Fibroblasts upregulate expression of adhesion molecules and promote lymphocyte retention in 3D fibroin/gelatin scaffolds

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Bioengineered scaffolds are crucial components in artificial tissue construction. In general, these scaffolds provide inert three-dimensional (3D) surfaces supporting cell growth. However, some scaffolds can affect the phenotype of cultured cells, especially, adherent stromal cells, such as fibroblasts. Here we report on unique properties of 3D fibroin/gelatin materials, which may rapidly induce expression of adhesion molecules, such as ICAM-1 and VCAM-1, in cultured primary murine embryonic fibroblasts (MEFs). In contrast, two-dimensional (2D) fibroin/gelatin films did not show significant effects on gene expression profiles in fibroblasts as compared to 3D culture conditions. Interestingly, TNF expression was induced in MEFs cultured in 3D fibroin/gelatin scaffolds, while genetic or pharmacological TNF ablation resulted in diminished ICAM-1 and VCAM-1 expression by these cells. Using selective MAPK inhibitors, we uncovered critical contribution of JNK to 3D-induced upregulation of these adhesion molecules. Moreover, we observed ICAM-1/VCAM-1-dependent adhesion of lymphocytes to fibroblasts cultured in 3D fibroin/gelatin scaffolds, but not on 2D fibroin/gelatin films, suggesting functional reprogramming in stromal cells, when exposed to 3D environment. Finally, we observed significant infiltration of lymphocytes into 3D fibroin/gelatin, but not into collagen scaffolds in vivo upon subcapsular kidney implantation in mice. Together our data highlight the important features of fibroin/gelatin scaffolds, when they are produced as 3D sponges rather than 2D films, which should be considered when using these materials for tissue engineering.

1. Introduction

Artificial tissue and organ engineering represents rapidly evolving research field, which may provide new therapeutic opportunities in the treatment of various diseases ranging from complete replacement of missing or damaged organ to tissue-specific delivery of cells, growth factors, vaccines, or drugs in vivo using bioengineered carriers [1–3]. One potential application of this approach is generation of artificial lymphoid tissues capable of attracting host-derived immune cells and inducing antigen-specific immune response. Such artificial organoids represent attractive tools for cancer immunotherapy [4,5] as well as for routine vaccinations [6]. However, there remain several unresolved issues regarding artificial lymphoid tissue generation with the key focus on the reconstruction of stromal and immune cell interactions.

Organ functionality requires cells to undergo a specific spatial distribution in the three-dimensional (3D) extracellular matrix (ECM) [7]. In order to reproduce this distribution in artificial tissues, various bioengineered 3D scaffolds have been developed [8–10]. Several types of biomaterials are considered suitable for scaffold production including synthetic [11,12] and natural [13] biopolymers. Physical and chemical properties of such biomaterials determine the bioactivity of fabricated scaffolds and can affect the expression program of the cells that interact

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with the scaffold [14–17]. For example, hydroxyapatite is attracting much attention because for its osteogenic properties [18], while tantalum-based 3D scaffolds support hematopoietic differentiation of embryonic stem cells [19]. Both synthetic and natural types of scaffolds are potentially being applicable for tissue engineering with specific tasks.

Despite the growing number of studies involving silk-based biomaterials, there is still a gap in our understanding of how these materials affect cell properties. Recent study demonstrated proinflammatory phenotype of human monocytes cultured on 3D fibroin scaffolds [20]. In line with that, proinflammatory properties of fibroin/gelatin, but not spidroin microparticles in the model of skin wound healing in mice were also reported [21]. In addition, an increase in ICAM-1 expression by murine embryonic fibroblasts (MEFs) cultured in 3D fibroin/gelatin scaffolds for several weeks were previously observed, although the exact mechanism for this effect was not defined [22]. In this study we uncover a peculiar property of 3D, but not of 2D fibroin, supplemented with gelatin, to induce a rapid expression of the adhesion molecules ICAM-1 and VCAM-1 in the primary cultured MEFs. These changes in stromal 3D fibroin/gelatin scaffolds both in vitro and in vivo. Together our data indicate that 3D fibroin/gelatin scaffolds can be beneficial for engineering of artificial lymphoid organs or for generation of biomaterial-based vaccines.

2. Materials and methods

2.1. Scaffold fabrication

Scaffolds were prepared from aqueous fibroin and gelatin solutions with minor modifications from previously described protocol [23]. Briefly, silk surgical threads (LCC “Optikum”) were boiled for 1.5 h in 0.03 M NaHCO₃ (pH 8.4), washed in distilled water and dried in order to remove residual sericin. Sericin-free silk was dissolved in a mixture of CaCl₂·C₆H₁₂O·H₂O (1:2.8 M proportion) for 5 h in water bath at 50 °C. Dissolved fibroin was centrifuged at 13,000 g and dialyzed using dialysis membrane (MWCO -12000 M, Orange scientific) against distilled water at 20 °C. After 10 dialysis cycles the solution was centrifuged for 20 min at 13,000 g.

Two-dimensional (2D) scaffolds were prepared using solution-casting method. Fibroin/gelatin (7:3) as 2% aqueous solution was applied as a thin layer to the surface of 35 mm culture dish and dried at room temperature for 2 days. Dried 2D fibroin/gelatin films were placed in 96% ethanol for 1 h, followed by gradual transfer to distilled water with decreased alcohol concentration by 10% every 15 min.

Three-dimensional scaffolds, 15 mm in diameter and 3.5 mm thick, were prepared in 24-well plates by adding 700 µL of 2% aqueous fibroin/gelatin (7:3) solution (3D F/G scaffolds) or 2% gelatin solution (3D G scaffolds) supplemented with 1% DMSO to each well. The plates were then stored at −20 °C for 7 days. Frozen scaffolds were soaked in 96% ethanol for 1 h, then samples were removed from the wells and transferred to 70% ethanol for storage. For implantation experiments 3D scaffolds were fabricated according to the same protocol, but employing 4 mm diameter plastic tube for casting. To generate F/G disks for implantation the resulting tube-shaped scaffolds were then cut to the final thickness of 1.5 mm and the diameter of 4 mm. 3D polystyrene scaffolds were purchased from Merck Sigma Aldrich. 3D collagen scaffolds were purchased from Advanced Biomatrix.

2.2. Mice

WT, TNF-deficient (TNF KO) [24] and IL-6-deficient (IL-6 KO) mice (generated from those described in Ref. [25]) on C57BL/6 genetic background were provided by the SSU “Biomodel,” a branch of the Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, housed under specific pathogen-free conditions on 12 h light/dark cycle at 20–23 °C and used for the experiments at the age of 8–10 weeks (weight of 20–22 g). GFP-transgenic mice (C57BL/6-Tg (CAG-EGFP)1Osb/J, The Jackson Laboratory) were kindly provided by Dr. N. Logunova from the Central Scientific Research Institute of Tuberculosis, Moscow, Russia. Animal manipulations were carried out in accordance with recommendations in the Guide for the Care and Use of Laboratory Animals (NRC 2011), the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, Council of Europe (ETS 123), and “The Guidelines for Manipulations with Experimental Animals” (the decree of the President of the Russian Academy of Sciences of April 2, 1980, no. 12000–496).

2.3. Mouse embryonic fibroblasts cultures

Primary mouse embryonic fibroblasts (MEFs) were isolated from WT, TNF KO, IL-6 KO and GFP-transgenic mice according to standard protocol [26]. Cells were cultured on 10 cm culture dishes for adherent cells (Greiner bio-one) in 10 mL of DMEM (ThermoFisher), supplemented with 100 U/mL of penicillin, 100 µg/mL of streptomycin, 2 mM l-glutamine, and 10% fetal bovine serum (all from ThermoFisher). To detach the cells from the dish, 0.25% trypsin 2 mM EDTA solution (ThermoFisher) was used. For experiments MEFs were seeded on 24-well plates in different conditions: standard culture plate (control). Primary MEFs were placed in incubator for 1 h to promote cell attachment. Then 900 µL of the medium were added to each well, resulting in 1 mL sample volume. For control and 2D F/G culture conditions 2 × 10⁵ cells/well in 1 mL total volume were seeded on standard of F/G-treated 24-well plates, respectively. At certain timepoints, culture medium was collected to analyze cytokine production, and scaffolds with cells were harvested in Trizol (ThermoFisher) for mRNA expression analysis. In supernatant transfer experiments culture medium from control, 2D- or 3D-cultured MEFs was collected at specified timepoints and placed over cells cultured under control conditions. In this case, cells were harvested using Trizol for mRNA analysis following 6 h of culture with transferred supernatants.

2.4. Lymphocyte adhesion assay

Mice, 6 to 8 weeks old, were euthanized by cervical dislocation and their spleens were collected into sterile RPMI1640 (ThermoFisher). Single cell suspensions were obtained by mashing the spleen and passing cells through 30 µm pre-separation filter (Miltenyi Biotec Inc.). For positive magnetic separation of CD4⁺ cells, splenocytes were incubated with L3T4 Microbeads (Miltenyi Biotec Inc.) for 15 min at 4 °C. After this, cells were treated with Cy3-conjugated secondary polyclonal antibodies (goat anti-rat IgG H + L) for 5 min at 4 °C. Cells were then washed once with PBS containing 0.5% BSA and 2 mM EDTA (pH 7.2). The amount of antibodies and beads were determined based on the initial cell count according to manufacturer’s protocol. Cells were maintained in chilled PBS containing 0.5% BSA and 2 mM EDTA (pH 7.2) throughout the incubations. For all magnetic cell separations, cells were transferred to LS Columns (Miltenyi Biotec Inc.) and MidiMACS following manufacturer’s instructions. Labeled cells were collected as the positive fraction for subsequent experiments.

Primary GFP⁺ MEFs were seeded at 2 × 10⁵ cells/well on 2D F/G-coated glass slides or in 3D F/G scaffolds and cultured for two days. The culture medium was then replaced with 2 × 10⁵ CD4⁺ cells were added to each well 16 h later. After 4 h of co-culturing MEFs with CD4⁺ cells at 37 °C and 5% CO₂, non-adherent cells were removed and the scaffolds were washed five times with DMEM to further eliminate unbound or loosely-bound cells. Samples were fixed with 4% paraformaldehyde and stained with DAPI (Thermo Fisher Scientific). Images were captured using Eclipse Ti-E microscope with the A1 confocal.
module (Nikon Corporation) and Plan Fluor 40×1.3 objective. Image analysis was performed using NIS-Elements and ImageJ software [27]. To determine the role of adhesion molecules in MEF–CD4+ cell interactions, MEFs were pre-exposed to 20 μM monoclonal antibodies against ICAM-1 (KAT1) or VCAM-1 (6C71) (generously provided by Dr. A. Kruglov, DRFZ, Berlin, Germany) for 45 min at 37 °C and 5% CO₂ prior to co-culturing them with CD4+ cells. 2D-cultured MEFs and non-seeded scaffolds were used as controls. All experiments were performed in duplicate wells.

2.5. Kidney subcapsular implantation

F/G or collagen (Advanced Biomatrix) scaffolds (4 mm in diameter) were used for implantation studies. Some scaffolds were first colonized with MEFs (10⁶ cells/scaffold) and cultured in complete DMEM in 96-well plates (200 μL of medium per sample) for one day. Control blank scaffolds without the cells were also cultured in the same medium for one day prior to implantation. On the day of implantation scaffolds were washed 3 times in 1 mL of sterile PBS prior to the start of surgical procedures. Implantation was performed according to the published protocol [28]. WT mice were anesthetized with 100 μL of 10% Zoletil/20% Rometar mixture in PBS and kept on heating pad until complete recovery from anesthesia. Each scaffold was cut in two equal parts, which were subsequently implanted under the kidney capsule (maximum 2 implants per kidney and total of 4 implants per mouse) and surgical sutures (Sterion, Russia) were applied to close the wounds. Three weeks following the implantation mice were euthanized and implanted scaffolds were collected and frozen in OCT Tissue Tech compound for subsequent IHC analysis. Some of the scaffolds were snap frozen in liquid nitrogen for subsequent gene expression analysis.

2.6. Intracellular signaling pathway inhibitors

MAPK inhibitors were purchased from Cell Signaling Technology: 10 μM of U0126 was used to inhibit ERK1/2, 50 μM of SP600125 was used to inhibit JNK1/2 and 10 μM of SB202190 was used to inhibit p38. In addition, 20 μM of JSH-23 (SigmaAldrich) was used to inhibit classical NFκB activation. All inhibitors were prepared as 1000x stock solutions in DMSO, thus, 0.1% DMSO was added to the control group. MEFs were exposed to the corresponding inhibitors for 30 min prior to being transferred to the scaffolds. Following 6-h incubation cells were harvested for gene expression analysis.

2.7. TNF inhibition

Murine TNF neutralizing antibody (clone XT3.11, BioXCell) and rat IgG1 isotype control (clone TNP6A7, BioXCell) were used at concentration of 2 μg/mL in the experiments with TNF inhibition. Antibodies were added at the time when cells were seeded on 3D F/G scaffolds or culture plate. After 6 h of incubation cells were harvested for gene expression analysis.

2.8. Gene expression analysis

RNA was isolated using TriZol (ThermoFisher) according to manufacturer’s instructions. RNA quantity and purity were analyzed by measuring A260, A230, and A280 using NanoPhotometer N50 (IMPLEN). For each sample, 1 μg of RNA was treated with DNase I (ThermoFisher) and subsequently used for reverse transcription with oligo-(dT) primers (ThermoFisher). Gene expression analysis was performed using RT-PCR kit (Evrogen) on 7500 RT-PCR System Machine (Applied Biosystems). Primer sequences specific for exon-exon junctions of RNA was treated with DNase I (ThermoFisher) and subsequently used for reverse transcription with oligo-(dT) primers (ThermoFisher). Gene expression analysis was performed using RT-PCR kit (Evrogen) on 7500 RT-PCR System Machine (Applied Biosystems). Primer sequences specific for exon-exon junctions of the target genes were designed using NCBI PrimerBlast software and are listed in Table 1. Aeb gene was used as internal reference. RT-PCR quality check was performed using melting curve analysis. Data analysis was performed using 2-ΔΔCt method [29]. In most cases, expression levels relative to control standard culture conditions are shown, unless otherwise stated.

### Table 1

| Gene              | Forward primer 5'-3'                          | Reverse primer 5'-3'                          |
|-------------------|-----------------------------------------------|-----------------------------------------------|
| Acb               | CTCTTGAGGCCAAGTACTCTGTG                        | TAAAGGCAGCTGTAACAGTCC                        |
| icam1             | AGTGGTGGAAGTGTTTCTT                          | TCAACGAGGACCATCAG                           |
| Vcam1             | GAGCCGCCACTAAAGGCGAA                         | TCTCTGGGAAAGATGAGTGCC                       |
| Tnf               | TGACCCAGCGTGACACCAC                           | CAAGGTTGAAACCCTAGGAC                       |
| It6               | AACCAGGGCGCTCTCATCT                            | TGCCATGCAACACTTCCTCT                        |
| Itg2              | ATTTTGAAGTTGAGGGCG                           | CACCGCCGGCTCTCTCTCA                        |
| icam4             | CTGGTCCAAAGAGCCAGCAAA                        | CACTCGGCCACCTTCCTCA                        |
| icam5             | CTGATCAAGGCGAGCTTTTGT                        | GGAGGCTGAGGAGGCTACG                        |
| Itg1              | GAGACGGCAGGAAAGGAGGAG                        | GGACGCTGAGGACACTTGT                        |
| Vegf              | CGGAGCCAGGAGGAGGAG                          | GGGATGATACGCGGGGAG                           |
| Vegfc             | GTGTCAGCCAGGAAAGGACCA                       | TTGCCCTCAGAAGAGCCAGCC                       |
| Kdr               | GCATACGGCCGTCCTGTGAC                        | TCCGACAGGACACTGAC                           |
| It1               | CATCATCGGCAACAAATCA                         | CACCAATCTGTCGCTTATT                        |
| It4               | ATTATCCAGGAAAGGCCAG                         | CGGCTAGTACCATGCTGC                         |
| Ccx13             | CTCTTCCAGGAGCAGCTTAT                       | CAGGGGGGAATCCTGCAAA                        |

* - Kindly provided by Dr. A. Kruglov, DRFZ, Berlin, Germany.

### Table 2

| Antibodies for immunofluorescent staining | Fluorophore | Clone | Dilution | Supplier             |
|------------------------------------------|-------------|-------|----------|----------------------|
| ICAM-1                                   | Cy5         | KAT1  | 1:300    | DRFZ*                |
| VCAM-1                                   | FITC        | 6C71  | 1:200    | DRFZ*                |
| CD4                                      | –           | RM4-5 | 1:200    | BD                   |
| CD3                                      | –           | 145-2C11 | 1:400  | Thermofisher         |
| R220                                     | –           | R3-682 | 1:400    | BD                   |
| CD31                                     | –           | S90   | 1:400    | BD                   |
| F4/80                                    | –           | BM8   | 1:400    | Thermofisher         |
| rat IgG                                  | Alexa Fluor | polyclonal | 1:200  | Jackson               |
| hamster                                 | Alexa Fluor | 546   | polyclonal | ImmunoResearch   |
| rabbit IgG                               | Alexa Fluor | 488   | polyclonal | ImmunoResearch   |
|                                         |             | 647   | polyclonal | ImmunoResearch   |

* - Kindly provided by Dr. A. Kruglov, DRFZ, Berlin, Germany.
2.9. Immunofluorescent staining

Samples of 3D F/G scaffolds or 2D F/G films with MEFs were fixed in 4% PFA and washed in PBST (PBS buffer with 0.1% Triton-X100) for 10 min. Next, non-specific binding was blocked with 0.3 M glycine, 1% BSA in PBST for 2 h. Primary antibodies to ICAM-1 and VCAM-1 (see Table 2) and SYTOX orange dye (3.125 μM, ThermoFisher) were added in PBST supplemented with 0.3 M glycine and 1% BSA and incubated for 45 min. After three washes in PBST, samples were mounted in Aqua-Poly/Mount (Polysciences, Inc.) and analyzed using confocal microscope Nikon Eclipse Ti-E A1 (Nikon Corporation) and objective lens CFI Plan Apo VC 20x/0.75.

Implanted scaffolds were fixed in 4% PFA and placed on a shaker for 30 min at +4 °C. Samples were then washed in PBST and blocked in

Fig. 1. MEFs, cultured on 3D fibroin/gelatin scaffolds, overexpress adhesion molecules. A – Time-dependent gene expression of Icam1 and Vcam1 in MEFs, cultured on control cell culture grade plastic plate, 2D F/G films or in 3D F/G scaffolds (n = 3). B – Immunofluorescent staining of ICAM-1 and VCAM-1 (green) on MEFs, cultured on 2D F/G films or in 3D F/G scaffolds for 24 h. Nuclei (red) are stained with SYTOX Orange. C – Gene expression of ICAM-family adhesion molecules in MEFs, cultured on 2D F/G films or in 3D F/G scaffolds for 6 h (n = 3). D – Gene expression of ICAM-1 and VCAM-1 ligand subunits (Itgal/Itgb2 and Itga4/Itgb1, respectively) in MEFs, cultured on 2D F/G films or in 3D F/G scaffolds for 6 h (n = 3). Expression of Itgal was not detected in MEFs. E – Icam1 and Vcam1 expression in MEFs, cultured for 6 h on control cell culture grade plastic plate, 2D F/G films, 3D F/G, 3D fibroin-only (3D F), 3D gelatin-only (3D G) or 3D polystyrene (3D PS) scaffolds (n = 5). Data from one representative experiment of at least three independent experiments is shown.*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns – not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
PBST with 1% skimmed milk and 2% BSA and placed on a shaker for 1 h at +4 °C. Primary antibodies (see Table 2) were added in PBST with skimmed milk and incubated overnight with shaking at +4 °C. After three subsequent washes in PBST (each for 2 h with shaking +4 °C), samples were incubated with secondary antibodies in PBST with skimmed milk overnight with shaking at +4 °C. After three more washing steps, samples were incubated with PBST containing DAPI for 1 h and were imaged using Leica TCS SP5 and Zeiss LSM 510 laser scanning microscopes. Images were analyzed with ImageJ software.

2.10. Immunohistochemistry

Frozen samples of implanted scaffolds were cut using cryotome HM 560 to generate 20 μm sections (ThermoFisher) then placed on glass slides, dried and fixed in cold acetone for 20 min. All subsequent steps were performed in moisture chamber to prevent slides from drying. Samples were washed and permeabilized in TBST (TBS buffer with 0.1% Tween 20) for 5 min. Non-specific binding was blocked by incubation in 5% BSA solution in TBST for 1 h. Next, primary antibodies (see Table 2) in TBST were added for overnight incubation at +4 °C. After three washes in TBST, secondary antibodies were added for 1 h at +4 °C. After final three washes in TBST, slides were mounted with FluoromountG with DAPI (ThermoFisher). Samples were analyzed using Leica TCS SP5 and Zeiss LSM 510 laser scanning microscopes. Images were analyzed with ImageJ software.

2.11. Cytokine production

TNF and IL-6 levels in supernatants collected from the primary MEFs cultures were measured using Mouse TNF-, IL-6, or IL-1β Ready-Set-Go! ELISA Kit (ThermoFisher) according to the manufacturer’s instructions.

2.12. Statistical analysis

Data analysis was performed in GraphPad Prism software and presented as mean ± SEM. One-way and two-way ANOVA with Holm-Sidak post-test were used to calculate statistical significance. p-values less than 0.05 were considered to be significant.

3. Results

3.1. Increased expression of ICAM-1 and VCAM-1 in primary MEFs cultured in 3D F/G scaffolds, but not on 2D F/G films

We previously established that fibroin/gelatin scaffolds supplemented with 30% gelatin induce potent growth of primary murine embryonic fibroblasts (MEFs) in vitro [30]. However, the impact of the scaffold culture conditions on the phenotype of the stromal cells was not previously investigated. The expression of adhesion molecules is known to be crucial for the function of stromal cells (i.e., fibroblasts), especially, for their contacts and interaction with the immune cells [31,32]. Among the adhesion molecules ICAM-1 and VCAM-1 appear the most relevant, since they mediate lymphocyte adhesion to endothelial cells in the peripheral tissues and lymphoid organs [33-35]. Moreover, ICAM-1/VCAM-1 expression levels correlate with lymph node stromal cell maturation starting with ICAM-1 VCAM-1 lymphoid tissue organizer cells (LTGo) differentiating to mature ICAM-1hiVCAM-1hi fibroblastic reticular cells [32]. Thus, we first aimed to determine whether 3D culture conditions on fibroin/gelatin scaffolds may affect the expression of ICAM-1 and VCAM-1 in MEFs.

To investigate the impact of both 3D microenvironment and fibroin/gelatin biomaterial, we setup three types of MEF cultures: a control culture on standard cell culture-grade plastic (control), two-dimensional fibroin/gelatin films (2D F/G) and three-dimensional fibroin/gelatin scaffolds (3D F/G). Interestingly, we found that 3D F/G scaffolds, but not 2D F/G films, induced rapid increase in mRNA expression of both ICAM-1 and Vcam1 genes by MEFs starting as early as 3 h of culture and reaching the peak mRNA level after 6 h (Fig. 1A). This effect was further confirmed at the protein expression level by using immunofluorescence staining after 24 h of incubation (Fig. 1B). Moreover, MEFs maintained upregulated expression profile of ICAM-1 and Vcam1 in 3D F/G scaffold even in 7-day cultures, while in 2D MEF cultures the expression of adhesion molecules remained low even in 3-day cultures, the latest time point analyzed before the cells reached full confluency (Suppl. Fig. 1A). Interestingly, among the ICAM family of adhesion molecules, only ICAM-1 was overexpressed in 3D-cultured MEFs at 6-h time point (Fig. 1C). Expression of the corresponding ligands for ICAM-1 (Igα/ Igβ2) and VCAM-1 (Igα4/Igβ1) was not affected by 3D culture conditions at 6 h (Fig. 1D). Tissue engineering requires vascularization of implanted scaffolds [36]. To reveal whether culturing of the cells in 3D F/G scaffolds could promote vessel growth upon implantation, expression of VEGF family growth factors, as well as their receptor subunits was analyzed in plate- and 3D-cultured MEFs. Significant upregulation of Vegfa, Vegfr3, and Vgfc was observed, while only Flt1 receptor subunit demonstrated increased expression in 3D culture system (Suppl. Fig. 1B). These data indicate that 3D F/G scaffolds could possibly promote vascularization upon implantation in vivo.

Previous studies on human monocytes suggested that the effects of 3D fibroin scaffolds may be due to specific changes in fibroin conformation in these scaffolds, rather than to 3D microenvironment per se [20]. To address this possibility, we performed additional experiments using commercially available 3D polystyrene scaffolds (3D PS) as 3D microenvironment control. Indeed, 3D PS-cultured MEFs failed to show an increase in ICAM-1 and VCAM-1 expression, suggesting that this effect was specific to 3D F/G scaffolds (Fig. 1E). Finally, since collagen and its derivatives can potentially alter expression of adhesion molecules by the cells [37], we analyzed MEFs cultured on 3D fibroin-only scaffolds and found the same upregulation of ICAM-1/VCAM-1 expression, suggesting that this effect was not related to gelatin moiety in the scaffolds (Fig. 1E), but rather was induced by specific properties of 3D fibroin itself. In line with this, no upregulation of Icam1 and Vcam1 was observed in MEFs cultured on 3D gelatin-only scaffolds (3D G, Fig. 1E).

Together our data indicate that 3D F/G scaffolds could possibly promote overexpression of adhesion molecules ICAM-1 and VCAM-1 in primary MEF cultures.

3.2. TNF is partially responsible for Icam1 and Vcam1 overexpression in MEFs cultured in 3D F/G scaffolds

We next investigated the mechanism driving the overexpression of adhesion molecules in 3D-cultured MEFs. First, we asked if this effect required any soluble mediators, produced by cells in response to 3D scaffolds, which induced the overexpression of adhesion molecules in autocrine manner. To test this hypothesis, we performed experiments with the transfer of conditioned medium from control, 2D- or 3D F/G-cultured MEFs to MEFs that were cultured in standard conditions (Fig. 2A). Strikingly, we observed a significant increase in expression of Icam1 and Vcam1 only in those MEFs that received conditioned medium from 3D F/G-cultured MEFs (Fig. 2B). Importantly, using conditioned medium from different time points, we observed clear time dynamics in the expression of adhesion molecules. Medium from MEFs cultured in 3D scaffolds for 2–3 h failed to induce significant expression of Icam1 and Vcam1. However, medium from MEFs cultured in 3D scaffolds for 6 or 24 h induced significant upregulation of Icam1 expression by MEFs, cultured under standard conditions (10-15-fold increase as compared to control cells). At the same time, moderate increase in Vcam1 expression was observed when using medium from MEFs, cultured in 2D scaffolds for 24 h (3-5-fold increase as compared to control cells). Thus, our data indicate that Icam1 and Vcam1 expression in 3D-cultured MEFs was induced by soluble mediators.

Various cytokines and stress factors are known to induce Icam1/Vcam1 expression in fibroblasts [38-40]. Since F/G microparticles...
Fig. 2. MEFs, cultured on 3D fibroin/gelatin scaffolds, produce TNF and downregulate production of IL-6. 

A – Schematic design of conditioned medium transfer experiment.

B – Expression of *Icam1* and *Vcam1* in MEFs, cultured on plate for 6 h following transfer of conditioned medium from MEFs, cultured on 2D F/G films or in 3D F/G scaffolds, for indicated timepoint (2–24 h) (n = 3).

C – Expression of *Tnf*, *Il6*, and *Il1b* genes in MEFs, cultured on control cell culture grade plastic plate or in 3D F/G scaffolds (n = 4).

D – TNF and IL-6 proteins in culture medium of plate- or 3D F/G-cultured MEFs (n = 4). IL-1β was not detected in analyzed samples.

E – *Icam1* and *Vcam1* expression in TNF KO and IL-6 KO MEFs, cultured on cell culture grade plastic plate or in 3D F/G scaffolds (n = 3). Data are relative to expression level in plate-cultured WT MEFs.

F – *Icam1* and *Vcam1* expression in MEFs cultured on cell culture grade plastic plate or in 3D F/G scaffolds in the presence of neutralizing anti-TNF antibody (αTNF) or isotype control (Iso) (n = 3). Data are relative to expression level in plate-cultured MEFs with isotype control antibody. Gene expression was analyzed after 6 h of culturing cells in above mentioned conditions. Data from one representative experiment of the two independent experiments is shown. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns – not significant.
have been previously characterized to induce moderate inflammatory response [21], we evaluated the expression of the three most prominent proinflammatory cytokines, known to induce ICAM-1 and VCAM-1 expression: TNF, IL-6, and IL-1β [41-43], and, surprisingly, found that only TNF was expressed in 3D F/G-cultured MEFs both at mRNA (Fig. 2C) and at protein level (Fig. 2D). Moreover, MEFs from TNF KO mice failed to overexpress Icam1 in 3D culture conditions (Fig. 2E), while Vcam1 expression was not affected. Strikingly, IL-6 KO MEFs demonstrated increased Icam1 expression at steady state, indicating that IL-6 is rather involved in downregulation of Icam1 (Suppl. Fig. 2A).

Finally, the role of TNF in upregulation of adhesion molecules was confirmed by using neutralizing anti-TNF antibody, which significantly suppressed both Icam1 and Vcam1 expression in 3D-cultured MEFs (Fig. 2F). Altogether, our data demonstrate that upregulation of Icam1 and Vcam1 expression by MEFs cultured in 3D F/G scaffolds was driven by soluble TNF.

3.3. Expression of adhesion molecules in MEFs cultured in 3D F/G scaffolds is mediated by JNK

Next, we aimed to assess the intracellular pathways responsible for adhesion molecule upregulation by MEFs, cultured in 3D F/G scaffolds. In order to do so, we performed experiments with selective inhibitors of key MAP kinases (ERK1/2, JNK1/2, p38), as well as a classical NFκB inhibitor. We found that selective inhibition of JNK resulted in abrogated Icam1 and Vcam1 expression in 3D F/G-cultured MEFs (Fig. 3A and B). In addition, Tfng expression was also reduced after inhibition of JNK (Fig. 3C), suggesting that activation of this kinase controls TNF expression by MEFs. Taken together, our data indicate that in MEFs cultured in 3D F/G scaffolds JNK mediates the expression of TNF, which, in turn, induces the expression of ICAM-1 and VCAM-1.

3.4. MEFs with overexpressed Icam1/Vcam1 promote lymphocyte adhesion and retention in 3D F/G scaffolds in vitro

ICAM-1 and VCAM-1 are important for lymphocyte adhesion during inflammatory responses [33-34], as well as during homeostatic recirculation through lymphoid tissues [35]. Thus, we hypothesized that overexpression of ICAM-1/VCAM-1 in 3D F/G-cultured MEFs may promote their interaction with immune cells and can potentially be used for artificial lymphoid tissue engineering. To test this idea in vitro, we performed co-culture assays in which we first established GFP+ MEFs culture on 2D F/G-coated glass surface or in 3D F/G scaffolds and then added CD4+ splenocytes, enriched for T lymphocytes (Suppl. Fig. 2B). The number of CD4+ cells which became adherent to MEFs after 4 h of co-culture was calculated for each culture condition using immunofluorescent staining and confocal microscopy (Fig. 4A). We found that only 3D F/G-cultured MEFs promoted sufficient CD4+ T cell adhesion (Fig. 4B) and observed clusters of lymphocytes, attached to MEFs in 3D F/G scaffolds (Fig. 4A). To verify that this interaction was mediated by adhesion molecules, we used monoclonal antibodies to block ICAM-1 or VCAM-1. We found that both antibodies could disrupt lymphocyte adhesion to MEFs in 3D F/G scaffolds (Fig. 4B), suggesting that both ICAM-1 and VCAM-1 are crucial for MEF-C4+ T lymphocyte interaction. Altogether, we demonstrated that 3D F/G-induced overexpression of adhesion molecules by MEFs is required for cell-to-cell interactions with CD4+ T lymphocytes in vitro and supports lymphocyte retention in the scaffold.

3.5. Implantation of 3D F/G scaffolds induces infiltration and clustering of lymphocytes in vivo

We established that 3D fibroin/gelatin scaffolds induced the activation of fibroblasts (i.e. MEFs) resulting in upregulation of adhesion molecules ICAM-1/VCAM-1 and increased adhesion of CD4+ T lymphocytes to MEFs. Our final goal was to determine whether this effect of 3D F/G scaffolds can influence their functional properties in vivo. The regenerative potential of these scaffolds was previously validated in tissue regeneration models [21,44,45]. However, the interaction of this biomaterial with the immune cells in vivo was not previously addressed. To investigate the interplay between immune system and 3D F/G scaffolds in vivo, we implanted the matrices under the kidney capsule of mice and analyzed the composition and distribution of immune cells in the implants after 3 weeks using immunohistochemistry. For controls we used well-described commercially available collagen scaffolds of comparable size which do not induce robust immune cell infiltration without additional stimulation [46].

Three weeks after transplantation both types of scaffolds were
sufficiently vascularized as revealed by CD31 immunostaining (Fig. 5A). Strikingly, only 3D F/G scaffolds induced significant infiltration of CD4+ T cells (Fig. 5B) and B220+ B cells upon implantation (Fig. 5C). Moreover, B cells in implanted 3D F/G scaffolds were clustered into primitive lymphoid follicles (Fig. 5E), while T cells were mainly distributed between such B cell clusters (Suppl. Fig. 3A). In contrast, no significant infiltration and/or clustering of lymphocytes were found in implanted collagen scaffolds as compared to F/G implants (Fig. 5E). Small numbers of B cells in implanted collagen scaffolds can possibly be explained by lower expression of B cell attracting chemokine Cxcl13 (Suppl. Fig. 3B). No other markers for mature lymphoid tissues (e.g. FDC-M1, PNAd, MAdCAM-1) were expressed by the cells in the implants (data not shown). At the same time, no difference in macrophage infiltration between the two types of scaffolds was observed (Fig. 5D). Altogether, our data suggested that 3D fibroin/gelatin scaffolds induce specific infiltration and clustering of adaptive immune cells upon implantation under the kidney capsule in vivo.

4. Discussion

Biomaterials are attracting a lot of attention due to potentially wide range of applications in translational medicine, including targeted drug delivery, regeneration or replacement of damaged tissue, bioengineering of artificial organs for transplantation, generation of surgical suture materials and others. In most cases, biomaterials are expected to behave as inert scaffolds or vehicles that lack their own biological activity and are either resorbed or excreted from the organism after some time. The important feature for biomaterials is their biocompatibility that includes lack of toxicity, pyrogenicity, and, at the same time, ability to support cell adhesion and growth that are crucial for regeneration and tissue engineering [47,48]. However, in addition to biocompatibility, some artificial materials can unexpectedly affect the surrounding cells and induce certain transcriptional and translational changes, which should be taken into account before such materials can be applied to clinical setting [49,50].

Among natural biopolymers used for scaffolding materials, the silk
Fig. 5. Implantation of F/G, but not collagen scaffolds, results in infiltration and clustering of lymphocytes in vivo. A – CD31 (red) immunohistochemistry staining of implanted 3D F/G or collagen scaffolds 3 weeks after implantation. Dotted yellow line indicates the border of the implant. Immunohistochemistry staining (yellow) for B220 (B), CD4 (C) and F4/80 (D) of implanted 3D F/G or collagen scaffolds 3 weeks after implantation. Nuclei (blue) are stained with DAPI. White arrows indicate clusters of T- and B-cells in 3D F/G implanted scaffolds, as well as clusters of macrophages in both types of implanted scaffolds. E – Immunofluorescent staining for B220 (red), CD3 (green) and DAPI (blue) of implanted 3D F/G or collagen scaffolds 3 weeks after implantation. Scanning was performed from the surface of the implant. Data from one representative experiment of at least three independent experiments is shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
proteins show several unique features that make them valuable for tissue engineering [51–54]. First, silk-based biomaterials demonstrate extremely high mechanical strength with, for example, dragline silk being five times stronger by weight than steel [55]. At the same time, silk polymers are characterized by high elasticity and increased resistance to compression as compared to other high performance fibers [51]. Silk is used to fabricate scaffolds in various forms, including 2D films, 3D sponges, hydrogels, micro- and nanoparticles, fibers. Several modifications of silk-based scaffolds have been described [30,56,57]. Among them modification with gelatin, a mixture of type I collagen partial hydrolysis products, was introduced to improve biocompatibility, as well as physical and chemical properties of silk fibroin scaffolds [23,58]. Importantly, gelatin modification introduces RGD (Arg-Gly-Asp tripeptide) sequences to the silk-based scaffolds [58], thus, improving cell adhesion, migration, and proliferation of cells on fibroin/gelatin (F/G) biomaterial as compared to pure fibroin (F), lacking RGD sequence. These properties of fibroin/gelatin scaffolds are useful for generating 3D scaffolds for tissue engineering and regenerative medicine. For example, fibroin/gelatin blends were recently employed for generation of bioinks, suitable for 3D printing of soft tissue implants [59].

In present study we uncovered novel biological effects of previously characterized biocompatible silk fibroin-based scaffolds both in vitro and in vivo. First, we found that three-dimensional (3D) fibroin or fibroin/gelatin scaffolds may induce rapid expression of adhesion molecules ICAM-1 and VCAM-1 in the primary culture of mouse embryonic fibroblasts (Fig. 1). Surprisingly, two-dimensional (2D) composition of fibroin/gelatin scaffolds (either as films or glass coating) did not induce significant changes in gene expression by MEFs. This data is in line with previously published report on the effect of 3D, but not 2D, fibroin scaffolds on human monocytes [20]. However, MEFs acquired changes in their expression program that were different from monocytes with decreased expression of IL-6 and increased expression of TNF and adhesion molecules (Figs. 1 and 2), suggesting a distinct type of effects of fibroin-based 3D scaffolds on different cell types. Intracellular signaling pathways responsible for these effects also appear to differ in MEFs as compared to human monocytes. Importantly, we found that inhibition of JNK can specifically suppress 3D F/G-mediated upregulation of ICAM-1/VCAM-1 in MEFs. Recently, activation of the classical NFkB by monomeric silk fibroin was detected in murine and human fibroblasts [60]. However, in our hands, inhibition of the classical NFkB did not affect the expression of adhesion molecules by MEFs, thus, supporting the idea that the effect of 3D F/G scaffolds is not mediated by cleaved soluble fibroin. At the same time, our data indicate that TNF is partially responsible for ICAM-1 and VCAM-1 expression in MEFs cultured in 3D F/G scaffolds, suggesting that other signaling pathways (including, possibly, JNK) apart from the classical NFkB may mediate this effect of TNF. Interestingly, the effect of macroscopic 3D scaffolds differs from 3D F/G microparticles, which induce expression of both TNF and IL-6 by MEFs, as recently reported [21]. Possibly, this reflects the differences in microenvironment in these two types of biomaterials, for example, reduced diffusion of oxygen in macroscopic 3D scaffolds as compared to microscopic particles.

Of note, one cannot completely rule out possible effects on cells and on gene expression of such physical properties as the pore size or the stiffness of bioengineered materials. There is evidence that cells cultured on stiff surface may alter the expression of ICAM-1 and VCAM-1 [61]. Physical and chemical properties of 2D F/G films and 3D F/G scaffolds used in this study were previously described [62]. The pore size of 3D scaffolds comprised of different materials used in this study was comparable, ranging from 200 μm in diameter for 3D polystyrene scaffolds to 220 ± 65 μm and 250 ± 80 μm for fibroin and fibroin/gelatin scaffolds, respectively. At the same time, the mechanical stiffness was different for the type of materials used, among which the most rigid was 3D polystyrene with shear modulus ∼ 3 × 10^6 kPa [62], compared to 9.0 ± 1.6 kPa for fibroin/gelatin and at 5.9 ± 1.1 kPa for fibroin.

Nevertheless, these differences in rigidity between fibroin and F/G-scaffolds did not significantly affect expression of adhesion molecules, since ICAM-1 expression was highly upregulated in both types of scaffolds, suggesting a stronger contribution from the fibroin itself, rather than from the differences in physical properties of bioengineered materials.

Adhesion molecules are important for stromal cell functions and are involved in their interactions with immunocytes [31,64]. In embryonic lymph node development, expression of ICAM-1 and VCAM-1 is significantly increased during the process of lymph node stromal cell maturation, reflecting the growing influx of lymphocytes into lymphoid sacs [32]. In line with this, we observed a drastic increase in adhesion of CD4^+ cells to MEFs cultured in 3D F/G scaffolds, but not in those cultured under conventional 2D conditions (Fig. 4). Since inhibition of ICAM-1 and VCAM-1 with specific antibodies abrogated CD4^+ cell attachment to MEFs, we concluded that this interaction was indeed ICAM-1/VCAM-1-dependent. Thus, our data strongly suggest that 3D F/G scaffolds can induce functional changes in stromal cells, which may affect their interactions with the immune cells. This property might be beneficial for generation of scaffold-based vaccines, for example, in the highly relevant field of tumor therapy [4,5]. One may expect that F/G scaffolds will be superior in attracting T lymphocytes and, thus, promoting antigen-specific immune responses as compared to other biomaterials, lacking the ability to rapidly induce and sustain the expression of adhesion molecules by stromal cells.

Of particular significance, our in vivo data suggested that F/G scaffolds promote infiltration and clustering of lymphocytes (Fig. 5, Suppl. Fig. 3). The fact that implanted scaffolds become well vascularized (Fig. 5A) suggests that they are not rejected upon implantation. Furthermore, 3D F/G scaffolds could promote expression of VEGF growth factors (Suppl. Fig. 1B) in fibroblasts, potentially promoting vessels growth. Interestingly, T cells and B cells in implanted F/G scaffolds acquired zonal distribution, similar to those observed in the secondary and tertiary lymphoid organs, although no markers specific for mature lymphoid tissue were upregulated in the implanted scaffolds (data not shown). Migration of immune cell requires the presence of chemokines that stimulate cell egress into the tissues and guide different cell types to their niches, maintaining zonal distribution of cells within the organ [65]. In lymphoid tissues CCL19 and CCL21 mainly govern migration of T cells to T-zones, while CXCL13 attracts B cells to the follicles [66]. Surprisingly, implanted F/G scaffolds induced significant expression of Cxcl13 (Suppl. Fig. 3B), that, presumably, drove B cell infiltration and clustering (Fig. 5B). At the same time, implanted collagen scaffolds showed reduced Cxcl13 expression and, consequently, contained a smaller number of B cells (Fig. 5B). Thus, upon implantation F/G scaffolds may induce the expression of lymphoid tissue-specific chemokine Cxcl13 and stimulate infiltration of lymphocytes. This feature can be employed for generating artificial lymphoid tissues that represent another potential therapeutic tool for vaccinations [67–70]. Our findings suggest that F/G scaffolds can provide suitable microenvironment for construction of such organs since they can promote lymphocyte infiltration, adhesion and proper initial clustering, which can be further guided and manipulated by specific chemokine and cytokine signaling. Altogether, we data uncover novel biological properties of 3D fibroin/gelatin scaffolds in vitro and in vivo which affect stromal cell phenotype and promote lymphocyte infiltration and adhesion inside the scaffolds.

**CRediT authorship contribution statement**

Maxim A. Nosenko: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing. Anastasia M. Moysenovich: Methodology, Investigation, Writing, Visualization, Resources. Anastasia Y. Arkhipova: Investigation. Kamar-Sulu N. Atretkhany: Investigation. Sergei A. Neshporov: Conceptualization, Writing, Funding acquisition. Marina S. Drutskaya: Conceptualization,
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Mikhail M. Moisenovich: Conceptualization, Methodology, Supervision, Project administration, Writing, Funding acquisition, Visualization, Resources.

Declaration of competing interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Dedicated to the memory of Dr. Mikhail Moisenovich.

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bioactmat.2021.03.016.

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