

**ISOLATION, MANIPULATION, AND TREATMENT WITH FASL+  
EXOSOMES**

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## ABSTRACT

A subset of B lymphocytes produce and secrete exosomes that contain both Fas ligand (FasL) and Major Histocompatibility Complex Class II (MHCII) molecules which can stimulate apoptosis of antigen-specific T helper lymphocytes. Standard ultracentrifugation techniques for isolating exosomes are low throughput and fail to recover a concentrated population of FasL+/MHCII+ exosomes, thus resulting in functionally diverse samples for experimental analysis. I investigated novel methods for purifying large quantities of mouse and human FasL+/MHCII+ exosomes by implementing density gradient ultracentrifugation and bead affinity purification. While exosomes were not separated by density, anti-IgM antibody-coated beads captured a dense population FasL+ mouse-derived exosomes, as did anti-CD63 for human-derived exosomes. I also studied ways to manipulate FasL production, exosome secretion, and other immunological suppressive mechanisms of human B cells by specifically inhibiting signaling pathways relevant to immunological function. Although none of the inhibitors affected FasL production, exosome secretion was effectively regulated by dimethyl amiloride. Finally, I used a mouse model of rheumatoid arthritis in order to test the immunosuppressive capacity of FasL+/MHCII+ exosomes *in vivo* and examine their potential role in clinical treatment. An injection of cII-loaded exosomes significantly reduced severity of arthritis in recipient mice compared to exosomes loaded with irrelevant gp39 peptide or no exosome treatment at all. By continuing to investigate methods of purifying FasL+/MHCII+ exosomes, manipulating B cells into secreting more FasL+ exosomes, and analyzing their physiological effect *in vivo*, these lethal macromolecules may prove to be ideal for treating autoimmune disease in a natural, effective, and antigen-specific manner.

## INTRODUCTION

Rheumatoid arthritis is an autoimmune disease that affects an estimated 1% of the United States population. The disease is characterized by an inflammatory response against the joints that results in chronic swelling, pain, and destruction of cartilage. This damage can eventually result in the complete loss of joint function. Historically, therapies for rheumatoid arthritis include anti-inflammatory drugs, steroids, surgery, and other “disease-modifying medications” [1]. Unfortunately, these options are often potent and non-specific, result in undesirable side effects, and focus on alleviating symptoms instead of curing disease. Currently, the leading drugs for treatment of rheumatoid arthritis are biological inhibitors of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), which must be injected regularly in order to maintain effectiveness [1]. Again, this highly inconvenient and suboptimal process, similar to historical therapies, acts mostly to relieve disease symptoms and has little impact on the source of the disease itself. Therefore, proposed improvements in therapies are driven by the philosophy of strictly targeting the malfunctioning immune cells that cause rheumatoid arthritis as a way to effectively treat the root problem of the disease.

The traditional model of the immune system involves T cell activation from antigen-presenting cells such as macrophages, dendritic cells, and B lymphocytes. Stimulation occurs when Major Histocompatibility Complex Class II (MHCII) molecules of antigen presenting cells bind with T cell receptors of T helper lymphocytes. The activation signal is further strengthened by other cell-cell interactions and cytokine mediation, resulting in the differentiation of lymphocytes into functional subsets specific to the presented antigen. In most cases, this process serves to protect the body from pathogens and infectious disease. However, in cases of autoimmunity, antigen-presenting cells display self-antigens that incorrectly direct T helper lymphocyte-mediated

destruction of the body. Usually, the process of immune tolerance results in the destruction of these self-reactive lymphocytes before they can proliferate, but in rare cases these lymphocytes persist.

There is much evidence indicating that the chronic inflammatory response of rheumatoid arthritis is directed by CD4<sup>+</sup> helper lymphocytes [2-4]. Since T helper lymphocytes are essential in protecting the body from pathogenic microorganisms, eliminating them entirely in an effort to prevent autoimmune reaction would result in a highly suppressed immune system and leave the individual vulnerable to infections and cancer. Therefore, the challenge lies in developing a technique that eliminates the subset of T helper lymphocytes responsible for the rheumatoid arthritis with both specificity and effectiveness while maintaining the others involved in healthy immune system function.

Recently, the discovery of a B cell subset that naturally expresses the death-inducing molecule Fas ligand (FasL) challenges the traditional model of the immune system and may offer the opportunity for ideal alternative therapies [5, 6]. FasL is a tightly regulated molecule that potently induces cell apoptosis and is found on a limited number of cell types, most notably Cytotoxic T cells and Natural Killer cells. Since B cells specifically and readily come into contact with T helper lymphocytes at high affinities due to their MHC Class II:TCR interaction, the expression of FasL on the B cell surface would present significant opportunities for inducing apoptosis within the associated T cell. Thus, in addition to their traditional stimulatory role in immune functioning, FasL<sup>+</sup> B cells have the potential to kill antigen-specific T helper lymphocytes naturally and effectively.

Previous studies have demonstrated the importance of these “Killer” B cells in preventing chronic inflammation in mice. Using a schistosome infection model, it was discovered that CD5+ B cells from the spleen express FasL and have the ability to induce death of T helper lymphocytes [7]. In addition, this study determined that maximal expression of FasL on these Killer B cells was dependant on IL-4, IL-10 and antigenic stimulation. Using a collagen-induced arthritis (CIA) model, my lab conducted a study that again demonstrated the ability for purified B cells to induce antigen-specific killing of T helper lymphocytes. Furthermore, it was discovered that increased disease severity correlates with reduced numbers of splenic FasL+ CD5+ B cells [8]. In 2004, it was shown that B6 female mice permanently accepted B6 male skin grafts when treated with B6 male spleen cells, while untreated female B6 mice or Fas deficient *lpr* mice readily rejected B6 male skin grafts [9]. This group went on to prove that FasL+ B cells were responsible for the induction of graft tolerance. From these experimental findings, there appears to be strong evidence that this subset population of FasL+ B cells exhibits a significant role in regulating chronic inflammation in mice, including inflammation resulting from arthritis.

Of considerable interest is the ability for these Killer B cells to produce and secrete exosomes. Exosomes are 30–100 nm diameter vesicles formed in the late endocytic compartment and naturally secreted by a large variety of both hematopoietic and nonhematopoietic cells [10]. The surface molecules of these small membrane vesicles are closely related to the cell from which they were produced. This phenomenon allows for signaling interactions that mimic the cell of origin. Exosomes from leukocytes, for example, have been shown to contain many of the important molecules involved in their regulatory signaling, both stimulatory and suppressive [11]. In this same line of reasoning, Killer B cell-derived exosomes may express FasL in addition to

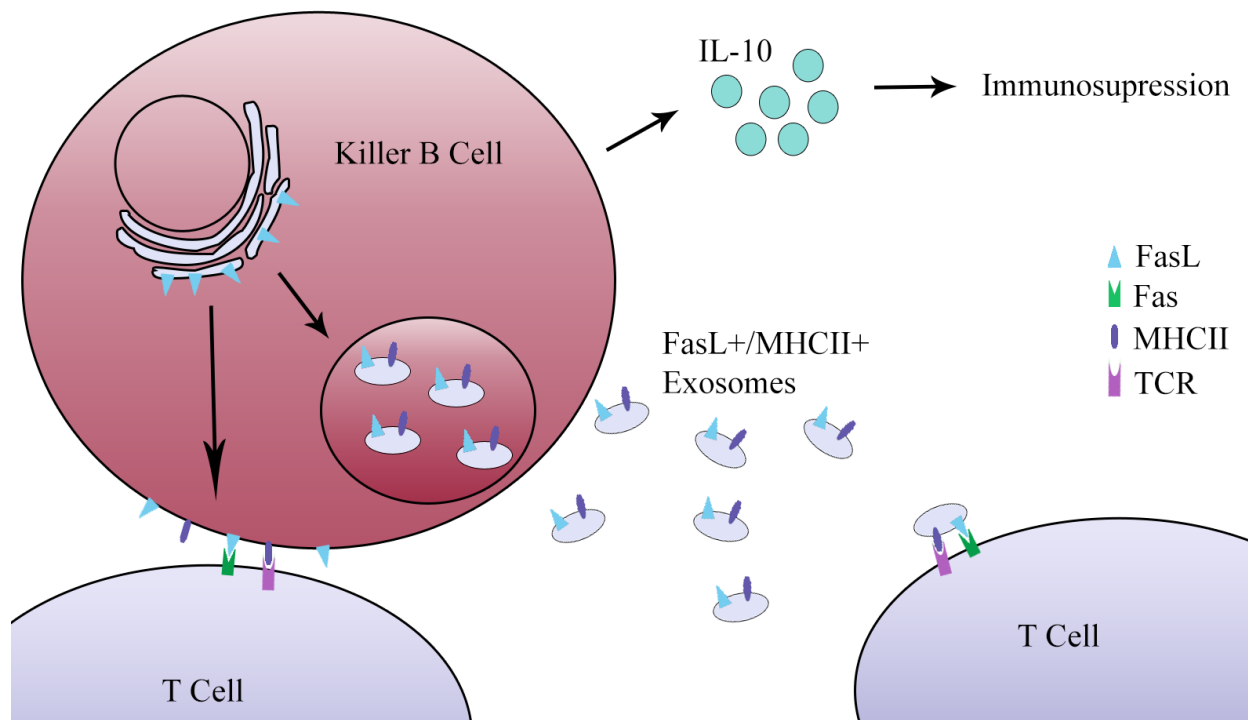


Figure 1: Model of Killer B Cell Immune Regulation

A subset of B cells naturally produces the immune regulatory cytokine IL-10 and the death-inducing molecule FasL, which gives them the unique capacity to kill antigen-specific T helper lymphocytes. By expressing FasL and MHCII on their surface, Killer B cells can directly induce T helper lymphocyte apoptosis. Additionally, the release of signaling vesicles in the form of FasL+/MHCII+ exosomes can achieve the same lethal result at a distance from the original cell.

MHCII on their surface, granting them similar lethal properties toward T helper lymphocytes (Figure 1).

Relatively recent studies have demonstrated the effective signaling capabilities of exosomes. In 2005, it was reported that exosomes collected from dendritic cells that had been transduced with an adenovirus carrying the IL-10 gene or simply treated with IL-10 were anti-inflammatory and able to suppress mouse CIA. A single injection of these exosomes into the paw of an arthritic mouse was shown to effectively reduce disease progression [12]. The same study also discovered that the surface expression of MHCII is vital for the therapeutic effect, as exosomes deficient in MHCII were unable to suppress delayed-type hypersensitivity response in mice immunized with keyhole limpet hemocyanin antigen [12]. Using a similar mouse model in a

later study, exosomes derived from dendritic cells that were genetically modified to express FasL were able to suppress inflammation and, when systemically injected, were similarly effective in treating CIA [13]. Lastly, a follow-up experiment revealed that blood-borne exosomes from mice immunized to keyhole limpet hemocyanin, when transplanted, were able to suppress a delayed-type hypersensitivity response in an antigen-specific manner [14]. The results of this study suggest that exosomes in the plasma were produced by MHCII<sup>+</sup> and CD11b<sup>+</sup> cells and suppress immune response in part through a Fas/FasL-dependent manner. It is important to note that while CD11b is a common cell marker for macrophages, it is also expressed on CD5<sup>+</sup> B1 cells, a subset that have been demonstrated to express FasL. While the authors speculated that the exosomes were derived from macrophages (due to the common cell marker), it is also possible that the origin of these FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes was from CD11b<sup>+</sup> Killer B cells.

***Previous Findings:***

In order to establish a Killer B cell equivalent for human studies, Dr. Steven Lundy and other members of my lab had previously screened a panel of lymphoblastoid cell lines (LCLs) that had been transformed with Epstein-Barr virus. Analysis of all LCL lysate samples by immunoblot showed constitutive intracellular expression of FasL. Previous studies in the lab utilized flow cytometry to demonstrate that these LCLs constitutively produced exosomes that simultaneously expressed both FasL and MHCII molecules on their surface (Figure 2, left panel). To determine if LCL-derived FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes could induce death in CD4<sup>+</sup> helper T cells, T helper lymphocytes (either activated or inactivated) were incubated with purified exosomes samples. Evidence showed that these exosomes induced a significant amount of apoptosis in activated CD4<sup>+</sup> T cells (Figure 2, right panel). Finally, to demonstrate that this killing is antigen specific, donor peripheral blood mononuclear cells were stimulated with tetanus toxoid (TT) peptide



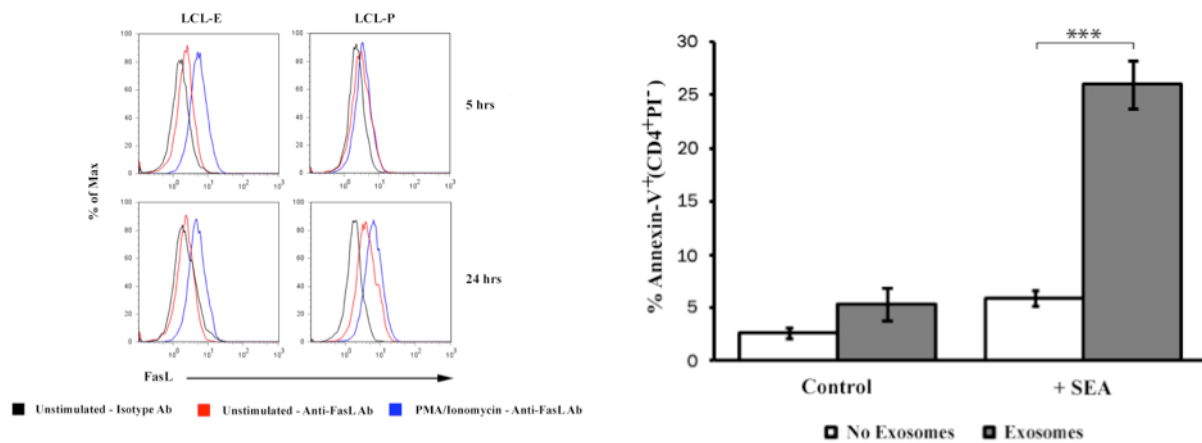


Figure 2: LCL-Derived Exosomes are FasL+/MHCII+ and Mediate Apoptosis of CD4+ T Cells in the Presence of Superantigen

Since exosomes are too small to be detected by standard flow cytometry, Strepavidin-coated beads that were coated with biotin-conjugated anti-MHCII were analyzed after capturing exosomes. LCLs were incubated for either 5 or 24 hours in the presence or absence of phorbol myristate acetate (PMA) and ionomycin. LCL-derived exosomes were collected by ultracentrifugation of culture supernatant at 35600 rpm. Beads were incubated with purified exosome samples so that only exosomes expressing MHCII would remain adhered after being washed. Exosomes that remained on the beads were stained with anti-FasL antibody and measured by flow analysis (Left Panel). CD4+ T cells were obtained and purified from the blood of the original donor using the Rosette Sep technique. Exosomes were incubated with T cells in the presence or absence of *S. aureus* enterotoxin A (SEA, a superantigen) for six days. Cells were analyzed by flow cytometry using CD4-PE, AnnexinV-FITC, and propidium iodide, and selected by CD4+ and propidium iodide negative (live T helper cells). The data were plotted as the percent of T cells positive for AnnV (an apoptosis marker)  $\pm$  the standard deviation of the quadruplicate samples. Exosomes demonstrated extensive killing of CD4+ T cells when activated by superantigen ( $p < 0.05$ , Right Panel).

before culturing the resulting activated T cells with the same donor's LCL-derived exosomes.

Apoptosis significantly increased for CD4+ T cells that were incubated with TT peptide compared to those that were not, and apoptosis was considerably blocked with the addition of anti-FasL antibody. These data show that exosome-mediated killing of CD4+ T cells is antigen specific and is induced in part by the FasL pathway (Figure 3).

Manipulation of these powerful and naturally occurring exosomes may very well be the solution to rheumatoid arthritis, among other autoimmune diseases. Lethal exosomes theoretically represent the ideal therapy by exclusively targeting and effectively killing only the malfunctioning antigen-specific T helper cells involved in autoimmunity. However, while recent experiments further reinforce the promising characteristics of these lethal exosomes, there are

currently limitations that must be overcome before they can be seriously considered for possible therapeutic techniques. First, collecting, identifying, and purifying exosomes is a long and tedious process. In addition, there is currently no effective protocol for extracting only the relevant exosomes without affecting their physiology. Because many of the exosomes released by B lymphocytes are not double positive for FasL and MHCII, simply collecting all exosomes results in a small lethal population within a functionally diverse mixture. Developing improved methods to separate large quantities of FasL+/MHCII+ exosomes without altering their functionality is one of the primary goals of the Lundy Lab.

Therefore, the focus of my research project was to target activated T cells for apoptosis by: 1) stimulating Killer B cells to secrete more FasL+/MHCII+ exosomes; and 2) exploring techniques for isolating, purifying, and quantifying FasL+/MHCII+ exosomes in higher throughput. Advancements in these areas are essential for making lethal exosome therapy a viable treatment option.

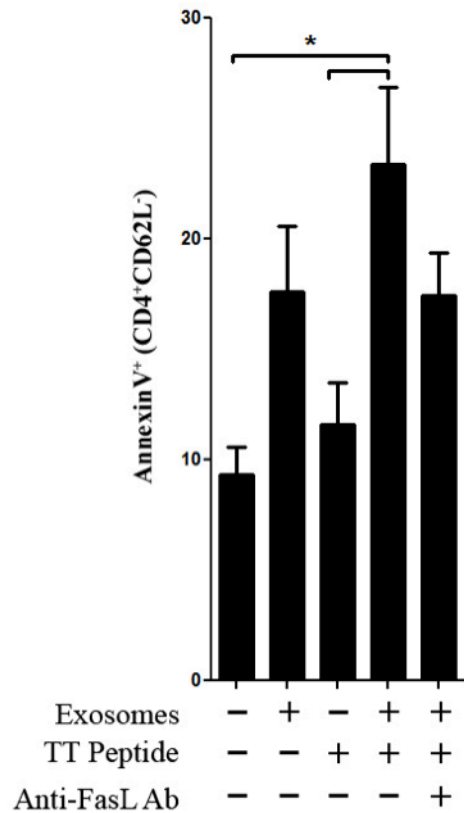


Figure 3: LCL-Derived Exosomes Mediate Apoptosis of CD4+ T Cells in a Peptide Antigen-Specific Manner

Exosomes were purified by ultracentrifugation and resuspended in culture media. CD4+ T cells were obtained by collecting peripheral blood mononuclear cells (PBMC) from the same donor that was used to generate the LCL. The PBMC were stimulated with TT peptide for 12 days in order to induce activation and enrichment of relevant T cells before being incubated with the LCL-derived exosomes. CD4+ T cells were cultured in the presence or absence of exosomes, TT peptide, and anti-FasL antibody (acts to block FasL signaling). After 16 hours of culture, flow cytometry was used to analyze apoptosis in the activated T cells as in Figure 2.

## MATERIALS AND METHODS

### *Mice:*

Wild-type DBA/1LacJ mice were obtained from the lab's breeding colony at the University of Michigan or purchased from Jackson Laboratories. cII-TCR transgenic and Xid mice were generated on the DBA/1LacJ background. Mice with the TCR transgene specifically recognize an immunodominant peptide of type 2 collagen (cII<sub>260-267</sub>) when presented by MHC class II I-A<sup>q</sup> molecules [8]. X-linked immunodeficiency mice (Xid) have a mutation in the Bruton's tyrosine kinase gene, resulting in a resistance to CIA induction and lack of antibody response to type II collagen [15]. The presence of either the transgene or Xid mutation was confirmed by polymerase chain reaction genotyping in all animals. All protocols involving animal subjects were approved by the University of Michigan Committee on the Use and Care of Animals.

### *Spleen Extract Collection:*

Mouse spleens were surgically removed and isolated in 5 mL PBS. Spleens were then disrupted and splenocytes were filtered through 70 µm nylon cell strainers. The resulting splenocyte and PBS solutions were then centrifuged at 1500 rpm for 5 minutes to pellet cells. Exosome-containing supernatant was removed and splenocytes were resuspended in PBS or culture media for experimental application.

### *Generation of Lymphoblastoid Cell Lines by Epstein-Barr Virus:*

B lymphocytes were collected from the peripheral blood of healthy human donors and isolated using Ficoll-Paque centrifugation. Cells were infected by EBV viral suspension and incubated for 2 hr in a 37 °C water bath. Next, 5 mL of RPMI-10 containing 1 µg/mL cyclosporine A was

added to the cell suspension, transferred to a culture flask, and placed in a humidified 37 °C, 5% CO<sub>2</sub> incubator for 6-8 weeks [16]. Cell lines (SL0, SL2, SL4, YHE, YHP) were positively screened for constitutive expression of FasL by immunoblot. These LCLs were grown continuously and regularly maintained at high cell densities in RPMI media containing antibiotics and 20% exosome-free fetal calf serum.

### ***Ultracentrifugation:***

In order to purify exosomes from cellular debris, exosome-containing supernatant from culture media or spleen extracts was first spun at 10,000 x g in a high-speed centrifuge. The resulting supernatant was collected and spun in a clean ultracentrifuge tube at 110,000 x g to create an exosome pellet. Removal of the protein-containing supernatant and resuspension of the pellet in a small volume of PBS resulted in a concentrated quantity of purified exosomes.

### ***Ultrafiltration:***

In order to concentrate exosome solutions into smaller volumes, samples were centrifuged through a 100 kDa filter at 4000 g and room temperature. Samples were generally kept separate; however, in some cases, several samples were periodically added to a single filter in order to concentrate them into a single exosome solution. Flow-through solutions were disposed and the remaining exosome samples were collected. Filters were washed with a minimal amount of PBS in order to collect any remaining exosomes.

### ***Three-Layer Density Gradient:***

This gradient, consisting of three densities, was constructed in an ultracentrifuge tube. First, 1 mL of 60% iodixanol in water (density 1.320) was added to the bottom of the tube. Next, 3 mL

of Ficoll (density 1.077) was carefully added above this, as to prevent mixing of the layers. Finally, the exosome solution was floated on top as the first layer, effectively creating a three-tiered gradient. The density gradient was centrifuged overnight (110,000 x g, 4 °C) and the three fractions were removed sequentially. Fractions from each gradient were pooled together and concentrated by ultrafiltration. The resulting samples were analyzed by immunoblot.

#### ***Multiple-Layer Density Gradient:***

Density gradient layers were constructed with different ratios of 60% iodixanol in water and PBS. Fractions were carefully added at a volume of 0.6 mL, in order of decreasing density, and without disturbing or mixing layers. The final gradient consisted of 7 iodixanol layers (densities of 1.037, 1.058, 1.079, 1.100, 1.160, 1.215, and 1.320) along with an uppermost exosome solution layer. The gradient was then centrifuged for 2 hours (110,000 x g, 4 °C) and fractions were individually collected. Fractions from multiple gradients were pooled and concentrated by ultrafiltration to a final volume of approximately 60 µL. Samples were analyzed by immunoblot.

#### ***Bead Affinity Purification:***

Fresh 6.7 µm streptavidin-coated beads were incubated separately with biotinylated antibodies against specific cell surface markers for 30 minutes at room temperature. Biotinylated antibodies used in experiments included anti-FasL (BD Pharmingen 555292, Biolegend 306403), anti-MHCII (BD Pharmingen 553622), anti-HLA-DR (Biolegend 307614), anti-CD63 (Biolegend 312008), anti-IgM (BD Pharmingen 553406), anti-CD5 (BD Pharmingen 553019), anti-CD1d (BD Pharmingen 553844), anti-CD40 (BD Pharmingen 553789), anti-CD3 (BD Pharmingen 553060), and anti-IcosL (BD Pharmingen 557788). Solution containing LCL or mouse-derived exosomes was added to these antibody-coated beads and incubated overnight. Unbound

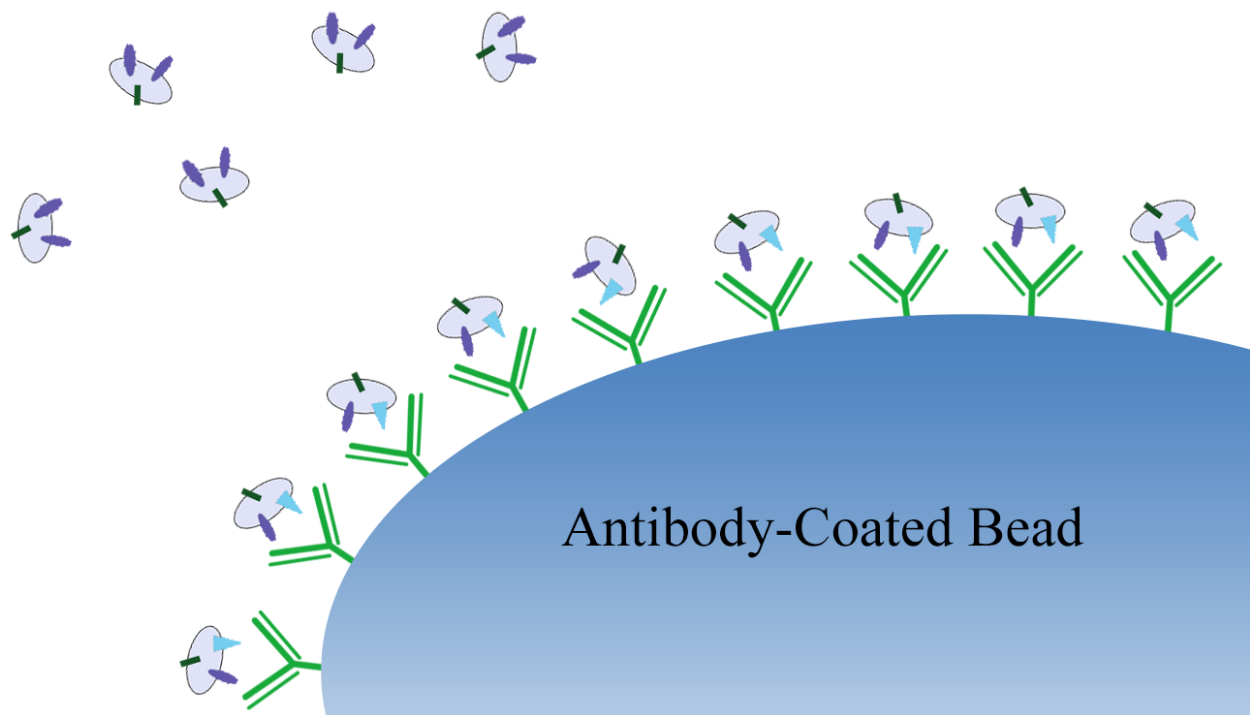


Figure 4: Bead Affinity Purification of Exosomes

LCL-derived exosomes and mouse-derived exosomes were incubated with beads coated in specific antibodies against surface markers. Exosomes that expressed the relevant surface markers were captured, while those that lacked the surface markers were washed away.

exosomes were washed away, and the remaining populations of bead-bound exosomes were lysed in 100  $\mu$ L 3X SDS buffer to be analyzed by immunoblot (Figure 4).

#### ***Enzyme-linked Immunosorbent Assay (ELISA):***

Cells from a human cell-derived lymphoblastoid cell line (LCL) were centrifuged at 1500 rpm for 5 minutes. After formation of a pellet, 5 mL of the exosome-containing supernatant was removed and distributed on 96-well flat-bottomed plates at no dilution, 1:2 dilution, or 1:4 dilution. Exosomes were captured using Purified  $\alpha$ HLA-DR (Biolegend 307602) at 2  $\mu$ g/mL and incubated on the plates overnight. Exosomes were then detected using Biotin  $\alpha$ HLA-DR (Biolegend 307614), Biotin  $\alpha$ FasL (Biolegend 306404), and Streptavidin-HRP (Biolegend 405210). Samples were developed with TCB reagent and stopped with 2N H<sub>2</sub>SO<sub>4</sub>. Absorbance was read at 450 nm wavelength.

Supernatant from cell cultures were also analyzed for production of IL-6, a pro-inflammatory cytokine, and IL-10, an immunosuppressive cytokine, using ELISA kits (BD Biosciences 555220, BD Biosciences 555157) following manufacturer's recommendations.

***Immunoblotting:***

Cell and exosome lysate samples were prepared by adding a quantity of 1X or 3X SDS detergent and incubated at 100 °C for 10 minutes. Samples were separated by SDS-PAGE gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% non-fat dry milk in 1X PBS and 0.05% Tween-20 and incubated with polyclonal rabbit anti-FasL IgG (Millipore AB16982), anti- $\beta$ -Actin (Cell Signaling 4970), anti-MHCII (Santa Cruz 59322), or anti-HLA-DR (Biolegend 307602). Antibody binding was detected with anti-rabbit IgG-HRP secondary antibody (Cell Signaling 7074) or anti-rat IgG-HRP secondary antibody (Santa Cruz 2006) and ECL reagent (Thermo Scientific).

***Chemical Stimulants and Inhibitors:***

Many chemicals were cultured with LCLs in order to characterize their impact on FasL expression and exosome secretion. Terreic Acid (Calbiochem 581810) is a selective inhibitor of Bruton's tyrosine kinase (BTK) catalytic activity that acts by binding the BTK pleckstrin homology domain ( $IC_{50} = 100 \mu M$ ). JAK2 Inhibitor II (Calbiochem 420132) specifically and directly inhibits JAK2 autophosphorylation by interacting with a solvent-accessible pocket near the activation loop ( $IC_{50} = 50 \mu M$ ). STAT5 Inhibitor (Calbiochem 573108) selectively interrupts the SH2 domain of STAT5 with minimal effect on the similar domains of STAT1, STAT3, or Lck ( $IC_{50} = 50 \mu M$ ). Cyclosporine A largely suppresses immune response by blocking evolutionary conserved signal transduction pathways, specifically calcineurin and the NFAT

transcription factors [17]. Dimethyl amiloride (DMA) was generously provided to the lab by Emilie Bourdonnay and Marc Peters-Golden's Lab and inhibits exosome secretion by targeting  $H^+/Na^+$  and  $Na^+/Ca^{2+}$  exchangers, thus disrupting calcium-signaled release mechanisms [18]. Monensin (BD GolgiStop 554724) acts oppositely to stimulate an influx of intracellular  $Ca^{2+}$  and promote exosome secretion [18].

### ***Collagen-Induced Arthritis Immunization and Arthritis Scoring:***

Type II collagen was dissolved overnight at a concentration of 2 mg/mL in sterile 0.05M Acetic Acid. A 1:1 emulsion of type II collagen was prepared in Complete Freund's Adjuvant (CFA) and mixed by transferring solution between two syringes until homogenous. Emulsion was injected into the base of the tail and mice were regularly observed for signs of arthritis.

Paws were regularly scored throughout the duration of *in vivo* experiments. Scores ranged from 0-3 for each paw, which were scored individually for every mouse. A score of 0 represented no inflammation. A score of 1 represented isolated inflammation in one or more digit. A score of 2 represented swelling and redness of the entire paw without involvement of the knee joint. A score of 3 represented major swelling of paws, inflammation in the knee joint, and loss of flexibility in the joints. Final scores ranged from 0-12 for each mouse.

### ***Adoptive Transfer of Exosomes in Arthritis Model:***

DBA mouse spleen extracts were passed through 0.22  $\mu$ m filters in order to remove splenocytes. The resulting exosome-containing solution was concentrated by ultrafiltration. The exosome solution was split into two equal parts, and incubated with either relevant cII peptide or control gp39 protein. Fourteen days following the stimulation of CIA, exosomes were injected into the



tail veins of male DBA mice. Mice were briefly observed for healthy activity and then regularly scored for arthritis in the subsequent days.

## RESULTS AND INTERPRETATION

### *Isolation and Purification of FasL+/MHCII+ Exosomes:*

Previously, LCLs were found to produce FasL+ exosomes by ultracentrifugation of culture supernatant and analysis of samples by immunoblot. One of primary aims of my research was to explore ways to quantify, isolate, and purify FasL+/ MHCII+ exosomes in higher throughput.

I initially tried to quantify the amount of FasL+/MHCII+ exosomes in a sample using ELISA technique. LCL-derived exosomes were collected from supernatant of SL2 cell solution and distributed onto 96-well plates containing anti-HLA-DR (human MHCII) capture antibodies.

The samples were then probed for FasL or HLA-DR and measured against standards by absorbance. When exosomes were captured by anti-HLA-DR, there were no measurable quantities of FasL+ exosomes detected by

anti-FasL (data not shown). There were, however, significant quantities of HLA-DR+ exosomes detected using ELISA method (Figure 5). This suggests that the majority of MHCII+ exosomes do not express FasL on their surface.

Since there is a relatively low detection rate of FasL+ exosomes compared to MHCII+ exosomes, it is important to find methods that increase the concentration of FasL+ exosomes. Separation of FasL+ exosomes from the larger

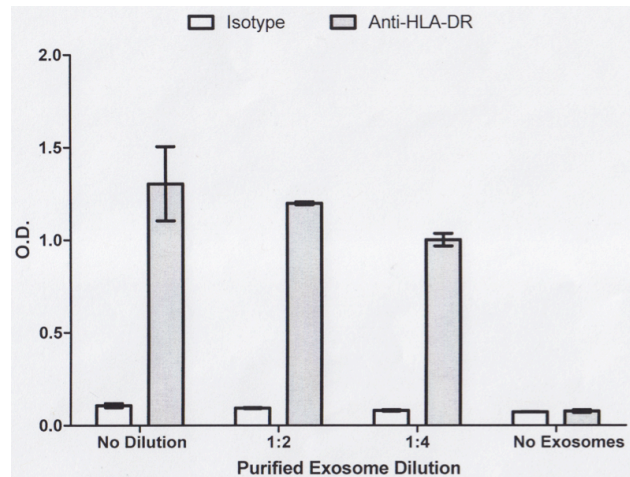


Figure 5: HLA-DR ELISA Detection for LCL-Derived Exosomes

SL2 cells were centrifuged at 1500 rpm for 5 minutes to pellet, and 5 mL of exosome-containing supernatant was removed. Plates were prepared with purified anti-HLA-DR capture antibodies and exosome-containing supernatant was distributed on the plates at equivalent volumes in stock, 1/2, or 1/4 dilution in quadruplicate. Captured exosomes were detected by anti-FasL or anti-HLA-DR antibodies and resolved using Streptavidin-HRP and TCB solution. Absorbance was read at 450 nm.

population would most effectively increase the prevalence of FasL+/MHCII+ exosomes within a given sample.

A previous study found that FasL+/MHCII+ exosomes might have a density different from the majority of MHCII+ exosomes. In an effort to isolate FasL+/MHCII+ exosomes from the larger population, a series of density gradients were constructed to separate the relevant exosomes by exploring their density-specific characteristics. The first density gradient was composed of three layers. Exosomes from DBA-Xid mouse spleens were collected and added onto a layer of Ficoll and a layer of 60% iodixanol in water. Mice were heterozygous for mutation (confirmed by PCR) and were not deficient in FasL expression. The exosomes were separated by ultracentrifugation and individual layers were carefully removed, pooled, and concentrated in

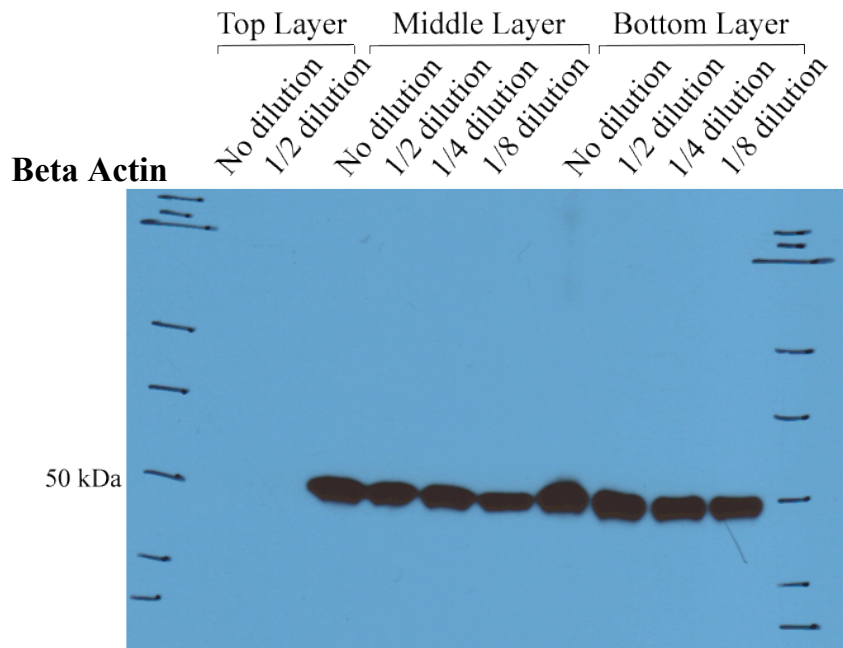


Figure 6: Beta Actin Detection of Exosome Protein from DBA-Xid Mice in Three-Layer Density Gradient

To isolate mouse-derived exosomes, 10 female DBA-Xid (heterozygous) mouse spleens were extracted and splenocytes were filtered through a 70  $\mu\text{m}$  nylon cell strainer. Cells were removed by passing solution through 0.22  $\mu\text{m}$  filter, and exosomes were isolated by ultracentrifugation technique. The exosome solution was floated on multiple gradients consisting of a 3 mL layer of Ficoll (density 1.077) and a 1 mL layer of 60% iodixanol in water (density 1.320). Gradients were spun overnight by ultracentrifugation and similar fractions were concentrated in 100 kDa filters. Lysis samples for each layer were created by adding 10  $\mu\text{L}$  3X SDS buffer to 20  $\mu\text{L}$  of each sample, which were analyzed by immunoblot at stock,  $\frac{1}{2}$ ,  $\frac{1}{4}$ , or  $\frac{1}{8}$  dilution.

100 kDa size filters. Layers were lysed by SDS buffer and probed for FasL and Beta Actin by immunoblot. Analysis indicated no evidence of FasL detection (data not shown). Beta Actin revealed large quantities of protein in the middle and bottom layer (Figure 6).

In order to resolve more distinct populations of exosomes, a multiple-layer density gradient was constructed.

Several layers with distinct densities were created by mixing 60% iodixanol in water and PBS in different ratios. Exosomes collected from the spleens of DBA-Xid mice were floated on top of the gradient and separated by ultracentrifugation. Individual fractions were removed, pooled, and concentrated in 100 kDa filters as previously indicated. Layers were lysed with SDS buffer and probed for FasL, MHCII, and Beta Actin by immunoblot (Figure 7). FasL detection revealed that the majority of exosomes expressing FasL remained in the top layer and did not move through the gradient. The same pattern was observed for MHCII expression as well. Actin detection, however, showed that there was a significant amount of protein in the top four layers, and an additional spike in

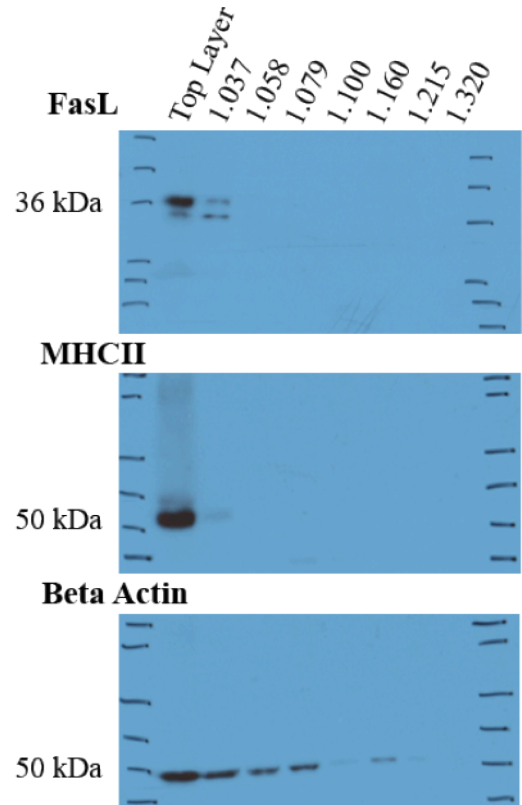


Figure 7: FasL, MHCII, and Beta Actin Detection of DBA-Xid Exosomes in Multiple-Layer Density Gradient

To isolate mouse-derived exosomes, 5 female DBA-Xid (heterozygous) mouse spleens were extracted and disrupted in PBS. Exosomes were collected following the same protocol as Figure 7. The exosome solution was floated on multiple gradients with fractions consisting of 60% iodixanol and PBS at densities of 1.037, 1.058, 1.079, 1.100, 1.160, 1.215, and 1.320 at a volume of 0.6 mL per layer. Gradients were spun overnight by ultracentrifugation and similar fractions were concentrated in 100 kDa filters. Lysis samples for each layer were created by adding 10  $\mu$ L 3X SDS buffer to 20  $\mu$ L of each sample, which were analyzed for FasL, MHCII, and Beta Actin by immunoblot.

the lower 1.160 density fraction. Although this does suggest a certain degree of separation, the multiple-layer density gradient was unable to isolate a distinct population of mouse-derived FasL+ and MHCII+ exosomes into a single density fraction.

The same multiple-layer density gradient was also used in an attempt to separate Lymphoblastoid Cell Line-derived exosomes. The gradient was constructed in the same way as previously; however, there were some differences in experimental protocol. One alteration was the purification of exosomes through ultracentrifugation before floating them on the density gradient, which contrasted with the previous technique of simply collecting supernatant from high-speed centrifugation. This change also allowed for resuspension of the exosome pellet into a relatively small volume of PBS. For this reason, the concentrated exosome solution was added

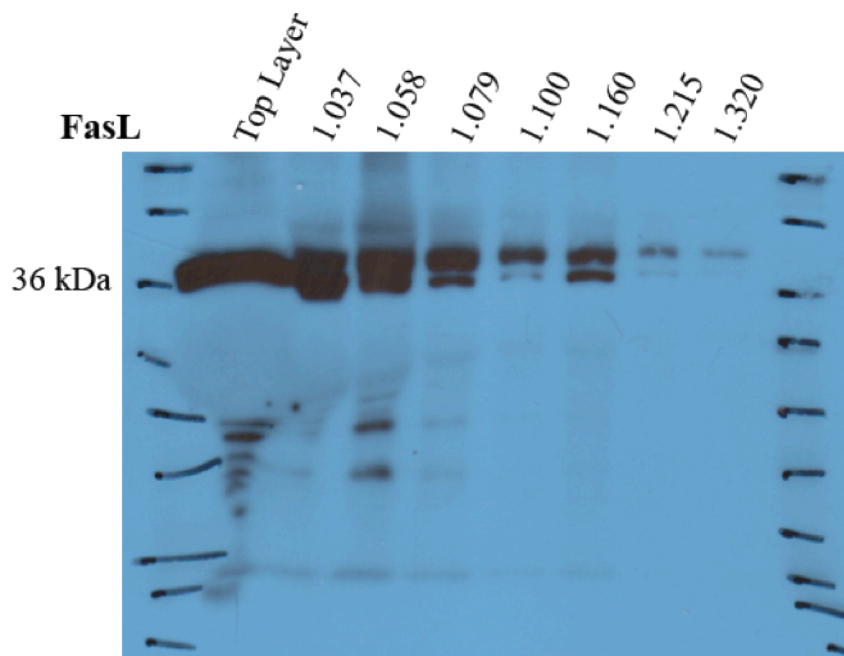


Figure 8: FasL Detection of LCL-Derived Exosomes in Multiple-Layer Density Gradient

To isolate LCL-derived exosomes, SL0 cells were centrifuged to pellet and exosome-containing supernatant collected. The solution was spun at 10,000 x g for 1 hour in a high-speed centrifuge, and the resulting supernatant was spun overnight in an ultracentrifuge at 110,000 x g and 4 °C. The exosome pellet was resuspended in 1 mL volume of PBS, which was floated on a single density gradient with fractions consisting of 60% iodixanol and PBS at densities of 1.037, 1.058, 1.079, 1.100, 1.160, 1.215, and 1.320 at a volume of 0.6 mL per layer. The gradient was ultracentrifuged for two hours. Fractions were collected and concentrated in 100 kDa filters for 4 hours at room temperature. Lysis samples were created by adding 10 µL 3X SDS buffer to 20 µL of each sample. Samples from each layer were analyzed for FasL by immunoblot. [19]

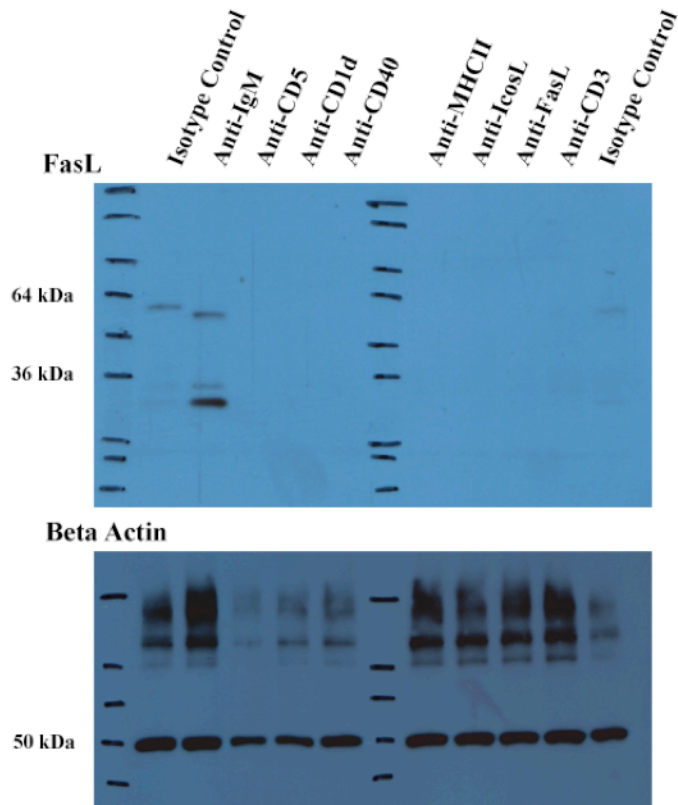


Figure 9: Immunoblot for DBA Mouse Exosomes Selected by Bead Affinity Purification

To isolate mouse-derived exosomes, DBA mouse spleens (n=5 mice) were removed and crushed in PBS. Cells were removed by centrifugation and cell-free supernatant was filtered through 0.22  $\mu$ m filters. The resulting suspension was separated and incubated overnight with 6.7  $\mu$ m streptavidin beads coated with antibodies specific to cell-surface markers. The beads were washed in PBS and pellets were lysed with 100  $\mu$ L 1X SDS buffer and probed for FasL and Beta Actin by immunoblot.

to survey which ones capture the FasL+ exosome population most effectively. DBA mouse exosomes were captured by antibody-coated beads, which were then washed, lysed, and probed for FasL and Beta Actin by immunoblot (Figure 9).

Anti-IgM was the only antibody capable of pulling down a population of FasL+ exosomes.

Interestingly, beads coated with anti-FasL and anti-MHCII did not capture a high-density FasL+ population, which may be related to the number of FasL-/MHCII+ exosomes in the solution as

to a single density gradient. Individual fractions were collected, lysed, and analyzed by immunoblot (Figure 8). Detection for FasL revealed a large amount of FasL expression by exosomes in the top layer, with decreasing expression within each subsequent fraction. The only exception to this pattern was a spike in FasL expression observed in the 1.160 density fraction. Ultimately, the density gradients were unable to separate FasL+/MHCII+ exosomes into a single, homogenous population.

The next technique that I explored for isolating FasL+/MHCII+ exosomes was bead affinity purification. First, streptavidin-coated beads were incubated with various antibodies against exosome surface markers

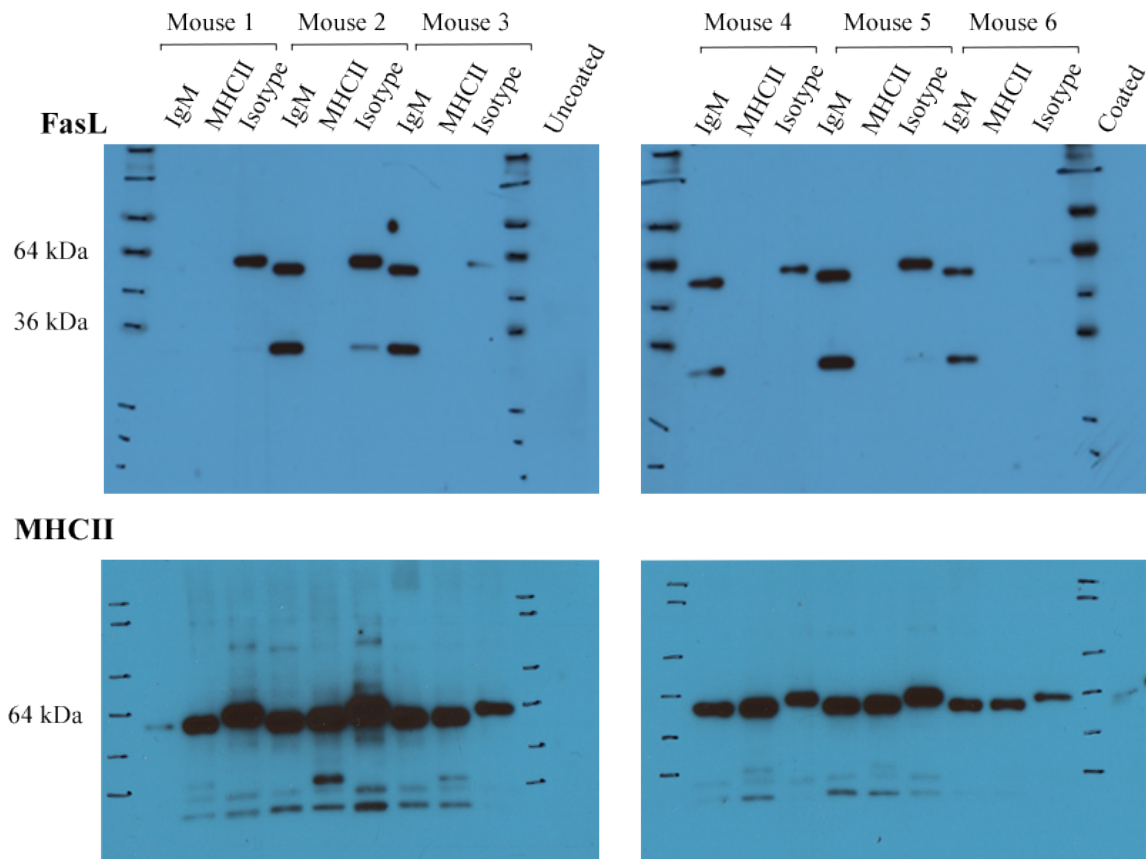


Figure 10: Immunoblot for Individual DBA Mouse Exosomes Selected by Bead Affinity Purification

To isolate mouse-derived exosomes, DBA mouse spleens (n=6 mice) were removed and individually crushed in PBS. Cells were removed by centrifugation and cell-free supernatant was filtered through 0.22  $\mu\text{m}$  filters. The resulting suspensions were kept separated and incubated overnight with 6.7  $\mu\text{m}$  streptavidin beads coated with anti-IgM and anti-MHCII antibodies. The beads were washed in PBS and pellets were lysed with 100  $\mu\text{L}$  1X SDS buffer and probed for FasL and MHCII by immunoblot.

well as the relatively weak interaction between the FasL and anti-FasL capture antibody. The band that appears for FasL detection of isotype control is larger than FasL and is likely an artifact of non-specific binding, which is evident from the strong Beta Actin signal detected in all conditions even though they mostly did not result in the binding of FasL+ exosomes.

Since bead affinity purification by the IgM cell marker showed promise in selecting for a dense population of FasL+/MHCII+ exosomes, the same protocol was followed on a series of individual DBA mouse spleens in order to test experimental consistency. Instead of an assortment of cell markers, beads were only coated with anti-IgM, anti-MHCII, or isotype

control antibodies. Uncoated beads cultured with exosomes as well as uncultured beads coated in a mixture of the antibodies were processed as controls. Exosomes were captured on beads, lysed, and analyzed for FasL and MHCII by immunoblot (Figure 10).

Anti-IgM antibody-coated beads captured a population of FasL<sup>+</sup> and MHCII<sup>+</sup> exosomes for 5 of 6 mice. The one exosome sample (Mouse 1) that failed to produce a concentrated FasL<sup>+</sup> population by anti-IgM capture also failed to bind a population of MHCII<sup>+</sup> exosomes, indicating that this sample may have been compromised and not a negative result. Anti-MHCII beads captured a population of MHCII<sup>+</sup> exosomes, but the amount of FasL on these exosomes were not within an observable range of detection. Again, this is likely explained by the presence of a relatively large FasL<sup>-</sup>/MHCII<sup>+</sup> population of exosomes within the exosome solution. Similar to the previous experiment, the isotype control appeared to bind selectively to a protein on the exosomes and was detected with the secondary antibody, producing bands at a larger size than expected for either FasL or MHCII.

The ability to isolate FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes by bead affinity purification was also tested on LCL-derived exosomes. SL0 and SL2 cell solution was collected and cells removed by centrifugation. The exosome-containing supernatant was incubated with anti-FasL, anti-HLA-DR, and anti-CD63 (a marker specific to secreted exosomes) antibody-coated beads. Coated and uncoated beads were again used as controls. The lysed samples were probed for FasL and HLA-DR by immunoblot (Figure 11).

Anti-FasL antibody-coated beads may have captured some FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes, but the bands are barely detectable by immunoblot analysis. Again, anti-HLA-DR beads were unable to capture a dense population of FasL<sup>+</sup> exosomes. However, anti-CD63 antibody-coated beads



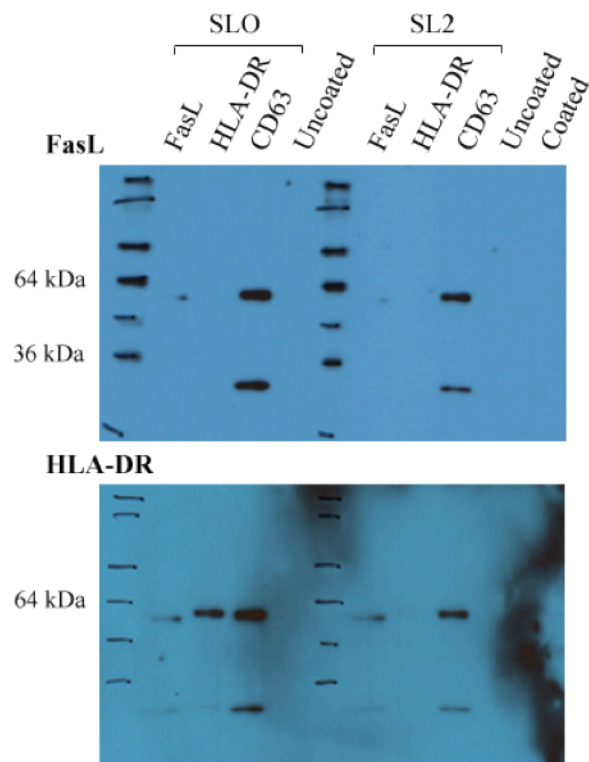


Figure 11: Immunoblot for LCL-Derived Exosomes Selected by Bead Affinity Purification

Culture supernatants from unstimulated LCLs were cleared of cells by centrifugation and put through a 0.22  $\mu\text{m}$  filter. The exosome solution was incubated overnight with 6.7  $\mu\text{m}$  streptavidin beads that were coated with anti-FasL, anti-HLA-DR, or anti-CD63 antibodies. These beads were then lysed with 100  $\mu\text{L}$  1X SDS buffer and probed for FasL and HLA-DR by immunoblot.

showed the ability to effectively capture a population of both FasL<sup>+</sup> and MHCII<sup>+</sup> exosomes, as indicated by the prominent bands for both SL0 and SL2 lysates. This is especially significant because it associates FasL and MHCII molecules with a marker specific to secreted exosomes.

### ***Manipulation of FasL<sup>+</sup> Expression and Exosome Secretion:***

In addition to collecting FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes, I conducted experiments to identify signaling pathways involved in FasL expression and exosome secretion. Manipulation of these pathways might result in a larger quantity of FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes available for collection and investigation as well as provide a better understanding for their immunological potential.

An initial survey of various inhibitors and their effects on FasL expression was conducted on the SL0 lymphoblastoid cell line. Cells were cultured with JAK2 Inhibitor II, STAT5 inhibitor, and Terreic Acid for 72 hours. Cells were collected by centrifugation and lysis samples were analyzed for FasL by immunoblot (Figure 12). Exosome-containing supernatant did not contain enough protein for observable detection of FasL expression (data not shown). When compared

to LCLs without inhibitor, JAK2 Inhibitor II and STAT5 Inhibitor did not affect intracellular FasL expression. Although it first appeared that Terreic Acid entirely suppressed FasL production in LCLs, a closer look at the cells revealed that the cells in culture were extremely

unhealthy, indicating that the lack of FasL signal may have been a consequence of cell death.

A follow up experiment involving serial dilutions of Terreic Acid concentrations was performed in order to evaluate Terreic Acid's effect on the immunological potential of LCLs in less lethal doses.

LCLs were cultured in the presence of several different concentrations of Terreic Acid (1.5625  $\mu$ M – 100  $\mu$ M) for 72 hours. Cells were collected by centrifugation and lysis samples were probed for FasL by immunoblot (Figure 13). Analysis revealed no change in intracellular FasL expression for LCLs when cultured by non-lethal concentrations of Terreic Acid (>25  $\mu$ M).

Culture supernatant was also measured for quantities of IL-6 and IL-10 by ELISA. There was little expression of IL-6 detected in the culture supernatants

of any inhibitor concentration (data not shown), indicating a low potential for inflammation.

However, there was a clear trend for IL-10 expression that was consistent with every cell line:

IL-10 expression positively correlated with Terreic Acid concentration (Figure 14). This is an

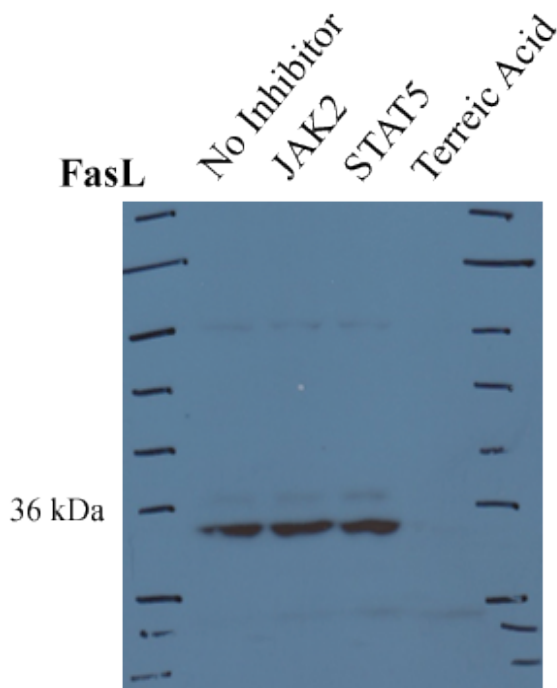


Figure 12: FasL Detection for Selective Inhibitors Acting on LCLs

SL0 cells were collected from culture flasks, washed, and distributed at 2 million cells per well on a 24-well plate. Chemical inhibitors were added at maximal inhibition and were tested in triplicate. Cell cultures incubated for 72 hours in a final volume of 2 mL exosome-free media. Eight million cells for each condition were then collected, lysed in 200  $\mu$ L 1X SDS buffer, and probed for FasL by immunoblot. The supernatant was also collected for exosome characterization, but there was not enough protein for observable results.

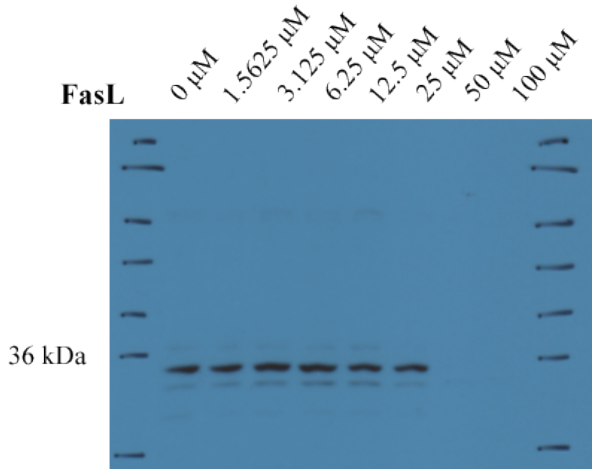


Figure 13: Terreic Acid has No Effect on FasL Production in LCLs

SL0 cells were collected from culture flasks, washed, and distributed at 1.5 million cells per well on a 24-well plate. Terreic Acid was added by serial dilution at final concentrations between 0 μM and 100 μM. Cells were incubated for 72 hours in a final volume of 1 mL media. Cells were then collected by centrifugation, lysed in 200 μL 1X SDS buffer, and probed for FasL by immunoblot.

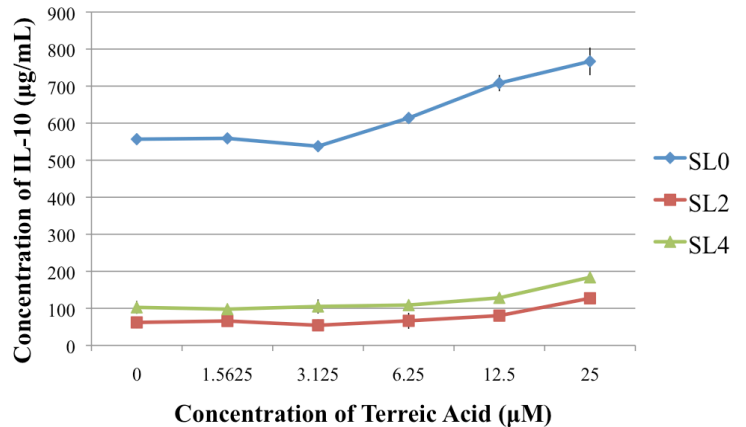


Figure 14: Terreic Acid Increases IL-10 Production in LCLs

SL0, SL2, and SL4 cells were collected from culture flasks, washed, and distributed at 1.5 million cells per well on a 24-well plate. Terreic Acid was added by serial dilution at final concentration between 0 μM and 100 μM. Cells were incubated for 72 hours in a final volume of 1 mL media. Cells were centrifuged to pellet and the remaining supernatants were collected for analysis by ELISA.

interesting result as it suggests an increase in immunosuppressive potential with an increase in Terreic Acid inhibition.

Several different LCLs were also cultured with Cyclosporine A to determine its effect on intracellular FasL and IL-10 production. Cells and corresponding supernatant were collected after incubation in the presence of absence of Cyclosporine A for 72 hours. Cell lysis samples were probed for FasL by immunoblot (Figure 15) and supernatants were analyzed for IL-10 by ELISA (Figure 16). There were no observable differences in intracellular FasL production for all cell lines. However, the presence of Cyclosporine A did result in a decrease of IL-10 production by the LCLs, indicating a decrease in immunosuppressive potential. Interestingly, the supernatant from the YHE cell line had an immeasurably high concentration of IL-10, even

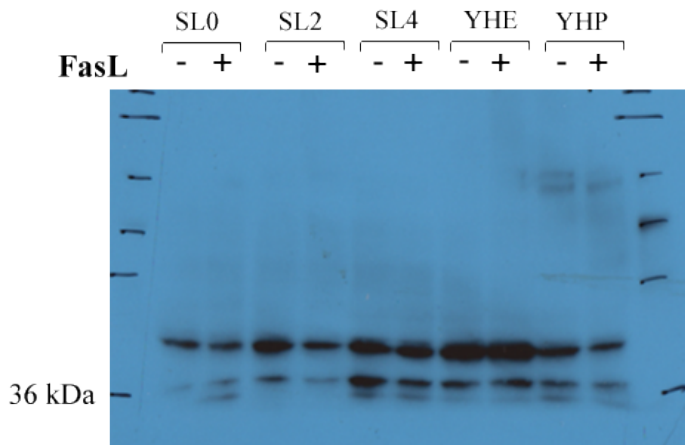


Figure 15: Cyclosporine A has No Effect on FasL Production in LCLs

SL0, SL2, SL4, YHE, and YHP cells were collected from culture flasks, washed, and distributed at 5 million cells per well on a 48-well plate. Cells were incubated for 72 hours in the presence or absence of Cyclosporine A at a concentration of 250 ng/mL with each condition repeated in triplicate. Cells were then collected by centrifugation, lysed in 100  $\mu$ L 1X SDS buffer, and probed for FasL by immunoblot.

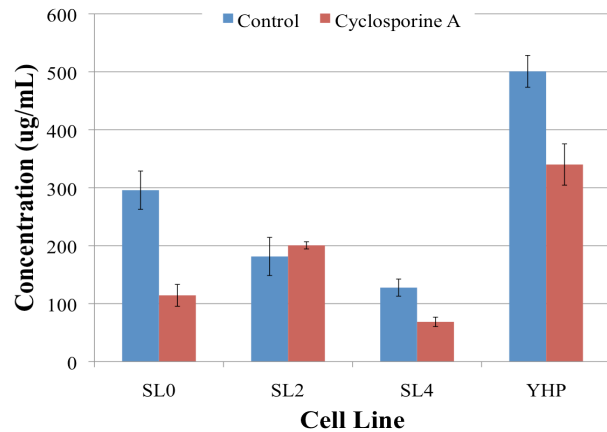


Figure 16: Cyclosporine A Decreases IL-10 Production in LCLs

SL0, SL2, SL4, and YHP cells were collected from culture flasks, washed, and distributed at 5 million cells per well on a 48-well plate. Cells were incubated for 72 hours in the presence or absence of Cyclosporine A at a concentration of 250 ng/mL with each condition repeated in triplicate. Cells were centrifuged to pellet and the remaining supernatants were collected for analysis by ELISA.

with Cyclosporine A inhibition. For this reason, the data from YHE are not presented in the graph.

Exosome secretion pathways were also investigated as potential targets of manipulation in order to produce more FasL+/MHCII+ exosomes. Dimethyl amiloride (DMA), an exosome secretion inhibitor, and Monensin, an exosome secretion promoter, were both employed as chemical agents in treating LCLs. Cells were cultured with various concentrations of DMA and a recommended concentration of Monensin. Cell lysate samples were collected and probed for FasL and Beta Actin by immunoblot (Figure 17).

Analysis of FasL band patterns revealed two distinct sets of bands, one occurring around 64 kDa and another at 36 kDa. Both bands were observed for the cell lysates, but the shorter band was

much more prominent. The larger band became more pronounced with an increase in DMA concentration. The larger band was also the only band detected for exosome lysates, and it became less pronounced with an increase in DMA concentration. The native form of FasL is presented on the surface of a membrane as a homotrimer, while its intracellular expression may be in monomeric form. This would explain the increased detection of the larger band as exosome secretion is inhibited and more trimeric FasL is retained within the cell. This would also explain the corresponding decrease in FasL expression observed in supernatant, as fewer exosomes would be secreted. There was no noticeable change in FasL expression in the presence of monensin.

### ***In Vivo Treatment of Arthritis:***

In order to determine the clinical effectiveness of FasL+/MHCII+ exosome treatment, exosomes from the spleen extract of immunized male DBA mice were transferred into arthritis-induced male DBA mice. Spleens were harvested and exosomes were incubated with either cII peptide or gp39 protein. DBA male mice were previously immunized with an established protocol for collagen-induced arthritis with a single injection of cII in

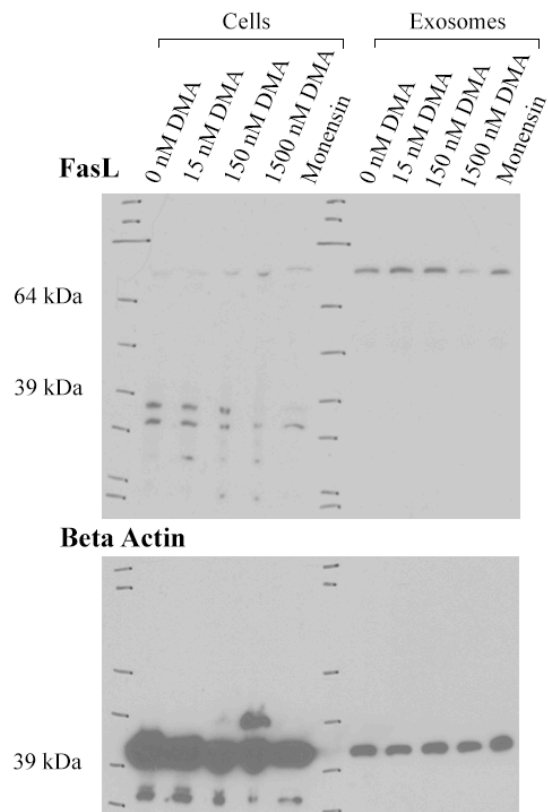


Figure 17: Immunoblot of Dimethyl Amiloride and Monensin Effect on FasL+ Exosome Production

SL0 cells were collected and evenly distributed at 5.75 million cells per well on a 12-well plate. DMA was added at final concentrations of 15  $\mu$ M, 150  $\mu$ M, and 1500  $\mu$ M. Monensin was added at a final concentration of 1  $\mu$ M. Cell culture conditions were done in duplicate and in a final volume of 3 mL exosome-free media. After incubating for 48 hours, cells were collected by centrifugation and lysed in 200  $\mu$ L 1X SDS. Exosomes were isolated by collecting culture supernatant, centrifuging it at 10,000 x g for 1 hour, and finally centrifuging the remaining supernatant at 110,000 x g into a pellet. Exosome lysis samples were prepared by adding 100  $\mu$ L 1X SDS directly to the pellet. Cell and exosome lysates were probed for FasL and Beta Actin by immunoblot.

Complete Freund's Adjuvant (CFA) to the base of the tail. After 14 days, mice were injected with cII-stimulated exosomes, gp39 exosomes, or no exosomes. Mice paws were regularly scored for arthritic swelling for 35 days and then humanely euthanized following University of Michigan Animal Care and Use Committee protocol. Spleens were removed and splenocyte counts were individually recorded (Table 1).

Mice receiving cII-stimulated exosomes experienced significantly less swelling than mice receiving gp39-stimulated exosomes or no exosome treatment (Figure 18). On average, mice with gp39-stimulated exosomes had a similar arthritic progression as mice not receiving exosome treatment. The final scores for each condition were 0-4 for cII-stimulated exosome treatment, 1-12 for gp39 exosome treatment, and 0-9 for no exosome treatment. While some mice from each condition had minor swelling, only the mice treated with gp39 exosomes or

Mouse	Treatment	Day 17	Day 25	Day 33	Day 42	Day 52	Total # of Splenocytes
1	No Exos	0,0,0,0	0,0,0,0	0,0,3,0	0,3,3,0	0,3,1,0	8.56 x 10 <sup>7</sup>
2	No Exos	0,0,0,0	0,0,0,3	0,0,0,3	0,3,0,3	0,3,0,3	9.04 x 10 <sup>7</sup>
3	No Exos	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,1,1	10.4 x 10 <sup>7</sup>
4	No Exos	0,0,1,0	0,0,1,1	0,0,1,1	0,3,2,3	0,3,3,3	9.76 x 10 <sup>7</sup>
5	No Exos	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	5.60 x 10 <sup>7</sup>
6	gp39 Exos	0,0,0,0	0,0,2,2	0,0,2,2	3,3,3,3	3,3,3,3	13.0 x 10 <sup>7</sup>
7	gp39 Exos	0,0,0,0	0,0,1,0	0,0,1,0	0,0,1,0	0,0,1,0	13.5 x 10 <sup>7</sup>
8	gp39 Exos	0,0,0,0	0,0,0,0	0,0,0,0	0,0,1,0	0,0,1,0	4.48 x 10 <sup>7</sup>
9	cII Exos	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	0,0,0,0	3.12 x 10 <sup>7</sup>
10	cII Exos	0,0,0,0	0,0,0,0	0,0,0,1	0,0,0,1	0,3,0,1	4.08 x 10 <sup>7</sup>
11	cII Exos	0,0,0,0	0,0,1,0	0,0,1,0	0,0,1,0	0,0,1,0	6.00 x 10 <sup>7</sup>

Table 1: Arthritic Scoring and Splenocyte Count for Male DBA Mice Receiving Transferred Exosomes

Male DBA mice were sacrificed and spleens harvested. Splenocytes were centrifuged to pellet and exosome-containing supernatant was removed. Exosomes were concentrated by ultrafiltration. Exosomes were then incubated with cII peptide or gp39 protein at a final concentration of 1:100 relative to exosome protein. Male DBA mice received a single injection of cII in CFA to the base of the tail in order to stimulate collagen-induced arthritis. After 14 days, mice were intravenously injected with 142.7 µg of cII-stimulated exosomes or gp39-stimulated exosomes in the tail vein, or not given treatment at all. Paw swelling was regularly recorded on a scale of 0-3 per paw, 0 indicating no swelling and 3 indicating severe swelling. Mice were sacrificed 35 days after injection of exosomes and splenocyte counts were recorded.

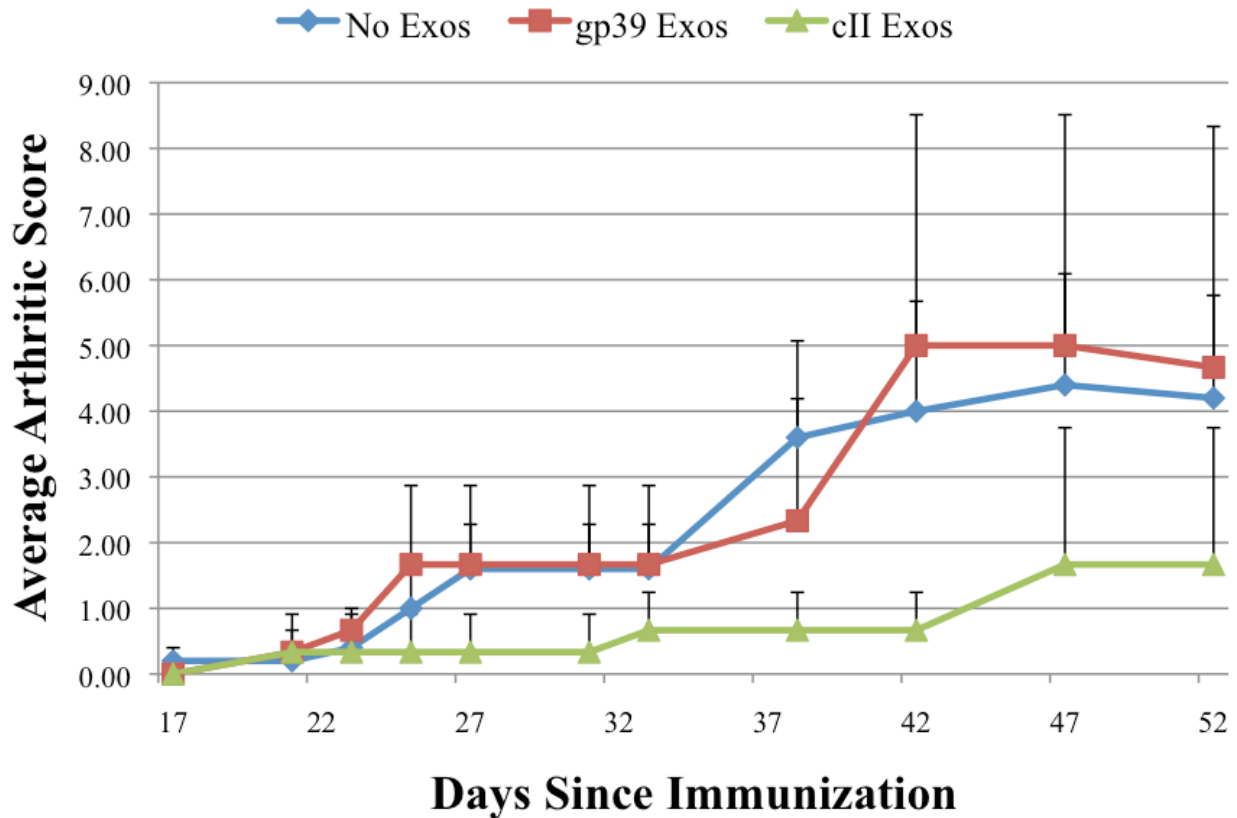


Figure 18: Average Arthritic Score for Mice Receiving cII, gp39, or No Exosomes

Total scores for each condition were averaged and plotted against days after initial immunization of male DBA mice. Error bars were calculated using standard error of the mean. Treatment by cII-stimulated exosomes resulted in a significantly lower score when compared to gp39 exosome treatment or no exosome treatment ( $p < 0.05$ , determined by Student's t-test).

without exosomes experienced severe arthritic symptoms and intense paw inflammation. Mice that received cII-stimulated exosomes also had significantly reduced cellularity of the spleen when compared to both other conditions ( $p < 0.05$ , determined by Student's t-test). This may be indicative of a suppressed immune response, which correlates with their reduced arthritic scores. These results show that stimulated splenic exosomes, containing a population of FasL+/MHCII+ exosomes, may be useful as a treatment for autoimmune disease.

## DISCUSSION

The ability of to isolate and quantify FasL+/MHCII+ exosomes is important for investigating the unique characteristics and physiological function of these lethal signaling vesicles. Ideally, researchers would use a pure, measured quantity of FasL+/MHCII+ exosomes when conducting experiments, both *in vivo* and *in vitro*, in order to accurately identify the cause of the experimental results. There are multiple ways to pursue quantification and purification, each exploiting unique qualities of the relevant exosomes. My experiments focused on three techniques in particular: ELISA, density gradient, and bead affinity purification.

ELISA is a well-known technique that can quantify the presence of cellular markers and substances in high-throughput. Unfortunately, this tool was not effective in detecting measurable amounts of FasL on captured exosomes. The detection of significant MHCII expression, however, suggests that there is a relatively large population of FasL-/MHCII+ exosomes in a given sample. This further reinforces the importance of discovering ways to isolate large quantities of FasL+/MHCII+ exosomes, as their lethal effects are probably diluted when existing as a minority in greater exosome populations.

Currently, the common technique used for separating exosomes from other cellular bodies is a series of centrifugation steps with a final ultracentrifugation at 110,000 x g, where the exosomes are ultimately pelleted. However, while this protocol effectively isolates exosomes from a solution, it does not distinguish among subset populations. Interestingly, a recent experiment in the lab indicated that the FasL+/MHCII+ exosome population might be characterized by a specific density. In an attempt to take advantage of this property, density gradients were constructed for the ultracentrifugation step of the protocol. Isolation by density gradient is



especially attractive because relevant exosomes can be easily rescued without damaging their physiological function.

None of the density gradients were successful in purifying a population of FasL+/MHCII+ exosomes. While the multiple-layer density gradient remained intact throughout ultracentrifugation, the three-layer gradient dissolved into a continuous gradient, which made it difficult to collect fractions. Collecting exosomes by ultracentrifugation before floating them on a single multiple-layer gradient seemed to improve the separation of exosomes. Unfortunately, analysis of the individual fractions revealed no distinct layer of FasL+/MHCII+ exosome population. Ultimately, it appears that the densities of FasL+/MHCII+ exosomes are not distinct enough to be exploited for isolation techniques.

Bead affinity purification seems to be a much more promising technique for isolating FasL+/MHCII+ exosomes. The successful capture of relevant mouse-derived exosomes by anti-IgM has significant implications, especially when considering the fact that IgM is a surface marker that is highly specific to B cells. This means that the dense population of FasL+ exosomes captured by anti-IgM beads were certainly secreted by B lymphocytes, an important selection tool for the future. Also, the ability to capture a FasL+ and MHCII+ population of LCL-derived exosomes by anti-CD63 (an exosome secretion marker) provides another target to exploit. It is important to note, however, that these bands need to be sequenced in order to verify that they are, in fact, FasL protein, and not the result of non-specific binding to IgG heavy and light chains.

Logistically, selection by bead affinity comes with both advantages and challenges. In terms of preparing samples, this technique conveniently bypasses time-consuming steps when compared

to the traditional protocol for collecting exosomes; instead of preparing exosome samples by ultracentrifugation, it is only necessary to centrifuge supernatant through a 0.22 micron filter before incubating with antibody-coated beads. Especially since CD63 is specific to exosomes, it is not necessary to prepare a pure sample of exosome suspension. Additionally, the same concept of exosome capture could be utilized in the form of a column, resulting in even greater throughput. A challenge in utilizing bead affinity purification, however, is removing exosomes from the beads without disrupting their structure or function. There are a few possibilities yet to be investigated, such as flooding beads with glycine or salting off the exosomes. In order to determine the impact of these protocols on their function, eluted exosomes would be tested with both *in vitro* killing assays and *in vivo* exosome transfers. The retrieval of exosomes without compromising their physiological function will be a focus of future studies.

An inherent limitation with bead affinity purification technique is the ability to identify exosomes that contain both FasL and MHCII on their surface. Ideally, this would be possible by capturing a population of exosomes with anti-MHCII antibodies and probing for FasL, or vice versa. However, in this case, FasL<sup>+</sup> exosomes were not effectively captured by anti-FasL antibody-coated beads or anti-MHCII antibody-coated beads due to weak FasL to anti-FasL interactions and a dense population of FasL<sup>-</sup>/MHCII<sup>+</sup> exosomes, as previously explained.

Although anti-IgM antibody-coated beads captured a population of exosomes that were positive for FasL and/or MHCII, it is impossible to say that the molecules were simultaneously present on individual exosomes. It is overwhelming probable, though, that the higher prevalence of FasL and MHCII in this specific population translates to a higher concentration of FasL<sup>+</sup>/MHCII<sup>+</sup> exosomes. In the future, other clones of anti-FasL antibodies that may possibly have higher affinities for surface FasL will be used in an attempt to capture a FasL<sup>+</sup> population of exosomes

and confirm the high prevalence of FasL+/MHCII+ exosomes. Other surface markers will also be investigated as targets for FasL+/MHCII+ exosome capture. Overall, these investigations, along with further testing on the retrieval of relevant exosomes, make bead affinity purification a promising technique for capturing lethal exosomes.

It was generally difficult to examine the inhibitors' effects on the expression of FasL on exosomes because there were low yields of protein, especially considering the relatively short incubation times. For this reason, many of the analyses were focused on measuring intracellular FasL and other extracellular immunological signaling structures, such as IL-10. While not ideal, a stimulatory or inhibitory effect on the production of intracellular FasL is likely an indication of the amount of surface FasL on secreted exosomes.

None of the inhibitors (Terreic Acid, JAK2 Inhibitor II, STAT5 Inhibitor, Cyclosporine A) were found to affect FasL production in LCLs. While there is no surprise that cyclosporine A (an immunosuppressant) impaired production of IL-10 in LCLs, it is interesting that Terreic Acid actually stimulated IL-10 production. At the moment it is difficult to offer an explanation for this result, and further study is necessary for understanding the reason for it.

One of the more elegant results is that of the exosome release inhibition and stimulation experiment. This was a rare case where there were enough exosomes to produce lysis samples for analysis by immunoblot. Unfortunately, treating LCLs with monensin did not cause a noticeable increase in the amount of FasL+ exosomes. Additionally, as expected, an increase in DMA resulted in a decrease in FasL+ exosome detection, as fewer exosomes were being secreted into the external media by LCLs. However, it is important to notice that there are two distinct bands at 64 kDa and 36 kDa. As the larger band on the exosome samples became fainter with an

increase of DBA concentration, the same band became visible in the corresponding cell lysate samples. This suggests that the larger band is strictly related to the surface expression of FasL, possibly as a covalently-linked homotrimer, while the smaller band is characteristic of the intracellular monomer. Therefore, it follows that the smaller band is completely absent from the exosome lysates samples because their FasL expression is entirely on the surface. Also, as an increasing number of exosomes are retained in the cell, the larger band indicative of surface expression becomes more prominent. In the future, bands will be confirmed as FasL trimer or FasL monomer by protein sequencing. In addition, more signaling pathways will continue to be investigated in order to better manipulate Killers B cells into producing and secreting higher quantities of FasL+/MHCII+ exosomes.

The clinical effect of exosomes is evident when reviewing the results of the mouse model *in vivo* experiment. Injection of cII-activated exosomes significantly reduced inflammation in the paws of arthritic mice compared to mice treated with gp39-stimulated exosomes or no exosomes. Also, mice that received cII-stimulated exosomes had significantly fewer splenocytes than the other mice. While fewer splenocytes is an indication of a suppressed immune response, whether this is a result of lethal exosomes targeting CD4+ helper T cells for destruction will need to be confirmed with cell staining and analysis by flow cytometry.

Interestingly, it seems that while the mice treated with cII-stimulated exosomes were largely protected for the first 25 days after injection, the symptoms worsened toward the end of the experiment. Currently, there is little information about the half-life of circulating exosomes, but it may be possible that the exosomes are cleared after this period of time and are unavailable for further protection against disease. Future studies will investigate other treatment schedules, such

as regular injection of stimulated exosomes, in order to improve the clinical effectiveness of exosomes on autoimmune disease.

However, although the treatment was successful, there is one major limitation on the conclusions that can be drawn from this study. The protocol for collecting exosomes was entirely dependent on separation by size; that is, ultrafiltration by 100 kDa filters. While it is likely that the majority of the solution was indeed exosomes, there may have been other small cellular bodies or particles in it as well. Furthermore, even if the solution was entirely composed of exosomes, there was no way of knowing the amount that were both FasL+/MHCII+. Even though it is highly suspected that the clinical effect is from the population FasL+/MHCII+ exosomes, it will be very difficult to make a definitive claim until techniques to properly isolate and purify relevant exosomes in large quantities are possible.

These studies in the manipulation, isolation, and clinical implementation of FasL+/MHCII+ exosomes illustrate the promise of exosomes in arthritis research and treatment. Lethal exosomes and their natural, effective, and specific killing capacities have wide-ranging implications for immunology. However, further investigations are necessary in order to demonstrate them as a viable treatment for autoimmune disease.

## **ACKNOWLEDGEMENTS**

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