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 0.2 µg/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-dimensional laser micro-textured silicon (Si) scaffold loaded with macrophages and activated in vitro with 1 µg/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-α, 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.

KEY WORDS: endotoxemia; lipopolysaccharide; peptidoglycan; si scaffolds; sepsis; immunosuppression

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 conditions. 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 and oxygen free radicals, stimulation of the inducible NO synthase, decrease of effector immune cells, and 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, and programmed death-1 inhibitors, as well as intravenous immunoglobulin (IVIG) treatment enriched or not with IgM, have been envisaged as potential treatments [10–14]. To this extent, 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), as well as 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 to as “vaccine-on-chip” technology. Thus, using as human serum albumin (HSA) conventional antigen, 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, 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 to as PG-mediated immunotherapy-on-chip), the present study inquired whether the mild stimulatory activity induced by the application of the PG to the immunotherapy-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+, and Foxp3+ cells and arginase-1 activity in the spleen of LPS-treated animals, as well as IL-6, IL-18, IL-10, TNF-α, 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 # 292,314, ADA ΨΗΘ47ΛΚ-Γ5Ψ). Six- to 10-week-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 — 5 μg/mouse — per day, E. coli O111:B4, purity > 99%, Sigma-Aldrich, Germany), which led to death of approximately half of the treated mice (LD50 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 a single-cell suspension, and examined for various sepsis specific markers.

Mice were divided in 5 groups including untreated controls, untreated naïve 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 immunotherapy-on-chip treatment were implanted with the PG-activated or non-activated Si scaffold (see below) to the left rear foot of anesthetized mice (Avertin, Sigma-Aldrich) and sutured
using 3–0 silk suture material (DemeTECH, 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 3 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 dH2O, and aliquots at the concentration of 3 mg/ml were stored at –20 °C.

**Silicon Scaffold Preparation**

Planar Si surfaces 5 × 5 mm² 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/cm².

**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 10⁷ cells/ml at a final volume of 2 ml in the presence of scaffold substrates (5 × 5 mm²), 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, PG was added to the cultures. After an incubation of 24 h, 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 (CellTrace 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 Carl Zeiss 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, 24 h after PG addition, the antigen was removed and T and B lymphocytes, isolated from the total spleen cell population, were added to the cultures (6.5 × 10⁶ 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 a Forma Scientific incubator at 37 °C in the presence of 5% CO₂.

**Antibodies**

Antibodies against mouse Foxp3-Alexa Fluor 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 1 μg/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, TX) were used at the concentration of 0.1 μg/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 mmol 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, and 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 Titertek ELISA photometer (DigiScan, ASYS Hitech 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 0.1 M sodium cacodylate buffer (SCB) and then incubated in the same solution for 15 min, twice. Samples were fixed using 2% glutaraldehyde and 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.

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 immunotherapy-on-chip system leading to macrophage proliferation and spreading on the Si scaffolds (Fig. 1). To this extent, Si scaffolds loaded with macrophages after a 24-h 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 “MATERIALS AND METHODS”). The results showed that PG induced the best proliferative activity at the concentration of 1 μg/ml (Fig. 1a, b). 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
Immunotherapy-on-Chip Against an Experimental Sepsis Model

Immunofluorescence staining, followed by confocal microscopy analysis of the activated Si scaffolds, could detect CD11+ macrophages (red) and on top CD4+ T cells (green) (Fig. 1d). In this system, B cells (CD19+) and non-specific IgG, while the activated Si scaffolds were washed and submitted to double immunofluorescence experiments for the detection of T-macrophage cell interactions.

**Fig. 1** *In vitro* behavior of PG-activated implants. Spleen cells were let to adhere for 24 h 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 square millimeter 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) 24 h 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). Serial sections from bottom (1) to top (6) are shown. Red arrows show some examples of CD4+ cells, which appear clearly in top sections, indicating thus their position vis-à-vis CD11+ cells. 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).
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 of approximately half of the treated mice (LD50 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 [15]. 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 “MATERIALS AND METHODS” and implanted 1 day after the initiation of the LPS treatment to mice. Animal behavior was evaluated during the treatment period and 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), and Gr1 by 45% (p = 0.0136) and increase the expression 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 of reversing the LPS-induced suppressive markers in the spleen (Fig. 2). The application of Si implant without a macrophage load did not alter any of the markers tested in these sets of experiments (data not shown).

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.
Fig. 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 1 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.
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, IL-18, 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 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). The application of Si implant without a macrophage load did not alter any of the factors tested in these sets of experiments (data not shown).

Implant Histology

Previous studies have shown that the histology of the excised implants differs in accordance to the antigenic 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 5 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, 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 implants 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, on a daily basis, within a range of grades from 0 to 5 (most severe), the appearance, level of consciousness, activity, response to stimulus, appearance of eyes, respiratory rate, and respiratory quality were evaluated. Adapting the MSS scoring [16, 19], animals acquired sepsis symptoms even 1 day after the LPS treatment, showing patches of hair piloerected, avoidance of standing upright, and slightly suppressed activity. The phenotypic recording showed that the LPS-treated animals that received the PG-activated implant reduced by 80% defined by MSS symptoms, while non-activated implants failed to rescue the endotoxemia phenotype (Fig. 5).

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
Immunotherapy-on-Chip Against an Experimental Sepsis Model

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

Fig. 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 “MATERIALS AND METHODS.” 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.
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 5 μg 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 h
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 1 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 for 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 IL-18, 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 for 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), 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-mediated-immunotherapy-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.

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AUTHOR CONTRIBUTION

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

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AVAILABILITY OF DATA AND MATERIALS

Not applicable.

CODE AVAILABILITY

Not applicable.

DECLARATIONS

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.

Conflict of Interest The authors declare no competing interests.

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