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Controlled Release of Cytokines Using Silk-biomaterials for Macrophage Polarization

Andrew R.D. Reeves¹, Kara L. Spiller², Donald O. Freytes³, Gordana Vunjak-Novakovic⁴, and David L. Kaplan¹

Andrew R.D. Reeves: andrew.reeves@tufts.edu; Kara L. Spiller: kls35@drexel.edu; Donald O. Freytes: dfreytes@nyscf.org; Gordana Vunjak-Novakovic: gv2131@columbia.edu; David L. Kaplan: david.kaplan@tufts.edu

¹Department of Biomedical Engineering, Tufts University, Medford, Massachusetts, USA

²School of Biomedical Engineering, Drexel University, Philadelphia, PA, USA

³New York Stem Cell Foundation Research Institute, New York, NY, USA

⁴Department of Biomedical Engineering, Columbia University, New York, NY, USA

Abstract

Polarization of macrophages into an inflammatory (M1) or anti-inflammatory (M2) phenotype is important for clearing pathogens and wound repair, however chronic activation of either type of macrophages has been implicated in several diseases. Methods to locally control the polarization of macrophages is of great interest for biomedical implants and tissue engineering. To that end, silk protein was used to form biopolymer films that release either IFN- γ or IL-4 to control the polarization of macrophages. Modulation of the solubility of the silk films through regulation of β -sheet (crystalline) content enabled a short-term release (4–8 hours) of either cytokine, with smaller amounts released out to 24 hours. Altering the solubility of the films was accomplished by varying the time that the films were exposed to water vapor. The released IFN- γ or IL-4 induced polarization of THP-1 derived macrophages into the M1 or M2 phenotypes, respectively. The silk biomaterials were able to release enough IFN- γ or IL-4 to repolarize the macrophage from M1 to M2 and vice versa, demonstrating the well-established plasticity of macrophages. High β -sheet content films that are not soluble and do not release the trapped cytokines were also able to polarize macrophages that adhered to the surface through degradation of the silk protein. Chemically conjugating IFN- γ to silk films through disulfide bonds allowed for longer-term release to 10 days. The release of covalently attached IFN- γ from the films was also able to polarize M1 macrophages *in vitro*. Thus, the strategy described here offers new approaches to utilizing biomaterials for directing the polarization of macrophages.

Correspondence to: David L. Kaplan, david.kaplan@tufts.edu.

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Introduction

The innate immune system is the first line of defense against pathogens upon infection or wounding. Macrophages clear dead cell debris from a wound, help fight pathogens, and present pathogen-associated antigens to T-cells to activate the adaptive immune response. While macrophages are extremely important for immune defense and wound healing, chronic activation of different types of macrophages has been associated with several diseases, including atherosclerosis^{1,2}, insulin resistance or metabolic syndrome^{3–5}, and cancer⁶. Treating these diseases by targeting macrophages systemically is not ideal, as the treatment can impair the immune system as a whole. Localized delivery of treatments for chronic macrophage activation is therefore highly desired. Furthermore, implanted devices or tissue engineered constructs can be functionally hampered by the foreign body response, which involves macrophage-derived giant cell formation and development of granulation tissue surrounding the implants. A film or thin coating on the device that can control macrophage response would be highly beneficial.

Acute inflammation occurs in a wound or at an implant site just after the deposition of fibrinogen and blood clotting. Following neutrophil infiltration monocytes are recruited to the site of the wound within the first 24 hours to start clearing away dead cell debris and phagocytose bacteria that may have entered the wound^{7,8}. As the monocytes infiltrate the wound site they begin to differentiate into macrophages⁹. Once at the wound site macrophages polarize into specialized cells for different purposes. Interferon-gamma (IFN- γ) is a cytokine that activates macrophages to polarize along the “classical pathway,” which generates inflammatory macrophages, termed M1 macrophage due to the concurrent emergence of type 1 helper T-cells (Th1)¹⁰. M1 macrophages incite further inflammation by secreting cytokines such as interleukin-1 β (IL-1 β), IL-6, IL-12 and tumor necrosis factor alpha (TNF- α)¹¹. Several markers of M1 macrophage differentiation have been found including C-C chemokine receptor type-7 (CCR7)¹². These CCR7⁺ M1 macrophages help to defend against bacterial infections, contribute to phagocytosis of dead cells, and recruit Th1 cells to the site of the infection. Diseases associated with chronic activation of M1 macrophages include atherosclerosis², obesity^{13,14}, and inflammatory bowel disease^{15,16}.

Following the acute inflammatory phase, M2 macrophages emerge at the wound site, corresponding to the emergence of Th2 cells¹⁷. M2 macrophages are considered anti-inflammatory because they secrete IL-10, cytokine ligand 13 (CCL13), and transforming growth factor-beta (TGF- β), which promote tissue regeneration^{18,19}. The M2 macrophages are polarized through the “alternative pathway” by activation of the IL-4 receptor with either IL-4 or IL-13²⁰. The M2 macrophages are commonly delineated from M1 macrophages by the expression of the mannose receptor CD206²⁰, as well as CD163 and CD209^{21–24}. M2 macrophages have been implicated in promoting tissue regeneration and proliferation²⁵. In addition, tumor associated macrophages (TAM) are believed to take on M2-like characteristics, including the M2 marker CD206, as they promote tumor progression^{26–28}.

One of the interesting properties of the different macrophage phenotypes is their plasticity. The ability for an M1 macrophage to become M2 or vice versa has been observed *in vitro* and *in vivo*^{2,29–31}. This leads to the possibility that macrophages can be converted using

implantable biomaterials to control a specific disease. For instance, conversion of M1 macrophages that are found abundantly in atherosclerotic plaques into M2 macrophages might result in a reduction of plaque obstruction of blood flow by inducing tissue remodeling surrounding the blood vessel. Similarly, conversion of TAMs to M1 macrophages may allow them to better attack the tumors rather than aiding in their proliferation. Materials that can locally release cytokines to differentiate macrophages without systemically affecting the immune system could be very beneficial for the treatment of the macrophage associated diseases.

Recently, decellularized bone scaffolds with IL-4 bound via biotin-streptavidin interactions were used to promote vascular genesis *in vivo* and to control macrophage polarization *in vitro*³². That study demonstrates the potential for cytokine manipulation of host macrophages responding to implanted scaffolds. Future materials that can be coated onto any scaffold or medical device and that can release cytokines for macrophage control may have broad applications and implications for the medical industry.

Silk fibroin is a protein derived from the cocoons of the *Bombyx mori* silk worms and has been used for centuries as a suturing material. Silk has more recently been used as a versatile material for other biomedical purposes such as for fabricating scaffolds for cells, and therapeutics^{33,34}, and for localized drug delivery into wounds^{35–37}. Silk fibers can be solubilized and processed into biomaterials that have an advantage over other commonly used implantable materials because they have the ability to stabilize drugs and proteins³⁸, have a slow and controllable degradation rate, and the degradation products are non-toxic^{39,40}.

The goal of the present study was to use thin layers of silk protein as films to modulate the release of cytokines to control macrophage responses. In the long run this approach is anticipated to help with the incorporation of tissue engineered constructs and medical device implants into the body and well as improving wound healing. Silk films were utilized in the study as they can be tailored to sustain the release of cytokines and also be processed in aqueous, ambient conditions, to preserve the functionality of the cytokines for these types of *in vitro* studies. The goal to control the polarization of macrophages would have value in fundamental *in vitro* inquiries of macrophage functions, as well as in chronic macrophage associated diseases. If successful, these approaches could be useful for treating a number of diseases from atherosclerosis to cancer by converting the M1 into M2 or vice versa.

Materials and Methods

Silk Degumming and Dissolving

Silkworm cocoons from *B. mori* were purchased from Tajima Shoji Co., LTD (Yokohama, Japan) and are processed into pure silk fibroin solution as previously described⁴¹. Briefly, cocoons are cut into small pieces and placed into boiling 0.02mM Na₂CO₃ for 30 minutes. After boiling, the fibers are washed in deionized water and left to dry in a fume hood overnight. The next day a solution of 9.3 M LiBr is added to the silk fibers at a ratio of 1:4 weight (g) to volume (mL) and then allowed to dissolve at 60°C for 4 hours. The silk solution is then dialyzed against 4 L deionized water for 3 days, changing the water 3 times

per day for a total of 7 changes. At that point the silk fibroin is centrifuged at 4,000 x g to remove particulate matter and is ready for experiments. To determine the concentration of the silk solution, 1mL of solution is added to a weigh boat and weighed before and after drying in a convection oven at 60°C overnight.

Silk Casting and Water Annealing

To create molds for the silk films to be cast onto, polydimethyl siloxane (PDMS) (GE Plastics, Pittsfield, MA) was cast into a petri dish at about 3 mm thickness and allowed to polymerize overnight at 60°C. Cylinders with 1.4 cm diameter were punched out of the polymerized PDMS. Silk solutions were added on top of the cylinders and allowed to dry overnight under constant air flow. Water annealing (WA) the silk films was performed as previously described with slight adjustments to time and temperature⁴². The dried silk films were placed in a vacuum chamber with a 1 L basin of water inside at 23°C. The vacuum chamber was sealed and a vacuum was pulled to -80 kPa. The vacuum line was shut to allow the water to evaporate and build the water pressure in the chamber. The amount of time in the WA chamber is specified in each experiment

Silk-aniline conjugation

Tyrosine residues on silk fibroin were conjugated to 4-(2-aminoethyl)-aniline, as previously described⁴³. Briefly, a 6% silk solution was dialyzed against borate buffer (100mM borate, 150 mM NaCl, pH 9.0) for 1 day prior to reaction. To create the diazonium salt of the aniline 34 mg of 4-(2-aminoethyl)-aniline (2.5×10^{-4} mol) (Sigma Aldrich) was initially dissolved in 1.25 mL acetonitrile (0.2 M). Then 625 μ L of the aniline solution was mixed with 625 μ L 1.6 M p-toluenesulfonic acid. The aniline/toluene sulfonic acid mixture was combined 1:1 with 625 μ L 0.8 M NaNO₂ on ice and stirred for 15 minutes to create the diazonium salt of the aniline. The diazonium-aniline solution (125 μ L) was added to 2 mL silk solution in borate buffer and 375 μ L dH₂O to bring the total volume to 2.5 mL. The reaction was allowed to proceed for 10 minutes. Reacted silk-aniline solution was dialyzed (Slide-A-Lyzer Dialysis Cassettes 3500K MWCO, Thermo Fisher, Rockford, IL) against deionized water for 24 hours to remove any unreacted diazonium-aniline. Films of silk-aniline were created by drying 10mg silk-aniline per 1.54 cm² on PDMS overnight (3.26 mg silk-aniline on a 0.8 cm diameter PDMS cylinder was used). Successful conjugation of the aniline to silk was measured by UV/vis spectrum between 200–550 nm showing an absorbance peak at 330 nm. Silk-aniline conjugation results in introduction of free amines to the silk and is from here on referred to as Silk-NH₂.

Sulfo-LC-SPDP conjugation to silk fibroin

Silk-NH₂ lyophilized protein was resuspended in phosphate buffered saline with 5mM EDTA, pH 8.0 (PBS/EDTA). To 1.16 mL Silk-NH₂ solution, 8 mg of sulfo-LC-SPDP (sulfosuccinimidyl 6-[3'-(2-pyridylthio)propionamido]hexanoate) (Thermo Fisher, Rockford, IL) was dissolved and incubated at room temperature for 1 hour to link the sulfo-LC-SPDP to the free amines, creating Silk-LC-SPDP. The Silk-LC-SPDP solution (80 μ L, or 4.8 mg silk) was then cast onto PDMS cylinders and allowed to dry into films overnight. Films were washed 3x with PBS for 20 minutes each to remove residual sulfo-LC-SPDP. Successful conjugation of sulfo-LC-SPDP to the Silk-LC-SPDP films was evaluated by

measuring the release of the pyridine-2-thione ring of the sulfo-LC-SPDP after treatment with 15 mg/mL dithiothreitol (DTT) for 30 min and measuring the absorbance at 343 nm, as per the manufacturer's directions.

IFN- γ conjugation with Traut's Reagent

IFN- γ was reconstituted at 1mg/mL in dH₂O. Traut's reagent (2 mg) was dissolved in 1 mL dH₂O and 4.6 μ L was added to 100 μ L IFN- γ solution and incubated at room temperature for 1 hour to add sulfhydryl groups to the free amines of IFN- γ (SH-IFN- γ). The reacted SH-IFN- γ solution was diluted in 400 μ L PBS and dialyzed against PBS for 24 hours to remove unreacted Traut's reagent. SH-IFN- γ solution (100 μ L) was added to Silk-LC-SPDP films and incubated overnight at 4°C to link the Silk-LC-SPDP films to SH-IFN- γ via disulfide bonds (Silk-SS-IFN). Recombinant IFN- γ does not contain any cysteine residues, and therefore there are no free sulfhydryl groups, so the release of the pyridine-2-thione ring from the SPDP conjugated to silk after reacting with the SH-IFN- γ , as measured by absorbance at 343 nm, was used as confirmation that Traut's Reagent introduced thiols to IFN- γ .

FTIR Analysis

As previously described⁴², Fourier transform infrared spectroscopy (FTIR) measurements were recorded with a Jasco FT/IR-6200 Spectrometer (Jasco Analytical Instruments, Easton, MD) with a MIRacle™ Attenuated Total Reflectance (ATR) crystal (Ge crystal; Pike Tech, Madison, WI). The wavenumber spectrum analyzed was from 600 to 4000 cm⁻¹ and beta-sheet content was measured within the amide I region from 1585–1710 cm⁻¹. OPUS 5.5 software was used for Fourier self-deconvolution using Lorentzian curves with a 13 bandwidth and resolution enhancement of 2. OriginPro 8.5 software was used for peak fitting of the deconvoluted IR spectrum. Area under the peaks was calculated at full-width at half-max and were used for quantitation of β -sheet content using the peaks within the 1616–1628 cm⁻¹ range and 1690–1710 cm⁻¹ range.

Solubility testing

Silk films (10 mg) with a diameter of 1.4 cm were placed in 1 mL 1X Phosphate Buffered Saline (PBS) (Gibco, Thermo Fisher, Grand Island, NY) in wells of a 12-well culture plate (Falcon, Corning,) and placed on a shaker for 24 hours. Silk solution (10mg) was added to 1 mL PBS as the 100% control. Standard curves were made by diluting silk 1:1 starting with 30 mg/mL down to 0.47mg/mL in PBS. Dissolved silk solutions (200 μ L) were added to 96-well UV microplates (Thermo Scientific) and the absorbance at 280 nm (A280) was measured.

IFN- γ and IL-4 Sources and Release

Recombinant human IFN- γ or IL-4 (200 ng) (Peprotech, Rocky Hill, NJ, USA) was added to the silk solution to be cast into a 10 mg silk film. After the films were dried and water-annealed they were placed in 12-well plates containing 1mL PBS and placed on a shaker at room temperature to allow release of the cytokines from the films. At each time point to be measured the full 1mL was removed from each well and a fresh 1mL PBS was added. All

samples were frozen at -80°C . To measure the concentration of IFN- γ or IL-4 in each sample ELISA kits (R&D Systems, IL-4 Duo Set, DY204; IFN- γ Duo Set, DY285) were used according to manufacturer protocols.

THP-1 cell culture and treatments with IFN- γ & IL-4 Films

The human acute monocytic leukemia cell line (THP-1) was purchased through ATCC. The cells are maintained in RPMI-1640 with 10% Fetal Bovine Serum (FBS) and 1% antibiotic-antimycotic (Gibco, ThermoFisher Scientific). For differentiation of THP-1 to macrophage a total of 5×10^5 cells were seeded per well of 24-well culture plate in RPMI/10% FBS containing 100 ng/mL (162 nM) or 10 nM phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich) as noted in the figure legends and allowed to attach to the plate overnight. The next day medium was changed to polarization medium or films containing 200ng of either IFN- γ or IL-4 were submerged in basal RPMI/10%FBS medium. M1 polarization medium was composed of RPMI/10% FBS containing 20 ng/mL IFN- γ and M2 polarization medium contained 20 ng/mL IL-4 instead of IFN- γ .

Gene Expression by Real-Time qPCR

To analyze gene expression, cells were lysed and RNA purified using the RNeasy Mini Kit (Qiagen). To generate cDNA, a total of 1 μg RNA was used for reverse transcription using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). Using a Stratagene Mx3000P RT-PCR machine. RT-qPCR was performed using either TaqMan probes with the TaqMan Advanced 2x Master mix (ThermoFisher Scientific) or primers with Power SybrGreen (ThermoFisher Scientific). All TaqMan probes were run with a cycle of 15s at 95°C and 30s at 60°C for 40 cycles. All primers were used at 1 μM and run with cycles of 15s at 95°C and 45s at 60°C for 40 cycles. Taqman probes and primers used are listed below.

TaqMan probes used in this study:

Gene	Assay ID
CD68	Hs02836816_g1
CCR7	Hs01013469_m1
CD206	Hs00267207_m1
GAPDH	Hs02758991_g1
Actin	Hs00969077_m1

Primers for RT-qPCR using SybrGreen:

Gene	Forward Sequence	Reverse Sequence
TNF	GAGGCCAAGCCCTGGTATG	CGGGCCGATTGATCTCAGC
IL1B	ATGATGGCTTATTACAGTGGCAA	GTCGGAGATTCGTAGCTGGA

Gene	Forward Sequence	Reverse Sequence
IL12	TGCCCATTGAGGTCATGGTG	CTTGGGTGGGTCAGGTTTGA
IL6	CCTGAACCTTCCAAAGATGGC	TTCACCAGGCAAGTCTCCTCA
CD68	CTTCTCTCATTCCCCTATGGACA	GAAGGACACATTGTACTCCACC
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC
CD36	AGCCTCATTTCACCTTTTG	TGGGTTTTCAACTGGAGAGG
CD163	TGTGGCCTGCATAGAGAGTG	TTCCCCAAAATGAGCAGAAC
CCL13	CTCAACGTCCCCTACTTGC	TCTTCAGGGTGTGAGCTTTCC
CD209	AATGGCTGGAACGACGACAAA	CAGGAGGCTGCGGACTTTTT
CD206	CTACAAGGGATCGGGTTTATGGA	TTGGCATTGCCTAGTAGCGTA
CCR7	TGAGGTCACGGACGATTACAT	GTAGGCCACGAAACAAATGAT

Immunofluorescent Staining

THP-1 macrophages were cultured in 24-well plates on tissue culture plastic. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then washed 3x in phosphate buffered saline containing 0.1% Tween-20 to permeabilize cells. Cells were then blocked with 5% BSA for 30 min in PBS. Primary antibodies for CCR7 (Novus Biologicals, NB100-712) or CD206 (Biorbyt, orb4941) were incubated at 1:100 in PBS with 1% BSA and 0.05% Tween-20 at 4°C overnight. Secondary antibodies (AbCam, ab150129, ab96883) were incubated at room temperature for 2 hrs. Images were taken with a Zeiss Axiovert 40 CFL.

Scanning Electron Microscopy

Silk films with THP-1 derived macrophages attached were treated with 0.25% trypsin for 10–15 minutes with constant agitation until cells were visibly detached and the films were then dried and sputter coated with platinum. Images were taken with a Zeiss Supra 55VP scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

Statistics

Results of RT-qPCR are represented as the mean $\pm$ standard deviation. Results of ELISA measurements are represented as mean $\pm$ SEM. To calculate the statistical significance between two experimental groups the Student's t-test was used. Regression analysis of release data measured by ELISA utilized either a two phase association algorithm for cumulative release data or a two phase exponential decay algorithm for raw data points. Two-way associative/decay regressions were chosen based on the assumption that the cytokines and silk have some interaction other than simple release into the medium. GraphPad Prism 5 was used for all regression analysis. All experiments were performed in triplicate or more. Differences between groups were considered statistically significant when $p < 0.05$.

Results

Solubility and cytokine release profile of silk films

Treatment of silk films with 90% methanol (MeOH) and water induces rapid β -sheet content, which causes the films to become insoluble⁴¹. However, solvents can cause conformational changes to proteins that lead to inactivity of the protein and thus solvents were avoided for the creation of silk films that contained the cytokines. The process of water annealing (WA) allows control of the β -sheet content of silk films, avoids brittleness⁴² and avoids solvent-induced protein inactivity. The solubility of the silk films and the release of cytokines loaded into the films was hypothesized to depend on the β -sheet content of the films.

Silk films consisting of 10 mg silk were dried overnight and crystallized via WA for 0–120 minutes, overnight, or treated with 90% methanol (MeOH) for 30 minutes, and the extent of β -sheet content was determined over time (Fig. 1A & B). FT-IR absorbance of the silk films was measured between 600 and 4000 cm^{-1} . The Amide I region of the IR spectrum (1585–1710 cm^{-1}) contains the IR absorbance that corresponds to β -sheet bonding (1616–1628 cm^{-1} and 1695–1705 cm^{-1})⁴². After peak fitting within the deconvoluted Amide I spectrum, the percentage contribution of each individual peak to the entire amide I region could be quantitated. The contribution of β -sheet peaks between 1618–1628 cm^{-1} and 1695–1705 cm^{-1} were calculated (Fig. 1B). At 60 minutes of WA the silk films began to show a shift in the β -sheet peaks. By 80 minutes of WA the β -sheet peaks contributed around 20% of the area under the curve of the Amide I region. Overnight WA showed an increase in β -sheet content to 25%, whereas treatment with methanol increased the β -sheet content to nearly 35%.

The solubility of the films after various WA times was determined by absorbance of the solutions containing the films via UV spectroscopy at 280 nm. The films were 100% soluble until a critical percentage of β -sheet content was achieved after 60 minutes of WA at which point the films were on average 64% soluble (Fig. 1C). After 80 minutes of WA time the films were only 20% soluble. After 100 minutes of WA the films were mostly insoluble. Therefore a WA time of 70 or 80 minutes was used herein to induce β -sheet content of 20%, which allowed the films to maintain structural integrity with 20% dissolution of the films for the release of the IL-4 or IFN- γ cytokines.

Silk solution containing 200ng IFN- γ or IL-4 were dried overnight to form films and then WA for 70 or 80 minutes to induce β -sheet content. Release of the IL-4 and IFN- γ was measured by ELISA over the course of 24 hrs in PBS to determine the release profile of the cytokines. An average of 36.0 ng (18%) of IFN- γ and 19.61 (9.8%) of IL-4 was rapidly released after 30 min from the 80 min WA films. The percentage of release within the first 30 min for both cytokines in the 80 min WA films nearly matched the solubility of the films indicating that the burst release of the cytokines was closely related to the solubility of the films. IFN- γ continued to be released out to 73.13 ng (36.5%) after the initial 30 min burst release in the 80 min WA films, whereas continued IL-4 release was more subdued with a final release of 39.63 ng (19.8%). The cumulative release data was fit with a two-phase associative non-linear regression and from that a best-fit plateau of the curve was calculated.

For IFN- γ release from 70 and 80 min WA films the plateaus were 89.19 ng and 76.02 ng, respectively, indicating a small but continuous release out past 24 hours. The plateaus for IL-4 release were calculated to be 116.8 ng for 70 min WA and 39.63 ng for 80 min WA, indicating a continuous release only up to 8 hours. Very little release occurred after 4 hours from any of the films. Two-way ANOVA was used to analyze the two groups of 70 vs 80 minute WA films for both cytokines and in both cases there was a significant ($p = 0.001$) difference between release after 70 vs 80 min WA.

Macrophage polarization with silk films

To determine the ability of the cytokine loaded silk films to polarize macrophages, the monocytic THP-1 cell line was used. The THP-1 cells were first induced to differentiate from monocytes into macrophages using PMA. Films were then submerged into the culture medium, without direct contact, and allowed to polarize the cells for another 48 hours. Macrophages treated with control films that did not have loaded cytokines adhered to the plate as small round clusters that did not spread (Fig. 2A). IFN- γ loaded films caused the macrophages to project many protrusions with many cells appearing dendritic. The IL-4 loaded films induced the cells to spread over a wider area than control but did not show the numerous projections observed with IFN- γ loaded films.

Using qPCR, the markers of M1 or M2 macrophage were investigated after treatment with silk films. CD68 is a marker of both monocytes and macrophage but is generally found to be increased after macrophage differentiation by PMA^{44,45}. After 16 hr treatment of the THP-1 cells with PMA, CD68 was found to be upregulated (Fig. 2B). As expected, IFN- γ loaded films induced strong upregulation of CCR7, an M1 macrophage marker (Fig. 2C). Treatment with IFN- γ loaded films also decreased the M2 marker CD206. Conversely, treatment with IL-4 loaded films increased expression of CD206, and decreased CCR7 expression. Further investigation of several other markers of M1 (TNFA, IL1B, IL12, IL6, and CD36) and M2 (CD163, CCL13, and CD209) were investigated as well after differentiation with two different concentrations of PMA, 10 nM (6.1 ng/mL) or 162 nM (100 ng/mL). In general all M1 genes were most strongly upregulated by IFN- γ treatment and M2 genes were most strongly upregulated by IL-4 treatment, however, CCL13 showed very strong upregulation by IFN- γ after treatment with 162nM PMA (Supplementary Fig. 1B). Immunofluorescent staining was performed on the differentiated THP-1 cells that had been treated with PMA or cytokines to look at CCR7 and CD206. There was high background fluorescence of CCR7 in all cells but CCR7 appeared slightly brighter in the IFN- γ treated cells (Supplementary Fig. 1A). The cells treated with IL-4 showed strong expression of CD206 while the control cells showed very little expression and IFN- γ treated cells were nearly completely devoid of CD206 staining (Supplementary Fig. 1A). Together these data indicate that IFN- γ and IL-4 loaded films can induce an M1 or M2 phenotype and morphology^{46,47}, respectively.

Control of macrophage plasticity with silk films

One of the hallmarks of macrophages is their plasticity. To determine if the cytokine release from silk films was potent enough to convert one polarized macrophage phenotype to the opposing phenotype a two-stage polarization program was set up. First, THP-1 cells were treated with PMA to induce them into macrophages, and then treated with either IFN- γ or

IL-4 directly for 48 hours. As a control, some wells of the THP-1 macrophages were lysed at this first time point and the expression of CCR7 or CD206 was evaluated. The remaining wells were then switched to medium with silk films containing the opposing cytokine for an additional 48 hours. For example, cells treated for 48 hours with medium containing 20 ng/mL IL-4 were switched to medium with films that were loaded with 200 ng IFN- γ for another 48 hours. Cells first treated with IFN- γ for 48 hours expressed very high levels of CCR7. When the medium was then switched to medium containing films loaded with 200 ng IL-4 the expression of CCR7 was dramatically reduced and the expression of CD206 was significantly increased (Fig. 3A). Conversely, when cells were first treated with IL-4 they showed strong expression of the M2 marker CD206 but when the medium was switched to contain IFN- γ loaded films the expression of CD206 was lost and CCR7 expression was upregulated (Fig. 3B). As a control, medium was also switched to contain either 20 ng/mL IL-4 or IFN- γ to determine the differences between adding the cytokines straight to the medium compared with the release of the cytokines from films. In all cases, the cytokines added directly to the medium had a similar effect.

Degradation of silk films by macrophages

To determine if macrophage can access cytokines trapped within an insoluble silk matrix, such as the non-solubilized portion of 80 min WA films, by degrading the silk protein, films with high β -sheet content were made that contained either IFN- γ or IL-4. Silk films were water annealed at 50°C, rather than 24°C as in the previous experiments, and for a duration of 2.5 hrs to induce more rapid β -sheet crystallinity, as has been reported previously⁴². Only 0.5% of IFN- γ and 0.1% of IL-4 that was loaded into the films was released indicating that the majority of the cytokines were still trapped within the silk films (Fig. 4A). The small amount released is likely to be surface bound cytokines and further demonstrates that the burst release from earlier experiments was most likely due to the solubility of the silk films and not surface bound cytokines. The β -sheet content of these high temperature and long water annealing times was measured to be an average of 27% (Fig. 4B), which is higher than the insoluble 25% β -sheet content observed at lower temperatures with 24 hours of WA.

To test if direct contact with macrophages and silk films could still result in macrophage polarization the higher β -sheet films were washed briefly to remove any surface bound cytokines. When the films were submerged in the medium without direct contact with the macrophages, the polarization of macrophages was comparable for the controls and either of the cytokine loaded films (Fig. 4C). However, when the macrophages were seeded directly onto the films a significant increase in expression of CCR7 with IFN- γ films, and CD206 with IL-4 films, was observed, as compared with control films lacking any cytokines (Fig. 4C). This experiment showed that the macrophages were able to access the cytokines embedded within the silk films, which led to their polarization even when the cytokines could not diffuse out of the films.

In order to gain access to the cytokines loaded into the high-content β -sheet films the macrophages must alter the film structure, likely through the degradation of the silk matrix. Macrophages were seeded onto high- β -sheet content films that contained IFN- γ in order to investigate if silk is degraded during polarization of the macrophages in direct contact with

the films. After 48 hours of cell attachment the films were incubated in trypsin to remove the cells and then dried and imaged under scanning electron microscopy. In areas where cells were attached many small pits were seen surrounding the cell as well as a general degradation of the surrounding area (Fig. 4D).

Covalent attachment of IFN- γ to silk films for long-term release

To treat diseases associated with chronic macrophage activation, such as atherosclerotic plaques or cancers associated with TAMs, a longer term release of the cytokines would be beneficial. To this end, IFN- γ was covalently attached to the silk films, as a proof of principle, through disulfide bonds that have been previously shown to hydrolyze at a constant rate in physiological conditions to prolong release of protein from biomaterials⁴⁸. Covalent attachment of two proteins can be accomplished by reacting free amines on the first protein with the linker molecule N-Succinimidyl 3-(2-pyridyldithio)-propionate (SPDP), which can then be reacted with free sulfhydryl groups on the second protein. Therefore a water soluble, long chain, SPDP analogue (sulfo-LC-SPDP) was used to conjugate IFN- γ to the surface of silk films (Fig. 5A–C).

There are 12 lysine residues (0.21%) in full length silk fibroin with which to conjugate sulfo-LC-SPDP. Initial attempts to conjugate sulfo-LC-SPDP to these lysine residues did not result in detectable amounts of conjugation. In order to functionalize silk fibroin with sulfo-LC-SPDP, the number of free amines capable of reactivity was increased through reaction of the diazonium salt of 4-(2-aminoethyl)-aniline with the silk solution (Fig. 5A). The reaction produced a red color in silk and an absorbance peak at 330 nm (Fig. 5D) indicating the reaction successfully covalently attached the aniline to the silk, herein referred to as Silk-NH₂. The sulfo-LC-SPDP was then reacted with the silk-NH₂, creating the silk-LC-SPDP solution, which was dried into films and treated with methanol to induce beta-sheet formation. The concentration of SPDP that reacted with silk was measured to be 6.85% of the total SPDP used (Fig. 5e). This indicated a 1.9:1 molar ratio between sulfo-LC-SPDP to silk fibroin (calculated using 125kD as the average molecular weight of 30 minute boiled silk fibroin⁴⁹).

The recombinant IFN- γ used does not contain any cysteines and therefore does not have any free sulfhydryl groups to react with the protected disulfide bond in the Silk-SPDP. To add sulfhydryl groups to IFN- γ the protein was reacted with Traut's reagent, which introduces sulfhydryl groups at free amines (SH-IFN- γ) (Fig. 5B). A solution of SH-IFN- γ was then added to each silk-SPDP film and allowed to react at 4°C overnight to generate silk films with covalently bound IFN- γ via disulfide bonds to the SPDP linker (Silk-SS-IFN). Absorbance at 343 nm was again measured to determine the amount of pyridine-2-thione released from the reaction of SH-IFN- γ with silk-LC-SPDP, which showed that an average of 11.1 μ g SH-IFN- γ had formed disulfide bonds, which shows that 111% $\pm$ 10.68% of the IFN- γ covalently bound to the silk-SPDP films (Fig 5E).

The release of IFN- γ was measured by ELISA over the course of 10 days in PBS (Fig. 6A). Though the total release of IFN- γ was only a fraction of that bound to the silk, the continuous release allowed for polarization of THP-1 derived macrophages to M1

macrophages as indicated by the increased expression of CCR7 and decreased expression of CD206 after 48 hours in culture with Silk-SS-IFN films (Fig. 6B).

Discussion

One goal for biomaterial and tissue engineered implants is to control the biological response, optimizing regenerative needs while reducing inflammatory pathways. Following implantation of a biomaterial, scaffold, or tissue, serum proteins will begin to adsorb to the surface allowing for immune cells to adhere and develop a response to the implant^{50,51}. Monocytes and macrophages are innate immune cells that mount part of the early response to implanted materials. Macrophages have been shown to be integral for wound repair and tissue regeneration at the site of an implantation⁵², and wounds in general^{53,54}. The acute phase of the macrophage response involves the release of inflammatory cytokines, reactive oxygen species, acid, and proteolytic enzymes⁵⁵. These acute, M1, macrophages are responsible for phagocytosing parasites and clearing dead cells from the wound, but they have also been shown to be involved with initiating a regeneration response⁵⁶. Following the M1 response a new subset of macrophage emerge, termed M2, at the site of a wound that release cytokines that are anti-inflammatory along with growth factors to promote tissue regeneration. In fact, activation of both M1 and M2 macrophage chronologically can lead to improved angiogenesis^{32,57}.

Given the dual importance of M1 and M2 macrophages, silk-based biomaterial films were created that could polarize both M1 and M2 macrophages from human THP-1 monocytic cells. These silk films were a proof-of-principle for further studies *in vitro* and *in vivo* on the effects of modulating macrophage responses to tissue engineering and medical device implants. M1 polarization of macrophages is commonly accomplished *in vitro* by treatment with IFN- γ , and M2 polarization can be accomplished with IL-4 treatment. Thus, the silk films were generated to release either IFN- γ or IL-4 in varying quantities based on the solubility of the silk films, or through hydrolysis of the linker molecule, sulfo-LC-SPDP, that covalently bound IFN- γ to the surface of the silk films.

The first method that was investigated here for controlling cytokine release from silk films was to modulate the β -sheet crystallinity and solubility of the films. In order to maintain structural integrity of the films but allow some of the films to dissolve, and thus release the cytokines, the solubility was kept near 20% by inducing β -sheet crystallinity for 80 minutes with water vapor. The semi-soluble films released enough cytokine to polarize THP-1 derived macrophages toward the M1 or M2 phenotype. Furthermore, insoluble silk films that were loaded with cytokines did not release significant amounts but were able to polarize macrophages when they were in direct contact with the film surface. The ability of the macrophages to become polarized on the surface of insoluble films indicated that the cytokines were still accessible to the macrophages. The silk films were imaged by SEM and the areas around the attached cells was found to have many small holes and general degradation suggesting that the ability to access the cytokines loaded into the insoluble films was accomplished through degradation of the biomaterial. Silk films have previously been shown to increase the stability of proteins at high temperatures and for long periods of time³³, and given the fact that macrophages were able to slowly degrade the silk protein to

access trapped cytokines means that cytokine loaded silk films may have a long term effect after implantation, even after the initial burst release. Altogether, these soluble and insoluble films demonstrated the potential for silk materials that are loaded with cytokines to modulate macrophage polarization both through release of the cytokines as well as direct contact and degradation to access trapped cytokines. This was, however, limited to *in vitro* work using a single monocytic leukemia cell line, so there are likely to be differences in release and polarization profiles in the complex environment found *in vivo*. Furthermore, the solubility of the silk films was originally hypothesized to control the rate at which cytokines were released, however the semi-soluble films always demonstrated a burst release within the first four hours and further release, though detectable, was minimal out to only 24 hours.

Another major role of macrophages is the formation of giant cells that encapsulate foreign material, such as implanted biomaterials and medical devices, and lead to the formation of granulation tissue. In fact a problem with insulin pumps is that the cannula can become occluded due to inflammation, granulation tissue, and fibrosis^{58,59}. Similarly, foreign body granulation tissue can form around stents after implantation limiting their effectiveness⁶⁰. Therefore, initial reduction of giant cell formation around implants is desirable. Modulation of macrophage phenotype through burst release from silk as well as sustained control of attached macrophage may be useful for preventing giant cell formation and cannula occlusion. Silk solutions have previously been demonstrated to be capable of coating various surfaces such as glass⁶¹, plastics⁶², and metal^{63,64}, and therefore the cytokine loaded silk films studied here could represent a good model for biomaterials and medical device coatings for the control of macrophage giant cell responses as well.

Macrophages are highly plastic cells that can change their functional phenotype from M1 to M2 and vice versa depending on the environment where they reside^{2,29-31}. In chronic diseases associated with macrophages the environment pushes the macrophages toward one or the other phenotype which exacerbates the disease. For instance, atherosclerotic lesions are associated with M1 macrophages and high levels of IFN- γ within the microenvironment of the lesion. The IFN- γ leads to M1 polarization of infiltrating monocytes and inhibits HDL-mediated cholesterol efflux, which further augments the formation of atherosclerotic plaques⁶⁵. Systemic administration of low levels of IL-4 ($1.1 \text{ ng g}^{-1} \text{ day}^{-1}$) to control the conversion of M1 to M2 has been tested in late stage mouse atherosclerotic plaque models (apoE^{-/-} and LDL receptor^{-/-}) but was not successful, showing no change in lesion size⁶⁶. However, in an early atherosclerosis model using mice fed a high-cholesterol diet for 16 weeks, injections of IL-4 showed a 90% reduction in lesion size⁶⁷. This indicates that exogenous IL-4 can have a positive effect that might be dependent on the stage of the lesion. The release of IL-4 from semi-soluble silk films *in vitro* showed the ability to repolarize the M1 macrophages toward the M2 phenotype and therefore may represent a potential treatment for atherosclerosis if coated onto stents. The large quantities of IL-4 released (~50 ng) and the localization of an implant may prove to be more beneficial for later stage atherosclerotic plaques as well for stents coated with a silk film loaded with IL-4. Though not performed in this study, it would be interesting to get a longer term release of IL-4 through chemical crosslinking of endogenous cysteines in IL-4 with sulfo-LC-SPDP modified silk.

Along the same line, implantation of IFN- γ loaded films at the site of a tumor may represent a novel strategy for treatment of solid tumors that have tumor-associated macrophages (TAMs), which have been shown to exacerbate tumor growth. Genetic ablation of TAMs with diphtheria toxin driven from the mouse macrophage-specific CD11b promoter was shown to reduce colorectal cancer growth and invasiveness⁶⁸. Furthermore, introduction of M1 macrophage into a tumor environment can reduce tumor growth⁶⁹. Thus conversion of the M2-like TAMs to an M1 state may have two levels of effect: 1) reduction of tumor growth and invasiveness through reduced numbers of M2 macrophages, and 2) clearance of the tumor by the M1 macrophages. Release of IFN- γ from the semi-soluble films was able to repolarize M2 macrophages towards M1 leading to the possibility that silk biomaterials could be used to deliver IFN- γ to tumors as well. However, a longer-term sustained release of IFN- γ may be necessary for the maintenance of an M1 state for TAM treatments or other chronic macrophage-associated diseases and thus the rapid burst release from the soluble silk-films was not considered ideal. Therefore, the creation of a sustained release system was attempted by conjugating IFN- γ to the silk film surface via the hydrolysable linker molecule sulfo-LC-SPDP. IFN- γ was detected out to 10 days and thus represents a longer-term implantable biomaterial than the semi-soluble silk films. However, the amount of IFN- γ released declined relatively quickly and a more sustainable and linear release is still desired for *in vivo* testing. The sulfo-LC-SPDP linker was not hydrolyzed as rapidly as expected so further studies will warrant testing different surface chemistries to allow for sustained and linear release of either IFN- γ or IL-4. To study the application of silk films for treatment of TAMs, the 4T1 mouse model of breast cancer would be appropriate because the 4T1 breast cancer cells have been clearly demonstrated to be dependent on infiltrating CD206+ macrophage for tumor growth⁷⁰. Implantation of IFN- γ films alongside implantation of the cancer cells in the flank of the mouse would be a potential way to study these films in cancer progression.

One of the limitations of this study was the use of THP-1 cells as a model for controlling macrophage polarization rather than *in vivo* experiments or with the use of primary peripheral blood monocyte derived macrophages (PBMCs). However, THP-1 cells have been demonstrated to have a similar response to IFN- γ and IL-4⁷¹ as do PBMCs^{72,73} so the choice of THP-1 cells may not affect the interpretation of the results dramatically. Another limitation is that phenotypic characterization was limited to gene expression, immunofluorescence of CCR7 and CD206, and morphologic changes without looking at phenotypic outcomes such as secreted cytokines at the protein level. Regardless of these limitations this study clearly shows that the silk films have the potential to polarize macrophages, which is an exciting area for further research and potential clinical application.

Conclusions

This study investigated the ability of silk films to release cytokines and control THP-1 macrophage polarization. Silk films loaded with either IFN- γ or IL-4 were able to polarize macrophages to the M1 or M2 macrophage phenotype, respectively. Release of the cytokines from the films was found to be correlated with the solubility of the silk films, which was in turn dependant on the percentage of β -sheet content in the films. Insoluble

films were also able to direct macrophage polarization after degradation of the silk material by the macrophages. Finally, silk films were functionalized to increase the duration of release out to 10 days by covalently attaching IFN- γ to the films through disulfide bonds. All together, this study showed that THP-1 macrophage polarization can be modulated through the controlled presentation of specific cytokines using silk films. This approach has interesting potential for related studies of polarized macrophages and their mechanisms of action *in vitro* and *in vivo*. Further, *in vivo* studies could be pursued to exploit this approach in a proactive way, to direct regeneration vs. rejection pathways via macrophage polarization, and to determine how to optimize such functional outcomes for specific implants, or for the study of specific diseases.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CCR7	C-C chemokine receptor 7
CD206	cluster of differentiation 206
CD68	cluster of differentiation 68
DTT	dithiothreitol
FBS	fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IFN-γ	Interferon Gamma
IL-12	Interleukin 12
IL-13	Interleukin 13
IL-1β	Interleukin-6 beta
IL-4	Interleukin 4
PMA	phorbol 12-myristate 13-acetate
SEM	scanning electron microscopy, or SEM, standard error of the mean
sulfo-LC-SPDP	sulfosuccinimidyl 6-[3'-(2-pyridyldithio)propionamido]hexanoate
TAM	tumor associated macrophage
Th2	T-helper 2
THP-1	human monocytic leukemia cell line

WA

water annealing

References

1. Hansson GK, Libby P. The immune response in atherosclerosis: a double-edged sword. *Nat Rev Immunol.* 2006; 6(7):508–519.10.1038/nri1882 [PubMed: 16778830]
2. Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston A-T, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A, et al. Macrophage Plasticity in Experimental Atherosclerosis. *PLoS ONE.* 2010; 5(1):e8852.10.1371/journal.pone.0008852 [PubMed: 20111605]
3. Aron-Wisnewsky J, Tordjman J, Poitou C, Darakhshan F, Hugol D, Basdevant A, Aissat A, Guerre-Millo M, Clément K. Human adipose tissue macrophages: m1 and m2 cell surface markers in subcutaneous and omental depots and after weight loss. *J Clin Endocrinol Metab.* 2009; 94(11): 4619–4623.10.1210/jc.2009-0925 [PubMed: 19837929]
4. Chawla A, Nguyen KD, Goh YPS. Macrophage-mediated inflammation in metabolic disease. *Nat Rev Immunol.* 2011; 11(11):738–749.10.1038/nri3071 [PubMed: 21984069]
5. Odegaard JI, Chawla A. Pleiotropic actions of insulin resistance and inflammation in metabolic homeostasis. *Science.* 2013; 339(6116):172–177.10.1126/science.1230721 [PubMed: 23307735]
6. Noy R, Pollard JW. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity.* 2014; 41(1):49–61.10.1016/j.immuni.2014.06.010 [PubMed: 25035953]
7. Silva MT. Macrophage phagocytosis of neutrophils at inflammatory/infectious foci: a cooperative mechanism in the control of infection and infectious inflammation. *J Leukoc Biol.* 2011; 89(5):675–683.10.1189/jlb.0910536 [PubMed: 21169518]
8. Daley JM, Brancato SK, Thomay AA, Reichner JS, Albina JE. The phenotype of murine wound macrophages. *J Leukoc Biol.* 2010; 87(1):59–67. [PubMed: 20052800]
9. Epelman S, Lavine KJ, Randolph GJ. Origin and Functions of Tissue Macrophages. *Immunity.* 2014; 41(1):21–35.10.1016/j.immuni.2014.06.013 [PubMed: 25035951]
10. Yin J, Ferguson TA. Identification of an IFN-gamma-producing neutrophil early in the response to *Listeria monocytogenes*. *J Immunol Baltim Md 1950.* 2009; 182(11):7069–7073.10.4049/jimmunol.0802410
11. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000prime Rep.* 2014; 6:13.10.12703/P6-13 [PubMed: 24669294]
12. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional Profiling of the Human Monocyte-to-Macrophage Differentiation and Polarization: New Molecules and Patterns of Gene Expression. *J Immunol.* 2006; 177(10):7303–7311. [PubMed: 17082649]
13. Hotamisligil GS. Inflammation and metabolic disorders. *Nature.* 2006; 444(7121):860–867.10.1038/nature05485 [PubMed: 17167474]
14. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW. Obesity is associated with macrophage accumulation in adipose tissue. *J Clin Invest.* 2003; 112(12):1796–1808.10.1172/JCI19246 [PubMed: 14679176]
15. Bain CC, Scott CL, Uronen-Hansson H, Gudjonsson S, Jansson O, Grip O, Williams M, Malissen B, Agace WW, Mowat AM. Resident and pro-inflammatory macrophages in the colon represent alternative context-dependent fates of the same Ly6Chi monocyte precursors. *Mucosal Immunol.* 2013; 6(3):498–510.10.1038/mi.2012.89 [PubMed: 22990622]
16. Abraham C, Cho JH. Inflammatory bowel disease. *N Engl J Med.* 2009; 361(21):2066–2078.10.1056/NEJMra0804647 [PubMed: 19923578]
17. Martinez FO, Gordon S. The M1 and M2 paradigm of macrophage activation: time for reassessment. *F1000Prime Rep.* 2014; 6:10.12703/P6-13
18. Martinez FO, Gordon S, Locati M, Mantovani A. Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol Baltim Md 1950.* 2006; 177(10):7303–7311.
19. Murray PJ, Allen JE, Biswas SK, Fisher EA, Gilroy DW, Goerdt S, Gordon S, Hamilton JA, Ivashkiv LB, Lawrence T, et al. Macrophage Activation and Polarization: Nomenclature and

- Experimental Guidelines. *Immunity*. 2014; 41(1):14–20.10.1016/j.immuni.2014.06.008 [PubMed: 25035950]
20. Gordon S. Alternative activation of macrophages. *Nat Rev Immunol*. 2003; 3(1):23–35.10.1038/nri978 [PubMed: 12511873]
 21. Fujisaka S, Usui I, Bukhari A, Ikutani M, Oya T, Kanatani Y, Tsuneyama K, Nagai Y, Takatsu K, Urakaze M, et al. Regulatory Mechanisms for Adipose Tissue M1 and M2 Macrophages in Diet-Induced Obese Mice. *Diabetes*. 2009; 58(11):2574–2582.10.2337/db08-1475 [PubMed: 19690061]
 22. Genin M, Clement F, Fattaccioli A, Raes M, Michiels C. M1 and M2 macrophages derived from THP-1 cells differentially modulate the response of cancer cells to etoposide. *BMC Cancer*. 2015; 15:577.10.1186/s12885-015-1546-9 [PubMed: 26253167]
 23. Fuentes-Duculan J, Suárez-Fariñas M, Zaba LC, Nograles KE, Pierson KC, Mitsui H, Pensabene CA, Kzhyshkowska J, Krueger JG, Lowes MA. A subpopulation of CD163 positive macrophages is classically activated in psoriasis. *J Invest Dermatol*. 2010; 130(10):2412–2422.10.1038/jid.2010.165 [PubMed: 20555352]
 24. Takahashi H, Hatta Y, Iriyama N, Hasegawa Y, Uchida H, Nakagawa M, Makishima M, Takeuchi J, Takei M. Induced differentiation of human myeloid leukemia cells into M2 macrophages by combined treatment with retinoic acid and 1 α ,25-dihydroxyvitamin D3. *PloS One*. 2014; 9(11):e113722.10.1371/journal.pone.0113722 [PubMed: 25409436]
 25. Xiao X, Gaffar I, Guo P, Wiersch J, Fischbach S, Peirish L, Song Z, El-Gohary Y, Prasad K, Shiota C, et al. M2 macrophages promote beta-cell proliferation by up-regulation of SMAD7. *Proc Natl Acad Sci*. 2014; 111(13):E1211–E1220.10.1073/pnas.1321347111 [PubMed: 24639504]
 26. Lewis CE, Pollard JW. Distinct role of macrophages in different tumor microenvironments. *Cancer Res*. 2006; 66(2):605–612.10.1158/0008-5472.CAN-05-4005 [PubMed: 16423985]
 27. Sangaletti S, Di Carlo E, Gariboldi S, Miotti S, Cappetti B, Parenza M, Rumio C, Brekken RA, Chiodoni C, Colombo MP. Macrophage-derived SPARC bridges tumor cell-extracellular matrix interactions toward metastasis. *Cancer Res*. 2008; 68(21):9050–9059.10.1158/0008-5472.CAN-08-1327 [PubMed: 18974151]
 28. Luo Y, Zhou H, Krueger J, Kaplan C, Lee S-H, Dolman C, Markowitz D, Wu W, Liu C, Reisfeld RA, et al. Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J Clin Invest*. 2006; 116(8):2132–2141.10.1172/JCI27648 [PubMed: 16862213]
 29. Stout RD. Functional plasticity of macrophages: reversible adaptation to changing microenvironments. *J Leukoc Biol*. 2004; 76(3):509–513.10.1189/jlb.0504272 [PubMed: 15218057]
 30. Davis MJ, Tsang TM, Qiu Y, Dayrit JK, Freij JB, Huffnagle GB, Olszewski MA. Macrophage M1/M2 Polarization Dynamically Adapts to Changes in Cytokine Microenvironments in *Cryptococcus neoformans* Infection. *mBio*. 2013; 4(3):e00264–13.10.1128/mBio.00264-13 [PubMed: 23781069]
 31. Mantovani A, Sica A, Sozzani S, Allavena P, Vecchi A, Locati M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol*. 2004; 25(12):677–686.10.1016/j.it.2004.09.015 [PubMed: 15530839]
 32. Spiller KL, Nassiri S, Witherel CE, Anfang RR, Ng J, Nakazawa KR, Yu T, Vunjak-Novakovic G. Sequential delivery of immunomodulatory cytokines to facilitate the M1-to-M2 transition of macrophages and enhance vascularization of bone scaffolds. *Biomaterials*. 2014; 37C:194–207.10.1016/j.biomaterials.2014.10.017 [PubMed: 25453950]
 33. Zhang J, Pritchard E, Hu X, Valentin T, Panilaitis B, Omenetto FG, Kaplan DL. Stabilization of vaccines and antibiotics in silk and eliminating the cold chain. *Proc Natl Acad Sci U S A*. 2012; 109(30):11981–11986.10.1073/pnas.1206210109 [PubMed: 22778443]
 34. Raja WK, Maccorkle S, Diwan IM, Abdurrob A, Lu J, Omenetto FG, Kaplan DL. Transdermal delivery devices: fabrication, mechanics and drug release from silk. *Small Weinhe Bergstr Ger*. 2013; 9(21):3704–3713.10.1002/smll.201202075
 35. Leal-Egaña A, Scheibel T. Silk-based materials for biomedical applications. *Biotechnol Appl Biochem*. 2010; 55(3):155–167.10.1042/BA20090229 [PubMed: 20222871]

36. Yucel T, Lovett ML, Kaplan DL. Silk-based biomaterials for sustained drug delivery. *J Controlled Release*. 10.1016/j.jconrel.2014.05.059
37. Gil ES, Panilaitis B, Bellas E, Kaplan DL. Functionalized silk biomaterials for wound healing. *Adv Healthc Mater*. 2013; 2(1):206–217.10.1002/adhm.201200192 [PubMed: 23184644]
38. Pritchard EM, Dennis PB, Omenetto F, Naik RR, Kaplan DL. Review physical and chemical aspects of stabilization of compounds in silk. *Biopolymers*. 2012; 97(6):479–498.10.1002/bip.22026 [PubMed: 22270942]
39. Rajkhowa R, Hu X, Tsuzuki T, Kaplan DL, Wang X. Structure and biodegradation mechanism of milled Bombyx mori silk particles. *Biomacromolecules*. 2012; 13(8):2503–2512.10.1021/bm300736m [PubMed: 22746375]
40. Wang Y, Rudym DD, Walsh A, Abrahamsen L, Kim H-J, Kim HS, Kirker-Head C, Kaplan DL. In vivo degradation of three-dimensional silk fibroin scaffolds. *Biomaterials*. 2008; 29(24–25):3415–3428.10.1016/j.biomaterials.2008.05.002 [PubMed: 18502501]
41. Rockwood DN, Preda RC, Yücel T, Wang X, Lovett ML, Kaplan DL. Materials fabrication from Bombyx mori silk fibroin. *Nat Protoc*. 2011; 6(10):1612–1631.10.1038/nprot.2011.379 [PubMed: 21959241]
42. Hu X, Shmelev K, Sun L, Gil E-S, Park S-H, Cebe P, Kaplan DL. Regulation of Silk Material Structure by Temperature-Controlled Water Vapor Annealing. *Biomacromolecules*. 2011; 12(5):1686–1696.10.1021/bm200062a [PubMed: 21425769]
43. Murphy AR, John PS, Kaplan DL. Modification of Silk Fibroin Using Diazonium Coupling Chemistry and the Effects on hMSC Proliferation and Differentiation. *Biomaterials*. 2008; 29(19):2829–2838.10.1016/j.biomaterials.2008.03.039 [PubMed: 18417206]
44. Hayden JM, Brachova L, Higgins K, Obermiller L, Sevanian A, Khandrika S, Reaven PD. Induction of monocyte differentiation and foam cell formation in vitro by 7-ketocholesterol. *J Lipid Res*. 2002; 43(1):26–35. [PubMed: 11792719]
45. Ma W, Liu Y, Ellison N, Shen J. Induction of C-X-C chemokine receptor type 7 (CXCR7) switches stromal cell-derived factor-1 (SDF-1) signaling and phagocytic activity in macrophages linked to atherosclerosis. *J Biol Chem*. 2013; 288(22):15481–15494.10.1074/jbc.M112.445510 [PubMed: 23599431]
46. Kuss M, Adamopoulou E, Kahle PJ. Interferon- γ induces leucine-rich repeat kinase LRRK2 via extracellular signal-regulated kinase ERK5 in macrophages. *J Neurochem*. 2014; 129(6):980–987.10.1111/jnc.12668 [PubMed: 24479685]
47. Chan WK, Cheung CC, Law HK, Lau YL, Chan GC. Ganoderma lucidum polysaccharides can induce human monocytic leukemia cells into dendritic cells with immuno-stimulatory function. *J Hematol Oncol* *J Hematol Oncol*. 2008; 1(1):9.10.1186/1756-8722-1-9 [PubMed: 18644156]
48. Kurimoto A, Tanabe T, Tachibana A, Yamauchi K. Keratin sponge: immobilization of lysozyme. *J Biosci Bioeng*. 2003; 96(3):307–309. [PubMed: 16233527]
49. Wray LS, Hu X, Gallego J, Georgakoudi I, Omenetto FG, Schmidt D, Kaplan DL. Effect of processing on silk-based biomaterials: reproducibility and biocompatibility. *J Biomed Mater Res B Appl Biomater*. 2011; 99(1):89–101.10.1002/jbm.b.31875 [PubMed: 21695778]
50. Jenney CR, Anderson JM. Adsorbed serum proteins responsible for surface dependent human macrophage behavior. *J Biomed Mater Res*. 2000; 49(4):435–447. [PubMed: 10602077]
51. Anderson JM, Rodriguez A, Chang DT. Foreign body reaction to biomaterials. *Semin Immunol*. 2008; 20(2):86–100.10.1016/j.smim.2007.11.004 [PubMed: 18162407]
52. Babensee JE, Anderson JM, McIntire LV, Mikos AG. Host response to tissue engineered devices. *Adv Drug Deliv Rev*. 1998; 33(1–2):111–139.10.1016/S0169-409X(98)00023-4 [PubMed: 10837656]
53. Koh TJ, DiPietro LA. Inflammation and wound healing: the role of the macrophage. *Expert Rev Mol Med*. 2011; 13:e23.10.1017/S1462399411001943 [PubMed: 21740602]
54. Brancato SK, Albina JE. Wound Macrophages as Key Regulators of Repair. *Am J Pathol*. 2011; 178(1):19–25.10.1016/j.ajpath.2010.08.003 [PubMed: 21224038]
55. Takemura R, Werb Z. Secretory products of macrophages and their physiological functions. *Am J Physiol*. 1984; 246(1 Pt 1):C1–C9. [PubMed: 6364825]

56. Forbes SJ, Rosenthal N. Preparing the ground for tissue regeneration: from mechanism to therapy. *Nat Med*. 2014; 20(8):857–869.10.1038/nm.3653 [PubMed: 25100531]
57. Spiller KL, Anfang RR, Spiller KJ, Ng J, Nakazawa KR, Daulton JW, Vunjak-Novakovic G. The role of macrophage phenotype in vascularization of tissue engineering scaffolds. *Biomaterials*. 2014; 35(15):4477–4488.10.1016/j.biomaterials.2014.02.012 [PubMed: 24589361]
58. Heinemann L, Krinelke L. Insulin infusion set: the Achilles heel of continuous subcutaneous insulin infusion. *J Diabetes Sci Technol*. 2012; 6(4):954–964. [PubMed: 22920824]
59. Patte C, Pleus S, Wiegel C, Schiltges G, Jendrike N, Haug C, Freckmann G. Effect of Infusion Rate and Indwelling Time on Tissue Resistance Pressure in Small-Volume Subcutaneous Infusion like in Continuous Subcutaneous Insulin Infusion. *Diabetes Technol Ther*. 2013; 15(4):289–294.10.1089/dia.2012.0319 [PubMed: 23427865]
60. Yutani C, Ishibashi-Ueda H, Suzuki T, Kojima A. Histologic evidence of foreign body granulation tissue and de novo lesions in patients with coronary stent restenosis. *Cardiology*. 1999; 92(3):171–177. 6967. [PubMed: 10754347]
61. Wang X, Hu X, Daley A, Rabotyagova O, Cebe P, Kaplan DL. Nanolayer biomaterial coatings of silk fibroin for controlled release. *J Control Release Off J Control Release Soc*. 2007; 121(3):190–199.10.1016/j.jconrel.2007.06.006
62. Liang M, Yao J, Chen X, Huang L, Shao Z. Silk fibroin immobilization on poly(ethylene terephthalate) films: comparison of two surface modification methods and their effect on mesenchymal stem cells culture. *Mater Sci Eng C Mater Biol Appl*. 2013; 33(3):1409–1416.10.1016/j.msec.2012.12.044 [PubMed: 23827589]
63. Elia R, Michelson CD, Perera AL, Harsono M, Leisk GG, Kugel G, Kaplan DL. Silk electrogel coatings for titanium dental implants. *J Biomater Appl*. 2015; 29(9):1247–1255.10.1177/0885328214561536 [PubMed: 25425563]
64. Gao Y, Wang Q, Cui X, Liu Y, Zheng T, Chen C, Sun C, Huang S, Wang X, Liu Y, et al. Controlled release of stromal cell-derived factor-1α from silk fibroin-coated coils accelerates intra-aneurysmal organization and occlusion of neck remnant by recruiting endothelial progenitor cells. *Int J Clin Exp Pathol*. 2014; 7(12):8366–8380. [PubMed: 25674201]
65. Panousis CG, Zuckerman SH. Regulation of cholesterol distribution in macrophage-derived foam cells by interferon-gamma. *J Lipid Res*. 2000; 41(1):75–83. [PubMed: 10627504]
66. King VL, Cassis LA, Daugherty A. Interleukin-4 Does Not Influence Development of Hypercholesterolemia or Angiotensin II-Induced Atherosclerotic Lesions in Mice. *Am J Pathol*. 2007; 171(6):2040–2047.10.2353/ajpath.2007.060857 [PubMed: 18055554]
67. Huber SA, Sakkinen P, David C, Newell MK, Tracy RP. T helper-cell phenotype regulates atherosclerosis in mice under conditions of mild hypercholesterolemia. *Circulation*. 2001; 103(21):2610–2616. [PubMed: 11382732]
68. Liu J, Zhang N, Li Q, Zhang W, Ke F, Leng Q, Wang H, Chen J, Wang H. Tumor-associated macrophages recruit CCR6+ regulatory T cells and promote the development of colorectal cancer via enhancing CCL20 production in mice. *PloS One*. 2011; 6(4):e19495.10.1371/journal.pone.0019495 [PubMed: 21559338]
69. Liu H, Wu X, Wang S, Deng W, Zan L, Yu S. In vitro repolarized tumor macrophages inhibit gastric tumor growth. *Oncol Res*. 2013; 20(7):275–280. [PubMed: 23879167]
70. Sun X, Gao D, Gao L, Zhang C, Yu X, Jia B, Wang F, Liu Z. Molecular imaging of tumor-infiltrating macrophages in a preclinical mouse model of breast cancer. *Theranostics*. 2015; 5(6):597–608.10.7150/thno.11546 [PubMed: 25825599]
71. Chanput W, Mes JJ, Savelkoul HFJ, Wichers HJ. Characterization of polarized THP-1 macrophages and polarizing ability of LPS and food compounds. *Food Funct*. 2013; 4(2):266–276.10.1039/C2FO30156C [PubMed: 23135314]
72. Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. The identification of markers of macrophage differentiation in PMA-stimulated THP-1 cells and monocyte-derived macrophages. *PloS One*. 2010; 5(1):e8668.10.1371/journal.pone.0008668 [PubMed: 20084270]
73. Schildberger A, Rossmannith E, Eichhorn T, Strassl K, Weber V, Schildberger A, Rossmannith E, Eichhorn T, Strassl K, Weber V. Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns following Stimulation with

Lipopolysaccharide, Monocytes, Peripheral Blood Mononuclear Cells, and THP-1 Cells Exhibit Different Cytokine Expression Patterns following Stimulation with Lipopolysaccharide. *Mediat Inflamm Mediat Inflamm*. 2013; 2013:e697972.10.1155/2013/697972

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Figure 1.

Characterization of silk film β -sheet content, solubility, and cytokine release profile. (A) Plots of the Fourier Self-deconvoluted FT-IR spectra; a', b'... g' = 0, 45, 60, 80, 100, 120 min, and 24 hr WA times, h' = 30 min MeOH treatment. Dotted lines represent the wavenumbers (cm⁻¹) for β -sheet absorption by FT-IR. (B) Quantification of β -sheet content within the Amide I region. (C) Solubility of silk films as measured by UV absorbance at 280 nm. (D) Release of IFN- γ and IL-4, as measured by ELISA after WA for 70 min and 80 min. Lines through data are the best-fit curves using a two-phase association regression. The R^2 values for IFN- γ films were 0.9859 and 0.6568 for 70 or 80 min WA, respectively. The R^2 values for IL-4 films were 0.2787 and 0.4328 for 70 and 80 min WA, respectively. Two way ANOVA showed high significance ($p < 0.0001$) between 70 min WA and 80 min WA for both IFN- γ and IL-4 films. Significant differences at each time point are represented as * = 0.05, ** = 0.001, *** = 0.0001 and determined by Bonferroni posttests. All experiments were done with $n \geq 3$ independently formed films.

Figure 2.

Macrophage polarization with silk films. (A) Morphology of THP-1 cells after PMA (162 nM) treatment for 16 hours followed by submersion of silk films containing cytokines (Control, IFN- γ , IL-4) in medium for another 48 hours. Scale bars represent 50 μ m. (B) Expression of CD68, a general macrophage marker, after PMA treatment as compared to THP-1 monocytes as control. (C) Expression of CCR7 (M1 marker) and CD206 (M2 marker) after treatment with IFN- γ or IL-4 loaded silk films. Student's t-test, * = $P < 0.05$ vs Control, $n=3$ triplicate wells with 3 independently formed silk films.

Figure 3.

Macrophage plasticity. (A) Macrophage were initially differentiated with 20 ng/mL IFN- γ for 48 hrs then medium was switched to films containing 200ng IL-4. Expression of CCR7 and CD206 was quantified by qPCR at 48 hrs after initial polarization and at 96 hrs after switching to films. (B) The inverse of (A) was also performed starting with 20 ng/mL IL-4 for 48 hours and switching to IFN- γ loaded films for an additional 48 hours. CCR7 and CD206 expression was quantified by qPCR. Error bars = s.d.; Student's t-test, * = $P < 0.01$ vs control, # = <0.05 vs 96 hr IFN- γ or IL-4 expression; $n=3$ triplicate wells with 3 independently formed films.

Figure 4.

Degradation of silk films by macrophage attachment. (A) Cumulative release of IFN- γ and IL-4 from silk films that were water annealed at 50°C for 2.5 hrs. Lines through data are the best-fit curves using a two-phase association regression. (B) Quantification of β -sheet content of films water annealed at 50°C for 2.5 hrs. (C) THP-1 cells were seeded on tissue culture plastic and were treated with PMA overnight. Films loaded with IFN- γ or IL-4 and WA for 2.5 hrs at 50°C were then submerged into the medium for 48 hours (indirect release). Alternatively, THP-1 cells were seeded directly onto films containing cytokines that had been WA for 2.5 hrs at 50°C (direct contact). CCR7 and CD206 expression was quantified by qPCR. (D) Macrophage degradation of silk films was imaged by SEM after removal of cells with trypsin. Scale bar = 10 μ m. Error bars in B–C = s.d., n=3 triplicate wells with 3 independently formed films; student's t-test, * = $P < 0.05$ vs Control.

Figure 5.

Covalent attachment of IFN- γ to silk films. (A) Step 1: conjugation of 4-(2-aminoethyl)-aniline to free tyrosine residues in the silk solution to generate functionalized silk with free amines (Silk-NH₂). Step 2: addition of sulfo-LC-SPDP to the free amines on the silk-NH₂ (Silk-LC-SPDP). (B) Reaction of IFN- γ with Traut's reagent adds sulfhydryl groups to IFN- γ (SH-IFN- γ). (C) Combining Silk-LC-SPDP with SH-IFN- γ allows disulfide bond formation and covalent attachment of IFN- γ to the silk films (Silk-SS-IFN). (D) The UV/Vis absorbance spectrum of silk and silk-NH₂ from 200–550 nm and normalized to the peak at 224 nm. Arrow points to aniline peak absorbance at 330 nm in Silk-NH₂. (E) Table showing the change in absorbance (ΔA) measured at 343 nm after treatment of Silk-LC-SPDP films with either DTT or SH-IFN- γ , along with the calculated moles of pyridine-2-thione (P2T) liberated after the reaction. The molecular weight (M.W.) of sulfo-LC-SPDP and IFN- γ used to calculate amount and percentage of total SPDP available for reaction and the amount IFN- γ that was reacted.

Figure 6. IFN- γ release and macrophage polarization from covalently functionalized films. (A) Release of IFN- γ from Silk-SS-IFN in PBS as measured by ELISA. A two phase associative decay $R^2 = 0.936$ was calculated based on the raw values rather than cumulative values, as shown in the insert. Error bars are SEM from three independently created silk films. (B) Expression of CCR7 and CD206, measured by qPCR. Error bars are s.d. Student's t-test, * = $p < 0.05$ vs Control, $n=3$ triplicate wells with 3 independently formed films.