Immunotherapy-on-chip Against an Experimental Sepsis Model

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Abstract

Lipopolysaccharide (LPS) is commonly used in murine sepsis models, which are largely associated with immunosuppression and collapse of the immune system. After adapting the LPS treatment to the needs of locally bred BALB/c mice, the present study explored the protective role of *Micrococcus luteus* peptidoglycan (PG) pre-activated vaccine-on chip technology in endotoxemia. The established protocol consisted of five daily intraperitoneal injections of 0.2mg/g LPS, allowing longer survival, necessary for a therapeutic treatment application. A novel immunotherapy technology, the so-called vaccine-on-chip consists of a 3-dimentional laser micro-textured silicon (Si)-scaffold loaded with macrophages and activated *in vitro* with 1μg/ml PG, which has been previously shown to exert a mild immunostimulatory activity upon subcutaneous implantation. The LPS treatment significantly decreased CD4+ and CD8+ cells, while increasing CD11b+, Gr1+, CD25+, Foxp3+ and class II+ cells. These results were accompanied by increased arginase-1 activity in spleen cell lysates and C-reactive protein (CRP), procalcitonin (PCT), IL-6, TNF-a, IL-10 and IL-18 in the serum, while acquiring severe sepsis phenotype as defined by the murine sepsis scoring. The *in vivo* application of PG pre-activated implant significantly increased the percentage of CD4+ and CD8+ cells, while decreasing the percentage of Gr1+, CD25+, CD11b+, Foxp3+ cells and arginase-1 activity in the spleen of LPS-treated animals, as well as all serum markers tested, allowing survival and rescuing the severity of sepsis phenotype. In conclusion, these results reveal a novel immunotherapy technology based on PG pre-activated micro-texture Si-scaffolds in LPS endotoxemia, supporting thus its potential use in the treatment of septic patients.

Introduction

Sepsis is a polyparametric condition, which back in the early ‘90s was defined as a systematic inflammatory response resulting in a variety of severe clinical symptoms [1], early in 2001 it was defined as “infection” [2] and today it is defined as life-threatening organ dysfunction caused by dysregulated response of the host to infection [3]. The establishment of the Sequential (sepsis-related) Organ Failure Assessment (SOFA) score [4] allowed early diagnosis and management, but yet sepsis remains a significant burden on health systems worldwide and therapy is still a wishful goal.

Sepsis is accompanied by impaired innate and adaptive immunity mediated by the development of immunosuppressive mechanisms [5], further abrogating homeostasis and worsening health condition. Such suppressive mechanisms were shown to include expansion of T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which exert their effects through production of suppressive factors including arginase-1, oxygen free radicals, stimulation of the inducible NO synthase etc, decrease of effector immune cells as well as imbalance of the inflammatory versus anti-inflammatory cytokine profile [6–9]. Due to the immunosuppressive nature of this pathology, immunostimulatory therapies, including granulocyte macrophage colony stimulating factor, interleukin 7, programmed death-1 inhibitors, intravenous immunoglobulin (IVIG) treatment enriched or not with IgM, have been envisaged as potential treatments [10–14]. To this extend, the application of IgG and IgM in a sepsis-like murine LPS-induced endotoxemia model significantly decreased the percent of myeloid-derived suppressor cells

(CD11b + Gr1 + cells), and regulatory T cells (CD25+, Foxp3 + cells), the arginase-1 activity in the spleen, while also decreasing IL-6, TNF-α and CRP levels in the serum, allowing survival to all animals tested [15].

Previous studies have shown that laser microstructured 3-dimensional (3D) silicon (Si) scaffolds loaded with macrophages/antigen presenting cells (APCs) activated with different antigenic stimuli may, upon implantation, confer various levels of immune activation to the host [16]. Such manipulation has been referred as "*vaccine-on-chip*" technology. Thus, using as conventional antigen human serum albumin (HSA), the application of the above technology led to high levels of HSA-specific antibody detection in the serum lasting for several months [17]. Although following a different histological behavior of the implant, the use of *Salmonella Typhimurium* as the antigenic stimulus to the "*vaccine-on-chip*" technology also encouraged the production of specific antibodies in the serum several weeks after implantation. The application of *M. luteus* peptidoglycan (PG) to this technology resulted in increased IgG levels in the serum, but no antigen specific antibody. This observation, along with the increased cytokine levels and white cell numbers had led to the hypothesis that *M. luteus* peptidoglycan was likely to act as a mitogen [18].

Taking advantage of the mitogenic activity of the peptidoglycan stimulated-macrophage-activated 3D Si-scaffold technology (referred as *PG-vaccine-on-chip*), the present study inquired whether the mild stimulatory activity induced by the application of the PG-vaccine-on-chip technology could rescue the LPS-induced endotoxemia. The results showed that such manipulation could indeed increase the percentage of CD4 + and CD8 + cells, while decreasing the percentage of CD11b+, Gr1+, CD25+, Foxp3 + cells and arginase-1 activity in the spleen of LPS-treated animals, as well as IL-6, IL-18, IL-10, TNF-a, PCT and CRP in the serum, rescuing the severity of sepsis phenotype and allowing survival to all animals tested.

Materials And Methods

Animal manipulation

BALB/c mice, purchased from Charles River (Milan, Italy), were maintained in the animal facility at the University of Crete (Crete, Greece) and their care was in accord with the Institution's guidelines. The applied protocol was approved by the national Bioethical Committee (Approval # 292314, ADA ΨΗΘ47ΛΚ-Γ5Ψ). Six to 10 weeks old males or females were used in all experimentations.

The sepsis mouse model consisted of a 5-day intraperitoneal injection of LPS (0.2 μg/g of body weight – 5μg/mouse- per day, *E. Coli* O111:B4, purity > 99%, Sigma-Aldrich, Germany), which led to death approximately half of the treated mice (LD_{50} dose) [15]. On the second day of LPS inoculation, mice were implanted with 3D Si scaffolds loaded with or without PG-activated macrophages (see below). Blood samples were collected before treatment initiation by tail sectioning (approximately 30 μl of blood). Mice were euthanized on day 6. Blood samples were collected via heart puncture and spleens were harvested, put in single cell suspension and examined for various sepsis specific markers.

Mice were divided in 5 groups including untreated controls, untreated controls (C) that received the PG-activated scaffold (C + PGsc), LPS-treated (LPS), LPS-treated that received the PG-activated scaffold (LPS + PGsc) and LPS-treated that received a non-activated scaffold (LPS + sc).

Mice that received the vaccine-on-chip therapy were implanted with the PG-activated or not Si-scaffold (see below) to the left rear foot of anesthetized mice (Avertin, Sigma-Aldrich) and sutured using 3 – 0 silk suture material (Deme TECH, USA). Implants were surgically excised on day 6. Implant histology was evaluated by scanning electron microscopy (SEM) analysis.

Sepsis was also scored using the Murine Sepsis Score (MSS), which takes into account animal appearance, level of consciousness, activity, response to stimulus, eyes, respiration rate and respiration quality [19]. According to this scoring, MSS greater than 10 predicts mortality within a few hours, while mice attaining a clinical score of three have 100% specificity for dying from sepsis during the experimental timeline.

Peptidoglycan

Peptidoglycan (PG; 3 mg; Sigma-Aldrich) 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.

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 [15]. The surface topology was obtained using a laser radiation density of 0.68 J/cm^2.

Fabrication of PG-activated implants

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 2ml in the presence of Si scaffold substrates (5×5 mm^2), placed at the center of the well. After 24h 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, PG was added to the cultures. After an incubation of 24 hours, the scaffolds were thoroughly washed in order to eliminate the residual antigen and implanted to animals. In some experiments, non-activated Si-scaffolds were implanted to LPS-treated mice.

In order to determine the proliferation of macrophages loaded onto the scaffold, CFSE (Cell trace CFSE cell proliferation kit, Molecular Probes) was added to the cultures and after fixation the cells were immunostained with a PE-conjugated anti-CD11b. 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). In another set of experiments, 24h after PG addition, the antigen was removed and T and B lymphocytes, isolated from the total spleen cell population, were added to the cultures ($6.5x10^6$ cells/ml).

After 4 days of incubation the scaffolds were submitted to double immunofluorescent staining with anti-CD11b and anti-CD4 and analyzed by confocal microscopy analysis using a 'Zeiss AxiosKop 2 plus' laser scanning confocal microscope as previously described [17]. Culture supernatants were tested for the presence of total or antigen-specific IgG antibody. All cells were grown in an incubator Forma Scientific at 37ºC in the presence of 5% CO_2.

Antibodies

Antibodies against mouse Foxp3-Alexafluor 488, CD8a (IgG2a) FITC-labeled, CD25 (IgG2a) PE-labeled, CD11b PE-labeled and IA/IE (IgG2b) FITC-labeled were purchased from Biolegend Inc (San Diego, CA). The anti- mouse Gr1-FITC conjugated antibody was purchased from ImmunoTools (Friesoythe, Germany). Anti-mouse CD4 (IgG2b) FITC-conjugated was purchased from BD bioscience (San Jose, CA). IgG isotype controls FITC- or PE-conjugated (Sigma, St. Louis, MO) were used in all immunofluorescence experiments. All antibodies were used at the concentration of 1μg/mL in immunofluorescence experiments. Finally, rat anti-mouse IL6 (IgG1, Biolegend Inc San Diego, CA), rat anti-mouse TNFa (IgG1k, Biolegend Inc, San Diego, CA), rat anti-mouse IL10 (IgG1k, Biolegend Inc, San Diego, CA) and goat anti-mouse IL-18 (Santa Cruz Biotechnology Inc, Dallas, Tex), were used at the concentration of 0.1μg/mL for ELISA experiments. Goat anti rat IgG (Fab fragment, Sigma-Aldrich), rabbit anti-goat IgG (Sigma-Aldrich) and rat anti-mouse IgG (Santa Cruz, CA, USA) secondary antibodies coupled to horseradish peroxidase, were used at the concentration of 0.02 μg/mL.

Arginase-1 activity

Arginase-1 activity was measured in spleen cells upon lysis, using an arginase detection kit (arginase activity assay kit, Sigma-Aldrich). In this assay, arginase catalyzes the conversion of arginine to urea and ornithine. The urea produced specifically reacts with the substrate to generate a colored product, proportional to the arginase activity present. One unit of Arginase is the amount of enzyme that will convert 1.0 mmole of L-arginine to ornithine and urea per minute at pH 9.5 and 37°C.

Detection of sepsis biomarkers in spleen cells

The percent of CD11b, Gr1, CD25, Foxp3, MHC-II, CD4 and CD8 positive cells in spleens of control or LPS-treated animals with or without implant application was evaluated by immunofluorescence experiments followed by flow cytometry analysis as previously described [20].

Detection of sepsis biomarkers in blood

Serum was collected from all animals before and after treatment and stored at -20°C until use. The levels of cytokines IL-6, IL-18, TNF-α and IL-10 were evaluated by ELISA, as previously described [15]. Optical density (OD) was measured at 450 nm using a Titertec ELISA photometer (Digiscan, ASYSHitech, GmbH; Engendorf, Austria). The detection of CRP and PCT in the serum was performed using the mouse CRP (C-reactive Protein) ELISA kit (Elabscience, Bethesda,MD; E-EL-M0053) and the mouse PCT (Procalcitonin) ELISA kit (Elabscience, E-EL-M2419) following the instructions of the supplier.

SEM analysis

Upon excision from the animals, scaffolds were washed with 0.1M sodium cacodylate buffer (SCB) and then incubated in the same solution for 15min, twice. Samples were fixed using a 2% glutaraldehyde, 2% formaldehyde in SCB fixative buffer for 1h at 4°C.All surfaces were washed twice (from 15min per time) with SCB 0.1M at 4°C, dehydrated using serially graded ethanol immersions (from 30, 50, 70, 90 to 100%) and incubated for 15min 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 15nm gold layer prior to observation. SEM analysis was processed with a JEOL JSM 6390LV Scanning Electron Microscope (SEM) operated at 15kV.

Statistical analysis

Data were analyzed with two-tailed Paired (*in vitro* experiments) or Unpaired *(in vivo* experiments) Student's T-test unless mentioned otherwise. 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).

Results

Previous studies have indicated that *M. luteus*-derived PG, when applied to the *vaccine-on-chip* technology drives a mild mitogenic response upon implantation. In order to evaluate whether such activity could rescue the LPS-induced endotoxemia, PG-activated implants were fabricated and applied to the experimental model.

Fabrication of PG-activated implants

Dose response experiments defined the best concentration of PG to be used in the vaccine-on-chip system leading to macrophage proliferation and spreading on the Si-scaffolds (Fig. 1). To this extend, Si-scaffold loaded with macrophages after a 24-hour culture of total spleen cells, were transferred to fresh cultures and pulsed with 0.1, 1, and 10 µg/mL PG. Upon removal of the antigen, cell proliferation was evaluated using CFSE staining (see Methods). The results showed that PG induced the best proliferative activity at the concentration of 1 µg/mL (Fig. 1a, 1b). Double staining experiments showed that the proliferating cells were indeed macrophages, as detected by an anti-CD11 monoclonal antibody (Fig. 1c).

In order to evaluate whether such construction could support antigen recognition by T cells and production of antibodies, upon elimination of the antigen, naïve T and B cells were added to the PG-activated macrophages loaded onto the Si-scaffolds and after a 4-day culture, supernatants were tested for the presence of antigen-specific and non-specific IgG, while the activated Si-scaffolds were submitted to double immunofluorescence experiments for the detection of T-macrophage cell interactions.

Immunofluorescence staining, followed by confocal microscopy analysis of the activated Si-scaffolds could detect contacts of CD11 + macrophages (red) and CD4 + T cells (green) (Fig. 1d). In this system, B cells (CD19+) cells could not be detected in contact with CD4 + cells, but they were present as non-

adherent cells within the culture, as detected by flow cytometry analysis (data not shown). However, one cannot exclude the possibility of the establishment of T-B cell interactions, since weak contacts could be disrupted during the experimental manipulation.

Interestingly, the system produced significant amounts of total IgG, but only limiting amounts of specific anti-PG antibodies (Fig. 1e), indicating the ability of PG to stimulate pre-existing B cell clones with different antigen specificities. The detection of IgG antibodies supports B cell activation in the described cellular system.

Application of PG-activated implants to the endotoxemia model

The experimental mouse model applied herein consisted of 5-day intraperitoneal injections of 5 μg/day LPS, which led to death approximately half of the treated mice (LD_{50} dose) [15]. It was mandatory to adapt the LPS-inducing endotoxemia protocols to the local animal facility and mice, since protocols described in the literature [21, 22] were fatal to the animals maintained in the local animal facility. In addition, since the aim of the present study was to apply immunostimulatory treatments to reverse LPS toxicity, it had to be assured that animals could survive long enough, in order for the experiment to be realized.

The PG-activated-scaffolds were fabricated as described in the section of Materials and Methods and implanted one day after the initiation of the LPS treatment to mice. Animal behavior was evaluated during the treatment period the MSS was calculated. On the sixth day, mice were euthanized and tested for the presence of inflammatory markers in the serum and suppressor populations of cells in the spleen.

Marker analysis of spleen cells

Spleen cells were submitted to immunofluorescent staining, followed by flow cytometry analysis. Study groups included untreated controls (C), untreated controls that received the PG-activated scaffold (C + PGsc), LPS-treated (S), LPS-treated that received the PG-activated scaffold (S + PGsc) and LPS-treated that received a non-activated scaffold (S + sc). As expected, the LPS treatment increased in a statistically significant manner the CD11b+ ($p = 0.0281$), Gr1+ ($p = 0.0003$), CD25+ ($p = 0.0002$), Foxp3+ ($p = 0.0488$) and class II MHC+ ($p = 0.0281$) cell populations as compared to untreated controls (Fig. 2). The application of the PG-activated implant reversed the above phenotype resulting in a statistically significant reduction of all suppressive markers. Thus, compared to the LPS-treated animals, those that received the PG-activated implant reduced CD11b + cells in the spleen by 46% ($p = 0.0450$), Gr1 + cells by 57% ($p = 0.0053$, CD25 + cells by 22% ($p = 0.0381$) and Foxp3 + cells by 40% ($p = 0.0182$) (Fig. 2).

Since the expression of Gr1 and CD25/Foxp3 mainly characterizes MDSCs and Treg respectively, the results indicated that the PG-activated implant could indeed rescue the suppressive state characterizing endotoxemia. By the same token, the increase of CD4 + cells by 69% ($p = 0.0317$) and CD8 + cells by 44% ($p = 0.0063$) fortifies the therapeutic role of the implant.

It is interesting to note that the application of PG-activated implant to control untreated animals did not alter any of the tested markers with the exception of class II MHC protein expression, which was increased by 48% ($p = 0.0227$) and correlates with the mild immunostimulation previously described [18].

The application of non-activated implant to LPS-treated animals could also decrease the expression of CD11b by 68% ($p = 0.0411$), CD25 by 25% ($p = 0.0371$), Foxp3 by 68% ($p = 0.0031$), Gr1 by 45% ($p = 0.0136$) and increase of CD4 + cells by 156% ($p = 0.0007$) but not CD8 + cells in the spleen. Although PG has been used as a mild mitogen to the system, it seemed that the scaffold itself could also provide immunostimulation capable in reversing the LPS-induced suppressive markers in the spleen (Fig. 2).

Arginase-1 activity, which has been considered to be part of the mechanisms mediating the immunosuppressive activity of MDSCs [18, 19, 21] was found to be increased by 7% ($p = 0.0396$) in the LPS-treated animals and decreased by 11% ($p = 0.0496$) after application of the PG-activated implant. In this case, the non-activated implant increased rather than decreased arginase-1 activity by 12% ($p = 0.0318$) as compared to the LPS-treated animals (Fig. 2), arguing against the therapeutic value of the non-activated scaffold.

Inflammatory marker analysis in serum

To further evaluate the protective effect of the PG-implant application against LPS-induced endotoxemia, the profile of serum inflammatory factors was analyzed. Thus, the levels of IL-6, IL18, TNF-a, CRP and PCT, as well as the levels of the anti-inflammatory immunosuppressive IL-10 were examined in all groups of animals tested herein. The results showed that the LPS-treatment increased in a statistically significant manner the levels of IL-6 by 57% ($p = 0.0049$), IL-18 by 43% ($p = 0.0062$), TNF-a by 174% ($p < 0.0001$), CRP by 90% ($p = 0.0035$) and PCT by 34% ($p = 0.0259$), while also increasing the levels of IL-10 by 95% ($p < 0.0001$) as compared to untreated controls (Fig. 3). The application of the PG-activated implant resulted in a statistically significant reduction of IL-6 by 58% ($p = 0.0004$), IL-18 by 45% ($p < 0.0001$), TNF-a by 61% ($p = 0.0004$), CRP by 55% ($p = 0.0187$) and PCT by 52% ($p = 0.0007$), while also decreasing the levels of IL-10 by 68% ($p = 0.0008$) as compared to the LPS treated animals (Fig. 3).

When applied to untreated control mice, the PG-activated implant showed an increase in the IL-6 (by 55%, $p = 0.0082$) and TNF-a (by 91%, $p < 0.0001$) serum levels, but not IL-18, IL-10, CRP or PCT. On the other hand, the application of non-activated implant to the LPS-treated animals could only rescue the production of TNF-a, CRP and PCT, but not IL-6, IL-18 or IL-10, the levels of which were similar to the LPS-treated mice (Fig. 3).

Implant histology

Previous studies have shown that the histology of the excised implants differs in accordance to the anitigenic stimulus [16]. In this case, in order to compare the LPS-induced endotoxemic mice that received the PG-activated or non-activated implants, which showed a similar profile for several of the markers tested, SEM analysis was performed to the implants that were excised five days after application. The results showed that PG induced a well structured morphology of the excised scaffold with vigorous

cellular activity and plenty of collagen depositions creating an organic membrane surrounding the implant (Fig. 4). On the contrary, non-activated scaffolds, although becoming populated by adherent macrophages and fibroblasts, they failed to develop collagen depositions within the same time period (Fig. 4). Therefore, it seems that although the non-activated implants could show some similarities with the PG-activated as to the markers tested, their behavior showed a delayed type of reaction as to the development of protective membranes, at least at the time of excision.

MSS evaluation

In order to evaluate the overall effectiveness of the treatment, the MSS was evaluated in all cases of the LPS treatment and therapy. Thus, in a daily basis, the appearance, the level of consciousness, activity, the response to stimulus, the appearance of eyes, the respiratory rate and respiratory quality were evaluated. The phenotypic recording showed that the LPS-treated animals that received the PG-activated implant reduced by 80% the defined by MSS symptoms, while non-activated implants failed to rescue the endotoxemia phenotype (Fig. 6).

Discussion

Sepsis is one of the most aggressive, life threatening situations, represented by a deregulated response to infection, impaired with immune suppression and multi-organ collapse. Nowadays, sepsis is being considered as an immunosuppressive disorder and therefore, immunostimulatory therapies are envisaged as potential treatments. Among the various experimental models, the present study focused on the LPS-induced endotoxemia model that displayed major sepsis-related immunosuppressive and inflammatory markers and survived long enough to allow application of a therapeutic treatment, which consisted of a PG-activated implant, previously described to exert a mild mitogenic effect to the host. The results showed that indeed implantation of a PG-activated scaffold to LPS-treated mice could reverse all tested markers of endotoxemia, while also significantly ameliorating animal morbidity.

Implant fabrication followed the previously described "*vaccine-on-chip*" technology according to which 3D laser micro-textured Si scaffolds are used to support autologous macrophage adherence, antigen seeding and natural antigen presentation *in vitro* and further activation of the immune response *in vivo* [16]. For the construction of the implants used herein, laser micro-structured Si scaffolds were loaded with naïve macrophages and the best conditions for PG-induced proliferation and subsequent lymphocyte activation were determined. Thus, seeding with 1 μg/ml PG provided the highest proliferation and spreading of macrophages onto the Si-scaffolds as visualized by CFSE staining, while also supporting non-specific antibody production upon addition of autologous naïve T and B lymphocytes to the system. The interaction of macrophages with T cells was visualized by double fluorescence experiments, followed by confocal microscopy analysis, indicating that this system could indeed activate T cells, which in turn could stimulate B cells, as verified by the presence of IgG antibodies to the culture supernatants. The ability of this system to induce non-specific antibody production was mandatory, since previous results had demonstrated the ability of IgG to decrease sepsis markers and allow animal

survival [15], while inoculation of IgM-enriched IgG to patients has been considered to reverse septic shock conditions [13].

The experimental model used herein consisted of 5-day intraperitoneal injections of 5μg LPS per day. As previously described, such manipulation allows half of the animals to survive long enough to allow the application of a treatment [15]. Most protocols in the literature using higher doses of LPS ranging from 1 to 25 mg/kg of body weight of BALB/c mice study endotoxemia 24 hours after LPS injection, which is not convenient for a treatment application [21, 23]. Following the above experimental model, animals showed a statistically significant increase of immunosuppressive cell populations in the spleen as evaluated by the expression of CD11b/Gr1 and CD25/Foxp3 markers, which characterize MDSCs and Tregs respectively, while also increasing arginase-1 activity that is known to mediate the suppressive effect of MDSCs [6–9]. These cell populations have been shown to expand in various pathological conditions including cancer and acute infectious diseases and in particular sepsis, when migrating to the periphery [6]. The LPS-induced endotoxemia also resulted in a decrease of effector CD4- and CD8-positive cells in the spleen, which also correlates with the septic profile [6]. In addition, such treatment increased the levels of CRP and PCT as well as the inflammatory cytokines IL-6, IL-18 and TNF-a in the serum, which classically increase during inflammation and are also associated with sepsis [21, 24]. The treatment also increased the immunosuppressive cytokine IL-10 in the serum, facilitating thus the establishment of the immunosuppressive state to the animals.

The application of the PG-activated implant one day after the LPS treatment initiation rescued all inflammatory and suppressive markers tested to control levels, while also restoring the levels of effector CD4- and CD8-positive cells in the spleen. Except from restoring endotoxemia markers, the PG-implant has been previously shown to induce non-specific IgG production [15], which could also play an additional therapeutic role to the model.

The application of the PG-activated implant to control untreated mice did not alter any of the markers in the spleen, except from the expression of class II MHC protein, which argues in favor of the mild immunostimulatory activity previously described [18]. However, such treatment of control mice increased the levels of IL-6 and TNF-a, but not IL18, IL-10, CRP or PCT, which could be proved useful to other pathologic conditions.

Interestingly, the application of non-activated implant to the LPS-treated animals, which was used as control to the system, could also rescue sepsis-associated markers in the spleen except from the effector CD8-positive cell population and arginase-1 activity, while also rescuing the levels of TNF-a, CRP and PCT, but not IL-6, IL-18 and IL-10 in the serum. Despite the similarities, when the non-activated implant was excised from the animals and submitted to SEM analysis, it displayed a quite different histology as compared to the PG-activated implant. The PG-activated implant showed thick cellular and membrane structures covering the scaffold with important collagen depositions, which as previously shown in the case of a conventional antigenic stimulus will support the development of blood vessels [17]. Non-activated scaffolds, although becoming populated by adherent cells (morphologically defined as

macrophages and fibroblasts), they failed to induce collagen depositions and protective membranes, at least at the specific time point tested.

Most importantly, the non-activated scaffolds failed to rescue the septic phenotype as evaluated by the MSS score, which, however, could be rescued almost to control levels by the PG-activated implants.

In conclusion, the results presented in this study showed that the “vaccine-on-chip” technology, using PG as the antigenic stimulus, could rescue the LPS-induced endotoxemia. In the context of personalized therapy, the “PG-vaccine-on-chip” could provide a controllable and safe management of the systematic inflammatory response characterizing sepsis. The rapid effectiveness of the proposed immunotherapy could be proved to rescue fatal morbidity to humans.

Declarations

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Conflicts of interest/Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

All authors contributed to the study. Conceptualization, data analysis, writing, funding acquisition and supervision was performed by Irene Athanassakis. Material preparation and data collection were performed by Ioanna Zerva and Katerina Bekela. All authors read and approved the final manuscript.

Ethics approval

The applied protocol was approved by the national Bioethical Committee (Approval # 292314, ADA ΨΗΘ47ΛΚ-Γ5Ψ).

Consent to participate

Not applicable

Consent for publication

Not applicable

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Figures

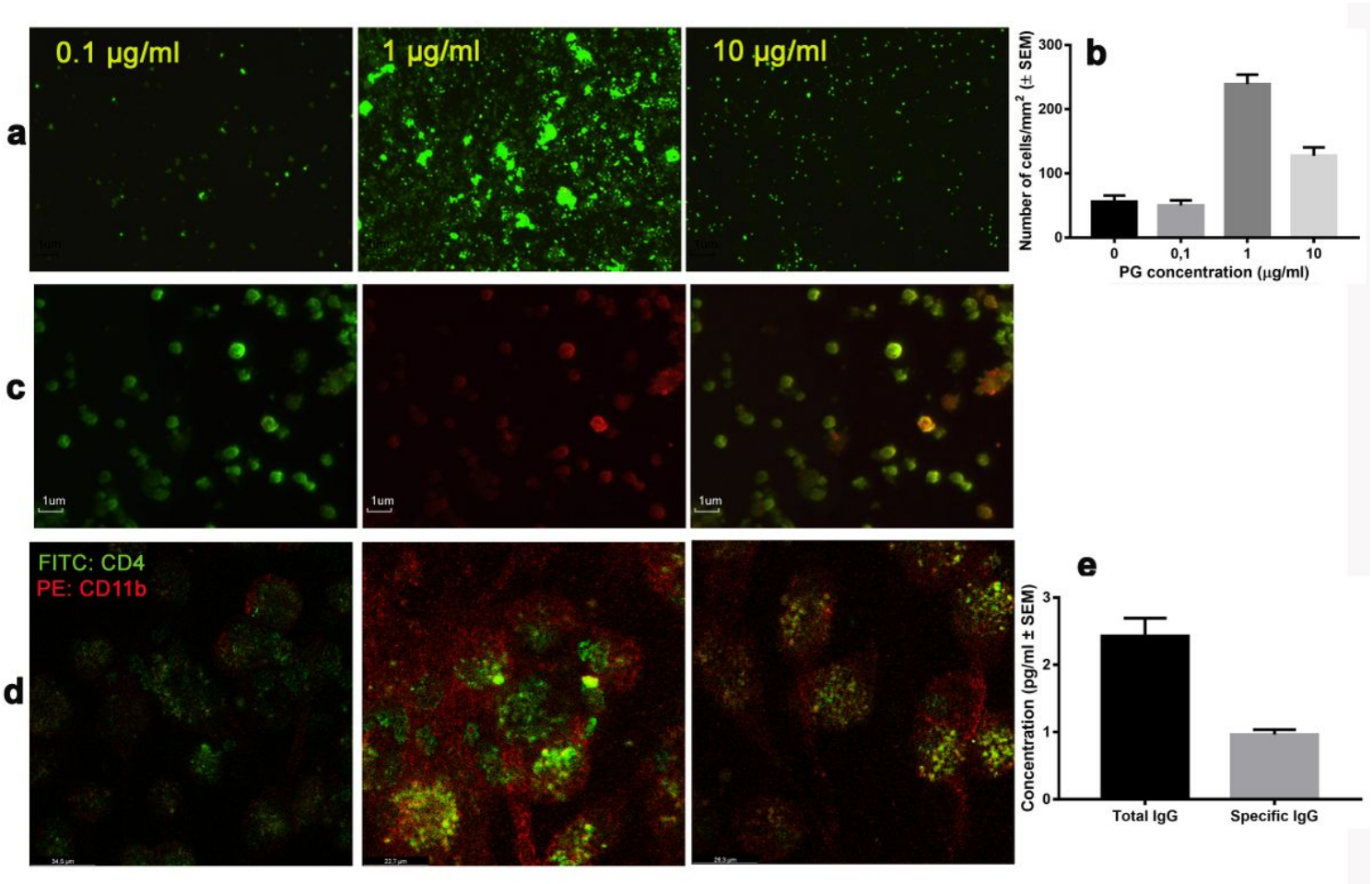

Figure 1

In vitro behavior of PG-activated implants. Spleen cells were let to adhere for 24-hours in the presence of Si scaffolds. After transferring scaffolds to new plates, PG was added to the cultures at concentrations of 0.1, 1 and 10 µg/ml. Proliferation of was assessed by CFSE staining (a). Quantification of the proliferating cells was evaluated by counting the numbers of nuclei and cells per mm2 of surface area stained with CFSE using the ImageJ analysis software (b). The results represent the mean of three experiments (±SEM). The CFSE-stained scaffolds (green) that were incubated with PG at the concentration of 1 µg/ml were also immunostained with PE-conjugated anti-CD11b and observed using an epifluorescence microscope (c). In another set of experiments, the PG-activated Si-scaffolds (1 µg/ml) 24h after PG addition, the antigen was removed and T and B lymphocytes, isolated from the total spleen cell population were further added to the cultures for 4 days and upon culture termination the scaffolds were submitted to double immunofluorescent staining with anti-CD11b (red) and anti-CD4 (green) and submitted to confocal microscopy analysis (d). Culture supernatants were tested for the presence of total or antigen-specific IgG antibody by ELISA (e). The results represent the mean of three experiments (±SEM).

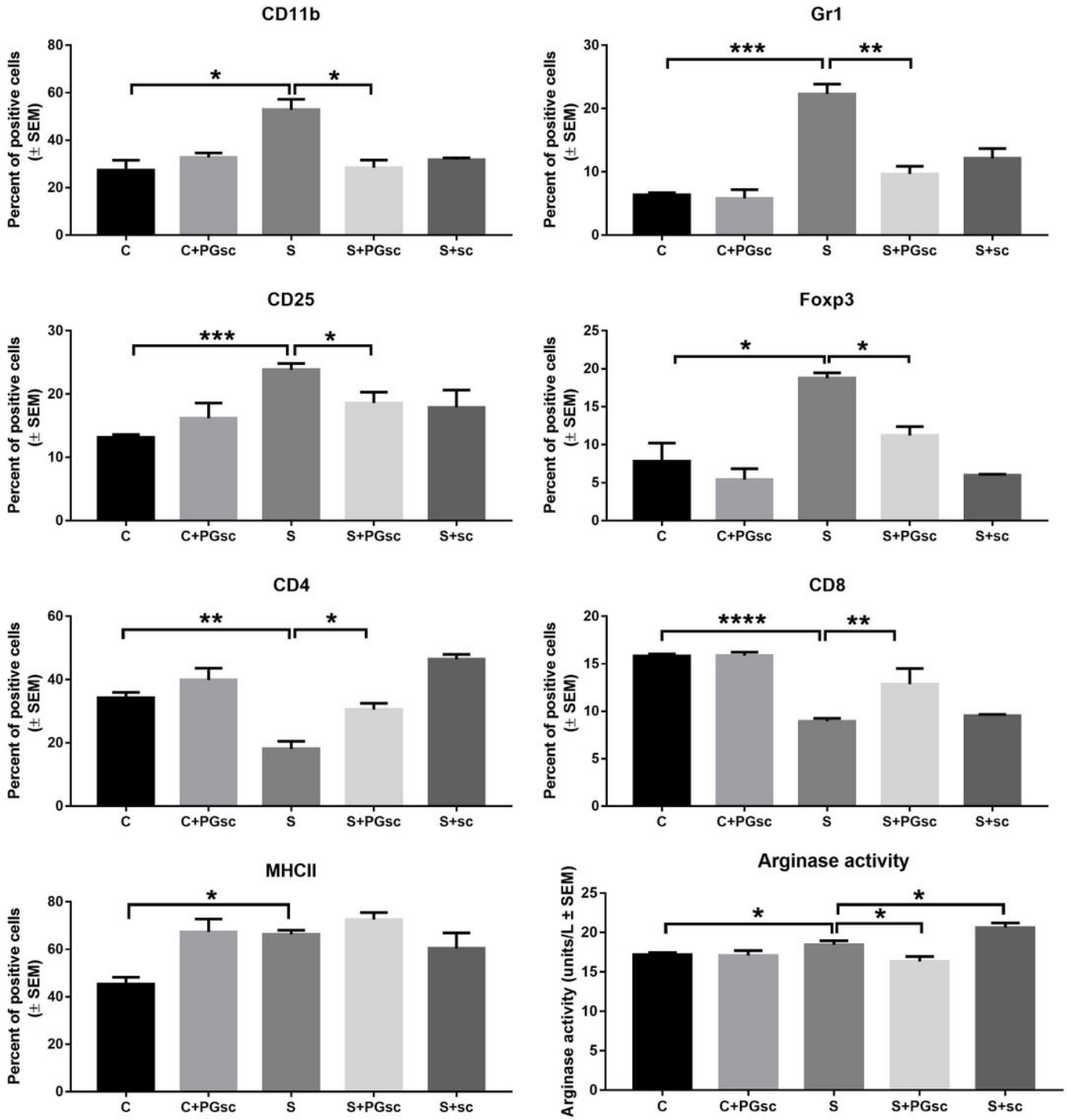

Figure 2

Immune marker analysis in spleen cells isolated from control (C) or LPS-treated (S) animals with or without implantation of PG-activated (PGsc) or non activated (sc) Si-scaffolds. BALB/c mice received daily intraperitoneal injections of 0.2 µg/g of body weight per day for 5 days and sacrificed on day 6. Implants were applied one day after the LPS initiation treatment. For implantation, mice were anesthetized and implanted to the rear left foot with control (sc) or PG-seeded (PGsc) macrophages

loaded onto the 3D microstructured Si scaffolds. Spleen cells were put in single cell suspension and upon lysis of erythrocytes the cells were submitted to immunofluorescence experiments followed by flow cytometry analysis. Arginase-1 activity was measured in spleen cells upon lysis, using an arginase detection kit. The results represent the mean of 5 experiments (± SEM). ****: p<0.0001, ***: p<0.001, **: p<0.005, *: p<0.01.

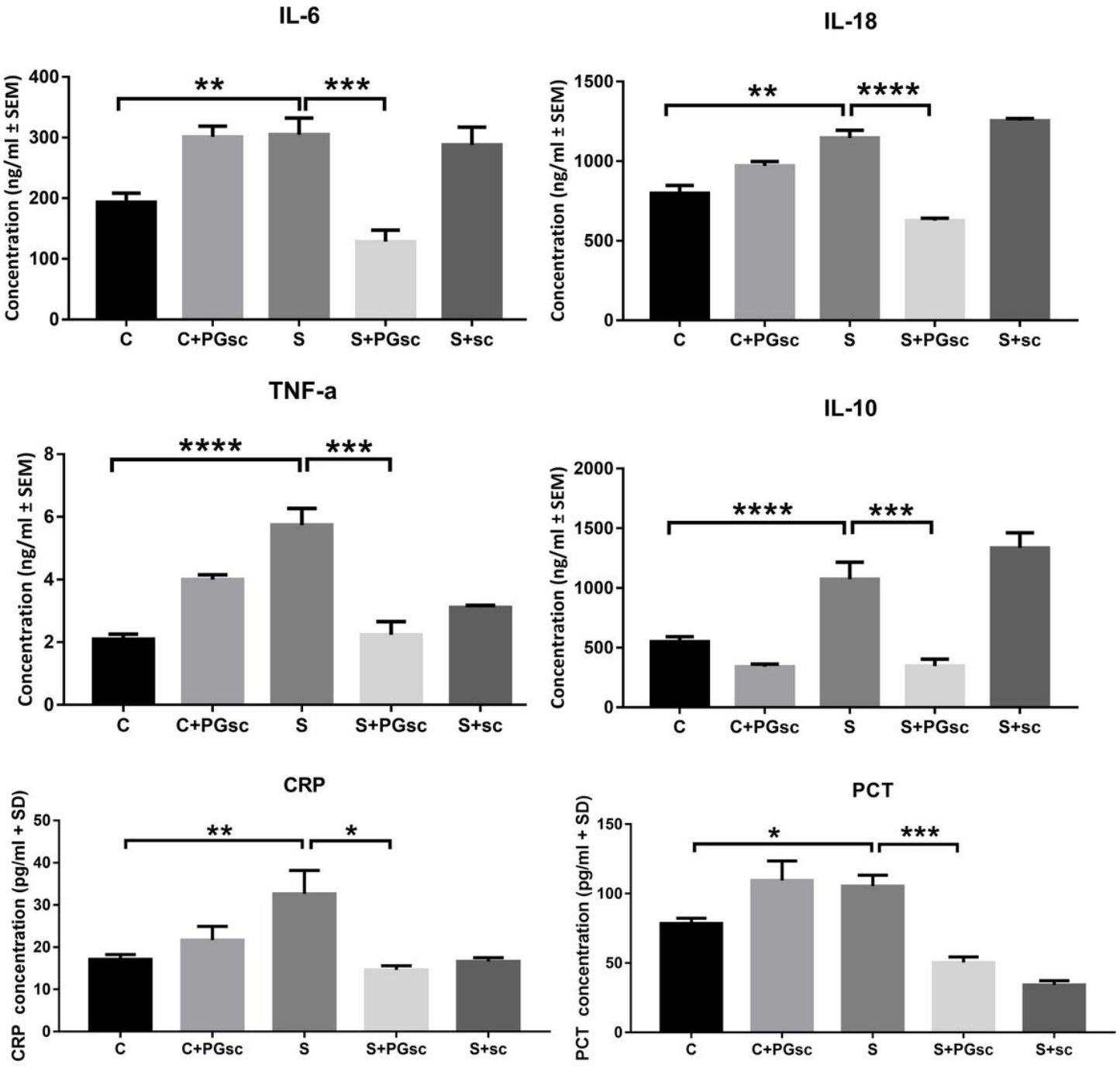

Figure 3

Detection of cytokine, CRP and PCT levels in the serum of control (C) or LPS-treated (S) animals with or without implantation of PG-activated (PGsc) or non activated (sc) Si-scaffolds. Cytokines were detected

in the serum by ELISA using specific monoclonal antibodies as described in the Methods section. The detection of CRP and PCT was performed using a mouse CRP ELISA Kit and a mouse PCT ELISA Kit respectively. The results represent the mean of 5 experiments (± SEM). ****: p<0.0001, ***: p<0.001, **: p<0.005, *: p<0.01.

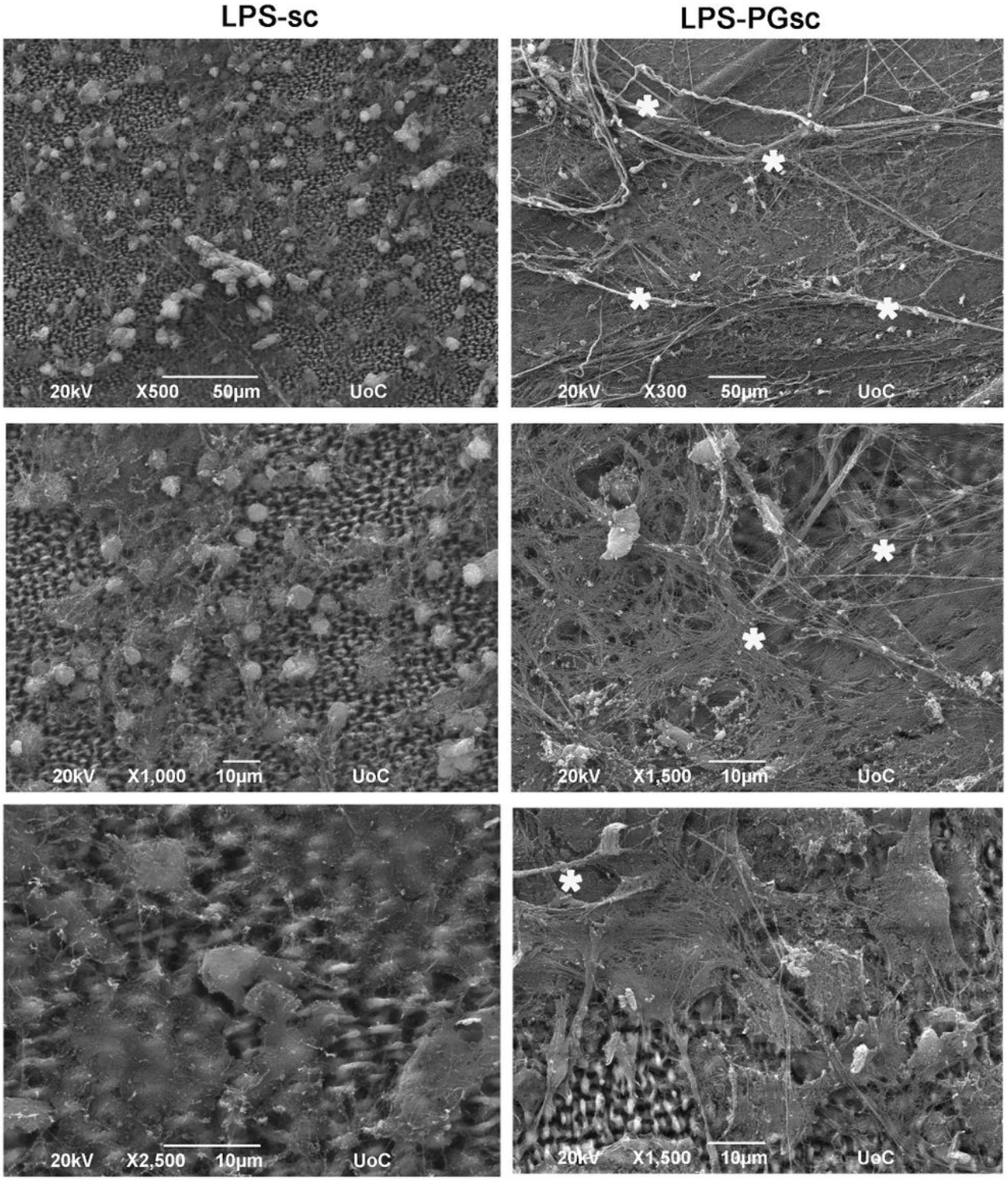

Figure 4

In vivo Si-scaffold-induced histology in LPS-treated mice implanted with PG-activated (LPS-PGsc) or non-activated (LPS-sc) Si-scaffolds. Implants were excided on day 6. Asterisks show collagen depositions. Panels show increasing magnification ranging from X300 to X2.500.

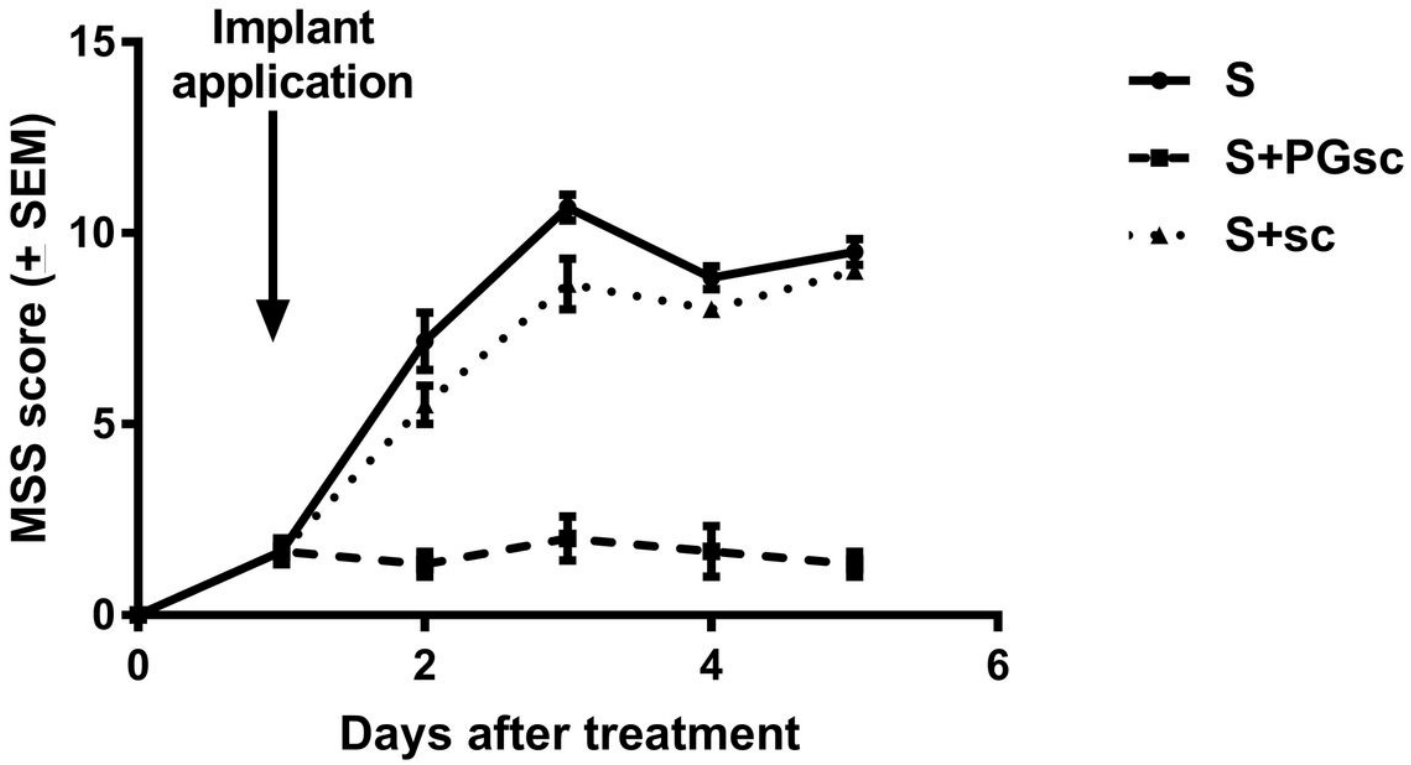

Figure 5

MSS scoring of LPS-treated mice implanted with PG-activated (S + PGsc) or non-activated (S + sc) Si-scaffolds. The number of mice examined in each case varied from 4 to 11. The results of MSS scoring are expressed as the mean of MSS ± SEM.