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Effects of Delayed Delivery of Dexamethasone-21-Phosphate via Subcutaneous Microdialysis Implants on Macrophage Activation in Rats

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Abstract

Macrophage activation is of interest in the biomaterials field since macrophages with an M(Dex) characteristic phenotype, i.e., CD68⁺CD163⁺, are believed to result in improved integration of the biomaterial as well as improved tissue remodeling and increased biomaterial longevity. To facilitate delivery of a macrophage modulator, dexamethasone-21-phosphate (Dex), microdialysis probes were subcutaneously implanted in male Sprague-Dawley rats. Dex localized delivery was delayed to the third day post implantation as means to alter macrophage activation state at an implant site. To better elucidate the molecular mechanisms associated with M(Dex) macrophage activation, CCL2 was quantified in dialysates, gene expression ratios were determined from excised tissue surrounding the implant, histological analyses, and immunohistochemical analyses (CD68, CD163) were performed. Dex delayed infusion resulted in the up-regulation of IL-6 at the transcript level in the tissue in contact with the microdialysis probe and decreased CCL2 concentrations collected in dialysates. Histological analyses showed increased cellular density as compared to controls in response to Dex delayed infusion. Dex delayed infusion resulted in an increased percentage of CD68⁺CD163⁺, M(Dex), macrophages in the tissue surrounding the microdialysis probe as compared to probes that served as controls.

Graphical Abstract

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The authors do not have any conflicts of interest to disclose.

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Keywords

CD68; CD163; Dexamethasone; Immunohistochemistry; Macrophage activation; microdialysis; rat

1. Introduction

Millions of different types of biomaterials (artificial heart valves, breast implants, implanted biosensors, pacemakers, and prosthetic joints) are implanted worldwide every year. Each of these different biomaterials elicits a foreign body reaction (FBR) which is an immune response to the implanted biomaterial [1]. While normal wound healing consists of hemostasis, inflammation, proliferation and remodeling, the FBR consists of acute inflammation, chronic inflammation, and the eventual fibrotic encapsulation of the biomaterial. This encapsulation results in the biomaterial being ‘walled off’ from the rest of the body, residing in its own microenvironment. For many biomaterials, this encapsulation poses no significant clinical concerns, but for other materials such as sensors, this encapsulation proves to be detrimental resulting in loss of function [2]. For this reason, much effort has been put into controlling or altering the FBR to eliminate or reduce the formation of the fibrotic encapsulation.

Macrophages play a role in both innate and adaptive immunity and can phagocytize foreign materials including microbes and cellular debris [3]. Beyond being phagocytic cells, macrophages play dueling roles in both driving and resolving inflammation, antigen presentation vs. scavenging, and tissue destruction vs tissue remodeling [4]. Macrophages have been historically-identified as playing a critical role in the outcome of implanted biomaterials and their FBR [5].

Macrophages are highly plastic cells exhibiting a wide range of phenotypes [6]. Macrophage activation is a term used to describe the ability of macrophages to change phenotypes in response to biochemical signals [7, 8]. Mills identified this phenotypic change as macrophage polarization and identified the extremes of these phenotypes along this plastic continuum as either M1 or M2 [9]. However, it is important to note that macrophage activation exhibits a continuum of states. M1 macrophages are classically activated by lipopolysaccharide (LPS), interferon gamma (IFN- γ), or tumor necrosis factor alpha (TNF- α). M1 macrophages secrete pro-inflammatory cytokines (IL-1 β , IL-6, IL-12, and TNF- α) and high concentrations of nitric oxide [8, 10]. M1 macrophages are characterized as being

pro-inflammatory, highly microbicidal, and efficient antigen presenting cells expressing high amounts of major histocompatibility complex II (MHC II) [11]. M2 macrophages consist of three subclasses, M2a,b,c, and are considered to be anti-inflammatory. M2 macrophages are induced by a variety of different modulators, IL-4 and/or IL-13 (M2a), immune complex, toll-like receptor, or IL-1 receptor ligation (M2b) and IL-10, glucocorticoids, and secosteroids (M2c) [11, 12]. M2c macrophages are considered to be anti-inflammatory, pro-tissue remodeling, and pro-wound healing. Macrophage activation has become of wide interest in the fields of biomaterials and regenerative medicine [13–16]. It has been postulated that by switching macrophages to a predominantly M2c activation state at an implant site, improved wound healing and improved implant integration into the host tissue will be achieved. Recently, a nomenclature change was requested to denote macrophage polarization as macrophage activation with the macrophage phenotype being termed according to the modulator which was used to elicit the macrophage, i.e., M(LPS) rather than M1[17]. In this paper, the M1/M2 nomenclature will be used as a broad categorization for comparisons to past literature while the modulator nomenclature, M(Dex), will be used to describe macrophages that were altered via the delivery of Dex through the implanted microdialysis probe.

Microdialysis sampling is a minimally-invasive, diffusion-based sampling technique [18]. The microdialysis probe consists of inlet and outlet tubing, inner cannula, and a semi-permeable membrane with a defined molecular weight cut-off (MWCO). Microdialysis sampling has been used *in vivo* to sample low molecular weight soluble analytes from the extracellular space (ECS) which are smaller than the MWCO of the probe membrane. This technique works by passing a fluid (perfusate), which is of physiological ionic strength and pH, through the inlet and down the inner cannula. Once the perfusate leaves the inner cannula, it passes by the membrane and exits through the outlet tubing and is collected as a dialysate. Any concentration gradient which exists between the perfusate and the ECS allows the diffusion of molecules into or out of the microdialysis probe. This feature allows microdialysis sampling to be used for the simultaneous collection of analytes from and delivery of modulators to the ECS [19]. Once collected, analytes in the dialysate can be quantified using a wide variety of chemical analysis methods.

Dexamethasone (Dex) is a synthetic glucocorticoid that is widely used as an anti-inflammatory and immunosuppressant drug [20, 21]. Once inside the cell, dexamethasone binds to glucocorticoid receptors which then translocate to the nucleus [22]. In the nucleus, Dex carries out its anti-inflammatory effects via two means: transactivation and transrepression [23]. Transactivation is the increase in the expression of certain anti-inflammatory genes including lipocortin-1 and the type II IL-1 receptor [24, 25]. Transrepression is the down regulation of pro-inflammatory genes through the direct binding of the glucocorticoid receptor to negative glucocorticoid receptor elements [26, 27] and by interfering with activator protein-1 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) [28, 29]. Through these actions Dex has been shown to down-regulate pro-inflammatory cytokines such as CCL2, IL-6, and TNF- α at the transcriptional level [30] as well as reducing CCL2, IL-6, and TNF- α protein concentrations in different animal models [31].

Chemokine (C-C motif) ligand 2 (CCL2) is a 13 kDa chemokine which exists as a monomer and homodimer at physiological concentrations [32]. CCL2 was formerly known as monocyte chemoattractant protein-1 (MCP-1) due to its ability to result in monocyte migration. CCL2 has been shown to be one of the primary factors for attracting monocytes to a wound site [33]. Once at the wound site the monocytes differentiate into macrophages. The activation state of these macrophages depends on the biochemical milieu present at the wound site.

Dexamethasone has been widely used as a release agent from biomaterials studies in an effort to improve integration of the biomaterial with the host tissue [34–36]. Dex has also been identified as a modulator which produces a phenotype that has characteristics of the former M2c macrophage [8, 11]. While much work has been done with incorporating Dex-release into implanted biomaterials, quantifying the molecular response, particularly *in vivo* attempts to shift macrophages to an M(Dex) phenotype has not been reported. Most work with Dex-releasing biomaterials uses standard histological, rather than molecular assessments, of implant/tissue outcomes. Our previous work has shown that Dex delivered immediately after microdialysis probe insertion is capable of shifting macrophages to a more CD163+ state, the former M2c designation [37]. This is consistent with other macrophage studies in rats with dexamethasone that have also targeted CD163+ cells [38]. However, there is a body of literature suggesting that an initial inflammatory response is critical for proper wound healing [39]. Therefore, we sought to determine if a different or even more optimal response in terms of converting macrophages to an M(Dex) state can be gained if the start of Dex infusion is delayed to allow the initial inflammatory response to commence. The resulting foreign body reaction to the implanted dialysis probes was characterized not only with standard histological means, but also immunohistochemical and molecular means at the gene and protein level. Thus, this work aimed to gain a better molecular understanding of macrophage-activation modulators effectiveness at controlling the foreign body reaction.

2. Materials and Methods

2.1 Chemicals

The following chemicals were used in this study: Anti-CD68 Antibody (Santa Cruz Biotechnology, Inc., Dallas, TX); Anti-CD163 Antibody (Santa Cruz Biotechnology, Inc., Dallas, TX); ApexTM Antibody Labeling Kits (Alexa Fluor 488 and Alexa Fluor 647) (Life Technologies, Carlsbad, CA); BD OptEIATM Rat MCP-1 ELISA Set (BD Biosciences, San Jose, CA); bovine serum albumin (BSA) (Rockland Immunochemicals, Gilbertsville, PA); chloroform (MP Biomedicals LLC, Solon, OH); dexamethasone-21-phosphate disodium salt (Dex) (Alfa Aesar, Ward Hill, MA); Dextran-500 (Sigma Aldrich, St Louis, MO); ethylene oxide (Anderson Sterilizers, Inc, Haw River, NC); Halt Protease Inhibitor (Pierce, Rockford, IL); Hoeschst 34580 (Sigma Aldrich, St Louis, MO); Horse Serum (Life Technologies, Carlsbad, CA); HPLC grade water (Fisher Scientific, Waltham, MA); isoflurane (Abbott Laboratories, North Chicago, IL); Optimal Cutting Temperature solution (Sakura[®] Finetek, Torrance, CA); povidone-iodine (Professional Disposables International Inc, Orangeburg, NY); Proteinase K (Qiagen, Venlo, Limburg); RNAlater (Life Technologies, Carlsbad, CA); Trizol (Life Technologies, Carlsbad, CA); Taqman[®] Gene Expression Assays (IL-6, Arg2,

CCL2, CD 163, CD 206, IL-1ra, IL-10, iNOS2, Lipo-1, TGF β -1, TNF- α , and Taf9b) (Life Technologies, Carlsbad, CA) and VetbondTM (3M, St Paul, MN). Ringer's solution contained 147 mM NaCl, 4.6 mM KCl, 2.3 mM CaCl₂, pH 7.4 and was prepared in HPLC-grade water. All other chemicals were reagent-grade or higher.

2.2 Microdialysis Procedure

CMA 20 microdialysis probes with a 10 mm 100 kDa molecular weight cut-off (MWCO) polyethersulfone membrane (Harvard Apparatus, Holliston, MA) were used for all microdialysis sampling procedures. All microdialysis probes were ethylene oxide sterilized (Anderson Sterilizers Inc, Haw River, NC) prior to use. A BAS Bee microdialysis pump (Bioanalytical Systems Inc, West Lafayette, IN) with 1 mL BAS syringes (Bioanalytical Systems Inc, West Lafayette, IN) were used to perform infusions. All perfusion fluids were autoclaved and filter sterilized prior to use. Post microdialysis probe implantation, the inlet and outlet lines were placed in a subcutaneous pocket at the anterior incision. Animals were then returned to the vivarium until three days post implantation. On days 3–6 post implantation, the animals were anesthetized, lines removed from the subcutaneous pocket, and the rat was placed in a CMA 120 freely moving collection system (CMA Microdialysis, Solna, Sweden) with the tubing lines were connected.

2.3 Surgical Procedure

All surgeries were performed on male Sprague Dawley rats (Harlan Laboratories, Indianapolis, IN) in a weight range of 280–315 g. Prior to surgery and post collection, animals were kept in a temperature controlled vivarium at 72°F. Animals were allowed access to food and water *ad libitum*. All surgical procedures and experiments were approved by the University of Arkansas Institutional Animal Care and Usage Committee (IACUC) and conformed to the NIH standards for the ethical treatment of animals.

Animals were initially anesthetized using an induction chamber and 5% isoflurane in 1 L min⁻¹ O₂. Animals were then moved to a nose cone where they were maintained using 3% isoflurane in 1 L min⁻¹ O₂ during the surgical procedure. Body temperatures were maintained using a CMA 150 temperature controller (CMA Microdialysis, Solna, Sweden) during surgical procedures. All surgical procedures were performed using aseptic technique. All tools were autoclaved prior to use.

The techniques for implanting microdialysis probes into the rats have been previously described [37, 40]. Two probes were implanted on opposite sides of the spine with approximately 2.5 cm separation between them. After collection techniques have commenced each day, the inlet and outlet tubing is connected with a tubing connector and placed in the subcutaneous pocket. The anterior incision was then closed using sterile Reflex wound clips (Fine Science Tools, Foster City, CA). On collection days, animals were anesthetized, the incision was swabbed with alcohol and the lines were removed. All lines were cleaned with alcohol prior to being placed in the subcutaneous pocket.

2.4 Collection Procedure

With two microdialysis probes implanted, one probe serves as a control and the other as the treatment (see Graphical Abstract). The control probe is perfused with Ringer's + 4% Dextran-500 + 0.1% BSA while the treatment probe is perfused with 20 $\mu\text{g mL}^{-1}$ dexamethasone-21-phosphate added. Thus each animal serves as its own control. Dexamethasone-21-phosphate was used as a derivative which is more water soluble than dexamethasone and is converted to dexamethasone by *in vivo* esterases [41]. Dextran-500 is an osmotic agent used to reduce fluid loss in high MWCO probes which has been shown to cause no additional inflammation at the implant site [40]. Bovine serum albumin (BSA) is used in the perfusion fluid to prevent non-specific binding to microdialysis materials [42]. Prior to implantation, microdialysis probes were flushed with sterile Ringer's. On the third day post implantation, animals were placed in a CMA 120 freely moving animal system. An initial flush was performed starting at 3 $\mu\text{L min}^{-1}$ and the flow rate was reduced by 0.5 $\mu\text{L min}^{-1}$ every 5 mins until a flow rate of 1 $\mu\text{L min}^{-1}$ was reached. Collections were then performed in 1 hr increments for 6 hours. All collection vials contained Halt Protease Inhibitor, were immediately placed on ice following collection, and stored at -80°C following the entire collection period. Following the final collection hour, a final flush was performed at 3 $\mu\text{L min}^{-1}$ for 30 min. For the final flush, Ringer's or Ringer's + 20 $\mu\text{g mL}^{-1}$ Dex were used as perfusion fluids for the control and treatment, respectively. On the seventh day post implantation, the animal was euthanized and the probe as well as the tissue surrounding the probe were harvested as previously described [40]. The probe and tissue surrounding it were then stored in optimal cutting temperature (OCT) solution and flash frozen using liquid nitrogen for histological and immunohistochemical analyses or the tissue surrounding the membrane was removed and stored in RNeasy lysis buffer for qRT-PCR analyses.

2.5 qRT-PCR Procedure

Tissue immediately surrounding (~1–2 mm) was harvested from the membrane portion of the probe and placed in RNeasy lysis buffer and stored on ice. Harvested tissue was stored at 4°C until RNA was extracted. RNA was then extracted from the tissue and purified using the Trizol method and an RNeasy Minikit (Qiagen, Venlo, Limburg). The integrity of the RNA was confirmed by comparing the 18s and 28s band ratios via gel electrophoresis. RNA was converted to cDNA using a high capacity reverse transcription kit (Life Technologies, Carlsbad, CA). Gene expression ratios were determined using Taqman gene expression assays (Life Technologies, Carlsbad, CA) using a 7500 Real Time PCR instrument (Life Technologies, Carlsbad, CA). Gene expression data were analyzed using REST Gene Quantification Software with Taf9b (Transcription initiation factor TFIID subunit 9B) being used as a control gene.

2.6 CCL2 Quantification

CCL2 was quantified in dialysates collected from both the control and treatment probes for each of the 6 collection hours as well as the initial flush period using a BD OptEIA™ rat MCP-1 ELISA set (BD Biosciences, San Jose, CA). The ELISA was performed per the manufacturer's protocol with the exception that 50 μL of sample were used and the remaining reagents were adjusted accordingly. Briefly, a 96 well plate was incubated

overnight with detection antibody, the plate was then blocked using assay diluent for 1 hour, standards and samples were then loaded and incubated for two hours, substrate solution was then incubated for 30 mins, and the reaction was stopped using stop solution. All the appropriate wash steps were performed per the manufacturer's protocol. Absorbance was then read at 450 nm and a reference of 570 nm was used via a Tecan Infinite M200 (Tecan, Maennedorf, Switzerland).

2.7 Immunohistochemistry Procedure

Monoclonal antibodies specific for Cluster of Differentiation (CD) 68 and CD163 (Santa Cruz Biotechnology, Dallas, TX) were used as a pan macrophage marker and M(Dex) marker, respectively. Fluorophore conjugation to the antibodies was performed using APEX Antibody Labeling Kits (Life Technologies, Carlsbad, CA). Alexa Fluor 488 was conjugated to CD68 and Alexa Fluor 647 was conjugated to CD163. Tissue sections were cut to a thickness of ~5 μm using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany). Sections were then mounted on microscope slides. Sections were fixed by placing them in cold methanol for 20 min at -20°C . The methanol was allowed to dry from the slide and the tissue sections were encircled with a hydrophobic pen. Blocking solution (PBS + 2% v/v horse serum + 0.05% (w/v) Tween 20 + 0.0001% (w/v) BSA) was then applied and slides were incubated in a humidity chamber for 30 min at room temperature. Once blocking was completed, slides were washed 4 times for 10 min each wash in phosphate buffered saline. Wash solution was then wicked away from the tissue sections using Kimwipes. Antibodies diluted in blocking solution were then applied to tissue sections, CD68 (1:125) and CD163 (1:50), and sections were incubated overnight at 4°C in a humidity chamber. Slides were then washed 4 times for 10 min per wash and excess wash solution was wicked away using a Kimwipe. A nuclear counterstain (Hoechst 34580) was then applied to the tissue sections and allowed to incubate at room temperature for 12 min in the dark. Slides were then washed 3 times for 5 min/wash and excess solution was wicked away using a Kimwipe. VECTASHIELD[®] (Vector Laboratories, Burlingame, CA) mounting media was then applied to tissue sections; cover slips were placed on slides and sealed. All images were obtained using a Leica TCS SP5 II confocal microscope (Leica Microsystems, Wetzlar, Germany). Images were then subjected to manual counts of cells staining positive for CD68 as well as CD68 and CD163. Using the data from the manual counts, the percentage of M(Dex) macrophages was determined.

2.8 Histological Analyses

Tissue sections (~5 μm) were obtained using a Leica CM3050 S cryostat (Leica Microsystems, Wetzlar, Germany) and mounted on microscope slides. Tissue sections were fixed in 10% neutral buffered formalin. Tissue sections were subjected to hematoxylin and eosin (H&E) as well as Masson's Trichrome stains. Images were obtained using a Zeiss Axioskop II plus microscope (Carl Zeiss Inc., Thornwood, NY) with Cannon EOS Digital Software for Rebel T2i camera.

2.9 Statistical Analysis

A Shapiro-Wilk test was performed to determine normal distribution of the data. For CCL2 concentrations, a Kruskal-Wallis ANOVA was performed with a Bonferroni post-hoc test to

determine significance. For the percent M(Dex) cells present, a two sample t-test was performed to determine significance. Origin 2015 statistical software was used for all statistical tests.

3. Results

3.1 qRT-PCR

Figure 1 shows the gene expression ratios of eleven different genes: Arginase (Arg₂), CCL2, CD163, CD206, Interleukin-1 receptor antagonist (IL-1ra), IL-6, IL-10, iNOS₂, Lipocortin-1 (Lipo-1), TGF- β 1, and TNF- α . Gene expression ratios were determined from tissue excised from around the microdialysis probe using qRT-PCR. IL-6, CCL2, IL-1ra, IL-10, Lipo-1, TGF- β 1, and TNF- α were chosen to determine if delayed Dex was able to alter the cytokines involved in the FBR at the gene level. IL-6 was found to be significantly up-regulated in response to delayed Dex treatment ($p < 0.001$). CCL2, IL-1ra, IL-10, Lipo-1, TGF- β 1, and TNF- α were not affected by delayed Dex treatment. Arg₂, CD163, CD206, and iNOS₂ were chosen to determine if Dex had any effect on indicators of macrophage activation state at the gene level and none of these were affected by delayed Dex treatment.

3.2 CCL2 Quantification

CCL2 concentrations were quantified in the dialysates collected from both control and treatment probes for each hour of the six hour collection period as well as the initial flush (Figure 2). Three days post implantation (Figure 2A), CCL2 concentrations were found to be highest in both the control and treatment dialysates during the initial flush with concentrations of $\sim 1500 \text{ pg mL}^{-1}$ and $\sim 1700 \text{ pg mL}^{-1}$, respectively. After the initial flush, CCL2 concentrations in the control dialysate dropped to $\sim 900 \text{ pg mL}^{-1}$ during the first hour of collection and remained within $\sim 200 \text{ pg mL}^{-1}$ of that concentration for the entirety of the collection period. In the treatment dialysate, CCL2 concentrations remained high, $\sim 2000 \text{ pg mL}^{-1}$, during the first hour of collection and then began to decrease over the remainder of the collection period with concentrations being $\sim 450 \text{ pg mL}^{-1}$ during the last hour of collection. No significant difference in CCL2 concentrations was seen in the treatment as compared to the control in any of the time points tested at three days post implantation.

Four days post implantation (Figure 2B), CCL2 concentrations were found to be the highest during the initial flush at $\sim 2750 \text{ pg mL}^{-1}$ and $\sim 2000 \text{ pg mL}^{-1}$ for control and treatment, respectively. After the initial flush, CCL2 concentrations in the control dialysate dropped and remained relatively constant, fluctuating from a high of $\sim 1500 \text{ pg mL}^{-1}$ to a low of $\sim 1000 \text{ pg mL}^{-1}$. In the treatment dialysate, CCL2 concentrations dropped during the first hour of collection to $\sim 1500 \text{ pg mL}^{-1}$. Interestingly, a spike in CCL2 concentration was seen during the second hour of collection in the treatment dialysate with values being the same as was seen in the initial flush, $\sim 2000 \text{ pg mL}^{-1}$. In hour 3, the concentration of CCL2 in the treatment dialysate fell to $\sim 450 \text{ pg mL}^{-1}$ and was found to be significantly lower than the concentration of CCL2 in the control dialysate. The concentration of CCL2 remained constant during hour 4 and showed a slight decrease in hours 5 and 6 with CCL2 concentrations being significantly lower in the treatment dialysates as compared to the control in hours 5 and 6.

Five days post implantation (Figure 2C), CCL2 concentrations were again found to be highest in the initial flush. After the initial flush, CCL2 concentrations reduced and were found to be relatively stable over hours 1–6 ranging from $\sim 1900 \text{ pg mL}^{-1}$ to $\sim 1200 \text{ pg mL}^{-1}$. In the treatment dialysate, CCL2 concentrations were found to steadily decrease from hours 1–6, ranging from $\sim 1300 \text{ pg mL}^{-1}$ to $\sim 200 \text{ pg mL}^{-1}$. In hours 3–6, the concentration of CCL2 was found to be significantly lower in the treatment dialysate as compared to the control dialysate.

Six days post implantation (Figure 2D), CCL2 concentrations were found to be $\sim 2400 \text{ pg mL}^{-1}$ and $\sim 2100 \text{ pg mL}^{-1}$ in the control and treatment dialysates during the initial flush, respectively. During hours 1–6, CCL2 concentrations remained fairly constant at $\sim 1500 \text{ pg mL}^{-1}$ with a range of $\sim 1750 \text{ pg mL}^{-1}$ - $\sim 1300 \text{ pg mL}^{-1}$ in the control dialysate. In the treatment dialysate, CCL2 concentrations were found to steadily decrease over the collection period from $\sim 1350 \text{ pg mL}^{-1}$ during hour 1 to $\sim 500 \text{ pg mL}^{-1}$ during hour six. Though CCL2 concentrations were seen to decrease over time in the treatment dialysate, significance was not reached in any of the collection periods tested.

3.3 Immunohistochemistry

Immunohistochemistry was used to investigate the density and number of total macrophages (CD68^+) compared with M(Dex) macrophages ($\text{CD68}^+\text{CD163}^+$) present in the tissue surrounding both control and treatment microdialysis probes. At lower magnifications (Figure 3A) few macrophages are seen surrounding the microdialysis probe in the control tissue. Further, there are minimal M(Dex), $\text{CD68}^+\text{CD163}^+$, macrophages seen with none seen less than $100 \mu\text{m}$ away from the microdialysis probe. In the tissue surrounding the treatment probe, at lower magnification, many macrophages are seen surrounding the probe with a high number of M(Dex) macrophages being present. At higher magnification (Figure 3B), few M(Dex) macrophages are seen in the tissue immediately surrounding the control probe. In the tissue surrounding the treatment probe, M(Dex) macrophages are seen immediately surrounding the probe with M(Dex) macrophages seen as close as $30 \mu\text{m}$ away from the membrane. The percent of M(Dex) macrophages found in the tissue surrounding both the control and treatment probes was determined via manual counts of macrophages staining CD68^+ as well as macrophages staining $\text{CD68}^+\text{CD163}^+$ in the high magnification (40x) images (Figure 3B). The percentage of M(Dex) macrophages found in the tissue surrounding the treatment probe was statistically higher than the percentage of M(Dex) macrophages found in the tissue surrounding the control probe (Figure 4).

3.4 Histology

Figure 5 shows the hematoxylin and eosin (H&E) and Masson's Trichrome analyses performed to determine the effects of delayed Dex infusion on the cellular density and collagen in the explanted tissue surrounding the microdialysis probe (7 days). The tissue surrounding the treatment probes was characterized as having a higher cellular density as well as more macrophages as compared to the control probe. Further, the tissue is better integrated surrounding the treatment probe as compared to the control as evidenced by the lack of acellular cytoplasmic material surrounding the treatment probe which is seen in the control. Of the cells seen surrounding the control probe, there was a higher number of

foreign body giant cells as compared to the treatment probe. The Masson's Trichrome showed there to be no appreciable difference in the amount of collagen seen in the tissue surrounding the treatment probe as compared to the control probe. Additionally, for both the control and treatment probes, few aggregated cells or foreign body giant cells were observed.

4. Discussion

Macrophages have long been recognized as pivotal cells for directing the foreign body reaction to implanted biomaterials [5]. Within the biomaterials community, there is a tremendous interest in modulating macrophage phenotype from what has been termed the M1, inflammatory phenotype, to the anti-inflammatory, M2, phenotype [13]. In order for wounds to heal properly, there are multiple stages through which a healing wound must pass [39]. In these stages, macrophages play many significant roles and removal of macrophages is detrimental to wound healing [43]. Early in the wound stage, macrophages release cytokines that attract additional leukocytes. Macrophages also clear neutrophils from the site allowing for the resolution of inflammation. It is believed that during this stage macrophages can begin to alter their phenotype to a reparative state stimulating tissue regeneration [6, 44]. Within this macrophage phenotype continuum at the host/biomaterial implant interface, there is an approximate three- to five-day window before the arrival of fibroblasts that begin to deposit collagen [45]. In this study, we chose to target altering macrophage phenotype within this approximate five-day window. While much work has been done to understand the different activation states of macrophages *in vitro*, understanding how shifts in macrophage activation states occurs *in vivo* is still lacking. Indeed, a review of many different studies aimed to affect or knockout different cells involved in wound repair concludes that no one cell type appears to be absolutely essential for repair, but inflammation is known to inhibit repair processes by either slowing down the process or causing excessive fibrosis [46].

Dexamethasone is a synthetic glucocorticoid known to have powerful anti-inflammatory and immunosuppressive effects. Dex has been widely used as a controlled-release agent in many different biomaterials studies as a means to dampen the fibrotic response [34, 47–50]. Macrophages possessing the M2c characteristic phenotype (CD163⁺) have been implicated as being positive for appropriate tissue remodeling at an implant site [51, 52]. Shifting macrophages to the M(Dex) (M2c) activation state has become of interest in the field of biomaterials as it is thought that by doing so, the biomaterial will better integrate into the surrounding tissue leading to reduced fibrosis, reduced scarring, and increased longevity of some biomaterials. Dexamethasone is known to be able to shift macrophages to an M2c activation state [8, 11]. This M2c [M(Dex)] characteristic phenotype has been shown to result in the increased expression of CD163 at the cell surface [53]. Additionally, since little is known about the time-course or optimum timing necessary for altering macrophage activation, we chose to investigate a 3-day post-implantation time for initiating Dex infusion. Others have suggested that immediate intervention to the wound healing response may not be the most effective way to control the FBR as interfering with normal wound healing processes leads to improper healing [54]. Following this rationale, allowing macrophages to enter the wound site prior to attempting to modulate their phenotype may prove to be more

effective in long-term reduced fibrosis, reduction in fibrotic capsule, and better integration of the biomaterial.

Delayed delivery of dexamethasone from the microdialysis probe resulted in the significant increase of IL-6 at the gene transcript level. Similarly, we showed in a previous study that immediate and daily infusion of Dex to a subcutaneous wound site in rats, resulted in the significant decrease in IL-6 gene transcription [37]. It should also be noted that IL-6 is known to exhibit either pro- and anti-inflammatory properties [55]. While this finding was unexpected, it may be due to the delayed delivery of Dex to the wound site. Additionally, while Dex has been widely used in biomaterials studies in the rat, direct measurements of cytokine gene or protein expression are lacking in the literature.

The delayed delivery of Dex to the wound site had no effect on the transcription levels of the remaining genes tested. In the cases of TNF- α and TGF- β 1, these results are not surprising as TNF- α levels have been shown to immediately rise following Dex administration [56] and the effects of Dex on TGF- β 1 have been shown to be highly variable [57–59]. These results were unexpected in the case of CCL2 which has been shown to be decreased in response to Dex in a rat pancreatitis model [60], and in response to locally delivered Dex to the subcutaneous space [37]. The inability of Dex to down-regulate CCL2 gene transcription may be due to the delayed delivery of the Dex, the concentration of the Dex delivered being insufficient to reduce CCL2, or a combination of the two. Further, this may be due to Dex being able to decrease CCL2 mRNA stability as opposed to transcription rates as has been shown *in vitro* [61].

In the case of IL-1ra, Dex has been shown to decrease transcript levels *in vitro* in human monocytes [62, 63]. Lipocortin-1 transcript levels have been shown to increase in response to dexamethasone treatment *in vitro* in rat astrocytoma cells [64]. In this study, no difference in the expression levels of IL-1ra or lipocortin-1 transcripts was observed. This may be due to the time course of the study in which tissue was harvested after four days of Dex infusion. In the case of lipocortin-1, previous up-regulation in gene transcription was seen four hours after Dex administration [64]. This might imply that the effects of Dex on transcript levels had already peaked and returned to basal levels at the time of tissue harvesting in this work. However, since direct protein concentration measurements were not performed, it is not possible to say if Dex had any effect on their concentrations.

The transcription levels of Arg₂, CD 163, CD 206, and iNOS₂ were chosen as markers of macrophage activation state. In M2c macrophages, protein levels of Arg₂, CD163, and CD206 are increased while iNOS₂ is decreased. Although there was no differential expression of these transcripts, this does not mean that there is no differential expression of the protein as it has been shown that transcript levels and protein levels are poorly correlated [65]. The immunohistochemical analyses show there to be an increase in the amount of CD163 protein present in response to Dex treatment. Since protein levels for Arg₂, CD206, or iNOS₂ were not measured in this study, it is inconclusive whether delayed delivery of Dex had an effect on their expression. Again, these differences may be due in part to the timing where CD163 transcript levels may have peaked prior to tissue harvest.

CCL2 is a chemokine responsible for the recruitment of monocytes to a wound site. This cytokine has been implicated as playing a significant role in wound repair and has recently been implicated in promoting healing in diabetic wounds by restoring macrophage response [66]. However, in other work, CCL2 knock-out mice have demonstrated an inability to form a fibrous capsule around implants [67]. In a previous study, when Dex was immediately infused through the dialysis probe, the concentration of CCL2 was decreased roughly three hours after infusion initiation [37]. However, this effect was not immediately apparent with the delayed Dex infusions. Here, the reduction in CCL2 concentrations was not altered until the second day (four days post implant) of Dex infusion. Additionally, the reduction in CCL2 concentration was also not as great as with immediate Dex infusion. Moreover, while some reductions in CCL2 concentrations were observed on the third day of infusion (5th day post-implantation), no significant alterations in CCL2 concentrations are observed on the fourth day (6th day post-implantation) of Dex infusion. While in all cases, the CCL2 concentrations decrease after initiation of the dialysis collection, what is notable is the initial collected concentrations in the flush collection. While many microdialysis sampling practitioners would generally not even analyze initial flush solutions through the probe, this is actually fluid that may be more representative of the approximate external concentrations residing in the extracellular fluid space (ECS) in the tissue in contact with the dialysis probe. The dead volume within the dialysis probe shaft is roughly 1 μL . However, if solutes diffuse into the shaft, they can continue to diffuse through the tubing lines. While these initial flush concentrations were variable from day to day, the average is roughly 2000 to 2500 pg mL^{-1} . The decreasing concentrations of CCL2 after initiation of dialysis indicate that CCL2 production in the ECS is not sufficient to compete with the dialysis removal process. However, the significant decrease in CCL2 with Dex-delivery in some days suggests bioactivity of the Dex at the tissue site. Why this decrease in CCL2 is not as significant as with the daily infusions of Dex that we have previously reported is not clear. The lack of difference in CCL2 concentrations between control and treatment six days post implantation may suggest that after three days of Dex infusion, higher concentrations of Dex are needed to illicit the same effect. This may also be due to the arrival of more CCL2-secreting cells, such as macrophages, to the tissue surrounding the treatment probe, as is seen in the histological analyses. These macrophages may be receiving competing endogenous signals from within the extracellular space such that addition of Dex does not effectively modulate the CCL2 levels.

Immunohistochemical analyses were performed to determine the effects of delayed Dex infusion on the amount of macrophages (CD68^+) present in the tissue surrounding the microdialysis probe as well as the amount of M(Dex) ($\text{CD68}^+ \text{CD163}^+$) macrophages present. The interesting finding here is that $\text{CD68}^+ \text{CD163}^+$ macrophages are not found adjacent to the dialysis probe, but rather further away ($\sim 100 \mu\text{m}$ in control tissue and $\sim 30 \mu\text{m}$ in treatment tissue). This is consistent with a recent scaffold implant study in rats that measured CD68 and CD206 (a pan M2 macrophage marker), but not CD163, where they observed CD68^+ cells near the scaffold/tissue interface and $\text{CD68}^+ \text{CD206}^+$ cells farther away.[68] While these findings are consistent, we note that CD206 was not quantified in this study. While all M2 macrophages would be expected to express CD206, we have not stained for this marker in this work and thus cannot say if this would hold true in this work. More

importantly, CD68⁺CD163⁺ macrophages are observed in both control and treatment tissue, but with more M(Dex) macrophages at the treatment site. This suggests that while the biomaterial itself may result in the previously described M2c activation state (CD68⁺CD163⁺), it is clear that adding Dex appears to promote a significantly higher wound healing macrophage phenotype. Additionally, few foreign body giant cells were observed in this study which is consistent with a recent non-degradable implant study in the rat [69].

Dexamethasone has been previously used extensively in biomaterials studies in an effort to reduce fibrosis where histological analyses were used as an endpoint [48–50, 70]. These studies have shown that tissue surrounding dexamethasone eluting materials are characterized as having reduced cellular density. We have previously reported that immediate infusion of Dex results in a loose capsule surrounding the dialysis probe resulting in difficulties in obtaining the tissue for histological analysis[37, 71]. However, with delayed Dex infusion, the results demonstrated an increased cellular density surrounding the treatment probe as compared to the control probe. Further, we found no difference in the amount of collagen present in the tissue surrounding the treatment probe as compared to the control probe. These differences may be attributed to three factors: 1) initial burst associated with other techniques, 2) the time course of Dex infusion, 3) the concentration of Dex used. While many controlled release biomaterials suffer from initial bursts of drug (dexamethasone), the microdialysis sampling approach allows a constant (zero-order) delivery of Dex. Further, controlled release biomaterials begin eluting drug immediately following implantation (the initial burst) and continuously deliver the drug over time. Our technique allows for the delivery of Dex for defined amounts of time with the ability to begin delivery at any given time post implantation. This is important due to increasing knowledge that an initial wounding response is necessary to elicit appropriate wound healing [39, 54]. Finally, the concentrations of Dex used in this study differ from the previously mentioned studies. In the previously mentioned studies, a concentration range from 5.3 µg-7 mg of Dex was used. In this work, ~3.6 µg of Dex was delivered per six hour collection period resulting in a total of ~14.4 µg of Dex being delivered over the entire collection period. It should be noted at this point that the ~14.4 µg of Dex does not take into account the Dex which was allowed to remain in the probe and freely diffuse out between collection periods. While our previous study showed ~10.6 µg of Dex to be sufficient to reduce cellular density around the treatment probe, this was in response to immediate Dex delivery following implantation [37] while in this study Dex was not delivered until three days post implantation. To our knowledge, this is the first study to look at the effects of delayed Dex infusion on a subcutaneous implant site. An additional unknown variable is that we do not know the range of Dex actually needed to induce macrophages to the CD68⁺ CD163⁺ state.

5. Conclusions

In this study, microdialysis sampling probes were implanted in the subcutaneous space of rats and used to locally deliver Dex over a four day period starting three days post probe implantation. The delayed delivery of Dex resulted in an up-regulation of IL-6 gene transcripts as well as a moderate decrease in collected CCL2 concentrations. Histologically, the delayed Dex treatment resulted in an increase in cellular density in the tissue

surrounding the microdialysis probe. Further, the delayed delivery of Dex was able to shift macrophages to an M(Dex) activation state (CD68⁺CD163⁺ macrophages (M2c) in the tissue). The use of modulators to shift the activation state of macrophages has focused primarily on *in vitro* studies. This is the first study to show that a delayed delivery of Dex can be used to shift macrophages to an M(Dex) activation state *in vivo* at a non-degradable implant site. However, the limitations of this present study include the explant time (7-days) and whether the appropriate macrophage phenotype would remain after suspending the infusion of modulator. Continuous release of modulator throughout the lifetime of an implant would likely not be feasible.

Acknowledgments

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Figure 1. Relative Gene Expression Ratios between Treatment and Control Tissue

Tissue samples from around the microdialysis probe were used to determine relative gene expression ratios of eleven different genes in response to delayed dexamethasone-21-phosphate (20 µg/mL) treatment. (n=3) **p 0.001 Error bars represent SEM.

Figure 2. Concentration of CCL2 Collected in Dialysate

Collections performed 3 days (A), 4 days (B), 5 days (C), and 6 days (D) post implantation. n=8 (for A, B, C) and n=6 (for D) where n represents the number of animals, *p < 0.05 with the error bars representing the SEM, F represents the initial flush.

Figure 3. Immunohistochemical Staining of Tissue Surrounding a Microdialysis Probe
 Immunohistochemistry was used to identify CD68⁺ (green) cells, CD163⁺ (red) cells, and nuclei (blue). Cells which show an overlap of colors (stain both red and green or yellow/orange) represent a M(Dex) macrophage. **A)** Tissue surrounding both control and treatment microdialysis probes at 20X magnification. **B)** Tissue surrounding both control and treatment microdialysis probes at 40X magnification. M indicates the microdialysis probe membrane. Scale bars in lower right of each image represent 100 μ m (20X) and 50 μ m (40X). Images representative of three animals.

Figure 4. Percent of CD68⁺ Macrophages which Stain CD68⁺CD163⁺ [M (Dex)] Macrophages Surrounding a Microdialysis Probe

Bar graph representing the percentage of M(Dex) macrophages found in the tissue surrounding both the control and treatment probe. Error bars represent the SEM where *p 0.01, n=12 where n represents the number of measurements.

Figure 5. Histological Staining of Tissue Surrounding a Microdialysis Probe

Top: H&E Stained tissue (Six panes) (nuclei-blue, eosinophilic structures-red, basophilic structures-purple, erythrocytes-bright red). **Bottom:** Masson's Trichrome stained tissue (Six panes) (nuclei-dark brown/black, cytoplasm pink/light red, collagen-blue) **A)** Control and treatment tissue immediately surrounding a microdialysis probe, 10X magnification, 100 μ m scale. **B)** Control and treatment tissue distal to the microdialysis probe, 10X magnification, 100 μ m scale. **C)** Control and treatment tissue immediately surrounding a microdialysis probe, 40X magnification, 20 μ m scale bar. Arrows mark some of the foreign body giant cells surrounding the probe. Images representative of three animals.