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Resveratrol Delivery from Porous Poly(lactide-co-glycolide) Scaffolds Promotes an Anti-Inflammatory Environment within Visceral Adipose Tissue

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Abstract

As biomaterial therapies emerge to address adipose tissue dysfunction that underlies metabolic disease, the immune response to these systems must be established. As a potential therapy, we are investigating resveratrol delivery from porous poly(lactide-co-glycolide) scaffolds designed to integrate with adipose tissue. Resveratrol was selected for its ability to protect mice and primates from high fat diet and broad anti-inflammatory properties. Herein, we report fabrication of scaffolds with high resveratrol loading that are stable and active for up to one year. *In vitro* release profiles indicate that drug release is biphasic with a burst release over 3 days followed by a plateau. Surprisingly, we find that PLG scaffold implanted into adipose tissue of mice promotes an anti-inflammatory environment characterized by high arginase-1 and low TNF- α and IL-6 compared to naïve unmanipulated fat. Resveratrol delivery from the scaffold augments this anti-inflammatory environment by decreasing monocyte and lymphocyte numbers at the implant site and increasing expression of IL-10 and IL-13, cytokines that promote healthy adipose tissue. In terms of therapeutic applications, implant of scaffolds designed to release resveratrol into the visceral fat decreases MCP-1 expression in mice fed a high fat diet, a molecule that drives both local and systemic inflammation during obesity. Taken together, resveratrol delivery to adipose tissue using poly(lactide-co-glycolide) scaffolds is a promising therapeutic strategy for the treatment of adipose tissue inflammation that drives metabolic disease.

Graphical Abstract

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Supporting Information

The supporting information is available free of charge on the ACS Publications website.

Resveratrol wavelength scan, resveratrol standard curve reproducibility, flow cytometry gating scheme, and detection of IL-6 using western blot (PDF link).

Keywords

scaffold; resveratrol; adipose tissue engineering; poly(lactide-co-glycolide); immune response to biomaterials

Introduction

While the adipose tissue is a common site for biomaterial supported delivery of endocrine cells (i.e. islets),¹⁻⁵ the field has yet to investigate biomaterial strategies to modulate this tissue's inherent endocrine function. Indeed, the adipose tissue regulates metabolism, immunity, reproduction, and many other processes required for homeostasis through the factors it secretes.⁶⁻⁹ Therefore, an ability to control adipose secretory function is a promising avenue to treat metabolic diseases. In devising platforms to modulate the adipose tissue, it is important to consider that (i) biomaterial implant causes local changes in immune cell populations¹⁰ and (ii) adipose tissue function is inextricably linked to the immune cells resident within it.^{11,12} Furthermore, excessive adipose tissue inflammation characterized by immune cell accumulation and inflammatory cytokine production is associated with metabolic disease.¹³⁻¹⁵ Thus, from a tissue engineering perspective, it is important to understand how the adipose tissue immune environment responds to biomaterial implants and develop strategies to modulate this response, if necessary.

The immune response to biomaterial implants within the adipose tissue remains largely uncharacterized; however, studies of subcutaneous biomaterial implant indicates there are three phases: acute inflammation, chronic inflammation, and wound healing.¹⁰ During the acute inflammation phase, proinflammatory cytokines such as tumor necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6), as well as chemokines, such as CXCL1 and monocyte chemotactic protein 1 (MCP-1), are secreted to recruit neutrophils and monocytes from the bloodstream and into the implant site.^{10,16} Chronic inflammation follows this phase and is characterized by the presence of macrophages, monocytes, lymphocytes, such as natural killer (NK) cells, T cells, and B cells, and foreign body giant cells at the biomaterial interface.^{10,16} The wound healing process begins with the resolution of inflammation through the increase of anti-inflammatory cytokines, such as interleukin-10 (IL-10), interleukin 4 (IL-4), and interleukin-13 (IL-13), downregulation of inflammatory mediators, and apoptosis of immune cells.¹⁶ In terms of developing implantable devices to modulate adipose tissue function, especially for treatment of metabolic disease, it would be beneficial to limit inflammatory cytokine expression upon device implant while simultaneously enhancing anti-inflammatory cytokines that might improve adipose tissue health.

In this study, we investigated resveratrol release from porous poly(lactide-co-glycolide) (PLG) scaffolds that integrate with the adipose tissue.⁵ Resveratrol, a naturally occurring polyphenol, was selected because it protects mice and primates from diet induced obesity, in part through its metabolic effects on adipose tissue.¹⁷⁻²¹ In addition, resveratrol decreases inflammation associated with biomaterial implant in bone, cartilage, and the vasculature.²²⁻²⁷ However, scaffold-based delivery of resveratrol to modulate the immune response to biomaterial implant in the adipose tissue has not been investigated. Therefore, we performed the following studies to investigate whether resveratrol delivery from PLG scaffolds would limit inflammation and speed its resolution following implant into visceral adipose tissue. We chose to deliver resveratrol directly from the scaffold instead of oral delivery because resveratrol suffers from poor bioavailability and requires frequent, large doses to address adipose tissue inflammation.^{17,18,20,28} To investigate therapeutic efficacy of our strategy, we determined whether resveratrol delivery from scaffolds could induce an anti-inflammatory response in proinflammatory environments including adipocytes challenged with TNF- α and the fat pads of mice fed a high fat diet.

Materials and Methods

Particle Fabrication.

Particles were produced by an oil-in-water emulsion-solvent evaporation technique. The oil phase consisted of a 3:1 mixture of dichloromethane (Sigma) and ethanol (Sigma) containing 6% w/v PLG (purchased from Evonik, 75:25 mole ratio lactide to glycolide, 0.76 dL/g) and 10 mg/mL of resveratrol (Sigma). The aqueous phase consisted of 1% w/v PVA (Sigma) dissolved in ultrapure water. The oil phase was homogenized with the aqueous phase using a 1:7 volume ratio with a benchtop homogenizer. The emulsion was then immediately added to ultrapure water and stirred for 5 hours to allow particles to harden and organic solvents to evaporate. Particles were collected via centrifugation, washed with ultrapure water, and lyophilized.

Scaffold Fabrication.

Scaffolds were fabricated by mixing PLG particles with 250-500 μ m NaCl particles in a 1:30 ratio and the mixture was then pelleted in a die. Scaffolds were gas-foamed using 800 psi CO₂ at room temperature in a pressure vessel. The salt porogen was removed by washing in ultrapure water. Complete salt removal was confirmed by scaffold weight and microscopy. Scaffolds that did not contain resveratrol were fabricated from particles produced using an oil phase that did not contain resveratrol or ethanol.

Scanning Electron Microscopy.

Particles or scaffolds were added to carbon adhesive tape on aluminum stubs and gold sputtered 3 times for 1 minute with a Denton Desk II Vacuum sputter coater. Compressed air was used to ensure a monolayer of particles prior to sputtering. Images were taken on a TESCAN Vega3 scanning electron microscope at 10kV.

Measuring Resveratrol Loading Capacity and Encapsulation Efficiency in PLG Particles and Scaffolds.

Particles and scaffolds were weighed and then dissolved in a defined volume of DMSO (Sigma) and analyzed for absorbance at 330 nm, which is the maximum absorbance wavelength for these solutions as determined by a wavelength scan (Fig. S1). PLG in the solution did not affect resveratrol's absorbance spectrum (data not shown). Absorbance values were then compared to a 9-point standard curve allowing for the interpolation of the unknown resveratrol concentration in the sample. Standard curves were highly reproducible between experiments (Fig. S2). Particle loading capacity (PLC) and scaffold loading capacity (SLC) was determined using eqs 1 and 2, respectively.

$$PLC\left(\frac{\mu g}{mg}\right) = \frac{M_{P-RSV}(\mu g)}{M_P(mg)} \quad (1)$$

$$SLC\left(\frac{\mu g}{mg}\right) = \frac{M_{S-RSV}(\mu g)}{M_S(mg)} \quad (2)$$

M_{P-RSV} and M_{S-RSV} is the mass of resveratrol measured in particles and scaffolds, respectively. M_P and M_S is the mass of particles and scaffold analyzed. To investigate the effect of storage on resveratrol loading capacity, particles or scaffolds were analyzed for resveratrol at 2 and 52 weeks after fabrication.

Resveratrol encapsulation efficiency was calculated for particles (EE_P) and scaffolds (EE_S) using eqs 3 and 4, respectively.

$$EE_P(\%) = \left(\frac{M_{P-Recovered}(mg) * PLC\left(\frac{\mu g}{mg}\right)}{M_{RSV-Emulsion}(mg)} \right) * 100 \quad (3)$$

$$EE_S(\%) = \left(\frac{M_S(mg) * SLC\left(\frac{\mu g}{mg}\right)}{M_{P-Scaffold Fabrication}(mg) * PLC\left(\frac{\mu g}{mg}\right)} \right) * 100 \quad (4)$$

$M_{P-Recovered}$ is the mass of particles recovered after particle fabrication, $M_{RSV-Emulsion}$ is the mass of resveratrol added to the emulsion, and $M_{P-Scaffold Fabrication}$ is the mass of particles used in scaffold fabrication.

3T3-L1 Cell Culture.

3T3-L1 fibroblasts (ATCC), were differentiated into adipocytes following ATCC's protocol, with slight modifications. Briefly, 3T3-L1 cells were seeded in 6 well plates at 80,000 cells/well or 24 well plates at 20,000 cells/well and cultured in DMEM (Corning Cellgro) supplemented with 10% Super Calf Serum (GemCell) and 1% Pen/Strep (Fisher). 48 hours after confluence was reached, media was exchanged with *differentiation media* consisting of DMEM supplemented with 10% FBS (Fisher), 10 $\mu g/mL$ bovine insulin (Sigma), 0.25 μM

dexamethasone (Sigma), and 0.5 mM isobutylmethylxanthine (Sigma). 48 hours after differentiation media was added, media was exchanged for DMEM supplemented with 10% FBS and 10 μ g/mL bovine insulin. After another 48 hours, media was exchanged for DMEM supplemented with 10% FBS. Cells were then cultured until treatment with particles or scaffolds, which occurred nine days after exposure to the *differentiation media*.

Particle Treatment of 3T3-L1 Adipocytes and Oil Red O Assay.

On day 9 of differentiation, 3T3-L1 adipocytes in 6-well plates were treated with 300 μ g/mL of PLG particles, PLG+RSV particles 2 weeks after fabrication, or PLG+RSV particles 52 weeks after fabrication. As a positive control, 3T3-L1 adipocytes treated with 60 μ M resveratrol. 48 hours later, cells were washed and then fixed using 10% formalin in PBS. Immediately before the experiment, particles were washed in 35% ethanol in water and then suspended in DMEM media.

An Oil Red O assay was performed on the fixed cells to visualize their lipid content. Cells were washed with ultrapure water followed by 60% isopropanol. Cells were then incubated with Oil Red O (Sigma) for 10 minutes at room temperature followed by 4 washes with ultrapure water to ensure removal of unbound Oil Red O. Images were taken on Nikon Eclipse Ci microscope with a 4x objective. To quantify the staining, Oil Red O was extracted from the cells by incubating them in 100% isopropanol for 10 minutes at room temperature. Absorbance of the isopropanol solution was then measured at 500 nm using a spectrophotometer. Absorbance was normalized to that of untreated differentiated cells.

Resveratrol *In Vitro* Release Assay.

To investigate resveratrol release, scaffolds were weighed and then incubated in ultrapure water at 37°C for 0.25, 1, 3, 7, or 14 days. At the end of the assay, scaffolds were retrieved from the incubator and stored in a desiccator until dry. Dryness was confirmed by scaffold weight. The amount of resveratrol remaining in the scaffold was then measured as described in the *Measuring Resveratrol Loading Capacity and Encapsulation Efficiency in PLG Particles and Scaffolds* section above.

Animal Care and Scaffold Implant.

All animal procedures were approved by the University of South Carolina Institutional Animal Care and Use Committee. Six-week-old male C57BL/6 mice were purchased from Jackson Labs. Mice were allowed to acclimate for 2 weeks. Prior to implant, all scaffolds were washed in 70% ethanol and then rinsed in sterile PBS. Mice were anesthetized with isoflurane and the surgical site was shaved and disinfected with betadine and alcohol. Following a lower abdominal midline incision one scaffold was wrapped into each epididymal fat pad (i.e. each mouse received two scaffolds). The abdominal wall was then closed with a running stitch, and the skin was closed with wound clips. Epididymal fat pads were collected for analysis 3, 7 or 14 days after implant.

Flow Cytometry.

Seven and 14 days after implant, epididymal fat pads were harvested following euthanasia and washed in ice cold PBS (Sigma). Fat pads were minced, digested in collagenase

(Liberase TL, Roche), and passed through a 100 μ m filter. The stromal vascular fraction was harvested by centrifugation, washed in MACS buffer (PBS, 0.5 mM EDTA, 30% BSA), and incubated with anti-CD16/32 prior to adding an antibody cocktail against extracellular antigens. The following antibodies were purchased from Biolegend: anti-CD45 clone 30-F11, anti-Ly6G clone 1A8, anti-F4/80 clone BM8, anti-NK1.1 clone PK136, anti-CD19 clone 6D5, anti-CD11b clone M1/70, anti-CD3 clone 17A2, and TruStain fcX (anti-CD16/32) clone 93. The following isotype controls were also purchased from Biolegend: mouse IgG2a clone MOPC-173; rat IgG2a clone RTK2758; rat IgG2b clone RTK4530. After antibody incubation, cells were washed, fixed, and analyzed using a FACS Aria flow cytometer (BD Biosciences). The number of CD45 cells in each flow cytometry sample was calculated using Bang's labs Flow Cytometry Absolute Count Standard, which was added prior to data acquisition. FlowJo software (Treestar) was utilized to compensate and analyze data. FMOs with isotype controls were used to determine specific antibody signal. The gating scheme used in the flow cytometry analysis is depicted in Figure S3.

Enzyme Linked Immunosorbent Assay (ELISA).

Following euthanasia, epididymal fat pads were harvested at 3, 7, or 14 days after scaffold implant, washed in ice cold PBS, frozen on dry ice, and stored at -80°C until homogenization. Prior to homogenization, tissues were weighed and then homogenized in a defined volume of RIPA buffer containing Halt Protease Inhibitor Cocktail (Thermo Fisher Scientific), PhosStop (Sigma), and phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Thermo Fisher Scientific) using a bench top homogenizer. Insoluble material was removed by centrifugation. Whole tissue homogenate samples were aliquoted and stored at -80°C until use.

All cytokines were measured in whole tissue homogenate using DuoSet ELISA kits (R&D Systems); however, several modifications were made to the manufacturer's instructions to improve sensitivity. A high binding 96 well plate was incubated with capture antibody overnight at 4°C . The plate was then blocked with 10% BSA for 3 hours at room temperature. Samples were incubated on the plate overnight at 4°C . Detection antibody incubation lasted for 3 hours at room temperature before incubation with streptavidin-HRP for 2 hours at room temperature. After incubation with the substrate for 20 minutes, stop solution was added to stop the enzymatic reaction, and absorbance was measured at 450 nm. Background was removed by measuring absorbance at 540 nm. A standard curve was used to determine each cytokine concentration in each sample.

Western Blotting.

Whole tissue homogenate was obtained as stated in the previous section. Total protein in the sample was quantified using the Pierce BCA protein assay kit (Thermo Fisher Scientific). 30–50 μ g of protein was separated by 8–15% SDS polyacrylamide gel electrophoresis, depending on targeted molecular weight, and transferred to 0.2 μ m nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing Tween-20 (TBST) for 1 hour at room temperature. Primary antibodies for Bax, Mcl-1, and IL-6 were purchased from Cell Signaling Technologies, CD206 was purchased from Abcam, and arginase 1 was purchased from Santa Cruz Biotechnology. Each primary

antibody was added to each blot at 1:1000 dilution, with the exception of arginase-1 which was added at 1:100 dilution, in 5% non-fat dry milk in TBST overnight at 4°C. Each membrane was then washed with TBST before incubation with a polyclonal secondary antibody (Abcam), or in the case of arginase-1 a monoclonal secondary antibody (Biorad), conjugated to horseradish peroxidase for 1 hour at room temperature. The blots were then developed with SuperSignal enhanced chemiluminescent substrate solution (Pierce). To verify equal loading of samples, blots were incubated in stripping buffer for 20 minutes and washed with TBST. The blot then underwent the same protocol as previously stated to probe for GAPDH (Cell Signaling Technologies). ImageLabs software was used to analyze the relative intensity of each protein.

High Fat Diet Feeding.

Mice were allowed to acclimate for 2 weeks prior to being placed on a 60% high fat diet (Research Diets D12492) while a control group was kept on a normal chow diet. The high fat diet was initiated 1 week prior to scaffold implant and the mice remained on the diet until the end of the study. Five weeks after scaffold implantation, mice were sacrificed under anesthesia and epididymal fat pads were collected for analysis. Fat pads were homogenized and analyzed for cytokine expression using ELISA.

In Vitro Model of 3T3-L1 Adipocyte Inflammation.

On day 9 of differentiation, PLG or PLG+RSV scaffolds were introduced into wells containing 3T3-L1 adipocytes. Prior to addition, all scaffolds were washed in 70% ethanol and then rinsed in sterile PBS. After 72 hours of pre-treatment with the scaffold, 10 ng/mL TNF- α was then added to each well and allowed to incubate for an additional 24 hours. Media was collected at this time point for further analysis. Differentiated cells not treated with the TNF- α served as a control. Cell media collected at the end of the experiment was analyzed for MCP-1 using a DuoSet ELISA kit (R&D Systems).

Statistics.

Comparisons between two or more groups over time were conducted with a two-way ANOVA followed by Tukey's multiple comparisons test. The p-values calculated by the two-way ANOVA, which address the effect of resveratrol, time, and the interactions between the two are reported as p_{drug} , p_{time} , and $p_{\text{interaction}}$, respectively. Comparisons between three groups at a single time point were made with a one-way ANOVA followed by Tukey's multiple comparisons test. Comparisons between two groups at a single time point were made with an unpaired t test. The number of samples analyzed, and the statistical analysis carried out are detailed in each figure legend. All analyses were completed using GraphPad Prism. In all figures, the error bars denote standard error of the mean (SEM).

Results

Characterization of Resveratrol Scaffolds.

Resveratrol loaded PLG particles (PLG+RSV) were prepared using a single emulsion and solvent evaporation technique. The morphology of these particles and particles containing only the polymer (PLG) are shown in Fig. 1A-B. The images show that the morphology of

PLG+RSV particles are irregular in shape compared to PLG particles, which are spherical. These particles were then used to fabricate scaffolds using a gas foaming and particulate leaching method. The structure of PLG+RSV and PLG scaffolds are shown in Fig. 1C-D. Both scaffolds contain a similar porous structure indicating that particle morphology does not have an impact on scaffold structure. During the gas foaming process, the polymer is saturated with high pressure CO₂ (800 psi) which leads to the nucleation and growth of gas pores in the polymer particles.²⁹ The polymer particles expand and fuse around the salt particles, resulting in a continuous matrix.²⁹ It is likely the gas foaming process is the reason why particle morphology does not impact the final scaffold structure.

The amount of resveratrol in particles and scaffolds was measured by dissolving the polymer in DMSO and measuring the absorbance of the solution at 330 nm. Resveratrol encapsulation within PLG particles is reproducible, with three representative batches containing 54 ± 2 , 61 ± 2 , and 59 ± 1 μg resveratrol/mg particles, respectively (Fig. 2A). Scaffold fabrication from these particles is also reproducible. After salt leaching, scaffolds made from the particles in Fig. 2A contained 35 ± 1 , 36 ± 3 , and 36 ± 3 μg resveratrol/mg scaffold (Fig. 2B) and weighed 2.29 ± 0.02 , 2.48 ± 0.17 , and 2.43 ± 0.07 mg (Fig. 2C). The amount of resveratrol in a scaffold can be calculated by multiplying the loading capacity (Fig. 2B) by the scaffold weight (Fig 2C), which was between 80 and 90 μg . Collectively, this data demonstrates we can fabricate resveratrol loaded scaffolds with reproducible drug payloads.

The drug encapsulation efficiency in the particles was $20.3 \pm 0.7\%$ (Table 1). This encapsulation efficiency was impacted by the mass of PLG particles we were able to recover following the single emulsion/solvent evaporation fabrication procedure. The mass yield for particles was typically 40%. Drug loading capacity dropped from approximately 60 $\mu\text{g}/\text{mg}$ for particles to approximately 35 $\mu\text{g}/\text{mg}$ for scaffolds, giving the scaffolds a drug encapsulation efficiency of $59.6 \pm 3.1\%$ (Table 1). Inspection of our fabrication procedure suggests that resveratrol is lost during the salt porogen removal step because scaffolds assayed immediately after gas foaming have a drug encapsulation efficiency of nearly 100% (data not shown).

Resveratrol stability, which is indicated by its absorption at 330 nm,³⁰ was studied by analyzing a fourth batch of particles and scaffolds shortly after fabrication and one year later (Fig. 3A-B). When particles were analyzed immediately after fabrication they contained 65 ± 0.5 μg resveratrol/mg particles and when this same batch of particles were analyzed one year later they contained 61 ± 6 μg resveratrol/mg particles (Fig. 3A). When scaffolds were analyzed directly after fabrication they contained 37 ± 0.6 μg resveratrol/mg scaffold and when scaffolds from the same batch were analyzed one year later they contained 36 ± 0.4 μg resveratrol/mg scaffold (Fig. 3B). These results indicate that resveratrol activity is not lost after one year when resveratrol loaded particles or scaffolds are kept in dry conditions and protected from light.

To confirm that resveratrol was active after one year of encapsulation in PLG, we investigated that ability PLG+RSV particles to induce lipolysis in differentiated 3T3-L1 adipocytes by measuring lipid content with an Oil Red O assay. We found that PLG+RSV

particles stored for either two weeks or 52 weeks significantly decreased lipid content of the adipocytes to a similar extent, while lipid content was not affected by the PLG particles (Fig. 3C). Representative microscopic images were used to validate the decrease in lipid staining in the PLG+RSV and RSV treated groups (Fig. 3D). Collectively, these results demonstrate that resveratrol remains bioactive after its encapsulation in PLG, and that it remains active for at least one year.

The resveratrol release profile of scaffolds over 14 days was characterized *in vitro* (Fig. 4A). At each time point scaffolds are removed from the assay and tested for resveratrol content. The initial amount of resveratrol in the scaffold was taken to be the average amount of resveratrol measured in three scaffolds from the same batch immediately after salt leaching. Resveratrol exhibited a biphasic release pattern (burst followed by a plateau), with approximately 50% of the drug released within the first 3 days. The amount of resveratrol released during the plateau phase accounted for approximately 10% of the initial resveratrol in the scaffold. The release profile indicated there was approximately 30% of the resveratrol present within the scaffold after 14 days. Importantly, this burst release of resveratrol could be beneficial in decreasing the acute inflammatory phase which occurs 1–5 days after injury. Minimal scaffold degradation (approximately 5% mass loss) was required for drug release over this time period (Fig. 4B).

Effect of Resveratrol on Immune Cell Infiltration Following Scaffold Implant.

To quantify the effect of resveratrol on immune cell infiltration into adipose tissue following scaffold implant, we employed flow cytometry. Total immune cells numbers were measured based on CD45 expression 7 and 14 days after implant (Fig 5A). As expected, PLG scaffold implant increased immune cell numbers at day 7. Contrary to our expectations, resveratrol did not decrease immune cell numbers at this time point relative to PLG scaffolds. Surprisingly, immune cell numbers significantly decreased from day 7 to day 14 for both scaffold groups and we could not detect differences between any of the groups at day 14 by Tukey's multiple comparison's test. The data indicate immune cell infiltration following PLG implant into fat largely resolves within two weeks without intervention. While not significant, immune cell numbers in the PLG+RSV group tended to be higher at day 7 and lower at day 14 compared to the PLG group.

To better understand these trends, seven immune cell populations were measured at 7 and 14 days: macrophages (F4/80), neutrophils (Ly6G), monocytes (CD11b), NK cells (NK1.1), T cells (CD3), NKT cells (NK1.1 CD3), and B cells (CD19) (Fig. 5B-C). For ease of interpretation, immune cell numbers for the scaffold groups are normalized by the naïve controls. At day 7, resveratrol delivery resulted in a significant increase in neutrophils with a concomitant decrease in T cells, NK cells, and NKT cells (Fig. 5B). At 14 days, all populations exhibited a trend of decrease, but only monocytes, NK cells, and B cells numbers were significantly lower in resveratrol groups. The data suggests that even though resveratrol does not decrease the total number of immune cells, it does have an impact on particular immune cell populations.

Flow cytometry revealed that macrophage number was not different between PLG and PLG +RSV at either time point; however, we hypothesized that resveratrol may modify

macrophage phenotype. To identify changes in macrophage phenotype, two molecules associated with M2 polarization, CD206 and arginase-1,³¹ were measured in scaffold homogenates 7 days after implant using western blot (Fig. 6A). At this timepoint, CD206 was unchanged in both the PLG and PLG+RSV scaffold groups compared to naïve fat (Fig. 6B). In contrast, arginase-1 was approximately 35-fold higher in both the PLG and PLG+RSV scaffold groups compared to naïve fat (Fig. 6C). The data indicate that resveratrol delivery does not impact CD206 or arginase-1 expression, but scaffold implant elevates arginase-1 expression above naïve levels.

Effect of Resveratrol on Cytokine Levels Following Scaffold Implant.

Cytokines control immune cell infiltration, residence, and survival in the tissue. Thus, we investigated the effect of resveratrol delivery on protein levels of pro-inflammatory cytokines MCP-1, TNF- α , IL-6, and CXCL1, as well as anti-inflammatory cytokines IL-4, IL-10 and IL-13 in whole tissue homogenates 3, 7, and 14 days after scaffold implant. There was no significant interaction ($p_{\text{interaction}}$) between drug treatment and time detected by 2-Way ANOVA for the analyses carried out in Figure 7 (Fig. 7H). MCP-1 exhibited the largest increase following PLG scaffold implant of all cytokines measured but was not affected by resveratrol (Fig. 7A). MCP-1 expression significantly decreased by day 14 as determined by Tukey's multiple comparisons test. Interestingly, scaffold implant, irrespective of drug payload, did not increase whole tissue levels of TNF- α , IL-6, or CXCL1 compared to naïve levels (Fig. 7 B-D). We did detect a significant effect of resveratrol on TNF- α ($p_{\text{drug}}=0.0147$) and CXCL1 ($p_{\text{drug}}=0.0168$) by Two-Way ANOVA (Fig. 7H), but drug delivery largely served to bring expression up towards naïve levels. To validate these findings, western blot was used to detect IL-6 in all groups. We were not able to detect IL-6 in any tissues by this method; however, IL-6 was detected in Raw 264.7 cells after incubation with LPS and Brefeldin A (Fig. S4).

In terms of anti-inflammatory cytokines, scaffolds, irrespective of drug payload, did not modulate IL-4 expression (Fig. 7E). On the other hand, there was a significant effect of resveratrol on IL-10 ($p_{\text{drug}}=0.0065$) and IL-13 ($p_{\text{drug}}=0.0023$), with resveratrol increasing expression of both these proteins. In addition, expression of both these molecules increased over time (Fig. 7F-G). Together, these results suggest that resveratrol's effect on IL-10 and IL-13 may be responsible for decreases in immune cell numbers at day 14 as measured by flow cytometry (Fig. 5B-C).

Effect of Resveratrol on Bax and Mcl-1 Following Scaffold Implant.

Apoptosis is increased in tissues during resolution of inflammation, reflecting the clearance of immune cells that are no longer needed.^{16,32} In addition, resveratrol increases immune cell apoptosis, especially in neutrophils.³³⁻⁴⁰ Thus, we measured the expression of Bax, a pro-apoptotic, and Mcl-1, an anti-apoptotic, protein in whole tissue protein homogenates 7 and 14 days after implant (Fig. 8A). Bax and Mcl-1 proteins were present in naïve fat (Fig. 8A), but only Bax levels increased with scaffold implant (Fig. 8B); Mcl-1 levels were largely unaffected (Fig. 8C). There was no significant interaction between treatment and time detected by 2-Way ANOVA for Fig. 8B or Fig. 8C. There was a trend for resveratrol to increase Bax levels, but it was not significant ($p_{\text{drug}}=0.0508$); however, over time Bax

expression did decrease ($p_{\text{time}}=0.0040$). Collectively, these results suggest that scaffold implant into the fat may increase rates of apoptosis since pro-apoptotic Bax increases and anti-apoptotic Mcl-1 is relatively unchanged; however, resveratrol's ability to alter Bax expression, if indeed it does, was difficult to detect.

Ability of Resveratrol to Induce Anti-Inflammatory Responses in Inflammatory Environments.

An important application of PLG+RSV scaffolds would be to induce an anti-inflammatory response in inflamed adipose tissue. To investigate the ability of PLG+RSV scaffolds to achieve this, we utilized a common model of adipose tissue inflammation in which mice are fed a high fat diet. Mice received PLG or PLG+RSV scaffolds in both epididymal fat pads one week after being fed a high fat diet. The mice were then fed the diet for 5 additional weeks prior to harvest of the epididymal fat. (Fig. 9A). Cytokine analysis was conducted using ELISA to measure MCP-1, IL-10, and IL-13 in the epididymal fat. High fat diet feeding resulted in an increase in pro-inflammatory cytokine MCP-1 and a decrease in anti-inflammatory cytokines IL-10 and IL-13 (Fig. 9B). The results demonstrate that PLG+RSV scaffolds, but not PLG scaffolds, significantly decreased MCP-1 expression in the epididymal fat. Interestingly, PLG+RSV scaffolds did not increase the expression of IL-10 and IL-13.

In order to investigate resveratrol's anti-inflammatory effect on adipocytes, we utilized an *in vitro* model of adipocyte inflammation. On day 9 of differentiation, cells were treated with PLG scaffolds, PLG+RSV scaffolds, or 60 μM RSV for 72 hours and then treated with 10 ng/mL TNF- α for 24 hours (Fig. 9C). Media was collected and analyzed for MCP-1 using ELISA. Differentiated cells that were treated with TNF- α secreted approximately 2.5-fold more MCP-1 compared to untreated differentiated cells (Fig. 9D). PLG scaffolds had no effect on MCP-1 secretion; however, PLG+RSV scaffolds significantly decreased MCP-1 compared to differentiated cells treated only with TNF- α or pre-treated with PLG scaffolds prior to TNF- α .

Discussion

This report presents the characterization of resveratrol loaded PLG scaffolds and their novel use in modulating the immune response to biomaterial implants in the adipose tissue. Porous PLG scaffolds containing resveratrol were fabricated using a gas foaming/particulate leaching method in which NaCl served as the sacrificial porogen. To our knowledge, this approach is not commonly used for the fabrication of small molecule loaded PLG scaffolds and our results provide further insight into drug loading and release kinetics of this approach. Our scaffolds contained 37 μg of resveratrol per mg of PLG, which is at least 4 fold higher loading than previous studies using biodegradable polyester scaffolds to deliver resveratrol.^{22,27} However, this scaffold fabrication method was suboptimal because removal of the salt porogen led to a 40% loss of resveratrol loaded in the scaffold. To improve drug loading efficiency of scaffolds, a non-particulate leaching method to promote scaffold porosity should be investigated. Nonetheless, resveratrol loaded scaffolds fabricated using this method exhibited a burst and plateau release profile and overall delivered a large amount

of resveratrol over 14 days compared to sintered resveratrol encapsulated PLG nanoparticles which delivered seven times less resveratrol in a sustained manner.²² In addition, resveratrol in our PLG scaffolds did not degrade over one year when stored at room temperature conditions as determined by measuring absorbance at the wavelength of trans-resveratrol, the active isomer of resveratrol.⁴¹ Furthermore, we confirmed this finding with a bioactivity assay that indicated both fresh PLG+RSV particles and those stored for one year induced lipolysis in 3T3-L1 adipocytes and to the same extent. The ability to maintain bioactivity while stored at room temperature is an advantage of small molecule delivery compared to other immunomodulatory strategies that require temperature-controlled storage. For example, proteins and viral vectors are kept at freezing temperatures to maintain activity, posing challenges during manufacturing, storage, and transportation once in the market.

Initially, *in vivo* studies investigated resveratrol delivery as a way to modulate the immune environment within adipose tissue following implant of PLG scaffolds. Resveratrol impacted the prevalence of monocytes and several lymphocyte populations over the time course of our experiments. Monocytes and lymphocytes play key roles in adipose tissue inflammation during obesity;⁴² therefore, the ability to modulate their number is significant to future therapies. Monocytes and lymphocytes are recruited to sites of inflammation by inflammatory cytokines. Resveratrol delivery increased expression of the anti-inflammatory cytokine IL-10, which inhibits inflammatory cytokine production and signaling in many cell types, and this effect may be responsible for the decrease in monocytes and lymphocytes recruited to the scaffold implant site. IL-10 is produced by macrophages (among other cells), with M2 polarized macrophages expressing IL-10 at higher levels than pro-inflammatory macrophages.⁴³ Thus, we hypothesized that resveratrol delivery increased macrophage polarization towards an M2 phenotype within the implant site. Surprisingly, we were not able to detect elevation of CD206 or arginase-1, markers of M2 macrophage polarization, following resveratrol delivery. However, there was a 35-fold increase in arginase-1 due to the PLG scaffold implant and it is likely that further upregulation via resveratrol was not possible. In addition to IL-10, resveratrol delivery increased expression of IL-13, which is not produced by macrophages, but by TH2 lymphocytes, mast cells, basophils, eosinophils, and IL2 cells.³¹ In particular, eosinophils, and IL2 cells are present in the adipose tissue and have the ability to shape immune responses favoring immune resolution rather than inflammation.⁴⁴

An ability to modulate the cytokine milieu in the adipose tissue would be useful for treating metabolic diseases associated with adipose tissue inflammation. Therefore, we investigated the efficacy of PLG+RSV scaffolds in mitigating adipose tissue inflammation that occurs in high fat diet fed mice. We found that resveratrol delivery decreased expression of MCP-1 in the adipose tissue of mice fed a high fat diet for 6 weeks. High fat diet elevates MCP-1 after 1 week,⁴⁵ and promotes inflammatory macrophage infiltration into the adipose tissue, which drives systemic insulin resistance.⁴⁶ Therefore, decreasing this cytokine is an important benchmark for treating adipose tissue inflammation. We also found that pre-treatment with PLG+RSV scaffolds prevented TNF- α induced MCP-1 secretion from differentiated 3T3-L1 adipocytes, suggesting that the decrease in MCP-1 *in vivo* was mediated, at least in part, by resveratrol acting on adipocytes. In contrast to healthy adipose tissue (Fig 9), PLG+RSV scaffolds did not increase IL-10 and IL-13 expression in adipose tissue from mice fed the

high fat diet; however, scaffold implant did not further decrease the expression of these anti-inflammatory proteins either, which were decreased by the high fat diet. Since adipose tissue expression of IL-10 and IL-13 protects mice against the detrimental effects of high fat diet,⁴⁷⁻⁴⁹, future studies will aim to identify small molecules that increase these factors when released from scaffolds implanted into the adipose tissue.

We hypothesize that the potential mechanism for resveratrol's protection against MCP-1 elevation is through its effects on the NF- κ B pathway, a regulator of cytokine production. Resveratrol inhibits the DNA binding activity of the NF- κ B complex through activation of Sirt-1, which inhibits NF- κ B transcription in 3T3-L1 adipocytes.⁵⁰ Interestingly, upon degradation, PLG produces lactic acid, a bioactive molecule that abrogates the pro-inflammatory response of macrophages stimulated with LPS via downregulation of the NF- κ B pathway through the GPR81 receptor.^{51,52} This is a plausible mechanism as to why PLG scaffold without drug loading, does not produce pro-inflammatory cytokine production upon implant into adipose tissue (Fig. 7). Furthermore, delivery of lactate via PLG implant accelerates the wound healing response.⁵³ Therefore, the delivery of resveratrol from a degrading PLG scaffold may synergistically promote an anti-inflammatory environment through the combinatorial actions of resveratrol and lactic acid on adipocytes and macrophages. Furthermore, PLG degradation creates an acidic environment. Resveratrol is most stable at pH less than 6.^{54,55} Therefore, this acidic environment could have beneficial effects on drug stability.

Notable was our finding that PLG scaffold implant did not increase expression of TNF- α and IL-6 in the epididymal adipose tissue relative to naïve fat and, in fact, these cytokines appeared to decrease. Generally, the literature reports that biomaterial implant increases these factors and an important metric for anti-inflammatory drug delivery is the ability of the delivered factor to decrease their expression. However, we note from an extensive literature search that rarely are the experimental groups receiving the "empty" biomaterial and the "drug-loaded" biomaterial compared to an unmanipulated tissue.^{2,56,57-62} Thus, in these cited studies it is possible that the "drug-loaded" group actually decreased inflammatory gene expression below what we call "naïve levels". On the other hand, we may have uncovered an attribute specific to adipose tissue. In a healthy state, the adipose tissue is biased towards anti-inflammation,⁶³ therefore we hypothesize that upon implant, the biomaterial invokes a series of signaling pathways that down regulate inflammatory mediators such as TNF- α and IL-6. In support of this hypothesis, we find that scaffold implant upregulated arginase 35-fold (Fig.6), an enzyme that inhibits production of TNF- α and IL-6.⁶⁴

A limitation of the study is that we did not conduct a dose response. In order to minimize animal use, we chose to deliver the maximal amount of resveratrol we could in a 2.5 mg scaffold, which we knew from previous work is well tolerated by the mouse.⁶⁵ While resveratrol ultimately had anti-inflammatory effects, the spike in neutrophils at the seven-day time point that occurred with its delivery was likely counterproductive. As we believe the neutrophil influx was the result of local resveratrol concentrations that were too high, future studies will investigate scaffolds with lower payloads of resveratrol, but also scaffolds with high payloads designed to release resveratrol more slowly.

Conclusion

Resveratrol can be encapsulated into PLG particles using a single emulsion solvent evaporation technique and these particles can be formed into porous PLG scaffolds using gas foaming followed by particulate leaching with NaCl as the sacrificial porogen. This method results in scaffolds with high resveratrol loading and drug release with a burst phase followed by plateau. In healthy tissue, resveratrol delivery augments an anti-inflammatory environment established by PLG scaffolds, characterized by high arginase-1 and low TNF- α and IL-6, through increased expression of IL-10 and IL-13. In terms of therapeutic applications, PLG+RSV scaffolds decrease MCP-1 expression in the fat of mice fed a high fat diet, an inflammatory molecule that spurs the chronic inflammation associated with obesity's comorbidities. Thus, we conclude that PLG scaffolds are a promising platform for the treatment of adipose tissue inflammation that drives metabolic disease, since the polymer alone induces anti-inflammatory responses and the scaffold is easily engineered for drug and cell delivery. Finally, we hypothesize that further investigation into the anti-inflammatory effects of PLG in the adipose tissue will lead to new drug targets and therapeutic strategies for metabolic disease and other syndromes associated with chronic inflammation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1: SEM images of resveratrol loaded PLG particles and scaffold.

(A-B) Scanning electron microscope images of PLG+RSV (A) and PLG (B) particles. (C-D) Scanning electron microscope images of scaffolds produced with PLG+RSV (C) and PLG (D) particles.

Figure 2: Resveratrol loading capacity of PLG particles and scaffolds.

(A) Resveratrol loading capacity of three representative batches of particles. Data is mean $\pm$ SEM from three independent measurements. (B) Resveratrol loading capacity of scaffolds fabricated from the particles depicted in (A). Data is mean $\pm$ SEM from three scaffolds. (C) Mass of scaffolds depicted in (B).

Figure 3: Resveratrol loading capacity and activity after yearlong storage.

(A) Resveratrol loading capacity in a fourth batch of particles measured 2 and 52 weeks after fabrication. Data is mean $\pm$ SEM from three independent measurements. (B) Resveratrol loading capacity of scaffolds made from the particles in panel A measured 2 and 52 weeks after fabrication. Data is mean $\pm$ SEM from three scaffolds. (C) Lipid content of differentiated 3T3-L1 adipocytes 48 hours after treatment with PLG particles, PLG+RSV particles that were 2 or 52 weeks old, or free resveratrol (60 μ M). Data is from three independent experiments and shown as absorption normalized to differentiated untreated 3T3-L1 adipocytes. Statistics conducted using a one-way ANOVA followed by a Tukey's multiple comparisons test. * indicates $p < 0.05$ compared to PLG. (D) Representative microscopic images of Oil Red O staining.

Figure 4: Resveratrol release from PLG scaffolds.

(A) Two-week *in vitro* release profile of resveratrol loaded PLG scaffolds. (B) Scaffold degradation during the release assay. Data is from 5 scaffolds per time point. Error bars denote SEM.

Figure 5: Effect of resveratrol on immune cell infiltration following scaffold implant.

(A) Number of CD45 cells harvested from fat tissue following PLG and PLG+RSV scaffold implant measured by flow cytometry. Statistics by two-way ANOVA with a Tukey's multiple comparison test. * indicates $p < 0.05$ compared to naïve at day 7. † indicates $p < 0.05$ compared to the corresponding group at day 7. (B-C) Immune cell numbers from tissue receiving PLG and PLG+RSV scaffolds normalized to naïve numbers at (B) 7 and (C) 14 days after implant. Statistics calculated using a t-test, * indicates $p < 0.05$ between PLG and PLG+RSV. N=8-9 scaffolds per treatment per time point.

Figure 6: Effect of resveratrol on expression of proteins associated with M2 macrophage polarization.

(A) Representative blot of CD206 and arginase-1 protein expression in adipose homogenates collected 7 days after scaffold implant. Naïve fat is included for reference. (B-C) Quantification of (B) CD206 and (C) arginase-1 expression from 6 mice per group. Statistics were determined using an unpaired t-test.

Figure 7: Effect of resveratrol on cytokine levels following scaffold implant.

(A) MCP-1, (B) TNF- α , (C) IL-6, (D) CXCL1, (E) IL-4, (F) IL-10, and (G) IL-13 were detected by ELISA in scaffold homogenate collected 3, 7, and 14 days after scaffold implant. Data is from 3-4 mice per condition. (H) A table of the p values determined by a two-way ANOVA. * indicates $p < 0.05$ compared to corresponding group at D7 and D14 determined by Tukey's multiple comparison.

Figure 8: Effect of resveratrol on Bax and Mcl-1 following scaffold implant.

(A) A representative blot of Bax and Mcl-1 protein expression in adipose homogenates collected 7 or 14 days after scaffold implant. Naïve fat is included for reference. (B-C) Quantification of (B) Bax and (C) Mcl-1 expression from 7 mice per group. (D) A table of the p values determined by a Two-way ANOVA.

Figure 9: Ability of resveratrol to induce anti-inflammatory responses in pro-inflammatory environments.

(A) Timeline of experiment for panel B. (B) MCP-1, IL-10, and IL-13 detected by ELISA in adipose homogenate 5 weeks after scaffold implant in mice fed a high fat diet. Data is from 3-4 mice per group. Statistics determined by one-way ANOVA with a Tukey multiple comparison test. * indicates $p < 0.05$ compared to HFD. (C) Timeline of experiment for panel D. (D). MCP-1 was detected by ELISA in media from 3T3-L1 adipocytes treated with scaffolds prior to treatment with TNF- α . $n=4$ per group. Statistics determined by one-way ANOVA with a Tukey multiple comparison test. * and # indicates $p < 0.05$ compared to no scaffold and PLG, respectively.

Table 1:

Resveratrol encapsulation efficiency for PLG particles and scaffolds.

	Drug Encapsulation Efficiency (%)
Particles	20.3 ± 0.7
Scaffolds	59.6 ± 3.1