all experiments thereafter.

The efficacy of laser-microstructured Si-scaffolds to support the development of an immune response against *S. Typhimurium* was initially tested *in vitro*. Thus, Si scaffold-loaded adherent spleen cells (mainly macrophages, see Supplement Fig. 1) were initially pulsed with the antigen and after 24 h the antigen was removed, virgin lymphocytes were added to the culture and incubated for a total of 4 and 7-days (see Methods). Antigen-specific IgG could be detected in the culture supernatants after 4 and 7 days of culture (Fig. 1B), but the IgG levels were 6-fold lower as compared to the previously studied antigenic stimulus of human serum albumin (HSA; data not shown). Scanning electron microscopy analysis showed that in the presence of antigen, the Si scaffold-loaded macrophages supplemented with T- and B-lymphocytes display significant activation as compared to the cultures without antigenic stimulus (Fig. 1C). Cell density, as well as the increased number of secretory vesicles could be considered as a direct proof for an ongoing immune response.

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2019.02.080.

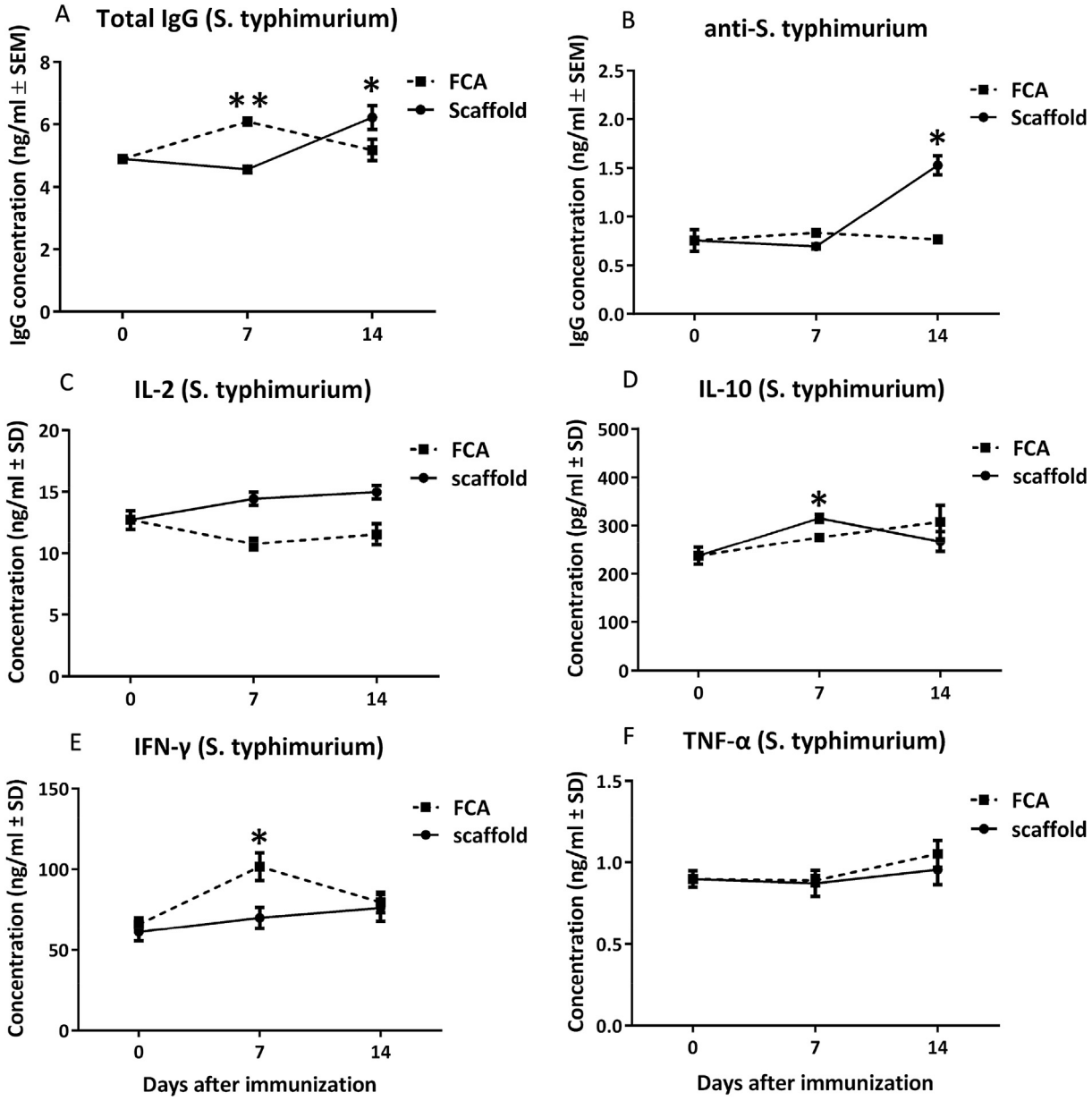

Fig. 2. *In vivo* immune response to *S. Typhimurium*. Upon anesthesia, antigen-seeded macrophages loaded onto 3D microstructured Si scaffolds were implanted to the rear left foot of young male mice and blood tests were conducted 7 and 14 days post-implantation. The results were compared to classical immunization protocols using Freud's complete adjuvant (FCA). Total (A) or antigen specific (B) IgG content in serum of scaffold implanted or FCA/antigen vaccinated animals was evaluated by ELISA The results represent the mean of 5 animals in each case (±SEM). Serum content for IL-2 (C), IL-10 (D), IFN-γ (E) and TNF-α (F) of animals implanted with activated Si scaffolds or FCA/antigen vaccinated without macrophages was tested by ELISA. The results represent the mean of 5 animals in each case (±SD). Concentrations have been calculated following standard curves for each protein tested. *: $p < 0.01$, **: $p < 0.005$.

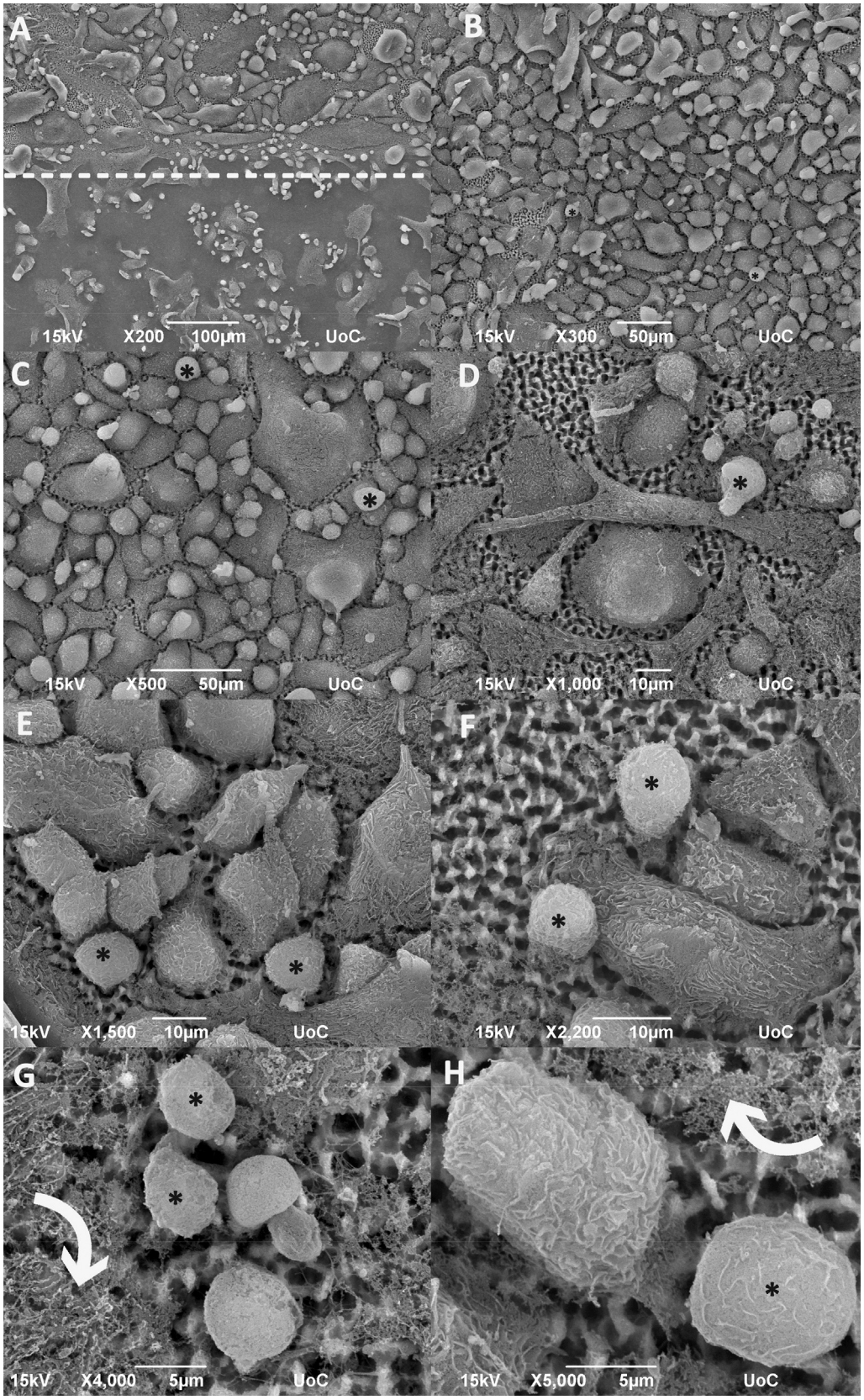

Fig. 3. *In vivo* Si-scaffold-induced histology in response to *S. Typhimurium*. Implants shown here were excided 14 after implantation. Panels show increasing magnification ranging from X200 to X5000 (A to H). The dashed line (A) indicates the limits of the Si scaffold. Arrows show depositions of extracellular matrix. Asterisks show some (not all) morphologically distinguished lymphocytes.

As already mentioned, the implantable 3D-Si scaffold technology for vaccine development consists of antigen-activated macrophage-loaded laser-microstructured Si-scaffolds. Following this technology, 3×10^6 *S.Typhimurium* cell fragments/ml were seeded to macrophages *in vitro* and after allowing antigen presentation to occur, the activated Si-scaffolds were implanted to 2-month old male BALB/c mice and removed 14 days later. Serum was collected on days 0, 7 and 14 after implantation and tested for specific antibody as well as IL-2, IL-10, IFN-γ and TNF-α production. In parallel experiments, mice were immunized following the conventional immunization protocol, where *S. Typhimurium* cell fragments were emulsified in FCA and administered intraperitoneally to mice.

It is interesting to note that the conventional immunization protocol increased by 24% ($p = 0.0039$) total IgG production after 7 days, but did not last, since it decreased to control levels 14 days post-immunization (Fig. 2A). On the contrary, implantable 3D-Si scaffold vaccine showed a delay as to the stimulation of total IgG production, which increased, however, by 27% as compared to non-immunized controls 14 days after implantation ($p = 0.0499$; Fig. 2A). Although the conventional immunization protocol failed to induce any specific anti-*S.Typhimurium* IgG, the implantable 3D-Si scaffold vaccine significantly increased by 102% ($p = 0.0251$) specific IgG production 14 days post implantation (Fig. 2B). Examining the cytokine alteration profile upon the immunization, it was observed that the implantable 3D-Si scaffold vaccine significantly increased IL-10 production by 33% ($p = 0.0153$; Fig. 2D), while the conventional FCA immunization protocol induced IFN-γ increase by 54% ($p = 0.0088$; Fig. 2E) in the serum 7 days post immunization, which decreased to control levels by day 14. The two immunization protocols did not show any statistically significant differences as to the levels of IL-2 and TNF-α production (Fig. 2C, F).

In order to examine the histological features caused by the implant, these were excised on day 14 and tissue histology was evaluated using SEM analysis (Fig. 3). Cell density on the *S. Typhimurium*-activated macrophages loaded 3D-Si scaffolds explants was definitely higher as compared to the adjacent tissues without scaffold (Fig. 3A; upper part as compared to the lower part of the picture). Adherent cells, comprising morphologically distinguished macrophages and fibroblasts almost completely covered the Si-scaffold surface as a monolayer (Fig. 3B–E). The activated Si-scaffold seemed to attract lymphocytes, which established contacts with the adherent cells (Fig. 3C–G; some examples marked with asterisk). Interestingly, the development of extracellular matrix could be observed (Fig. 3G, E; arrows) indicating that cells started to create their own microenvironment for growth and function. However, this morphology was different from the one previously reported, when the antigenic stimulus was HSA [11], where the macrophage and fibroblast network was more complex, with long cytoplasmic extensions and multilayer organization (Suppl. Fig. 2A). Indeed, in the case of the HSA antigenic stimulus, specific antibody production was 2-fold higher as compared to *S. Typhimurium* extracts (Suppl. Fig. 2B, [11]). The use of whole cell extracts, as compared to the pure HSA previously used, could be an important difference vis-à-vis the two antigenic stimuli.

To this extend, the parameter that was thereafter examined was peptidoglycan, which is an essential compound of the pathogen's envelope and is involved in protective mechanism facilitating penetration to the host's cells [15]. Although some unique amino acid differences may occur among peptidoglycans of different species, their antigenic behavior should not be much different. Therefore, peprtidoglycan from *Micrococcus luteus* was studied following a similar protocol as to the one described above.

3.2. Development of immune response against peptidoglycan

Dose response experiments initially defined the most effective peptidoglycan (PG) concentration for spleen cell activation, which was determined to be 1 μg/ml and was used at all experiments thereafter (Fig. 4A). It is interesting to note that the proliferative activity of spleen cells in response to PG was 2-times higher than the one induced by whole *S. Typhimurium* extracts, accompanied by a 3-fold increased production of non-specific IgG as compared to the specific anti-PG IgG (Fig. 4B), when applied to the *in vitro* stimulation of the 3D-Si scaffold system described above.

When PG was applied to the *in vivo* vaccination systems, as describe for *S. Typhimurium* for 7 days, it was shown that the PG-seeded macrophages loaded onto the 3D-microstructured Si scaffolds could induce a 3-fold higher total IgG production as compared to the conventional FCA vaccination protocol (Fig. 4C). Thus, the activated 3D-Si scaffold system and the conventional FCA immunization protocol increased by 43% ($p = 0.0017$) and 13% ($p = 0.0462$) non-specific IgG production as compared to untreated controls. However, none of the vaccination protocols could induce specific anti-PG IgG production (Fig. 4D).

Examining the cytokine levels in the serum of these animals, it was observed that the conventional FCA immunization protocol significantly increased IL-10 ($p = 0.0025$) and IFN-γ ($p = 0.0028$), while the PG-activated 3D-Si scaffold immunization system significantly decreased the production of IL-2 ($p = 0.0176$), IFN-γ ($p = 0.0051$) and TNF-α ($p = 0.0122$). These results could explain the differential responsiveness as to the antibody production of the two protocols (see discussion).

Compared to the antigenic stimulus of *S. Typhimurium*, histological analysis of the excised PG-activated Si scaffolds showed a different morphology of the adherent layer (Fig. 5). Macrophages and fibroblasts were not structured in monolayers and cells were showing dynamic cytoplasmic extensions. Although the presence of lymphocytes was obvious, it was not easy to distinguish cell interactions with the adherent layer, indicating that the behavior and responsiveness of the 3D-microstructured Si scaffolds tightly depends on the nature of the antigenic stimulus and consequently the activation state of antigen presenting cells.

4. Discussion

The currently available thyphoid vaccines display restricted effectiveness and have been considered as "treaveling" vaccines. Taking advantage of the previously described implantable vaccine technology consisting on 3D laser-microstructured Si scaffolds loaded with antigen-seeded macrophages, the present study used as antigenic stimuli whole extracts of *S. Typhimurium* or peptidoglycan, which is an fundamental component of the pathogen's envelop, and demonstrated the development of an immunogenic and mitogenic immune response respectively, which were shown to be more effective than the conventional vaccination protocols.

Looking at *S. Typhi* and its virulent analogue in mice *S. Typhimurium* morphology, similar features can be observed. The outer membrane comprises Vi capsular polysaccharide antigens, which are nowadays used as a subunit vaccine and suffer from moderate efficacy [4]. Peptidoglycan, which forms a thin layer between the outer and inner membrane, plays an important role in pathogen's survival, since it has been considered to act as a "transition sensor" for habitalization to environmental conditions [15]. When whole attenuated pathogens are used for vaccination, the presence of polysaccharides at the outer membrane can be considered to induce mitogenic responses, which although they are initially protective, their duration is short and does not induce memory to the

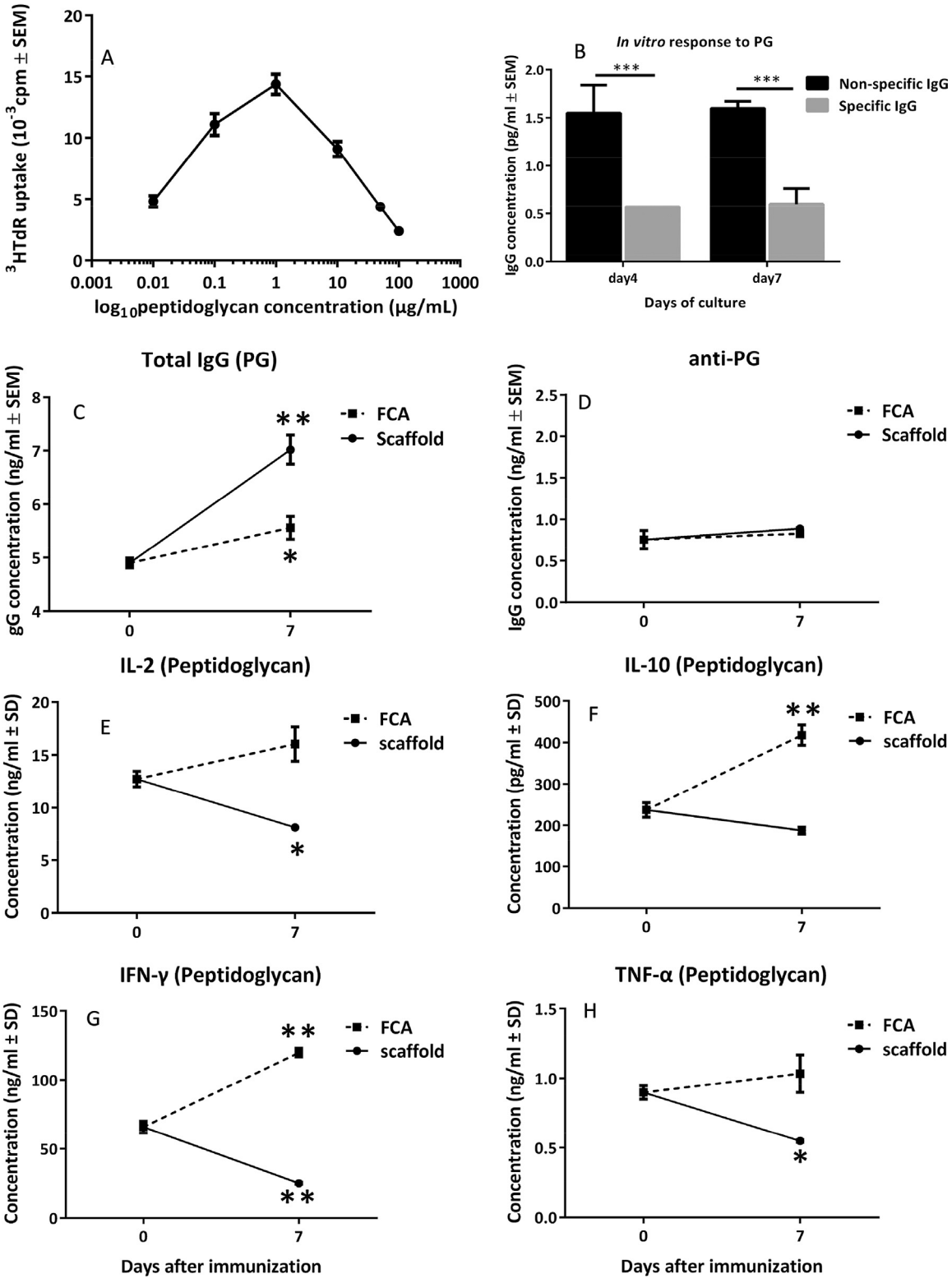

Fig. 4. Immune response to peptidoglycan. The most effective dose to be used was determined by total spleen cell proliferation assays as assessed by ^{3}HTdR uptake (A). The results represent the mean of 3 experiments and are expressed as cpm ± SEM. For evaluating the ability of peptidoglycan to mount a specific immune response *in vitro*, spleen macrophages were cultured on laser patterned micro-structured Si-substrates for 24 h, transferred to new plates and seeded with 1 μg/ml peptidoglycan for 24 h and after antigen elimination lymphocytes (6.5×10^6 cells/ml) were added to the cultures and incubated for 4 or 7 days. Culture supernatants were tested for the presence of specific or non-specific IgG by ELISA (B). The results represent the mean of three experiments and are expressed as pg/ml ± SEM. For the *in vivo* experiments, mice were anesthetized and implanted to the rear left foot with antigen-seeded macrophages loaded onto 3D microstructured Si scaffolds. Blood tests were conducted 7 days post-implantation. The results were compared to classical immunization protocols using Freud's complete adjuvant (FCA). Total (C) or antigen specific (D) IgG content in serum of scaffold implanted or FCA/antigen vaccinated animals was evaluated by ELISA. The results represent the mean of 5 animals in each case (±SEM). Serum content for IL-2 (E), IL-10 (F), IFN-γ (G) and TNF-α (H) of animals implanted with activated Si scaffolds or FCA/antigen vaccinated without macrophages was tested by ELISA. The results represent the mean of 5 animals in each case (±SD). Concentrations have been calculated following standard curves for each protein tested. *: $p < 0.01$, **: $p < 0.005$, ***: $p < 0.001$.

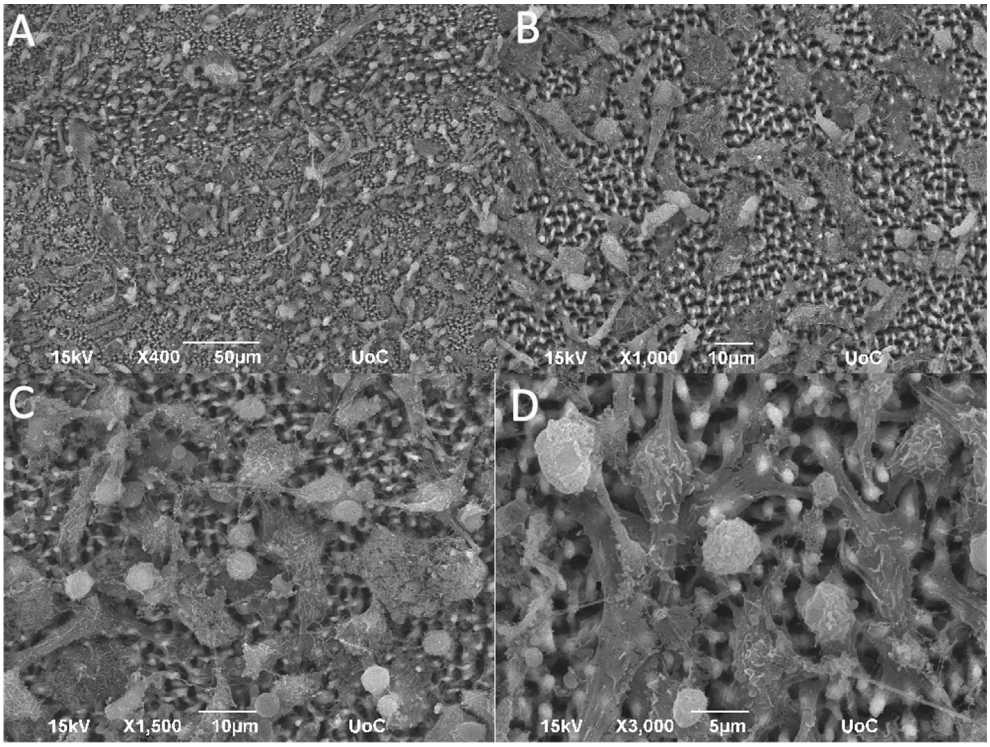

Fig. 5. In vivo Si-scaffold-induced histology in response to peptidoglycan. Implants shown here were excided 7 days after implantation. Panels show increasing magnification ranging from X400 to X3000 (A to D).

host, explaining thus the moderate efficacy of the vaccines. A similar explanation can be applied to the Vi subunit vaccine.

The application of the recently developed implantable 3D-laser micro-structured Si-scaffold vaccines has been suggested to avoid virulence (since antigen pulsing takes place *in vitro* and not *in vivo*), to induce moderate systemic immunostimulation necessary, to support the development of specific immune response, and allow natural antigenic epitope selection for each host depending on the individual's MHC polymorphism. Therefore, in the present study, this technology was initially applied using an antigenic stimulus of whole cell extracts of *S. Typhimurium*. The ability of the 3D-laser micro-structured Si-scaffold technology to support the development of immune response against *S. Typhimurium* was initially tested *in vitro*, where it was shown that specific anti- *S. Typhimurium* IgG could be detected in the culture supernatants after 4 and 7 days of culture, while SEM analysis of the scaffolds showed a definite activation of immune cells in the presence of the antigen.

When antigen-seeded macrophages loaded onto the 3D Si-scaffolds were implanted to mice, specific antibody production could be obtained 14 days after implantation. Parallel experiments using conventional FCA vaccination protocols, showed that although FCA-antigen could increase total IgG production 7-days after immunization, this dropped to control levels at 14-days post immunization, while this protocol was unable to mount a specific anti-*S. Typhimurium* response. The cytokine profile showed an increase of IL-10 in the case of implantable vaccination 7 days after implantation, which dropped to control levels thereafter. The increase of the immunosuppressive IL-10 could be responsible for the absence of specific anti-*S. Typhimurium* IgG, 7 days post implantation. In the case of conventional vaccination, IFN-γ was shown to significantly increase 7 days after immunization, which could be responsible for total IgG production. In these experiments, the levels of IL-2 and TNF-α did not show any alterations with the two vaccination protocols. Morphological studies on the excised scaffolds 14 days after implantation showed the development of a well structured adherent monolayer, establishing multiple contacts with lymphocytes in favor to the immune response development.

Comparing the antigenic stimulus of *S. Typhimurium* with the previously studied HSA antigen [11], important differences could be observed. The levels of specific antibody production was higher in the case of HSA, while scaffold histology upon excision was showing a multilayer development with long and complex cytoplasmic extensions compared to the cell structured monolayer observed in the case of *S. Typhimurium.* That was an important observation showing that the behavior of adherent cells on the Si-scaffold was highly dependent on the antigenic stimulus. However, the lower immunogenicity of *S. Typhimurium* as compared to HSA was a puzzling result. Since whole cell extracts were used in this protocol, one would expect a higher responsiveness due to the multiple antigenic determinants present in the preparation. Therefore, these observations led to the hypothesis of the presence of both stimulatory and suppressive components in the vaccination preparation, which would affect the overall activity. To this extend, peptidoglycan was applied as an antigen to the vaccination protocols.

As already mentioned, PG is a fundamental component of the pathogen's envelop and could exert some positive or negative effects. Although the PG used in these experiments derived from a different microorganism, its antigenic properties were not considered to significantly differ from *S. Typhimurium.* Surprisingly, PG was shown to induce a mitogenic rather than a specific immunogenic response, which could also explain the short duration of the available vaccines for *S. Typhi.* Although both vaccination protocols applied herein (conventional FCA vaccination and implantable antigen –activated macrophage-loaded Si scaffolds) induced total IgG production, but were unable to mount a specific anti-PG response. The two protocols differed as to the cytokine profile they induced. Thus, the conventional protocol increased

IL-10 and IFN-γ production, while the implantable vaccine protocol decreased IL-2, IFN-γ and TNF-α production, indicating a suppressive rather than a stimulatory activity towards specific antibody production. Histological analysis of the excised scaffolds showed a restricted layer of adherent cells with cytoplasmic extensions, where however, it was difficult to distinguish promotion of significant cell contacts with lymphocytes.

In conclusion, the presented results showed that the application of whole *S. Typhimurium* cell extracts to the implantable 3D-laser micro-structured Si-scaffold technology could mount a specific immune response, while a conventional immunization protocol could only induce non-specific IgG production. None of the vaccination protocols studied was able to mount a specific immune response to peptidoglycan, indicating that such component, in any type of vaccine, could rather act as mitogen, and could therefore explain the poor outcome of vaccines comprising such components in their preparations. Last, but not least, the presented results showed a differential behavior of antigen presenting cells in accordance to the antigenic stimulus. The same Si scaffold could allow differential adherent layer structure depending on the activation state of the cells. Tailoring the micro/sub-micron 3D structures and chemistry of Si scaffolds, one could envisage altering cell behavior according to the needs and final goals of the user.

Declaration of interests

The authors declare that they have no known competing financial interests

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References

[1] House D, Bishop A, Parry C, Dougan G, Wain J. Typhoid fever: pathogenesis and disease. Curr Opin Infect Dis 2001;14:573–8.
[2] Meiring JE, Gibani M, TyVAC Consortium Meeting Group. The Typhoid Vaccine Acceleration Consortium (TyVAC): Vaccine effectiveness study designs: accelerating the introduction of typhoid conjugate vaccines and reducing the global burden of enteric fever. Report from a meeting held on 26–27 October 2016, Oxford, UK. Vaccine 2017;35:5081–8.
[3] Garmory HS, Brown KA, Titball RW. *Salmonella* vaccines for use in humans: present and future perspectives. FEMS Microbiol Rev 2002;26:339–53.
[4] Pham OH, McSorley SJ. Protective host immune responses to Salmonella infection. Future Microbiol 2015;10:101–10.
[5] Mastroeni P, Villarreal-Ramos B, Hormaeche CE. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect Immun 1993;61:3981–4.
[6] Garai P, Gnanadhas DP, Chakravortty D. *Salmonella enterica serovars* Typhimurium and Typhi as model organisms, Revealing paradigm of host-pathogen interactions. Virulence 2012;3:377–88.
[7] Makela PH, Hormaeche CE. Immunity to Salmonella. In: Kaufmann SHE, editor. Host response to intracellular pathogens. Austin, Tex: R. G. Landes Company; 1997. p. 143–66.
[8] Mittrucker HW, Kauffman SH. Immune response to infection with Salmonella typhimurium in mice. J Leukoc Biol 2000;67:457–63.
[9] Garcia-del Portillo F, Stein MA, Finlay BB. Release of lipopolysaccharide from intracellular compartments containing Salmonella typhimurium to vesicles of the host epithelial cell. Infect Immun 1997;65:24–34.
[10] Zerva I, Simitzi C, Ranella A, Stratakis E, Fotakis C, Athanassakis I. 3-dimensional laser structured scaffolds improve macrophage adherence and antigen-specific response. Procedia Eng 2013;59:211–8.
[11] Zerva I, Simitzi C, Siakouli-Galanopoulou A, Ranella A, Stratakis E, Fotakis C, et al. Implantable vaccines: *In vitro* antigen presentation enables *in vivo* immune response. Vaccine 2015;33:3142–9.
[12] Ranella A, Barberoglou M, Bakogianni S, Fotakis C, Stratakis E. Tuning cell adhesion by controlling the roughness and wettability of 3D mocro/nano silicon structures. Acta Biomater 2010;6:2711–20.
[13] Simitzi C, Efstathopoulos P, Kourgiantaki A, Ranella A, Charalampopoulos I, Fotakis C, et al. Semiperiodic discontinuous anisotropic microconical substrates as a new model scaffold to control directionality of neuronal network outgrowth. Biomaterials 2015;67:115–28.
[14] Van Goethem E, Poincloux R, Gauffre F, Maridonneau-Parini I, Le Cabec V. Matrix architecture dictates three-dimensional migration modes of human macrophages: differential involvement of proteases and podosome-like structures. J Immunol 2010;184:1049–61.
[15] Quintela JC, de Pedro MA, Zöllner P, Allmaier G, Garcia-del Portillo F. Peptidoglycan structure of Salmonella typhimurium growing within cultured mammalian cells. Mol Microbiol 1997;23:693–704.