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Colleen McLoughlin
Virginia Commonwealth University

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IN VIVO IMMUNOTOXICOLOGICAL EVALUATION OF ELECTROSPUN
POLYCAPROLACTONE (EPCL) AND INVESTIGATION OF EPCL AS A DRUG
DELIVERY SYSTEM FOR IMMUNOMODULATORY COMPOUNDS

A dissertation submitted in partial fulfillment of the requirements for the degree of
Doctor of Philosophy in Biomedical Engineering at Virginia Commonwealth University.

by

COLLEEN ELIZABETH MCLOUGHLIN
B.S., Virginia Commonwealth University, 2005

Director: GARY L. BOWLIN, PH.D.
PROFESSOR, DEPARTMENT OF BIOMEDICAL ENGINEERING
Co-Director: KIMBER L. WHITE, JR. PH.D.
PROFESSOR, DEPARTMENT OF PHARMACOLOGY AND TOXICOLOGY

Virginia Commonwealth University
Richmond, Virginia
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List of Abbreviations

AAGM1	anti-asialo GM1
AFC	antibody forming cell
ANOVA	analysis of variance
B-cell	B lymphocyte
C	Celsius
<i>C. albicans</i>	<i>Candida albicans</i>
CD	cluster of designation
CFA	complete Freund's adjuvant
CIA	collagen-induced arthritis
CMI	cell-mediated immunity
CPM	counts per minute
CPS	cyclophosphamide
CTL	cytotoxic T lymphocytes
DBA/2	stimulator mouse for mixed leukocyte response
DEU	dexamethasone-equivalent units
DEX	dexamethasone

DTH	delayed-type hypersensitivity
E:T	effector-to-target
EBSS	Earle's balanced salt solution
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetate
ELISA	enzyme-linked immunosorbent assay
EPCL	electrospun polycaprolactone
EPCL-DEX	electrospun polycaprolactone with dexamethasone
FITC	fluorescein isothiocyanate
g	grams
IFA	incomplete Freund's adjuvant
h	hour
hBMSCs	human derived bone marrow stem cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HFP	1,1,1,3,3,3-hexafluoro-2-propanol
i.p.	intraperitoneal
i.v.	intravenous
IACUC	institutional animal care and use committee
Ig	immunoglobulin
IgM	immunoglobulin M

kg	kilogram
KLH	keyhole limpet hemocyanin
L	liter
LDC	lidocaine
Log ₂	logarithm to the base 2
LPS	lipopolysaccharide
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MCV	Medical College of Virginia
MEM	minimum essential media
mg	milligram
mg/kg	milligram per kilogram
ml	milliliter
MLR	mixed leukocyte response
mM	millimolar
NA	naïve
NK	natural killer
NTP	National Toxicology Program
OD	optical density

PAMPs	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PC	positive control
PCL	polycaprolactone
PDO	polydioxanone
PE	phycoerythrin
PI	propidium iodide
PMN	polymorphonuclear
PRP	platelet-rich plasma
PRRs	pattern recognition receptors
RBCs	red blood cells
RPMI	Roswell Park Memorial Institute
s.c.	subcutaneous
SE	standard error
SEM	scanning electron micrograph
SH	sham control
SF	silk fibroin
sRBC	sheep red blood cell
T-cell	T lymphocyte
TF	tissue factor

TH	T helper cells
VCU	Virginia Commonwealth University
VH	vehicle
x g	times gravity
YAC-1	target cell for natural killer cell assay
μCi	micro Curie
μl	microliter

Abstract

IN VIVO IMMUNOTOXICOLOGICAL EVALUATION OF ELECTROSPUN
POLYCAPROLACTONE (EPCL) AND INVESTIGATION OF EPCL AS A DRUG
DELIVERY SYSTEM FOR IMMUNOMODULATORY COMPOUNDS

By Colleen Elizabeth McLoughlin, Ph.D.

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Engineering at Virginia Commonwealth University.

Virginia Commonwealth University, 2012

Major Director: Gary L. Bowlin, Ph.D.
Professor, Department of Biomedical Engineering

Co-Director: Kimber L. White, Jr., Ph.D.
Professor, Department of Pharmacology and Toxicology

Electrospun materials have potential use in many biomedical applications such as soft tissue replacements or as scaffolds to target drug delivery to local sites. Electrospinning is a polymer processing technique that can be used to create materials composed of fibers with diameters ranging from the micron to the nanoscale. We investigated the effects of microfibrinous and nanofibrinous electrospun polycaprolactone

(EPCL) on innate, cell-mediated, and humoral components of the immune system. Results demonstrated that in both young (12 week) and old (6 month) mice, EPCL had no effect on various immune parameters. With its lack of immunotoxicity, EPCL presents an excellent polymer scaffold for use in delivering drugs to local sites. Drug delivery studies focused on using EPCL nanofiber scaffolds with the known immunosuppressive compound dexamethasone (DEX) incorporated within the matrix. The ability of the EPCL-DEX scaffold to suppress cell-mediated immunity (CMI) was evaluated using the delayed-type hypersensitivity (DTH) response to *Candida albicans*. Preliminary studies were conducted following subcutaneous implantation of a single disk (6-mm or 3-mm diameter) with 3, 10, 30, or 100 % w/w DEX in EPCL in the thigh region. Based on footpad swelling, dose-responsive suppression of the DTH was observed based on DEX equivalent units (DEU) at all but the lowest dose. The animals that received the high dose (100% in 6-mm) had decreased spleen weights, however no change in spleen weight was observed at the lower doses. Thymus weights were only affected at the four highest doses. These preliminary results suggest that implantation of a drug-containing electrospun scaffold may achieve local immunosuppression without systemic toxicity. Finally, we evaluated the EPCL-DEX scaffold in an acute inflammatory model (keyhole limpet hemocyanin) and a mouse model of rheumatoid arthritis (collagen induced arthritis). While similar trends were observed in the other models, the EPCL-DEX system achieved greatest success in the DTH model.

CHAPTER 1: Introduction

Tissue engineering, nanomedicine, and nanotoxicology

Tissue engineering is an interdisciplinary field requiring participants from engineers, biologists, immunologists, and clinicians, amongst others, which has grown widely since the late 1980s. Tissue engineering aims to create therapies that can improve, maintain, or restore tissue function, typically through the creation of a scaffold intended to mimic native tissue [Boland et al. 2004a]. The goal in creating a scaffold is generally to mimic both the structure and function of the extracellular matrix (ECM). An important characteristic is size and since the ECM has fibers on the order of nanometers this leads to the development of nanomaterials for use in tissue engineering. Nanobiomaterials and tissue-engineered products can also be considered a part of the larger field of nanomedicine.

The use of nanomaterials in medicine can offer many advantages but also cause concern about adverse effects due to the potential for the same characteristics that make nanomaterials attractive for use can lead to unique toxicity profiles. In addition to tissue engineering therapies, nanomedicines include therapies for drug delivery and diagnostic tools [Kagan et al. 2005]. Common materials range from metals to carbon-based nanomaterials, metal-based nanomaterials, biologicals, nanoceramics and nanocomposites, to nanopolymers [Zhao and Castranova 2011]. The focus here is on nanofibrous polymers, and biocompatibility and toxicity

testing of nanoparticles, with emphasis on immune responses to biomaterials and immunotoxicity.

Nanofibrous polymers and processing techniques

Due to the limitation of conventional polymer processing techniques being unable to achieve fiber diameters smaller than 10 μ m, methods of nanofiber generation have been developed which include self-assembly, phase separation, and electrospinning. Self-assembly allows for the creation of the smallest fiber diameters (5-8nm), however, it involves a difficult laboratory procedure which is limited to a small number of polymers. Phase separation offers several advantages including high control over architecture, batch-to-batch consistency, and a simple process, however the technique is limited to few polymers and produces a low yield. Electrospinning has been highly favored by researchers because it is easy and inexpensive, can be used with a large variety of natural and synthetic polymers, and provides the ability to control fiber size on the nano to micron scale, however disadvantages include that the smallest fiber sizes tend to be larger than other methods and the use of organic solvents [Barnes et al. 2007].

Biocompatibility and toxicity testing of nanoparticles

Biocompatibility testing, including most often *in vitro* cytocompatibility and hemato-compatibility tests, can provide important initial insight for biomaterial testing. According to the Williams definition of biocompatibility for tissue-engineering products “The biocompatibility of a scaffold or matrix for a tissue-engineering product refers to the ability to perform as substrate that will support the appropriate cellular activity, including the facilitation of molecular and mechanical signaling systems, in order to optimize tissue regeneration, without

eliciting any undesirable effects in those cells, or inducing any undesirable local or systemic responses in the eventual host” [Williams 2003]. In spite of this definition, the term “biocompatibility” pervades the literature with inconsistent use and is often applied without knowledge of the *in vivo* response that may include systemic effects unable to be assessed *in vitro*. Of particular interest for nanopolymers is the potential of degradation products on the nanoscale to have adverse effects. Upon implantation of biomaterials, immune cells immediately come in to contact with the material and therefore the immune response is of particular importance [Ratner et al. 2004].

The immune system and response to biomaterials

The immune system protects the body from infection through physical barriers (skin), cells, and organs. The immune system is divided into two main branches, innate immunity, and acquired immunity. Innate immunity is the first to respond and includes skin, macrophages, neutrophils, natural killer cells, and complement proteins. Innate immune responses are directed against generic pathogens. Pathogens have pathogen-associated molecular patterns (PAMPs) which are recognized by innate immune cells through pattern recognition receptors (PRRs). The innate response is rapid. In contrast to the innate immune response, the acquired immune response is specific to antigen, and is slow to respond following the first contact with a new antigen. The cellular components, T-cells and B-cells can form antigen-specific memory and upon re-exposure respond quickly to the antigen [Janeway, 2008]. Upon implantation of a biomaterial, injury and inflammation occurs and neutrophils and monocytes infiltrate the site. Due to protein absorption on the biomaterial surface, complement activation occurs activating macrophages. The acute inflammatory phase is followed by a chronic inflammatory and foreign

body reaction both mediated by macrophages [Franz et al. 2011]. Currently, researchers are exploring ways to change the immune response to biomaterials at the surface through surface modifications, incorporation of bioactive molecules, and through incorporation of drugs and growth factors [Franz et al. 2011]. However, the focus of immune response testing remains primarily in the surface phenomenon and little attention has been given to potential systemic immunomodulatory effects of biomaterials/nanopolymers.

In several works by Smith et al. an immunotoxicological approach was used to study several electrospun polymers, primarily blends of polydioxanone and collagen and polydioxanone and elastin, on immune cell function responses *in vitro* [Smith et al. 2007, Smith et al. 2009, Smith et al. 2010]. Results indicated that some electrospun polymers can potentially cause immunotoxicity to innate and acquired immune responses *in vitro*. Recently, work has been done to examine an electrospun polymer, polycaprolactone, (or EPCL) for potential immunotoxicity *in vivo*. McLoughlin et al. studied both microfibrinous and nanofibrinous EPCL and found neither configuration affected innate or acquired immune functions tested [McLoughlin et al. 2012].

CHAPTER 2: Research Design

Rationale and Major Hypotheses

The research goal was to investigate the immunomodulatory effects of EPCL utilizing both micron and nano-sized fiber diameters, *in vivo*, in both young adult (3 months of age) and aged (6-month-old) mice. Based on these results showing a lack of effect with either fiber size on either age group, a preliminary study was conducted to establish doses of dexamethasone-loaded EPCL able to deliver the drug locally to an inflamed joint in both an acute and a systemic inflammatory model without systemic effects (including spleen and thymus weights). To this end, we proposed the following hypothesis: *in vivo* exposure to dexamethasone-loaded electrospun polycaprolactone will dose-responsively cause suppression of local joint and footpad swelling in mouse inflammatory models with minimal systemic immunosuppression.

Specific Aims

In order to test the hypotheses four specific aims were developed. To test the hypothesis that nanofibrous EPCL would cause greater dose-responsive immunosuppression than microfibrous EPCL and that aged animals would be more susceptible to the immunotoxic effects specific aim 1 was developed. Specific aim 1 was to determine the effects of electrospun polycaprolactone materials on various innate and acquired immune responses following *in vivo*

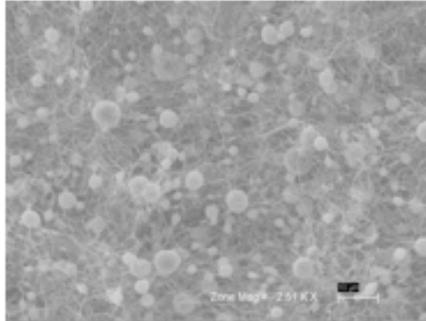
exposure of 3-month-old (young adult) B6C3F1 mice to determine effects using nano fiber diameter electrospun materials and effects using micron fiber diameter materials. In order to simulate an older individual we used 6-month-old B6C3F1 mice and examined both innate and acquired immune responses following *in vivo* exposure to electrospun polycaprolactone graft material and compared effects of nano and micron fiber diameters on the immune responses. Specific aims 2-4 were developed to test the hypothesis that *in vivo* exposure to dexamethasone-loaded electrospun polycaprolactone will dose-responsively cause suppression of local joint and footpad swelling in mouse inflammatory models with minimal systemic immunosuppression. Specific aim 2 was to determine doses to be used in acute and systemic inflammatory models using the acquired immune model of delayed-type hypersensitivity in response to *C. albicans*. Specific Aim 3 was to determine the dose-response and time-course effects of dexamethasone (DEX) loaded EPCL (DEX-EPCL) in the acute inflammatory model keyhole limpet hemocyanin (KLH). Specific Aim 4 was to determine dose-response and time-course effects of dexamethasone (DEX) loaded EPCL (DEX-EPCL) in a mouse model of rheumatoid arthritis: collagen-induced arthritis (CIA). Additionally, the CIA model was developed in the B6C3F1 mouse strain.

Establishing Scaffolds

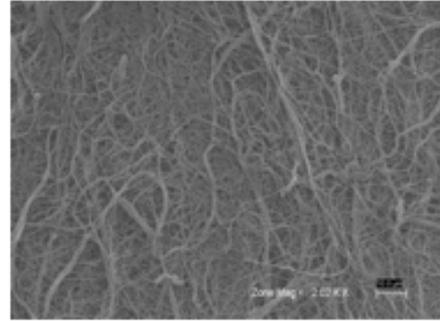
EPCL was chosen based on results by Smith et al. that a 50:50 blend of PDO:PCL was immunosuppressive in the Mishell Dutton assay, an *in vitro* test of humoral immunity [Smith et al. 2007]. Additionally, by choosing a single synthetic polymer the question of the role of fiber size was more easily addressed without a protein component or multiple polymers adding covariables. In order to test whether fiber diameter would play a role in immunotoxicity of EPCL

scaffolds, the concentration of polymer was varied and fiber sizes of resulting scaffolds were measured using ImageJ (NIH) software. Results shown in Figure 1 demonstrate that with a low concentration of 50 mg/mL PCL electrospinning occurred, with a high concentration of 250 mg/mL large fused fibers occurred, primarily nanofibrous scaffolds resulted with a concentration of 80 mg/mL and microfibrinous scaffolds resulted with a concentration of 200 mg/mL.

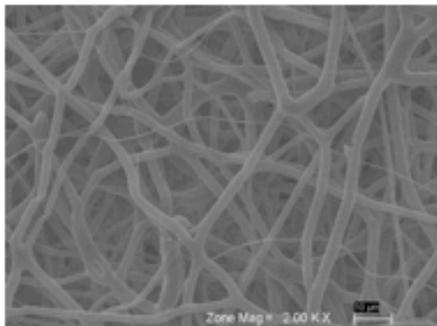
Electrospraying
50 mg/mL



Nanofibrous EPCL
80 mg/mL



Microfibrous EPCL
200 mg/mL



Fused Fibers
250 mg/mL

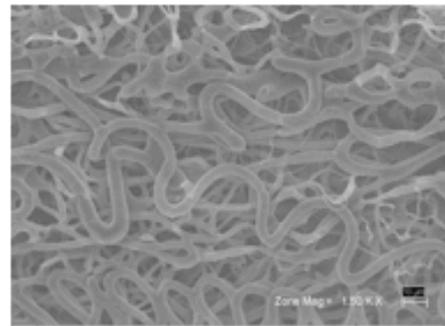


Figure 1. Nanofibrous and microfibrinous EPCL scaffolds were achieved by varying polycaprolactone concentration.

CHAPTER 3: Immunotoxicological Investigation in Young Animals

The following manuscript has been published in Biomedical Materials. The work demonstrates the lack of effect on innate, humoral, and cell-mediated immune endpoints following *in vivo* implantation of EPCL in young mice.

Evaluation of innate, humoral, and cell-mediated immunity in mice following *in vivo* implantation of electrospun polycaprolactone

Colleen E. McLoughlin^{1,2,3}, Matthew J. Smith², Wimolnut Auttachoat², Gary L. Bowlin¹, and Kimber L. White, Jr.²

¹Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA

²Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA

³To whom all correspondence should be addressed
Email mcloughlince@vcu.edu

Abstract

Electrospun polycaprolactone (EPCL) is currently being investigated for use in tissue engineering applications such as vascular grafts. However, the effects of electrospun polymers on systemic immune responses following *in vivo* exposure have not previously been examined.

The work presented evaluates whether EPCL in either a microfibrillar or nanofibrillar form affects innate, humoral, and/or cell-mediated immunity using a standard immunotoxicological testing

battery. Holistic *in vivo* endpoints examined include the antibody-forming cell assay (AFC or Plaque Assay) and the delayed-type hypersensitivity (DTH) response to *C. albicans*. In addition, natural killer cell cytotoxic activity was assessed using an *ex vivo* assay and splenic cell population phenotypes were analyzed by flow cytometry for material exposure-related changes. Results indicated that 28-day subcutaneous implantation of EPCL, either in microfibrinous or nanofibrinous forms, did not affect the systemic functions of the immune system in 12-16 week old female B6C3F1 mice.

Introduction

Electrospinning is being utilized by many researchers because it has proven to be a cost effective, relatively easy method to create complex three-dimensional tissue-engineered structures, with specific mechanical properties, and with fiber sizes in the micron down to the nanometer range. Native extracellular matrix (ECM) fibers are on the order of 50-500 nm, a size that researchers strive to replicate. The advent of electrospinning biomaterials has attracted many researchers to use the technology because it allows for the creation of small ECM sized fibers; where previous technology allowed only for fiber diameters down to 10 μm [Boland et al. 2004a]. Although electrospun polymers are being widely investigated for use in many biomedical applications, the immunomodulatory effects of electrospun biomaterials implanted *in vivo* have not been comprehensively examined. Previously, *in vitro* studies of electrospun materials have shown effects on both the innate and acquired immune responses [Smith et al. 2009, Smith et al. 2010]. The current work is the first to evaluate *in vivo* effects on the immune system by an electrospun synthetic polymer, polycaprolactone (PCL). Additionally, the potential role of fiber diameter in the toxicity of electrospun materials was examined. To this end, the

studies presented herein were designed to evaluate the potential dose-responsive effects of microfibrinous and nanofibrinous electrospun polycaprolactone (EPCL) on the innate and acquired immune system following *in vivo* subcutaneous (s.c.) implantation. The approach utilized was a standard immunotoxicological testing battery as recommended by Luster et al. using well-known, validated assays that have been shown to be sensitive to detecting immunomodulatory effects [Luster et al. 1988, Luster et al. 1992].

Polycaprolactone has potential for use in many soft tissue applications due to its many favorable properties. Polycaprolactone is a synthetic biodegradable polymer that is semicrystalline, has a low melting point (59-64°C), high solubility, and low glass transition temperature (-60°C). PCL is degraded by hydrolysis through cleavage of ester bonds similar to the degradation of other biodegradable polymers, such as poly(lactic acid) and poly(glycolic acid), but at a comparatively slow rate of over one year. PCL can be used to easily form copolymers, which allows for the manipulation of various properties [Kohn et al. 2004]. PCL has been studied in basic toxicological studies for use in an implantable contraceptive device (the Capronor system) without any systemic toxic effects observed [Pitt 1990]. The local and inflammatory responses to biomaterials are very important to characterize. To this end, several *in vivo* and *in vitro* studies have been conducted to evaluate the inflammatory response to nanofibrinous polycaprolactone and have shown an inflammatory response does occur with fibrous capsule formation regardless of topographical structure [Giavaresi et al. 2006]. However, the goal of the work presented in this manuscript was to evaluate the systemic immune response to EPCL.

EPCL is currently being investigated for use in cartilage, bone, urologic, nerve, and vascular tissue engineered constructs [Alves da Silva et al. 2010, Fang et al. 2010, Kundu et al.

2011, Hong and Kim 2010, McClure et al. 2010]. Surprisingly, even though EPCL is being investigated extensively for a large range of applications, little work has been done to evaluate immune responses outside of biocompatibility assays, which are typically *in vitro* tests, which are not holistic. Biocompatibility as defined by Williams is a term meant for a material used in a specific application even though the term is often used as general material property [Williams 2003]. PCL is generally considered biocompatible and nontoxic due to studies conducted for its use in a contraceptive device (the Capronor system, an inch long tube of PCL filled with levonorgestrel [Hypotenuse 1988]). However holistic evaluation of PCL electrospun with micron or nanofibers has not previously been extensively evaluated following *in vivo* implantation [Pitt 1988]. PCL was chosen for evaluation in these studies based on previous finding by Smith et al. that a 50:50 blend of polydioxanone and PCL was immunosuppressive in the Mishell-Dutton assay [Smith et al. 2007]. Additionally, a single polymer was chosen in order to test if fiber size would impact immunotoxicity based on results by Boland et al. that demonstrated improved biocompatibility test results with poly(glycolic acid) electrospun scaffolds with smaller mean fiber diameters and results of Sanders et al. showing that smaller diameter polymer fibers had reduced fibrotic capsule thickness [Boland et al. 2004b, Sanders et al. 2000]. It should be noted the mean fiber diameter of the “nanofibrous” implants does not meet the prevalent definition of a nanomaterial, which as defined by the EU is less than 100 nm [Liden 2011]. However, individual fiber diameters vary and the terms nanofibrous and microfibrinous have been used to differentiate between the form with fiber diameters predominately above one micrometer and the form with fibers predominately less than one micrometer. In addition, the less than 100 nm definition for nanomaterials has been controversial and yet to be formally adopted by regulators outside of the EU [Liden 2011].

The literature provides few reports on EPCL with respect to immune function. Wolfe et al. evaluated acute monocyte tissue factor (TF) expression *in vitro* for silk, EPCL, electrospun PDO, e-PTFE by an In-Cell western assay [Wolfe et al. 2010]. Results demonstrated low levels of TF expression for EPCL and suggested that EPCL is unlikely to cause greater risk of acute thrombotic occlusion as compared to e-PTFE, which is currently used clinically in vascular replacement applications. Williamson et al. evaluated human vascular endothelial cell attachment, proliferation, and bioactivity *in vitro* with an electrospun composite of PCL and polyurethane [Williamson 2006]. Results indicated that endothelial cells attached to the PCL surface forming a monolayer, proliferate, and normally release von willebrand factor and nitric oxide. Nottelet et al. were able to successfully replace the abdominal aorta in Sprague-Dawley rats with an EPCL graft. Their findings, after 12 weeks of implantation, were that the grafts were patent and fully endothelialized (however with some intimal hyperplasia) and that cellular infiltration by macrophages and fibroblasts had occurred [Nottelet et al. 2009]. Overall, these studies suggest that EPCL has excellent blood compatibility and cytocompatibility, however no conclusions regarding its immunocompatibility can be drawn.

The immune system, with innate and acquired (cell mediated and humoral) immunity, is composed of cells, organs, and soluble factors which protect the body from foreign and infectious agents. Innate immunity includes the skin, phagocytic cells (such as macrophages and polymorphonuclear cells (PMN)), natural killer (NK) cells, and various soluble factors including those of the complement system. Innate immunity is the first defense against pathogens but is not specific to the pathogen. Acquired immunity is divided into cell-mediated immunity (T cell) and humoral immunity (B cell/antibody-mediated). Acquired immune responses are specific to the pathogen (antigen) but are slower to respond. Each component of the immune system has a

specific function, yet all parts are integrated with each other and with other organ systems, including the endocrine and nervous systems. Inappropriate modulation of the immune system can result in immunosuppression and decreased ability of the host to defend itself against pathogens. Alternatively, an enhanced response to “self” antigens can result, which may lead to the formation of autoimmunity [Janeway 2008].

The work presented evaluates the potential of both microfibrinous and nanofibrinous EPCL to produce immunomodulatory effects following 28 days of exposure *in vivo* in 12-16 week old female B6C3F1 mice. Splenocyte phenotypic changes were evaluated using flow cytometry, and innate immunity was assessed using the natural killer cell activity assay. To assess effects on humoral immunity, the holistic measure of the T-dependent antibody response (AFC assay) and the sheep red blood cell (sRBC) ELISA were used. Finally, cell-mediated immunity was evaluated using the holistic delayed-type hypersensitivity response.

Materials and Methods

Scaffold Preparation

PCL (molecular weight 65 000 kDa, Sigma-Aldrich) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP; TCI America) at concentrations of 80 mg/mL and 200 mg/mL for nanofibrinous and microfibrinous scaffolds, respectively. In order to obtain equal thickness (approximately 0.3 mm), 8 mL was used for nanofibrinous and 2 mL for microfibrinous scaffolds. Using a KD Scientific syringe pump, solutions were dispensed at 8 mL/h (from a 10 mL syringe for nanofibrinous and a 3 mL syringe (Becton Dickinson) for microfibrinous with an 18 gauge blunt tip needle) and electrospun onto a rectangular mandrel (7.7 cm x 3.2 cm x 30.5 cm). A fixed voltage of 25 kV was applied to the needle by a Spellman CZE1000R high voltage power supply

(Spellman High Voltage Electronics Corp., Hauppauge, NY). The grounded mandrel had a rotational speed of 500 rpm and translational speed of 2 cm/s over a translational distance of ± 3.75 cm. Circular pieces 6 mm diameter (representing 32 mm² surface area) were punched using a single hole paper punch (Harold's Business Supply, Richmond, VA) and then disinfected in 100% ethanol for 30 min followed by three 10-minute washes in physiological saline.

Scanning Electron Microscopy

Representative samples were sputter-coated with gold (Model 5550; Electron Microscope Sciences) and scanning electron microscopy (SEM, Zeiss EV050) was used to obtain micrographs. Micrographs were analyzed to determine fiber size using ImageJ (NIH) by taking 80 random measurements from throughout the image and obtaining the mean and standard error.

Animal Husbandry and Exposure

All animal procedures were conducted under an animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University (VCU). Female B6C3F1 (Taconic Farms) were housed four per cage, and started on study between 12-16 weeks old. Animals were fed NTP 2000 diet and provided tap water ad libitum. Vivarium conditions were maintained at 21-24°C and 40–70% relative humidity, with a 12-h light/dark cycle. Mice were determined to be free of *M. pulmonis*, coronavirus, and Sendai virus by murine Immunocomb test kits (Charles River). Following randomization, eight mice were assigned to each dose group. For each implant, a small incision (approximately 1 cm) was made on the ventral side below the zyphoid process and above the acetabulofemoral joint. By blunt dissection using Miltex 18-780 forceps (Thomas Scientific, Swedesboro NJ) a small pocket was

prepared s.c. to insert the material. The incision sites were closed using surgical staples. The low dose animals received 1 implant (total surface of 32 mm²), the middle dose received 2 implants (64 mm²), and the high dose 4 implants (128 mm²). Sham (negative control) animals received the same surgical manipulations as the high dose animals, however they did not receive any implants. Animals were administered lidocaine topically (lidocaine has been tested in the laboratory and shown to have no effect on the holistic AFC and IgM ELISA response to sRBCs following 3 days of exposure) on each incision site daily for 3 days after surgery, and staples were removed on or after 5 days following surgery. During this postoperative period, animals were closely monitored for signs of infection with no infections encountered during the studies.

Toxicological Studies

Animal body weights were obtained at the start and end of study to determine the effects of EPCL on body weight. When the endpoint required the excision of the spleen, the spleens were excised and weighed. In addition, a study was conducted for each form of EPCL where the thymus, lungs, liver, spleen, and kidneys with adrenals were excised and weighed and evaluated as both absolute and percent of body weight.

Splenocyte Single-cell Suspension Preparation

On day 29 of implantation, mice were anesthetized by CO₂ inhalation and then euthanized by cervical dislocation. Spleens were excised and placed in 3 mL of Earle's Balanced Salt Solution (EBSS) with 15 mM HEPES (GibcoBRL, Grand Island, NY, USA). Splenocyte suspensions were prepared by mincing the spleen between the frosted ends of two microscope slides, washing the slides with media and then returning all of the suspension to 5 mL test tubes.

Samples were centrifuged at 300g for 10 min and resuspended in RPMI 1640 (GibcoBRL, Grand Island, NY, USA) complete medium with 10% fetal bovine serum (Hyclone). Cell counts were determined using a ZBII Coulter® counter in the presence of ZAP-OGLOBIN II lytic reagent (Coulter Corporation, Miami, Florida).

Positive Controls

Cyclophosphamide (CPS, Sigma-Aldrich) at a concentration of 50 mg/kg in phosphate buffered saline (PBS) was given to animals on the last 4 days of the exposure period by intraperitoneal (i.p.) injection as a positive control for the AFC assay, DTH response, and splenocyte phenotypic analysis, and spleen and thymus weights. Rabbit anti-asialo GM1 (AAGM1, Wako BioProducts, Richmond, VA) was administered by intravenous (i.v.) injection (0.2 mL) after a 1:10 dilution in sterile physiological saline on day 28 as positive control for the natural killer cell activity.

Natural Killer Cell Activity

Natural killer cell activity was assessed using a modified version of the assay described by Reynolds and Herbman [Reynolds and Herbman 1981]. The positive control group received anti-asialo GM1 (0.2 mL at a 1:10 dilution) i.v. once on day 28. Splenocyte suspensions, effector cells, were adjusted to 1×10^6 cells/mL. T cell lymphoma YAC-1 cells, target cells, were labeled with ^{51}Cr and diluted to 1×10^5 cells/mL. In a 96-well plate, 0.1 mL of effector cells and then 0.1 mL of target cells were added to yield six target:effector cell ratios (200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1) with two replicates per ratio for each animal. Twelve wells with target cells only received either medium or Triton X-100 for measurement of spontaneous and maximum

release, respectively. After a four-hour incubation, plates were centrifuged at 250g for 10 min and 0.1 mL of supernatant was removed from every well and counted on a Wallac 1480 Wizard® 3” gamma counter (Perkin Elmer, Turku, Finland). Percent cytotoxicity was then determined as the difference of the experimental and spontaneous releases divided by the difference of the maximum and spontaneous releases. The natural killer cell activity assay was conducted once for each form of EPCL [Reynolds and Herbman 1981].

Antibody Forming Cell Assay

A modified version of the Jerne and Nordin hemolytic plaque assay was used to measure the primary IgM antibody-forming cell (AFC) response to sheep red blood cells (sRBC) as described by White et al. [Jerne and Nordin 1963, White et al. 2010]. Positive control animals received CPS given i.p. on days 25- 28. Mice were immunized i.v. 4 days prior to sacrifice with 7.5×10^7 sRBCs. Splenocyte suspensions were prepared in EBSS and an aliquot of cells was added to a test tube containing guinea pig complement, sRBC and warm agar (46–48°C). The mixture was placed in a petri dish, covered with a microscope cover slip and incubated at 37°C for 3 hours. Cell counts were performed, and the developed plaques were counted using a Bellco® plaque viewer. The data were expressed as specific activity (AFC/ 10^6 spleen cells) and total activity (AFC/spleen).

sRBC ELISA

The IgM enzyme-linked immunosorbent assay (ELISA), as previously described by Temple et al., was used to determine primary IgM serum titers to sRBC from the same animals used in the plaque assay [Temple et al. 1993]. At the time of sacrifice, blood was obtained by

cardiac puncture via 1 mL syringes (Becton Dickinson) with 23 gauge needles and place into borosilicate glass tubes to clot. After at least 2 hours of clotting, the serum was collected. Briefly, the day before the assay was conducted Immulon-2® microtiter plates were coated with 100 µl (5 µg/mL/per well), of sRBC membrane preparation containing high-salt release antigens and incubated at 4°C. Between steps plates were washed with 0.01% Tween 20 in PBS. Serum from each animal was diluted (1:8 or 1:2 for positive controls) in the Tween/PBS buffer and after blocking 100 µl of the serum was added to each well with 100 µl of buffer. Each serum sample was assayed as 10 serial two-fold dilutions. Plates were incubated for 1 hour at room temperature and 100 µl of horseradish peroxidase-conjugated anti-mouse antibody (Southern Biotech, Birmingham, AL) was added and plates were incubated for an additional 1 hour. Finally, peroxidase substrate (2,2'-azino-di[3-ethyl-benzthiazoline-6-sulfonic acid]) (Sigma-Aldrich) was added and the absorbance was read after 45 min at 405 nm on a THERMOmax™ microplate reader (Molecular Devices Corp., Sunnyvale, CA). The mean absorbance was obtained using SoftMax® Pro (Molecular Devices Corp., Sunnyvale, CA) by comparison with the linear portion of the standard curve to find the value at 0.5 optical density (OD). Titers are defined as the reciprocal of the serum dilution that has an OD value of 0.5, and were determined for each animal. One sRBC ELISA was done for each form of EPCL.

Delayed-Type Hypersensitivity Assay

The DTH assay was measured in response to *Candida albicans*, as described by Smith and White [Smith and White 2010]. On day 21 following surgery, mice were sensitized with 1×10^7 formalin fixed *Candida albicans* organisms by s.c. injection (100 µl into the right flank. A challenge only negative control group remained unsensitized. On day 29, all animals were

challenged by injection of 40 μ l (1.1 mg/mL) of *Candida albicans* chitosan antigen in the right footpad. Prior to the challenge, a pre-challenge measurement was taken of the right foot using a digital micrometer (Mitutoyo Corp., Tokyo, Japan). Twenty-four hours later, the thickness of the right footpad was measured. The mean change in footpad thickness was calculated for each mouse (post-challenge minus pre-challenge thickness) and was expressed as footpad swelling in mm x 100. The DTH was conducted twice for both microfibrinous and nanofibrinous EPCL.

Flow Cytometric Analysis

The percentages and total number of lymphocyte subsets in the spleen were measured using flow cytometric analysis in a single set of animals (n=8) for microfibrinous and nanofibrinous EPCL [Guo et al. 2001]. All antibodies used were obtained from BD Pharmingen (San Diego, CA., USA) and diluted 1:80 in staining buffer. Antibodies used were rat anti-mouse CD4–PE, anti-mouse CD8a– FITC, anti-mouse CD3– FITC, anti-mouse CD25–FITC, anti-mouse NK1.1-PE, and anti-mouse Mac3-FITC. Splenocyte suspensions were labeled in the presence of anti-CD16/32 (Mouse FC block) for 30 min on ice. Following initial staining with antibody and washing with staining buffer, propidium iodide (PI, Sigma-Aldrich) solution was added (non-viable cell label). A Becton Dickinson FACScan® Flow Cytometer was used for enumeration where log fluorescence intensity was read gated on PI (100 μ L of 0.005 mg/mL) to eliminate dead cells. A forward scatter threshold high enough to eliminate red blood cells was used. Data were analyzed using CellQuest™ software (Becton Dickinson, San Jose, CA). Isotype-matched irrelevant antibodies were used as controls.

Statistical Analysis

Data were evaluated by using a parametric one-way analysis of variance (ANOVA). The Dunnett's t-Test, a *post hoc* comparison test, was used to compare and determine differences between the treatment and sham control groups.

Results

Fiber Size

As seen in Figure 2, scanning electron micrographs of EPCL showed that scaffolds with micron diameter fibers (left panel) and submicron diameter fibers (right panel) were achieved by modifying the concentration of the polymer solution. As measured, the mean fiber diameter with a concentration of 200 mg/mL of PCL (microfibrous) was $1.7\pm 0.1\ \mu\text{m}$ while with a concentration of 80 mg/mL of PCL (nanofibrous) the mean was $0.23\pm 0.03\ \mu\text{m}$.

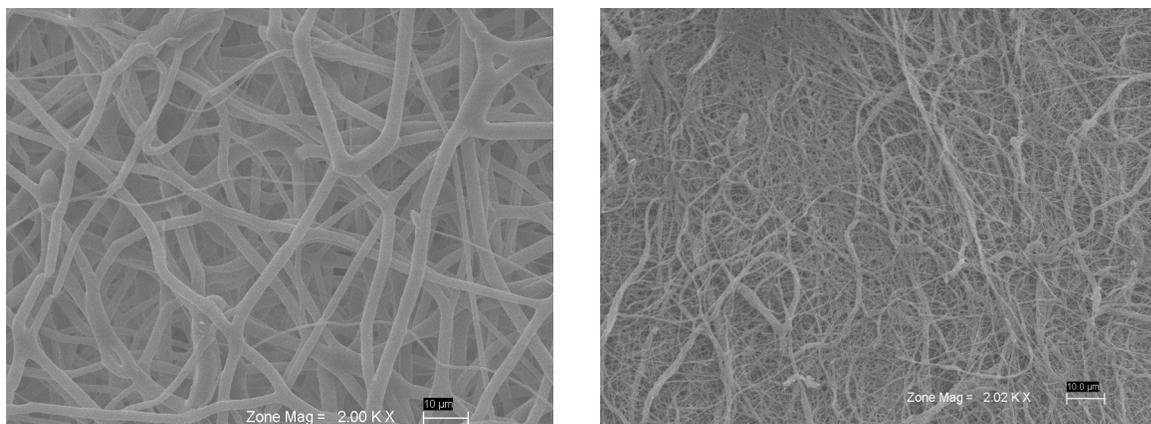


Figure 2. Scanning electron micrographs of electrospun polycaprolactone with micron diameter fibers (left panel) and submicron diameter fibers (right panel) achieved by modifying the concentration of the polymer solution. The mean fiber diameter with a concentration of 200 mg/mL of PCL is $1.7\pm 0.1\ \mu\text{m}$ with a concentration of 80 mg/mL of PCL the mean is $0.23\pm 0.03\ \mu\text{m}$.

Effect of EPCL on Body and Organ Weights

Subcutaneous implantation of microfibrinous or nanofibrinous EPCL for 28 days had no effect on body weights in 12-16 week old B6C3F1 mice (data not shown). No overt signs of toxicity or mortalities occurred. Organ weight measurements were evaluated as both absolute organ weights and organ to body weight ratio (relative weights) for the liver, spleen, lung, thymus, and kidneys with adrenals. Organ weights were evaluated in animals also used for the AFC study therefore spleen weights are slightly increased for all groups due to immunization with sRBC as compared to unimmunized animals. No significant differences were observed between the sham controls and EPCL implanted animals for any organs evaluated.

Effect of EPCL on Splenocyte Phenotype

As shown in Table 1, following analysis by flow cytometry, microfibrinous EPCL did not affect percentages of B cells, T cells, or macrophages at any dose level. The percent of natural killer cells was slightly increased with both 1 (10% increase) and 4 implants (8% increase). The percentage of macrophages was decreased (21%) in the same study for the naïve control. With 4 implants, the absolute numbers of T cells, T cell subsets, B cells, and NK cells increased, and with 1 implant, the absolute number of NK cells was increased. However, in this study, the total number of spleen cells was increased with 1 and 4 implants, therefore it is not surprising that absolute numbers of spleen cell subsets were increased as well. Because the increase in cell numbers observed in this single study is inconsistent with other studies, these effects are not believed to be biologically relevant.

As seen in Table 2, nanofibrinous EPCL did not affect the percentage of T cells at any dose level. The percentage of B cells was slightly increased with 1, 2, and 4 nanofibrinous implants

when compared to sham control, however, the sham control in the study had a slightly lower percentage of B cells than both the naïve group and historical controls. The total spleen cell number was decreased in the study with one implant and therefore decreases were seen in the absolute number of spleen cell subsets (CD8, NK, macrophages). The absolute number of NK cells was decreased with 1, 2, and 4 implants, and % NK cells was decreased with 1 and 4 implants only. The results do not demonstrate dose-responsive changes in phenotypic markers and are not believed to be biologically relevant. Naïve controls in the nanofibrous study had decreased NK cell numbers (24%) and decreased macrophage numbers (22%) compared to the sham control. As expected, in both studies, CPS-treated animals had increased T cell and macrophage numbers and decreased absolute T, T subset, and NK numbers. Anti-asialo GM1 exposed animals had minimal numbers of NK cells.

Table 1. Splenocyte subsets following microfibrinous EPCL implantation. Results are presented as mean \pm S.E. for absolute or percent cells. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group. N/C = not conducted. Only appropriate positive controls were used for each cell type.

Splenocyte subsets	Naïve	Sham	Microfibrinous polycaprolactone (mm ²)			CPS 50 mg kg ⁻¹	AAGM1 1:10	
			32	64	128			
Absolute ($\times 10^6$)	B Cell (Ig ⁺)	72.1 \pm 3.5	70.3 \pm 4.0	82.9 \pm 3.4	78.1 \pm 4.8	91.6 \pm 3.3**	23.2 \pm 1.7**	N/C
	T Cell (CD3 ⁺)	42.1 \pm 2.9	41.2 \pm 2.6	51.2 \pm 3.5	50.3 \pm 2.9	58.8 \pm 2.8**	29.2 \pm 2.0**	N/C
	TH (CD4 ⁺ CD8 ⁻)	25.5 \pm 1.2	25.8 \pm 1.4	30.5 \pm 1.1	32.0 \pm 2.2*	37.3 \pm 1.8**	18.7 \pm 1.2**	N/C
	TC (CD4 ⁻ CD8 ⁺)	12.1 \pm 0.9	13.1 \pm 0.7	15.4 \pm 0.8	16.7 \pm 1.3*	18.1 \pm 1.1**	10.4 \pm 0.6*	N/C
	T (CD4 ⁺ CD8 ⁺)	0.10 \pm 0.02	0.10 \pm 0.02	0.10 \pm 0.03	0.10 \pm 0.03	0.15 \pm 0.02	0.11 \pm 0.01	N/C
	NK (NK1.1 ⁺ CD3 ⁻)	5.0 \pm 0.2	4.5 \pm 0.4	6.8 \pm 0.4**	5.7 \pm 0.6	7.4 \pm 0.3**	N/C	0.66 \pm 0.04**
Per cent	MΦ (Mac-3 ⁺)	3.0 \pm 0.1**	4.5 \pm 0.4	4.9 \pm 0.2	5.0 \pm 0.7	6.1 \pm 0.3	3.4 \pm 0.3*	N/C
	B Cell (Ig ⁺)	56.5 \pm 1.0	55.6 \pm 0.9	54.4 \pm 0.9	53.5 \pm 1.3	53.3 \pm 0.7	40.4 \pm 0.8**	N/C
	T Cell (CD3 ⁺)	32.9 \pm 1.4	32.6 \pm 0.8	33.2 \pm 1.7	34.6 \pm 0.9	34.2 \pm 1.0	51.1 \pm 1.3**	N/C
	TH (CD4 ⁺ CD8 ⁻)	20.0 \pm 0.7	20.5 \pm 0.6	19.9 \pm 0.6	21.9 \pm 0.6	21.7 \pm 0.4	32.8 \pm 0.9**	N/C
	TC (CD4 ⁻ CD8 ⁺)	9.5 \pm 0.4	10.4 \pm 0.3	10.0 \pm 0.4	11.4 \pm 0.4	10.5 \pm 0.4	18.3 \pm 0.5**	N/C
	T (CD4 ⁺ CD8 ⁺)	0.07 \pm 0.02	0.08 \pm 0.01	0.07 \pm 0.02	0.07 \pm 0.02	0.09 \pm 0.03	0.19 \pm 0.02**	N/C
Spleen cell number ($\times 10^6$)	NK (NK1.1 ⁺ CD3 ⁻)	4.0 \pm 0.2	3.6 \pm 0.2	4.4 \pm 0.2*	3.9 \pm 0.2	4.3 \pm 0.2*	N/C	0.38 \pm 0.03**
	MΦ (Mac-3 ⁺)	2.4 \pm 0.2**	3.5 \pm 0.2	3.2 \pm 0.1	3.4 \pm 0.3	3.5 \pm 0.1	6.0 \pm 0.4**	N/C
		127.6 \pm 5.5	126.6 \pm 7.3	154.4 \pm 6.1*	145.9 \pm 8.6	172.2 \pm 7.0**	57.5 \pm 4.2**	174.1 \pm 8.3**

Table 2. Splenocyte subsets following nanofibrous EPCL implantation. Results are presented as mean \pm S.E. for absolute or percent cells. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group. N/C = not conducted. Only appropriate positive controls were used for each cell type.

Splenocyte subsets	Naïve	Sham	Nanofibrous polycaprolactone (mm ²)			CPS 50 mg kg ⁻¹	AAGM1 1:10
			32	64	128		
Absolute ($\times 10^6$)							
B Cell (Ig ⁺)	124.2 \pm 7.9**	68.7 \pm 4.1	65.3 \pm 4.6	54.9 \pm 12.2	81.7 \pm 4.2	34.1 \pm 2.5**	N/C
T Cell (CD3 ⁺)	64.8 \pm 3.9**	41.9 \pm 2.3	34.8 \pm 2.2	38.2 \pm 1.8	40.2 \pm 2.9	33.0 \pm 1.8	N/C
TH (CD4 ⁺ CD8 ⁻)	40.3 \pm 2.5**	26.0 \pm 1.3	22.1 \pm 1.3	25.3 \pm 0.7	25.9 \pm 1.8	19.7 \pm 1.0*	N/C
TC (CD4 ⁻ CD8 ⁺)	19.9 \pm 1.2**	14.1 \pm 0.7	10.4 \pm 0.6**	12.7 \pm 0.5	12.5 \pm 0.9	10.4 \pm 0.5*	N/C
T (CD4 ⁺ CD8 ⁺)	0.24 \pm 0.02**	0.10 \pm 0.03	0.15 \pm 0.01	0.15 \pm 0.04	0.19 \pm 0.03	0.15 \pm 0.02	N/C
NK (NK1.1 ⁺ CD3 ⁻)	7.3 \pm 0.6	7.0 \pm 0.6	4.0 \pm 0.4**	5.2 \pm 0.2**	5.4 \pm 0.3*	N/C	1.3 \pm 0.2**
MΦ (Mac-3 ⁺)	12.4 \pm 1.2	11.6 \pm 0.3	6.9 \pm 0.5**	7.9 \pm 0.8**	11.4 \pm 0.9	6.0 \pm 0.4**	N/C
Per cent							
B Cell (Ig ⁺)	52.8 \pm 1.5**	40.2 \pm 2.3	48.9 \pm 1.2**	51.0 \pm 1.4**	49.1 \pm 1.3**	40.4 \pm 1.8	N/C
T Cell (CD3 ⁺)	27.6 \pm 0.6	24.7 \pm 1.7	26.1 \pm 0.6	25.7 \pm 1.0	24.0 \pm 1.2	39.3 \pm 0.8**	N/C
TH (CD4 ⁺ CD8 ⁻)	17.2 \pm 0.4	15.2 \pm 0.8	16.6 \pm 0.5	17.1 \pm 0.7	15.5 \pm 0.6	23.5 \pm 1.0**	N/C
TC (CD4 ⁻ CD8 ⁺)	8.5 \pm 0.2	8.3 \pm 0.5	7.9 \pm 0.4	8.6 \pm 0.2	7.4 \pm 0.4	12.6 \pm 0.9**	N/C
T (CD4 ⁺ CD8 ⁺)	0.1 \pm 0.0	0.06 \pm 0.03	0.12 \pm 0.01	0.1 \pm 0.03	0.11 \pm 0.01	0.18 \pm 0.03**	N/C
NK (NK1.1 ⁺ CD3 ⁻)	3.1 \pm 0.2**	4.1 \pm 0.2	3.0 \pm 0.2**	3.5 \pm 0.1	3.3 \pm 0.2*	N/C	0.69 \pm 0.1**
MΦ (Mac-3 ⁺)	5.3 \pm 0.4**	6.8 \pm 0.3	5.2 \pm 0.3**	5.3 \pm 0.4**	6.8 \pm 0.4	6.9 \pm 0.3	N/C
Spleen cell number ($\times 10^6$)	235.7 \pm 15.5**	171.6 \pm 6.5	133.3 \pm 7.8**	148.8 \pm 5.7	166.7 \pm 7.9	84.2 \pm 4.3**	194.8 \pm 8.6

Effect of EPCL on Innate Immunity: NK Cytotoxicity

Results are shown as percent cytotoxicity (Figure 3). Neither microfibrinous or nanofibrinous EPCL had effects on natural killer cell activity in 12-16 week old female B6C3F1 mice. As expected, animals that received AAGM1 had minimal cytotoxicity as compared to sham control. In the nanofibrinous EPCL study, percent cytotoxicity in the naïve group was lower than for the sham at five of the six effector:target ratios (all except 200:1), the reason for this is unclear, however the appropriate control group for the NK study was the sham control group.

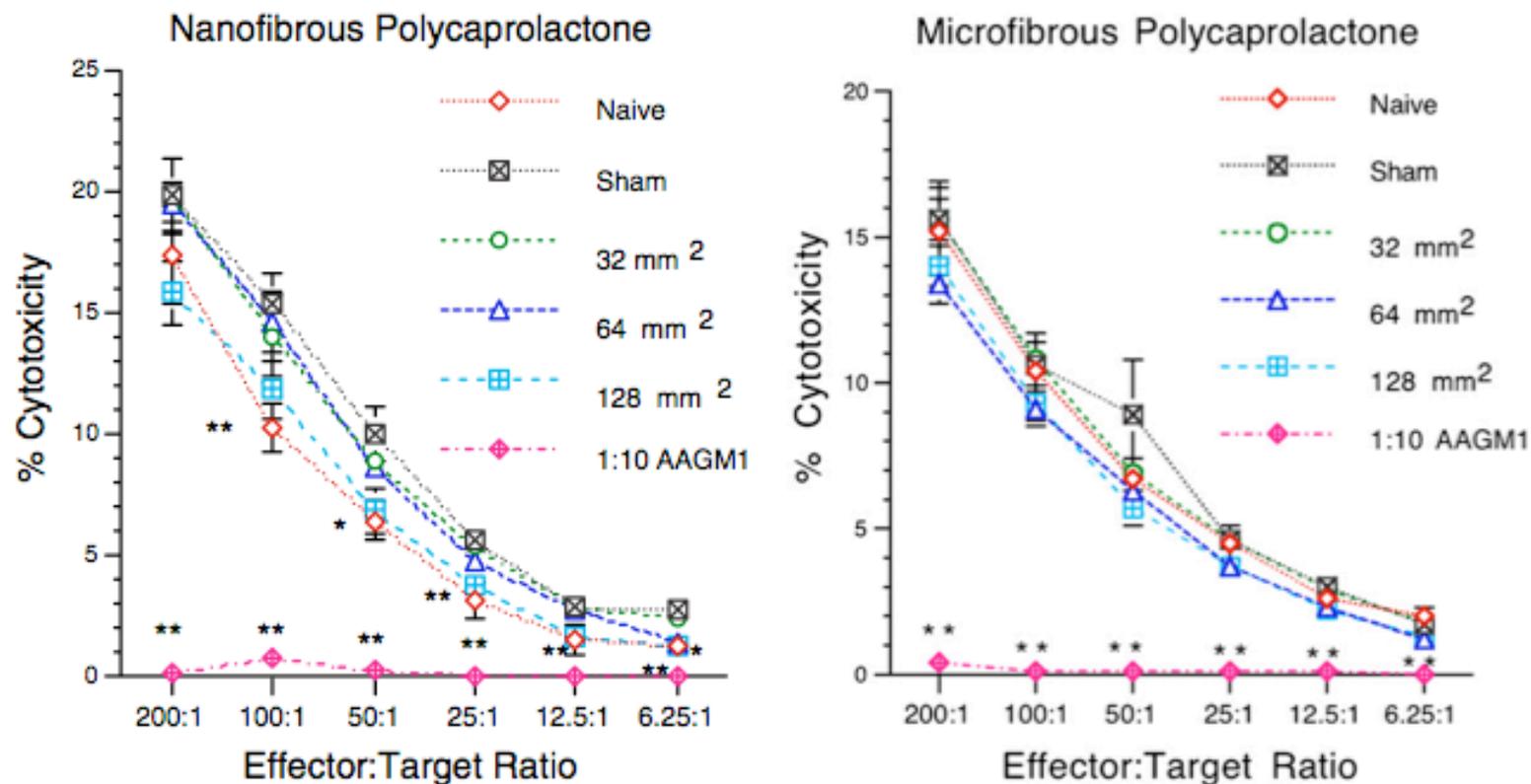


Figure 3. Natural Killer cell activity. Results are presented as mean \pm S.E. for percent cytotoxicity. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Effect of EPCL on Humoral Immunity: AFC

The results are presented as AFC/ 10^6 spleen cells (specific activity) and total spleen activity. As shown in Figures 4 and 5, neither microfibrinous or nanofibrinous EPCL significantly affected the AFC response to sheep erythrocytes on either specific activity or total spleen activity in 12- 16 week old female B6C3F1 mice. Results represent the percent control mean obtained from combining three studies, each of which utilized seven or eight animals per group. Since the comparisons of sham control means between three studies conducted were found to be significantly different, the studies were combined and presented as percent control. CPS significantly suppressed the AFC response as expected.

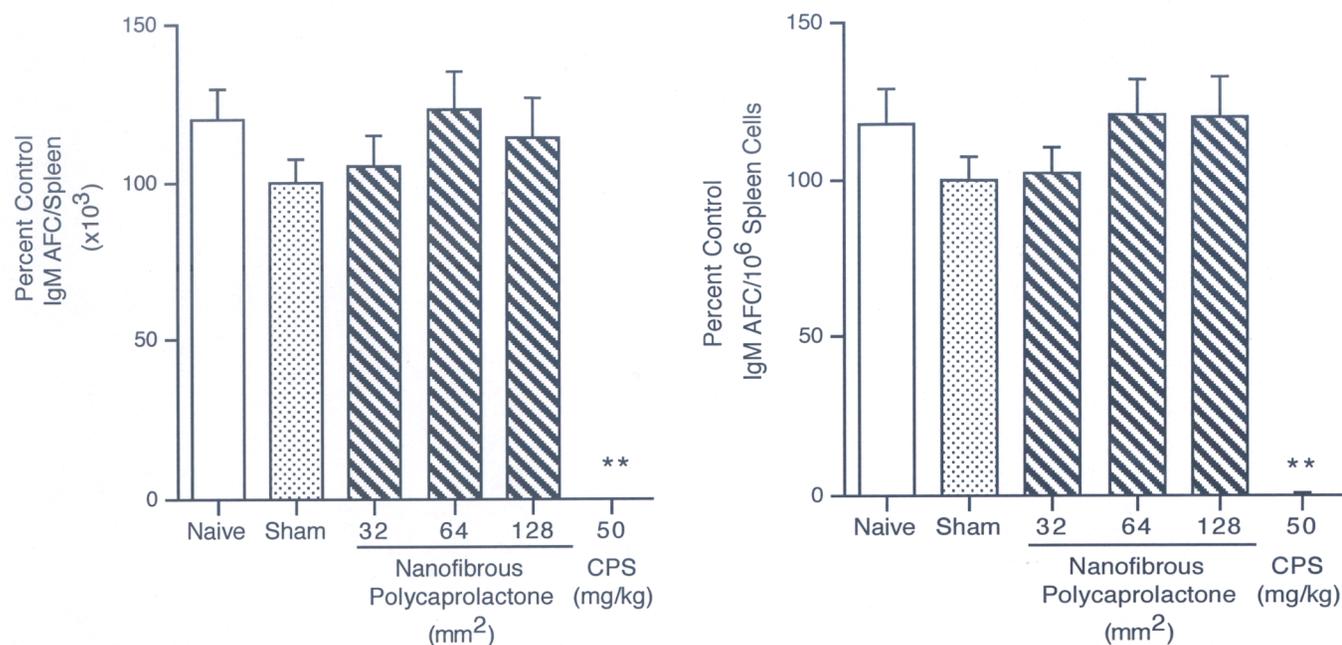


Figure 4. Spleen IgM Antibody-Forming Cell (AFC) Response to Sheep Erythrocytes. Female B6C3F1 mice were exposed to microfibrous of nanofibrous EPCL for 28 days and were immunized with sRBCs on day 25. Results are presented as percent control mean \pm S.E. for the AFC/10⁶ Spleen Cells (specific activity) and AFC/Spleen (total spleen activity). Results represent the mean obtained from combining three studies, each of which used seven or eight animals per group. Comparisons of sham controls means between three studies were conducted using ANOVA 243 \pm 21 vs. 290 \pm 58 vs. 293 \pm 55 for specific activity and 1401 \pm 76 vs. 1767 \pm 242 vs. 1036 \pm 198 for total spleen activity. On days 25-28, 50 mg/kg cyclophosphamide (CPS) was administered as a positive control by i.p. injection. The level of statistical significance is denoted as ** $p < 0.01$ as compared to the sham control group.

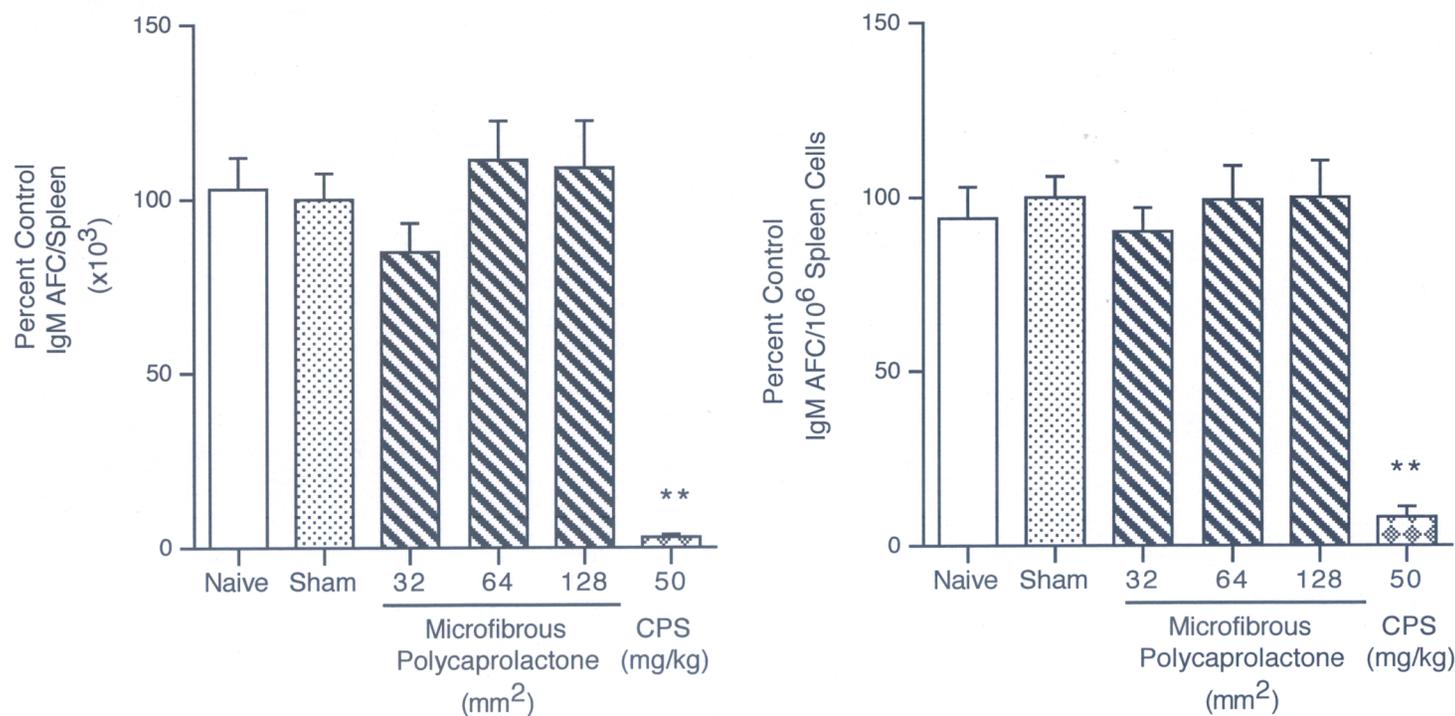


Figure 5. Spleen IgM Antibody-Forming Cell (AFC) Response to Sheep Erythrocytes. Female B6C3F1 mice were exposed to microfibrous or nanofibrous EPCL for 28 days and were immunized with sRBCs on day 25. Results are presented as percent control mean \pm S.E. for the AFC/ 10^6 Spleen Cells (specific activity) and AFC/Spleen (total spleen activity). Results represent the mean obtained from combining three studies, each of which used seven or eight animals per group. Comparisons of sham controls means between three studies were conducted using ANOVA 753 ± 69 vs. 261 ± 12 vs. 290 ± 58 for specific activity and 3077 ± 268 vs. 1322 ± 46 vs. 1036 ± 198 for total spleen activity). On days 25-28 50 mg/kg cyclophosphamide (CPS) was administered as a positive control by i.p. injection. The level of statistical significance is denoted as ** $p < 0.01$ as compared to the sham control group.

Effect of EPCL on Humoral Immunity: sRBC Primary IgM ELISA Response

sRBC ELISA results are expressed as mean $\text{Log}_2(\text{Titer})$ as shown in Figure 6.

Consistent with the AFC results, neither microfibrinous or nanofibrinous EPCL produced any significant effects compared to the sham control. Positive control mice, which received CPS, had a significant decrease in the serum IgM response to sRBC compared to the sham control.

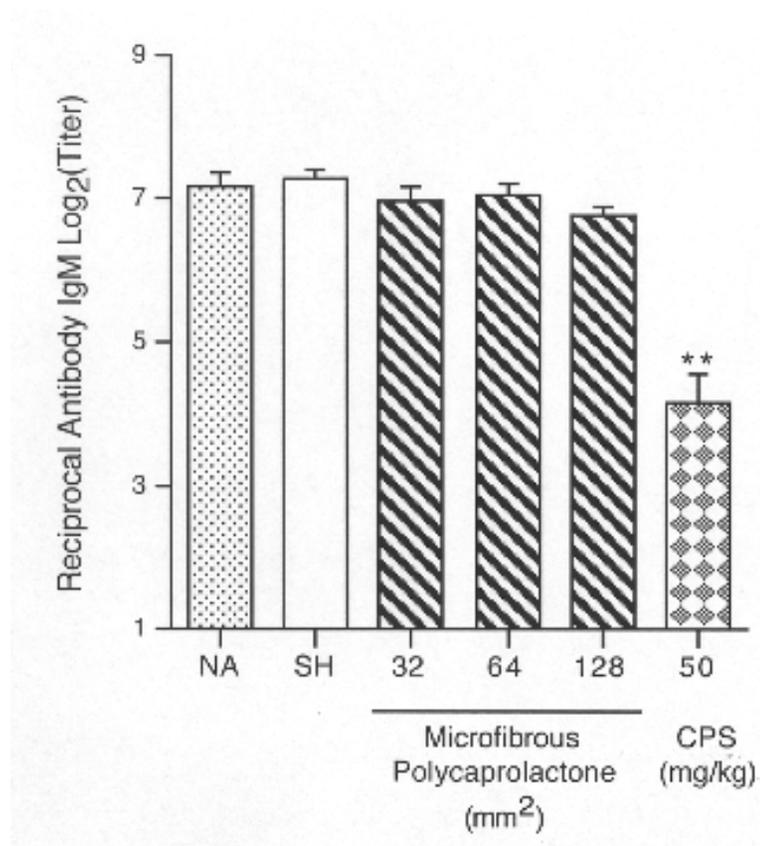
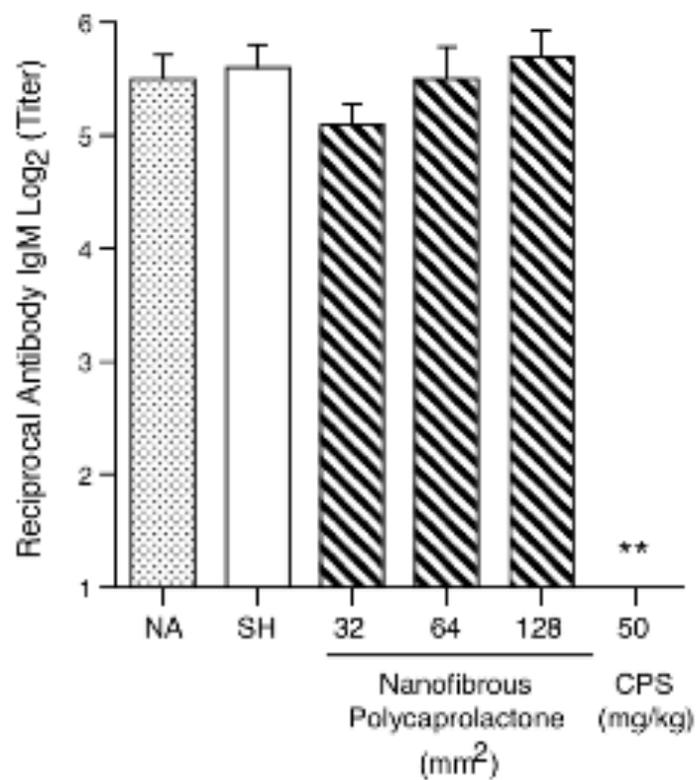


Figure 6. sRBC ELISA. Results are presented as Titer (Log₂) Mean \pm S.E. Level of statistical significance is denoted as ** < p 0.01 as compared to the sham control group.

Effect of EPCL on Cell-mediated Immunity: DTH

The DTH results are presented as mean footpad swelling (Figure 7). Subcutaneous implantation of microfibrinous or nanofibrinous EPCL did not affect the DTH response to *C. albicans* in 12-16 week old female B6C3F1 mice. As expected, footpad swelling of the challenge only group was significantly different from the sham control group, which had received both sensitization and challenge. CPS, the positive control, significantly suppressed the footpad swelling when compared to the sham control.

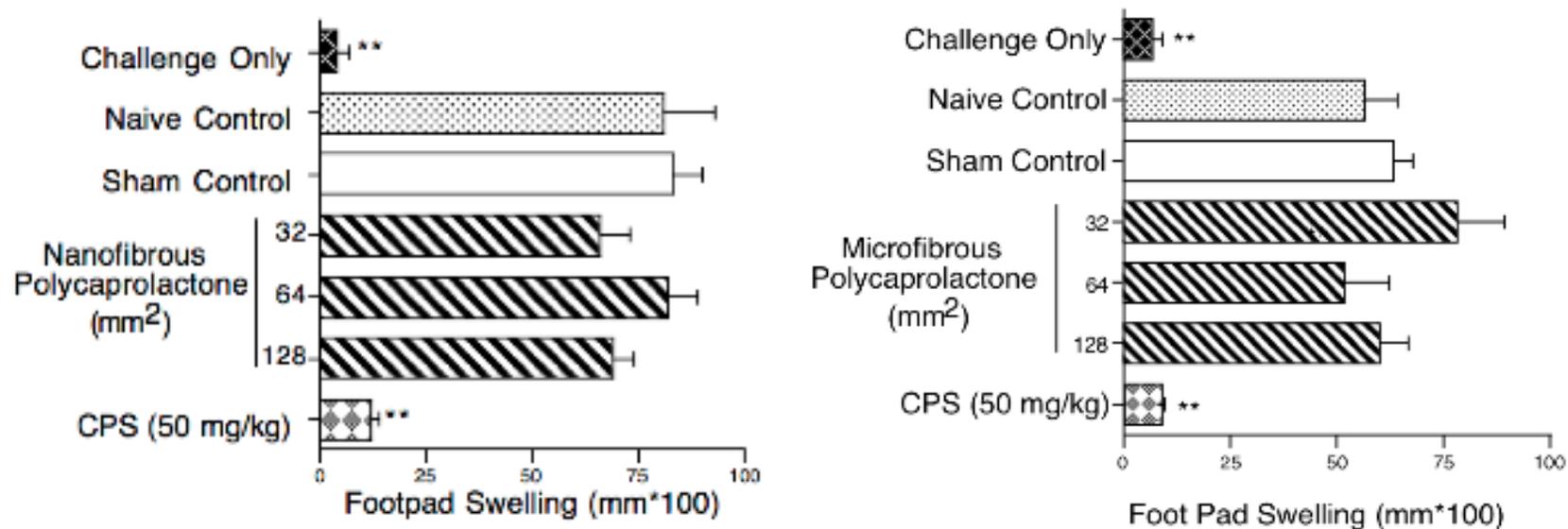


Figure 7. Delayed type hypersensitivity response to *C. albicans*. Results are presented as Mean \pm S.E. footpad swelling (mm*100). Level of statistical significance is denoted as ** $p < 0.01$ as compared to the sham control group.

Discussion

This work represents the first comprehensive *in vivo* evaluation of an electrospun polymer for immunotoxicological effects, evaluating the three major components of the immune response. Previously, electrospun polymer blends (PDO:elastin and PDO:collagen) have been evaluated *in vitro* by Smith et al. [Smith et al. 2009, Smith et al. 2010]. Both PDO:elastin and PDO:collagen blends produced effects on innate, humoral, and cell-mediated immunity *in vitro* as described below.

The synthetic polymer silicone has been studied extensively in multiple forms (gel, elastomer, fluid, with polyurethane cover) for immunotoxicological effects following *in vivo* exposure by s.c. implantation. Results from these studies indicated that silicone (and polyurethane covered silicone) did not affect any endpoint tested, with the exception of NK cell activity, which was decreased with the silicone gel. However, the authors indicated the decrease was minimal, inconsistent and was not considered to contraindicate the use of silicone gel in implant materials [Bradley et al. 1994, NTP 1992].

Smith et al. assessed electrospun PDO:elastin and PDO:collagen *in vitro* for effects on innate immunity using the NK assay, macrophage phagocytosis, ROS production, and nitric oxide production [Smith et al. 2009, Smith et al. 2010]. Natural killer cell activity was significantly suppressed following exposure to both PDO:elastin and PDO:collagen blends *in vitro*, while other endpoints were unaffected. Following *in vivo* exposure to EPCL neither microfibrillar or nanofibrillar EPCL affected NK activity. Although naïve controls differed from sham controls when nanofibrillar EPCL was evaluated for NK activity, this was not repeated in the microfibrillar study and does not appear to be biologically significant.

Effects on humoral immunity were also assessed for PDO:elastin and PDO:collagen blends by Smith et al. using the Mishell-Dutton assay—the *in vitro* analogue to the AFC assay [Smith et al. 2009, Smith et al. 2010]. Blends of both PDO:elastin and PDO:collagen significantly suppressed the number of antibody forming cells per culture. Additionally, Smith et al. found that a 50:50 blend of PDO:PCL significantly suppressed humoral immunity in the Mishell-Dutton assay [Smith et al. 2007]. In contrast, the present findings showed that electrospun PCL does not suppress humoral immunity in either the AFC assay or the IgM serum ELISA.

Cell-mediated immunity was evaluated *in vivo* using the DTH response to *C. albicans*, which is a holistic functional assay. Following exposure to microfibrillar and nanofibrillar PCL, no effects were observed on footpad swelling, the endpoint of the DTH response. This assay has been indicated to be more sensitive than *ex vivo* assays (such as the MLR or cytotoxic T lymphocyte [CTL] assay) [Smith et al. 2011, White et al. 2012]. In contrast, Smith et al. have previously found that *in vitro* exposure to PDO:elastin and PDO:collagen blends affected both T and B cell proliferation using lymphoproliferative assays (MLR, F(ab')₂, anti-CD3) [Smith et al. 2009, Smith et al. 2010]. No biologically significant differences occurred in splenocyte phenotypes when evaluated for both microfibrillar and nanofibrillar EPCL exposure *in vivo*. Although sporadic differences did occur, none of these changes were dose-responsive and therefore are not believed to be biologically relevant. Functional holistic assays also confirm the lack of biological effect on these cell types.

Although Smith et al. evaluated different polymers and blends, those works suggested that electrospun polymers could be immunosuppressive. The work presented here was conducted in order to evaluate the dose-response effects of a single electrospun polymer following *in vivo*

exposure as opposed to *in vitro*. When converted based on percent surface area (SA) a single implant would have an equivalent surface area of 6800 mm², or a circular disc with a diameter of 9-cm in humans. Therefore, the highest dose tested (four 6 mm implants in the mouse) would be equivalent to four 9 cm implants in a human. This level of exposure is relevant for many potential applications for EPCL. For example, this exposure level (approximately 272 cm² SA) would be comparable to vascular graft with 4 mm inner diameter that is 2 m long. All results suggest that neither microfibrinous or nanofibrinous EPCL suppress innate, humoral, or cell-mediated immunity and that EPCL appears to have excellent immunocompatibility.

Conclusions

Although testing of local and inflammatory response are routinely conducted for biomaterials, potential effects on the systemic immune system are not often tested. The work presented is the first immunotoxicological evaluation of an electrospun polymer *in vivo*. These findings show that both microfibrinous and nanofibrinous EPCL had no biologically significant effects on any of the parameters examined. Although other endpoints, such as the mononuclear phagocytic system, remain to be evaluated, this work represents a promising start to obtaining more in-depth assessments of the *in vivo* immunocompatibility and safety of electrospun polymers. In addition, in this work, no difference was observed between microfibrinous and nanofibrinous materials, thereby suggesting that neither configuration for EPCL biomaterials is more immunotoxic than the other. EPCL therefore is a material with excellent immunocompatibility deserving further investigation for tissue engineering applications.

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CHAPTER 4: Immunotoxicological Investigation in Aged Animals

The following manuscript has been prepared for submission to Food and Chemical Toxicology.

The work demonstrates the lack of effect on innate, humoral, and cell-mediated immune endpoints following *in vivo* implantation of EPCL in aged mice.

Evaluation of immunity in aged mice following *in vivo* implantation of electrospun polycaprolactone

Colleen E. McLoughlin^{1,2}, Matthew J. Smith², Wimolnut Auttachoat², Gary L. Bowlin¹, and Kimber L. White, Jr.²

¹Department of Biomedical Engineering, Virginia Commonwealth University, Richmond, VA

²Department of Pharmacology and Toxicology, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, VA

³Author to whom all correspondence should be addressed
Email: mcloughlince@vcu.edu

Keywords: immunotoxicity, electrospinning, polycaprolactone, delayed type hypersensitivity response, mice, tissue engineering, aging

Short title: *In vivo* immunotoxicological evaluation of electrospun polycaprolactone in aged mice

Abstract

Recently our laboratory reported the lack of effects on innate, humoral, and cell-mediated immunity in young adult mice following *in vivo* exposure to electrospun polycaprolactone (EPCL). Electrospinning is a polymer processing technique being widely investigated to create scaffolds for tissue engineering. EPCL in particular has potential use in applications ranging from vascular grafts to drug delivery. When the target population is aged, which is the case for many of the applications for EPCL, immunosenescence can play a role in the potential immunotoxicity of a xenobiotic. Therefore, this work evaluated the potential for EPCL in a microfibrinous and a nanofibrinous to produce immunosuppressive effects in aged female B6C3F1 mice. Multiple components of innate, humoral, and cell-mediated immunity were evaluated. Endpoints examined included the antibody-forming cell assay (AFC or Plaque Assay) and the delayed-type hypersensitivity (DTH) response to *C. albicans*. An *ex vivo* assay of natural killer cell cytotoxic activity was used to assess innate immunity. Additionally, spleen cell phenotypes were analyzed by flow cytometry. All results indicated that the systemic immune functions in 24-28 week old female B6C3F1 mice were not affected following 28-day subcutaneous implantation of EPCL in either in microfibrinous or nanofibrinous form.

Introduction

Electrospinning is a popular polymer processing technique often used to create scaffolds for tissue engineering applications. We have previously reported the lack of effect following subcutaneous implantation of a synthetic electrospun polymer, electrospun polycaprolactone (EPCL), in both microfibrinous and nanofibrinous form, in young adult B6C3F1 mice [McLoughlin et al. 2012]. These results were in contrast to the work of Smith et al. that showed

immunomodulatory effects on both innate and acquired immunity following *in vitro* exposure of mouse spleen cells to electrospun materials [Smith et al. 2010, Smith et al. 2009].

Aging causes significant changes in both the innate and acquired immune systems. With age, hematopoietic stem cells, which give rise to B cells, T cells, and NK cells, have decreased function and ability to differentiate into immune cells. Macrophages and neutrophils show a decline in phagocytic capability with age, while NK cells increase in number but decrease in their ability to kill tumor cells and virally infected cells. Furthermore, cytokine and chemokine production is altered and it has been suggested that macrophages and fibroblasts produce more pro-inflammatory cytokines. Lower numbers of pro- and pre-B cells are seen in the aged immune system, and antibodies produced by B cells have lower affinity. Naïve T cells tend to have reduced ability to proliferate and defects in cytokine production. Memory T cells developed when animals are young continue to function well, but memory T cells originating from older animals have decreased responses. With these significant changes the immune system of old animals needs to be evaluated separately from that of young adult animals [Aw et al. 2007].

Although differential susceptibility of the developing immune system [Burns-Naas et al. 2008] is often investigated, the role of the aged immune system in xenobiotic immunotoxicity has been largely unexplored. Oughton et al. evaluated phenotypic changes of immune cells in the spleen and blood in aged mice alongside a study of long-term exposure to tetrachlorodibenzo-p-dioxin (TCDD). Results demonstrated age-related increases in effector and memory T cells and fewer naïve T cells in both the spleen and peripheral blood as well as decreases in the number of CD4⁺ T cells. In spite of thymic involution, changes in thymic cell phenotypes did not occur [Oughton et al. 1995]. Duffy et al. examined age related differences in immune responses to polychlorinated biphenyl (PCB) exposure in Japanese medaka fish. Specifically, the authors

reported that juvenile fish were more susceptible to PCB-induced effects on innate immunity (superoxide production), however age-related differences in humoral immunity (AFC response) were not as prevalent [Duffy et al. 2002, Duffy et al. 2003]. Studies by Luebke et al. and Vos et al. have shown that, following TCDD and bis(tris-n-butyltin) oxide (TBTO) exposure in aged animals, the aged immune system can provide protection from parasite infection when the clearance is T cell dependent [Luebke et al. 1999, Vos et al. 1990]. However, Luebke et al. have shown that the difference in susceptibility to increased or decreased host resistance due to age is model dependent, as aged animals may be more susceptible than young when humoral immunity plays a greater role [Luebke et al. 2000]. Dortant et al. explored age-related differences between young adult (12 week old) and old (27-30 month old) SPF wag rats following exposure to Ochratoxin A (OTA). The authors found similar kidney and brain toxicity profiles in both age groups, determined the old rats were more susceptible than young rats to OTA exposure based on clinical and pathological data, and finally concluded that the immune system is not the primary target of OTA [Dortant et al. 2001].

The lack of immunotoxicity with EPCL in young animals was an important finding toward the potential for using such electrospun tissue engineered devices in a clinical setting. However, if a tissue engineering product's intended patient population is elderly with potential age-related immunosenescence, the material's potential to produce immunosuppression in this population needs to be addressed. The work presented herein was undertaken to test the hypothesis that EPCL in a microfibrillar or nanofibrillar form would cause immunosuppressive effects in aged mice. Validated assays were chosen that have been shown to be sensitive for detecting immunomodulatory effects in a similar manner to the immunotoxicological testing battery approach suggested by Luster et al. [Luster et al. 1988, Luster et al. 1992].

As previously reported, EPCL was prepared in microfibrinous and nanofibrinous form by modifying the concentration of the polymer solution [McLoughlin et al. 2012]. Scanning electron micrographs were obtained and the mean fiber diameters were measured using ImageJ software (NIH). Microfibrinous EPCL had a mean of 1.7 ± 0.1 μm and nanofibrinous had a mean of 0.23 ± 0.03 μm .

The potential of both microfibrinous and nanofibrinous EPCL to produce immunomodulatory effects following 28 days of exposure *in vivo* in 24-28 week old female B6C3F1 mice was evaluated. Splenocyte phenotypic changes in T cells and T cell subsets, B cells, natural killer cells, and macrophages were evaluated using flow cytometry. Innate immunity was assessed using the natural killer cell activity assay. A holistic measure of the T-dependent antibody response (TDAR) was conducted using the AFC assay. In addition, serum antibody levels to the T-dependent antigen, sheep red blood cells (sRBC) were also used to assess effects on the TDAR. Effects on cell-mediated immunity were evaluated using a holistic assay evaluating the delayed-type hypersensitivity response to *C. albicans*.

Materials and Methods

Scaffold Preparation

As previously reported, PCL (molecular weight 65 000 kDa, Sigma-Aldrich) was dissolved in 1,1,1,3,3,3-hexafluoro-2-propanol (HFP; TCI America) at concentrations of 80 mg/mL (nanofibrinous) and 200 mg/mL (microfibrinous) and scaffolds were prepared by electrospinning [McLoughlin et al. 2012]. Implants were obtained by punching circular pieces 6 mm diameter (representing 32 mm² surface area) using a single hole paper punch (Harold's

Business Supply, Richmond, VA). The implants were disinfected in 100% ethanol for 30 min followed by three 10-minute washes in physiological saline.

Animal Husbandry and Exposure

All animal procedures were conducted under an animal protocol approved by the Institutional Animal Care and Use Committee (IACUC) at Virginia Commonwealth University. Animals were fed NTP 2000 diet, provided tap water ad libitum, and vivarium conditions were maintained at 21-24°C and 40–70% relative humidity, with a 12-h light/dark cycle. Sentinel animals were tested for and found to be free of *M. pulmonis*, coronavirus, and Sendai virus by murine Immunocomb test kits (Charles River). Female B6C3F1 (Taconic Farms) were housed four per cage, and were placed on study between 24-28 weeks of age. Mice were randomized, and eight mice were assigned to each dose group. Surgical procedures have been described previously [McLoughlin et al. 2012]. Briefly, for each implant, a 1 cm incision was made on the ventral quadrant and a small pocket was prepared for insertion of EPCL by blunt dissection. Incisions were closed using surgical staples. The low dose animals were implanted with 1 EPCL disc (total surface of 32 mm²), the middle dose received 2 discs (64 mm²), and the high dose 4 implants discs (128 mm²). Sham (negative control) animals received the same surgical manipulations as the high dose animals, however, they did not receive any implants. Animal body weights were obtained at the start and end of study.

Positive Controls

For the AFC assay, DTH response, and splenocyte phenotypic analysis, positive control animals received 50 mg/kg cyclophosphamide (CPS, Sigma-Aldrich) by intraperitoneal (i.p.)

injection on days 25-28. For the natural killer cell activity assay, positive control animals were administered a 1:10 dilution in sterile physiological saline of rabbit anti-asialo GM1 (AAGM1, Wako BioProducts, Richmond, VA) by intravenous (i.v.) injection (0.2 mL) on day 28.

Natural Killer Cell Activity

Natural killer cell activity was assessed using a modified version of the assay described by Reynolds and Herbman, as previously described [Reynolds and Herbman 1981]. In 96-well plates, splenocyte suspensions (effector cells) and ^{51}Cr -labeled T cell lymphoma YAC-1 cells (target cells) were incubated for four hours at six effector:target cell ratios (200:1, 100:1, 50:1, 25:1, 12.5:1, 6.25:1) with two replicates per ratio for each animal. Twelve wells with target cells only received either medium or Triton X-100 for measurement of spontaneous and maximum release, respectively. After incubation, plates were centrifuged at 250g for 10 min, and 0.1 mL of supernatant was removed from every well and counted on a Wallac 1480 Wizard® 3" gamma counter (Perkin Elmer, Turku, Finland). Percent cytotoxicity was then calculated as (experimental - spontaneous release) / (maximum - spontaneous releases).

Antibody Forming Cell Assay

As described by White et al. a modified version of the Jerne and Nordin hemolytic plaque assay was used to measure the primary IgM antibody-forming cell (AFC) response to sheep red blood cells (sRBC) [White et al. 2010, Jerne and Nordin 1963]. Mice were immunized i.v. on day 25 with 7.5×10^7 sRBCs. Splenocyte suspensions were prepared in EBSS and an aliquot of cells was added to a test tube containing guinea pig complement, sRBC and warm agar (46–48°C). The mixture was placed in a petri dish, covered with a microscope cover slip and

incubated at 37°C for 3 hours. Cell counts were performed, and the developed plaques were counted using a Bellco® plaque viewer. The data were expressed as specific activity (AFC/10⁶ spleen cells) and total activity (AFC/spleen).

sRBC ELISA

The same group of animals that was used for the AFC assay was used to determine primary IgM serum titers to sRBC using the IgM enzyme-linked immunosorbent assay (ELISA), as previously described by Temple et al. [Temple et al. 1993]. At the time of sacrifice, blood was obtained by cardiac puncture using 1 mL syringes (Becton Dickinson) with 23 gauge needles and placed into borosilicate glass tubes to clot. After at least 2 hours, the serum was collected and stored frozen at -20°C until thawed for analysis. Briefly, the day before the assay was conducted, Immulon-2® microtiter plates were coated with 100 µl per well of 10 µg/mL of sRBC membrane preparation containing high-salt release antigens and incubated overnight at 4°C. 0.05% Tween 20 in PBS was used as the assay buffer. Serum from each animal was diluted (1:8 or 1:2 for positive controls) and each serum sample was assayed as 10 serial two-fold dilutions. The secondary antibody used was horseradish peroxidase-conjugated goat anti-mouse IgM antibody (Southern Biotech, Birmingham, AL) and the peroxidase substrate used was 2,2'-azino-di[3-ethyl-benzthiazoline-6-sulfonic acid] (Sigma-Aldrich). After 45 min of substrate incubation at room temperature, the absorbance was read at 405 nm on a THERMOmax™ microplate reader (Molecular Devices Corp., Sunnyvale, CA). The mean absorbance was obtained using SoftMax® Pro (Molecular Devices Corp., Sunnyvale, CA) by comparison with the linear portion of the standard curve to find the value corresponding to 0.5 optical density (OD). Titers were

determined for each animal and are defined as the reciprocal of the serum dilution having an OD value of 0.5.

Delayed-Type Hypersensitivity Assay

The DTH response to *Candida albicans* was measured as described by Smith and White [Smith and White 2011]. On day 21 following surgery, mice were sensitized with 1×10^7 formalin fixed *Candida albicans* organisms by s.c. injection (100 μ l) into the right flank. A challenge only negative control group remained unsensitized. On day 29, prior to the challenge, a pre-challenge measurement was taken of the right foot using a digital micrometer (Mitutoyo Corp., Tokyo, Japan). All animals were subsequently challenged by injection of 40 μ l (1 mg/mL) of *Candida albicans* chitosan antigen in the right footpad. Twenty-four hours later, the thickness of the right footpad was measured. The mean change in footpad thickness was calculated for each mouse (post-challenge minus pre-challenge thickness) and data expressed as footpad swelling in mm x 100.

Flow Cytometric Analysis

The percentages and total number of lymphocyte subsets in the spleen were measured using flow cytometry [Guo et al. 2001]. All antibodies used were obtained from BD Pharmingen (San Diego, CA., USA) and diluted 1:80 with staining buffer which include rat anti-mouse CD4-PE, anti-mouse CD8a-FITC, anti-mouse CD3- FITC, anti-mouse CD25-FITC, anti-mouse NK1.1-PE, and anti-mouse Mac3-FITC. Isotype-matched irrelevant antibodies were used as controls. Spleen single-cell suspensions (1×10^6 cells/well) were dually labeled with 100 μ L fluorescein isothiocyanate (FITC)-conjugated and phycoerythrin (PE)-conjugated antibodies in

the presence of anti-CD16/32 (Mouse FC block) for 30 min on ice. Following initial staining with antibody and washing with staining buffer, propidium iodide (PI, 100 μ L of 0.005 mg/mL, Sigma-Aldrich) solution was added as a non-viable cell label. A Becton Dickinson FACScan® Flow Cytometer was used for enumeration where log fluorescence intensity is read gated on PI to eliminate dead cells and a forward scatter threshold high enough to eliminate red blood cells. Data were analyzed using CellQuest™ software (Becton Dickinson, San Jose, CA).

Statistical Analysis

All results were evaluated in JMP® software using a parametric one-way analysis of variance (ANOVA), and Dunnett's Test was used to compare differences between the treatment and sham control groups. Additional control groups, including naïve and positive controls, were tested using a separate ANOVA and compared with the sham control using the Dunnett's t-Test.

Results and Discussion

Effect of EPCL on Splenocyte Phenotype

As shown in Table 3, following analysis by flow cytometry, microfibrinous EPCL did not affect absolute number or percentage of T cells, T cell subsets, macrophages or natural killer cells at any dose level. The percentage of B cells was slightly increased with 1 implant (5% increase), however, the absolute number of B cells was not affected. The slight increase in percentage of B cells only occurring only at the low dose is not likely to be treatment-related and is not believed to be biologically relevant.

As seen in Table 4, nanofibrous EPCL did not affect the absolute number or percentage of B cells, T cells, T cell subsets, or natural killer cells at any dose level. The percentage and absolute number of macrophages was decreased with 1, 2, and 4 nanofibrous implants when compared to sham control. However, these decreases were not dose-responsive and do not appear to be treatment-related.

Table 3. Splenocyte subsets following microfibrinous EPCL implantation. Results are presented as mean \pm S.E. for absolute or percent cells. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group. N/C = not conducted. Only appropriate controls were used for each cell type.

	Splenocyte Subsets	Microfibrinous Polycaprolactone (mm ²)					CPS 50 mg/kg	AAGM1 1:10
		Naïve	Sham	32	64	128		
Absolute (x10 ⁶)	B Cell (Ig ⁺)	94.8 \pm 4.9	106.4 \pm 4.6	104.6 \pm 6.1	110.8 \pm 5.3	95.4 \pm 4.8	26.5 \pm 2.6**	N/C
	T Cell (CD3 ⁺)	40.5 \pm 2.4	46.4 \pm 2.3	38.4 \pm 2.7	39.5 \pm 2.3	38.7 \pm 2.5	27.3 \pm 1.4**	N/C
	TH (CD4 ⁺ CD8 ⁻)	26.9 \pm 1.6	29.7 \pm 1.5	26.1 \pm 1.6	28.5 \pm 1.6	27.9 \pm 1.9	17.0 \pm 0.8**	N/C
	TC (CD4 ⁻ CD8 ⁺)	17.3 \pm 1.3	18.7 \pm 0.8	14.7 \pm 1.2	14.8 \pm 1.3	15.9 \pm 0.9	12.4 \pm 0.8**	N/C
	T (CD4 ⁺ CD8 ⁺)	0.16 \pm 0.03	0.22 \pm 0.04	0.17 \pm 0.01	0.18 \pm 0.04	0.14 \pm 0.04	0.16 \pm 0.02	N/C
	NK (NK1.1 ⁺ CD3 ⁻)	4.4 \pm 0.3	4.2 \pm 0.3	4.7 \pm 0.3	4.7 \pm 0.2	4.3 \pm 0.2	N/C	0.9 \pm 0.1**
	MΦ (Mac-3 ⁺)	2.6 \pm 0.3**	4.9 \pm 0.3	3.9 \pm 0.2	4.1 \pm 0.3	4.3 \pm 0.6	5.2 \pm 0.3	N/C
Percent	B Cell (Ig ⁺)	57.8 \pm 1.1	58.2 \pm 0.5	61.4 \pm 0.6*	60.6 \pm 1.1	57.5 \pm 1.1	41.5 \pm 1.9**	N/C
	T Cell (CD3 ⁺)	24.7 \pm 0.7	25.4 \pm 0.6	22.7 \pm 1.2	21.7 \pm 1.0	23.5 \pm 1.5	43.8 \pm 2.5**	N/C
	TH (CD4 ⁺ CD8 ⁻)	16.4 \pm 0.6	16.2 \pm 0.4	15.6 \pm 0.7	15.6 \pm 0.7	16.8 \pm 5.8	27.3 \pm 1.4**	N/C
	TC (CD4 ⁻ CD8 ⁺)	10.4 \pm 0.4	10.2 \pm 0.2	8.8 \pm 0.7	8.1 \pm 0.7	9.6 \pm 0.5	20.1 \pm 1.4**	N/C
	T (CD4 ⁺ CD8 ⁺)	0.10 \pm 0.02	0.12 \pm 0.02	0.10 \pm 0.00	0.10 \pm 0.02	0.09 \pm 0.02	0.25 \pm 0.03**	N/C
	NK (NK1.1 ⁺ CD3 ⁻)	2.7 \pm 1.9	2.3 \pm 0.2	2.8 \pm 0.1	2.6 \pm 0.2	2.6 \pm 0.1	N/C	0.5 \pm 0.0**
	MΦ (Mac-3 ⁺)	1.6 \pm 0.2*	2.7 \pm 0.1	2.3 \pm 0.1	2.3 \pm 0.2	2.5 \pm 0.3	8.3 \pm 0.5**	N/C
Spleen Cell Number (x10 ⁶)		164.8 \pm 3.5	182.6 \pm 7.4	170.2 \pm 9.7	182.7 \pm 8.0	166.1 \pm 7.9	63.1 \pm 3.5**	174.2 \pm 8.9

Table 4. Splenocyte subsets following nanofibrous EPCL implantation. Results are presented as mean \pm S.E. for absolute or percent cells. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group. N/C = not conducted. Only appropriate controls were used for each cell type.

	Splenocyte Subsets	Nanofibrous Polycaprolactone (mm ²)					CPS 50 mg/kg	AAGM1 1:10
		Naïve	Sham	32	64	128		
Absolute (x10 ⁶)	B Cell (Ig ⁺)	89.3 \pm 4.8**	118.5 \pm 5.4	113.2 \pm 5.4	100.4 \pm 3.9	100.0 \pm 7.8	39.5 \pm 3.1**	N/C
	T Cell (CD3 ⁺)	35.3 \pm 2.7	37.0 \pm 2.2	40.6 \pm 3.3	38.9 \pm 2.1	39.2 \pm 2.6	34.3 \pm 3.3	N/C
	TH (CD4 ⁺ CD8 ⁻)	21.7 \pm 1.6	26.2 \pm 1.3	24.6 \pm 3.1	26.0 \pm 1.8	24.0 \pm 1.8	22.7 \pm 1.9	N/C
	TC (CD4 ⁻ CD8 ⁺)	13.3 \pm 1.4	12.5 \pm 0.9	12.0 \pm 2.0	12.5 \pm 0.5	11.8 \pm 0.5	11.8 \pm 1.2	N/C
	T (CD4 ⁺ CD8 ⁺)	0.08 \pm 0.03	0.15 \pm 0.04	0.07 \pm 0.03	0.11 \pm 0.03	0.09 \pm 0.03	0.06 \pm 0.02	N/C
	NK (NK1.1 ⁺ CD3 ⁻)	4.4 \pm 0.4*	5.9 \pm 0.4	6.2 \pm 0.3	5.1 \pm 0.2	4.9 \pm 0.5	N/C	0.8 \pm 0.3**
	MΦ (Mac-3 ⁺)	3.1 \pm 0.5*	5.3 \pm 0.6	2.7 \pm 0.5**	2.8 \pm 0.3**	3.1 \pm 0.4**	3.1 \pm 0.7*	N/C
Percent	B Cell (Ig ⁺)	55.3 \pm 1.2	58.5 \pm 1.4	59.3 \pm 1.6	53.8 \pm 1.2	57.2 \pm 1.4	43.9 \pm 1.7**	N/C
	T Cell (CD3 ⁺)	21.8 \pm 0.9	18.7 \pm 1.0	21.3 \pm 1.4	20.9 \pm 1.2	22.6 \pm 1.0	38.0 \pm 1.3**	N/C
	TH (CD4 ⁺ CD8 ⁻)	13.8 \pm 0.6	13.2 \pm 0.5	12.9 \pm 1.4	13.9 \pm 0.9	13.8 \pm 0.6	24.1 \pm 1.1**	N/C
	TC (CD4 ⁻ CD8 ⁺)	8.4 \pm 0.6**	6.3 \pm 0.4	6.3 \pm 1.0	6.8 \pm 0.3	6.9 \pm 0.5	13.0 \pm 0.7**	N/C
	T (CD4 ⁺ CD8 ⁺)	0.10 \pm 0.02	0.08 \pm 0.02	0.04 \pm 0.02	0.06 \pm 0.02	0.06 \pm 0.02	0.07 \pm 0.02	N/C
	NK (NK1.1 ⁺ CD3 ⁻)	2.7 \pm 0.1	3.0 \pm 0.2	3.3 \pm 0.2	2.7 \pm 0.1	2.9 \pm 0.2	N/C	0.6 \pm 0.2**
	MΦ (Mac-3 ⁺)	2.0 \pm 0.3	2.7 \pm 0.3	1.4 \pm 0.2**	1.5 \pm 0.1**	1.8 \pm 0.2*	3.3 \pm 0.7	N/C
Spleen Cell Number (x10 ⁶)		161.7 \pm 9.1*	197.8 \pm 6.0	191.9 \pm 10.1	186.7 \pm 5.4	172.3 \pm 9.1	89.9 \pm 6.5**	165.1 \pm 9.7*

Effect of EPCL on Innate Immunity: NK Cytotoxicity

Results of natural killer cell percent cytotoxicity are shown in Figure 8. Neither microfibrinous nor nanofibrinous EPCL had effects on natural killer cell activity in 24-28 week old female B6C3F1 mice. The positive control animals, which received AAGM1, had minimal cytotoxic activity.

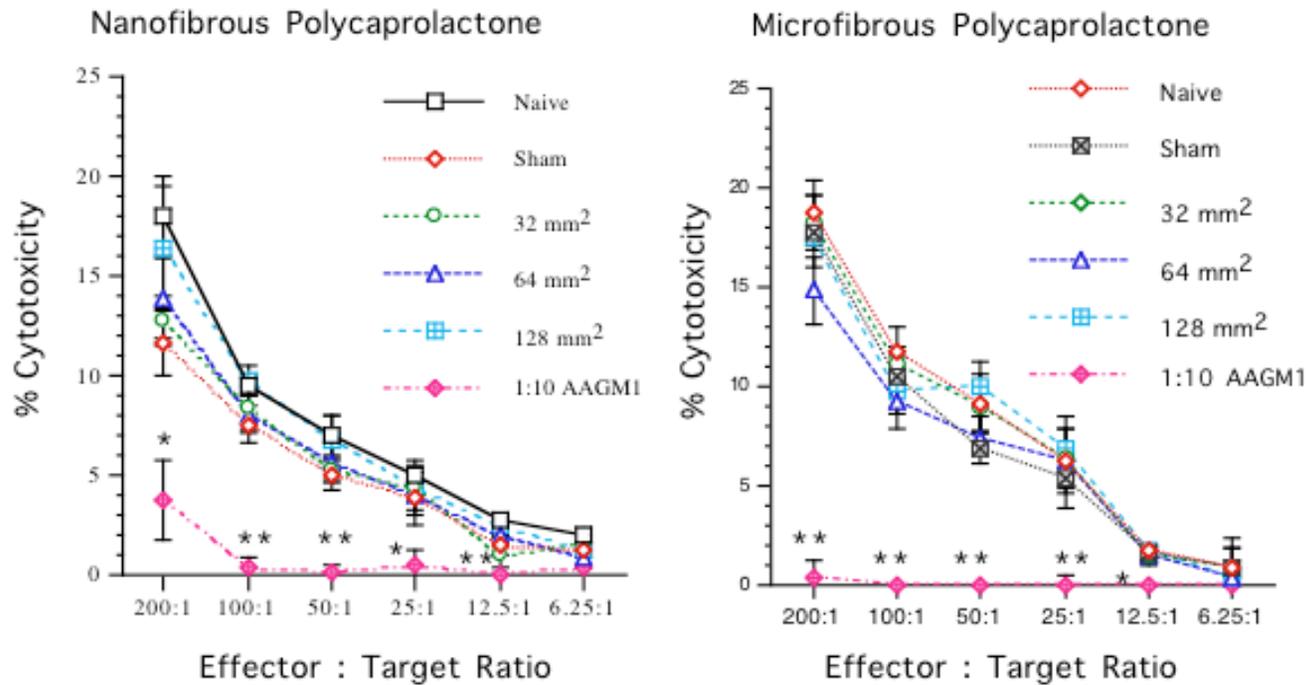


Figure 8. Natural Killer cell activity. Results are presented as mean \pm S.E. for percent cytotoxicity. Anti-asialo GM1 (AAGM1) was administered to positive control animals by i.v. injection on day 28. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Effect of EPCL on Humoral Immunity: AFC and sRBC ELISA

AFC assay results are presented in Figure 9 and 10 as AFC/10⁶ spleen cells (specific activity) and total spleen activity. Microfibrous EPCL did not significantly affect the AFC response to sheep erythrocytes when evaluated as either specific activity or as total spleen activity in 24-28 week old female B6C3F1 mice (Figure 9). Animals exposed to nanofibrous EPCL did demonstrate a statistically significant decrease in total activity at the middle dose, and a slight, albeit not statistically significant, decrease can be observed at each dose level. Results were obtained from combining two studies, each of which utilized seven or eight animals per group. CPS significantly suppressed the AFC response as expected.

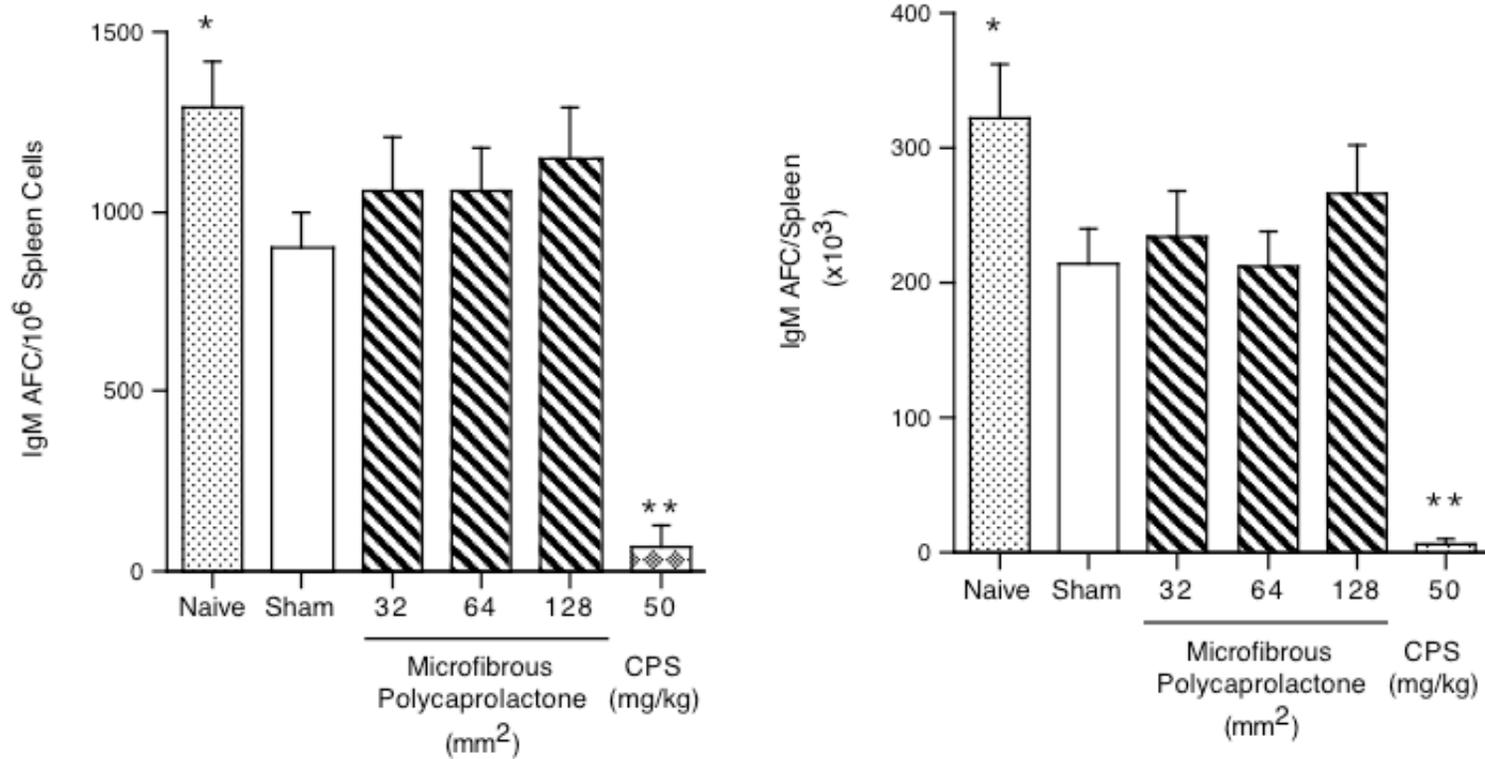


Figure 9. Spleen IgM Antibody-Forming Cell (AFC) Response to Sheep Erythrocytes. Female B6C3F1 mice were exposed to microfibrinous or nanofibrinous EPCL for 28 days and were immunized with sRBCs on day 25. Results are presented as percent control mean \pm S.E. for the AFC/10⁶ Spleen Cells (specific activity) and AFC/Spleen (total spleen activity). On days 25-28, 50 mg/kg cyclophosphamide (CPS) was administered as a positive control by i.p. injection. The level of statistical significance is denoted as ** $p < 0.01$ as compared to the sham control group.

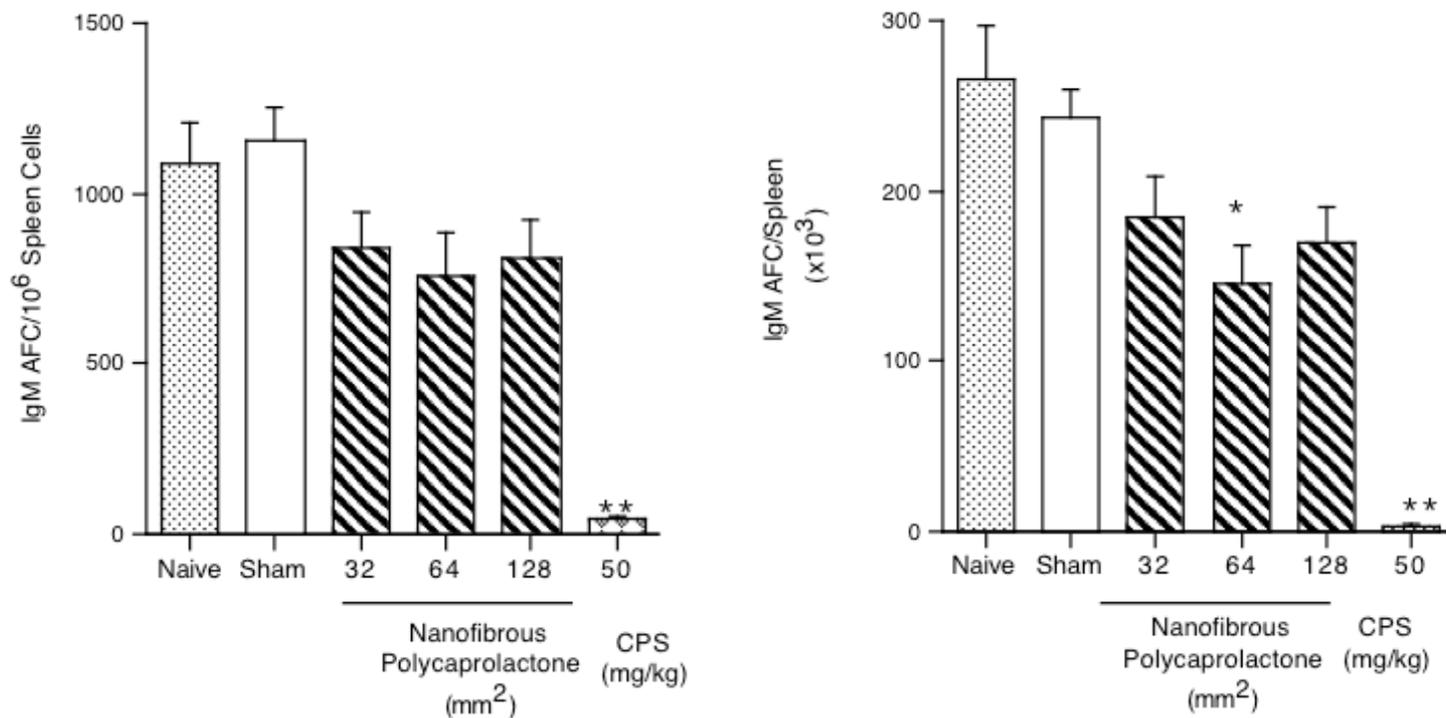


Figure 10. Spleen IgM Antibody-Forming Cell (AFC) Response to Sheep Erythrocytes. Female B6C3F1 mice were exposed to microfibrinous or nanofibrinous EPCL for 28 days and were immunized with sRBCs on day 25. Results are presented as percent control mean \pm S.E. for the AFC/10⁶ Spleen Cells (specific activity) and AFC/Spleen (total spleen activity). On days 25-28, 50 mg/kg cyclophosphamide (CPS) was administered as a positive control by i.p. injection. The level of statistical significance is denoted as ** $p < 0.01$ as compared to the sham control group.

Shown in Figure 11 are sRBC ELISA results, expressed as mean $\text{Log}_2(\text{Titer})$. Neither microfibrinous or nanofibrinous EPCL produced any significant effects compared to the sham control. Positive control mice, which received CPS, had a significant decrease in the serum IgM response to sRBC compared to the sham control.

Taken with results from the sRBC ELISA, other measures of immune function, and lack of a dose-responsive trend with nanofibrinous EPCL in the AFC assay it is not believed to be toxic to humoral immunity in aged animals.

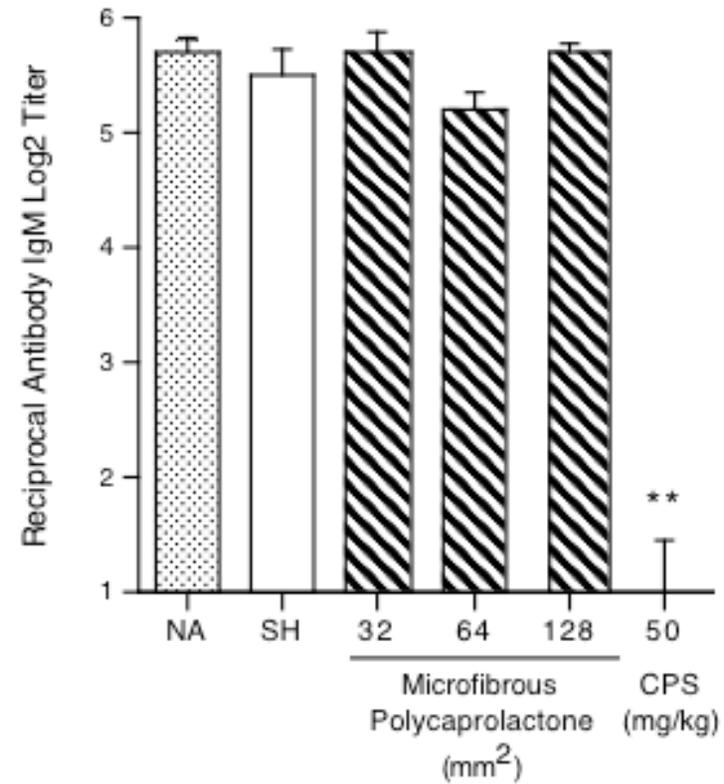
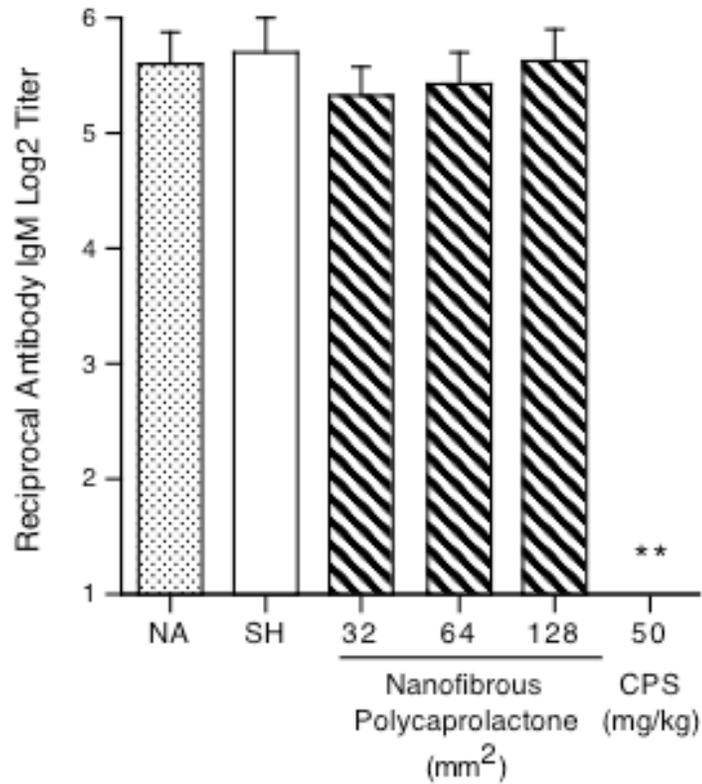


Figure 11. sRBC ELISA. Results are presented as Titer (Log₂) Mean ± S.E.. Level of statistical significance is denoted as ** p < 0.01 as compared to the sham control group.

Effect of EPCL on Cell-mediated Immunity: DTH

Results of the DTH response are shown in Figure 12 and presented as footpad swelling in mm x 100. Surgical implantation of microfibrinous or nanofibrinous EPCL did not affect the DTH response to *C. albicans* in 24-28 week old female B6C3F1 mice. The positive control group, which received CPS, had significantly decreased footpad swelling when compared to the sham control. Additionally, footpad swelling of the challenge only group was significantly less than the sham control group, which had received both sensitization and challenge.

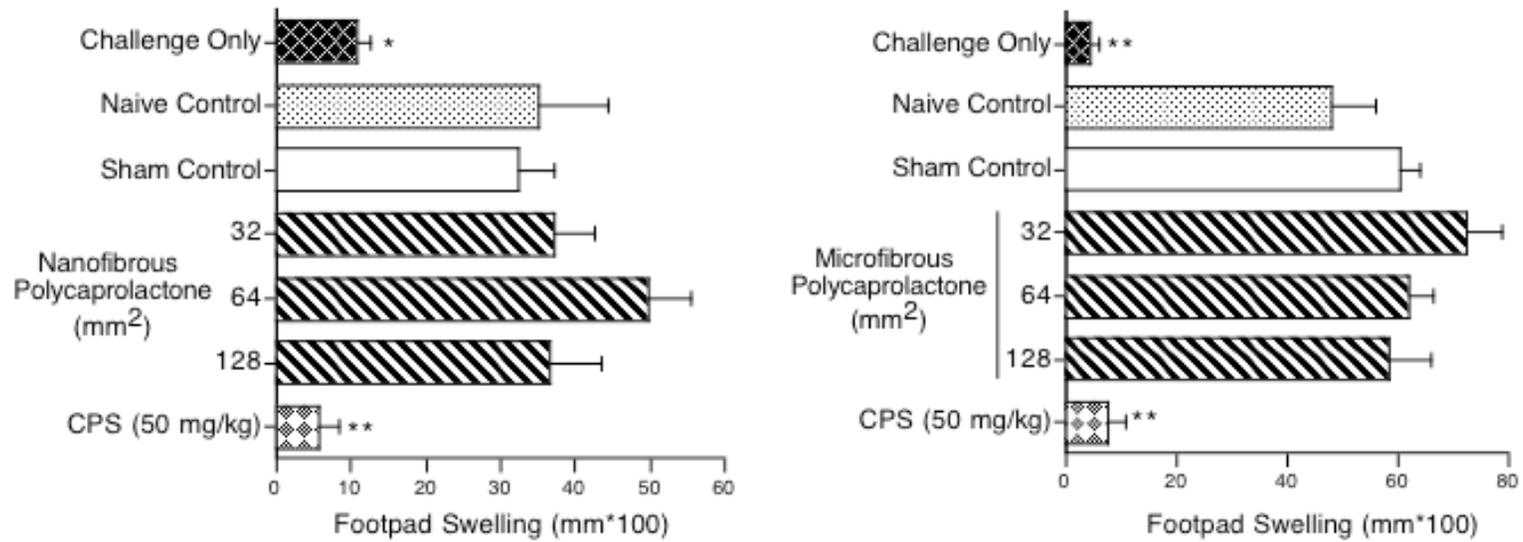


Figure 12. Delayed type hypersensitivity response to *C. albicans*. Results are presented as Mean \pm S.E. footpad swelling (mm*100). Level of statistical significance is denoted as ** $p < 0.01$ as compared to the sham control group.

Conclusions

The work was undertaken to examine aged animal immune responses to EPCL due to the potential use in applications intended for an aged patient population. Overall, results from innate, cell-mediated, and humoral immune tests demonstrate microfibrinous and nanofibrinous EPCL do not affect immune responses in aged animals. Therefore, studies indicate that EPCL is a material with excellent immunocompatibility deserving further investigation for tissue engineering applications including those for elderly populations.

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CHAPTER 5: Additional Studies of Lidocaine and EPCL

Lidocaine

A topical analgesic was required following survival surgery by the VCU IACUC. A preliminary study was permitted to test the chosen analgesic. Analgesics, which can include opioids, non-steroidal anti-inflammatory drugs, and local analgesics, can interfere with the immune system [Piersma et al. 1999]. Due to the immunodulatory potential of these medications they are typically not used in immunology research [Piersma et al. 1999]. Lidocaine was found to have the least documented immunomodulatory effects of the considered analgesics. A study by Dickstein et al. showed effects on lymphocyte proliferation assays when mice were exposed to 0.25 mg/10 g body weight lidocaine by i.p. injection four times a day for 7 days [Dickstein et al. 1985]. In the presented studies, animals were exposed to a small dab of lidocaine on each incision site once a day for three days. In order to determine if the lidocaine would interfere with the studies, a study was conducted where animals were exposed to lidocaine for three days and on day 15 of the study the antibody forming cell assay was conducted. Three groups were used, a naïve group (NA) which received no treatment, a sham surgery group (SH) which received the

same sham surgery as conducted in the EPCL studies without lidocaine, and a lidocaine (LDC) group which received sham surgery and three days of lidocaine treatment. As shown in Figure 13, the lidocaine treatment did not affect the AFC response.

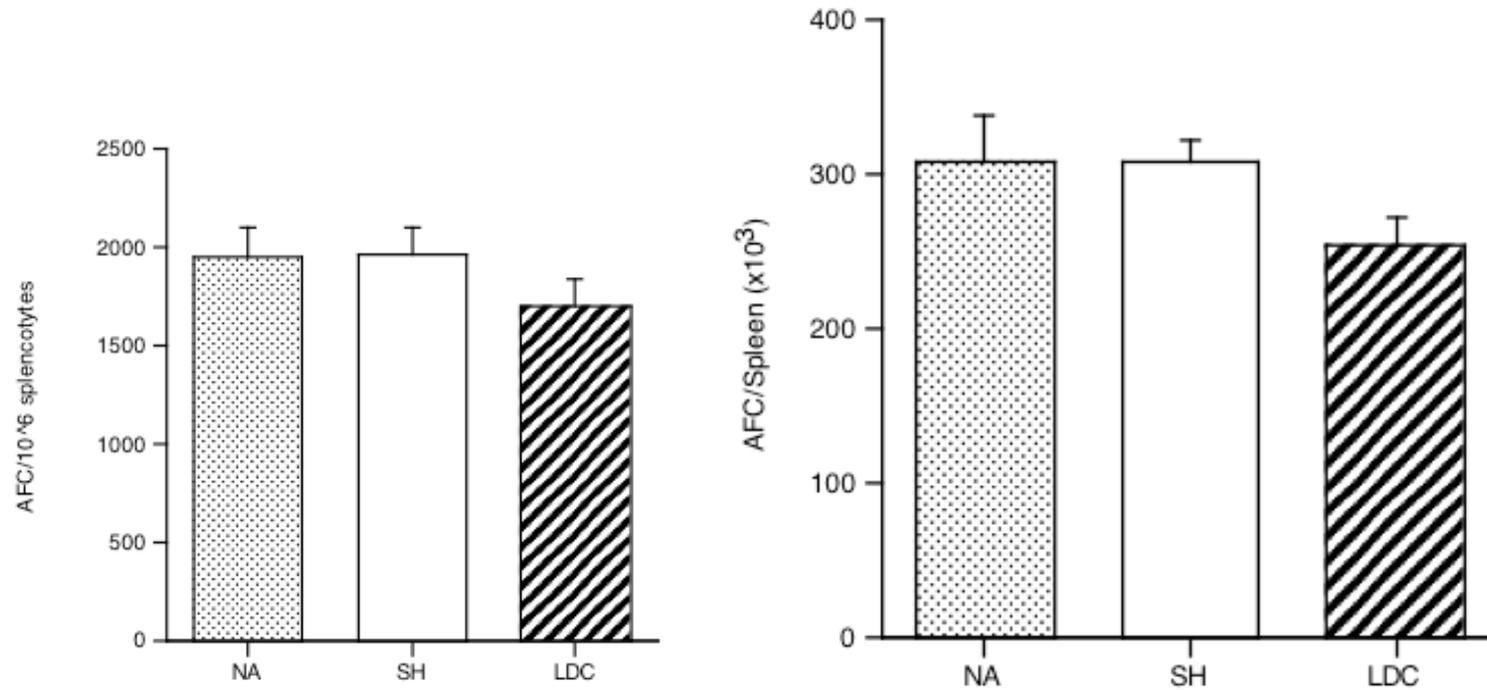


Figure 13. Antibody forming cell response following surgical treatment and lidocaine exposure. Groups tested were naïve (NA), sham control without lidocaine (SH) and sham control with lidocaine treatment for 3 days (LDC).

Additional EPCL Studies

Additional endpoints were evaluated but not pursued for all combinations of fiber size and age. Endpoints included were B-cell and T-cell lymphocyte proliferation assays.

Methods

The lymphocyte proliferation assays anti-CD3, concanavlin A (con A), and lipopolysaccharide (LPS), and the mixed leukocyte response (MLR), were conducted on young animals following exposure to microfibrinous EPCL. Assays were conducted on day 29 and have been described previously by Guo et al. [Guo et al. 2001].

Anti-CD3 Antibody Mediated T-cell Proliferation

On day 29 animals were humanely euthanized and splenocytes were prepared and diluted in EBSS to 2×10^6 cells/mL. 96-well BioCoat T-Cell activation plates were used, to stimulate T-cell proliferation anti-CD3 antibody coated plates were used and for controls uncoated control plates were used (BD Biosciences, San Jose, CA). 200 μ l of splenocytes and 100 μ l of RPMI were added to both the stimulated and unstimulated wells (4 replicates per animal) and plates were incubated for 3 days at 37°C with 5% CO₂ and 95% humidity. On day 2 of incubation, the cells were pulsed with ³H-thymidine (50 μ Ci/mL, 20 μ l/well). On day 3 of incubation, 18-24 hours after pulsing, cells were harvested using a Tomtec Harvester 96 Mach IIM (Tomtec, Hamden, CT) onto Wallac filtermats (Wallac, Turku, Finland), and scintillation fluid was added and the filtermats were counted on a 1450

Microbeta Trilux liquid scintillation and luminescence counter (Perkin Elmer, Gaithersburg, MD). The incorporation of ^3H -thymidine into the proliferating cells was used as the endpoint of the assay, and the data expressed as counts per minute (CPM)/ 2×10^5 cells.

Concanavalin A Stimulated T-Cell Proliferation

The con A assay was performed similarly to the anti-CD3, splenocytes were added at 2×10^6 cells/well to wells with con A (Amersham Pharmacia, Bjorkgatan, Sweden) at four dilutions 10, 5, 2.5, or $1.25 \mu\text{g}/\text{mL}$ (with 3 replicate wells for each concentration and 3 for unstimulated controls) and plates were incubated, pulsed and harvested in the manner as anti-CD3. The endpoint for the con A assay was also incorporation of ^3H -thymidine into the proliferating cells and expressed as cpm/ 2×10^6 cells.

F(ab')₂ + IL-4 Stimulated B-Cell Proliferation

The F(ab')₂ + IL-4 assay was performed similarly to the anti-CD3, splenocytes were added at 2×10^6 cells/well to wells with media only, or F(ab')₂ antibody fragment only, (Accurate Chemical, Westbury, NY) or F(ab')₂ with 10 ng/mL IL-4 (R&D Systems, Minneapolis, MN) and plates were incubated, pulsed and harvested in the manner as anti-CD3. The endpoint for the con A assay was also incorporation of ^3H -thymidine into the proliferating cells and expressed as cpm/ 2×10^6 cells.

Lipopolysaccharide Stimulated B-cell Proliferation

In the LPS assay splenocytes were added at 2×10^5 cells/well to wells with 100 $\mu\text{g/mL}$ LPS derived from *Salmonella typhosa* (Sigma Aldrich, St. Louis, MO) (with 4 replicate wells for stimulated and 4 wells for unstimulated controls) and plates were incubated, pulsed and harvested in the manner as anti-CD3. The endpoint for the LPS assay was also incorporation of ^3H -thymidine into the proliferating cells and expressed as cpm/ 2×10^5 cells.

The Mixed Leukocyte Response

The one-way mixed leukocyte response to DBA/2 spleen cells measures the proliferation of T cells in response to an allogeneic stimulus (the DBA/2 mouse spleen cells) [Guo et al. 2001]. On day 29, animals were humanely euthanized and splenocytes from exposed mice (responders) were prepared, diluted and added to U-bottom 96-well plates at a concentration of 1×10^5 cells/well. The allogeneic (stimulator) cells from DBA/2 mice were treated with mitomycin C ($0.5 \text{mg}/2 \times 10^8$ cells) (Sigma Aldrich, St. Louis, MO) for 45 minutes at 5% CO_2 and 37°C to stop proliferation. The stimulator cells were then washed four times with EBSS and diluted in RPMI at a concentration of 4×10^6 cells/mL. Each plate row contains 4 wells of unstimulated responder cells with RPMI and 4 wells of responder plus stimulator cells. The cells were co-cultured for 5 days with 5% CO_2 and 37°C . On day 4 of incubation the cells were pulsed with ^3H -thymidine ($50 \mu\text{Ci/mL}$, 20 $\mu\text{l/well}$). On day 5 of incubation, 18-24 hours after pulsing, cells were harvested using a Tomtec Harvester 96 Mach IIM (Tomtec, Hamden, CT) onto Wallac filtermats (Wallac,

Turku, Finland), and scintillation fluid was added and the filtermats were counted on a 1450 Microbeta Trilux liquid scintillation and luminescence counter (Perkin Elmer, Gaithersburg, MD). The incorporation of ^3H -thymidine into the proliferating cells was used as the endpoint of the assay, and the data expressed as cpm/ 1×10^5 cells.

Positive Control

For all lymphocyte proliferation assays CPS was used as the positive control administered at 50 mg/kg by i.p. injection on days 25-28.

Results

Anti-CD3 Antibody Mediated T-cell Proliferation

As shown in Figure 14, two studies were conducted in young animals following exposure to microfibrinous EPCL using the anti-CD3 antibody mediated T-cell proliferation assay. In the first study, naïve and sham controls and EPCL treated groups were not different. In the second study, the sham control was significantly lower than the naïve response and the 2-implant group was significantly increased above the sham control. In both studies, the positive control significantly suppressed the response.

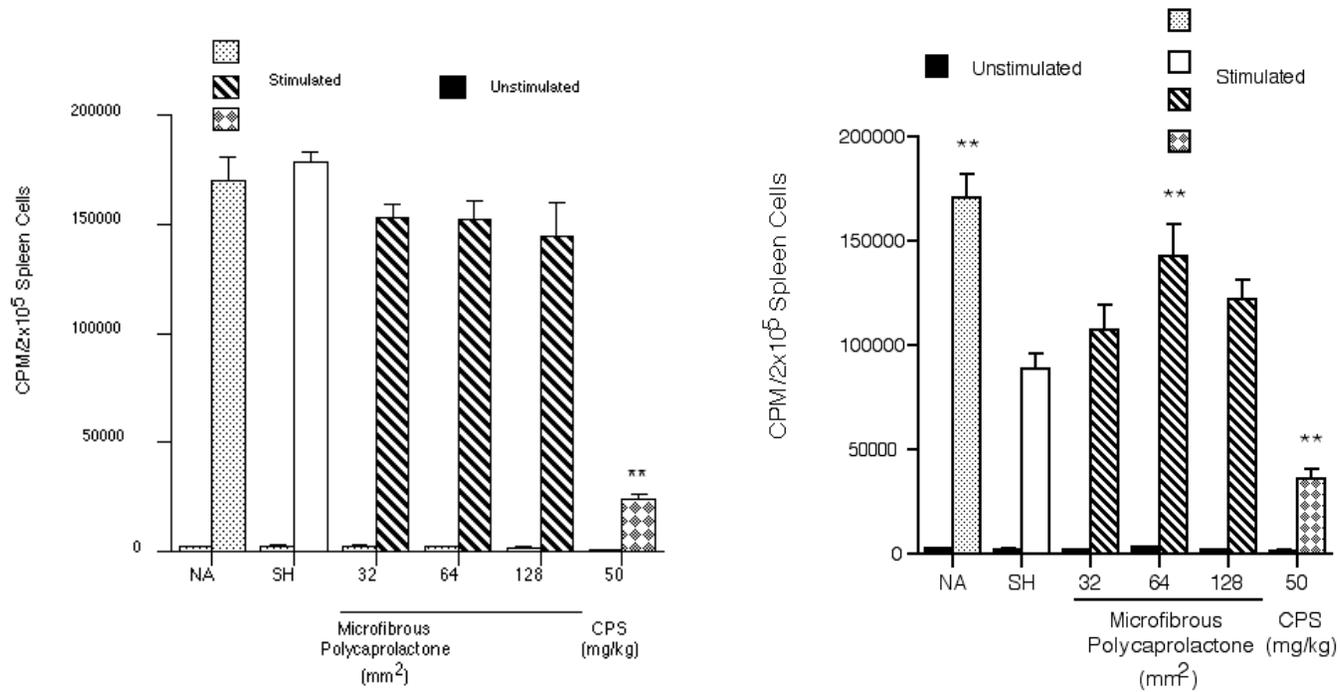


Figure 14. Proliferation response to anti-CD3. Results are presented as mean \pm S.E.. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Mixed Leukocyte Response

As shown in Figure 15, two studies were conducted in young animals following exposure to microfibrinous EPCL using the mixed leukocyte response. In the first study, naïve and sham controls and EPCL treated groups were not different. In the second study, the sham control was significantly lower than the naïve response and the 1-implant and 2-implant groups were significantly increased above the sham control. In both studies, the positive control significantly suppressed the response.

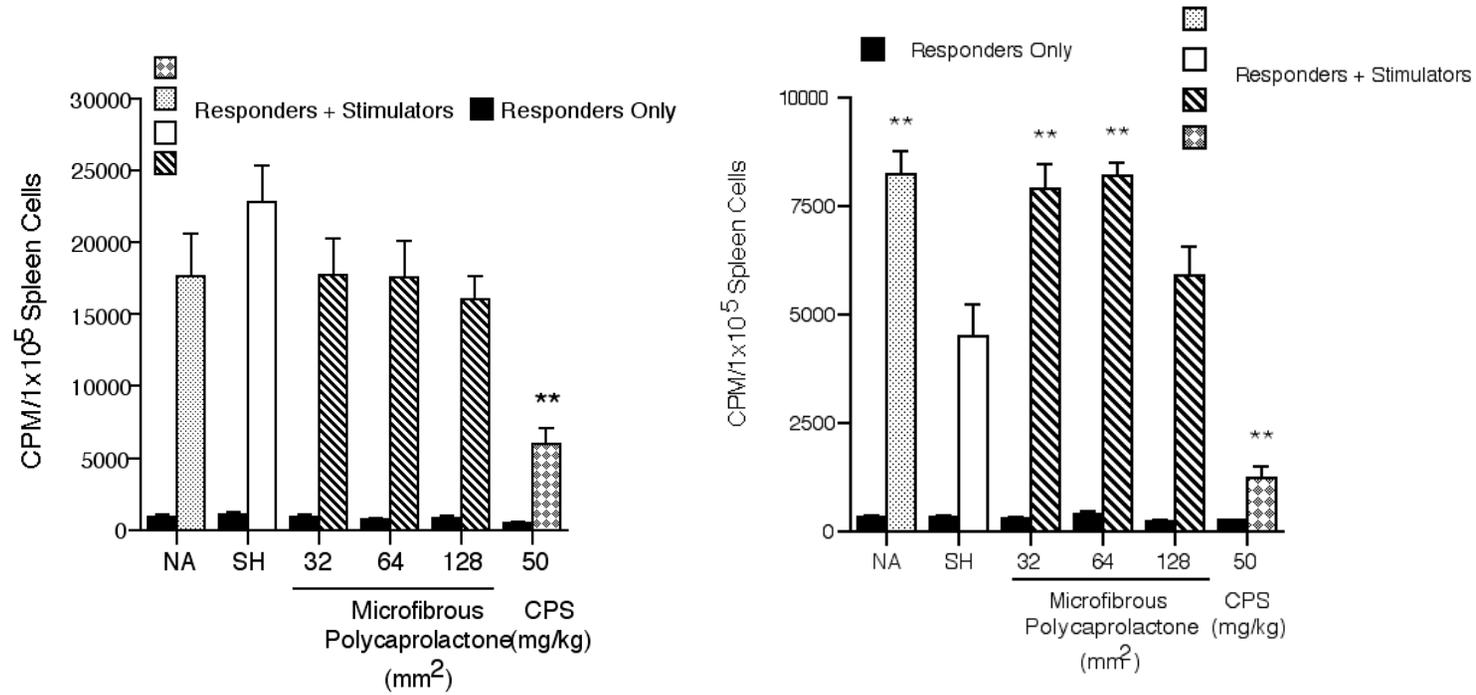


Figure 15. Mixed leukocyte response. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Concanavalin A Mediated T-cell Proliferation

As shown in Figure 16, one study was conducted in young animals following exposure to microfibrinous EPCL using the concanavalin A mediated t-cell proliferation assay. In the study, naïve and sham controls and EPCL treated groups were not different. The positive control significantly suppressed the response.

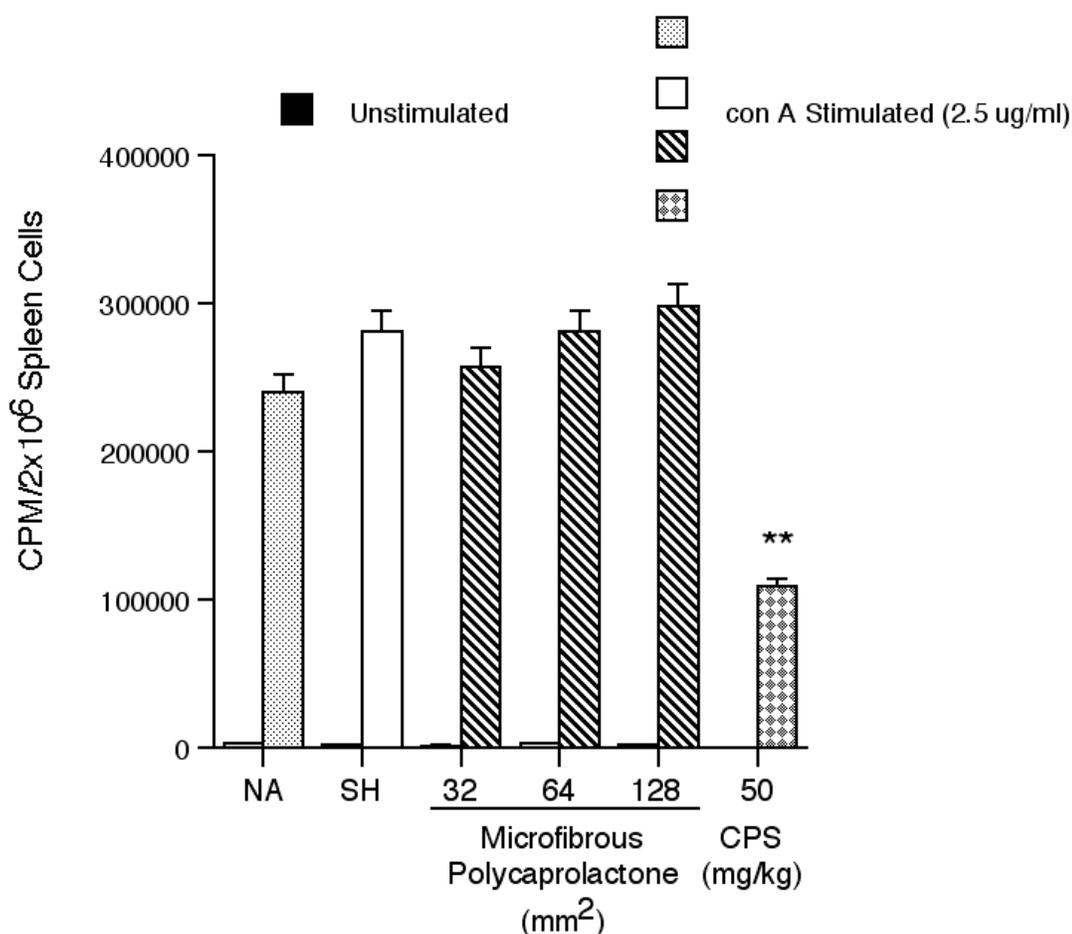


Figure 16. Concanavalin A stimulated proliferation. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

F(ab')₂ + IL-4 Stimulated B-Cell Proliferation

As shown in Figure 17, one study was conducted in young animals following exposure to microfibrous EPCL using the F(ab')₂ + IL-4 stimulated B-cell proliferation assay. In the study, the naïve control was significantly higher than the sham control. The 2-implant EPCL group was significantly lower than the sham control and the positive control significantly suppressed the response.

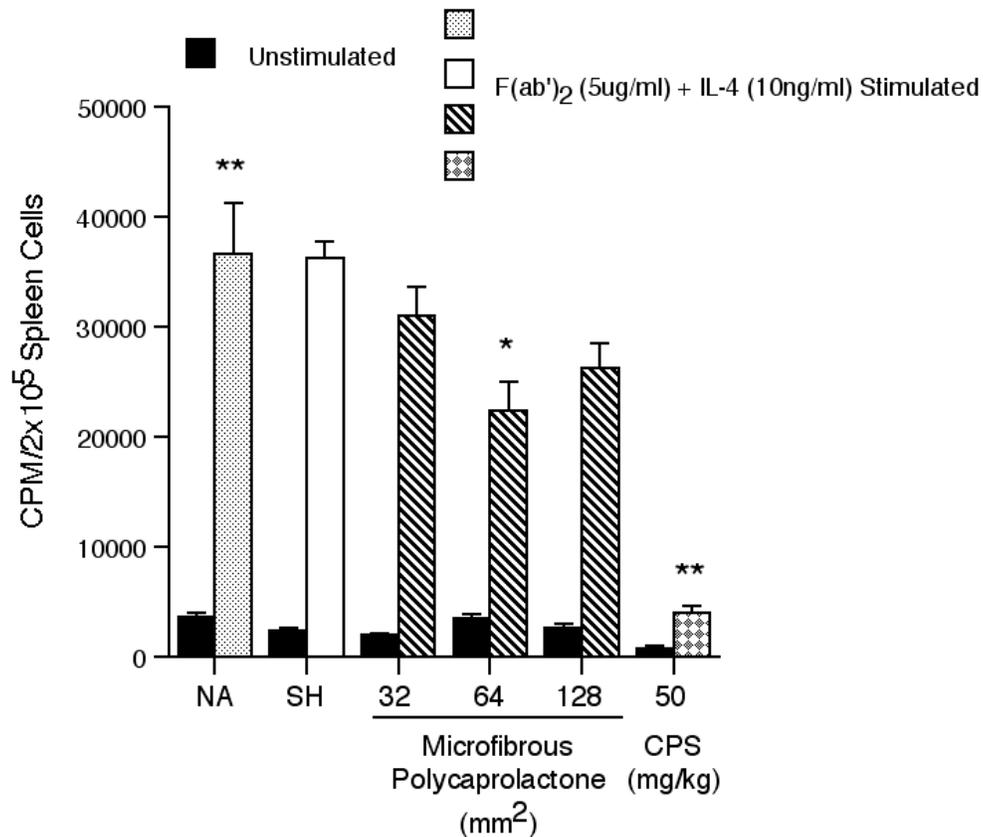


Figure 17. F(ab')₂ + IL-4 simulated proliferation. Results are presented as mean ± S.E. The level of statistical significance is denoted as **p < 0.01 or *p < 0.05 as compared to the sham control group.

Lipopolysaccharide Stimulated B-Cell Proliferation

As shown in Figure 18, one study was conducted in young animals following exposure to microfibrinous EPCL using the lipopolysaccharide stimulated B-cell proliferation assay. In the study, the naïve control was significantly higher than the sham control. The EPCL groups were not significantly different than the sham control and the positive control significantly suppressed the response.

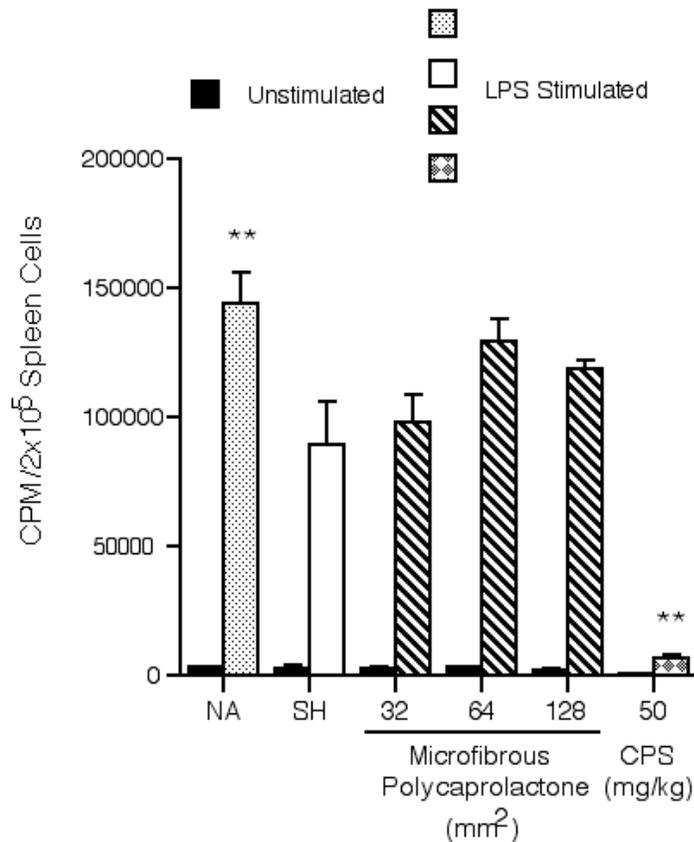


Figure 18. Lipopolysaccharide stimulated proliferation. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Discussion

The lymphocyte proliferation assays are used to measure T-cell and B-cell proliferation by *ex vivo* stimulation. Normal physiological T-cell stimulation occurs through antigen-specific binding to the T-cell receptor. CD3 is a pan T-cell marker and T-cell receptor subunit that can activate T-cell proliferation with appropriate co-stimulation (provided in the spleen suspension by antigen-presenting cells) [Pitcher et al. 2005, Janeway 2008]. Mitogens such as con A are capable of causing polyclonal lymphocyte proliferation, con A specifically stimulates T-cells [Janeway 2008]. In the mixed leukocyte response, responder T-cells proliferate in response to “non-self” allogeneic T-cells, the DBA/1 stimulator cells. Lipopolysaccharide is a mitogen, which stimulates polyclonal B cell proliferation [Janeway 2008]. F(ab')₂ stimulates the B-cell receptor (IgM antibody immobilized on the B-cell surface), however alone cannot stimulate antibody production, the addition of IL-4 can provide the necessary signal for cross-linking and B-cell proliferation [Parker et al. 1980].

Results from these studies were inconsistent. In the first anti-CD3 study no differences were observed between sham and EPCL-treated groups, with the EPCL-treated groups having slightly lower means than the sham control. In the second anti-CD3 study, the sham control response was lower than the naïve response and EPCL-treated groups were higher and more similar to the naïve response with the 2-implant group significantly higher than the sham. Taken together it is unlikely that these results are biologically significant. Similar results were obtained in the MLR studies; in the second MLR study both the 1-implant and 2-implant groups were significantly higher than the sham control.

In the third T-cell proliferation assay, con A, no differences were observed between naïve, sham, or EPCL-treated groups. Considering consistent results of no effect in the holistic assay the delayed-type hypersensitivity response to *C. albicans*, which requires T-cell proliferation, the overall evidence suggests that T-cell responses are not affected by EPCL.

The B-cell proliferation assays also resulted in non-dose responsive results. In the F(ab₂)' + IL-4 assay, the naïve group was significantly higher than the sham control group, and of the EPCL-treated groups a significantly lower response was seen in the 2-implant group. In the LPS assay, a significantly higher response was seen in the naïve group over the sham control while no differences were seen between the sham and EPCL-treated groups. General trends in these studies were opposite, where 2-implant and 4-implant EPCL-treated groups in the F(ab₂)' + IL-4 study were lowest, in the LPS study the 2-implant and 4-implant groups had higher means than the sham and 1-implant groups. Taken with consistent results of no effect in young animals in the holistic AFC assay, which requires antibody production, the overall evidence suggests that B-cell responses are not affected by EPCL.

The lymphocyte proliferation studies were conducted only with the young animals and microfibrinous EPCL given the overall weight of evidence that EPCL does not cause immunotoxicity in the holistic assays requiring lymphocyte function. Additionally, Sigma-Aldrich stop producing the original lot of PCL, remaining PCL from the original lot was used to complete aged animal studies.

New PCL Lot

Due the discontinuation of the original lot of PCL (molecular weight 65,000 kDa), the concentration of 50 mg/mL for nanofibrous scaffolds was established for the new lot (molecular weight 70,000-90,000 kDa) as shown in Figure 19. Mean fiber diameter was determined to be $0.56 \pm 0.03 \mu\text{m}$. As seen in Figure 20, the second lot of PCL did not affect the *c. Albicans* DTH.

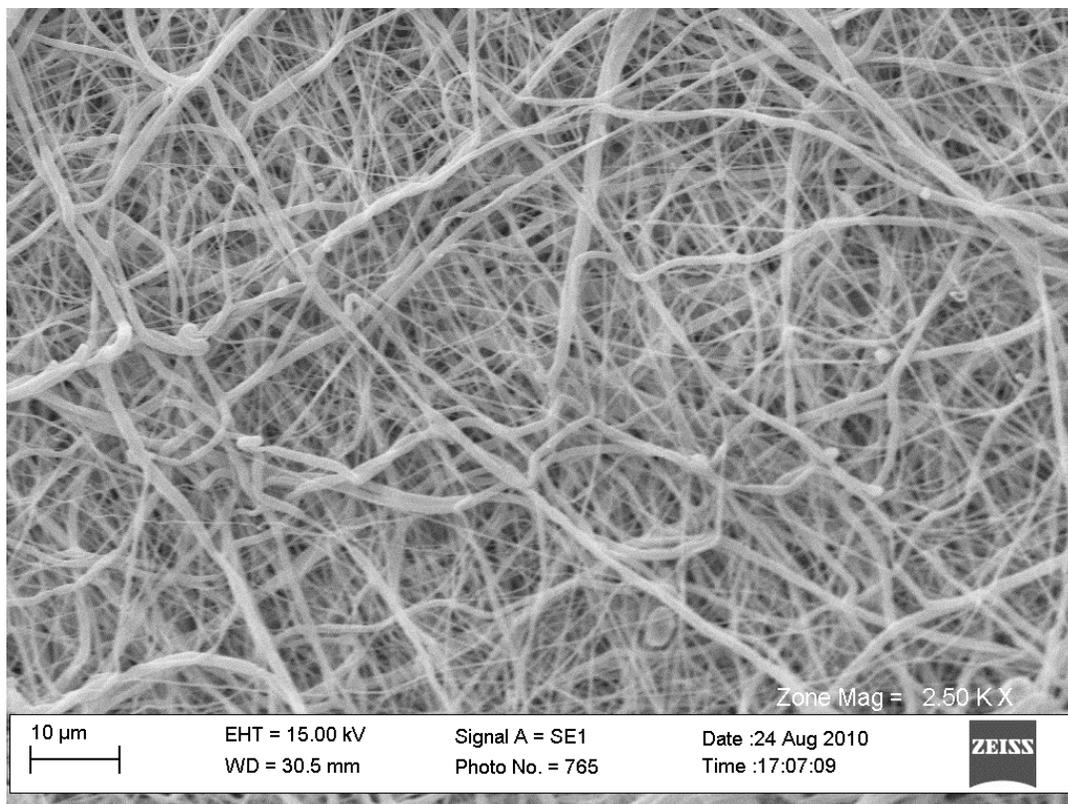


Figure 19. Scanning electron micrograph of nanofibrous EPCL with the second lot of PCL.

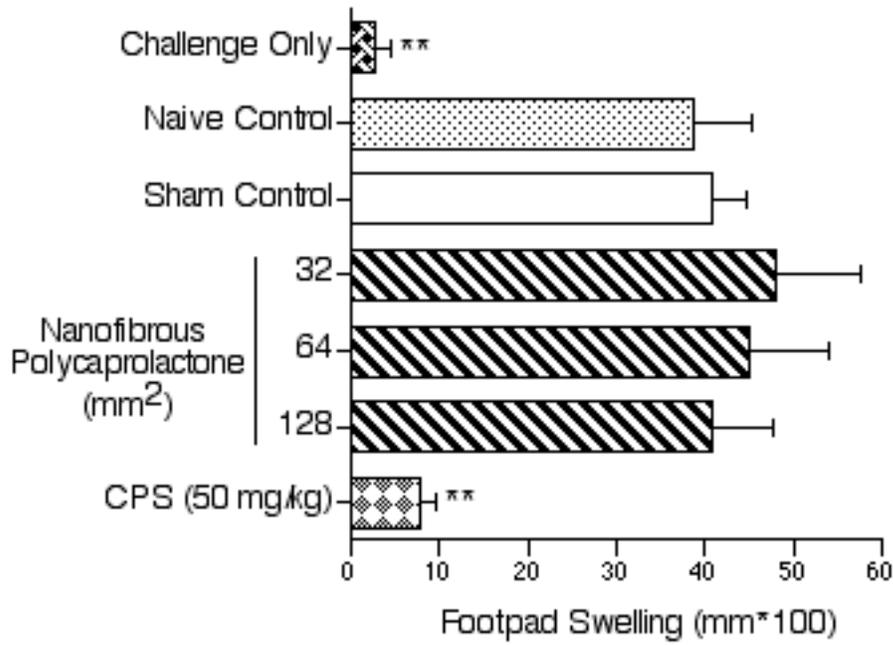


Figure 20. Results of c. Albicans DTH following exposure to the second lot of EPCL.

CHAPTER 6: Determination of Systemic Dexamethasone Effects

Introduction

Dexamethasone (DEX) is a known immunosuppressive agent, which has been studied, in rodent models. Studies by Munson et al. and White et al. in mice have demonstrated that DEX is suppressive in the DTH to sRBC and *c. Albicans*, respectively [Munson et al. 1982, White et al. 2012]. In order to determine endpoints to be evaluated in studies with dexamethasone-loaded EPCL (DEX-EPCL) various endpoints were evaluated following subcutaneous injection of dexamethasone. The endpoints evaluated included, body and organ weights, the antibody forming cell assay, hematology parameters, bone marrow cell counts, and the delayed-type hypersensitivity response.

Methods

Mice were treated with vehicle (4.75% ETOH in PBS) or Dexamethasone (0.01, 0.03, 0.1, or 0.3 mg/kg) by subcutaneous injection daily for 8 days. The positive control, cyclophosphamide (CPS), was administered i.p. on days 5-8.

The body and organ weights, and delayed-type hypersensitivity response were conducted as previously described in Chapters 1 and 2.

Bone marrow counts were obtained by removing femurs, resecting skin and muscle, and cutting without breaking the bone at both joints. The bones were placed in 15mL conical tubes with MEM medium (GibcoBRL, Grand Island, NY, USA) until flushing. To flush the femurs, a 23G needle was used to puncture the distal end of the femur and the marrow plug was flushed with 2.5 mL of MEM medium. Cells were centrifuged at 1200 ± 100 rpm ($300 \pm 10 \times g$) for approximately 10 minutes at $2-8^{\circ}\text{C}$ and cells were resuspended in 1mL of MEM medium. Cells were counted with a ZBII Coulter® counter.

For hematology analysis, on the day of sacrifice, animals were anesthetized with CO_2 and blood was collected from the retro-orbital sinus into EDTA tubes. Total numbers of leukocytes, leukocyte differentials, erythrocyte number, hemoglobin concentration, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and platelet number were determined using a Hemavet® hematology analyzer.

Results and Discussion

Spleen and Thymus Weights

The spleen and thymus weights of animals exposed to dexamethasone by s.c. injection at all dose levels were significantly decreased compared to vehicle control as shown in Figure 21. The positive control group, 4 days of 50 mg/kg cyclophosphamide

also significantly suppressed both spleen and thymus weights. These results indicate that spleen and thymus weights would be sensitive endpoints for detecting systemic immunosuppression from DEX and were chosen as endpoints for DEX-EPCL studies.

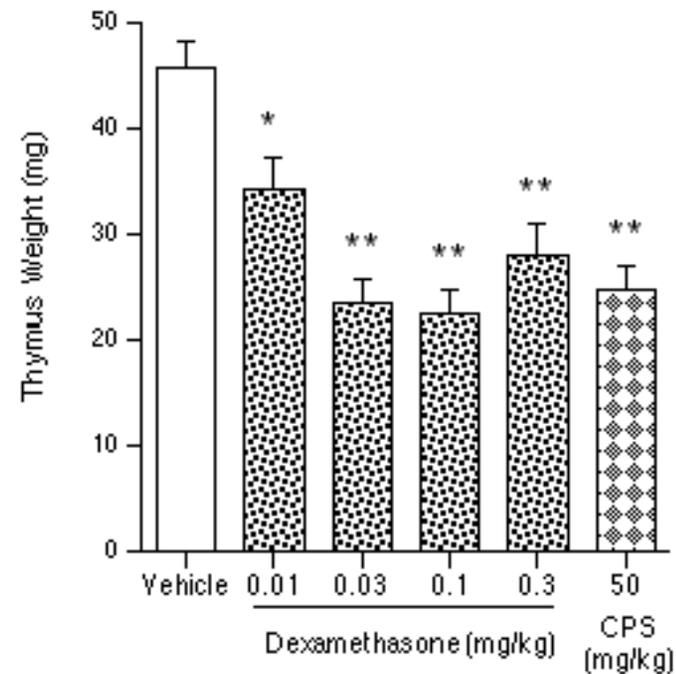
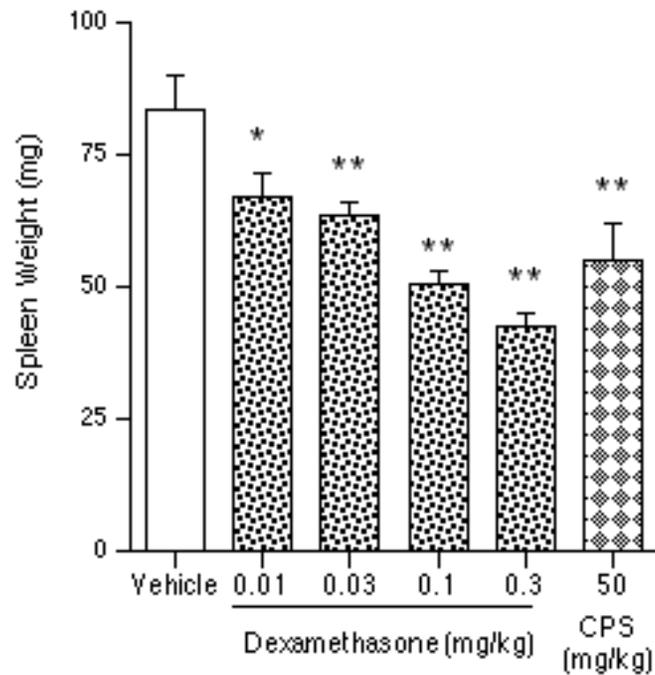


Figure 21. Spleen and thymus weights mice were treated with vehicle (4.75% ETOH in PBS) or dexamethasone by subcutaneous injection daily for 8 days. The positive control, cyclophosphamide (CPS), was administered i.p. on days 5-8. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the vehicle control group.

Delayed-Type Hypersensitivity Response

Following exposure to dexamethasone by s.c. injection the lowest dose (0.01 mg/kg) did not have significantly decreased swelling the DTH response to *C. albicans*. A dose-responsive decreasing trend was seen and all remaining doses significantly decreased footpad swelling. The positive control group, 4 days of 50 mg/kg cyclophosphamide also suppressed the response and the challenge-only group had minimum swelling, both groups were significantly different from the vehicle control. These results indicated that the *c. Albicans* DTH model would be an appropriate model for use in the DEX-EPCL studies.

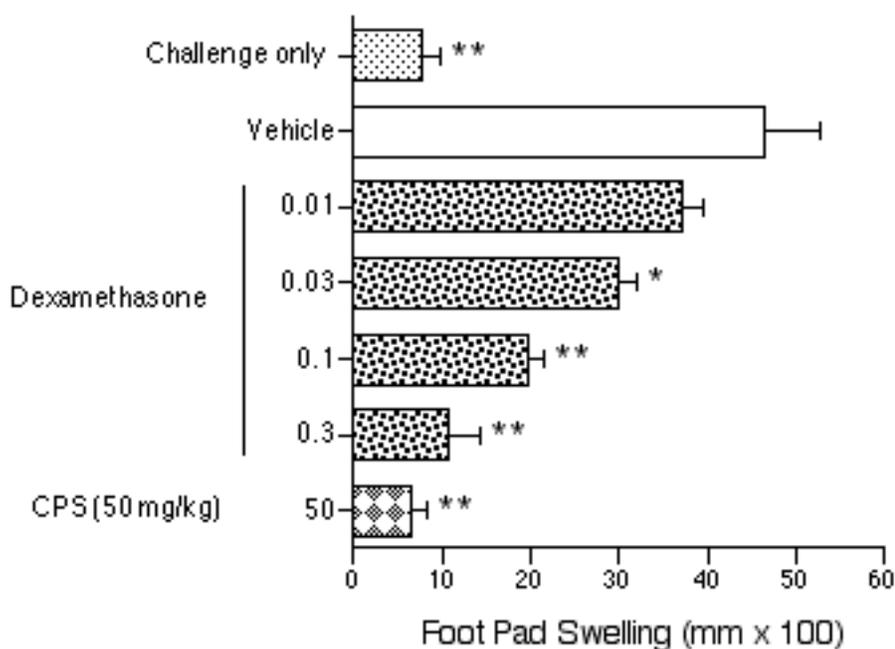


Figure 22. Delayed-type hypersensitivity response to *C. albicans*. Results are presented as mean \pm S.E. for footpad swelling (mm*100). The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Bone Marrow Cellularity

Bone marrow cellularity was not decreased from vehicle control at any dose tested following 8 days of dexamethasone exposure by s.c. injection. Therefore, bone marrow cellularity was not used in evaluating DEX-EPCL. The positive control group, 4 days of 50 mg/kg cyclophosphamide significantly suppressed bone marrow cell number.

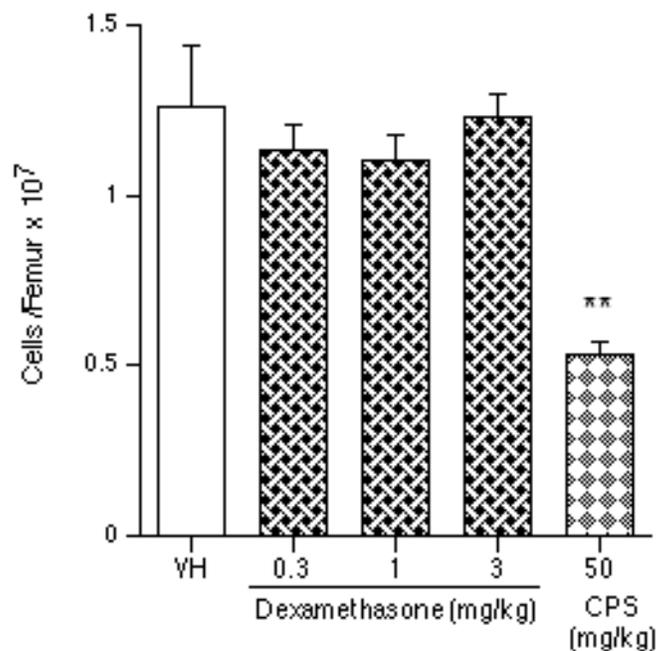


Figure 23. Bone marrow cellularity. Results are presented as mean \pm S.E. for bone marrow cell number. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Hematology Parameters

The results of hematology parameters of animals exposed to dexamethasone by s.c. are shown in Table 5. MCV was the only parameter affected at all dose levels. Percent lymphocytes and total red blood cells (RBC) were increased at the highest dose. HCT was slightly decreased at the lowest dose. Since changes in hematology parameters were relatively small and changes for all dose levels was only observed for one parameter hematology was not used as an endpoint in DEX-EPCL studies.

Table 5. Hematology parameters. Results are presented as mean \pm S.E. The level of statistical significance is denoted as **p < 0.01 or *p < 0.05 as compared to the sham control group.

	VH	Dexamethasone (mg/kg)			CPS (mg/kg)
		0.3	1	3	50
Total WBC (k/uL)	5.1 \pm 0.6	3.3 \pm 0.4	4.0 \pm 0.4	4.3 \pm 0.6	3.1 \pm 0.4*
% Lymphocytes	60.7 \pm 2.8	62.0 \pm 1.7	55.1 \pm 1.6	40.5 \pm 2.3**	62.2 \pm 2.7
% Neutrophils	23.8 \pm 0.9	27.0 \pm 1.4	30.5 \pm 0.9*	49.3 \pm 3.2**	20.7 \pm 2.0
% Monocytes	10.0 \pm 2.3	7.0 \pm 0.8	7.0 \pm 0.6	5.9 \pm 0.5	8.9 \pm 0.7
% Eosinophils	3.9 \pm 0.6	3.1 \pm 0.9	5.4 \pm 0.8	3.4 \pm 1.1	3.9 \pm 0.6
% Basophils	1.5 \pm 0.4	1.0 \pm 0.4	1.9 \pm 0.4	1.0 \pm 0.3	2.3 \pm 0.3
Total RBC (M/uL)	9.7 \pm 0.3	9.8 \pm 0.4	10.1 \pm 0.3	11.2 \pm 0.5*	9.3 \pm 0.6
HB (g/dL)	14.5 \pm 0.5	14.3 \pm 0.7	14.8 \pm 0.5	16.4 \pm 0.6	13.5 \pm 0.9
HCT %	53.3 \pm 1.8	52.6 \pm 2.3*	54.5 \pm 1.7	60.4 \pm 2.4	50.7 \pm 3.2
PLT (k/uL)	833 \pm 119	1075 \pm 95	924 \pm 118	1275 \pm 111	1045 \pm 82
MCV	55.1 \pm 0.3	53.9 \pm 0.2*	54.1 \pm 0.2*	54.0 \pm 0.4*	54.3 \pm 0.2
MCH	15.1 \pm 0.3	14.7 \pm 0.2	14.7 \pm 0.3	14.7 \pm 0.1	14.5 \pm 0.1
MCHC	27.4 \pm 0.6	27.2 \pm 0.4	27.2 \pm 0.6	27.2 \pm 0.3	26.7 \pm 0.2

CHAPTER 7: Studies on Dexamethasone-loaded EPCL Scaffolds

Introduction

According to the Center for Disease Control (CDC), in 2007-2009 50% of adults age 65 or older and 22% of all adults had been diagnosed with arthritis. The most common form is osteoarthritis, however rheumatoid arthritis and other conditions (gout, fibromyalgia) are also highly prevalent [CDC 2010]. Arthritis leads to joint inflammation and degradation, which can lead to the need for total joint replacement in order to maintain ability to conduct vital activities such as walking and bending [CDC 2010]. Current treatments for arthritis include oral, (i.e. systemic) administration with methotrexate, glucocorticoids, and/or biologicals. Due to systemic administration, all of these treatments have potential systemic immunomodulatory effects, which can lead to increased susceptibility to infection [Klarenbeek et al. 2010].

This work was undertaken to explore a novel drug delivery system to locally deliver immunomodulators to inflamed joints while aiming to reduce or eliminate systemic effects. The polymer studied, electrospun polycaprolactone (EPCL), was chosen because it

is biodegradable and previous studies have shown it is immunocompatible [McLoughlin et al. 2012]. Having demonstrated that EPCL alone does not adversely effect any of the immune components evaluated, including the delayed-type hypersensitivity (DTH) response we chose to study EPCL loaded with the known immunosuppressive compound, dexamethasone, as a potential drug delivery system.

Dexamethasone has been incorporated into various non-electrospun materials and devices for local anti-inflammatory activity and drug delivery. Silva-Cunha et al. incorporated dexamethasone into PCL through lyophilization and compression into discs for intravitreal devices and found that these were well-tolerated in rabbit eyes with prolonged drug release [Silva-Cunha et al. 2009]. Ward et al. examined dexamethasone with low release in pigs with subcutaneously implanted biosensors and found that decreases in local inflammatory responses were possible without systemic effects [Ward et al. 2009]. Pital et al. combined dexamethasone with vascular endothelial growth factor (VEGF) in PLGA microsphere/PVA hydrogel composites and along with decreasing local inflammation were able to promote neo-angiogenesis otherwise inhibited by dexamethasone [Pital et al. 2007].

Electrospun polymers, including EPCL have been studied as drug delivery systems for a range of compounds including antibiotics, growth factors, and nucleic acids. Techniques for drug loading of electrospun polymers include: post-electrospinning modification (drug absorption or covalent coupling to the scaffold following electrospinning), direct electrospinning of a drug/polymer solution, coaxial electrospinning of a core containing drug/polymer and polymer shell. Each technique has unique benefits,

the method chosen here was direct polymer/drug electrospinning, which tends to lower burst release compared to post-electrospinning modification [Meinel et al. 2012]. Kenawy et al. studied the release of tetracycline hydrochloride from two polymers individually, poly(ethylene-co-vinylacetate) (PVA) and poly(lactic acid) (PLA) and from a 50:50 blend of the two. Release profiles were characterized using UV-VIS and results indicated that release was sustained over approximately 5 days [Kenawy et al. 2002]. Sell et al. examined the release of platelet-rich plasma (PRP) from silk fibroin (SF), PGA, and PCL electrospun scaffolds and found that protein release occurred for up to 35 days *in vitro* and enhanced scaffold bioactivity [Sell et al. 2011]. Martins et al. electrospun DEX with PCL to promote osteogenic differentiation of human bone marrow-derived stem cells (hBMSCs) and found that the drug had sustained release for over 15 days and promoted an osteoblastic phenotype [Martins et al. 2010].

In the present work, three models were used to examine the ability of DEX-EPCL scaffolds to reduce local inflammatory effects and determine systemic immunomodulatory effects. The delayed-type hypersensitivity response to *C. albicans*, keyhole limpet hemocyanin induced footpad swelling, and a collagen-induced arthritis model. Local effects were assessed by measuring footpad swelling and spleen and thymus weights were measured as systemic endpoints.

Materials and Methods

Scaffold Preparation and Animal Husbandry

Electrospinning was conducted as described previously, however due to change in polycaprolactone (PCL, molecular weight 70,000-90,000 kDa Sigma-Aldrich) lot and molecular weight the PCL concentration used was 50 mg/mL. Dexamethasone (DEX, Sigma-Aldrich) was added to the polymer solution approximately 20 minutes before electrospinning at 3, 10, 30, or 100% w/w. Materials were disinfected by overnight exposure to UV light in a biological safety cabinet. Preliminary studies were conducted by implanting animals with 6-mm or 3-mm disks of DEX-EPCL obtained using biopsy punches (Fisher Scientific). Animal husbandry was conducted as previously described in Chapters 1 and 2. The DEX-EPCL was implanted as close to the inflamed joint as possible.

Delayed-Type Hypersensitivity Studies

In the DEX-EPCL studies with the DTH model, animals were sensitized and 24 hours later implanted. Eight days later, footpads were pre-measured and all animals were challenged. The pilot study was conducted with 6-mm implants containing 65, 100, or 200% DEX-EPCL and 4 animals per group. Subsequently, studies were conducted with 3, 10, 30, and 100% DEX-EPCL and 6-mm or 3-mm disks with eight animals per group. In order to compare between studies DEX-Equivalent Units were established, as shown in Figure 24, by multiplying the concentration of DEX by surface area of the implant. Spleen

and thymus weights were obtained for all studies. The positive control used in the DTH studies was 50 mg/kg CPS given the last four days.

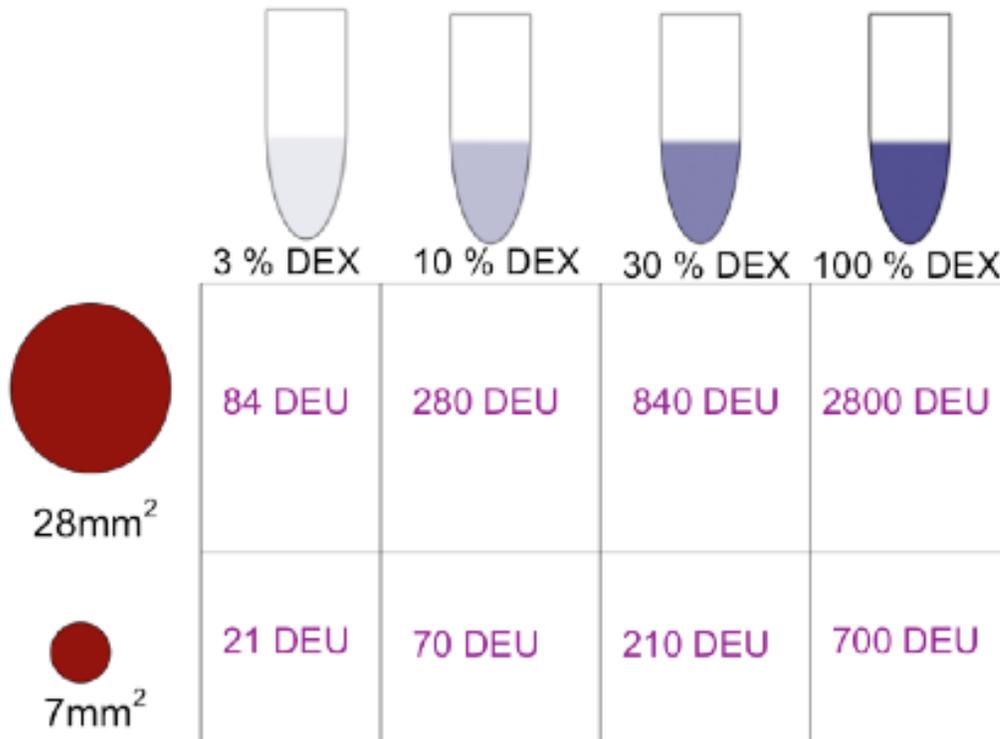


Figure 24. DEX-equivalent units.

Keyhole Limpet Hemocyanin Studies

Mice were implanted with DEX-EPCL approximately 24 hours after KLH injection (200 µg/mouse) in the right footpad. Two studies were conducted, in the first study footpad measurements were taken on days 1, 2, 4, and 7 after surgery and in the second study on days 1, 2, 3 and 4. Spleen and thymus weights were obtained only for the second study.

The positive control used in the studies was dexamethasone given by s.c. injection. In the first study, a single injection of 0.5 mg/kg was given the same day as surgeries were conducted, which was insufficient to reduce footpad swelling. The positive control was given again on days 4 and 6 of the study (3 and 5 days after implantation). In the second study, the dose was increased to 1mg/kg and given on both the day of and the day after surgery.

Collagen-Induced Arthritis Studies

Arthritis was induced as described by Bevaart. Mice were first injected at the base of the tail with 100 µl of an emulsion of chicken collagen type II (Chondrex, Inc. Redmond, WA) and complete Freund's adjuvant (CFA, Chondrex, Inc. Redmond, WA). A booster injection of collagen and incomplete Freund's adjuvant (IFA, Chondrex, Inc. Redmond, WA) was given 3 weeks later [Bevaart et al. 2010]. A preliminary study was conducted to establish the time course in the B6C3F1 mouse. A second study was conducted for DEX-EPCL exposure. The positive control used in the DEX-EPCL study was 1 mg/kg DEX given by s.c. injection given on days before measurement.

Results

Delayed-Type Hypersensitivity Study 1

As shown in Figure 25, a decreasing trend was seen in the spleen weights of the 3 DEX-EPCL groups with a significant decrease at the highest dose, thymus were unaffected

and the positive control group was significantly decreased in both spleen and thymus weights.

There was a decreasing trend with all DEX-EPCL groups in the DTH response to *C. albicans* as shown in Figure 26 with a significant decrease at the highest dose.

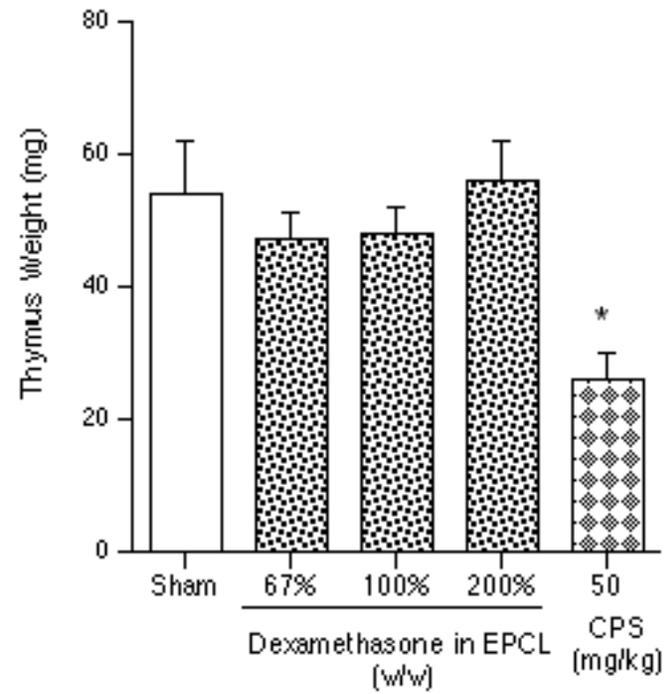
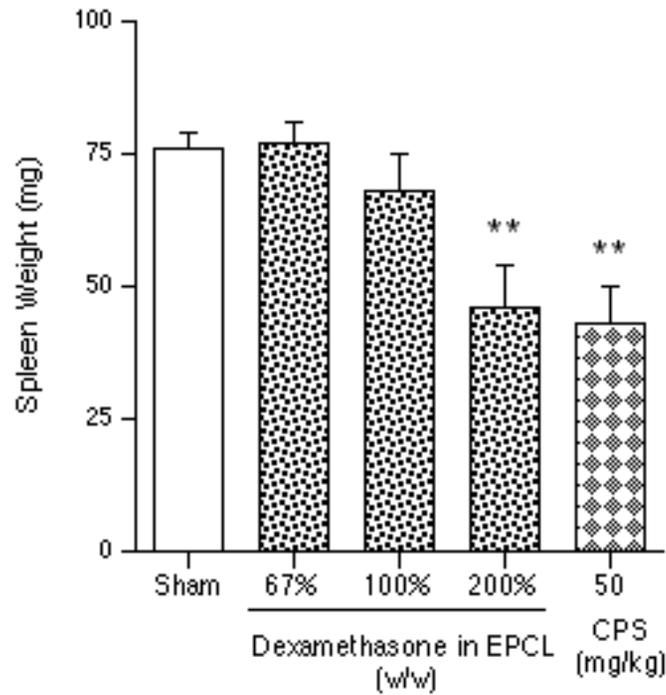


Figure 25. Spleen and Thymus weights. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

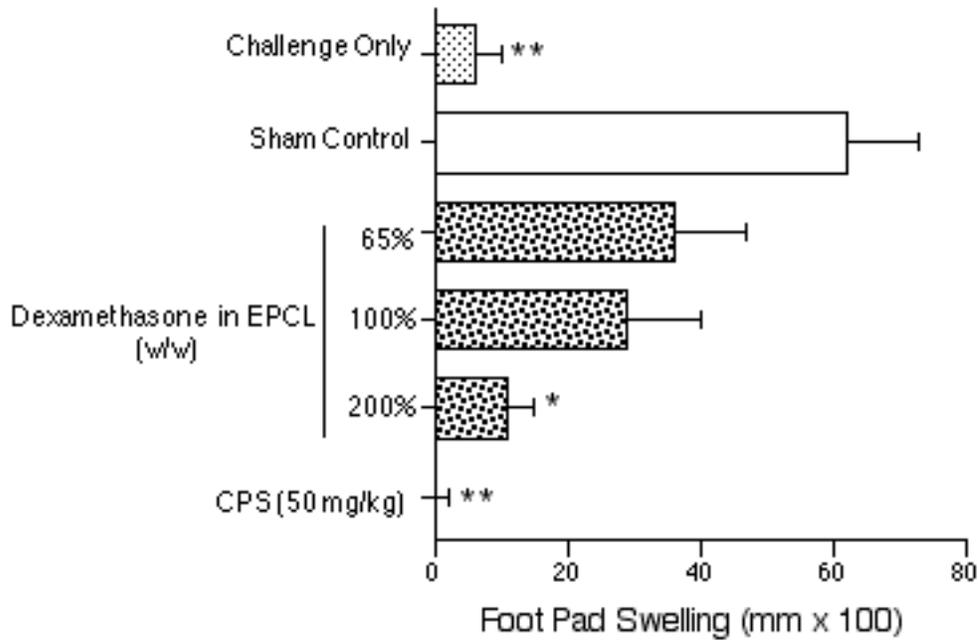


Figure 26. Delayed-type hypersensitivity response to *C. albicans*. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Delayed-Type Hypersensitivity Studies 2 and 3

In Figures 27-29 the results from DEX-EPCL studies 2 and 3 are shown according to DEUs and presented in percent control. The lowest dose, 21 DEU did not result in decreases in footpad swelling, thymus weight, or spleen weight. The next 3 higher doses, 70, 84, and 210 DEU resulted in a decrease in footpad swelling without decreases in spleen or thymus weight. The groups 280, 700, and 840 DEU resulted in decreases in footpad swelling and decreases in thymus weight. The highest dose, 2800 DEU resulted in decreases in footpad swelling, spleen, and thymus weight. Based on these results the 3-mm implants were chosen for further study.

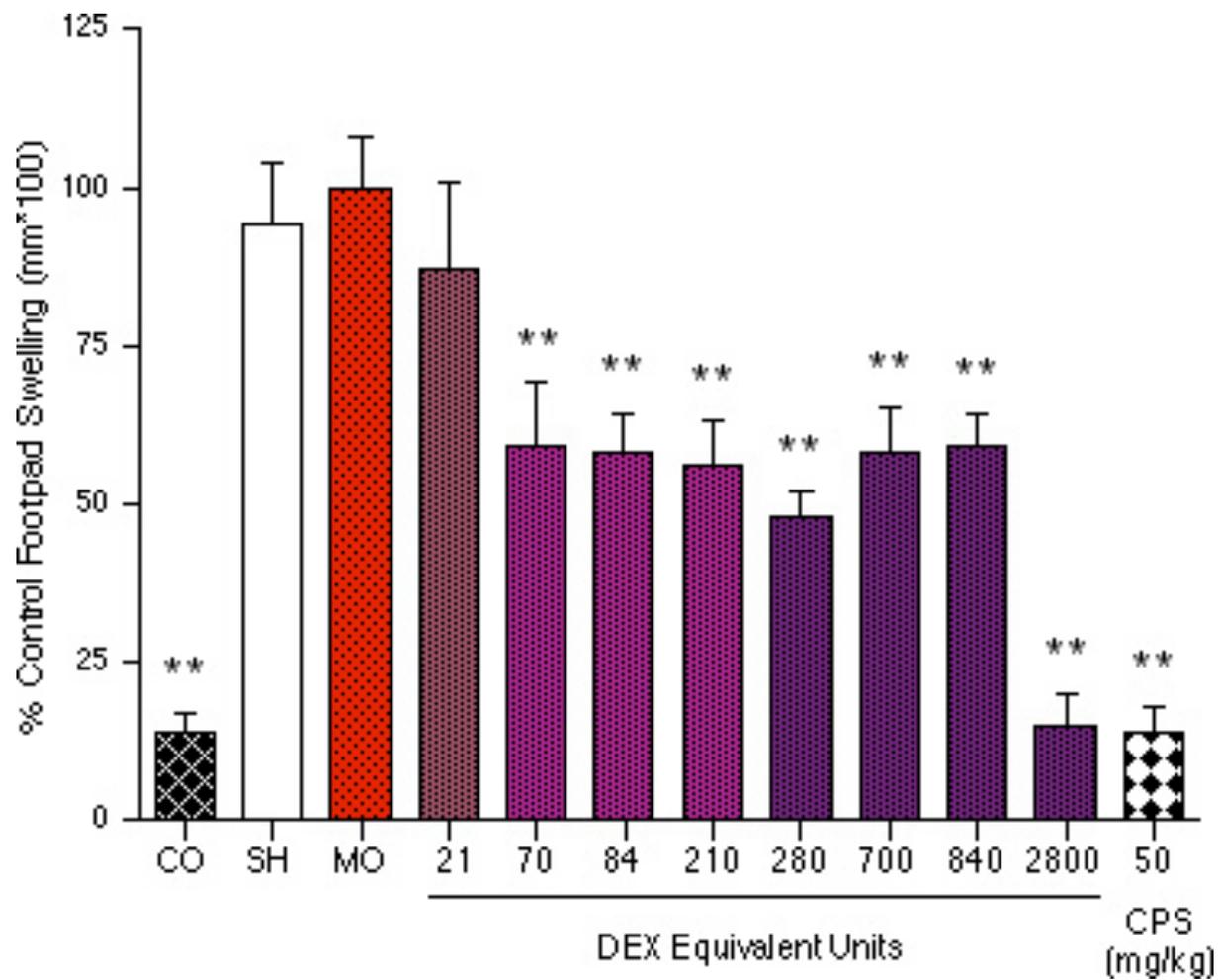


Figure 27. DEX-EPCL Studies 2 and 3. Footpad swelling. Results are presented as % control mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the material only control group.

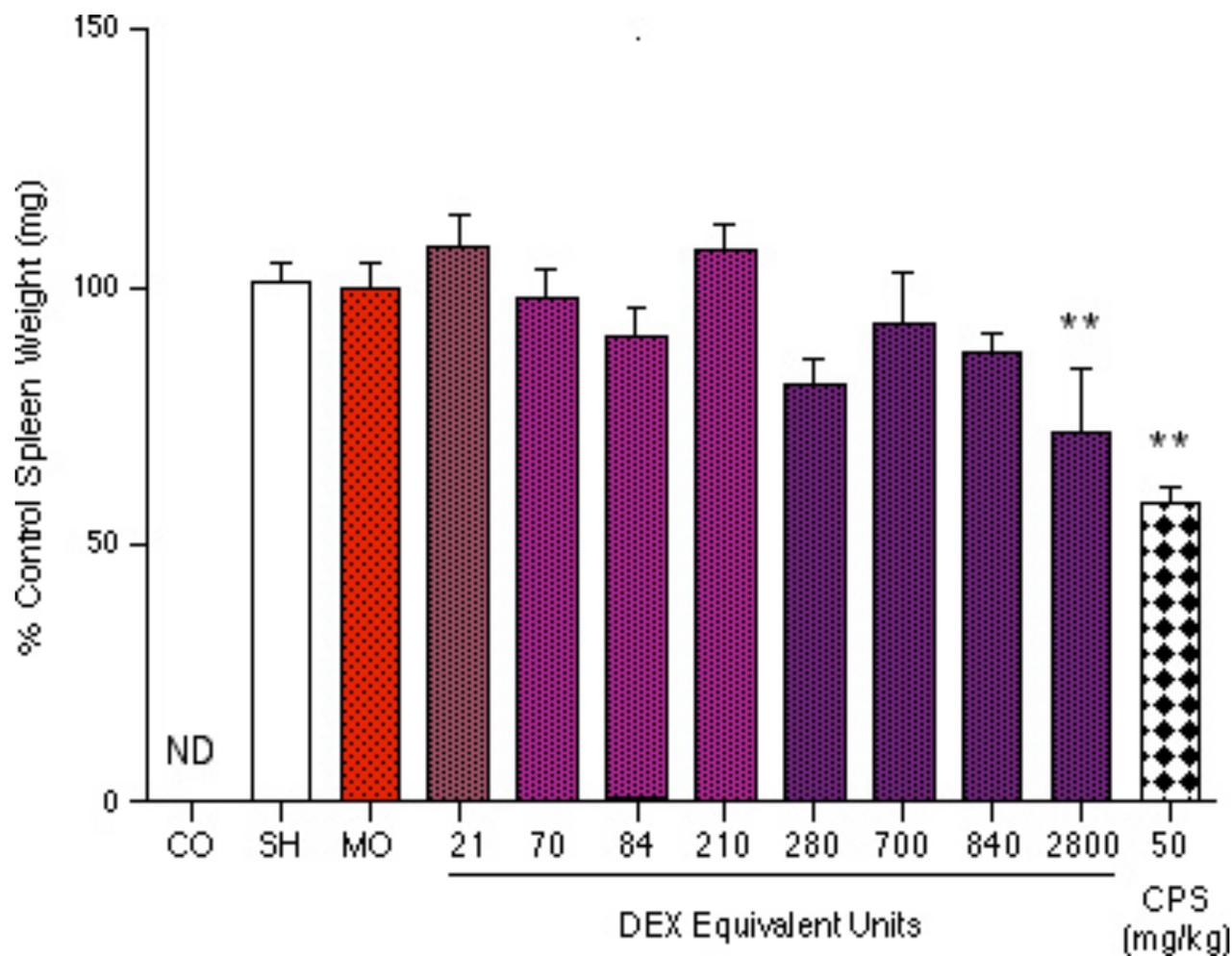


Figure 28. DEX-EPCL Studies 2 and 3. Spleen weight. Results are presented as percent mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the material only control group.

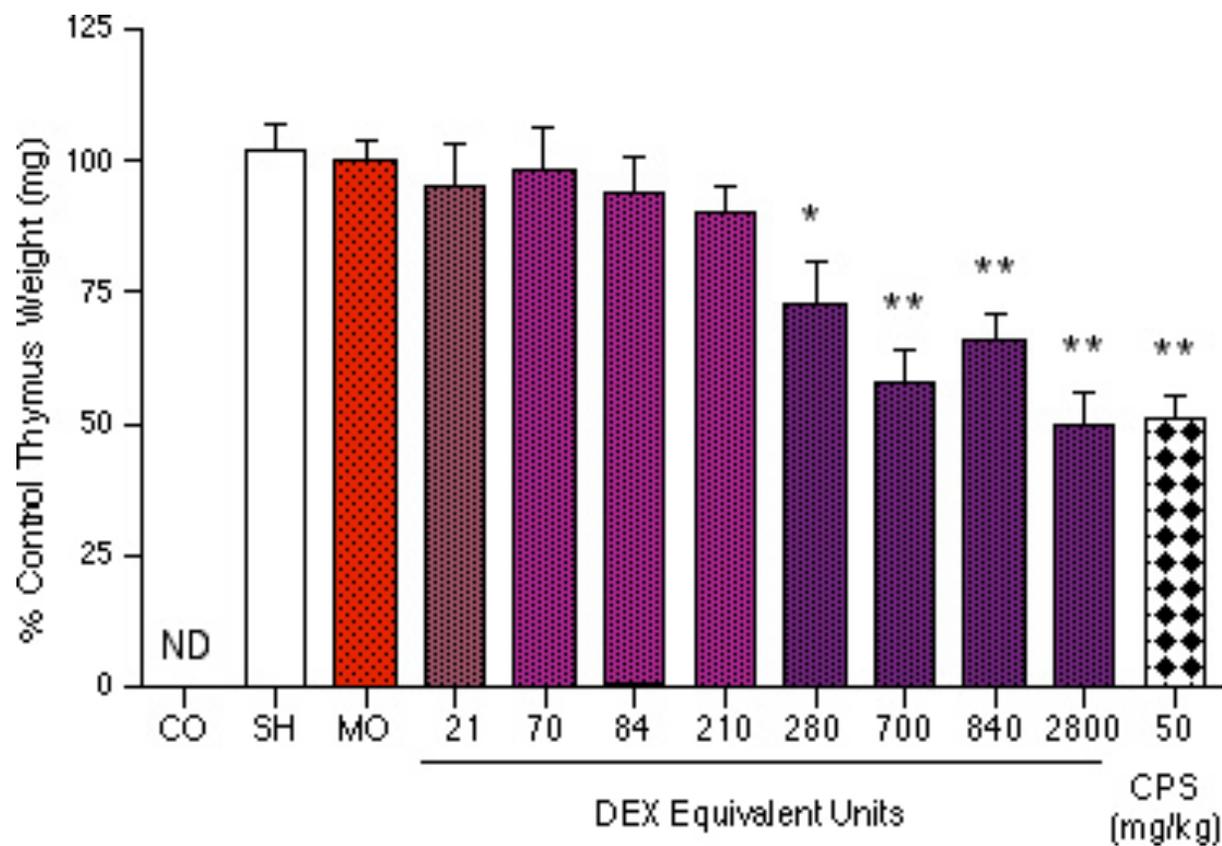


Figure 29. DEX-EPCL Studies 2 and 3. Thymus weight. Results are presented as percent control mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the material only control group.

Keyhole Limpet Hemocyanin Studies

KLH study 1, shown in Figure 30 (top panel), showed little difference between DEX-EPCL treated groups and the material-only (MO) control in days 1, 3, or 6 days after implantation (which occurred on day 1 in the graphs). At day 3 the high dose, 700 DEU had significantly reduced footpad swelling compared to control. On day 6 after implantation the two highest doses, 210 and 700 DEU had significantly reduced footpad swelling from material only control, however the MO response by day 6 had decreased to less than half the peak response. The positive control was decreased.

KLH study 2, shown in Figure 30 (bottom panel), showed similar results to KLH study 1. The naïve control was significantly less than the material only control on the day of implantation (day 1 in the graphs). On day 2 after implantation the high dose, 700 DEU, had significantly decreased footpad swelling and on day 3 after implantation 3 of the DEX-EPCL groups 70, 210, and 700 had significantly decreased footpad swelling compared to the material control. The peak response in study 2 was much lower than in study 1 and by day 3 after implantation the MO response was similar to study 1 at day 7. The spleen and thymus weights were significantly decreased at the end of study by the high doses of DEX-EPCL (210 and 700 DEU) and by the positive control.

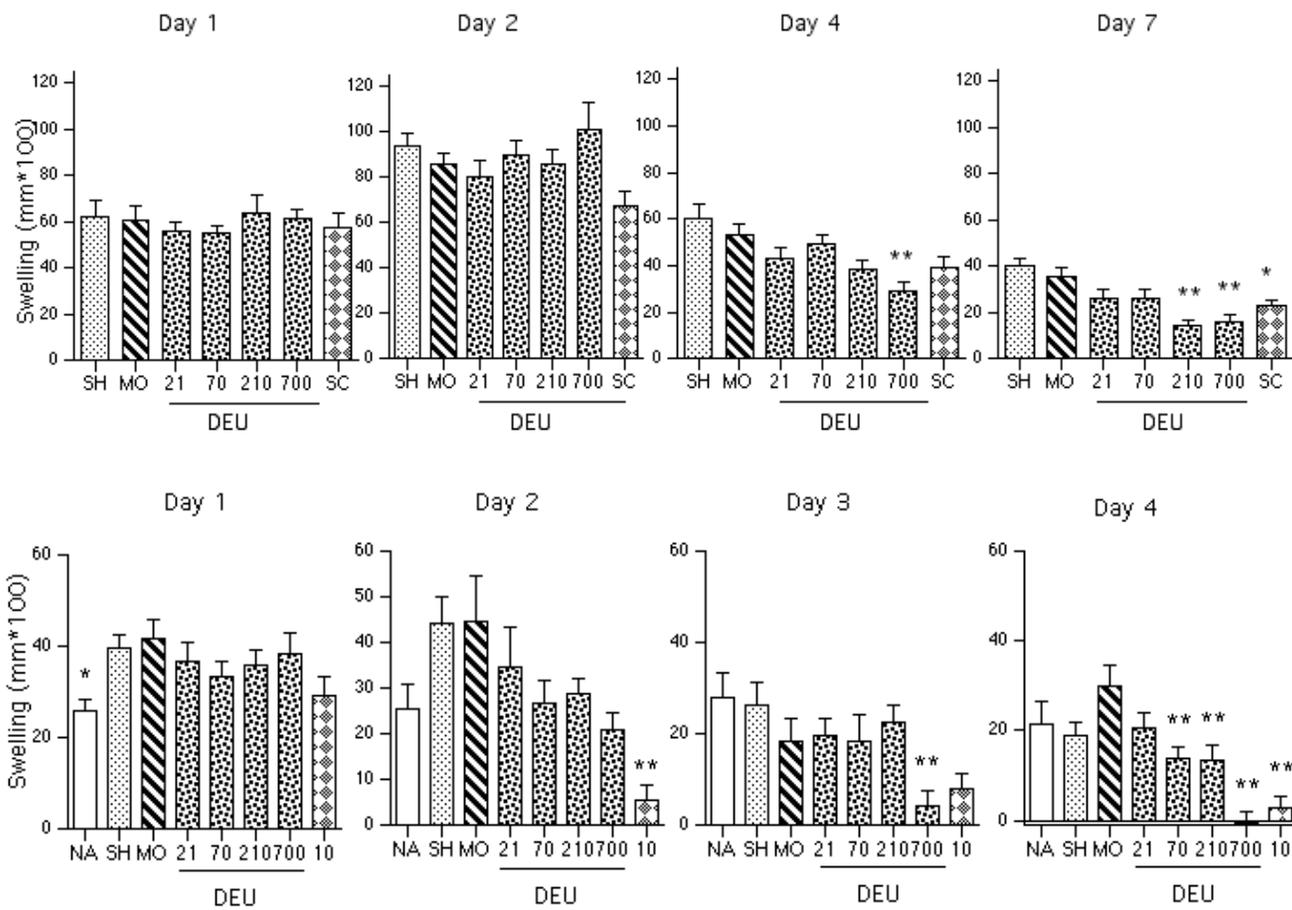


Figure 30. KLH Studies 1 (top panel) and 2 (bottom panel) results of footpad swelling (mm*100). Results are presented as mean ± S.E. The level of statistical significance is denoted as **p < 0.01 or *p < 0.05 as compared to the sham control group.

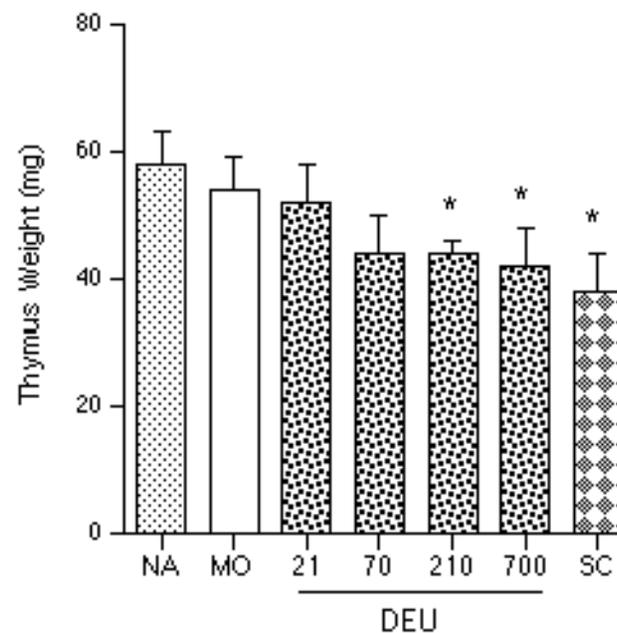
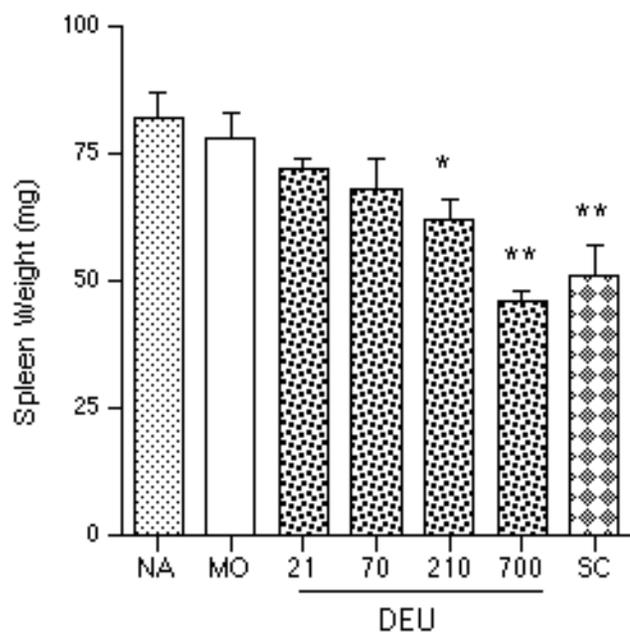


Figure 31. KLH Study 2. Spleen and Thymus weights. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

Collagen-Induced Arthritis Study 1

A pilot study of the collagen-induced arthritis model was conducted in the B6C3F1 mouse in order to determine feasibility and time course compared to the prototypic strain used in the model, the DBA/1. 78 B6C3F1 mice were used and 18 (23% incidence) developed inflammation in at least one paw starting at approximately 2-3 weeks after the second injection. Consistent with the DBA/1 model swelling persisted over an additional 3-4 weeks.

Collagen-Induced Arthritis DEX-EPCL Study

In the CIA DEX-EPCL, study animals that developed arthritis in at least one paw were implanted 4 weeks after the second injection. In the study, 106 B6C1F3 mice were injected and 27 developed arthritis (25% incidence). Animals were randomized and distributed throughout the groups, the 84 DEU group had 3 animals while all other groups had n = 4. As seen in Figure 32, body and thymus weights were unaffected for all groups. Spleen weights were significantly decreased for the positive control, 21, 210, and 700 DEU groups. Footpad measurements were obtained on days 3, 4, 5, 11, 12, 13, 14, 18, and 20 after implantation as seen in Figures 33-35. No significant differences were found in change in footpad swelling between measurement days and the day of implantation as compared to the material only group. Starting on day 13 most groups tended to have an increase in footpad swelling over the day of implantation.

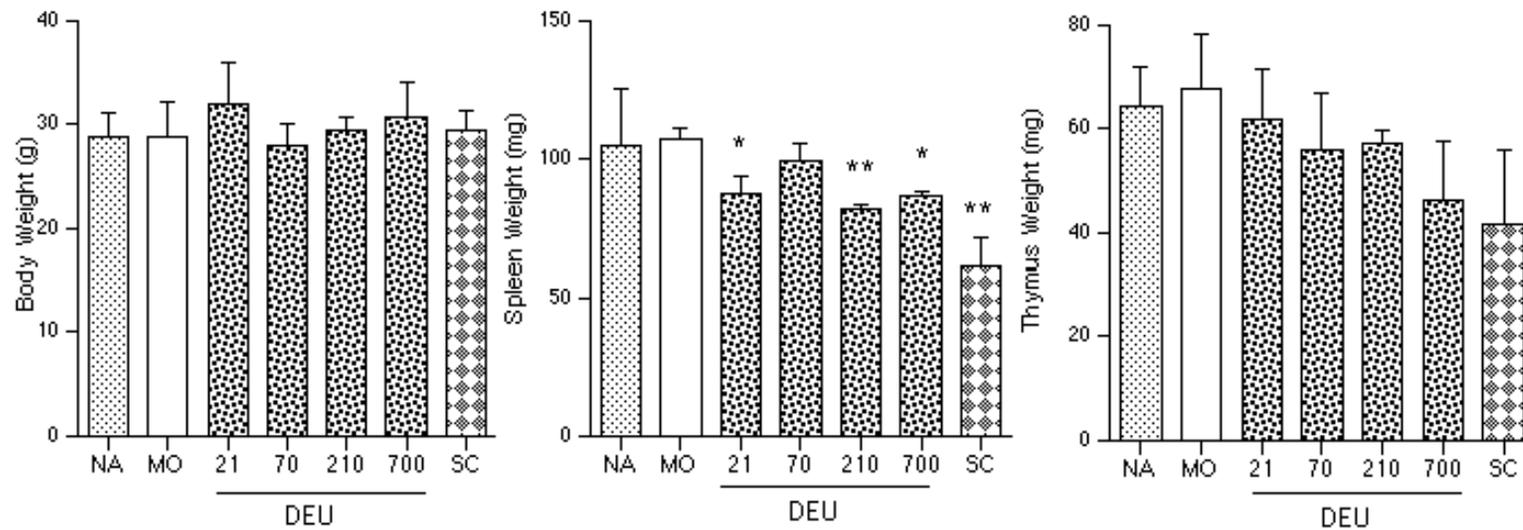


Figure 32. CIA Study 2 Body, Spleen, and Thymus weights. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the sham control group.

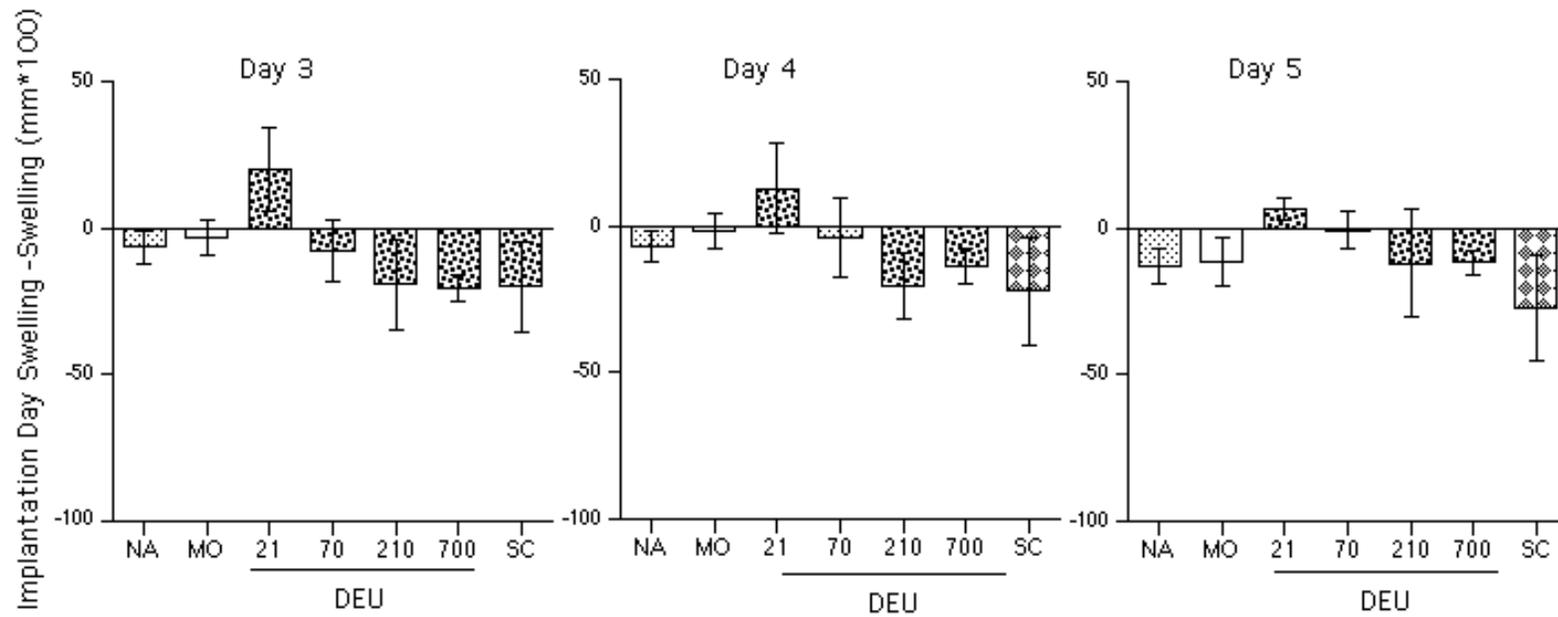


Figure 33 CIA DEX-EPCL Study Footpad swelling Days 3, 4, and 5. Results are presented as mean \pm S.E. The level of statistical significance is denoted as ** $p < 0.01$ or * $p < 0.05$ as compared to the material only control group.

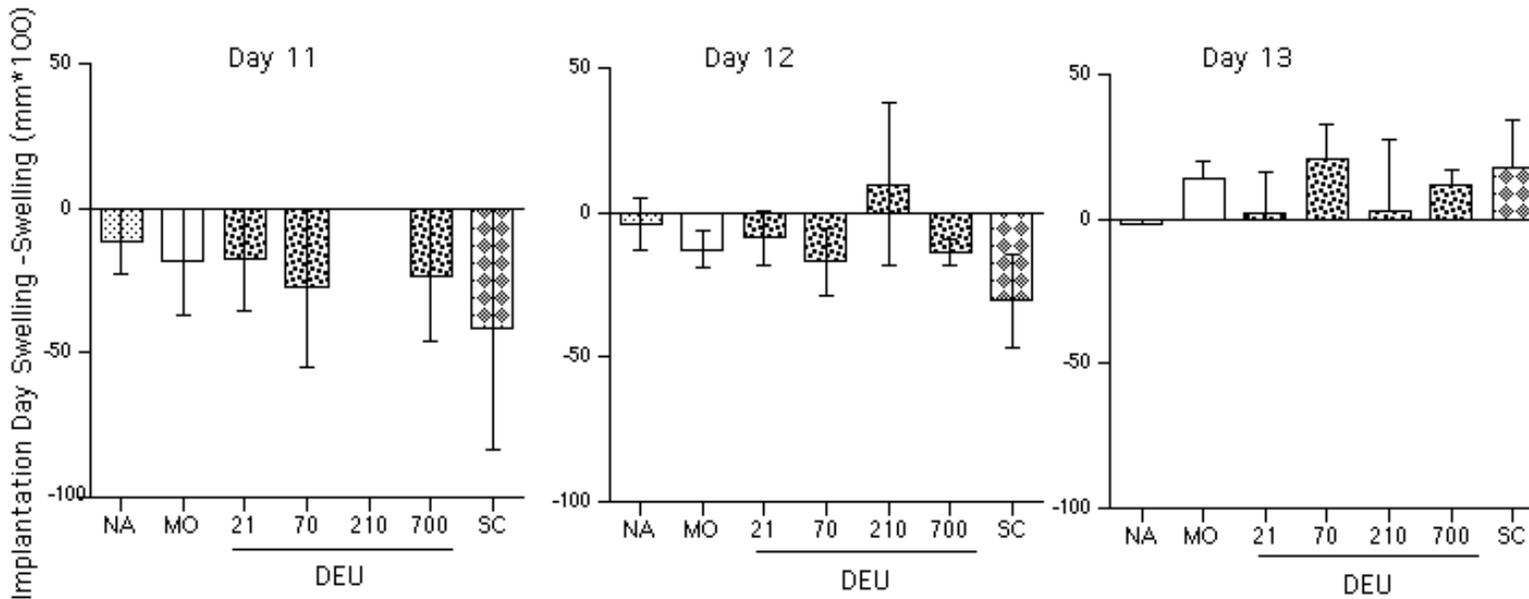


Figure 34. CIA DEX-EPCL Study Footpad swelling Days 11, 12, and 13. Results are presented as mean \pm S.E. The level of statistical significance is denoted as **p < 0.01 or *p < 0.05 as compared to the material only control group.

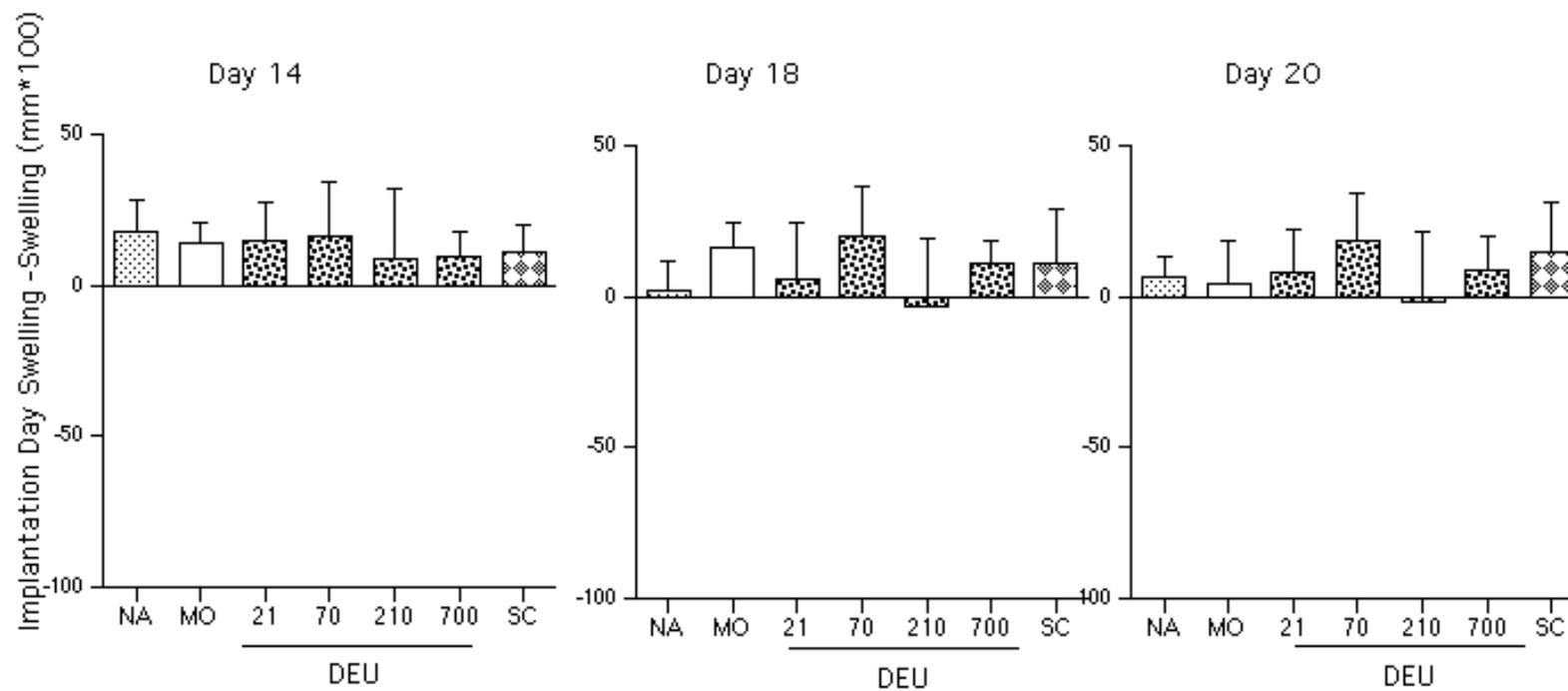


Figure 35. CIA DEX-EPCL Study Footpad swelling Days 14, 18, and 20. Results are presented as mean \pm S.E. The level of statistical significance is denoted as **p < 0.01 or *p < 0.05 as compared to the material only control group.

Discussion

Dexamethasone is a potent glucocorticoid. Glucocorticoids are used to treat autoimmune and inflammatory diseases and act on components of both the adaptive and innate immune response. Glucocorticoids decrease inflammation by enhancing opsonization and scavenger system activity and therefore enhancing clearance of antigens, toxins, dead cells, and microorganisms. Additionally, glucocorticoids downregulate cytokines (IL-1 β , TNF α , IL-6, IL-8, IL-12, and IL-18) and chemokine release while promoting anti-inflammatory cytokines (IL-10 and TGF β) and receptors for pro-inflammatory cytokines. In T-cell dependent responses, glucocorticoids promote a humoral Th2 immune response over a cell-mediated Th1 immune response by blocking IL-12 production by monocytes and dendritic cells and enhancing IL-10 secretion by macrophages. Glucocorticoids have varied effects on T-cell survival and apoptosis that are dependent on the T-cell type, activation state, and relative time of exposure to activation. The outcome may be due to whether signaling occurs through the T-cell receptor alone, glucocorticoid receptor alone, or during concomitant signaling [Franchimont 2004].

The delayed-type hypersensitivity response to *c. albicans* has been shown to primarily involve cell-mediated immunity with no major involvement of humoral immunity [Smith and White 2010]. Result from the DEX-EPCL studies indicated that the drug delivery system was able to successfully significantly decrease footpad swelling in the model which is likely due to a primarily Th1 response. The model was likely the most successful because the implants were present prior to challenge priming the immune system towards a Th2 over a Th1 response. A dose (70 DEU) was found that was able to

successfully decrease footpad swelling without effects on spleen or thymus weights. From these results the 3-mm implants were chosen for further study.

The keyhole limpet hemocyanin model is likely an inflammatory response caused by an influx of neutrophils and macrophages [Engstrom et al. 2009]. In the second KLH study, a similar successful response was seen with the 70 DEU group on day 3 after implantation where footpad swelling was decreased without effects on spleen or thymus weights. Variability in the model itself (responses between studies, and inconsistency between controls in study 2) and relatively fast waning phase decrease the usefulness in evaluating the DEX-EPCL system.

The collagen-induced arthritis model and genetic and immunological processes in mice with the H-2^q susceptible haplotype, including the prototypic strain the DBA/1, are well characterized. The likely mechanism of induction of arthritis starts with a T-cell response, promoted by mycobacteria components of the CFA, occurring in the draining lymph nodes at the site of collagen/IFA challenge. Naïve T-cells are then promoted to differentiate into Th1 cells which promotes isotype switching and production of IgG2a by collagen-specific B-cells. Antibodies enter the joint and bind collagen, which activates complement in turn activating blood vessel endothelium and allowing entry of T-cells, monocytes/macrophages, and neutrophils. Cytokine produced by Th1 cells activate macrophages causing the release of TNF- α promoting further migration from the blood into the joint. IL-1 is produced in the joint by macrophages and neutrophils leading to tissue destruction by infiltrating cells and synoviocytes, chondrocytes, and fibroblasts. A positive feedback continues due to release of joint antigens and inflammatory mediators,

which persists even as T-cell numbers decrease [Luross et al. 2001]. Dexamethasone given i.p. at for 5 days at high dose has been shown to be effective in the CIA model. In the same study by Kang et al. responses were shown to improve with co-administration of IL-4 [Kang et al. 2000]. The mechanism of induction of arthritic paws in the B6C3F1 mouse is unknown and may differ from that of the H-2^q mice. The results shortly following implantation of DEX-EPCL did have trends that could if tested and repeated with a larger sample size result in suppression of footpad swelling over MO controls. However, the cause of all groups having increased footpad swelling, although small in number, during later timepoints is unknown and is in contrast to H-2^q mice that would begin to see waning footpad swelling in the model overall by that time. Further study of the CIA model in the B6C3F1 mouse is warranted.

Conclusion

The DEX-EPCL studies indicate that the system has potential for local drug delivery of immunomodulators while reducing or eliminating systemic effects. The DEX-EPCL system was highly successful in the *C. albicans* delayed-type hypersensitivity response. The keyhole limpet hemocyanin model again pointed to the 70 DEU dose as a dose capable of local immunosuppression without systemic effects, however model was inconsistent. No statistically significant effects were observed in the collagen-induced arthritis model and further study of the model in the B6C3F1 mouse may provide further insight.

CHAPTER 8: Conclusions and Future Work

Results indicate that EPCL is an excellent polymer for immunocompatibility. EPCL was shown to have no effect on young or aged animals in a microfibrinous and a nanofibrinous form. Studies were conducted based on results from *in vitro* studies by Smith et al. suggesting that electrospun polymers could potentially cause immunosuppression [Smith et al. 2007, Smith et al. 2010, Smith et al. 2011]. Several disadvantages exist with *in vitro* testing including: direct contact of the material with the cells in culture, the production and lack of removal of cellular waste products, limited exposure time, and lack of signals and cells found *in vivo*. Currently, *in vitro* immunotoxicity testing can not be directly correlated with *in vivo* exposure and these disadvantages may explain the contrast in results presented compared to the work of Smith et al. [McLoughlin et al. 2012, Smith et al. 2007, Smith et al. 2010, Smith et al. 2011].

Preliminary drug delivery studies are promising that EPCL may be a good candidate for a local drug delivery system for immunomodulators. The work could be continued in many different directions. The studies presented only address the effects of EPCL after 28 days of implantation where the polymer does not present macroscopic signs of degradation, studies of long-term implantation and the role of degradation and

degradation products would present important information on the long-term safety of EPCL. Chronic inflammation at the implant site may also be a factor in long-term implantation and may be lessened or altered with the presence of dexamethasone.

Through *in vitro* testing and *in silico* modeling the parameters of the drug delivery system could be optimized. The *in vitro* release profile could be characterized using a method similar to Martins et al. DEX-EPCL could be placed in saline and incubated at 37°C and media samples taken at various timepoints. The presence of dexamethasone could then be measured using UV-VIS at 242 nm and concentration calculated from a standard curve [Martins et al. 2010]. The *in vitro* release profile could then be used in drug delivery modeling and parameters such as DEX concentration and scaffold thickness could be optimized. Following the *in vitro* and *in silico* work the new system could then be tested in the *in vivo* models.

Additional types of compounds could be incorporated, such as antibodies or antibodies directed against cytokines. Similarly, multiple types of treatment could be incorporated and a combinatorial product optimized. For example, IL-4 in combination with dexamethasone has been shown to increase efficacy in the CIA model [Kang et al. 2000]. Different polymers could also be used, by incorporating a faster degrading polymer drug release may occur more quickly. The amount of polymer encapsulation should be characterized to determine if there is a physical barrier to drug delivery. Finally, ideally the work could continue into creation of a tissue engineering therapy for rheumatoid arthritis where damaged cartilage could be replaced or regenerated.

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VITA

Colleen Elizabeth McLoughlin was born September 22, 1983 in Providence, Rhode Island and she is a citizen of the United States of America. Colleen graduated from Jamestown High School in Williamsburg, Virginia in 2001 and subsequently attended Virginia Commonwealth University for her Bachelor of Science degree in Biomedical Engineering. In the fall of 2005 Colleen began working as a technician in Dr. Kimber L. White, Jr.'s immunotoxicology laboratory at the Medical College of Virginia. In 2007 Colleen began pursuing a Doctor of Philosophy degree in Biomedical Engineering at Virginia Commonwealth University.

During graduate school Colleen has been a teaching assistant for Electrical Engineering and Quantitative Physiology as well as taught lectures in Tissue Engineering. In addition to authoring papers on her dissertation research she has co-authored a book chapter on monitoring effects of nanomaterials on the immune system and papers and other publications from work on projects such as nanoparticle titanium dioxide and the validation of the *C. albicans* delayed-type hypersensitivity model.

Colleen is highly active in the Society of Toxicology and has served as the student representative for the last two years for the National Capital Area Chapter. Colleen has also served on the Graduate Student Leadership Committee, Professional Development Subcommittee, and on the Continuing Education Committee. Colleen is also active in the Women in Toxicology Special Interest Group and the Immunotoxicology Specialty Section of SOT. In 2010 Colleen was the third place winner of the Bern Schewtz award given by the National Capital Area Chapter for her poster presentation at the annual SOT meeting. In 2011 Colleen received the Women in Toxicology Graduate Student Achievement Award for academic achievement, leadership, and service.