The role of 3D structure and protein conformation on the innate and adaptive immune responses to silk-based biomaterials

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1. Introduction

Silk protein produced by Bombyx mori silkworm has widely been used as biomaterial for centuries. Unique features of silk such as mechanical strength, ease of chemical modification to impart biofunctionalization and controlled degradation rate, made it an attractive candidate for biomedical applications. Silk has been used in different forms such as 2D film, hydrogels, sponges [1] and fibrous scaffolds [2] in tissue engineering and drug delivery, and applications in bioelectronics and biomedical sensing are upcoming [3]. In spite of unprecedented applications of silk as biomaterial, its biocompatibility has not been fully explored.

Biocompatibility of a biomaterial is generally assessed by studying immune responses generated upon the interaction of biomaterials with the cells of immune system including both innate and adaptive immunity. Innate immunity is the non-specific first line of defense including a variety of cellular and molecular components, which initiate immune recognition and respond to foreign materials. On the other hand, adaptive immunity comes into action later, following the initial exposure to the antigen and responds with a high degree of specificity and the generation of memory. Both pathways are highly interconnected.

Monocytes are important players in innate immunity. They express transmembrane receptors, such as Toll like receptors (TLRs), which facilitate their interaction with the biomaterials as the foreign material and respond to challenges by a variety of mechanisms including phagocytosis, generation of reactive oxygen species and nitrogen intermediates and secretion of pro-inflammatory cytokines including IL1β, IL6 and TNFα. These cytokines in turn may participate in the activation of T lymphocytes.

Silk filaments have been used as surgical sutures since long time. Recently, however, silk sutures have been replaced by synthetic polymeric sutures, due to undesirable immunogenicity and difference in degradation rates upon implantation at different sites [1,4–7].
Native silk fiber has been acknowledged to be an allergenic agent [8,9] causing Type I allergy [10], such as asthma and specific increase of IgE levels, but also delayed allergic responses. Upon sonication, silk fibers produced by silkworm release soluble factors, which were found to be responsible for induction of pro-inflammatory cytokines and increased phagocytosis [11]. The outer layer of silk, namely sericin (serine-rich glycoproteins), has been indicated to be responsible for allergic reactions or pro-inflammatory effects of silk [12,13]. Although a recent report has suggested a possible role of fibroin in the induction of Type I allergic reactions [10], most studies report minimal immune stimulation by fibroin component. Fibroin membrane was found to activate macrophages to a lesser extent as compared to polystyrene and poly(2-hydroxyethyl)methacrylate, since it did not support proper adhesion and spreading of macrophages, resulting in reduced secretion of IL1β in comparison to the other two polymers [14]. Panialiatis et al. [15] studied the interaction of macrophages with silk fibers, as well as insoluble silk fibroin particles (10–200 μm), along with collagen and black dyed braided silk suture. No significant amount of TNF was found to be released from a murine leukemia derived macrophage-like cell line (RAW 264.7) when cultured with silk fibers both in short term and prolonged culture, but TNF release was increased in presence of fibroin particles in a dose dependent manner [15]. Meinelt et al. [16] reported comparable immunogenic properties of silk films under in vitro and in vivo conditions. In vivo biocompatibility studies of silk fibroin reported nominal inflammatory reactions without fibrosis and lymphocyte invasion [17].

In the above studies the possible roles of secondary conformation of silk protein, and overall architecture of the biomaterials have been largely overlooked. However, major factors governing immunogenicity of any biomaterial could also include molecular size, shape, morphology, surface chemistry, surface topography, surface stiffness, implantation site and processing induced degradation products [18–22]. For instance, Poly-vinyl pyrrolidone (PVP) with a 10,000 Da MW is a strong immunogen [23]. The role of total surface area to volume ratio of the biomaterial may also be crucial, as a higher inflammatory reaction was reported to be induced by particles <20 μm in diameter as compared with particles >50 μm [18,19]. Bartneck et al. [21] studied the effect of material morphology in three-dimensional (3D) nanofibers with different porosity and surface chemistry in comparison with 2D substrate on the macrophage phenotype and observed the expression of anti-inflammatory markers upon stimulation in 2D system and pro-inflammatory markers in 3D system.

Here we explored innate and adaptive immune responses generated in vitro against silk biomaterials used in well documented different physico-chemical forms.

2. Materials and methods

2.1. Preparation of silk biomaterials

B. mori silk cocoons were cut into small pieces and degummed by boiling in 0.02 M Na2CO3 for 60 min to remove the outer sericin layer of silk. The supernatant containing sericin was collected and dialyzed for 48 h against deionized distilled water to obtain 13% w/v sericin solution. Sericin solution was cast on teetion coated plates and dried overnight in oven at 40 °C. The dried films were made insoluble by overnight treatment with 70% ethanol.

After degumming, the insoluble fibroin fibers were rinsed in distilled water and then dried for at least 12 h and dissolved in 9.3 mL LiBr at 60 °C for 4 h to obtain a 20% w/v pure fibroin solution of dried silk. The silk–LiBr solution was dialyzed in water using dialysis cassette (Pierce, M.W.C.O. 3.5 kDa) to obtain 8% w/v pure silk fibroin solution. The solution was then concentrated against 15% PEG for 4 h to obtain 13% w/v silk fibroin solution. Bidimensional (2D) fibroin films were prepared by casting the fibroin solution on teetion coated plates and slowly dried overnight in an oven at 40 °C. 3D fibroin scaffolds were prepared by lyophilizing the silk fibroin solution in 24 well plates. The silk solution was frozen in liquid nitrogen (−196 °C) for 24 h. The frozen membranes were then lyophilized for 24 h in a LABCONCO Lyophilizer, Kansas City, MO, USA) and treated with 70% ethanol for overnight. Biopsy punches were used to cut cylindrical scaffold of particular dimensions (Table 1).

2.2. Scanning electron microscopy (SEM)

Silk fibroin and sercin films and 3D fibroin scaffolds were analyzed for the presence of pores by SEM. The cultured silk samples were fixed with 4% formaldehyde, dehydrated using alcohol gradients and sputter coated with gold. All samples were examined by a Zeiss EVO S5 Scanning electron microscope (Oberkochen, Germany). Pure size of the scaffolds was calculated (n = 5) using Image J software (NIH, USA).

2.3. Attenuated total reflectance–Fourier transformed infrared spectroscopy (ATR-FTIR)

Infrared spectra for silk fibroin films, 3D fibroin scaffolds and sercin films were measured on Attenuated Total Reflectance FTIR model Alpha-2F, Bruker (Ettlingen, Germany). All spectra were taken in the spectral range of 4000–500 cm−1 by accumulation of 264 scans and with a resolution of 4 cm−1. The IR spectra of silk fibroin, 3D fibroin and sercin films covering the amide I region 1600–1700 cm−1 were deconvoluted using Peakfit v4.12 software (Systat Software Inc. San Jose, CA, USA). After normalizing the spectra, relative area under each peak was used to identify secondary structures in the sample. Peak absorption bands were assigned as follows: 1605–1615 cm−1 Tyr-enriched chains or aggregated strands, 1610–1625 and 1696–1704 cm−1 as β-sheet sheet structures; 1640–1650 cm−1 as random coil conformation; 1650–1662 cm−1 as α-helices; and 1660–1695 cm−1 as β-turns [24].

2.4. X-ray diffraction (XRD)

X-ray diffraction was performed by (X Pert PRO PANalytical – D1022, Almelo, The Netherlands) with CuKα radiation (1.5405 Å) from 0° to 50° [28]. The X-ray source was operated at 40 kV and 30 mA.

2.5. Contact angle measurement

The wettability of the samples was measured by adding a water droplet of 30 μL on 20 mm × 15 mm films (sericin and fibroin) and cylindrical 3D fibroin having a diameter of 4 mm and 2 mm height. The contact angle was measured by using a tangent placed at the intersection of the liquid and solid phase. The angles were measured at 5 different areas for each sample to calculate means and SD.

2.6. Atomic force microscopy (AFM)

The elastic moduli at the nanometer scale were mapped quantitatively using a FlexAFM ARTIDIS (Nanosurf AG, Liestal, Switzerland) and a Nanowizard I (JPK Instruments, Berlin, Germany) atomic force microscopes as described earlier [25]. Briefly, standard rectangular cantilevers with sharp pyramidal tips (nominal tip radius 20 nm, nominal k = 6.33 N/m; custom made cantilever, University of Neuchâtel, Switzerland) were used for stiffness measurements. The spring constant of each cantilever was determined using the thermal noise method [26]. Each sample was measured at 18 or more random spots comprising a scan area of 1800 μm2, with an indentation force of 1 pN at a frequency of 3 Hz. Generated maps with force–distance curves of at least 16 × 16 points were analyzed using the Oliver & Pharr method for calculating the dynamic elastic modulus (stiffness) in a custom-developed Labview software (National Instruments, Austin, TX, USA), as reported earlier [2,27].

2.7. Surface area measurement

The specific area of the silk biomaterials was calculated using a surface area analyzer (Micromeritics, ASAP 2010, USA). Nitrogen gas was used as adsorbate and liquid nitrogen as the cooling media for adsorption/desorption isotherms of samples. The surface areas of the 2D fibroin and 2D sercin film and 3D fibroin scaffolds were calculated as single point from the Brunauer–Emmett–Teller (BET) isotherm using adsorption points.

2.8. Isolation of human peripheral blood cells

Peripheral blood was collected from consenting healthy donors, with ethical approval from University Hospital of Basel, Switzerland. Fifty milliliters of blood were collected in 250 mL plastic tubes containing 6.7 mL HEPES buffered saline (Plasmon, Basel, Switzerland) containing 6.7 mL HEPES buffered saline (Plasmon, Basel, Switzerland) containing 3.2 mL heparin 250 units/mL (Heparit, Coop, Switzerland) and 1.1 mL 10% FCS (Plasmon). Peripheral blood mononuclear cells (PBMCs) were isolated using density gradient centrifugation on Histopaque-1077 (Sigma-Aldrich, St. Louis, USA) and washed twice in PBS. The total cell number was counted using a Coulter Counter. PBMCs were distributed in 24 well culture plates. Table 1 Dimensions of scaffolds used.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Weight</th>
<th>Size</th>
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<tbody>
<tr>
<td>Sericin film</td>
<td>1.8 mg</td>
<td>0.3 × 0.3 cm²</td>
</tr>
<tr>
<td>Fibroin film</td>
<td>1.8 mg</td>
<td>0.3 × 0.2 cm²</td>
</tr>
<tr>
<td>3D fibroin</td>
<td>1.8 mg</td>
<td>0.2 × 0.1 × 0.1 cm³</td>
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were diluted in PBS at a ratio of 1:2, layered onto Histopaque 1077 (Sigma, St. Louis, MO, USA) and centrifuged at 2400 rpm for 20 min to obtain peripheral blood mononuclear cells (PBMC). Cells were then washed once with PBS and once with MACS buffer (PBS; 2% FCS, 2 mM EDTA). The CD14+ monocytes were separated by positive cell sorting using anti-CD14-conjugated microbeads (Milteny Biotech, Bergisch Gladbach, Germany) according to techniques recommended by the manufacturer. Monocytes were then suspended at a 4 × 10^6 cells/ml concentration in RPMI 1640 supplemented with Kanamycin (100 μg/ml), Hepes buffer (10 mM), sodium pyruvate (1 mM), Glutamax (1 mM), non-essential amino acids and 10% FCS (all from Invitrogen, Basel, Switzerland). CD4+ and CD8+ T cells were also immuno-nomagnetically separated from the CD14+ fractions of the PBMC.

2.9. Culture of CD14+ cells with silk biomaterials

The biomaterials, namely, 2D sericin film, 2D fibroin film, 3D fibroin, collagen Ultrafoam (microfibrillar collagen sponge, bovine source, Bard Davol, Inc., Zurich, Switzerland) and tissue culture plates (TCP; Falcon, Allschwil, Switzerland) as negative control, and LPS (from Escherichia coli O111B4 100 ng/ml, Invitrogen, San Diego, CA, USA) as positive control, were placed in the 24 well plates and incubated for 18 h with 4 × 10^5 cells/ml concentration in RPMI 1640 supplemented with Kanamycin, Hepes, sodium pyruvate, Glutamax and non-essential amino acids, 10% FCS, at 37°C up to 18 h.

The biomaterials were then cultured with CD14+ cells under similar conditions for up to 5 days and cells and culture supernatants were sampled at 1, 3, and 6 days of culture to evaluate cytokine gene expression and protein release (see below).

2.10. Transwell experiments

For transwell experiments silk based biomaterials were sterilized using 70% ethanol and washed with PBS and along with Ultrafoam and LPS were put in the top wells/inserts of transwell plates with 3.0 μm pores (Corning, Corning NY) with 200 μl media in it and CD14+ cells were added to the bottom wells with 4 × 10^6 cells/well in presence of 800 μl of RPMI 1640 at 37°C up to 18 h. Cells and supernatants were collected for the analysis of cytokine gene expression and release, as detailed above.

2.11. Analysis of pro-inflammatory signal transduction pathways

Different inhibitors of inflammatory signal transduction pathways were used to obtain insights into mechanisms underlying biomaterial recognition by monocytes and the generation of immune reactivity. Polymyxin B (PMB) (Calbiochem, Merck, Bergisch Gladbach, Germany) according to techniques recommended by the manufacturer. Monocytes were then suspended at a 4 × 10^6 cells/ml concentration in RPMI 1640 supplemented with Kanamycin, Hepes, sodium pyruvate, Glutamax and non-essential amino acids, 10% FCS, at 37°C up to 18 h. The biomaterials were then cultured with CD14+ cells under similar conditions for up to 6 days and cells and culture supernatants were sampled at 1, 3, and 6 days of culture to evaluate cytokine gene expression and protein release.

2.12. Quantitative real time PCR (qRT-PCR)

Total RNA was isolated by using Nucleospin RNA II kit (Macherey–Nagel, Düren, Germany) following manufacturer’s protocol. Purified mRNA was reverse transcribed into cDNA by priming 1 μl of total RNA with 1 μl (200 μg/ml) of Oligo dt (Roche Diagnostics, Mannheim, Germany) at 65°C for 10 min followed by immediate transfer in ice. A mixture of 1 μl dNTP mix (10 mM), 4 μl first-strand buffer (5×), 2 μl DTT (0.1 mM) and 1 μl M-MLV reverse transcriptase (200 units) (all by Invitrogen Ltd, Paisley, UK) was added and incubated at 37°C for 1 h.

Expression of IL1β and IL6 genes was analyzed by 7300 Real Time PCR system (Applied Biosystems, Rotkreuz, Switzerland) according to manufacturer’s protocol, using specific primers and probes (Assays on Demand, Applied Biosystems). The expression of genes of interest was normalized to GAPDH house keeping gene. Expression levels were calculated using the 2^(-ΔΔCT) method with GAPDH as a reference gene [28].

2.13. Cytokine release

Culture supernatants were collected for cytokine release analysis by quantitative ELISA assays, using antibody pairs and standards specific for IL6 (BD PharMingen, San Diego, CA, USA) and IL1β (eBioscience, San Diego, CA, USA) detection according to instructions from manufacturer. Plates were read at 405 nm in a Spectramax 190 plate reader (Molecular Devices, Germany) and analyzed using SOFTmax software (Molecular Devices, Sunnyvale, CA, USA).

2.14. T cell cultures

Silk biomaterials were incubated with CD4+ or CD8+ T cells in the presence or absence of autologous CD14+ at a 5:1 ratio (5 × 10^5 cells/well) in RPMI 1640 supplemented with Kanamycin, Hepes, sodium pyruvate, Glutamax and non-essential amino acids supplemented with 5% human serum (Blutbank Basel, Switzerland) at 37°C for 18 h.

Cells were then collected and CD69 and CD25 expression was evaluated by flow cytometry (FACS Calibur, Becton-Dickinson, Allschwil, Switzerland) by using specific fluorochrome labeled monoclonal antibodies. IFNγ gene expression (IFNγ) was analyzed by qRT-PCR (7300 Real Time PCR system, Applied Biosystems, Rotkreuz, Switzerland) in the presence of specific primers and probes (Assays on Demand, Applied Biosystems), as detailed above.

2.15. Statistics

Statistical significance was calculated by independent t tests using SPSS software (SPSS Inc., an IBM Company, Chicago, USA) and was considered significant at p < 0.05.

3. Results

3.1. Monocyte responsiveness to 3D fibroin scaffolds

Pre-clinical “in vitro” assessment of biocompatibility of newly developed biomaterials is frequently performed using established human cell lines derived from myeloid leukemia cells [15,29]. However, due to their malignant nature and long term culture, these cells might fail to reliably mirror functional features of normal, untransformed cells of the innate immune system. Therefore, we used freshly purified peripheral blood monocytes to assess their responsiveness upon incubation with a lyophilized 3D fibroin scaffold generated for regenerative medicine applications. LPS, Ultrafoam collagen gel (UF) and standard tissue culture plates (TCP) were used as positive and negative controls, respectively.

Monocyte activation is typically associated with the expression of genes encoding pro-inflammatory cytokines, including IL1β and IL6 specific genes, as well as with their protein secretion. We observed that overnight incubation in the presence of a 3D fibroin scaffold was able to induce a significant expression of genes encoding IL1β and IL6 pro-inflammatory cytokines (Fig. 1A,B) and the secretion of the corresponding proteins (Fig. 1C,D).

3.2. Generation and physical characterization of silk biomaterials

Since “in vitro” data indicated that a fibroin based 3D scaffold was able to activate human monocytes, we hypothesized that biomaterial architectures, and their inherent biochemical composition might be responsible for immune activation. To test this hypothesis we generated silk fibroin biomaterials as 2D film and in 3D form, and sericin films. These materials were characterized by different techniques to gain insights into their morphology, surface chemistry, topography and wettability with potential relevance in the activation of immune competent cells.

SEM images showed that sericin and fibroin films have smooth surface and no pores (Fig. 2A,B) whereas the 3D fibroin scaffolds have rough surfaces, consisting of flats and grooves, (Fig. 2C) and are porous in nature with non-uniform pore diameter (129.88 ± 29.22 μm; n = 5). The BET surface area analysis for the silk based biomaterials indicated that they were all in the same range (Fig. 2H). Indeed, the surface area was found to be 0.0578 m²/g for 2D sericin, 0.0529 m²/g for 2D fibroin and 0.0531 m²/g for 3D fibroin.

ATR-FTIR was used to characterize the silk biomaterials in terms of macromolecular conformation. The infrared spectral absorption by the peptide backbones of amide I (1700–1600 cm⁻¹) and amide II (1600–1500 cm⁻¹) are usually assigned for the determination of silk protein conformation [24,30]. Sericin film in spectra a (Fig. 3A) showed its characteristic amide I and amide II peaks at 1622 cm⁻¹ and 1521 cm⁻¹, respectively. These bands however, may be attributed to the characteristic β-sheet structure induced by methanol treatment. 2D silk fibroin film in spectra b showed the presence of characteristic C=O stretching for amide I as observed at 1644 cm⁻¹.
revealed the appearance of the diffraction peaks at 20.7° and a strong spacing of 4.27 Å. The percentage of secondary components was then calculated using Fourier self-deconvolution (FSD) of the infrared spectra of the amide I region. In sericin, the 2D scaffold includes a 51.6% strong β-sheet macromolecular organization, 28.9% aggregated strands (Fig. 3D). Taken together, the IR spectra indicate that 2D silk fibroin predominantly exists in silk I conformational state. The percentage of the secondary N—H bending for amide II at 1519 cm⁻¹, and C—N stretching for amide III at 1228 cm⁻¹. Spectra c for 3D silk fibroin showed that the presence of characteristic C=O stretching for amide I is observed at 1622 cm⁻¹, the secondary N—H bending for amide II at 1506 cm⁻¹, and C—N stretching for amide III is visible at 1229 cm⁻¹ (Fig. 3A). Taken together, the IR spectra indicate that 2D silk fibroin film predominantly exists in silk I and 3D silk fibroin exists in silk II conformational state. The percentage of the secondary components was then calculated using Fourier self-deconvolution (FSD) of the infrared spectra of the amide I region to determine the percentage of secondary components. In sericin, the percent of β-sheet content is 37.6% with only 3.1% of random coils (Fig. 3B). The fraction of secondary structure within 2D fibroin film is 27.1% α-helices, 12.2% random coils, 8.7% β-sheet (weak) or aggregated β-strands and 7.9% β-turns (Fig. 3C). The 3D fibroin scaffold includes a 51.6% strong β-sheet crystal structure showing molecular aggregation with more packed structure and higher crystallinity and 28.9% aggregated strands (Fig. 3D).

XRD analysis confirmed the existence of different macromolecular conformations in 2D fibroin film and 3D fibroin scaffold. Indeed, the silk fibroin film made by casting the solution in teflon coated plates and slow drying at 40 °C exhibited silk I conformation showing the characteristic peak at 12.2°, 19.7° and 24.7° [31–33]. However, upon lyophilization and alcohol treatment the 3D fibroin scaffold matrix showed β-sheet macromolecular organization as evident by displacement from the silk I peak (12.2°, 19.7°) to the silk II characteristic peak at 20.7° (Fig. 3E). XRD data of sericin film revealed the appearance of the diffraction peaks at 20.7° with a strong spacing of 4.27 Å and 26.8° and a medium spacing of 3.32 Å. Furthermore, according to Bragg law (i=2d sin θ) the average distance (d) for 2D silk film was observed to be at 4.1 Å, consistent with silk I structure, and at 4.2 Å (silk II) in 3D fibroin [31–33].

The contact angle measured for sericin film and fibroin film was found to be 78.3° ± 2.4° and 67.3° ± 4.1° (Fig. 3F,G) respectively. Notably, the random coiled conformation of 2D fibroin film made it more hydrophilic as compared to sericin. The contact angle for 3D fibroin matrix could not be measured, due to its porous nature, this material absorbed the water droplets immediately upon contact. However, the presence of β-sheet conformation in 3D fibroin would make it comparatively more hydrophobic.

AFM analysis further validated SEM observations. Silk films possessed smooth surface, whereas 3D fibroin scaffold consisted of aggregated and flat features (Fig. 4A). Furthermore, AFM analysis showed that the 2D fibroin film exhibited the highest stiffness having moduli of 26 × 10⁵ Pa as compared to sericin and 3D fibroin, whose stiffness were 18 × 10⁵ Pa and 6 × 10⁵ Pa, respectively (Fig. 4B).

3.3. Monocyte response to silk materials of different architecture and conformation: cytokine gene expression and cytokine release

In order to address differential responsiveness of untransformed human cells to silk based materials of different architecture and conformation, we first explored monocyte adhesion by SEM. SEM images showed that purified peripheral blood CD14+ monocytes adhered well to the sericin film (Fig. 2D,E) and to the 3D fibroin scaffold (Fig. 2F,G) but minimal adhesion to the fibroin film was observed as compared to other matrices. The monocytes attached to non-porous sericin film displayed spread shape with extended filopodia (as shown by the arrows), whereas monocytes adhering to the porous 3D fibroin matrix appeared to be spherical in shape.
Silk derived materials were then incubated overnight in the presence of CD14+ monocytes, in order to assess their differential immunostimulatory ability. Ultrafoam did not induce cytokine gene expression levels significantly higher than those detectable in cells from unstimulated cultures. On the other hand, as expected, high IL1β gene expression was detectable in cells stimulated with LPS positive control (Fig. 5A). Sericin film also induced a relevant IL1β gene expression. Minimal IL1β gene expression was observed in 2D fibroin film stimulated cells. Most importantly, we observed that IL1β gene expression in 3D fibroin stimulated cells was significantly higher (p < 0.05) as compared to that detectable in cells cultured over 2D fibroin film (Fig. 5A).

Similarly, minimal levels of IL6 gene expression were observed in 2D fibroin film stimulated cells whereas sericin showed the highest immunostimulatory capacity, followed by 3D fibroin (Fig. 5B). The expression levels of both cytokines were found to be dramatically decreased after 3 days cultures in all samples including LPS stimulated cells and reached baseline levels after day 6 (Fig. 5A,B).

Gene expression data were corroborated at the protein level by quantifying IL1β and IL6 production by ELISA after overnight culture. IL1β levels were found to be 1.7 ng/ml in supernatants from sericin stimulated cultures, as compared to 2.3 ng/ml for 3D fibroin and 0.007 ng/ml for 2D fibroin film (p < 0.05, Fig. 6A,B). However, cytokine release after day 3 was found to be reduced in all experimental groups and after day 6 cytokine levels in culture supernatants were virtually negligible (Fig. 6A,B). Taken together, these data indicate that architecture and protein conformation of silk-based biomaterials play important roles in monocyte activation.

### 3.4. Transwell experiments

To verify whether the ability of the biomaterials under investigation could be due to the presence of soluble factors, transwell experiments were performed. Biomaterials were placed on the upper chamber of 3.0 μm transwell filters, and the CD14+ cells were seeded in the lower chamber. In these culture conditions, expression of IL1β and IL6 at both gene and protein level was only observed upon LPS stimulation (Fig. 7A–D). These data indicate that a direct contact is required between silk-based biomaterials and the immune cells to evoke responses.

### 3.5. Signaling pathways involved in monocyte response to silk biomaterials

To gain insights into specific signal transduction mechanisms underlying monocyte responsiveness to 3D fibroin scaffold we
added a panel of defined inhibitors to our cultures. Polymyxin B is a cyclic cationic polypeptide antibiotic which blocks the biological effects of gram negative lipopolysaccharide (LPS) through binding to its toxic component, negatively charged lipid A. PDTC inhibits activation of NF-κB, a transcription factor mediating a variety of inflammatory responses. LY294002 is a potent, cell permeable inhibitor of phosphatidylinositol 3-kinase (PI3K) known to regulate TLR-mediated inflammatory responses, whereas SB202190 is a p38 MAPK inhibitor. Since reagents were diluted in DMSO or water, both products were used as negative control.

In the presence of PDTC, IL6 production by CD14+ cells induced by all stimuli was inhibited, consistent with the involvement of NF-κB in the production of pro-inflammatory cytokines induced by silk-based biomaterials. Polymyxin B inhibited IL6 production in LPS stimulated cultures, but failed to affect IL6 production induced by sericin and 3D fibroin scaffold. SB202190 p38 MAP kinase inhibitor significantly decreased IL6 production in all cultures, whereas LY294002 PI3K inhibitor was always ineffective (Fig. 8).

3.6. T cell responsiveness to silk materials

To extend our investigation to cells from the adaptive immune system, we cultured purified CD4+ and CD8+ T cells together with silk based biomaterials in the presence of autologous CD14+ cells as antigen presenting cells. T cell activation was monitored by testing the expression of CD69 and CD25 lymphocyte activation markers and the expression of IFNγ gene. None of the biomaterials under investigation was able to induce a significant upregulation of phenotypic markers or cytokine gene expression (data not shown).

4. Discussion

Silk has been extensively used as a biopolymer in tissue engineering and regenerative medicine. However, the critical issue of silk protein’s ability to stimulate the immune system remains largely unexplored. Cells of the adaptive immune system are responsible for the long term responsiveness to antigenic challenges and for the establishment of immunological memory. In contrast, cells belonging to the innate immune system represent the first line defense against microorganisms and effectively respond to exogenous and endogenous “danger” signals. Interestingly, immune response has been shown to be dictated by surface properties intrinsic to biomaterials including size, shape, surface chemistry and topography [16–21].

In the present study we evaluated the capacity of silk biomaterials in different secondary conformations to activate human
monocytes and T cells. Importantly, in this study we did not use established human cell lines derived from myeloid leukemia cells [15,29] as responder cells. Instead, freshly purified peripheral blood monocytes from healthy donors were used to generate data more closely mirroring interactions presumably occurring “in vivo”. Furthermore, to explore the role played by conformational and structural features of silk-based biomaterials, we developed a 2D silk fibroin film, which was mainly in silk I state (α-helices), and a 3D fibroin scaffold, which was predominantly in silk II β-sheet conformation. ATR-FTIR studies and the deconvolution of the amide I region showed the predominance of silk I conformation within the 2D fibroin film along with some fractions of other conformations, whereas the strong absorption band of 3D fibroin at 1622 cm⁻¹ was consistent with the β-sheet conformation (silk II) [24,30]. These findings were further validated by XRD studies, as the diffraction peak and the spacings obtained in XRD confirmed the existence of amorphous structure (silk I) in 2D silk fibroin film and the transition of silk I to silk II (β-sheet conformation with crystalline structure) in 3D silk fibroin scaffold. Our data are consistent with earlier reports showing that fibroin films formed by slow drying at low temperature (40 °C) are mainly in silk I conformation [34,35]. However, presence of silk I conformation may generate concern regarding stability of the matrix in water or media. Within the gland of silkworm, fibroin protein solution assumes random coil conformation and it is soluble in water. Just before extrusion silk assumes insoluble silk I conformation (α-helices, β-turn and weak β-sheet crystal structure), whereas after extrusion it is insoluble in water due to formation of crystalline β-sheet (silk II conformation) [35]. In order to address the issue of water insolubility of fibroin film, we conducted FTIR and DSC studies before and after immersing silk biomaterials in PBS for 6 days. No significant difference in the fractions of random coil, β-sheet, α-helices, and β-turns was observed in the fibroin film after 6 days as detectable by Fourier self deconvoluted spectral analysis of amide I peak (data not shown). Secondly, we conducted DSC study to investigate difference in stability of silk I and silk II crystals, using 2D fibroin film before or after PBS treatment for 6 days. DSC curve of 2D fibroin film showed an endothermic peak at 100 °C due to the presence of silk I crystals (which is a hydrated structure) (Supplementary figure) [35]. Consistent with earlier reports the 2D fibroin film showed two degradation peaks at around 252 °C and 259 °C [35]. The degradation peak around 257–260 °C is related to the stability induced by the β-sheet crystals, while the other degradation peak around 247–252 °C is due to the silk I structure. These findings are consistent with a reasonable stability
of silk I crystals. Thirdly, previous reports indicate that a fibroin film generated by using the same water annealing and slow drying method utilized in this study, and displaying a similar silk I content, was water stable [34,35].

Furthermore, we calculated the surface area of all the silk biomaterials using BET isotherm based surface area analyzer. This study confirmed that all three materials were having comparable ranges of surface area.

We then focused on the response of freshly isolated CD14+ monocytes to different silk-based biomaterials developed. Monocytes cultured with silk biomaterials adhered to sericin film and 3D fibroin but minimal attachment to fibroin film was observed. Monocytes attached to sericin film maintained spread morphology with extended filopodia while they maintained a rounded morphology on the 3D fibroin substrate. This finding is largely consistent with data from previous studies where non-porous sub- strate supported the spread shape of monocytes, eventually forming a sheet on the substrate, whereas porous substrate supported circular cellular morphology similar to that observed in our sericin film [20]. McBane et al. [36] also reported spread monocyte morphology on 2D polyurethane films whereas more rounded appearance was detectable on the 3D polyurethane scaffolds. Young et al. [20] also showed that non-porous hydrophobic surface supported lesser monocyte attachment but higher amount of cytokines were produced as compared to porous hydrophilic surface. Thus, our findings are also consistent with a partial role of porosity and wettability of the matrices in the modulation of monocyte behavior.

Stiffness of underlying matrix could be another important factor to influence monocyte responses such as adhesion, spreading and activation [37,38]. Earlier studies reported that stiffer matrices favored macrophage attachment and spreading as compared to the lower moduli matrices [37]. In our study we observed that although 2D fibroin film was the stiffest material, followed by sericin film and 3D fibroin scaffold, cell attachment was maximal in sericin film and 3D fibroin with nominal attachment to fibroin film. However, the cell morphology on the silk matrices closely reminded studies by Blakney et al. [37], who reported similar rounded morphology of macrophages on lower moduli substrate whereas spread shape morphology with protruding filopodia upon culture on stiffer matrices. Furthermore, the expression of the three major pro-inflammatory cytokines TNFα, IL1β and IL6 was lowest for the softest material. One of the conclusions in our study is that IL1β and IL6 gene expression and protein release were maximal upon stimulation by 3D fibroin, having lower moduli as compared to 2D fibroin film. This suggests that stiffness of the biomaterial plays minimal roles in the induction of immune response by silk-based biomaterials.

We then addressed the role of biomaterial surface properties and protein conformation on immune cell activation. Activation of monocytes by silk biomaterials led to the expression of IL1β and IL6 genes and to the production of the corresponding cytokines. However, the level of expression significantly varied with the type of biomaterials and was found to be the highest in CD14+ cells stimulated by sericin film, followed by 3D fibroin and then 2D fibroin film. The reason for the weaker immune response to 2D fibroin film and strong response to 3D fibroin is likely to be represented by differential protein conformation. Earlier studies reported that peptides are poor immunogens but protein aggregates or supramolecular assembly of proteins are strongly immunogenic [39,40]. The strong immunogenic properties of peptides assembled by fibrillizing in the generation of strong antibody responses were reported by Rudra et al. [39]. Furthermore, prolonged T cell independent B cells response was observed to be induced by high molecular weight polymers multiple repeat epitope units [41]. Thus, in our study 2D fibroin film having mainly silk I conformation with reduced β-sheet was a poor immuno-stimulator as compared to the 3D fibroin with higher β-sheet content, e.g. more assembled structure acting as a strong immunostimulus. This indicates that cytokine induction is more governed by the protein conformation rather than by other properties such as wettability, porosity and stiffness of the material.

Most remarkably, the elicitation of these effects critically required physical contact between monocytes and silk materials, and was not detectable in cultures performed under trans-well conditions. These data clearly indicate that the well documented physicochemical differences between the materials under investigation are critical to their capacity to stimulate innate immune responses.

A recent study [21] has reported that macrophages cultured over 2D substrate express CD163, an M2 anti-inflammatory marker [42] and 27E10, an M1 pro-inflammatory marker upon culture on a 3D substrate. However cells cultured on the 2D substrate released pro-inflammatory cytokines whereas cells cultured on 3D substrate released M2-like factors with a decreased production of pro-inflammatory cytokines. Importantly, in our study we have
observed that none of the materials under investigation, regardless of its conformation was able to induce expression or release of IL10, a typical M2 cytokine (data not shown).

We found that the immunostimulatory effects of silk based materials were characterized by a characteristic kinetic, with high induction of pro-inflammatory cytokines during the first day of culture, rapidly declining in the following days, and becoming undetectable by day 6 of culture. Notably macrophage or bone marrow stem cell reactivity to silk materials was reported to be poor in prolonged cultures [16]. Accordingly, silk induced IL1β production was reported to be increased upon up to 3 day culture and then gradually decreased as compared to poly-glycolic acid (PGA) [43]. Our observations largely correlate with these studies regarding the poor long term pro-inflammatory potential of silk biomaterial.

We further analyzed the signaling pathways involved in the activation of monocytes upon interaction with the silk biomaterials. NF-κB transcription factor is largely involved in regulating a number of pro-inflammatory pathways. Indeed, by performing our experiments in the presence of specific inhibitors, we found that NF-κB and p38 MAP kinase critically contribute to monocyte activation stimulated by silk materials. Importantly, Polymyxin B, a highly effective inhibitor of endotoxin-mediated monocyte activation was unable to inhibit IL6 production induced by 3D Fibroin, thus ruling out the possibility of endotoxin contamination behind our observations. On the other hand, NF-κB and p38 MAP kinase play key roles in monocyte activation driven by Toll-like receptors (TLR) triggering. Therefore, future research is required to clarify whether the immunostimulatory ability of 3D silk-based biomaterials also resides in their capacity to trigger selected TLR.

Most interestingly, in our studies we were unable to detect a significant T cell responsiveness to silk-based biomaterials. However, the slow kinetics of the induction of T cell responses, might have failed to be adequately mirrored in our experiments. Furthermore, we cannot exclude that T cell responsiveness might only be detectable in healthy donors with an important previous exposure to the materials under investigation. Additional investigations are warranted to address these issues.

Fig. 7. Direct contact requirement for monocyte activation by silk-based biomaterials. Peripheral blood CD14+ monocytes from healthy donors were purified and cultured overnight in the presence of different silk-based biomaterials or control biomaterials in transwell plates with chambers separated by 3.0 µm pore membranes, preventing direct cell/biomaterial contact. Total cellular RNA was then extracted, reverse transcribed and amplified in the presence of primers and probes specific for IL-1β (panel A), IL-6 (panel B) and GAPDH housekeeping gene, used as reference. Culture supernatants were also harvested and their cytokine content was quantified by ELISA. Data refer to duplicate samples from an experiment out of two independently performed with cells from different healthy donors.
5. Conclusion

Our data strongly support the notion that physical characteristics and protein conformation play decisive roles in the induction of pro-inflammatory responses in monocytes upon stimulation with silk-based biomaterials. The presence of silk II conformation with higher β-sheet content in 3D fibroin proved to be more immunogenic as compared to 2D fibroin film with silk I conformation. Furthermore they provide a simple set of experimental approaches suitable for the initial pre-clinical evaluation of the bio-compatibility of materials of potential use in tissue regeneration.

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Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.biomaterials.2013.07.018.

References