

SPECIFIC AIMS

Surgical removal of the spleen, or splenectomy, is often a medically necessary procedure in patients who have suffered severe damage to the organ. This procedure is common for patients with a ruptured spleen, a cancer of the spleen or surrounding tissues such as leukemia, or an autoimmune disorder such as idiopathic thrombocytopenic purpura (ITP), which causes antibodies to target host platelets. Because the spleen is classified as a non-vital organ, post-operative patients generally live their lives without a spleen. However, the spleen plays key roles in both adaptive and innate immune responses; therefore, patients without spleens have an increased susceptibility to many bacterial and fungal infections. Additionally, the spleen is very important for blood filtration, where it is responsible for metabolizing hemoglobin and recycling iron.

Autologous stem cells must be used for a spleen transplant to be successful, in order to avoid the use of immunosuppressants, which are required after all traditional organ transplants, and would render the spleen's primary function useless. To do this, the spleen will be decellularized using a surfactant solution, leaving behind only a protein matrix with decellularized vasculature. Next, we aim to take advantage of the naturally occurring process known as splenosis, in which damaged fragments of spleen attach elsewhere, regenerate, and revascularize, creating what is known as a splenic nodule. Homogenized splenocytes will be inserted into the decellularized matrix, where we hypothesize they will grow to form a functional spleen. This method would allow for reseeded of the spleen matrix with the patient's own cells. Finally, the created organ will be transplanted into mice, where it will be evaluated *in vivo*. In the attempt to create a bioartificial spleen, the following specific aims are proposed:

Aim 1: Create a decellularized three-dimensional scaffold from a mouse spleen using various detergents

Hypothesis: Widely used detergents for decellularization will successfully create a splenic scaffold which is devoid of cellular material but still retains the overall protein structure of a spleen.

Method: Spleens of healthy mice will be carefully excised after the mouse is sacrificed. The spleen will be decellularized using perfusion decellularization. Different detergents, such as sodium dodecyl sulfate (SDS) and Triton X-100, will be injected into the splenic artery in varying concentrations to determine optimal conditions for decellularization. Optimal conditions will be defined as retaining less than 5% of the original cellular material, quantified by DNA content, as well as retaining the protein structure of the spleen. The structure and vasculature will be analyzed after decellularization using CT scans and histological samples stained with markers for different extracellular proteins.

Aim 2: Repopulate the decellularized spleen with homogenized splenocytes, through the natural process of splenosis.

Hypothesis: Splenocytes from a homogenized spleen will repopulate a decellularized matrix, creating a fully functional spleen, complete with red and white pulp, in a manner similar to that which naturally occurs during splenosis.

Method: A healthy rat spleen will be crushed with cell strainers to create a homogenous, single cell suspension. The suspension of splenocytes will then be introduced to a decellularized spleen, via injection into the splenic artery. Residual splenocytes in splenectomy patients have been shown to undergo splenosis and generate spleen-like organs *in vivo*, and we hypothesize the homogenized splenocytes will behave in a similar manner once introduced onto the scaffold.

Aim 3: Retransplantation and experimental evaluation of the autologous bioartificial spleen in mice

Hypothesis: The bioartificial spleen created through Aim 1 and Aim 2 will retain key characteristics present in normal healthy spleens, including: structure of viable splenic tissue, proper red blood cell (RBC) morphology, sufficient immunoarchitecture, and functional immunoprotection for the host.

Method: Mice will be randomly assigned to 1 of 4 groups: sham control, total splenectomy, splenectomy with partial spleen implant, and splenectomy with implantation of the bioartificial spleen created through Aim 1 and Aim 2. The structure and function of the bioartificial spleens will be evaluated using scanning electron microscopy, peripheral blood smears, T-cell and B-cell counts, and intravenous bacterial challenge using *Streptococcus pneumoniae*.

Long-term, this proposal aims to create working spleens from decellularized tissue reseeded with either stem cells or splenocytes, allowing for spleen implantation without the need for immunosuppressive drugs, which inhibit the spleen's functions. This research would constitute the first major research effort to create an artificial spleen, as well as the first which aims to take advantage of splenosis for spleen regeneration. The creation of a functional spleen implant will greatly improve the quality of life for patients who would typically undergo splenectomy, and this proposal is a first step towards this goal.

SIGNIFICANCE

The spleen is classified as a non-essential organ, meaning that humans can live without it. As a result, little research has been done with regards to creating artificial spleens. The spleen is highly compartmentalized and performs a variety of different functions in the body. Between the red pulp and the white pulp, it has blood filtration functionality, and contributes significantly to both adaptive and innate immunity [Mebius]. The red pulp has also been shown to contribute to erythropoiesis. Within the body, the spleen is one of the largest sites of blood filtration, and it removes old erythrocytes from circulation [Mebius, Steineger]. Approximately 22,000 splenectomies were performed in 2005 in the US [DeFrances], with an average cost of \$20,800 [Gwilliam].

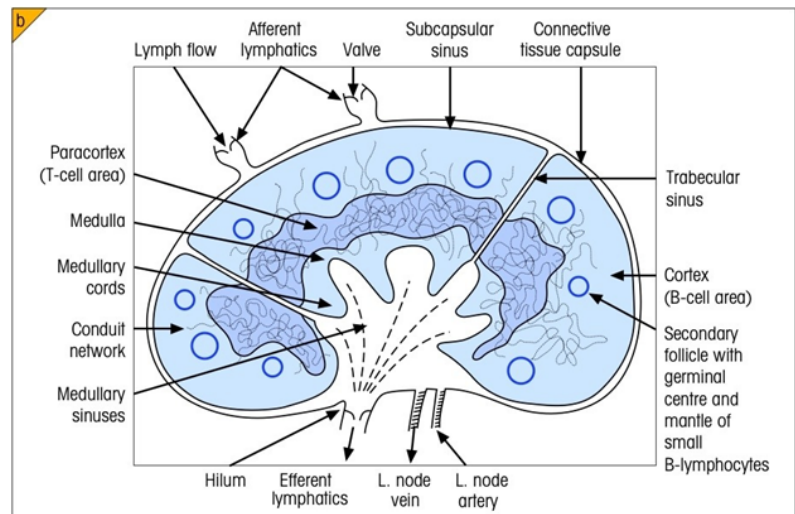


Figure 1: Diagram of a spleen, showing its high degree of compartmentalization

Several diseases and conditions can lead to the onset of hyposplenism, which is a reduction in splenic function, or even asplenia, the complete loss of splenic function. Common diseases leading to this diagnosis include rheumatoid arthritis and celiac diseases [Di Sabatino]. These conditions increase the risk of sepsis and other infections in suffering individuals; this is due to loss of white pulp function, which is responsible for storage of mature lymphocytes. It is estimated that 1.5 million people in the United States suffer from rheumatoid arthritis [Myasoedova], and another 3 million are affected by celiac diseases [Namatovu]. These two groups represent large populations of people at risk for developing immune complications due to hyposplenism, and this artificial splenic implants would improve their lives by removing the need to rely on antibiotics.

Patients who have impaired splenic function or no spleen at all are very dependent on antibiotics as their source of protection against bacterial infections [Kaplinsky]. In the past, antibiotics were able to satisfactorily control bacterial infections; however, currently, one of the greatest medical crises is the development of multi-drug resistant bacteria. These bacteria are evolving resistance to antibiotics faster than new antibiotics are being produced [Dewar]. This is of particular concern for patients who have undergone a splenectomy because the spleen plays a significant role in dealing with bacterial infection. As antibiotics begin to fail, these patients lose their only line of defense. A fully functional spleen transplant will provide them with a much greater chance of surviving a small bacterial infection. Also, antibiotics are not specific to pathogenic bacteria; they disrupt the human microbiome, resulting in digestive problems for patients. Fully functional artificial spleens would improve the quality of life for these patients.

Table 1. Comparison of Hazard Functions Between Groups

Groups Compared	Ratio of Hazard Functions
Splenectomized versus sham-operated	2.27 ($p < 0.005$)
Splenectomized versus whole-spleen autotransplant	2.49 ($p < 0.001$)
Splenectomized versus half-spleen autotransplant	2.25 ($p < 0.005$)
Whole-spleen autotransplant versus sham-operated	0.91 (NS)
Half-spleen autotransplant versus sham-operated	1.01 (NS)
Whole-spleen autotransplant versus half-spleen autotransplant	0.92 (NS)

NS, not significant.

In mouse studies, even partial spleens provide significantly better bacterial protection than no spleen, and greatly decreased the incidence of death [Dickerman]

Recently, a correlation has also been found between loss of splenic function and elevated blood glucose levels, suggesting that removal of the spleen results in an increased risk of diabetes mellitus [Ley]. This study suggests that the spleen has an extra, previously unknown function in the storage of pancreatic stem cells [Ley], and by replacing the spleen, this function could be restored.

Once the decellularization and reseeding process is developed, it can readily be applied to a porcine spleen as a scaffold which can be reseeded with human cells from part of the patient's original spleen, resulting in a relatively inexpensive spleen implant.

INNOVATION

1. The first attempt to decellularize the spleen and retain its protein matrix with decellularized vasculature, and one of the first attempts on a lymphatic organ.

Our goal here is to be the first group to decellularize a spleen by using a perfusion washing system that will remove all cellular components from the organ while retaining the complex compartmentalization and vasculature of the spleen. The spleen is one of the softest tissues in the body, so successfully decellularizing the organ without damaging the extracellular matrix represents both a difficult and exciting challenge.

Additionally, not much work has been done with engineering replacements for the lymphatic system in general [Niklason]. Creating an artificial spleen would be useful in not only providing patients with replacement spleens but also with creating models for *in vitro* studies regarding the spleen or the lymphatic system in general.

2. Using the naturally occurring process of splenosis to reseed the decellularized matrix in order to create a fully functional artificial spleen.

Splenosis offers an exciting alternative to stem cells for splenic regeneration. Splenosis is a typically a benign condition, occurring in 67% of patients with splenic rupture [De Vuysere, Lüdtke], in which cells that have broken off from the spleen metastasize through the body, often ending up in the liver or gut [Kang, Bock]. Hundreds of splenic nodules are formed, and grow to typically a couple cm in size [Kang, Fleming]. These splenic nodules preserve some red pulp function, with typically low immunoresponsive white-pulp function; however there is at least one documented case of a patient with splenosis who had splenocytic tissue indistinguishable from normal spleen, and full immune capabilities [Hathaway].

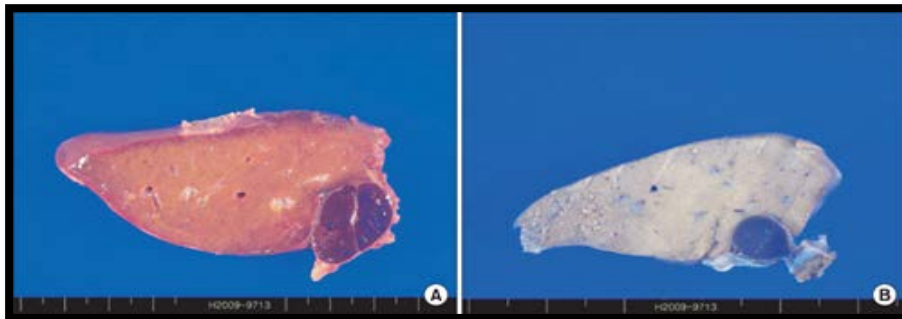


Figure 2: 2.3x1.9 cm and 0.7x0.6 cm splenocytes, found located within slices of liver tissue in a 28 year old male. 5 total of this size and larger were found within the liver [Kang].

Additionally, splenosis would allow for the patient's own damaged spleen cells to be used to create a new, healthy spleen, eliminating the need for stem cell harvesting; of course this is provided that their spleen contains some amount of healthy spleen cells. Finally, splenosis is known to occur in mice [Dickerman], and so animal models can be used for these studies.

We will be the first people to attempt to control the process of splenosis by confining it to a 3-D scaffold of natural tissue. This will not only allow for a functional spleen replacement, but it will also grant insight into the intricacies of splenosis. Once more is known about splenosis, that information could be utilized in comparison to metastatic disease or tissue regeneration.

APPROACH

Aim 1: Create decellularized three-dimensional scaffold from mouse spleen using various detergents.

Hypothesis: Widely used detergents for decellularization will successfully create a splenic scaffold which is devoid of cellular material but still retains the overall protein structure of a spleen.

1. Determine optimum operating parameters for perfusion decellularization of mouse spleen
2. Analysis of resulting decellularized murine spleen scaffold

The goal of this aim is to develop a method to create a decellularized scaffold that maintains the structure and compartments of a spleen. Other organs such as hearts, lungs, and kidneys have been successfully decellularized using sodium dodecyl sulfate (SDS) and Triton X-100 [Baptista, Sullivan]. These protocols have already been established, but little work has been done with the spleen and other lymphatic organs. Spleens have much lower values for Young's modulus when compared to the other organs in the body, and are therefore much softer [Carter, Levental]. Thus, the organ may respond differently to decellularization and the process must be developed specifically for the spleen.

To efficiently remove cellular material from the spleen, the technique of perfusion decellularization will be utilized, which has been shown to have more success than previously used methods [Baptista]. First, a mouse will be sacrificed and its spleen will be carefully excised without damaging the organ. Deionized water will then be perfused through the splenic artery and vein for two days in a sterile environment. This prepares the organ for perfusion of detergent.

1. Determine optimum operating parameters for perfusion decellularization of mouse spleen

Triton X-100 is a non-ionic, weaker detergent, which has been shown to work with some soft tissues at a concentration of 1%; SDS is a stronger, ionic detergent which has also been used with some success with concentrations between 0.1% and 5% [Sullivan]. Since the spleen is one of the softest tissues in the body, lower concentrations of detergent will likely be preferable to avoid damage to the scaffold. All detergent solutions will be diluted in 1x phosphate-buffered saline (PBS). Triton X-100 will be tested in concentrations of 0.5% and 1% along with 0.1% ammonium hydroxide in both cases, and separately, SDS will be tested in concentrations of 0.25% and 0.1%. Along with concentration of detergent, decellularization time will also be altered. Images of the organs will be taken every 6 hours to monitor the decellularization process visually, and different spleens will be perfused with each detergent for 12, 24, 48 and 72 hours. After detergent perfusion, deionized water will once again be perfused for two days to rinse away any detergent. Then, DNase will be perfused for 12 hours through the decellularized scaffold to degrade any residual DNA. To remove DNase and degraded DNA, the scaffold will be perfused one final time with 1x PBS for 2 hours.

2. Analysis of resulting decellularized murine spleen scaffold

To visualize the remaining vasculature following decellularization, the decellularized scaffolds as well as freshly excised spleens will be imaged using CT scans and compared. For more a more quantifiable analysis, histological samples will be taken of decellularized and native spleens and immunostained for collagen, glycosaminoglycans, laminin, and fibronectin, which are all components of the extracellular matrix (ECM). Samples of the tissue will also be used for DNA quantification to determine the efficiency of the decellularization. The DNA will be isolated and quantified using the Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) and the Quant-iT High-Sensitivity DNA Assay Kit (Invitrogen Corp., Carlsbad, CA). In order for the scaffold to be non-immunogenic, a successful level of DNA remaining after decellularization will be defined as 5% or less of the native tissue.

Potential Problems:

The splenic tissue may be too soft to decellularize easily with the given concentrations of detergents. In the event that not enough matrix is left behind at the lowest concentrations of detergent, the procedure will be repeated with half the concentration of detergent. If the opposite is true and the organ is not visibly decellularizing, the concentration of detergent will be doubled before repeating the experiment.

Aim 2: Repopulate the decellularized spleen with homogenized splenocytes, through the natural process of splenosis.

Hypothesis: Splenocytes from a homogenized spleen will repopulate a decellularized matrix, creating a fully functional spleen, complete with red and white pulp, in a manner similar to that which naturally occurs during splenosis.

1. Reseed, first with mouse endothelial cells to restore vasculature, then with homogenized splenocytes
2. Characterize and optimize the creation of the artificial spleen

1. Reseed, first with mouse endothelial cells to restore vasculature, then with homogenized splenocytes

First, the mouse spleen will be homogenized into a cellularly-intact slurry. To do this, the spleen will be placed between two pieces of mesh and a flat surface, such as the back of a syringe, will be used to repeatedly crush it. This mesh will be greater than 320 microns in diameter—twice the size of the larger cells in the spleen [Thompson]—to allow small clumps of cells through without breaking too many. However, different mesh sizes will be tested, both to decrease cell breakage and optimize later reseeded. A 6.8 pH buffer containing nutrients required for cell survival will be used for intermittent storage, as this is the pH which the spleen naturally buffers itself towards without the presence of blood [Levesque].

Because the size of the splenic nodules observed in splenosis is known to be limited by the blood supply [Fleming], the decellularized tissue will be revascularized before reseeded with splenic tissue. Modifying a method used by Song et al. to successfully regenerate the vasculature of a decellularized rat kidney, human umbilical venous endothelial cells (HUVECs) will be inserted through the primary splenic artery. Despite being human derived, these cells have not proven to cause problems in rodent models [Song]. After vascular regeneration, the homogenized spleen, complete with all cell types, will be reseeded into the decellularized spleen. The primary insertion method tested will be to insert the slurry, along with recirculating mouse blood, through the reformed vascularization via the splenic vein and artery, using a pressure gradient to increase spleen cell penetration through the decellularized scaffold. It was found that a pressure gradient of 40 cm H₂O did not cause any damage to reseeded kidney cells using this method [Song]; therefore this pressure will be used as a starting point. Vascular endothelial growth factor (VEGF), a well-known inducer of vascular permeability [Weis], will also be added to the slurry in order to allow better penetration of splenic cells through the endothelial membrane.

2. Characterize and optimize the creation of the artificial spleen

If reseeded is successful, slices of created spleen will be analyzed visually for red and white pulp contents; for a normal spleen, red pulp comprises approximately 76-79% by volume [Pochedly]. If red pulp content is too high—which is likely due to the mechanism of splenosis [Kang]—then it may be possible to decrease red pulp growth rates by increasing the level of oxygen. Because red pulp is associated with erythropoiesis, the process by which red blood cells are produced, it may grow slower when oxygen content is high and less red blood cells are needed [Kwok].

Because splenic cells are able to regenerate on their own through splenosis, we aim to find an optimum implantation mass, which we define as the minimum mass of splenic tissue required for full spleen regeneration. If it is true that the entire spleen cell mass is not required, as hypothesized, then partially diseased or even cancerous spleens may possibly be regenerated via this method. A disease or cancer could be cleansed after homogenization, when one does not have to worry about drug side effects on the body, theoretically causing a once unhealthy spleen to be reborn as a healthy one.

Potential Problems

Splenosis is not well characterized *in vitro*, and it is possible that there are *in vivo* factors not present in the decellularized matrix that are required for it to occur. If this is the case, blood from splenectomized mice, which would be rich in any splenosis-enhancing factors, could be circulated while the artificial spleen is growing.

Additionally, there would likely be FDA resistance to implantation of once diseased spleen cells back into the body. It is possible that additional tests would have to be done to ensure full remission of splenic disease before this type of procedure could become wide-spread.

Aim 3: Retransplantation and experimental evaluation of the autologous bioartificial spleen in mice

Hypothesis: The bioartificial spleen created through Aim 1 and Aim 2 will retain key characteristics present in normal healthy spleens, including: structure of viable splenic tissue, proper red blood cell (RBC) morphology, sufficient immunoarchitecture, and functional immunoprotection for the host.

1. Implant the recellularized bioartificial spleen into the omentum of mice.
2. Analyze RBC morphology to detect presence of abnormal RBCs in peripheral blood.
3. Perform cell counts on blood samples to determine amounts of T-cells and B-cells.
4. Challenge mice with intravenous *Streptococcus pneumoniae* to determine levels of host immunoprotection.

1. Implant the recellularized bioartificial spleen into the omentum of mice.

We seek to implant the recellularized organs into 10-12 week old mice to assess the function compared to controls. Mice will be randomly assigned to one of four groups: (1) sham control, (2) total splenectomy, (3) splenectomy with partial spleen implant, or (4) splenectomy with implantation of the bioartificial spleen. The sham control is analogous to a placebo-controlled drug trial; it seeks to isolate specific outcomes due to the treatment. Total splenectomized patients are used to determine whether our proposed artificial spleen has *in vivo* function. The procedure performed in group 3 mice represents a currently studied practice for post-splenectomized patients and has been shown to regenerate some splenic function related to blood filtration, but the retention of immunoprotection is very limited with this procedure [Pisters]. The results of the following evaluations will allow us to discover how our implant performs compared to the normal spleen and partial splenic transplant.

We will implant homogenized splenocytes into an omental pouch grafted into the greater omentum of mice from group 3. This process also relies on splenosis to occur in order to generate functional splenic nodules, and has been shown to occur *in vivo* in mice [Pisters]. Bioartificial spleens will be implanted directly into the omentum of mice belonging to group 4. The omentum is a large layer of fat which drapes over and attaches to all of the visceral organs; the omentum was chosen as the desired site of transplantation due to its rich blood supply and source of growth factors and cytokines [Alvarez]. Omental implants have been shown to result in better regeneration and immunologic function in cases where splenocytes were implanted into an omental pouch [Alvarez], and we hypothesize that the increased blood supply will improve vascularization of the bioartificial organ, and improve immunoprotection.

2. Analyze RBC morphology to detect presence of abnormal RBCs in peripheral blood.

The spleen filters the blood, metabolizing hemoglobin and recycling iron; it is also responsible for removing abnormal red blood cells from the blood stream. Abnormal blood cells can be found in splenectomized patients, and we will use peripheral blood smears to analyze the morphology of the blood of patients each week to determine if the spleens are functioning to remove abnormal RBCs.

Blood will be drawn from the patients and a small drop of venous blood will be placed on a glass microscope slide to be analyzed. This drop of blood droplet will then be spread out using the cover slide to draw the drop away, leaving behind a thin layer of blood. The slide will be left to dry for ten minutes and then placed into an automated hematology analyzer for staining and cell counting [Shelton]. Abnormal RBCs that are indicative of splenosis are codocytes, or “target cells” as they are aptly named for their target-like shape, and Howell-Jolly Bodies [Lüdtke]. The presence of either of these cell types would be indicative of splenectomy, and lack of these abnormal RBCs would show that the artificial spleen retains its blood filtration functionality.

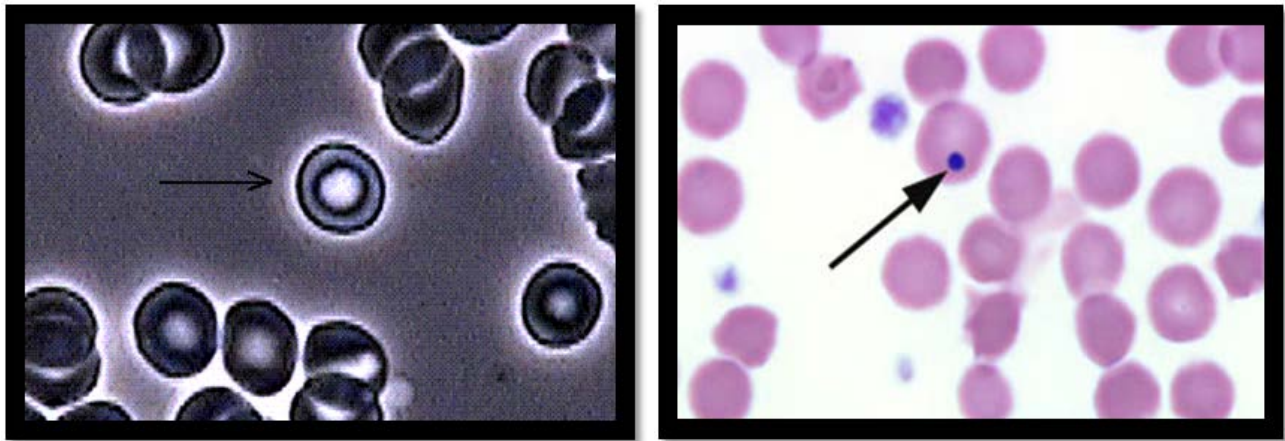


Figure 3: Pictured are examples of abnormal red blood cells amongst healthy RBCs: codocyte (left) and Howell-Jolly Body (right) [Corazza].

3. Perform cell counts on blood samples to determine amounts of T-cells and B-cells.

The white pulp of the spleen is responsible for the storage and production of B-lymphocytes and T-lymphocytes, therefore a patient having a functional spleen will have higher counts of lymphocytes in the peripheral blood [Shelton]. We will assess the immunoarchitecture of the patients by analyzing blood samples weekly as in the prior case. Whole blood samples will be drawn and then incubated in the presence of fluorochrome-conjugated monoclonal antibodies (mAbs) against specific cell surface receptors. Next RBCs will be lysed using ACK Lysing Buffer (Life Technologies, Grand Island, NY) and the remaining sample will be passed through a multiparametric flow cytometer. Using CD4 to screen for T-cells and CD19 for B-cells, the respective lymphocytes will be selected for and percentages and cell counts can be reported for each of the markers/lymphocytes [Goldeck].

4. Challenge mice with intravenous *Streptococcus pneumoniae* to determine levels of host immunoprotection.

From prior research it has been shown that while autologous spleen transplants are able to retain red pulp function, the white pulp function is rather limited [Pisters]. After determining the quantity of T-cells and B-cells in the peripheral blood of the mice, we will assess the immunoprotection provided to the host by the bioartificial spleen transplant. Mice will be intravenously exposed to *Streptococcus pneumoniae* Type III 8 weeks post-operation, and then will be tested on both (1) microorganism clearance, and (2) host mortality rate.

The LD₅₀ (number of bacteria necessary to kill 50% of the mice) will be determined for the sham control group by varying the dosage of pneumococci from 10¹, 10², 10³, 10⁴, 10⁵, 10⁶, 10⁷, and 10⁸ colony-forming units (CFU). The number of living mice will be recorded for 10 days, or longer if necessary [Yother]. After determining the LD₅₀ for the sham control mice, this same number of CFU will be injected into each mouse in each of the 4 groups. Two mice from each group will be sacrificed immediately following intravenous injection of *S. pneumoniae*, as well as at 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, 24 hours, and 48 hours following exposure [Jay]. These mice will be used to determine the levels of microorganism clearance. Additionally, overall host mortality rates will be compared between the different groups. We hypothesize that the bioartificial spleen will provide host immunoprotection greater than the mice with partial splenic transplants, and comparable to that of the sham control group.

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