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Stabilized Interleukin-4-Loaded Poly(lactic-co-glycolic) Acid Films Shift Proinflammatory Macrophages toward a Regenerative Phenotype *in Vitro*

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Abstract

Macrophages are immune cells involved in wound healing and tissue regeneration; however, the sustained presence of proinflammatory macrophages in wound sites impairs healing. In this study, we shifted peritoneal macrophage polarization away from a proinflammatory (M1) phenotype through exposure to stabilized interleukin-4 (IL-4) in poly(lactic-co-glycolic acid) films in combination with topographical guidance from electrospun poly-L-lactic acid fibers. To our knowledge, this was the first study to stabilize IL-4 with bovine serum albumin (BSA) within a biomaterial. When IL-4 was coloaded with BSA for stabilization, we saw increased IL-4 bioactivity compared to no added stabilization, trehalose stabilization, or murine serum albumin stabilization. We observed increased elongation of peritoneal macrophages, increased RNA expression of anti-inflammatory marker arginase-1, increased ratio of interleukin-10/interleukin-12 p40 RNA, and decreased protein expression of proinflammatory markers (interleukin-12 p40

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Author Contributions

A.M.Z. wrote the manuscript with contributions from A.R.D., T.M.M., and D.L.P. A.M.Z. conducted macrophage experiments; A.R.D. worked on material fabrication and assessed drug released, and T.M.M. conducted imaging and morphology assessments. D.L.P. fabricated scaffolds. A.M.Z., A.R.D., T.M.M., D.L.P., A.N.K., R.A.K., M.R.L., and R.J.G. discussed experimental work and have reviewed and have given approval to the final version of the manuscript.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI:10.1021/acsabm.8b00769.

Full materials and equipment list, coverslip imaging map, polarization control data, IL-4 immobilization test, and detailed statistical analyses (PDF)

Notes

The authors declare no competing financial interest.

and RANTES) compared to controls. Taken together, these results suggest the macrophages were less proinflammatory and were a more pro-resolving phenotype. When stabilized with BSA, IL-4-loaded films effectively shift macrophage polarization state and are thus promising scaffolds to reduce inflammation within *in vivo* injury models.

Graphical Abstract:

Keywords

macrophage; inflammation; interleukin-4; poly(lactic-co-glycolic acid); cytokine stabilization

INTRODUCTION

Macrophages are phagocytic cells that perform maintenance functions such as clearing senescent erythrocytes and removing cellular debris during tissue remodeling. These cells also act as immune effector cells and are responsive to signals produced in an injury environment.¹ On the basis of environmental cues, macrophages exhibit a spectrum of phenotypes; these range from a classically activated, proinflammatory M1 phenotype with antimicrobial and phagocytic activity to alternately activated, M2 phenotypes that contribute to wound healing, cell proliferation, growth factor production, angiogenesis, and extracellular matrix synthesis.^{1–6} At early time points after injury, the population of macrophages is overwhelmingly proinflammatory.^{2,6} This population shifts, or is partly replaced by, M2 macrophages during the normal course of wound healing. A persisting M1 population delays tissue regeneration and wound healing. For example, Nahrendorf et al. suggest that increased levels of Ly-6C^{hi} (proinflammatory) macrophages following myocardial infarction may compromise myocardial healing.⁷ Similarly, in spinal cord injury, the population of M1 macrophages dramatically outweighs M2 macrophages 2–4 weeks postinjury; this proinflammatory environment results in neuronal death and reduced neurite outgrowth, severely inhibiting nerve regeneration.^{3,8} Proinflammatory macrophages also contribute to chronic skin wounds;⁹ in a model of chronic venous leg ulcers, Sindrilaru et al. found that iron-induced M1-polarized macrophages produced toxic levels of reactive oxygen species that damaged fibroblast DNA, ultimately impairing wound healing.¹⁰

Despite the detrimental effects of persistent M1 macrophages, global depletion of macrophages impairs regeneration of various tissue types.^{11–16} Inflammation precedes, and indeed is required for, the shift toward resolution.¹⁷ Thus, the strategy of skewing macrophage polarization instead of depleting macrophages may enhance wound healing and

tissue regeneration in chronic inflammatory conditions. Common pharmacological strategies to dampen inflammation include steroids, nonsteroidal anti-inflammatory drugs, and antioxidants, but these often demonstrate mixed efficacy and have nonspecific activity resulting in detrimental side effects.^{18–20} A more promising strategy is to deliver cytokines to shift macrophage polarization toward a desired direction. Anti-inflammatory cytokines such as interleukin-4 (IL-4) have been used to shift the M1:M2 ratio to favor the reparative M2 phenotype, particularly in cases of nervous system injury and disease. In a murine T11 contusion model, IL-4 was injected intraspinally 48 h postinjury and shifted macrophages closer to an M2 phenotype; this preserved neurons and myelin and improved locomotor function.²¹ Similarly, intracerebroventricular injections of IL-4 following middle cerebral artery occlusion (cerebral ischemia) resulted in improved functional outcomes *in vivo*.²²

Some of the main challenges of working with cytokines is their transient nature (serum half-life of approximately 19 min²³) and their helical hydrophobic regions that promote aggregation, which can result in increased immunogenicity.²⁴ Studies have used a variety of agents to stabilize the tertiary structure of proteins and cytokines, including the disaccharide trehalose (Tre),^{25,26} bovine serum albumin (BSA),²⁶ the phosphoprotein casein,²⁶ and human serum albumin.²⁷ Others have demonstrated success in stabilizing and delivering IL-4 using various biomaterial strategies, primarily as coatings for implants. In an attempt to shift the local population to a less proinflammatory state, studies have incorporated IL-4 into silk films,²⁸ star polyethylene glycol hydrogels,²⁹ decellularized bone scaffolds via biotinylation,³⁰ gelatin-heparin microspheres,³¹ coatings on titania nanotubes,^{32,33} gelatin hydrogels coated onto titanium surfaces,³⁴ and chitosan/dermatan sulfate-coated polypropylene meshes.³⁵ In this study, we aim to combine the benefits of molecule stabilization and biomaterial delivery. For the first time presented in literature, our approach involves the codelivery of IL-4 with either stabilizing protein or disaccharide within a biomaterial film to maintain IL-4 bioactivity to ultimately shift macrophage polarization *in vitro*.

To prevent the detrimental effects of a persistent M1 macrophage population at an injury site, we aimed to shift peritoneal macrophage (PMAC) polarization from an M1-polarized state to a less proinflammatory phenotype. IL-4, along with a stabilizing agent, was incorporated into poly(lactic-co-glycolic) acid (PLGA) films with electrospun poly-L-lactic acid (PLLA) fibers on top. PLGA is a biocompatible, FDA-approved polymer that can be used to provide a localized IL-4 delivery with a tunable release rate.³⁶ PLGA has been used in nano- and microparticle form to deliver cytokines and other therapeutics.^{37,38} In addition to the film itself, IL-4 was further stabilized using Tre, BSA, and murine serum albumin (MSA). Electrospun fibers were added to the IL-4 delivery systems to provide scaffolding that is similar to the extracellular matrix³⁹ but more importantly to promote elongated macrophage morphology, which is associated with an M2 phenotypic shift.⁴⁰ The stabilized IL-4, along with the aligned electrospun fibers, should have a combined anti-inflammatory effect on M1 macrophages. The bioactivity of IL-4 was determined *in vitro* by studying PMAC gene and protein expression in addition to cell morphology. IL-4 release from the film was assessed using a sandwich enzyme-linked immunosorbent assay (ELISA). We hypothesized that the stabilization of IL-4 via disaccharide or protein within the polymer film would increase IL-4 bioactivity and, when combined with electrospun fibers, would

shift the macrophages to a less proinflammatory state. Development and assessment of this combinatorial biomaterial *in vitro* will aid in future optimization of this scaffold for *in vivo* implantation into various injury models.

MATERIALS AND METHODS

All material and equipment information are listed in the Supporting Information (Table S1 and S2).

Film Casting.

Poly(lactic-*co*-glycolic) acid [PLGA; L:G 50:50; 10% (w/w) in 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP)] was mixed on a stir plate with a magnetic stir bar for 3 h. For initial experiments (corresponding to Figure 2), IL-4 (1 μ g in 10 μ L water) was added directly to the polymer solution (200 μ L). Next, 50 μ L of the IL-4-PLGA solution were drop cast onto each 15 $\times$ 15 mm coverslip with a final mass of 238 ng IL-4/film. Coverslips were dried overnight using the conventional vacuum line attached to the chemical fume hood prior to electrospinning fibers on top. Films without IL-4 were used as controls.

Remaining experiments (corresponding to Figures 3–5 and Table 3) include additional steps to stabilize IL-4. Again, PLGA [10% (w/w) in HFIP] was mixed on a stir plate with a magnetic stir bar for 3 h. IL-4 (1 μ g in 10 μ L water) or water was diluted 1:1 with either trehalose (TRE; 200 mM stock in water), bovine serum albumin (BSA; 2% stock in water), murine serum albumin (MSA; 2% stock in water), or water (control) and incubated for 1 h on ice on a shake plate (Table 1). Tre and BSA were used as they have previously demonstrated the ability to stabilize cytokines and other proteins.^{25,26} MSA was studied to prevent potential immunogenic effects possible with BSA as the macrophages were extracted from mice. Concentrations were based off of the minimum values used by Farzamfar et al. then increased to enhance stabilization.²⁶

Once the polymer solution was homogeneous, the IL-4 solutions were combined with the polymer solutions by trituration (1 μ g IL-4/220 μ L total polymer solution). To fabricate films, 50 μ L of film solution were dispensed onto each 15 $\times$ 15 mm glass coverslip at a final mass of 228 ng IL-4/film. H₂O-only films and Tre-, BSA-, or MSA-only films were used as controls. Films were then placed in a stronger vacuum (~50 mTorr) overnight to remove solvent. IL-4-loaded films were fabricated fresh before each cell experiment (n = 4–6 independently fabricated batches).

Electrospinning.

The electrospinning setup and protocol used in this study have been previously described in the literature.⁴¹ In brief, films on 15 $\times$ 15 mm glass coverslips were secured to the electrospinning collection wheel (22 cm diameter) with double-sided tape. A solution consisting of 12% (w/w) PLLA in CHCl₃ was then electrospun onto the films using the following electrospinning parameters: an applied voltage of 15 kV, needle tip-to-wheel collection distance of 5 cm, ambient relative humidity of 21%, wheel rotational speed of 1500 rpm, and a collection time of 7 min. After electrospinning, film/fiber scaffolds were

sterilized via ethylene oxide gas exposure for 12 h and degassed in a tissue culture cabinet for 48 h at room temp.

Scanning Electron Microscopy and Fiber Morphological Analysis.

Films with electrospun fibers were imaged via scanning electron microscopy (SEM) to assess fiber collection, diameter, and alignment on the scaffolds. Prior to SEM, scaffolds were sputter coated with an approximately 0.5 nm layer of platinum using a Technics Hummer V Sputter Coater. Scaffolds were then imaged using an FEI Versa 3D Dual Beam SEM with an accelerating voltage of 2 kV, working distance of ~10 mm, and spot size of 5.0. Five images were taken of each scaffold to ensure a representative sample of fibers were available for analysis. Fiber images were analyzed using FIJI software. For fiber alignment, lines were drawn parallel to the fibers, and the angles were measured. Alignment data are reported as a histogram of the angle of deviation from the median angle. Fiber diameter was measured by drawing a line perpendicular to the fiber orientation and measuring the length. Fiber surface coverage was determined by counting the number of fibers in a given field of view, multiplying by the average fiber diameter and the field of view length, and dividing by the total field of view area. For all analyses, at least five fields of view for at least three independent batches of fibers ($n = 3$) were analyzed.

Peritoneal Macrophage Isolation and Culture.

Jackson Laboratory C57BL/6 mice were bred in house at Albany Medical Center, Albany, NY. All animal care and procedures were approved by the Albany Medical Center Institutional Animal Care and Use Committee (IACUC). To elicit macrophage recruitment to the peritoneum, mice were injected i.p. with sterile thioglycollate (3% in water, autoclaved then oxidized). Seventy-two hours later, sterile phosphate buffered saline (PBS, 10 mL) was injected to the peritoneum using a 23-gauge needle, and the peritoneum was gently massaged to release the macrophages. After the cell suspension was removed, contaminating red blood cells were lysed using ACK lysis buffer (150 mM NH_4Cl , 10 mM KHCO_3 , and 0.1 mM Na_2EDTA with a pH of 7.2–7.4). The cell suspension was centrifuged (1700 rcf, 6 min) and resuspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with fetal bovine serum (FBS; 10% v/v) and gentamicin (50 $\mu\text{g}/\text{mL}$). The cell suspension was plated onto untreated Petri dishes (15 cm diameter) for 4 h to enrich for peritoneal macrophages (PMACs) by selective adhesion. Adherent PMACs were washed twice with PBS to remove unattached cells. The PMACs were released with 1.5 mM ethylenediaminetetraacetic acid (EDTA) in PBS (12 min, 37 °C). The majority of the population was identified as macrophages ($\text{CD11b}^+\text{Ly6C}^+\text{Ly6G}^-$) by flow cytometry. The cell suspension was centrifuged (1700 rcf, 6 min), resuspended in DMEM containing FBS and gentamicin, and plated onto untreated 15 cm diameter Petri dishes (24 h, 37 °C). One dish was M1-polarized using interferon- γ (IFN- γ ; 100 ng/mL), while a control dish was left untreated (M0) to confirm the polarization of M1 PMACs. After 24 h, PMACs were lifted with 1.5 mM EDTA/PBS as above and resuspended in DMEM containing FBS and gentamicin and applied to film-fiber scaffolds.

Peritoneal Macrophage Plating.

For quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and cytokine bead array experiments, polydimethylsiloxane (PDMS) molds were placed around the edges of fiber-film scaffolds to ensure complete interaction of PMACs with the scaffold. M1-polarized PMACs were seeded at a density of 5×10^5 cells/coverslip within the PDMS mold for 24 h, as significant changes in RNA expression for these polarization genes are observable after 24 h.⁴² This high seeding density was used to ensure detectable levels of RNA and protein were expressed. For morphological analysis, PMACs were seeded onto the 15×15 mm scaffolds at a density of 15×10^3 cells/coverslip for 24 h to ensure individual cells were available for morphological analysis.

qPCR Polarization Assay.

qPCR was used to assess the polarization state of PMACs in response to the scaffolds through RNA expression of M1 and M2 markers. PMACs were lysed with Trizol 24 h after seeding onto the scaffolds. Pursuant to the manufacturer's instructions, RNA was extracted. Using the qScript cDNA SuperMix, RNA was reverse transcribed to make cDNA. The PCR was run with the primer sets listed in Table 2; the primers were designed using BLAST analyses. The cDNA was amplified using PerfeCTa SYBR Green FastMix ROX and an Applied Biosystems 7300 Real Time PCR System. The relative RNA expression was calculated using the $\Delta\Delta C_t$ method with data normalized to β -actin and the M1-polarized PMACs on control scaffolds (Table 1). For each condition, one coverslip from each batch of cells (derived from $n = 4$ –6 animals and plated on $n = 4$ –6 separate scaffold batches) was used to assess gene expression.

Cytokine Multiplex Array Polarization Assay.

The cytokine multiplex array was also used to assess the polarization state of PMACs by quantifying the protein expression of M1 and M2 cytokines secreted by the PMACs. Before PMACs were lysed for PCR, the cell culture supernatants were collected and frozen at -80°C . A Bio-Plex Pro Magnetic Bead-Based Multiplex Assay was used to study protein expression of the following cytokines: interleukin-1 β (IL-1 β), interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-12 p40 (IL-12 p40), interleukin-12 p70 (IL-12 p70), C-X-C motif chemokine ligand 1 (KC), C-C motif chemokine ligand 2 (MCP-1), C-C motif chemokine ligand 5 (RANTES), and granulocyte macrophage colony-stimulating factor (GM-CSF). These are all common cytokines and chemokines that are secreted postinjury, affecting cells proximal to the injury site and propagating the inflammation response;^{43,44} all but IL-10 are associated with a proinflammatory state. Experiments were conducted per manufacturer's instructions. All data were normalized to the M1 controls. For each condition, one coverslip from each batch of cells (derived from $n = 4$ –6 animals and plated on $n = 4$ –6 separate scaffold batches) was used to assess protein expression.

Immunocytochemistry.

Immunocytochemistry was used to characterize the morphology of PMACs on various scaffolds. The PMACs seeded at a lower density were fixed with paraformaldehyde (4% v/v) for 10 min after 24 h in culture. Cells were incubated in a BSA (5% w/v) blocking buffer

with Triton X-100 (0.4% v/v) for 1 h. PMACs were then treated with DAPI (4',6-diamidino-2-phenylindole, 1:1000) to visualize the nuclei and Alexa Fluor 488 Phalloidin (1:400) to visualize polymerized actin in PBS with BSA (5% w/v) and Tween 20 (0.1% v/v) for 1 h.

Fluorescence Microscopy.

Fluorescently stained PMACs were imaged using Metamorph Premier 7.7.3.0 imaging software and an Olympus IX-81 confocal microscope with a metal halide lamp (120 W). Cells labeled with DAPI and phalloidin were imaged using a 20× LUC Plan FLN objective and DAPI and fluorescein isothiocyanate (FITC) filter sets, respectively. Four fields of view per coverslip (~.168 mm²) were imaged (cells from $n = 4$ animals plated onto $n = 4$ separate scaffold batches). Randomly chosen fields of view in the center of each quadrant of the coverslip were used to represent the overall plated cell population (as shown in Figure S1). Every cell in each image was analyzed, excluding cells sharing a border with other cells, so individual cell morphology could be best characterized.

Morphology Analysis.

The morphology of the PMACs was determined using ImageJ 1.49v software. Briefly, the background was subtracted using the sliding paraboloid with a rolling ball radius = 50.0 pixels. Subsequently, the image threshold was established; the image was made binary and watershed to separate overlapping cells, and cell major and minor axes were measured. The lengths of the major and minor axes of the cell, measured in pixels, were used to calculate the aspect ratio of each cell. The aspect ratio of PMACs are represented as a plot of boxplots. Median aspect ratio was calculated for each condition as the aspect ratios of the cells exhibited a skewed distribution. A minimum of 150 cells from each treatment group were analyzed.

IL-4 Release Quantification.

Scaffolds were placed into 1 mL of PBS at 37 °C. The supernatant was collected and replaced with 1 mL of fresh PBS after 2, 8, 12, and 24 h and stored at -80 °C until all samples were collected. A Standard ABTS ELISA Development Kit (sensitivity of 20–2000 pg/mL) was used to assess levels of IL-4 release in the supernatants. Experiments were completed per the manufacturer's protocols. All data were normalized to PBS only controls. At least 3 separate batches of scaffolds were used to complete these experiments ($n = 3$ independently fabricated scaffolds with 2 technical replicates per condition).

Statistical Analysis.

Most data were reported as mean $\pm$ standard error of the mean with the exception of fiber alignment, which was reported as a histogram of fiber angles and PMAC morphology data which were represented by a plot of boxplots. All statistical analysis was done using Minitab 17. As PCR data sometimes varied by an order of magnitude, data were first logarithmically transformed. A linear regression model was used to analyze the data, and ANOVA was used to assess significance of the model. If a coefficient in the model had a p value of < 0.05 , the contribution of the term was deemed significant. Due to unequal variances, the cytokine

bead array data were analyzed using a Welch's ANOVA with post hoc Games–Howell Simultaneous Tests for Differences of Means. PMAC morphology data were not normally distributed, so Mood's Median Test was used to compare PMAC aspect ratio. For all tests, $p < 0.05$ was deemed significant ($n = 3$ –6 independently fabricated film-fiber scaffold batches and cells from 4 to 6 individual animals per condition for biological replicates). Please refer to the Supporting Information for more detailed statistical analysis information (Tables S3–S14).

RESULTS AND DISCUSSION

In this study, IL-4-loaded PLGA films with PLLA fibers electrospun on top of the films were fabricated to shift PMACs to a less proinflammatory state. We first used SEM to assess fiber physical characteristics on the surface of the film (Figure 1A). Fibers were highly aligned with the majority of fibers within 5° of the median fiber angle (Figure 1B); this aligned morphology is ideal to promote elongation of macrophages, which can shift macrophages to a more M2-like phenotype.⁴⁰ Fibers had an average diameter of $2.25 \pm 0.26 \mu\text{m}$ and average surface coverage of $53.5 \pm 7.5\%$, enabling PMAC interaction with both the PLLA fibers and the IL-4-containing PLGA film. The physical microscale characteristics of these film-fiber scaffolds were consistent between batches.

After an M1 (proinflammatory) PMAC phenotype was confirmed (Figure S2), M1-polarized PMACs were allowed to interact with control and IL-4-loaded scaffolds for 24 h, and the polarization state was assessed using qPCR. Canonical anti-inflammatory (Arg-1 and IL-10) and proinflammatory (iNOS and IL-12 p40) polarization state markers were assessed, and all data were normalized to the control scaffold conditions. Both soluble IL-4 ($T = 10.88$, $p < 0.001$) and IL-4-loaded PLGA film-fiber scaffolds ($T = 9.45$, $p < 0.001$) had increased Arg-1 expression compared to the PMACs on control scaffolds (Figure 2A). Exposure to soluble IL-4 did not alter iNOS gene expression compared to the M1 control ($T = -1.28$, $p = 0.236$) (Figure 2B). We hypothesized that we would also see no change or a decrease in iNOS gene expression when M1-polarized PMACs were exposed to IL-4-loaded film-fiber scaffolds. Surprisingly, M1-polarized macrophages on IL-4-loaded film-fiber scaffolds had significantly increased iNOS expression ($T = 2.50$, $p = 0.037$) (Figure 2B). Exposure to soluble IL-4 resulted in an approximately 20-fold increase in the ratio of IL-10/IL-12 p40 (anti-inflammatory/proinflammatory cytokine) compared to M1-polarized controls, suggesting a significant anti-inflammatory shift in polarization state ($T = 3.65$, $p = 0.007$) (Figure 2C). However, M1-polarized PMACs cultured directly on the IL-4-releasing scaffolds exhibited no change in the ratio of IL-10/IL-12 p40 gene expression compared to the M1-polarized controls ($T = -0.89$, $p = 0.399$) (Figure 2C).

In this experiment, we hypothesized that IL-4-loaded film-fiber scaffolds would shift M1-polarized PMACs to a less proinflammatory state. We rejected this hypothesis after observing increased iNOS gene expression and no change in the gene expression ratio of IL-10/IL-12 p40. In contrast, soluble IL-4 did not cause an increase in iNOS expression and resulted in a significant increase in IL-10/IL-12 p40. This suggests that the bioactivity of IL-4 was compromised following incorporation into film which may have increased IL-4 immunogenicity and lead to unexpected increases in proinflammatory marker expression

(Figure 2). The changes in immunogenicity of IL-4 based on the delivery mechanism suggest that IL-4 may be denatured or forming aggregates during film fabrication. Maas et al. demonstrated that heat denaturation of the protein ovalbumin increases the immune response of mice.⁴⁵ Our process of drop casting films using organic solvent, desiccation of films, and surface exposure of IL-4 may be having a similar denaturation effect. Postdenaturation, IL-4 may have aggregated within the HFIP solution. Both phenomena may cause immunogenic effects.²⁴ Thus, we aimed to further stabilize the IL-4-loaded into the films to increase the bioactivity and reduce the immunogenicity. To better preserve the bioactivity of IL-4, we modified the fabrication technique for remaining experiments. IL-4 was incubated with either Tre, BSA, MSA, or H₂O on ice on a shake plate for 1 h prior to incorporation into the PLGA/HFIP solution (Table 1). Once the films were drop cast, we used a stronger vacuum (~50 mTorr) to ensure HFIP removal.

After altering our fabrication procedures, qPCR was used again to assess polarization state of M1-polarized PMACS 24 h postplating on scaffolds with increased measures to stabilize IL-4. Arg-1 expression increased in PMACs cultured on control surfaces with soluble IL-4 ($T = 7.21$, $p < 0.001$), IL-4-only film-fiber scaffolds ($T = 5.44$, $p < 0.001$), IL-4/Tre film-fiber scaffolds ($T = 3.96$, $p < 0.001$), IL-4/BSA film-fiber scaffolds ($T = 8.08$, $p < 0.001$), and IL-4/MSA film-fiber scaffolds ($T = 7.39$, $p < 0.001$) (Figure 3A). When IL-4 was coloaded with BSA or MSA, Arg-1 expression increased to a level similar to soluble IL-4 exposure, suggesting improved bioactivity compared to films without added protein. There were no significant differences in iNOS expression compared to the M1 control (Figure 3B). Lastly, the ratio of IL-10/IL-12 p40 expression was assessed, and only the IL-4/BSA films resulted in a cytokine ratio that was significantly greater than the M1 control ($T = 2.65$, $p = 0.012$) (Figure 3C). Together, these results suggest that film-fiber scaffolds loaded with BSA-stabilized IL-4 demonstrate the greatest efficacy in shifting macrophage polarization away from a proinflammatory phenotype.

Trends in RNA expression were supported by cytokine protein expression. The supernatant from the cells cultured on the surfaces for 24 h was collected to assess cytokine release. IL-12 p40 (the subunit involved in mediating T cell responses⁴⁶) protein expression was significantly lower when M1-polarized PMACs were cultured on control surfaces with soluble IL-4 ($T = -5.67$ and $p = 0.026$) and IL-4/BSA surfaces ($T = -6.00$ and $p = 0.020$) compared to the M1 controls (Figure 4A). Protein expression of RANTES, a chemokine involved in the recruitment of T, natural killer, and other immune cells,^{47,48} also exhibited the same trends for PMACs with a soluble IL-4 treatment ($T = -5.95$ and $p = 0.021$) and PMACs cultured on IL-4/BSA scaffolds ($T = -4.76$ and $p = 0.052$) though not statistically significant (Figure 4C). As both IL-12 p40 and RANTES are involved with the recruitment and activation macrophages, eosinophils, and T cells, downregulation of these specific cytokines may be beneficial in dampening chronic inflammation by reducing the population of inflammatory cells, particularly activated cells, at a wound. Wound healing is often hindered by lingering populations of M1-polarized macrophages weeks postinjury. Thus, controlling this population of cells may facilitate wound healing by making the local environment less proinflammatory.

Additional chemokines and cytokines were also surveyed. The protein levels of IL-10, IL-12 p70, and GM-CSF were generally below the detection limits of the instrument (data not shown). There were no significant changes in the levels of IL-6, MCP-1, and KC (Figure 4B, 4D, and 4E, respectively). Data also show that protein levels of IL-12 p40, IL-6, RANTES, and KC trend higher on BSA-only films compared to IL-4/BSA films (Figures 4A–C and E). This suggests that BSA causes an immunogenic effect due to a difference in species origin;⁴⁹ however, IL-4 exposure masks this response. Despite the potential immunogenic effect of BSA alone, our results support that when IL-4 is combined with BSA, the bioactivity of IL-4 increases, resulting in downregulation of some proinflammatory cytokines and chemokines.

As previous studies have demonstrated that M2 macrophages have an elongated morphology while M1-polarized cells are more circular,⁴⁰ we assessed PMAC morphology by quantifying the aspect ratio (length/width) of each cell. The sample images below show cells on each scaffold type (Figure 5). The summary graph displays boxplots of the aspect ratios of PMACs on the different scaffolds. PMACs on control scaffolds with soluble IL-4 (X^2 [1, N = 418] = 7.60, p = 0.006), IL-4-releasing scaffolds (X^2 [1, N = 503] = 6.47, p = 0.011), IL-4/Tre scaffolds (X^2 [1, N = 453] = 19.71, p < 0.001), and IL-4/BSA scaffolds (X^2 [1, N = 478] = 11.64, p = 0.001) all demonstrated significantly greater elongation compared to M1-polarized macrophages on control scaffolds (Figure 5J). This further supports that there was a phenotypic shift away from a proinflammatory state.

Each coverslip was surveyed by taking one image in the center of each quadrant, as shown in Figure S1. These populations were less dense than at the center of the coverslip and enabled the morphology analysis of individual cells to avoid quantifying aspect ratios that may be altered through cell–cell interactions. However, it was observed that fields in the center with overlapping cells exhibited greater aspect ratios. Thus, it is possible that the reported aspect ratios are an underestimate for each population of cells.

Our *in vitro* tests suggest that IL-4 stabilized using BSA remained bioactive and shifted macrophage polarization with a 24 h exposure. Thus, we aimed to characterize the IL-4 release kinetics from unstabilized and stabilized films using an ELISA over the course of 24 h. We anticipated an initial burst release during the first 24 h because drug release from PLGA films begins with a diffusion phase for hydrophobic and hydrophilic small molecules.⁵⁰ However, levels of IL-4 released were 10⁴-fold lower than what was loaded, and the amount of released IL-4 varied greatly between films. IL-4/Tre films and IL-4/MSA films had undetectable levels of IL-4, while IL-4-only and IL-4/BSA films released low pM levels of IL-4 (Table 3). To ensure the limited IL-4 detection was not a result of the assay, we also used matrix-assisted laser desorption ionization (MALDI) and a cytometric bead array to confirm whether IL-4 was eluted into solution; again, IL-4 was undetectable (data not shown).

Cytokine therapeutics are generally challenging to deliver due to their hydrophobic helical regions.²⁴ In this study, IL-4 released into the aqueous media may have aggregated, preventing ELISA antibody detection of IL-4 after release from the scaffold. The low levels of detected IL-4 but significant shifts in PMAC polarization suggest that at least some of the IL-4 is bioactive and may be immobilized in the film. Reeves et al. demonstrated that

exposure to IFN- γ or IL-4 released into media was ineffective at increasing the expression of inflammation markers. However, when these cytokines were immobilized within silk films, there was a significant increase in the expression of M1 marker, CCR7, and M2 marker, CD206, respectively.²⁸ This agrees with our study where despite the observation of minimal IL-4 release, PMACs shifted away from an M1 phenotype. Potential immobilization of IL-4 at the PLGA film surface would provide direct contact between IL-4 and the seeded cells, producing an anti-inflammatory shift. We attempted to confirm immobilization of IL-4 within the films using fluoroldehyde labeling of IL-4, but results were inconclusive (Figure S3). Future studies would benefit from additional characterization of IL-4 localization and the effect of IL-4 incorporation on material properties. Differential scanning calorimetry (DSC) may elucidate whether the incorporation of IL-4 and other stabilization components results in changes in PLGA crystallinity and ultimately the stability of IL-4.

qPCR and cytokine multiplex assay results show that PMACs shift away from a proinflammatory phenotype when exposed to soluble IL-4 or cultured on IL-4/BSA films. While IL-4/BSA films demonstrated similar efficacy to soluble IL-4, there are additional benefits to biomaterial delivery of IL-4. Loading IL-4 in a film could enable local delivery of the cytokine to an inflamed injury region. By delivering a drug locally, it can be administered in lower, safer amounts and avoids risks of interactions with healthy cells elsewhere in the body that often occur with systemic drug delivery. Through manipulation of material properties, the release of cytokine from scaffolds can be tuned for short-term or long-term delivery.⁵¹ These advantages support the use of these combined BSA/PLGA films to stabilize cytokines for drug delivery. While BSA is commonly used to stabilize proteins for research use, to our knowledge, BSA has not been used for stabilization of IL-4 during delivery and provides a promising strategy to maintain bioactivity.

BSA has a history of efficacy in protein stabilization, and that may play a role in maintaining IL-4 bioactivity through the scaffold fabrication process. Chang et al. demonstrated that BSA stabilizes beta-galactosidase by binding with hydrophobic regions to prevent thermal inactivation at 64 °C.⁵² Similarly, BSA inhibited insulin and alcohol dehydrogenase aggregation in response to thermal stress at 55 °C or treatment with the reducing agent, DTT, by complexing with these proteins.⁵³ While MSA appeared to stabilize IL-4 to some extent, it was not as effective at maintaining IL-4 bioactivity as BSA. MSA is a member of a similar order to rabbit, *Rodentia/Lagomorpha*; a study by Majorek et al. showed that rabbit serum albumin has residues that differ from BSA and horse serum albumin and also a ligand binding site that does not appear to be conserved in the other serum albumins.⁵⁴ These differences in rabbit serum albumin may be conserved in MSA and may impair MSA's ability to bind to IL-4 and prevent aggregation.

Tre is also well-studied for its protein stabilization ability. Tre is a disaccharide produced by bacteria, fungi, plants, and lower animals under stress due to its ability to protect proteins and other molecules in cases of extreme temperature, desiccation, and oxidative stress. Tre may offer stabilization in the dried state by trapping the protein and preventing unfolding and replacing water to stabilize the native structure. In solution, Tre may stabilize the protein by compacting it with its large hydration radius.⁵⁵ A study by Hédoux et al. demonstrates

that Tre decreases the exposure of hydrophobic moieties of BSA while also stabilizing the hydrogen bond network with water around the BSA, further stabilizing BSA's conformation.²⁵ However, we observed that Tre did not maintain IL-4 bioactivity as well as BSA, ultimately preventing an anti-inflammatory shift of PMACs. As previous studies demonstrated that increased protein stability is seen with increased amounts of Tre (and likely fewer protein-protein interactions),⁵⁶ coloaded with greater amounts of Tre could increase IL-4 bioactivity. However, this may be insufficient as trehalose has a lower surface activity than BSA.

In this study, PLGA was chosen from preliminary tests to deliver IL-4 as it was hypothesized to provide a burst release of IL-4 to quickly target the acute proinflammatory response.⁵⁰ Despite the efficacy in shifting the macrophage response, it appears that little IL-4 was released from the films. Future studies can assess polymers that have different degradation rates, different types of drug release kinetics, and different protein stabilization properties to determine whether bioactivity of the cytokine is improved and whether the change in cellular response is enhanced. Additionally, as macrophages span a variety of phenotypes that are induced by various stimuli, future studies could examine the use of other M2-shifting cytokines such as IL-10. IL-4 shifts macrophages to a more wound healing, pro-proliferation phenotype, while IL-10 shifts macrophages to promote tissue repair and extracellular matrix synthesis.^{1,3} Depending on the type of tissue or stage of injury, different cytokine treatments may be more beneficial in the healing process. These anti-inflammatory scaffolds should be assessed in a variety of injury models to assess the anti-inflammatory efficacy.

CONCLUSIONS

We aimed to shift M1-polarized PMACs to a less proinflammatory state using an IL-4-releasing PLGA film coupled with electrospun fibers. We attempted to further stabilize IL-4 using various coloaded molecules; IL-4 coloaded with BSA was most effective at maintaining bioactivity and preventing an immunogenic response as demonstrated by an anti-inflammatory shift in PMAC RNA and protein expression. However, IL-4 released into PBS was at nearly undetectable levels. Taken together, our results suggest that the IL-4 may be immobilized on the PLGA surface and is able to interface with macrophages, thereby shifting their polarization state. Future studies should assess the efficacy of these combined scaffolds to reduce inflammation and enhance wound healing and tissue regeneration in various injury and disease models.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

Arg-1	arginase-1
BSA	bovine serum albumin
MCP-1	C–C motif chemokine ligand 2
RANTES	C–C motif chemokine ligand 5
KC	C–X–C motif chemokine ligand 1
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN- γ	interferon- γ
IL-1β	interleukin-1 β
IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10
IL-12 p40	interleukin-12 p40
IL-12 p70	interleukin-12 p70
MALDI	matrix-assisted laser desorption ionization
MSA	murine serum albumin
PMAC	peritoneal macrophage
PBS	phosphate buffered saline
PLGA	poly(lactic-co-glycolic acid)
PLLA	poly-L-lactic acid
PDMS	polydimethylsiloxane

qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
SEM	scanning electron microscopy
Tre	trehalose

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Figure 1.

Electrospun PLLA fibers are aligned on PLGA films. (A) SEM image of electrospun PLLA fibers on drop cast PLGA film, scale bar = 40 μm . (B) Histogram displaying fiber alignment as the percentage of PLLA fibers with a given angle of deviation from the median fiber angle ($n = 3$ batches of fibers with five fields of view per batch).

Figure 2.

IL-4 loaded PLGA films are less efficacious at shifting M1- polarized PMACs to a less proinflammatory state compared to soluble IL-4. qPCR was used to assess PMAC polarization state in response to soluble IL-4 and IL-4 released from PLGA films using the

Ct method. All data were normalized to β -actin and M1 controls (black). Summary graphs display RNA fold change of: (A) Arg-1, (B) iNOS, and (C) IL-10/IL-12 p40. Data are represented as a mean $\pm$ standard error of the mean ($n = 4-6$ animals per condition on $n = 4-6$ independently fabricated batches of fibers). Statistical differences compared to the M1 controls were determined by first logarithmically transforming the data then fitting all data with a linear regression model (* $p < 0.05$, ** $p < 0.001$).

Figure 3.

IL-4 stabilized with BSA is more effective at shifting M1-polarized PMACs to a less proinflammatory state compared to all other groups. qPCR was used to assess PMAC polarization state in response to IL-4 that was stabilized via various strategies using the

Ct method. All data were normalized to β -actin and M1 controls (black). Summary graphs display RNA fold change of: (A) Arg-1, (B) iNOS, and (C) IL-10/IL-12 p40. Data are represented as a mean $\pm$ standard error of the mean ($n = 4-6$ animals per condition on $n = 4-6$ independently fabricated batches of fibers). Statistical differences compared to the M1 controls were determined by first logarithmically transforming the data then fitting all data with a linear regression model (* $p < 0.05$, ** $p < 0.001$).

Figure 4.

Soluble IL-4 and IL-4 stabilized with BSA demonstrate similar efficacy in shifting macrophages to a less proinflammatory state compared to M1 controls. A cytokine multiplex assay was used to assess PMAC polarization state in response to IL-4 that was stabilized via various strategies. All data were normalized to the M1 controls (black). Summary graphs display protein fold change of: (A) IL-12 p40, (B) IL-6, (C) RANTES, (D) MCP-1, and (E) KC. Data are represented as a mean $\pm$ standard error of the mean ($n = 4-6$ animals per condition on $n = 4-6$ independently fabricated scaffolds). Statistical differences compared to the M1 controls were determined by Welch's ANOVA and post hoc Games-Howell Simultaneous Tests for Differences of Means ($*p < 0.05$).

Figure 5.

IL-4 causes elongation of PMACs. Sample images of M1-polarized PMACs on various scaffolds (scale bar = 100 μm). Macrophages are stained with phalloidin (green). (A) PMACs on a control scaffold, (B) PMACs on a control scaffold with soluble IL-4, (C) PMACs on an IL-4-loaded film, (D) PMACs on an IL-4/Tre-loaded film, (E) PMACs on an IL-4/BSA-loaded film, (F) PMACs on an IL-4/MSA-loaded film, (G) PMACs on a Tre-loaded film, (H) PMACs on a BSA-loaded film, and (I) PMACs on an MSA-loaded film. (J) Summary boxplots of PMAC aspect ratios on each scaffold type (coverslips from $n = 4$ animals with a minimum of 4 fields of view per animal and 150 or more PMACs per treatment group). Statistical differences of conditions compared to PMACs on control scaffolds were determined using Mood's Median Test (* $p < 0.05$, ** $p < 0.001$).

Table 1.Control and Enhanced IL-4 Stabilization Strategy Scaffolds Studied ^a

control film-fiber scaffolds	IL-4-releasing film-fiber scaffolds
H ₂ O	
H ₂ O + soluble IL-4	IL-4/H ₂ O (IL-4)
H ₂ O/Tre (Tre)	IL-4/Tre
H ₂ O/BSA (BSA)	IL-4/BSA
H ₂ O/MSA (MSA)	IL-4/MSA

^a All scaffolds are PLGA films with poly-L-lactic acid (PLLA) fibers on top. Experimental films also contain IL-4 with either H₂O, Tre, BSA, or MSA.

Table 2.

Primer Sets Used to Study Macrophage Polarization^a

gene	sense	antisense
<i>β</i> -actin (endogenous control)	TTCCAGCCTTTCCTTCTTGG	AGTAATCTCCTTCTGCAATCC
inducible nitric oxide synthase (iNOS; M1)	TCTATCAGGAAGAAATGCAGG	CACCAAGCTTCTTCAATGTGG
interleukin-12 p40 (IL-12 p40; M1)	AGCACTCCCCCATTCCTACTT	CACGCAGACATTCCCGCC
arginase-1 (Arg-1; M2)	GGAAAGCCAATGAAGAGCTG	GCTTCCAACTGCCAGACTGT
interleukin-10 (IL-10; M2)	TGTGAAAATAAGAGCAAGGCAGTG	GCCTTGTAGACACCTTGGT

^aThese are canonical markers used to distinguish M1 and M2 macrophages.

Table 3.Cumulative IL-4 Released (pg) into PBS over the Course of 24 h Was Assessed Using Sandwich ELISA^a

	2 h	8h	12 h	24 h
IL-4	0	5.45 ± 9.45	11.55 ± 20.00	20.03 ± 34.69
Tre	0	0	0	0
BSA	0	0	21.77 ± 37.71	21.77 ± 37.71
MSA	0	0	0	0

^a
^a *n* = 3 separate batches of film-fiber scaffolds per condition.