Mitigating the Foreign Body Reaction Associated with LARS Artificial Ligaments Through Delivery of IL-4

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Significance

The anterior cruciate ligament (ACL) is the most frequently injured ligament in the knee [1]. Most often, the ACL is sprained or torn while playing sports that involve sudden stops or changes in direction, jumping, and landing, such as soccer, basketball, and skiing [2]. Once injured, the ACL will not heal on its own because it is surrounded by synovial fluid and lacks significant vascularization [1]. In the United States, approximately 200,000 to 250,000 ACL injuries occur every year, with as many as 90% of patients undergoing ACL reconstruction [3], [4]. The ACL repair market size is expected to grow exponentially (Figure 1).

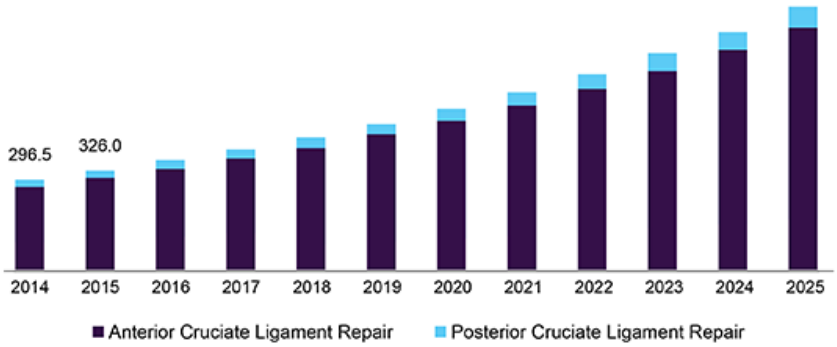

Figure 1: United States cruciate ligament repair procedures market size, by procedure type, 2014-2025 (000' procedures). The ACL repair market size is expected to grow exponentially due to an increase in sports-related knee injuries and demand for minimally invasive procedures [5]. The high prevalence of ACL injuries places a big burden on the United States healthcare system with an estimated 1 billion USD spent annually on reconstructive surgeries [6]. The original figure can be found in [5].

Over 90% of ACL reconstructions are done using patellar or hamstring tendon autografts, however this treatment has been associated with donor site morbidity and delayed return to sports, among other complications [1], [7]. To overcome these limitations, there have been many attempts to fabricate artificial ligaments for the treatment of ACL injuries [8]. The use of synthetic materials for ACL reconstruction is a promising treatment approach due to their abundant supply and strength, lack of harvest site pathology, and potential for accelerated rehabilitation [7]. Additionally, synthetic materials possess highly tunable properties that can be precisely adjusted during the manufacturing process to suit the intended application [9]. **Regardless of these advantages, so far no artificial ligament has been demonstrated to be a viable alternative to tendon autografts [1]. This is in part due to the development of a foreign body reaction which restricts artificial ligaments from widespread use [8].**

The foreign body reaction can negatively impact the biocompatibility of a biomaterial and lead to implant failure [10]. This reaction is composed of granulation tissue and foreign body giant cells that form as a result of a prolonged presence of pro-inflammatory (M1) macrophages at the implant site [11]. The lack of knowledge surrounding the inflammatory processes associated with implanted biomaterials made it challenging to design biocompatible materials that could withstand long-term use. Previous artificial ligament designs focused on achieving desired mechanical properties and did not incorporate mechanisms that limit inflammatory responses and promote healing. This led to the failure of many artificial ligaments and their subsequent removal from the market [7].

There is strong evidence to suggest that early-stage macrophage polarization towards an anti-inflammatory/ regulatory (M2) phenotype mitigates the foreign body reaction and promotes integration of non-degradable implants [12]**. By facilitating the release of an M2-polarizing cytokine from artificial ligaments, a more effective implant can be engineered which promotes healing and thereby results in a lower incidence of post-surgical complications and a quicker patient recovery time. The proposed solution will give patients another surgical option that can be used to reconstruct the ACL and will overcome the drawbacks of autografts and current artificial ligaments by preventing donor site morbidity and reducing pain associated with the host inflammatory response.** This project will provide further insight into the design and manufacture of immunomodulatory biomaterials. Additionally, it will advance an understanding of the effectiveness of chemical cues in polarizing immune cells and achieving a desired response. If the artificial ligament is successful in reducing the foreign body reaction, the engineering methodology can potentially be applied to other synthetic biomaterials and promote the innovation of additional solutions. The foreign body reaction has been associated with all artificial ligaments to date, making the development of biocompatible materials that minimize the degree of this immune response a critical research endeavor [8].

Innovation

The Ligament Augmentation and Reconstruction System (LARS) artificial ligament is a prosthesis made from polyethylene terephthalate (PET) that has recently been associated with a clinically significant degree of foreign body reaction. In an effort to improve design, the LARS Company has worked to optimize its production techniques by eliminating the use of all cytotoxic chemicals that may trigger a foreign body reaction. The microporosity of the ligament has also been adjusted to facilitate faster fibroblastic ingrowth and to prevent the production and release of wear particles [8]. Despite these improvements, the foreign body reaction remains a concern. **Current approaches to minimize the degree of foreign body reaction associated with LARS and other PET ligaments focus on coatings to improve osseointegration and wear resistance [13], [14]. However, the presence of these coatings may still elicit chronic inflammatory events that eventually lead to implant failure (Table 1).**

Table 1: **Approaches to mitigate the foreign body reaction associated with artificial ligaments.** Previous coatings applied on artificial ligaments aimed to improve osseointegration and enhance wear resistance, however they did not directly modulate inflammation [13], [14]. The proposed solution will directly mitigate the host inflammatory response by promoting M2 macrophage polarization, which should foster healing and improve biomaterial integration into the host tissue.

	Current Approaches	Proposed Approach
Mechanism of Action	Reduction or prevention of wear particle release through rapid bone ingrowth into the bone-ligament interface [13]	Macrophage modulation towards an anti-inflammatory/regulatory (M2) phenotype through the release of IL-4
Features	▪ Improved osseointegration ▪ Enhanced wear resistance ▪ Chronic inflammation may result from wear of intra-articular segment	▪ Should promote biomaterial integration into the host tissue ▪ Should foster healing ▪ Intended to mitigate inflammatory events that occur at the time of biomaterial implantation

Macrophages can be activated towards a pro-inflammatory (M1) phenotype or an anti-inflammatory/regulatory (M2) phenotype in response to environmental signals [11]. Biomaterials tend to elicit a persistent M1 macrophage response, which if unresolved, leads to a severe foreign body reaction that negatively affects biomaterial integration [9], [11]. Modulation of macrophage phenotype at the implant site has been shown to mitigate the foreign body reaction associated with polypropylene meshes commonly used for soft tissue reconstruction. The release of IL-4 from layer by layer (LbL) coated meshes promoted M2 macrophage polarization *in vitro* and *in vivo* and encouraged healing [12]. **This project seeks to improve the design of current LARS artificial ligaments by utilizing a LbL technique to incorporate IL-4 onto coated ligaments (Figure 2).**

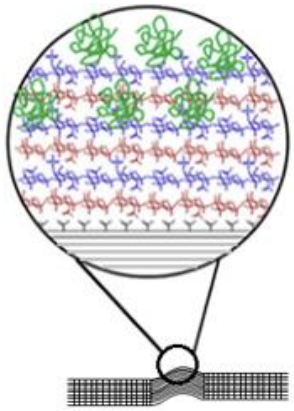

Figure 2: **IL-4 loaded LARS artificial ligament.** A zoomed-in view of the LARS ligament shows the LbL coating in blue and red and the IL-4 cytokines in green. Modified from [12].

The resolution of inflammation is critical to the success of an implant as persistent inflammation can lead to the formation of foreign body giant cells which negatively impact biomaterial longevity. The concept of IL-4 loaded coatings is not novel, however this approach has not yet been investigated for artificial ligaments, which contributes to the innovation of the solution presented in Figure 2. **Unlike previous coatings, the IL-4 loaded coatings on the LARS ligament will directly mitigate the host inflammatory response by promoting M2 macrophage polarization, which should foster healing and improve biomaterial integration into the host tissue [12].**

The role of macrophages in determining the success of an implant has only recently received considerable attention [12]. Whilst the initial lack of data may have impacted the development of immunomodulatory solutions, newfound popularity in immunoengineering approaches has caused a surge in related research. The corresponding appeal of immunoengineering strategies has rendered the development of macrophage-modulating biomaterials a feasible technique to pursue at this time.

Approach

Feasibility

The modulation of macrophage phenotype using IL-4 has been demonstrated by several studies [12], [15]–[19]. *In vitro*, M2 macrophages can be induced via IL-4 at concentrations as small as 0.5 ng/mL (Figure 3).

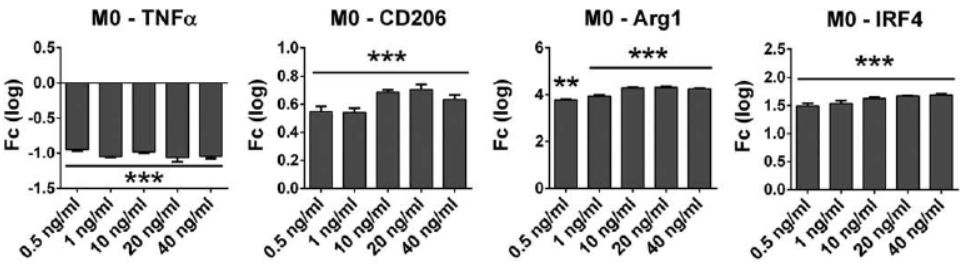

Figure 3: **Low concentrations of IL-4 induce an M2-like phenotype.** Exposing macrophages to 0.5 ng/mL of IL-4 *in vitro* caused significant downregulation of TNF-α, which is a pro-inflammatory cytokine produced by M1 macrophages. Exposure to IL-4 also caused an upregulation of the following M2 macrophage markers: CD206, Arg1, and IRF4. Modified from [15].

Specific Aims

The development of the proposed IL-4 loaded LARS ligament will be accomplished through the following Specific Aims:

- **Specific Aim 1**: **Investigate techniques for introducing a negative charge onto the surface of LARS ligaments.** A negatively charged PET surface is essential to enable subsequent LbL assembly of oppositely charged polyelectrolytes which are incorporated with IL-4.

  Sub Aim 1a: Assess the viability of maleic anhydride plasma treatment in producing a negatively charged PET surface. This technique has been successfully used to generate carboxyl groups on the surfaces of poly(tetrafluoroethylene) and polypropylene substrates [12], [20].

  Experiment: The PET ligament will be placed into a microwave reactor chamber along with maleic anhydride powder. The chamber will be purged with argon gas to reach a steady state pressure of 250 mTorr, after which microwave radiation will be initiated to induce plasma formation. At this point, the maleic anhydride is covalently attached to the PET and will be hydrolyzed to generate carboxyl groups by rinsing and subsequently boiling the artificial ligament in distilled water [20].

  Sub Aim 1b: **Assess the viability of low-pressure air plasma treatment in oxygenating a PET surface and thereby producing a negative charge** [21]**.** This plasma treatment approach has previously been used to produce hydrophilic groups on the surface of PET films [22].

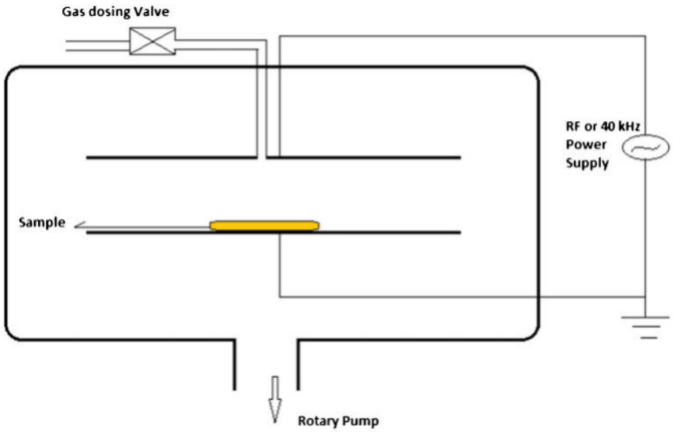

Figure 4: Schematic of low-pressure air plasma treatment. This approach involves the use of two parallel electrode plates situated in a low-pressure chamber. A rotary pump is used to obtain the necessary low pressure. The sample is placed on the electrode maintained at ground potential and dry air is used as the plasma forming gas. The original figure can be found in [23].

Experiment: The experimental set-up for low-pressure air plasma treatment consists of a cylindrical stainless steel chamber that contains two parallel electrode plates (Figure 4). A rotary pump will be used to obtain the necessary low pressure in the chamber. The PET artificial ligament will be placed on the electrode maintained at ground potential and dry air will be used as the plasma forming gas [23].

Benchmarks for Success: Contact angle measurements will be done to analyze the hydrophilicity of the PET surface. A contact angle of less than 90° indicates a hydrophilic, and therefore charged, surface. Additionally, analysis of the PET ligaments by X-ray photoelectron spectroscopy (XPS) should show a decreased C to O atomic ratio after plasma treatment which would suggest the formation of C-O and C=O bonds present in hydrophilic carboxyl groups [22].

- **Specific Aim 2**: **Investigate the characteristics and performance of the IL-4 loaded LbL coating.** A polyelectrolyte coating can be deposited on the negatively charged LARS ligament using the LbL procedure (Figure 5). Correct loading of the IL-4 within the coating is necessary to ensure a stable release over time. Additionally, an appropriate number of layers needs to be determined to ensure the effective polarization of macrophages.

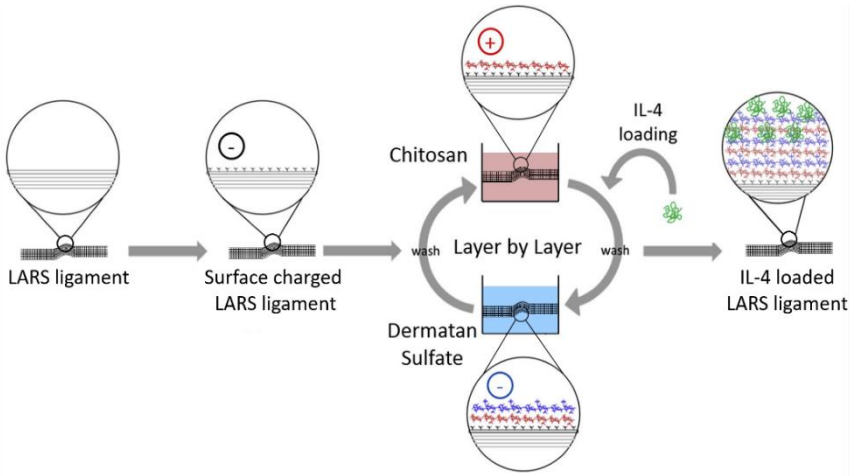

Figure 5: The creation of an IL-4 loaded ligament involves several steps. After a negative charge is established, the LbL approach is used to deposit oppositely charged polyelectrolyte solutions to produce a coating that will incorporate IL-4. Chitosan and dermatan sulfate have previously been used in an LbL approach to coat polypropylene meshes [12]. Once substances are dissolved to generate polyelectrolyte solutions, the PET ligament will be dipped in chitosan, washed with deionized water, and air dried. A similar process will be used to deposit a dermatan sulfate layer. After a 10-bilayer coating is created, an IL-4 (1.5 µg/mL) – dermatan sulfate (2 mg/mL) mixture will be made to incorporate IL-4 [12]. Modified from [12].

Sub Aim 2a: Verify that IL-4 is loaded and evenly distributed throughout the coating using immunolabeling and confocal microscopy.

Experiment: In order to block non-specific adsorption of antibodies, IL-4 loaded, coated (no IL-4), and pristine LARS ligaments will be immersed in a bovine serum albumin solution. Next, ligaments will be placed in an anti-IL-4 primary antibody solution, followed by a fluorescently tagged secondary antibody solution. Confocal microscopy will then be used to visualize the autofluorescence generated by the PET ligament, as well as the fluorescence due to the loaded IL-4 [12].

Sub Aim 2b: Determine the number of layers necessary to enable controlled release of IL-4 only at early stages of the host response.

Experiment: ELISA assays can be used to quantify IL-4 release over time to help determine the number of layers necessary to enable controlled release of IL-4 only at early stages of the host response (up to 14 days). A previous study showed that a 40-bilayer coating containing IL-4 was capable of releasing the cytokine during this timeframe [12]. This study proposes to test 30, 35, 40, and 45-bilayer coatings containing IL-4 to (1) verify the results of the previous study and (2) optimize the release profile of the coated ligament. The ligament with the coating that releases IL-4 up to 14 days of the host response will be selected for use in further studies.

Benchmarks for Success: The coating should release the majority of IL-4 (approximately 90%) at early stages of the immune response (up to 14 days) as this was found to improve resolution of the foreign body reaction and decrease fibrotic capsule formation *in vivo* as demonstrated by a former study [12].

- **Specific Aim 3**: **Test the performance of IL-4 loaded LARS ligaments *in vitro* and *in vivo*.** *In vitro* testing is required for preliminary assessment of the IL-4 loaded LARS ligaments in a cost- and time-efficient manner whereas *in vivo* studies are necessary to more accurately assess the safety and efficacy of the IL-4 loaded ligaments.

Sub Aim 3a: Verify M2 macrophage polarization *in vitro* in response to the IL-4 loaded LbL coating.

Experiment: Mouse bone marrow-derived macrophages will be exposed to a sample of pristine, coated (no IL-4), and IL-4 loaded LARS ligament for 72 hours. The macrophages will be immunolabeled against Arg1, an M2 macrophage specific marker, to determine the effectiveness of IL-4 in polarizing macrophages towards the M2 phenotype. The number of macrophages positive for Arg1 can be determined using the CellProfiler software [12].

Sub Aim 3b: Utilize a rabbit implantation model to test the ability of the IL-4 loaded LARS ligament to promote an early-stage M2 macrophage polarization *in vivo*.

Experiment: Native ACL from rabbits will be replaced with pristine, coated (no IL-4), and IL-4 coated LARS segments. The LARS segments and surrounding tissue will be harvested at 7 and 14 days post-implantation and sectioned. The sections will be immunolabelled against Arg1 (M2 marker) and iNOS (M1 marker) primary antibodies followed by fluorescent secondary antibodies which can be visualized using fluorescence microscopy to assess macrophage number, location, and phenotype. The number of M1 and M2 macrophages can be quantified using the ImageJ analysis program [12].

Sub Aim 3c: Utilize a rabbit implantation model to examine fibrous encapsulation of the IL-4 loaded LARS ligaments.

Experiment: A rabbit implantation model will also be used to examine fibrous encapsulation of the IL-4 loaded LARS ligaments. Native ACL from rabbits will be replaced with pristine, coated (no IL-4), and IL-4 coated LARS segments which will be harvested 90 days post-implantation. After sectioning the ligaments, Masson's trichrome staining protocol will be performed. The stained sections will be examined using fluorescence microscopy to visualize the degree of fibrous capsule formation and images can be analyzed using ImageJ to quantify capsule area and mean thickness [12].

Benchmarks for Success: A successful IL-4 loaded LARS ligament will elicit a significant increase in M2 macrophages and a corresponding decrease in capsule area and thickness compared to both pristine and coated (no IL-4) segments.

Potential Problems and Alternative Approaches

The primary challenge of this study is ensuring that IL-4 is released only at early stages of the host response. A previous study that utilized the described LbL procedure to incorporate IL-4 onto polypropylene meshes was able to achieve a sustained release of IL-4 over a period of 14 days. It is well-known that fibrous encapsulation of a biomaterial occurs after a period of 2-4 weeks and that prolonged IL-4 release may contribute to fibrosis [9], [19]. Thus, it is critical that the majority of the IL-4 is released within 14 days to reduce the extent of fibrous encapsulation. In the same study, a 1.5 µg/mL IL-4 mixture was coated onto the surface of polypropylene meshes to create layers containing IL-4. Meshes coated with 40 bilayers containing IL-4 at this concentration were able to release 2.25 ng/mL of IL-4 after 72 hours and achieve significant M2 macrophage polarization *in vitro*. It has been shown that M2 macrophages can be induced via IL-4 at concentrations as small as 0.5 ng/mL, so optimizing the dose of IL-4 should be investigated to reduce the potential for adverse effects [15].

Biomaterials tend to elicit a persistent M1 macrophage response, which if unresolved, leads to a severe foreign body reaction that negatively affects biomaterial integration [9], [11]. Thus, it is thought that early-stage M2 macrophage polarization will reduce the extent of the foreign body reaction by promoting implant integration and healing. However, it is important to note that M1 macrophages play an important role in cleaning the wound site and promoting an environment that is amenable to the healing process. Thus, a better healing response may be achieved by varying the concentration of IL-4 or by delaying its release to enable M1 polarization followed by M2 polarization.

Project Timeline

A project timeline is presented in Figure 6 below. It is expected that the project will take approximately 3 years to complete.

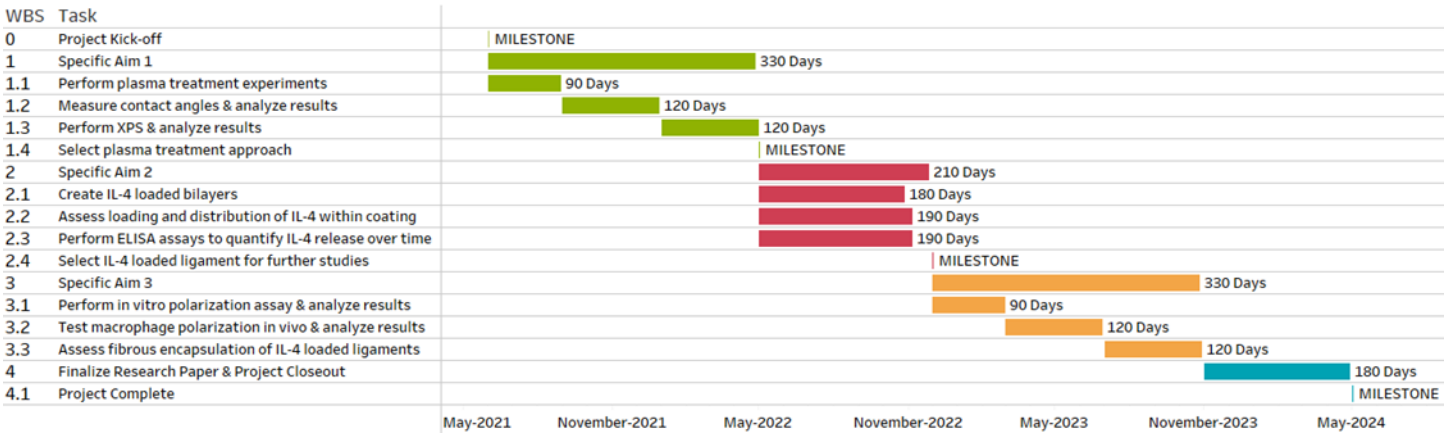

Figure 6: Work breakdown structure (WBS) and project timeline. It is expected that it will take almost 2.5 years to complete the Specific Aims, which are each shown in a different colour. Approximately 6 months is allocated to finalizing the research paper and closing out the project.

Conclusion

The ACL repair market size is expected to grow exponentially due to an increase in sports-related knee injuries and demand for minimally invasive procedures [5]. The majority of ACL reconstructions are done using tendon autografts, however this treatment has been associated with donor site morbidity and delayed return to sports, among other complications [1], [7]. The use of synthetic materials for ACL reconstruction is a promising treatment approach, but so far no artificial ligament has been demonstrated to be a viable alternative to autografts [1]. This is in part due to the development of a foreign body reaction which restricts artificial ligaments from widespread use [8].

This project seeks to test the efficacy of promoting the M2 macrophage phenotype in an effort to mitigate the foreign body reaction associated with LARS artificial ligaments. A LbL technique will be used to coat ligaments in alternating polyelectrolyte layers that enable sustained release of IL-4. The IL-4 will directly mitigate the host inflammatory response by promoting M2 macrophage polarization, which should foster healing and improve biomaterial integration into the host tissue [12]. The development of the proposed IL-4 loaded LARS ligament will be accomplished through three Specific Aims and will take approximately 3 years to complete. If the artificial ligament is successful in reducing the foreign body reaction, the next step would be to optimize the concentration of IL-4 within the coating to reduce the potential for adverse effects.

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