PHENOTYPE AND FUNCTION OF PROINFLAMMATORY T HELPER 17 CELLS AND IMMUNOSUPPRESSIVE REGULATORY T CELLS IN THE PATHOBIOLOGY OF EARLY OSTEOARTHRITIS

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Laura Elizabeth Keller

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PHENOTYPE AND FUNCTION OF PROINFLAMMATORY T HELPER 17 CELLS AND IMMUNOSUPPRESSIVE REGULATORY T CELLS IN THE PATHOBIOLOGY OF EARLY OSTEOARTHRITIS

Laura Elizabeth Keller, Ph.D.

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OA is the most common cause of disability in the United States, and evidence over the last several decades suggests that OA is not simply a "wear and tear" disease of articular cartilage. The majority of OA patients present with low-grade inflammation wherein inflammatory cytokines and chemokines released from cartilage and synovium home macrophages and $CD4^+$ T cells to the joint in chronic OA, with no information available for early OA. The overall goal of this project was to investigate the opposing roles of immunosuppressive Regulatory T (Treg) cells and proinflammatory T Helper 17 (Th17) cells in early osteoarthritis (OA), and to identify potential timing and novel targets for immunotherapeutic intervention.

It has previously been demonstrated that Tregs are enriched within the synovial membrane of patients with chronic OA, but their anti-inflammatory functions are suppressed, and Tregs are not able to restore joint homeostasis. The purpose of the first study was to determine if an enriched population of Tregs actively secreting anti-inflammatory IL-10 would be able to resolve inflammation induced by IL-1β in an equine in vitro, co-culture model of OA. Results of this study determined that Tregs alone were unable to restore metabolic and catabolic imbalance but were able to do so in the presence of anti-IL-6 neutralizing antibody, and that it was significantly influence by the chondrocyte/synoviocyte and Treg donors. The results of this study demonstrate that IL-6 is a promising therapeutic target in early OA, that a personalized medicine approach will need to be taken anti-IL-6 immunotherapy.

The purpose of the second study was to determine a timeline for infiltration of Tregs and Th17 cells into the synovial fluid during progression of posttraumatic OA (PTOA) using flow cytometry. This study utilized synovial fluid samples from horses with varying severity of naturally occurring PTOA and with healthy joints. The use of the horse was pivotal for this study, because control samples are typically not available from humans, and small animal models do not provide sufficient volumes of synovial fluid for analysis. The results of this study demonstrated, for the first time, plasticity of Tregs to a Th17-like Treg phenotype, with secretion of Th17 cytokine, within the joint during moderate PTOA, and that Tregs and Th17 cells are already present in the synovial fluid of healthy joints. Furthermore, Th17 cells were more populous in more severe disease compared to Tregs. This knowledge is critical to determine timing and targets of existing and approved immunotherapeutics that could be translated for the treatment of OA. These results warrant further studies to determine the driving factors for Treg phenotype plasticity and Th17 cell homing to and maintenance within the joint.

The purpose of the third study was to identify pathways and proteins associated with persistent inflammation in early PTOA as potential therapeutic targets in the prevention of PTOA. Synovial fluid samples were collected from human patients at 1-week and 4-weeks post anterior cruciate ligament (ACL) reconstruction surgery and patients were stratified into two groups based on IL-6 concentration in synovial fluid at 4-weeks post-ACL reconstruction as those who were non-responders (>316 pg/mL IL-6) to surgery and those who were responders (<316 pg/mL IL-6). Samples were analyzed using liquid chromatography-mass spectrophotometry. The results of this study indicated that those who are non-responders to ACL reconstruction have a high abundance of proteins related to the complement pathway and release of primary, azurophilic granules by neutrophils into synovial fluid. This demonstrates that non-responders to ACL reconstruction can be identified by IL-6 concentrations in synovial fluid and reveals novel targets for immunotherapeutic intervention in the prevention of PTOA following ACL reconstruction.

BIOGRAPHICAL SKETCH

Laura Elizabeth Keller was raised in Pipersville, Pennsylvania and graduated from high school in the Palisades School District in 2011. Laura attended The Pennsylvania State University College of Agricultural Sciences and received her Bachelor of Science degree in Animal Science (Science option) in 2015. While at Penn State, Laura joint the Penn State Equine Research Team to gain more experience in animal-focused research and worked in the equine nutrition lab of Dr. Burt Staniar. Starting in August 2016, Laura spent a year working as a lab technician for Dr. Bettina Wagner at Cornell University's College of Veterinary Medicine, where she was a part of the Equine Herpesvirus-1 research taking place within the lab. Laura then stayed at Cornell to pursue her Ph.D. in the Biological and Biomedical Sciences Graduate Program, with a major concentration in Immunology and Infectious Disease, and a minor concentration in Translational Medicine.

This thesis is dedicated to my parents,

Lorraine Pierce Holowach Keller
&
Jeffrey Karl Keller

for providing me with inspiration and guidance, and unwavering love and support.

And to my best friend and love of my life, Richard Paul Bardell.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

ADAMTS	A disintegrin and metalloproteinase with thrombospondin motifs
ACL	Anterior cruciate ligament
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CCL	C-C motif chemokine ligand
ConA	Concanavalin A
CTL	Cytotoxic T lymphocyte
CXCL	C-X-C motif chemokine ligand
DMEM	Dulbecco's modified eagle's medium
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IL	Interleukin
LC-MS/MS	Liquid chromatography-tandem mass spectrophotometry
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MMP	Matrix metalloproteinase
NGAL	Neutrophil gelatinase-associated lipocalin
OA	Osteoarthritis
OARSI	Osteoarthritis Research Society International
PBL	Peripheral blood lymphocyte
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
POTE E	POTE ankyrin domain family member E
PsA	Psoriatic arthritis

PTOA	Posttraumatic osteoarthritis
RA	Rheumatoid arthritis
RPMI	Roswell Park Memorial Institute medium
Th	T helper cell
Treg	Regulatory T cell
WOMAC	Western Ontario and McMaster Universities

CHAPTER 1

GENERAL INTRODUCTION[1]

Goals of the dissertation research and thesis overview

The overall goal of this thesis project was to investigate the role of Regulatory T (Treg) cells and T Helper 17 (Th17) cells as therapeutic targets for disease-modifying immunotherapy in the pathogenesis of early osteoarthritis (OA). This introductory chapter provides relevant background related to phenotype and function of T cell populations that infiltrate the joint during development of OA, and T cell-targeted immunotherapies that offer novel intervention options to mitigate OA.

The first aim of this dissertation research was to develop an equine *in vitro* culture system to determine how Tregs affect joint homeostasis and the reciprocal effects on Treg plasticity. Chapter 2 describes the development of a novel tri-culture system of chondrocytes, synoviocytes, and Tregs to reveal why Tregs are ineffective at offsetting inflammation in acute OA. We found that Tregs secrete chondroprotective IL-10 and IL-4 cytokines into conditioned media, and increase *TIMP1* gene expression in chondrocytes and synoviocytes, but do not mitigate the catabolic effects of IL-1β and IL-6. Chapter 3 expands upon this work, in which the aim was to determine if the neutralization of IL-6 would enhance Treg function in mitigating OA progression. We found that αIL-6 antibody and Tregs in combination decreased *MMP13* gene expression and restored *Acan* gene expression in chondrocytes, and that restoration of joint homeostasis was more successful by Tregs in combination with αIL-6 antibody. The significance of this work is that it describes a mechanism by which Tregs fail to restore joint homeostasis and provides a target for immunotherapeutics in the mitigation of OA. The second aim of this thesis, presented in Chapter 4, was to define a timeline for infiltration of Treg and Th17 cells into synovial fluid during posttraumatic OA (PTOA) progression. We used naturally occurring PTOA in equine patients undergoing arthroscopic surgery for PTOA. The goal of this aim was to use flow cytometry and multiplex

[1] This chapter has been adapted from: Keller LE, Fortier LA, Tait Wojno ED. T Cells in Early Osteoarthritis. In: Lattermann C, Madry H, Nakamura N, Kon E, eds. *Early Osteoarthritis.* Springer; 2021.

ELISA to establish a baseline and profiles for T cell phenotype and function during the initiation and progression of PTOA to assess the roles of Treg and Th17 cells in PTOA. This information would provide timing and targets for development and application of immunotherapeutics to mitigate disease progression. We found that Treg populations were in low abundance in healthy synovial fluid, and populations did not change during disease progression, but that both Th17 cells and Th17-like Tregs were increased in moderate PTOA, contributing to joint catabolism and inflammation. These findings indicate that cytokines and chemokines that contribute to the homing and maintenance of Th17 cells and phenotype instability of Tregs are targets for the prevention of PTOA progression.

The third aim of this thesis, presented in Chapter 5, was to identify pathways and proteins associated with persistent inflammation in early PTOA as potential therapeutic targets in the prevention of PTOA. Synovial fluid samples from human patients 1-week and 4-weeks post-anterior cruciate ligament (ACL) reconstruction surgery were analyzed using an unbiased, bottom-up proteomics approach. We found that proteins related to the complement system, neutrophil degranulation, and cartilage catabolism had a higher abundance ratio in synovial fluid from patients with high IL-6 concentrations at 4-weeks postop, and presumed persistent inflammation, providing both novel targets for the prevention of PTOA, and targets for the rapid translation of therapies already approved by the FDA for use in other diseases.

The final chapter of this thesis presents a discussion of the significance of our findings in the context of the current state of OA research and therapy. Future research directions are explored.

Background and Significance

Historically, OA was considered a wear and tear disease initiated and propagated by biomechanical processes resulting in degeneration of articular cartilage. However, there is also strong evidence demonstrating involvement of inflammation and the immune system in the pathogenesis of OA throughout the disease process. Histological studies reveal that over 50% of patients with OA have a mononuclear cellular infiltrate in the synovial fluid and synovium that consists of lymphocytes, monocytes, and dendritic cells derived from peripheral blood.[1,2] Acutely following joint injury, pro-

inflammatory chemokines and cytokines are released from the cartilage and the synovium and attract a variety of immune cells to the joint, the majority of which are macrophages and T lymphocytes.[3] Through the release of cytokines and chemokines and cell-to-cell interactions, T cells modify the inflammatory joint environment and influence the progression of disease.

This chapter will focus on the role of T cells in *early* OA, because only in the early phases of OA can true disease intervention and disease prevention occur. Herein, we will lay the foundation for how cytokines and chemokines released from chondrocytes and synoviocytes home T cells to the inflamed joint acutely following injury, and how the specific T cell subtypes can influence disease progression. We will discuss T cell behavior within the synovium, including activation and proliferation in antigen-dependent or independent manners, and why these events in early OA are critical for sustained T cell responses within the joint. We will explore the biology of different T cell subsets within the joint that can act to mitigate or propagate disease progression dependent upon their phenotype, and how the cytokine environment of the joint can reciprocally polarize T cell phenotype, potentially exacerbating the T cell inflammatory response. Finally, we will discuss how further exploration of the interplay between T cells and joint dysfunction will inform the development and utilization of targeted immunotherapies early in disease to mitigate OA. Throughout this chapter, we will convey the need to further explore how T cell functions within the joint during early OA influence disease progression and can potentially be manipulated to mitigate OA to prevent joint destruction.

Orchestration of the T Cell Response in OA

T cell infiltration into an inflamed joint is emerging as a hallmark of OA. This infiltration is considered abnormal because there are very few tissue resident T cells within the synovium or synovial fluid of a healthy joint.[4] While a small population of T cells may reside in a healthy joint and play a role in maintaining joint homeostasis, an inflammatory event is needed to initiate infiltration of pathogenic T cells into the joint. T cell activation can occur in both an antigen-dependent and -independent manner.

The presence of mono- and oligo-clonal populations of T cells within the synovium of OA patients points to an antigen-specific proliferation of T cells within the joint itself.[2] Additionally, T cells from the peripheral blood of some patients with OA activate and proliferate in response to chondrocyte and synoviocyte membrane antigens, suggesting that self-specific T cells exist in the circulation of OA patients as well.[5] Moreover, T cells from patients with OA have been found to recognize specific amino acid sequences from aggrecan, which is a major constituent of normal articular cartilage, but can also function as an auto-antigen within the joint.[6] Taken together, these data suggest that OA is characterized by aberrant systemic and local joint T cells that are driven by joint-derived antigens.

T cells are part of the adaptive immune system. They are derived from hematopoietic stem cells in the bone marrow that differentiate into lymphoid progenitor cells which migrate to the thymus and commit to the T lymphocytes lineage. During development, diverse T cell receptors are generated through germline DNA rearrangement. These T cell receptors can recognize virtually any antigen. The process of negative selection largely deletes T cells that strongly recognize self-antigen,[7] but this process is not perfect, and some self-reactive T cells can develop.[8] While still in the thymus, T cells either mature into $CD4^+$ helper T (Th) cells, which are the predominant cell type in an OA joint, or $CD8^+$ cytotoxic T cells. These mature T cells that are still naïve to antigen then leave the thymus and travel to secondary lymphoid tissues where they can be activated by an antigen-presenting cell, typically a dendritic cell.[9]

Three signals are required for the activation and proliferation of naïve T cells. First is the signal received when a T cell receptor recognizes its cognate antigen presented by an antigen presenting cell in the context of major histocompatibility complex (MHC). Second is co-stimulatory signaling in which a co-stimulatory molecule on the T cell, like CD28, binds a member of the B7 receptor family on the antigen presenting cell. Finally, the T cell must also encounter IL-2 for proliferation, and other cytokines that support activation and polarization. This three-step process creates a significant barrier for inappropriate T cell activation to occur, thus preventing the proliferation of T cells that might otherwise recognize auto-antigens. Moreover, if a T cell binds a specific antigen alone without receiving a co-stimulatory signal, it will become anergic and unable to respond to antigen in the future. Interestingly, T

cells co-cultured with fibroblast-like synoviocytes that are able to present antigen-loaded MHC II adopt an anergic phenotype. This suggests that, although fibroblast-like synoviocytes are capable of presenting antigen to T cells, they are unable to activate naïve T cells because they lack co-stimulatory molecules.[10]

Initial T cell priming in OA is likely to occur in a lymph node local to the joint. In this scenario, dendritic cells in the joint carry antigen to the lymph node, or alternatively, dendritic cells in tissues proximal to the damaged joint pick up antigens that have drained out of the damaged joint and then migrate to the lymph node.[11] However, during ongoing disease, there may be other modes of antigen presentation and persistent T cell activation. Lymphoid nodular aggregates and lymphoid follicles containing macrophages, T cells and B cells can be found in the synovium of patients in all stages of OA.[12] There is evidence in RA that auto-antigens are presented to T cells by antigen presenting cells within the synovium.[13] A rabbit medial meniscectomy model suggests that this may also be true for OA. At weeks 2 and 4 post-meniscectomy, large numbers of mature dendritic cells were present in lymphoid aggregates within the synovium.[14] A recent study in a mouse model of load-induced arthritis found that the total number of T cells in the inguinal lymph node were significantly increased within 1-2 weeks of loading.[15] This suggests that while initial T cell activation by dendritic cells likely occurs in local lymph nodes, it may then be perpetuated in the synovium.[11]

Cytokines in the microenvironment during priming determine the fate of $CD4^+$ T cells and polarize them to one of a number of functional subsets or fates.[16] These $CD4^+$ T cell fates include Th1, Th2, Th17 and Regulatory (Treg)(**Figure 1.1**). Th1 cells develop in response to IFN-γ and IL-12, which cause downstream activation of the T-bet transcription factor and induce Th1 cells to secrete IFN-γ and TNF-α. They activate phagocytic cells and are involved in the elimination of intracellular pathogens. IL-4 activates the transcription factor GATA3 and directs naïve cells to a Th2 fate. Th2 cells coordinate the immune response towards extracellular pathogens including helminths and predominantly secrete IL-4, IL-5, and IL-13. Th17 cells are responsible for immunity against extracellular bacteria and fungi through

secretion of IL-17A, IL-21, and IL-22. IL-1β, IL-6 and IL-23 activate the RORγt transcription factor, driving the emergence of the Th17 phenotype. Treg cells develop in one of two ways. Natural Tregs develop in the thymus and Induced Tregs develop in the periphery under the influence of TGF-β and IL-2. Treg development and function is directed by the transcription factor Foxp3 that supports downstream secretion of IL-10 and TGF-β. Tregs are critical for tolerance to self- and foreign-antigen and resolution of inflammation.[17] Activation by antigen recognition initiates T cell proliferation and differentiation prior to homing to sites of inflammation, such as an OA joint, where they carry out their effector functions.

As T cells are activated and adopt specific fates (see **Figure 1.1**)[17], these subsets also express specific chemokine receptors that mediate the ability of T cells to respond to chemokines that direct immune cell migration to and within tissues. In the context of OA, chemokines produced from inflamed cartilage and synovium promote T cell homing to the joint. In addition, the vasculature of the inflamed synovium becomes highly positive for E-selectin, which promotes extravasation of immune cells from the peripheral blood into the joint.[2]

Recent studies have revealed that specific chemokines are key mediators responsible for immune cell homing in early OA, including CCL5, CCL17, CCL20, and CXCL12.[18,19] CCL5 is a potent T cell chemoattractant which binds to CCR1, CCR3, and CCR5, all of which can be expressed by T cells.[20–22] CCL5 knockout mice are partially protected from cartilage injury as a result destabilization of the medical meniscus-induced OA compared to wildtype mice.[23] CCL17 induces chemotaxis in T cells through interactions with CCR4, which is expressed only on specific $CD4^+$ T cell subtypes, including Th17 and Tregs.[24] CCL17 blockade in mice with collagenase-induced arthritis resulted in reduced pain and OA.[25] Synoviocytes from OA patients secrete CCL20, which is strongly chemotactic for lymphocytes and binds to CCR6.[26] CXCL12 is another potent chemokine for lymphocytes that is closely associated with radiographic severity of OA.[27] Additionally, CXCL12 can enhance the effects of certain pro-inflammatory cytokines, including IL-17A, on fibroblast-like synoviocytes.[28]

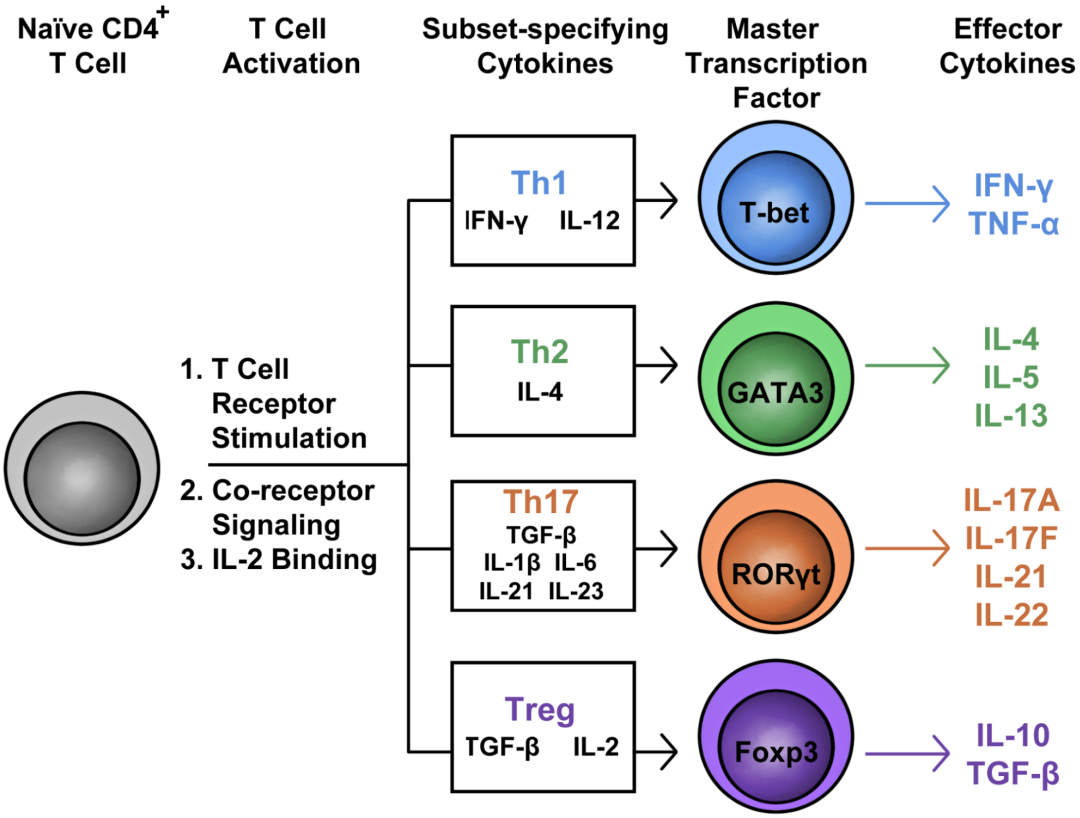

Figure 1.1 CD4$^+$ T cell fates are determined by cytokine environment during differentiation. Naïve T cells must receive three signals in order to activate and proliferate. This includes binding of the T cell receptor to the appropriate MHC class and cognate antigen for activation, co-receptor signaling to increase survival signal to the T cell, and finally T cells must bind IL-2 in order to proliferate. Subset-specifying cytokines released by mature antigen presenting cells activate subset-specific master transcription factors, determining T cell fate and effector functions.

Because different T cell subsets exhibit specific receptors, the cytokines released by cartilage and synovium during early OA will affect which subtypes are homed to the joint, subsequently playing a role in disease pathogenesis.

The aforementioned chemokines and cytokines orchestrate priming and homing T cells to the joint where they elicit their effector functions through several mechanisms including secretion of cytokines and cell-to-cell interactions (**Figure 1.2**). T cells can mediate the progression of OA by affecting both stromal and immune cells within the joint. These effector functions of T cells within the inflamed, early OA joint could be targeted therapeutically on a patient-to-patient basis to interrupt the course of disease before irreversible joint damage has occurred.

$CD8^+$ Cytotoxic T Cells (CTL)

Cytotoxic T lymphocytes (CTL) express the CD8 co-receptor and perform cell-mediated immunity. CTLs kill harmful cells, including cancer cells and cells carrying intracellular pathogens. CTLs recognizing self- and non-self-antigens presented by MHC class I, which is found on all nucleated cells. CTLs carry out their effector functions through two main actions. First is release of anti-viral and anti-tumor cytokines, primarily IFN-γ and TNF-α. Second is by directly killing cells, either through release of cytotoxic granules or by Fas/FasL interactions.[29] In the synovium of OA joints, $CD8^+$ T cells are present, but at significantly lower numbers than $CD4^+$ T cells.[30]

Interestingly, in OA, there is an increase in the $CD4^+$:$CD8^+$ ratio, and a decrease in the total number of CTLs in the patients' peripheral blood.[30] In a mouse model of anterior cruciate ligament transection, $CD8^+$ T cells infiltrated synovial fluid of afflicted joints within 30 days and persisted for 90 days. Additionally, $CD8^+$ T cells expressed TIMP1, a regulator of matrix metalloproteinases and disintegrin-metalloproteinases, which helps to maintain extracellular matrix composition, and the number

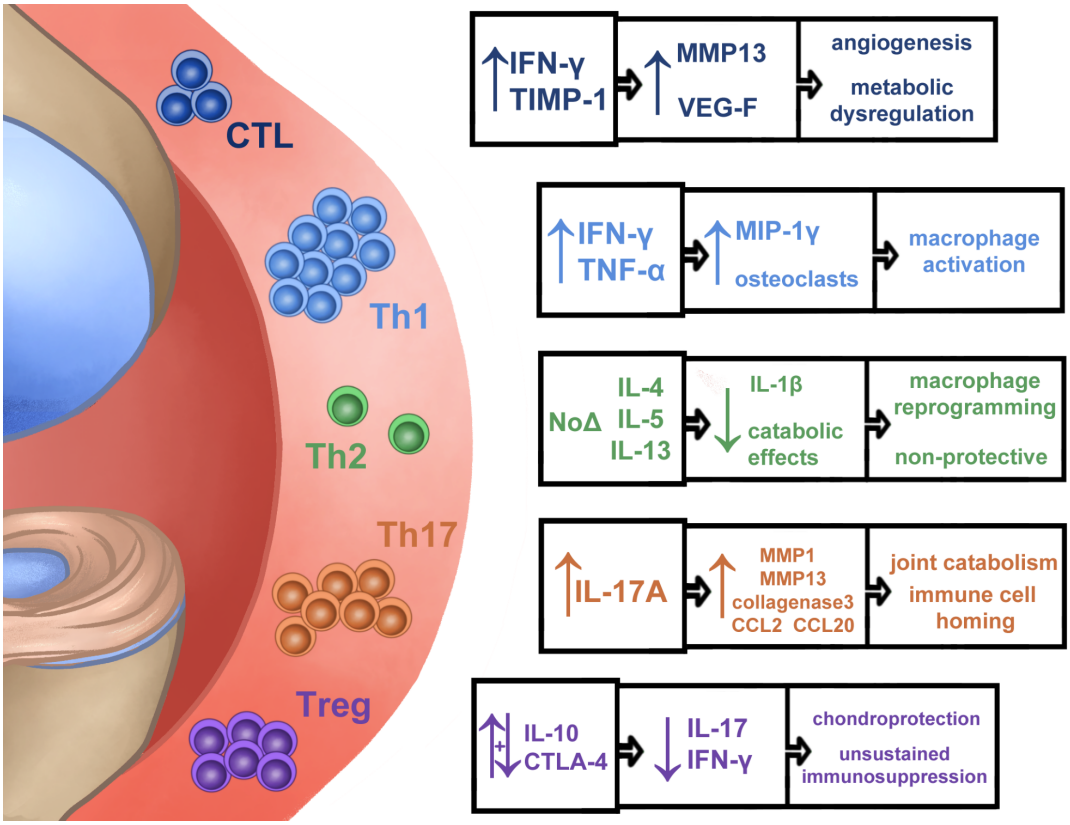

Figure 1.2. T cells alter joint homeostasis in a subset-specific manner. T cells are homed to the joint by inflammatory cytokines released by joint tissues, where they then carry out their effector functions and contribute to loss of joint homeostasis. Cytotoxic T lymphocytes are not abundant in the joint but contribute to increased vascularization and matrix degradation. Th1 cells are the most abundant T cell subtype within the joint but appear to carry out their effector functions mainly through macrophage polarization and activation. Th2 cells are found sporadically and sparsely within the OA joint and do not appear to offer protection against cartilage breakdown. Th17 cells contribute significantly to matrix degradation as well as synovial inflammation while further contributing to immune cell homing to the joint. Tregs provide early immunosuppression but are unable to restore joint homeostasis and ultimately cannot sustain their effector functions to mitigate OA progression.

of $CD8^+$ T cells expressing TIMP1 correlated positively with disease severity. Moreover, increased TIMP1, VEGF, and MMP-13 in the synovium correlated with $CD8^+$ T cell activation.[31] $CD8^+$ T cells therefore may contribute to imbalance of joint metabolism through dysregulation of both TIMP1 and MMP13, and angiogenesis leading to synovial inflammation.

Aside from these studies, $CD8^+$ T cells in the OA joint have remained somewhat unexplored. In RA, $CD8^+$ T cells are detected in synovium prior to clinical symptoms.[32] Within RA synovial fluid, there is an accumulation of autoreactive $CD8^+$ T cells that are clonally related[33] and are associated with disease severity and breakdown of self-tolerance. Conversely, suppressor $CD8^+$ T cells in the joint may play a role in disease mitigation by inhibiting functions of autoreactive $CD4^+$ T cells.[34] Additional studies into $CD8^+$ T cells will aid in understanding their contribution to OA initiation and progression, and potentially reveal new therapeutic options for OA patients.

T Helper 1 Cells (Th1)

Th1 cells, driven by IL-12 and IFN-γ and controlled by T-bet to produce IFN-γ,[17] are the most abundant T helper cell subset in the synovial fluid and synovium of patients with OA.[4] And although there are fewer Th1 cells in the synovium of patients with OA compared to RA, the Th1 cells present in both types of diseased tissue expressed similar transcript levels of IFN-γ when stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin.[35] Th1 cells in the synovial fluid of patients with OA also secrete higher concentrations of IFN-γ than circulating Th1 cells in the peripheral blood upon PMA and ionomycin stimulation.[36] Within 30 days of anterior cruciate ligament transection in a mouse model of OA, IFN-γ^+ cell numbers increased in the synovium, and subsequently decreased by 90 days post-induction. This was associated with an increase in MIP-1γ and number of osteoclasts, while CD4 knockout mice had lower concentrations of MIP-1γ and slower cartilage degeneration.[37]

Importantly, while Th1 cells are the most abundant $CD4^+$ T cell subtype in the OA joint, they may not be the most inflammatory. *In vitro*, Th17 cells induce synthesis of IL-6, IL-8, MMP-1 and MMP-3 in synovial fibroblasts from patients with early RA, whereas Th1 and naïve $CD4^+$ T cells do not.[38] It has also been reported that in patients with RA, Th1 cells and related cytokines are only significantly increased in peripheral blood in late-stage disease, while Th17 cells and their related cytokines are significantly elevated throughout disease progression.[39]

While the role of Th1 cells to the progression of OA remains unclear, their orchestration of the effector functions of macrophages and monocytes is likely partially responsible for continued inflammation within the joint. However, evidence from RA would suggest that the contribution of Th1 cells to rheumatic disease is more prominent during later disease stages, and that other $CD4^+$ T cell subtypes, including Th17 cells, are more important drivers of pathogenesis early in the disease process.

T Helper 2 Cells (Th2)

Th2 cells that respond to IL-4 and produce IL-4, IL-5, IL-9, and IL-13 under the control of GATA3 are involved in mucosal immunity and the immune responses to extracellular pathogens and tissue repair,[40] but it is currently unclear whether or not Th2 cells are important contributors to the pathogenesis of OA. In the synovium of patients undergoing total knee replacement, neither IL-4 nor IL-5 mRNA transcripts were found in any of the 18 patients.[35] Moreover, synovial fluid cells from OA patients stimulated with PMA and ionomycin did not express levels of IL-4 that were detectable by RT-qPCR after 24 or 72 hours of stimulation.[41] Additional studies have failed to find Th2-related transcripts within the joints of OA patients.[42] However, using flow cytometry, low numbers of $CD4^+$IL-4^+ cells have been found in the synovial fluid of OA patients[36] and appear in similar frequencies within the synovium when compared to RA patients.[43] IL-4^+ cells were also found in all three layers of the synovium using immunohistochemistry, albeit at very low numbers when compared to IFN-γ^+ cells or $CD4^+$ cells in total.[4]

Therefore, Th2 infiltration into the joints of OA patients appears to be sparse and sporadic, but within these patient subsets they may aid in disease mitigation through reprogramming of macrophages towards an anti-inflammatory and reparative phenotype, or by secretion of cytokines that can protect tissues of the joints from pro-inflammatory and catabolic cytokines. Overall, further investigation is required to elucidate the role of Th2 cells in early OA.

T Helper 17 Cells (Th17)

Th17 cells secrete IL-17 family cytokines, IL-22 and GM-CSF in response to IL-1β, IL-6 and IL-23 and under the control of RORγt, and are implicated in a variety of chronic inflammatory and autoimmune diseases, including rheumatoid arthritis (RA) and psoriatic arthritis (PsA).[44] Th17 cells were discovered as a distinct T helper subset in 2005, and so have not been well scrutinized under the lens of OA, but *in vitro* and *in vivo* models of OA and RA indicate that they play a considerable role in OA initiation and progression.[45]

In human patients with OA, IL-17A was significantly increased in synovial fluid compared to undetectable in unmatched, healthy controls.[30] Furthermore, an increase in IL-17A within the joint has been positively correlated with pain and severity of disease in patients with knee OA.[46] In OA patients with inflamed synovium, gene and protein expression of IL-17A and IL-22 were increased in inflamed regions compared to non-inflamed OA synovium, and correlated with release of IL-6 and IL-23.[47] This indicates that, not only are Th17 cells present and active within the joint, but that joint inflammation is associated with their maintenance. Additionally, in early joint trauma (ACL tear), the soluble form of IL-17 receptor A, which transduces IL-17 signaling, was increased 128% in synovial fluid at a mean of 14 days post-injury compared to 6 days post-injury.[48]

The presence of elevated IL-17A concentrations in the joint is thought to contribute directly to joint inflammation, tissue remodeling, and loss of function. *In vitro*, IL-17A treatment of human cartilage

explants or synoviocytes activated NF-κB and led to increased synthesis of MMP-1, MMP-13,[49] and collagenase-3, all of which contribute to matrix loss.[50] Importantly, in a co-culture system of T cells and synovial fibroblasts from early RA patients, Th1 cells did not elicit the same catabolic responses that Th17 cells did, suggesting that Th17, not Th1, responses may be more responsible for joint destruction in OA.[38] In addition, IL-17A enhanced expression of IL-6 and IL-8 from synovial fibroblasts, aiding in the maintenance of the Th17 phenotype and perpetuating immune cell homing to the inflamed joint.[51] Moreover, IL-17A further promoted T cell recruitment by upregulating expression of CCL2 and CCL20 expression in synovial fibroblasts.[52] These data suggest that Th17 cells, particularly through their ability to produce large amounts of IL-17, are important players in the loss of joint homeostasis in OA.

Studies in murine models are consistent with these *in vitro* and *ex vivo* findings in humans. In murine models of collagen-induced arthritis, treatment with anti-IL-17A neutralizing antibody reduced, though did not eliminate, synovitis and cartilage damage.[53] Furthermore, arthritis was considerably diminished in IL-17A-deficient mice compared to wildtype mice. Not only did fewer IL-17A-deficient mice develop arthritis, but those than did had lower arthritis scores.[54] Of note, other immune cell types, such as local synovial macrophages, participate in the orchestration of Th17 responses, where they promote differentiation and maintenance of Th17 cells within the synovium.[55] Mouse models have also shed light on the contribution of other Th17-derived factors, such as IL-22, to arthritic disease. For example, IL-22 mRNA and protein expression were increased during onset of antigen-induced arthritis in mice, while use of an anti-IL-22 antibody and IL-22 deficiency in mice attenuated pain and reduced synovitis, suggesting that targeting of multiple cytokines released by Th17 can reduce arthritis symptoms.[56]

Taken together, data from human OA and RA patients, as well as *in vitro* and *in vivo* models, suggest that Th17 cells and cytokines play a role in the establishment and progression of OA. IL-17A has been shown to have a direct inflammatory role on synoviocytes and chondrocytes by initiating and perpetuating catabolism and homing of additional immune cells to the inflamed joint. It will be critical to

continue investigation of Th17 cells in early OA in order to find targets for which new therapeutics can be made, or for which existing therapeutics can be implemented to mitigate OA progression.

Regulatory T Cells (Tregs)

TGF-β and IL-2 induce activation of the Foxp3 transcription factor in Regulatory T cells, which can occur in the thymus or periphery to give rise, respectively, to natural or induced Tregs. Tregs produce suppressive cytokines such as IL-10 and TGF-β and dampen immune activation through cell-to-cell interactions and by acting as an IL-2 "sink" to prevent IL-2-associated activation of auto-reactive naïve T cells. Tregs are key players in a multitude of autoimmune and inflammatory diseases, with disease emergence and progression often associated with a lack of Tregs at critical sites or a failure of Tregs to control or arrest ongoing T cell activation.[57]

In patients with mild to severe OA, there is an increase in the percentage of cells in peripheral blood that exhibit a Treg phenotype. However, when stimulated with PMA and ionomycin, these cells were significantly inhibited in their ability to secrete IL-10.[58] The inability of T cells to carry out their effector functions can be indicative of overstimulation and subsequent exhaustion. Within the context of OA, this could be a potential consequence of chronic inflammation in the joint. Evidence from RA patients would also suggest that peripheral Tregs have a reduced ability to suppress aberrant activation of effector $CD4^+$ T cells through cell-to-cell interactions. This is due to defects in expression of the immune checkpoint molecule CTLA-4, which competitively binds to B7 family members on antigen presenting cells, blocking effector T cell activation.[59]

Not only are Tregs enriched within the peripheral blood of OA patients, they are enriched within the synovium and synovial fluid. There is evidence that Tregs infiltrate the joint during the acute phase of inflammation and are highly active in this phase. Following acute ACL tear, IL-10 increased in synovial fluid, but waned as early as three months post-injury.[60] Tregs within the synovium of patients with

chronic OA displayed an activated effector memory phenotype compared to peripheral blood Tregs, which displayed a resting central memory phenotype.[61] Moreover, IL-10 transcripts were detected in the synovium of nearly all OA patients.[35] Taken together, these data indicate that Tregs are present during initiation of inflammation, persist in the joint, and may actively attempt to suppress inflammation but are unable to return joint homeostasis.

Animal models support the role of Tregs and IL-10 in chondroprotection. IL-10 knockout mice with collagen-induced arthritis developed more severe arthritis scores than wildtype mice, which was associated with an increase in production of Th1 and Th17 cytokines, and polarization of macrophages towards an M1 phenotype.[62,63] In a rabbit model of OA, intra-articular injection of synoviocytes overexpressing IL-10 through retroviral gene transfer five days post-excision of the medial collateral ligament plus medial meniscectomy improved histological scores compared to controls.[64] While absence of IL-10 leads to more severe arthritis, presence and over-expression of IL-10 does not appear to mitigate disease progression in the long term, suggesting that Treg cytokines alone are not sufficient to resolve inflammation.

The continued progression of OA suggests that Treg secreted factors and Treg cell-to-cell contact-mediated suppressor functions are not sufficient to mitigate disease progression. This is in spite of early Treg migration to and activation within the inflamed joint. Furthermore, evidence suggests that Treg activity is dampened as disease progresses, rendering these cells unable to mount suppressive functions that could help control inflammation in the joint to promote repair, thus contributing to OA pathogenesis and failure of disease mitigation.

Th17:Treg Phenotype Plasticity

$CD4^+$ T helper cell lineages were originally thought to be stable; however plasticity between Th17 and Treg phenotypes have now been described in multiple contexts including uveitis and scleritis, as well as RA.[65] This instability, within the context of normal physiological conditions, aids in overcoming infections, preventing collateral tissue damage, and resolution of inflammation.[66]

However, when plasticity becomes unregulated, it can lead to uncontrolled inflammatory T cell responses. While transcription factors RORγt and Foxp3, respectively, drive Th17 and Treg phenotype and function, the cytokine microenvironment can activate the reciprocal transcription factor, leading to phenotype plasticity (**Figure 1.3**).[67,68] The result is that T cell function is altered through simultaneous activation of both transcription factors, and cells are subsequently able to acquire the capabilities of both subsets; secreting Th17 cytokines while eliciting Treg suppressor functions.[69]
Evidence from diseases specifically affecting the joint, including RA and PsA, suggests this plasticity could be involved in failure of OA resolution as well.

In chronic diseases, persistence of inflammatory cytokines, including IL-1β, IL-6 and IL-23, can lead to destabilization of the Foxp3 transcription factor in Tregs. These cytokines promote expression of RORγt, yet the resultant Th17-like Tregs also maintain Foxp3 expression, though these cells do not always fully maintain the effector functions of true Tregs.[69] Evidence in mice supports a role for synoviocytes during the induction of Treg phenotype plasticity. For example, in a mouse model of collagen-induced arthritis, $Foxp3^+$ T cells secrete IL-17 following incubation with rheumatoid fibroblast-like synoviocytes, indicating that fibroblast-like synoviocytes from the inflamed joint are sufficient to induce conversion of local $Foxp3^+CD4^+$ T cells to $Foxp3^+CD4^+IL\text{-}17A^+$ cells, exacerbating early inflammation.[70]

Increased Th17-like Treg cells can be found in the blood of RA patients and is positively correlated with an increase in Th17 cells in the peripheral blood.[71] A parallel enrichment of Th17 cells in the peripheral blood of OA patients suggests that they also exhibit an increase in peripheral blood Th17-like Tregs.[72] Although these Th17-like Tregs begin to secrete IL-17A, they are still capable of suppressing effector T cell proliferation *ex vivo*. Conversely, Th17-like Tregs within the joint of RA patients do not maintain suppressor functions, and through secretion of IL-17A, likely contribute to disease progression. There is an upregulation of IL-1β and IL-6 in the synovial tissues following injury,

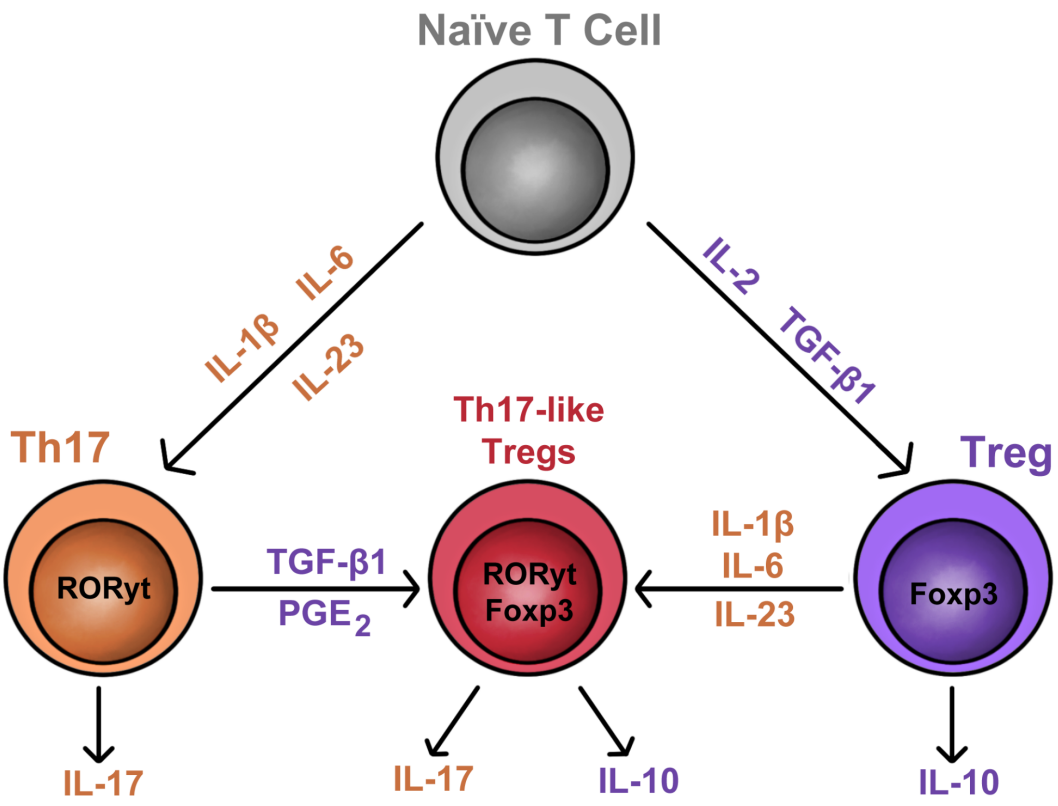

Figure 1.3. Th17:Treg plasticity may contribute to disease pathogenesis. Although T helper cell phenotypes were thought to be terminal and stable following naïve T cell differentiation, there can be plasticity between Th17 cells and Tregs. Cytokines within the OA joint can activate the reciprocal transcription factor, leading to an intermediate cell type that secretes Th17 cytokines and, in some contexts, is also capable of carrying out Treg suppressor functions. Instability in Treg phenotype may play a role in loss of joint homeostasis and continued catabolism.

and an upregulation of IL-23 in the peripheral blood of OA patients, further suggesting that Treg phenotypic switching is involved in the pathogenesis of OA.[73,74]

While pro-inflammatory cytokines can lead to Treg phenotype switching, during the resolution phase of inflammation, anti-inflammatory cytokines can induce Th17 cells to convert to a regulatory phenotype through upregulation of Foxp3. In a mouse model of colitis, during resolution of inflammation, high concentrations of TGF-β1 decreased RORγt activity in a dose-dependent manner and caused Th17 cells to transdifferentiate into IL-17A^{+}Foxp3^{+} cells, which simultaneously secrete IL-17A and IL-10.[75] Furthermore, TGF-β1 and PGE_2 secreted by mouse and human tumor cells induced Foxp3 expression and subsequent suppressor functions in Th17 cells.[76] Retinoic acid, a driver of Foxp3 activation in inducible Tregs, has also been implicated in suppression of Th17 phenotype.[77] However, it is not clear how retinoic acid concentrations vary within the joint, and whether increased retinoic acid contributes more strongly to cartilage destruction or immunomodulation in OA.[78] Regardless, increased concentrations of TGF-β1 and PGE_2 observed in joints of OA patients suggest that there is potential to drive infiltrating Th17 cells to express Foxp3 and limit the pro-inflammatory and pro-catabolic functions of Th17 cells.[18,79]

Observations made of IL-17A^{+}Foxp3^{+} cells and Th17-like Tregs in other diseases suggests that phenotype plasticity between Th17 and Tregs may play a role in OA pathogenesis. Investigating plasticity in Th17 and Treg phenotype will potentially increase our understanding of how T cells respond to the local joint environment in a context-dependent manner, which will be an important step towards developing and applying immunotherapies for early OA.

T Cell-Targeted Immunotherapies for OA

During progression of OA, homeostasis is lost in favor of a catabolic state, where catabolism is defined as progressive and irreversible joint destruction and pain. It is now accepted that disease

modification must occur early before this destruction becomes irreversible.[80,81] Immunotherapy is the use of treatment that targets the immune response, which can include stimulation or suppression, in order to modify disease progression. In the case of autoimmune disease, including RA, immunotherapies that suppress and block aberrant immune function have been successfully implemented for several decades to protect the patient from chronic pain and joint destruction.[82] While some of the key pathways in OA could similarly be targeted using existing immunotherapies, thus far, immunotherapy has not been a mainstay of OA treatment due to inconsistent patient results. For example, use of the anti-TNF-α monoclonal antibody (mAb) therapy adalimumab failed to reduce pain and symptoms in patients with erosive hand OA, but did improve joint stiffness and Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) score, which quantifies pain, stiffness, and physical function of joints, in patients with knee OA.[83] Currently, there are therapies available and in use for other diseases that target several key areas of the T cell response, including T cell homing, T cell activation and maintenance, and T cell effector functions (**Figure 1.4**). A number of these could be leveraged to treat various aspects of the inflammatory response in OA to limit or perhaps even reverse joint destruction. However, understanding more about T cells in the pathogenesis of OA will be important for targeted use of immunotherapies in OA.

As stated previously, acutely following joint damage, chondrocytes and synoviocytes release a cascade of cytokines and chemokines that not only affect the local joint environment, but also home immune cells, including T cells, to the damaged joint. One potential method to reduce T cell-induced inflammation within the joint is to stop T cell trafficking to the joint by either blocking chemokines or their receptors. In RA, treating patients with an antagonist against CCR1, which is expressed by T cells and binds CCL5, reduced the number of $CD4^+$ and $CD8^+$ T cells within the synovium after only 14 days of treatment and significantly reduced the number of tender and swollen joints.[84] Although all patients in the study had firmly established disease, application of such a therapy to early OA may restore homeostasis and promote complete repair of damaged tissue. In a murine model of collagenase-induced OA, treatment with mAb therapy targeting the T cell chemoattractant CCL17 ameliorated pain and

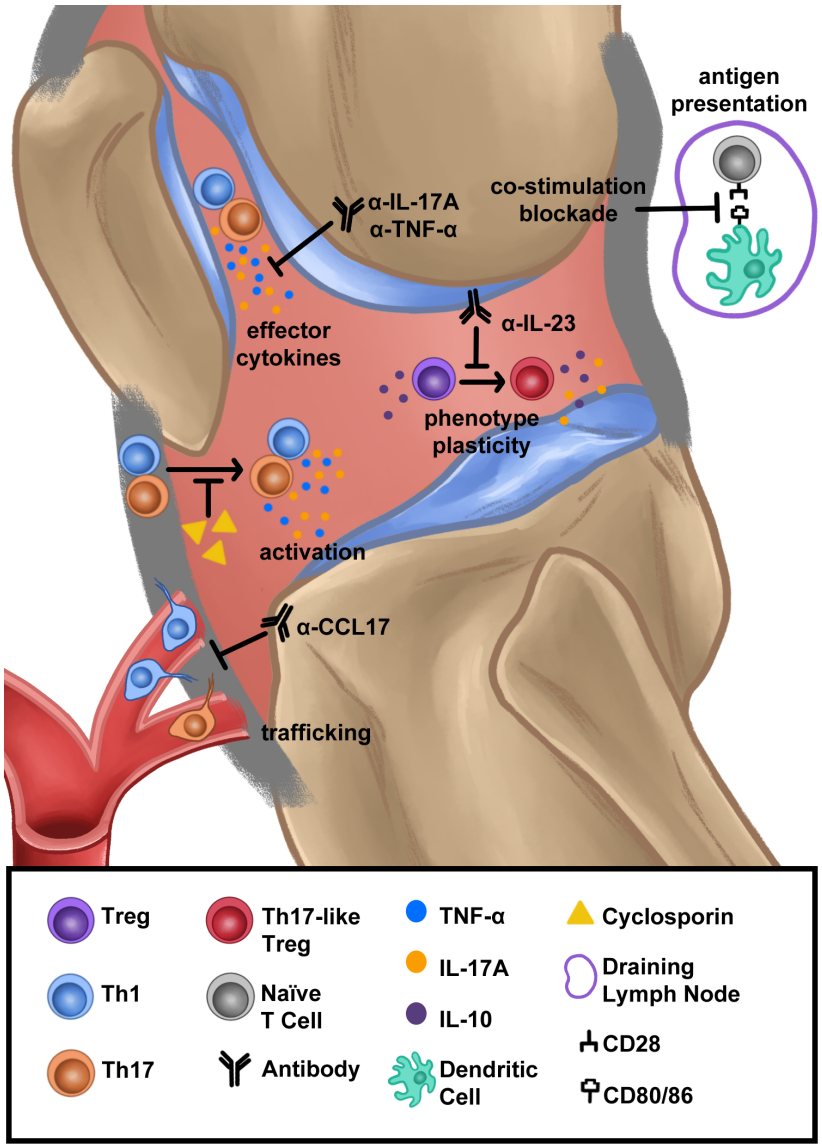

Figure 1.4. Immunotherapies that target different aspects of T cell response offer new intervention options for OA mitigation. There may be missing opportunities to rapidly translate existing immunotherapies for use in OA. Therapies that target antigen presentation to T cells, T cell trafficking, activation, phenotype plasticity, and effector cytokines are already available and approved for use in other T cell-mediated diseases and could be used to limit or mitigate progression of OA if applied at the right time during disease initiation.

significantly reduced histological score and osteophyte size.[25] Thus, blockade of T cell trafficking chemokines and their receptors may present viable options for OA mitigation.

Another approach to modifying disease progression in OA is to use therapies that target T cell activation. This can be achieved through several pathways, including blockage of extracellular signaling, or stopping downstream transcription factor activation. One approach to limit T cell activation is to block co-stimulation. Used for the treatment of RA, Abatacept is a fusion protein composed of the extracellular domain of the immune checkpoint molecule CTLA-4 and the Fc portion of IgG1. Abatacept shuts down T cell activation by antigen presenting cells, and within the context of RA, helps to prevent T cell recognition of self-antigen, a driving factor of the disease.[85] It is also possible to target T cell activation more directly. For example, cyclosporin is an immunosuppressant used in the treatment of chronic diseases, such as RA and Crohn's disease, that targets calcineurin, which is a signaling molecule critical for T cells to elicit effector functions.[86] Calcineurin activates nuclear factor of activated T cell cytoplasmic (NFATc), which upregulates downstream T cell responses. These therapies hold potential to mitigate and alleviate OA symptoms within the context of aberrant T cell activation. However, considering previous failures and mixed results of immunotherapy in OA patients, it will be pertinent to target patients who actively present with T cell-driven OA or else intervention is likely to be ineffective.

A third approach is to target inflammatory cytokines that are secreted by activated T cells. For example, anti-IL-17A therapy has been met with success in the treatment of RA and PsA where, as with OA, IL-17A secreted by Th17 cells is increased in the SF compared to healthy patients. In PsA patients, treatment with anti-IL-17A therapy reduced radiographic disease progression, effectively inhibiting structural degeneration of the joint.[87] Furthermore, RA patients in some clinical trials who did not respond to anti-TNA-α had reduced disease severity following treatment with anti-IL-17A mAbs.[88] Although mAb therapy targeting the Th1 cytokine TFN-α has previously produced mixed results in OA patients, it is possible that this is because treatment was applied too late in the progression of OA.[83] While treatment of anti-TNF-α therapy did not appear to mitigate disease nor reduce clinical symptoms of

several cohorts of patients with end-stage hand OA, it did yield promising results in patients with knee OA with a Kellgren-Lawrence grade of 2-3. Treated patients had significant improvements in WOMAC pain score, stiffness, and function.[89]

A final approach is the targeting of cytokines that maintain T cell phenotype and/or promote plasticity toward a pro-inflammatory phenotype. Of note, anti-IL-23 and combination anti-IL-12/23 antibodies that target the p40 region common to both cytokines have undergone phase II and phase III clinical trials in RA and PsA patients. IL-23 and IL-12 drive and maintain Th17 and Th1 phenotypes, respectively. In PsA patients, anti-IL-12/23 therapy inhibited radiographic progression of joint damage. However, in patients with RA, while there was numerically higher improvement in tender and swollen joints following anti-IL-12/23 treatment, neither the aforementioned treatment nor anti-IL-23 treatment significantly improved RA symptoms.[90] This may be partially explained by the findings that, in a murine model of collagen-induced arthritis, IL-12-driven Th1 activity was not responsible for collagen-induced arthritis, but IL-23 was responsible for T cell-mediated flare-ups, indicating that timing of anti-IL-23 is critical for mitigation of inflammation driven by T cells. [91]

Taken together, findings from use of immunotherapies in OA and related diseases warrants further exploration of their application early in the OA disease process. This is before irreversible joint destruction has occurred, when there is still the possibility of mitigating catabolism and returning the joint to homeostasis. However, the dynamic nature of OA also calls for a better understanding of T cell involvement during early stages and progression of disease, so that we are able to not only to identify targets for immunotherapies, but also timing of when those therapies will be most effective at mitigating disease.

Conclusion

The immune response is one of a number of critical factors that contribute to disease pathogenesis of OA. There is mounting evidence that T cell populations are altered not only in the synovium and

synovial fluid of those afflicted with OA, but also within the peripheral blood. Although a variety of $CD8^+$ and $CD4^+$ T cells infiltrate the joint acutely following injury, work in human patients and in animal models indicates that Th17 and Treg effector functions and phenotype plasticity within the joint environment play critical roles in the balance between catabolism and anabolism subsequent to joint damage.

Understanding how T cells contribute to OA initiation and progression presents the opportunity to use immunotherapies that successfully modulate T cell activities as has been done in other inflammation-mediated diseases, including RA and PsA. To date, there have been mixed outcomes of clinical trials using immunotherapies in OA patients, perhaps because the application of these therapies targeted the wrong T cell population or because of T cell plasticity, or more simply, they were used too late in the disease process, when cartilage damage is complete and irreversible. Therefore, it is important that the role of T cells in early OA continue to be investigated to yield new insights into OA as an immune-mediated disease. This will be critical for identifying novel immunotherapies that can truly modify the course of OA and mitigate disease progression.

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CHAPTER 2

REGUALTORY T CELLS PROVIDE CHRONDROPROTECTION THROUGH INCREASED TIMP1, IL-10, AND IL-4, BUT CANNOT MITIGATE THE CATABOLIC EFFECTS OF IL-1β AND IL-6 IN A TRI-CULTURE MODEL OF OSTEOARTHRITIS

Laura E. Keller[1], Elia D. Tait Wojno[2], Laila Begum[1,] Lisa A. Fortier[1]

[1]Cornell University, College of Veterinary Medicine, Department of Clinical Sciences

[2]University of Washington, Department of Immunology

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Abstract

Objective: To gain insight into Treg interactions with synovial tissues in early OA, an equine tri-culture model of OA was used to test the hypothesis that Tregs, in the absence of T Helper 17 cells, are sufficient to resolve inflammation elicited by IL-1β.

Methods: To model normal and OA joints, synoviocytes were co-cultured with chondrocytes in a transwell system and +/- stimulated with IL-1β. Tregs were activated and enriched, then added to co-cultures, creating tri-cultures. At culture end, synoviocytes and chondrocytes were analyzed for gene expression, Treg Foxp3 expression was reexamined by flow cytometry, and conditioned media were evaluated by ELISA.

Results: Tregs increased IL-10 and IL-4 in tri-culture media and increased *TIMP1* gene expression in synoviocytes and chondrocytes. Tregs increased IL-6 in conditioned media and *Il6* gene expression in synoviocytes, which was additive with IL-1β. In chondrocytes, addition of Tregs decreased *Col2b* gene expression while *Acan* gene expression was decreased by IL-1β and addition of Tregs. IL-17A was detected in tri-cultures. CCL2 and CCL5 were increased in tri-cultures.

Conclusions: In a tri-culture model of OA, addition of Tregs resulted in conditions conducive to chondroprotection including increased concentration of IL-10 and IL-4 in conditioned media and increased gene expression of *TIMP1* in both chondrocytes and synoviocytes. However, there was increased concentration of the catabolic cytokine IL-6, and decreased gene expression of *Col2b* and *Acan* in IL-1β-stimulated chondrocytes. These results suggest that blocking IL-6 could enhance Treg function in mitigating OA progression.

Introduction

In osteoarthritis (OA), immune cells from the peripheral blood infiltrate into the synovial membrane and synovial fluid of the affected joint[1]. Inflammatory cytokines and chemokines such as CCL2 and CCL5 are released from chondrocytes and synoviocytes with resultant chemotaxis of immune cells such as macrophages and T cells[2,3]. Activation and polarization of synovial macrophages contributes to inflammation, pain, and joint destruction[4]. T cells also regulate the OA disease process with infiltration of pro-inflammatory T Helper 17 (Th17) cells[5,6] and enrichment of anti-inflammatory Regulatory T (Treg) cells in the joint[7,8]. Because of their opposing roles in immunity and inflammation, an imbalance between pro-inflammatory Th17 and immunomodulatory Treg cells has been implicated in many autoimmune diseases including rheumatoid arthritis and psoriatic arthritis[9].

Tregs suppress proliferation, activation, and cytokine production by CD4+ T cells and CD8+ T cells to maintain immune homeostasis [10]. Tregs play a critical role in the maintenance of organismal homeostasis and are found to be tissue-resident in multiple tissues throughout the body including adipose tissue, skeletal muscle, and the colonic lamia propria[11]. Within skeletal muscle, Tregs promote tissue regeneration in the face of acute or chronic injury[12]. Tregs within synovial tissue may play a similar role in the absence of infection and autoimmunity as they accumulate in the synovial fluid and membrane of joints with early[13] and end-stage OA[8]. When activated, Tregs control magnitude and length of immune responses through several mechanisms. This includes cell-to-cell signaling through immune checkpoints CTLA-4 and PD-L1 and secretion of soluble mediators including IL-10, IL-35, and TFG-β1[14]. The anti-inflammatory and anabolic cytokine IL-10 is strongly associated with Treg function[15]. IL-10 plays a role in the prevention of autoimmune disease by downregulating secretion of pro-inflammatory cytokines from effector T helper cells and reducing expression of co-stimulatory molecules on macrophages, and correct temporal release of IL-10 is critical for resolution of inflammation.[16]

In chondrocytes, IL-10 treatment inhibits synthesis of IL-1β and TNF-α and suppresses proliferation and expression of NF-κB in cartilage collected from patients with end-stage OA.[17] In IL-1β - stimulated chondrocytes, IL-10 does not reduce *MMP13*,[18] but it can increase TIMP1 secretion from IL-1β stimulated synoviocytes[19] providing a potential mechanism by which Tregs could restore metabolic balance to a joint. Animal models also support a role for Tregs and IL-10 in chondroprotection. Retroviral transduction of chondrocytes with IL-10 conveys protection from IL-1β-induced *ADAMTS4* but not *MMP13* gene expression[18]. In a rabbit model of post-traumatic OA, intra-articular injection of synoviocytes overexpressing IL-10 five days post-injury resulted in improved histological cartilage scores[20]. Data from human patients are consistent with these studies. Following acute anterior cruciate ligament tear, IL-10 is increased in synovial fluid, but then decreases as early as three months post-injury[21]. Also in early OA, Tregs are enriched within the synovial membrane compared to synovial fluid and blood[13], even compared to these compartments in end-stage OA[8].

Despite an increasing knowledge about the dynamics of Tregs and the role of IL-10 and TGF-β1 in early and chronic OA, little is known about how the milieu of cytokines and chemokines secreted by Tregs affects chondrocyte and synoviocytes. Further, the reciprocal relationship is unknown, that is how an inflammatory articular environment affects Treg phenotype and function. Understanding how and why Tregs are ineffective at mitigating OA progression could reveal new insights into immunotherapeutics for OA. To address this knowledge gap, we used a novel *in vitro* model of OA based on a transwell co-culture system and tested the hypothesis that Treg anti-inflammatory function in the absence of pro-inflammatory Th17 cells is sufficient to resolve inflammation and catabolism elicited by IL-1β in an *in vitro* model of OA.

Materials and Methods

Identification of native Treg population in equine blood

Equine blood was collected to a final concentration of 40 U/ml heparin with approval from the Institutional Animal Care and Use Committee (n=6; 6-12 years of age). Peripheral blood lymphocytes (PBL) were isolated as previously described[22] and analyzed for surface and intracellular expression of CD4, CD25 and Foxp3 by flow cytometry. Tregs were identified as Foxp3-expressing cells within the $CD4^{+}CD25^{hi}$ gate[23]. Fluorescence was measured using a BD FACSymphony A5 Cell Analyzer (BD, Franklin Lakes, NJ) and analyzed with Flowjo software (TreeStar, Inc, Ashland, OR) with fluorescence-minus-one controls.

In vitro enrichment and activation of Treg populations

To obtain equine Tregs for *in vitro* studies of joint homeostasis, $CD4^{+}CD25^{hi}$ cells, which includes populations of Treg and effector T cells, were sorted (from the same 6 horses as above) using flow cytometry (**Figure 2.1a**) and further differentiated into activated Tregs (**Figure 2.1b**). Differentiation/activation was achieved by treatment of $CD4^{+}CD25^{hi}$ cells with concanavalin A (conA, 5 μg/mL; Sigma-Aldrich), rHu TGF-β1 (2 ng/mL; R&D Systems, Minneapolis, MN), and rHu IL-2 (100 U/mL; Peprotech, London, UK) as previously described [23] in modified RPMI medium containing 10% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) and basic fibroblastic growth factor (bFGF; 1 ng/mL) (**Figure 2.1c**)[22]. Medium was replenished after day three, and cells were harvested at day six for addition to co-cultures as described below. Flow cytometry was also performed for Foxp3 to verify differentiation of $CD4^{+}CD25^{hi}$ cells into Tregs.

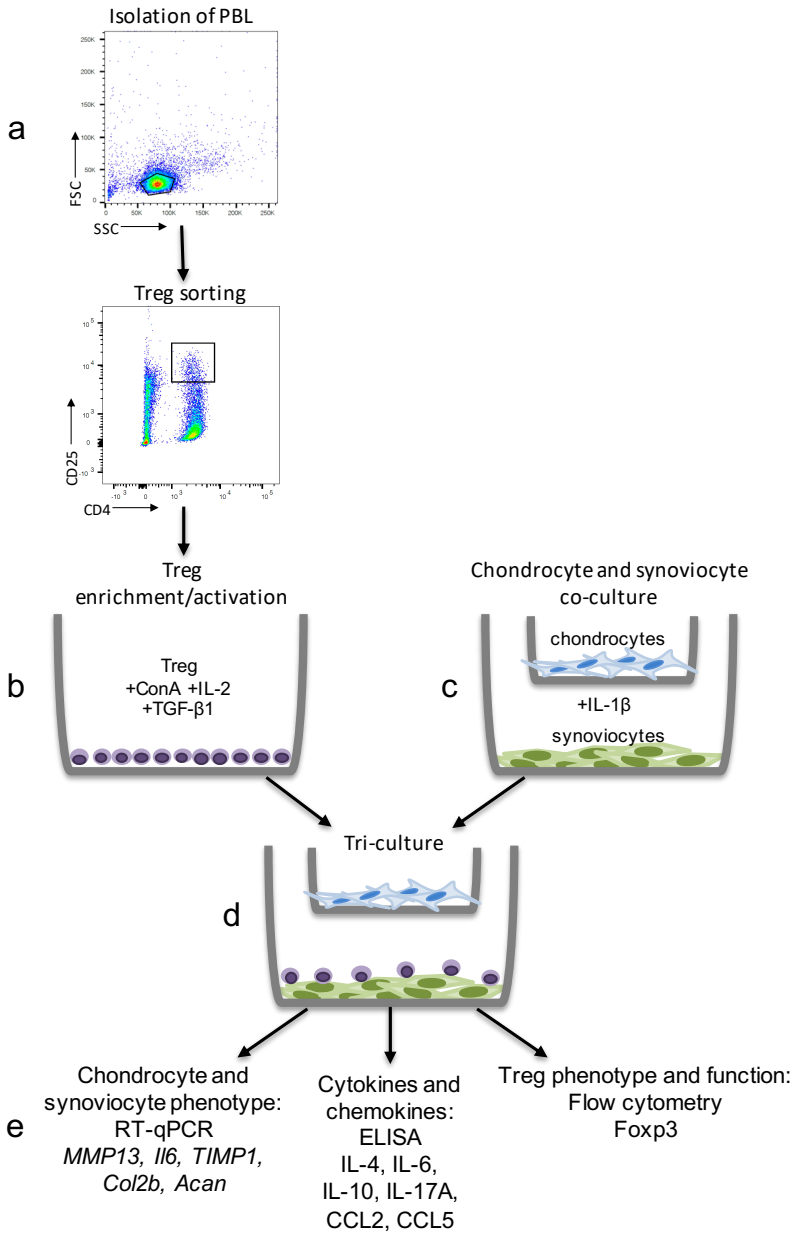

Figure 2.1. Study design overview. **a)** Peripheral blood lymphocytes (PBL) were isolated from equine (n=6) blood and Tregs were sorted using flow cytometric activated cell sorting. **b)** ConA, TGF-β1, and IL-2 were used to activate and differentiate Tregs and up-regulate protein expression of Foxp3 and secretion of anti-inflammatory cytokines. **c)** Simultaneously, synoviocytes/chondrocytes co-cultures were established and treated with IL-1β. **d)** Tregs were added in direct Treg-synoviocyte contact to create tri-cultures. **e)** Analysis of gene expression of catabolic (MMP13, IL-6) and anabolic (TIMP1, Col2b, Acan) genes was performed. Cytokines (IL-4, IL-6, IL-10, IL-17A) were analyzed in conditioned media samples. Flow cytometry was used to characterize Treg phenotype (Foxp3) to assess phenotype stability.

Co- and tri-cultures

Co-cultures of P0 chondrocytes and P2 synoviocytes from a single donor horse (1 year of age) were established with synoviocytes on the bottom of the tissue culture well and chondrocytes on the membrane insert (**Figure 2.1d**) (pore size 0.4 μm; Millipore, Burlington, MA). Chondrocytes were kept at P0 to maintain phenotype and avoid dedifferentiation[24], and synoviocytes were passaged twice to reduce presence of synovial macrophages[25] to avoid MHC II interactions with non-matched Tregs. Additionally, use of cryopreserved chondrocytes and synoviocytes allowed for use of a single donor horse to reduce donor-to-donor variability. Co-cultures were maintained for 24 hours in DMEM containing 10% FBS, 25 mM HEPES, ascorbic acid (50 μg/mL), α-ketoglutaric acid (30 μg/mL), L-glutamine (300 μg/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Co-cultures were treated with or without rEq IL-1β (10ng/mL; R&D Systems) for 24 hours, washed with PBS, and medium was replenished without IL-1β. Intraarticular injection of IL-1β is used to model OA in horses by inducing synovitis and MMP activity[26], and GAG loss at IL-1β 10 ng/mL[27].

To establish tri-cultures, the *in vitro* enriched and activated Tregs were plated in direct contact with the synoviocytes in co-culture (**Figure 2.1e**). After 24 hours, conditioned media samples were collected and Tregs were washed off the synoviocytes and centrifuged at 400xg for 5 minutes to pellet Tregs and clear conditioned media samples of cells and debris. Synoviocyte cultures were then observed by light microscopy to confirm removal of Tregs from synoviocyte surface. Conditioned media samples were stored at -80°C for subsequent chemokine and cytokine analyses. Tregs were washed with PBS/BSA, then fixed and stained for Foxp3 (Supplemental Methods). Total RNA was isolated from synoviocytes and chondrocytes for gene analysis.

Outcome analyses

In chondrocytes and synoviocytes, expression of genes involved in joint homeostasis were quantified by RT-PCR (**Figure 2.1e**) using equine-specific primers and probes (**Figure S2.1**). Total gene copy number was determined using absolute quantitative PCR derived from a standard curve used for each gene at time of analysis and were normalized to 18S.

Cytokines and chemokines in the conditioned media were measured using multiplex assays for equine cytokines (IL-4, IL-10 and IL-17A) and chemokines (CCL2, CCL5) as previously described[28]. Concentrations of IL-6 were also measured according to manufacturer directions (R&D Systems).

Statistical analyses

Gene expression and cytokine/chemokine concentrations in conditioned media were analyzed using a generalized linear model with horse as a random effect. To compare cytokine concentration and Foxp3 expression pre- and post-Treg differentiation, a paired Wilcoxon non-parametric test was used. Tukey's post-hoc was used with p values ≤ 0.05 were considered significant. Statistical analyses were performed using JMP Pro 15 (SAS Institute, Cary, NC).

Cell staining for flow cytometric identification of Tregs

Isolated PBL were labeled with goat anti-human CD25 (R&D Systems) at 4° for 30 minutes, followed by donkey anti-goat immunoglobulin G-Phycoerythrin (Invitrogen, Carlsbad, CA) as a secondary antibody, and mouse anti-equine CD4 (Washington State University, Pullman, WA) conjugated to Alexa Fluor 488 (Invitrogen). For extracellular staining and wash steps, phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 0.02% sodium azide (VWR, Radnor, PA) (PBS/BSA) was used. Cells were then fixed, permeabilized and stained for Foxp3 using the eBioscience Foxp3 Transcription Factor Staining Buffer Set (eBioscience, San Diego, CA) per the

manufacturer's instructions. Cells were stained with rat anti-mouse Foxp3 eFluor 450 (eBioscience) at 4° for 30 minutes.

Results

Treg differentiation and activation

Enrichment of Tregs was confirmed by increased expression of Foxp3 in the $CD4^{+}CD25^{hi}$ population. In the stimulated group, 79±3% of $CD4^{+}CD25^{hi}$ cells expressed Foxp3 compared to 47±5% in the naïve unstimulated population ($p<0.0001$). (**Figure S2.2**).

Chondrocyte and synoviocyte responses to Tregs

Pro- and anti-inflammatory and catabolic gene expression in synoviocytes and chondrocytes - As expected, addition of IL-1β to co-cultures increased gene expression of *MMP13* in synoviocytes ($p=0.02$) and chondrocytes ($p=0.0002$) compared to controls (**Figure S2.3**). Addition of Tregs did not affect gene expression of *MMP13* in IL-1β-stimulated synoviocytes ($p=0.18$) or chondrocytes ($p=0.35$). Gene expression of *Il6* was similarly increased by IL-1β in synoviocytes ($p=0.001$), and chondrocytes ($p=0.005$) (**Figure 2.2**). Surprisingly, addition of Tregs in the absence of IL-1β also increased gene expression of *Il6* in synoviocytes ($p=0.0025$), but not chondrocytes ($p=0.80$). Tregs and IL-1β together had an additive effect on *Il6* gene expression compared to IL-1β alone in synoviocytes ($p=0.0002$), but not chondrocytes ($p=0.50$), suggesting independent mechanisms for stimulation of *Il6* gene expression by IL-1β and Tregs. Gene expression of the matrix-sparing glycoprotein *TIMP1* was significantly increased when Tregs were added to co-cultures. *TIMP1* was increased in synoviocytes ($p<0.0001$) and chondrocytes ($p<0.03$) in the presence or absence of IL-1β, suggesting a possible mechanism for mitigating extracellular matrix catabolism (**Figure 2.3**).

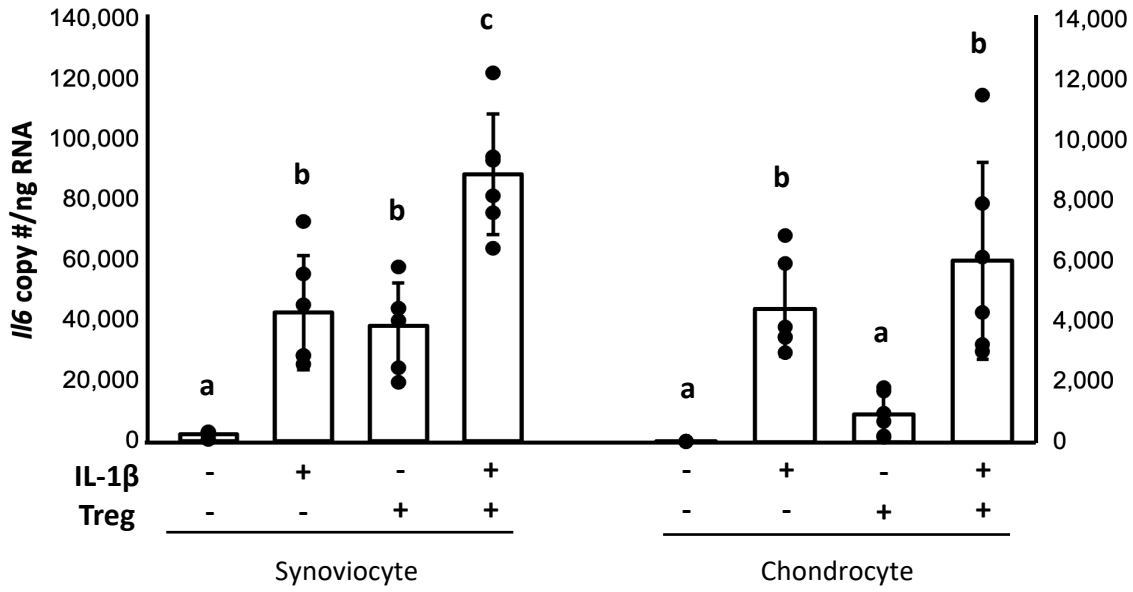

Figure 2.2 IL-1β or Tregs alone increased *Il6* in synoviocytes and appeared to act synergistically on *Il6* gene expression. Tregs did not similarly increase *Il6* in chondrocytes either alone or following stimulation with IL-1β. GLM with Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$.

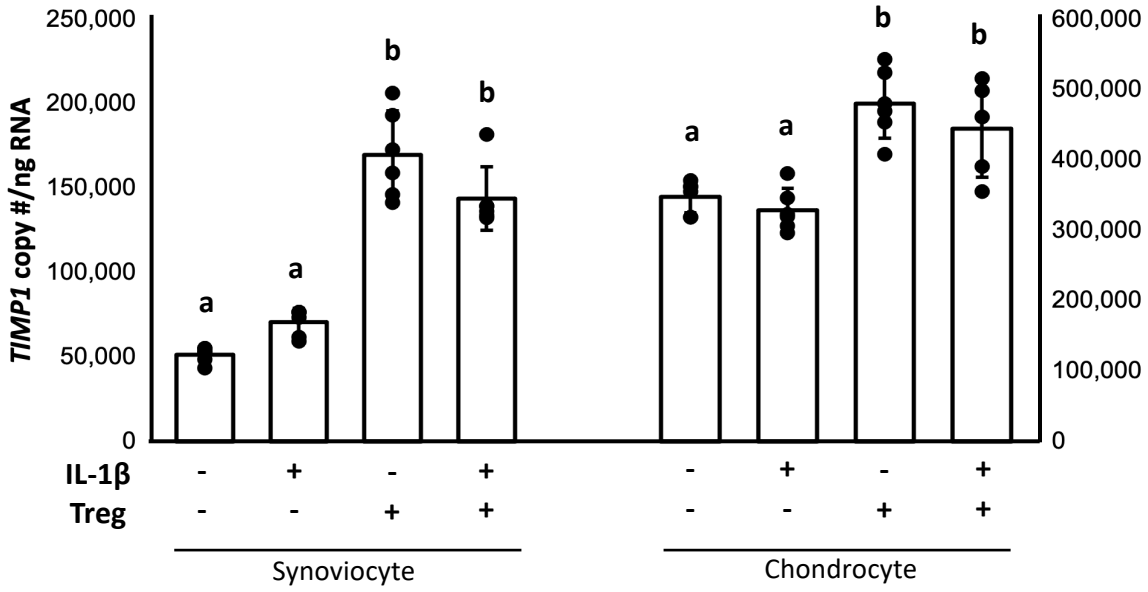

Figure 2.3 Treg soluble factors promoted *TIMP1* gene expression in both synoviocytes and chondrocytes regardless of stimulation with IL-1β. *TIMP1* was increased in synoviocytes in both tri-culture groups as Tregs promoted restoration of catabolic imbalance. Chondrocytes followed the same trend, suggesting that increased *TIMP1* gene expression could be contributed to factors within the Treg secretome. GLM with Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$.

Matrix gene expression in chondrocytes – Gene expression of *Col2b* was significantly decreased by IL-1β ($p<0.0001$), and to a lesser extent by Tregs alone in the absence of IL-1β ($p=0.0002$) (**Figure 2.4a**). The combination of IL-1β and Tregs was not additive and resulted in decreased *Col2b* gene expression to a level not different from IL-1β alone ($p=0.9$). For *Acan,* gene expression was unchanged by the addition of Tregs alone ($p=0.013$) (**Figure 2.4b**). In cultures treated with Tregs in addition to IL-1β, gene expression was significantly decreased compared to control cultures ($p=0.0005$) but was not different from IL-1β treated cultures ($p=0.30$).

Secretion of cytokines and chemokines and growth factors into tri-culture conditioned media

Cytokines IL-6, IL-10, IL-4, and IL-17A - Protein concentration of the catabolic cytokine IL-6 was increased in a similar, but not identical pattern to IL-6 gene expression in chondrocytes and synoviocytes. Both IL-1β ($p<0.0001$) and Treg-stimulated co-cultures ($p=0.007$) had increased IL-6 in the conditioned media samples compared to co-culture controls (**Figure 2.5a)**. Tregs did not increase protein secretion of IL-6 to the same extent as IL-1β stimulated cultures. Similar to gene expression, the effects of IL-1β and Tregs appear to be additive ($p<0.0001$).

The anabolic cytokine IL-10 is indicative of Treg function[15]. The addition of Tregs significantly increased IL-10 in conditioned media samples and was not affected by IL-1β treatment (**Figure 2.5b**; $p<0.001$). This suggests that Treg were phenotypically functioning and stable in the conditions of this study. Th2 cells characteristically secrete IL-4 which was significantly increased in cultures with Tregs and unaffected by the addition of IL-1β (**Figure 2.5c**). Interestingly IL-4 was below the limit of detection in co-cultures with no Tregs present. Like IL-4, IL-17A, which is secreted by Th17 effector cells, was Treg-dependent and detected in tri-cultures, but not co-cultures, and was unaffected by IL-1β (**Figure 2.5d**).

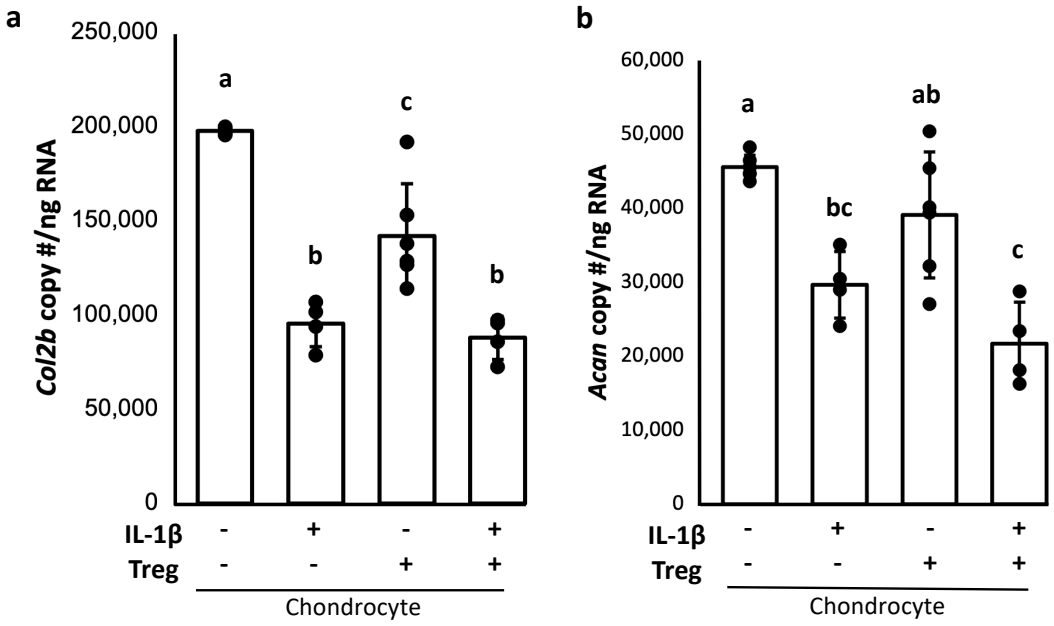

Figure 2.4 Tregs alone or in combination with IL-1β decrease expression of structural protein in cartilage **a)** Tregs alone decreased gene expression of *Col2b* in chondrocytes despite the presence of IL-10 and IL-4. **b**) Addition of Tregs to IL-1β-stimulate chondrocytes further decreased gene expression of *Acan*, promoting degradation of cartilage tissue. GLM with Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$.

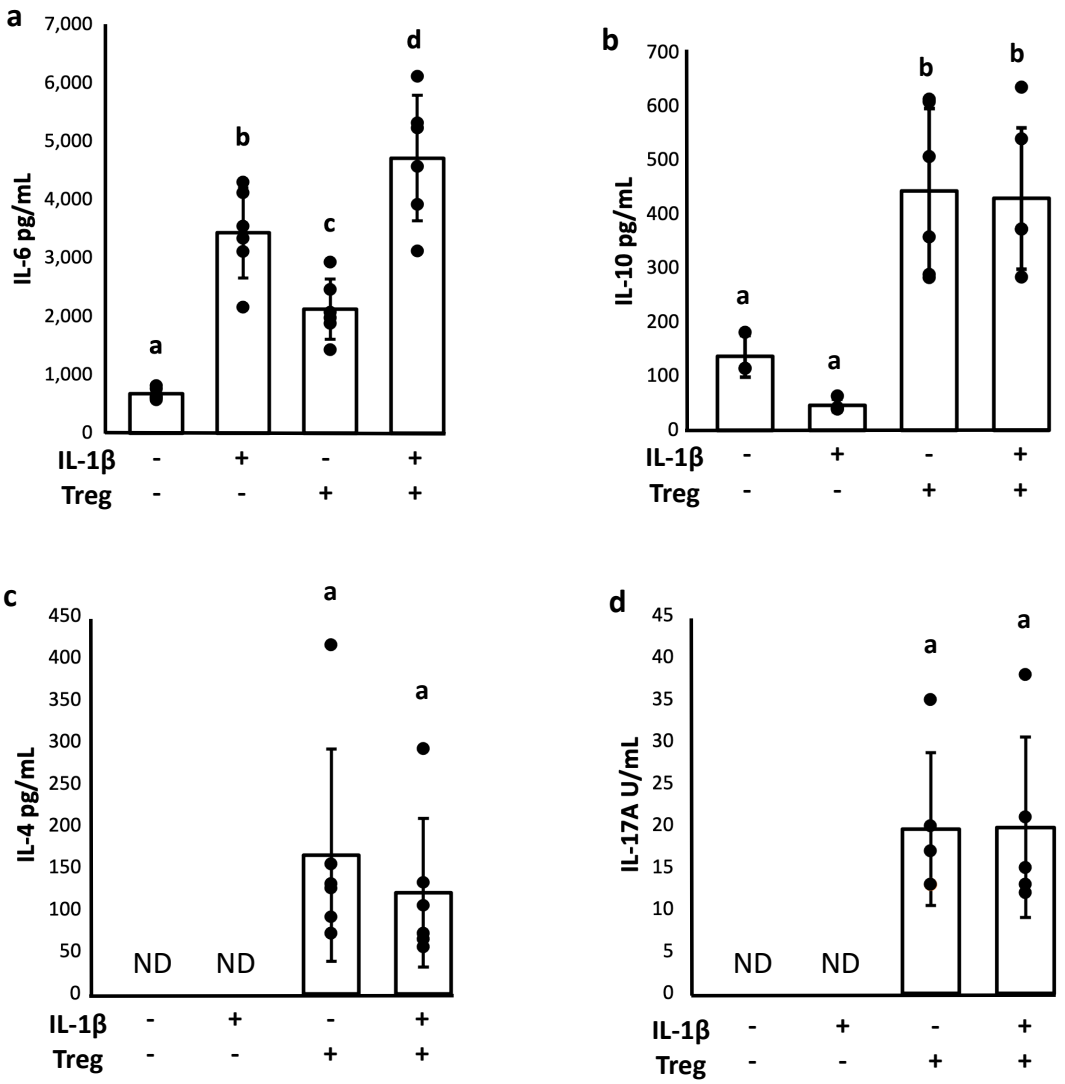

Figure 2.5 Tregs simultaneously increase concentrations of chondroprotective and anti-inflammatory cytokines in conditioned media samples while promoting secretion of pro-inflammatory IL-6 by chondrocytes and synoviocytes. Chemokine and cytokine concentrations in conditioned media samples following co- and tri-culture. **a)** IL-6 concentrations followed trends of *Il6* gene expression in synoviocytes, confirming its secretion following upregulation of gene expression. **b)** Enriched Tregs secreted high, unchanged concentrations of IL-10, regardless of inflammatory environment. **c)** Residual Th2 cells within the enriched Treg population secreted IL-4 in tri-cultures. **d)** IL-17A was detected in conditioned media samples, but inflammatory environment did not promote its secretion by T cells. GLM with Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$. ND = not detected.

Chemokines CCL2 and CCL5 – In OA, CCL2 and CCL5 are increased in synovial fluid and thought to be secreted by injured chondrocytes and synoviocytes. Both CCL2 and CCL5 were present at very low concentrations in co-cultures and significantly increased tri-cultures suggesting that activated Tregs were the main source of these chemokines (**Figure 2.6**). CCL2, but not CCL5 was decreased in tri-cultures treated with IL-1β.

Tregs phenotype and function after tri-culture

At the end of culture, Tregs were removed and Foxp3 expression was reassessed. Pre-culture, Foxp3 expression was (79±3%) and significantly decreased in both control (66±4%; $p<0.0001$) and IL-1β-treated (65±3%; $p<0.0001$) tri-cultures which were not different from each other ($p=0.98$).

Discussion

The purpose of this study was to determine if Tregs alone were sufficient to mitigate inflammation and matrix catabolism in an *in vitro* model of early OA. A tri-culture model was used to study the effects of Tregs on chondrocytes and synoviocytes, and to study the reciprocal relationship of how Treg phenotype and function are affected by joint inflammation. Our hypothesis was that Treg anti-inflammatory function would be sufficient to resolve inflammation and catabolism elicited by IL-1β. The use of equids to establish a tri-culture system allowed for sufficient collection of Tregs from peripheral blood and matched, cryopreserved chondrocytes and synoviocytes which would not have been possible if using a small animal or human model. Further, the horse is an established model for OA with cartilage thickness and biomechanical loading approximating that of humans[29,30].

A highly enriched Treg population failed to completely restore homeostasis in IL-1β-treated chondrocytes or synoviocytes. Incomplete differentiation of residual effector T cells that continued secreting pro-

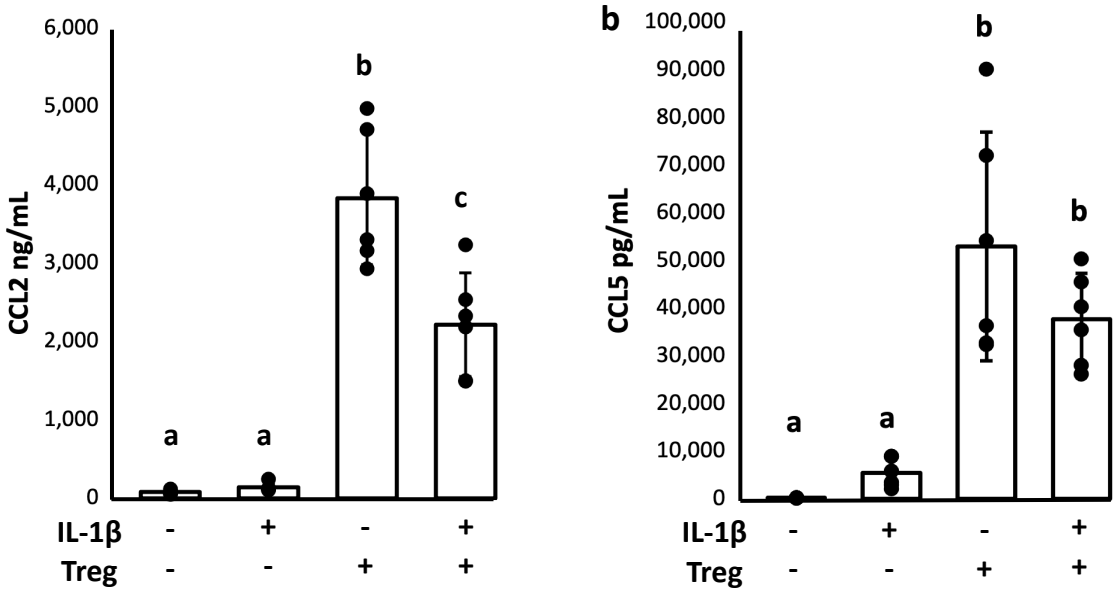

Figure 2.6 Tregs secrete high concentrations of macrophage and T cell chemoattractants. **a)** CCL2 concentration was not affected by IL-1β stimulation alone. Addition of Tregs increased CCL2 which was then diminished when both IL-1B and Tregs were in the tri-cultures. **b)** CCL5 was similarly unaffected by IL-1B alone and significantly increased with the addition of Tregs. Addition of IL-1B did not decreased Treg-induced CCL5 concentration. GLM with Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$.

inflammatory cytokines or Treg instability could have been a contributing factor. The T cell population at the beginning of the experiment was 72-92% Tregs after differentiation based on Foxp3 expression. In conditioned media samples, detection of IL-4 and IL-17A suggests that residual Th2 (IL-4) and Th17 (IL-17A) cells were present in the Treg-enriched population used in this study, or that some Tregs demonstrated phenotypic instability. A varied T cell phenotype is a more realistic representation of cellular infiltration into the joint during OA than a pure Treg population. Even if a pure Treg population could have been achieved, there was evidence that Treg phenotype was not stable during culture duration. At study end, about 10% fewer cells were expressing Foxp3 in both IL-1β-stimulated and control tri-cultures, suggesting destabilization in Treg function or Treg death. Treg destabilization, wherein the presence of pro-inflammatory factors, including IL-6, cause Tregs to lose Foxp3 expression and suppressive functions in favor of a pro-inflammatory phenotype, has been previously described[31]. The reduction in Foxp3 expression following tri-culture was surprising, as it has previously been reported that induced Tregs maintain immunosuppressive phenotype and function when interacting with TNF-α-treated synoviocytes from rheumatoid arthritis patients undergoing knee arthroscopy or synovectomy[32]. Differences in induced Treg stability between experiments could have been a result of variations in synoviocyte populations and stimulation methods or that, in those experiments, Tregs were continually stimulated with anti-CD3/CD28-coated beads, simulating interactions with antigen-presenting cells in T cell activation, whereas in the experiments of this study, Tregs did not receive continuous ConA stimulation during tri-culture.

Tregs were unable to alter IL-1β-induced *MMP13* gene expression in either chondrocytes or synoviocytes. MMP13 is well known as an extracellular matrix-degrading enzyme and is highly expressed in synovium and cartilage of OA joints and is therefore studied as a biomarker and potential target for OA treatment[33]. Unlike other MMPs, MMP13 can cleave intact type 2 collagen *in vivo*[34], and decreases gene expression of *Col2a* and *Acan in vitro*[35] so the inability of Tregs to affect *MMP13*

gene expression is a significant shortcoming in the concept of Tregs as a target or Treg secretome as a treatment for OA. The extracellular activity of MMPs is specifically antagonized by TIMPs including TIMP1[36]. *TIMP1* gene expression was increased by addition of Tregs in both chondrocytes and synoviocytes even in the presence of IL-1β. However, TIMP1 alone is not sufficient for chondroprotection as shown by addition of exogenous TIMP1 to IL-1β stimulated chondrocytes that resulted in decreased MMP3 concentration but did not protect against matrix catabolism[37]. Similarly, in the present study, despite an increase in *TIMP1* gene expression in Treg-containing cultures, neither *Col2b* nor *Acan* expression were protected from IL-1β.

Detection of IL-4 in tri-culture conditioned media samples was unexpected. IL-4 is secreted by Th2 cells and plays a critical role in wound healing by promoting alternate activation of macrophages to an anti-inflammatory M2 phenotype[38]. Within the context of OA, IL-4 alone or in combination with IL-10 protects cartilage in a dose-dependent manner by rescuing cartilage proteoglycan synthesis and release and reducing secretion of IL-1β and TNF-α by cartilage explants exposed to whole blood[39]. However, a subsequent study by the same group revealed that IL-4/IL-10 administration into joints of hemophilic mice following joint bleeds did not prevent an increase in cartilage Osteoarthritis Research Society International (OARSI) Score following Safranin-O Fast-Green staining, nor did it prevent an increase in synovial inflammation as determined by Valentino visual bleeding score. It was suggested that failure of IL-4/IL-10 administration to control inflammation within this context was the short half-life of these two molecules (<2 hours after intravenous injection). However, continuous secretion of IL-4 and IL-10 by activated Tregs within the tri-cultures would suggest there is another mechanism leading to failure of these cytokines to prevent IL-1β-induced decrease in *Col2b* and *Acan* in chondrocytes.

The chondroprotective effects of IL-4 and IL-10 may also have been negated by IL-6, which was increased by IL-1β and Tregs alone and further when in combination. IL-6 enhances synthesis of MMP13

in synoviocytes and chondrocytes[40] while suppressing collagen type II and aggrecan synthesis in chondrocytes[41]. In synovial fluid and serum from patients with OA, IL-6 concentration correlates with disease severity.[42] Secretion of IL-6 into the conditioned media samples of the present study may be responsible for the failure of Tregs to reduce *MMP13* gene expression in chondrocytes and synoviocytes and protect *Col2b* and *Acan* gene expression in chondrocytes. Moreover, IL-1β increases expression of membrane-bound IL-6 receptor on chondrocytes, which may explain why Tregs do not rescue *Col2b* gene expression and appear to have a synergistic effect with IL-1β on the decrease in *Acan* gene expression. Increased IL-6 concentration in conditioned media samples of Treg-containing cultures was unexpected. If IL-6 within this tri-culture system is responsible for the suppression of Treg anti-inflammatory functions, then neutralizing IL-6 or blocking its receptor could remove the inhibitory effects of IL-6 on Treg functions. Anti-IL-6 therapy has been met with success in the treatment of rheumatoid arthritis, and mice treated with anti-IL6 and/or anti-IL-6R antibody therapy markedly reduces post-traumatic OA, laying the foundation for its use as a therapy to treat OA[42,43]. If Tregs alone did not lead to the increase in IL-6 in control tri-culture, residual Th17 cells may have promoted increased IL-6 secretion by synoviocytes through IL-17A[44].

IL-17A was detected in cultures where Tregs were present and was independent of IL-1β. IL-17A leads to joint catabolism through increased synthesis of MMP1 and MMP13 by chondrocytes and synoviocytes[5]. The most likely source of IL-17A was residual effector Th17 cells remaining in the $CD4^{+}CD25^{hi}$ population rather than a result of Treg phenotype plasticity in the inflammatory environment. Concentrations of IL-10 and IL-17A were unchanged in tri-cultures treated with IL-1β suggesting that, although there was a decrease in $Foxp3^{+}$ Tregs following tri-culture, this was not due to plasticity resulting from promotion of a Th17 phenotype despite the OA environment of the IL-1β-stimulated tri-culture.

Concentrations of CCL2 and CCL5 were considerably higher in tri-cultures compared to co-cultures and CCL2, but not CCL5, was significantly decreased in tri-cultures stimulated with IL-1β. CCL2 recruits monocytes and T cells to sites of injury as part of a normal acute inflammatory response. In a mouse model of OA, the CCL2/CCR2 axis was shown to be involved in the recruitment of pro-inflammatory macrophages to the inflamed joint[45]. In CCL2 knockout mice, M2 macrophages were nearly absent compared to controls, further demonstrating a role for CCL2 in maintaining an M1/M2 balance[46]. CCL2 released by Tregs in the tri-cultures of the present study would not only act as a powerful monocyte chemoattractant but could also maintain homeostasis of M1/M2 phenotype of monocytes recruited to the joint. The CCL5/CCR5 axis is involved in T cell migration and recruitment, and maintenance of M2 phenotype in tumor-associated macrophages[47]. CCL5 is secreted by several cell types, including T cells following activation[48] and fibroblasts when stimulated with IL-1β[49]. CCL5 is also secreted by tumor cells in order to recruit Tregs to suppress pro-inflammatory effector T cells within the tumor microenvironment[50]. Secretion of CCL5 within the tri-culture by Tregs could be an attempt to recruit additional Tregs to the inflamed joint environment to suppress inflammation, as well as maintain or modify M1/M2 balance within the joint. The failure of Tregs to suppress IL-1β-induced increase in *MMP13* in synoviocytes and chondrocytes and prevent decrease of *Col2b* and *Acan* in chondrocytes may be due to the requirement of an intermediate step, such as monocytes or macrophages, to elicit anti-inflammatory effects on tissues within the joint[51]. One of the limitations of this study is that synoviocytes were cultured specifically to reduce presence of synovial macrophages in order to avoid MHC II crosstalk with Tregs from non-matched donors.

Conclusion

An activated and enriched Treg population was not sufficient to mitigate IL-1β-induced inflammation and catabolism in synoviocytes and chondrocytes despite secretion of anti-inflammatory cytokines IL-4 and IL-10. This indicates that either Tregs alone are not sufficient to restore joint homeostasis, or that the

additional inflammation induced by IL-6 or IL-17A inhibited Tregs from restoring homeostasis within the tri-culture. Considering the results of this study, IL-6 is likely a contributing factor to Treg failure to mitigate inflammation. Future directions will include reducing the function of IL-6 *in vitro* in order to determine if it is significantly contributing to failure of Tregs to mitigate OA.

Author contributions

LEK designed the study, performed data collection, analyzed the data, and drafted the manuscript. EDTW and LAF contributed to study design, data interpretation, and manuscript preparation. LB participated in data collection and reviewed the manuscript. All authors approved the final version of the manuscript.

Conflict of interest

None.

Role of the funding source

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None.

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Table S2.1. Equine-specific Taqman® primers and probes used in gene expression analysis with a Viia 7 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Gene	Abbreviation	Function	Sense	Anti-sense	Probe
18S ribosomal RNA	*18S*	Ribosome structural unit	GGCGTCCCCCAACTTCTT	AGGGCATCACAGACCTGTTATTG	TGGCGTTCAGCCACCCGAGATT
Collagenase 3	*MMP13*	Matrix metalloproteinase	TGAAGACCCGAACCCTAAACAT	GAAGACTGGTGATGGCATCAAG	CAAAACACCAGACAAATGCGATCCTTCCTTA
Interleukin 6	*Il6*	Inflammatory cytokine	AGTAACCACCCCTGACCCAACT	TGTTGTGTTCTTCAGCCACTCA	CCTGCTGGCTAAGCTGCATTCACAGA
Tissue inhibitor of metalloproteinases 1	*TIMP1*	Inhibitor of matrix metalloproteinases	TGGAGAGCCTCTGCGGATAC	CCGGCGATGAGAAACTCTTC	CCACAGGTCGGAGAACCGCAGC
Collagen 2 isoform b	*Col2b*	Cartilage extracellular matrix	CGCTGTCCTTCGGTGTCA	CTTGATGTCTCCAGGTTCTCCTT	TCCGGCAGCCAGGACCGAA
Aggrecan	*Acan*	Cartilage extracellular matrix	GATGCCACTGCCACAAAACA	GGGTTTCACTGTGAGGATCACA	CCGAGGGTGAAGCTCGAGGCAA

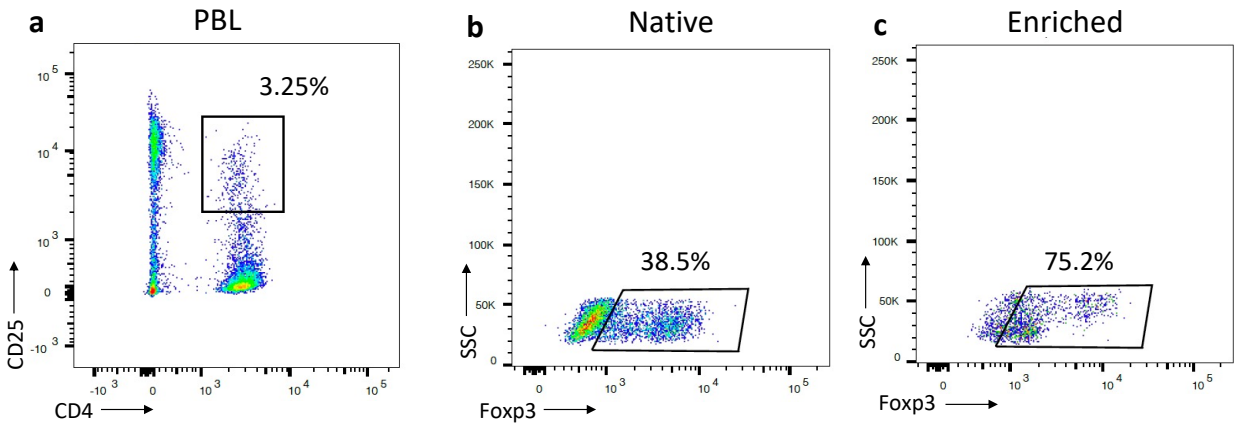

Figure S2.1. Treg activation and enrichment. **a)** Peripheral blood lymphocytes (PBL) were sorted based on expression of CD4 and CD25. **b**) Tregs, defined as $CD4^{+}CD25^{hi}$ in this native population account for 39-51% of this population based on Foxp3 expression. **c)** Treg were analyzed again following incubation with ConA, rHu TGF-β1 and rHu IL-2 to confirm enrichment of the $Foxp3^{+}$ Treg population.

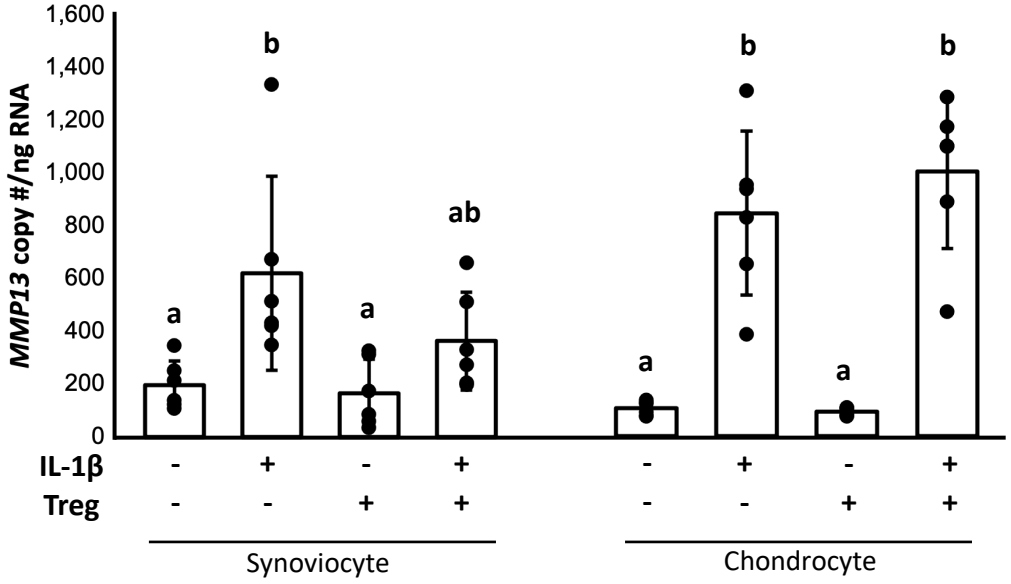

Figure S2.2. Addition of IL-1β increased expression of *MMP13* in synoviocytes and chondrocytes, but Tregs were not able to fully able to rescue this effect in synoviocytes and did not affect *MMP13* expression in chondrocytes. GLM with Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$.

CHAPTER 3

INTERLEUKIN-6 NEUTRALIZATION AND REGULATORY T CELLS ARE ADDITIVE IN CHONDROPROTECTION FROM IL-1β-INDUCED INFLAMMATION

Laura E. Keller[1], Elia D. Tait Wojno[2], Lisa A. Fortier[1]

[1]Cornell University, College of Veterinary Medicine, Department of Clinical Sciences

[2]University of Washington, Department of Immunology

Abstract

Objective: Gain insight into IL-6 as a mechanism of Treg failure to resolve inflammation in OA and test the hypothesis that neutralization of IL-6 will enable Treg anti-inflammatory function to resolve inflammation and catabolism elicited by IL-1β in an *in vitro* model of OA**.**

Methods: To model normal and OA joints, synoviocytes were co-cultured with chondrocytes in a transwell system and +/- stimulated with IL-1β and +/- treated with αIL-6 neutralizing antibody. Tregs were activated and enriched, then added to co-cultures, creating tri-cultures, which were repeat treated +/- αIL-6 antibody. At culture end, synoviocytes and chondrocytes were analyzed for gene expression, Treg Foxp3 expression was reexamined by flow cytometry, and conditioned media were evaluated by ELISA.

Results: Treatment with αIL-6 did not affect control synoviocyte/chondrocyte gene expression or Treg secretion of IL-4, IL-10, or IL-17A. Tregs increased IL-10 and IL-4 in tri-culture media. IL-6 neutralization in combination with Tregs, reduced IL-1β-stimulated synoviocyte *MMP13* gene expression to control levels and restored *Acan* gene expression in chondrocytes. Tregs alone increased *TIMP1* gene expression in synoviocytes and chondrocytes. Neutralization of secreted IL-6 decreased *Il6* gene expression chondrocytes and synoviocytes.

Conclusions: In a tri-culture model of OA, neutralization of IL-6 restored Treg anti-inflammatory functions. Treatment of IL-1β stimulated co-cultures with αIL-6 and Tregs reduced *MMP13* gene expression and increased *TIMP1* gene expression, suggesting that Tregs in the absence of IL-6 are capable of protecting the joint from IL-1β-induced catabolism. Additionally, αIL-6 antibody mitigated IL-6 positive feedback loop, indicating αIL-6 therapy as a promising therapeutic target for OA mitigation.

Introduction

Inflammation is increasingly recognized as a key driver of osteoarthritis and is present in the majority of patients suffering from OA.[1–3] Immune cells that infiltrate the joint such as macrophages[4] and T cells,[5] induce inflammation through cell-to-cell interactions and the production of cytokines that can alter the function of other cell types. When recruited and activated within the joint, macrophages and T cells produce cytokines that directly act on synoviocytes and chondrocytes to initiate and propagate cartilage damage in OA and offer an attractive therapeutic target. Despite success in other inflammation-mediated diseases such as rheumatoid and psoriatic arthritides, immunotherapies targeting inflammatory cytokines are less effective when treating idiopathic/degenerative OA.[6] This could be due to targeting an inappropriate molecule, or the appropriate molecule at the wrong time.

T cell populations within the joint include anti-inflammatory subsets, such as Regulatory T cells (Tregs),[5] that are responsible for metabolic[7] and immune homeostasis.[8] Tregs are typically characterized by nuclear expression of the master transcription factor Foxp3, extracellular expression of immune checkpoints PD-L1 and CTLA-4, and secretion of anti-inflammatory cytokines IL-10 and TGF-β1.[8] IL-10 increases synthesis of *TIMP1* in IL-1β-stimulated synoviocytes[9] and inhibits synthesis of IL-6 and TNF-α in OA chondrocytes[10]. Despite enrichment of Tregs within the joint,[11] inflammation and cartilage degeneration persist and lead to joint destruction. This persistent inflammation, when driven by the persistence of specific inflammatory cytokines, including IL-1β and IL-6, can lead to destabilization of the Foxp3 transcription factor in Tregs, compromising their anti-inflammatory functions, including secretion of IL-10.[12]

The pro-inflammatory cytokine IL-6 is elevated in the synovial fluid of patients with end-stage OA[13] and acute[14,15] and chronic posttraumatic OA[16,17] and has been identified as a factor correlating with OA

progression and severity of cartilage destruction.[17–19] IL-6 is secreted by activated synovial fibroblasts[20] and synovial macrophages[21] and acts synergistically with IL-1β to enhance secretion of catabolic matrix metalloproteinases (MMPs), further contributing to joint destruction[22]. Therapeutics targeting IL-6 and its receptor (IL-6R), such as Tocilizumab, have been used successfully in the treatment of rheumatoid arthritis by suppressing bone erosion, lowering Clinical Disease Activity Index, and reducing serum MMP-3 concentrations.[23] Although Tocilizumab was no more effective than placebo in relieving pain in patients with hand OA, a recent study by Blair et al. identified patient-dependent response to Tocilizumab therapy,[24] suggesting that use of therapy targeting IL-6/IL-6R in OA patients might be best prescribed on an personalized medicine basis.

The mechanisms behind Treg failure to restore joint homeostasis remain unclear but provide potential insights into immunotherapeutics to address inflammation in early OA. Because IL-10 and IL-6 have opposing roles in inflammation and metabolic balance in early osteoarthritis, neutralization of IL-6 in the presence of elevated IL-10 secreted by Tregs may lead to restoration of joint homeostasis. To investigate whether excess IL-6 within the joint environment contributes to failure of Tregs to mitigate inflammation and catabolism within the joint, this study utilized a tri-culture model of OA to test the hypothesis that neutralization of IL-6 will enable Treg anti-inflammatory function to resolve inflammation and catabolism elicited by IL-1β in an *in vitro* model of OA.

Materials and Methods

Identification of native Treg population in equine blood - Equine blood was collected to a final concentration of 40 U/ml heparin with approval from the Institutional Animal Care and Use Committee (n=6; 4-12 years of age). Peripheral blood lymphocytes (PBL) were isolated as previously described[25] and analyzed for surface and intracellular expression of CD4, CD25 and Foxp3 by flow cytometry. Tregs

were identified as Foxp3-expressing cells within the $CD4^{+}CD25^{hi}$ gate.[26] Fluorescence was measured using a BD FACSymphony A5 Cell Analyzer (BD, Franklin Lakes, NJ) and analyzed with FlowJo software (TreeStar, Inc, Ashland, OR) with fluorescence-minus-one controls.

In vitro enrichment and activation of Treg populations - To obtain equine Tregs for *in vitro* studies of joint homeostasis, $CD4^{+}CD25^{hi}$ cells, which includes populations of Treg and effector T cells, were sorted (from the same 6 horses as above) using flow cytometry and further differentiated into activated Tregs. Differentiation/activation was achieved by treatment of $CD4^{+}CD25^{hi}$ cells with concanavalin A (ConA, 5 μg/mL; Sigma-Aldrich), rHu TGF-β1 (2 ng/mL; R&D Systems, Minneapolis, MN), and rHu IL-2 (100 U/mL; Peprotech, London, UK) as previously described[26] in modified RPMI medium containing 10% fetal bovine serum (FBS), 0.1 mM 2-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL) and basic fibroblastic growth factor (bFGF; 1ng/mL).[25] Medium was replenished after day three, and cells were harvested at day six for addition to co-cultures as described below. Flow cytometry was also performed for Foxp3 to verify differentiation of $CD4^{+}CD25^{hi}$ cells into Tregs.

Co- and tri-cultures - Co-cultures of P1 chondrocytes and synoviocytes (n=3; 1-4 years of age) were established with synoviocytes on the bottom of the tissue culture well and chondrocytes on the membrane insert (pore size 0.4 μm; Millipore, Burlington, MA). Co-cultures were maintained for 24 hours in DMEM containing 10% FBS, 25 mM HEPES, ascorbic acid (50 μg/mL), α-ketoglutaric acid (30 μg/mL), L-glutamine (300 μg/mL), penicillin (100 U/mL), and streptomycin (100 μg/mL). Co-cultures were treated with or without rEq IL-1β (10ng/mL; R&D Systems) and with or without αEqIL-6 (1.5μg/mL; R&D Systems) for 24 hours, washed with PBS, and medium was replenished without IL-1β and with αEqIL-6 in wells which had previously been treated.

To establish tri-cultures the *in vitro* enriched and activated Tregs were plated in direct contact with the synoviocytes in co-culture. After 24 hours, conditioned media samples were collected with

cOmplete™ Protease Inhibitor Cocktail Tablets (F. Hoffmann-La Roche AG, Basel, Switzerland) and Tregs were washed off the synoviocytes and centrifuged at 400 *xg* for 5 minutes to pellet Tregs and clear conditioned media samples of cells and debris. Synoviocyte cultures were then observed by light microscopy to confirm removal of Tregs from synoviocyte surface. Conditioned media samples were stored at -80°C for subsequent chemokine and cytokine analyses. Tregs were washed with PBS/BSA, then fixed and stained for Foxp3 (Supplemental Methods). Total RNA was isolated from synoviocytes and chondrocytes for gene analysis.

Chondrocytes and synoviocyte gene expression – Following culture, RNA was isolated from chondrocytes and synoviocytes for analysis of genes involved in joint homeostasis by RT-PCR using equine-specific primers and probes (**Table S3.1**). Total gene copy number was determined using absolute quantitative PCR derived from a standard curve used for each gene at time of analysis and were normalized to 18S.

Cytokine analyses – Cytokines in conditioned media were measured using multiplex assays for equine cytokines (IL-4, IL-10 and IL-17A) as previously described.[27] Concentrations of free IL-6 were measured according to manufacturer directions with an ELISA that utilized the same α-equine IL-6 polyclonal goat IgG as was used for IL-6 neutralization (R&D Systems).

Cell staining for flow cytometric identification of Tregs - Isolated PBL were labeled with goat anti-human CD25 (R&D Systems) at 4° for 30 minutes, followed by donkey anti-goat immunoglobulin G-Phycoerythin (Invitrogen, Carlsbad, CA) as a secondary antibody, and mouse anti-equine CD4 (Washington State University, Pullman, WA) conjugated to Alexa Fluor 488 (Invitrogen). For extracellular staining and wash steps, phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO) and 0.02% sodium azide (VWR, Radnor, PA)

(PBS/BSA) was used. Cells were then fixed, permeabilized and stained for Foxp3 using the eBioscience Foxp3 Transcription Factor Staining Buffer Set (Invitrogen) per the manufacturer's instructions. Cells were stained with rat anti-mouse Foxp3 eFluor 450 (Invitrogen) at 4° for 30 minutes.

Statistical analyses - Gene expression and IL-6 concentration in conditioned media were analyzed using a generalized linear model with synoviocyte and/or chondrocyte donor as a random effect. IL-4, IL-10, and IL-17A concentrations in conditioned media were analyzed using a generalized linear model with Treg donor as a random effect. To compare Foxp3 pre- and post-Treg activation and enrichment, a paired Wilcoxon non-parametric test was used. Tukey's post-hoc was used with p values ≤ 0.05 were considered significant. Statistical analyses were performed using JMP Pro 15 (SAS Institute, Cary, NC).

Results

Treg differentiation and activation - Enrichment of Treg was confirmed by increased expression of Foxp3 in the $CD4^{+}CD25^{hi}$ population following activation and differentiation. Following stimulation with ConA, rHu IL-2 and rHu TGF-β1, 72±6% of $CD4^{+}CD25^{hi}$ cells expressed Foxp3 compared to 33±3% in the naïve, unstimulated population ($p<0.0001$).

Gene expression in synoviocytes and chondrocytes – Addition of IL-6 neutralizing antibody had no effect on control gene expression in chondrocytes or synoviocytes. Treatment with αIL-6 antibody simultaneously with IL-1β stimulation reduced *MMP13* expression in synoviocytes (**Figure 3.1A**; $p<0.0001$) and chondrocytes (**Figure 3.1B**; $p<0.0001$) by one-third. Addition of Tregs to IL-1β-stimulated co-cultures decreased *MMP13* expression in synoviocytes ($p=0.02$) but not chondrocytes ($p=0.90$). Treatment of IL-1β-stimulated co-cultures with both αIL-6 antibody and Tregs further reduced *MMP13* gene expression in synoviocytes to a level not different from controls ($p=0.7$) but did not

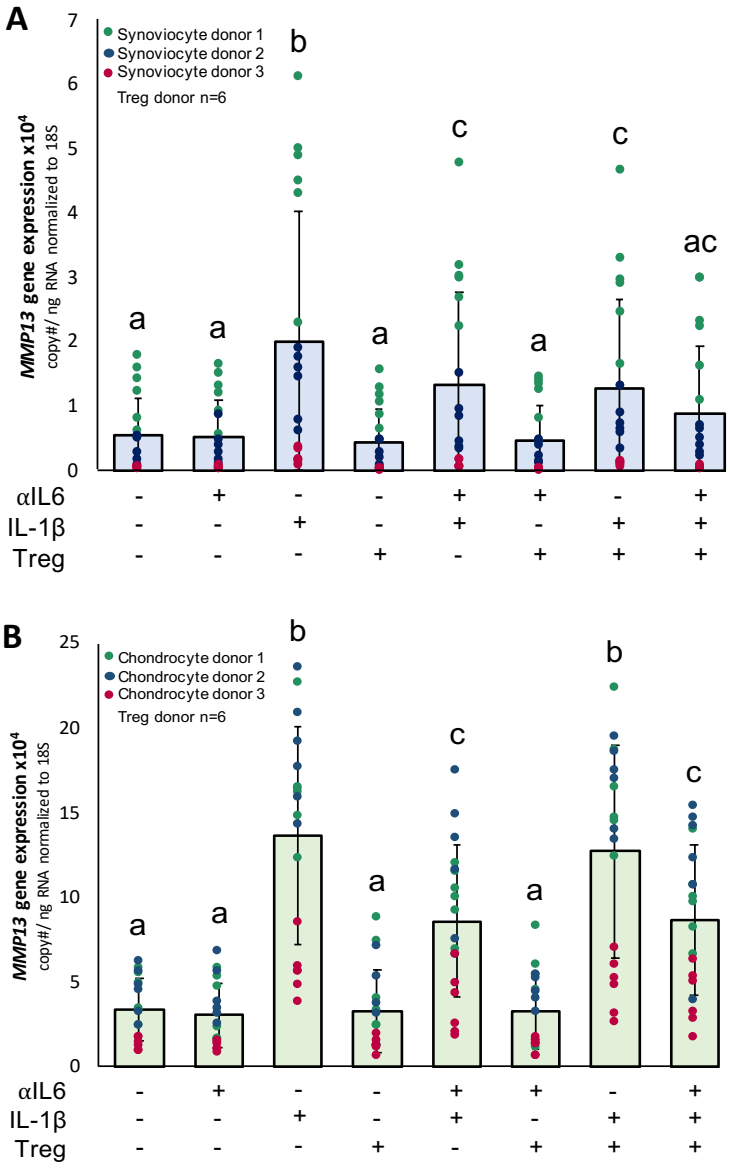

Figure 3.1. Tregs and αIL-6 decreased *MMP13* gene expression in IL-1β stimulated chondrocyte/synoviocyte co-cultures. Stimulation of chondrocyte/synoviocyte co-cultures with IL-1β increased gene expression of *MMP13* in synoviocytes (A) and chondrocytes (B) which was significantly decreased by approximately one-third in both cell types by addition of αIL-6 antibody. **A)** Tregs similarly decreased IL-1β-stimulated synoviocyte *MMP13* gene expression by approximately 50%. Together, αIL-6 antibodies and Tregs reduced *MMP13* expression in synoviocyte to control levels. **B)** Addition of Tregs had no effect on chondrocyte *MMP13* gene expression in the presence or absence of αIL-6 antibodies. GLM with synoviocyte/chondrocyte donor as random effect followed by Tukey's post-hoc with, groups that do not share a letter are statistically different, $p<0.05$.

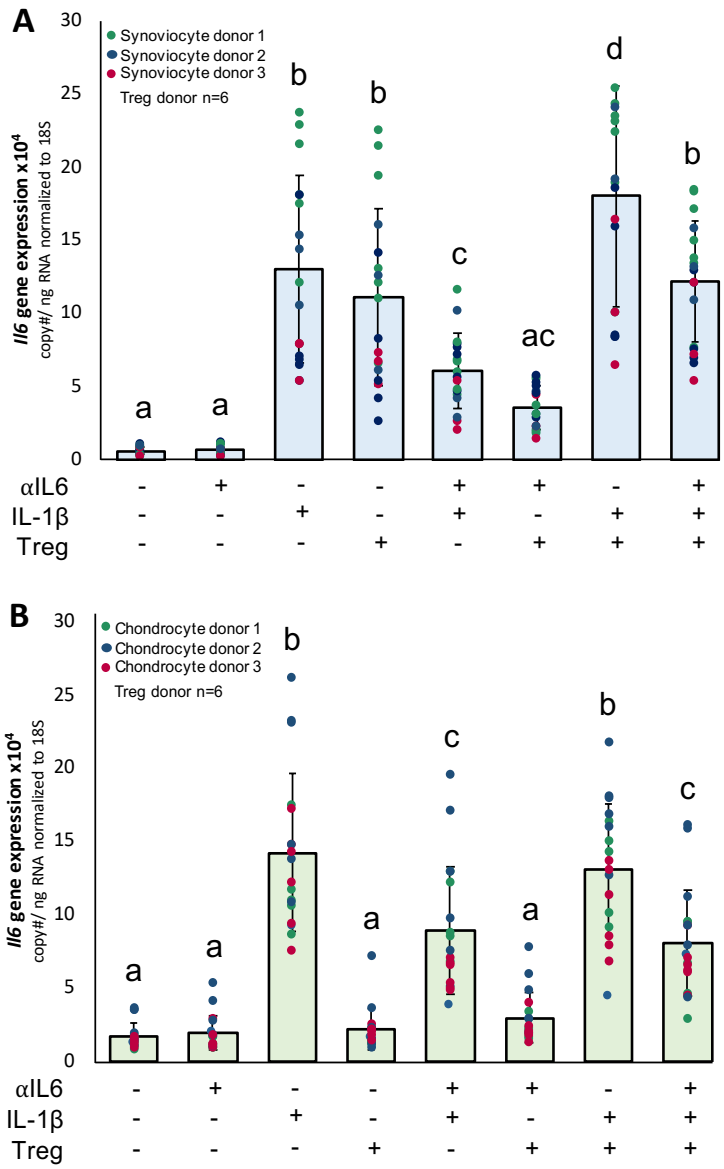

Figure 3.2. Tregs increase *Il6* expression in synoviocytes, and αIL-6 antibody reduces *Il6* gene expression in both chondrocytes and synoviocytes. IL-1β increased *Il6* gene expression in synoviocytes (A) and chondrocytes (B). **A)** Addition of Tregs increased *Il6* gene expression in synoviocytes and was additive with IL-1β. Treatment with αIL-6 antibodies decreased synoviocytes *IL6* gene expression following IL-1β stimulation and in synoviocyte/chondrocyte/Treg tri-cultures. **B)** Treatment with αIL-6 antibodies reduced *Il6* expression in co- and tri-cultures stimulated with IL-1β. GLM with synoviocyte/chondrocyte donor as random effect followed by Tukey's post-hoc with, groups that do not share a letter are statistically different, $p<0.05$.

similarly affect chondrocytes. (p=0.9). Treg donor did not affect *MMP13* gene expression, but synoviocyte donor (p<0.0001) and chondrocyte donor (p<0.0001) did have a significant effect.

Both IL-1β and Tregs (p<0.0001) increased *Il6* expression in synoviocytes (**Figure 3.2A**; p<0.0001), but only IL-1β increased *Il6* expression in chondrocytes (**Figure 3.2B**; p<0.0001). Surprisingly, addition of αIL-6 antibody to IL-1β-stimulated co-cultures decreased expression of *Il6* in both synoviocytes (p<0.0001) and chondrocytes (p<0.0001) suggesting a positive feedback loop between secreted IL-6 and *Il6* gene expression. Addition of Tregs to IL-1β-stimulated co-cultures further increased *Il6* gene expression in synoviocytes by nearly 140% (p=0.01), but this effect was rescued by addition of αIL-6 antibody (p=0.0001). Chondrocytes in IL-1β-stimulated tri-cultures treated with αIL-6 antibody had similar *Il6* gene expression to IL-1β-stimulated co-cultures treated with αIL-6 antibody (p=0.9), whereas *Il*6 gene expression in synoviocytes was similar between co-cultures stimulated with IL-1β (p=0.9) and control tri-cultures (0.90) compared to tri-cultures stimulated with IL-1β and treated with αIL-6 antibody. Treg donor did not affect *Il6* gene expression, but synoviocyte donor (p<0.0001) and chondrocyte donor (p<0.0001) did have a significant effect.

Addition of Tregs to all culture conditions increased gene expression of *TIMP1* by nearly two-fold in synoviocytes (**Figure 3.3A**; p>0.004), and αIL-6 antibody did significantly change this expression pattern. Chondrocytes exhibited a similar increased in *TIMP1* expression following addition of Tregs, with the exception of IL-1β-stimulated tri-cultures (**Figure 3.3B**; p=0.2), which were not different than controls. Treg donor did not affect *Il6* gene expression, but synoviocyte donor (p=0.002) and chondrocyte donor (p<=0.02) did have a significant effect.

As anticipated, IL-1β decreased *Col2b* gene expression in chondrocytes by half (**Figure 3.4A**; p<0.0001). Tregs similarly decreased *Col2b* expression by about 40% (p=0.003) compared to controls. Addition of αIL-6 antibody to IL-1β-stimulated co-cultures did not rescue *Col2b* expression in chondrocytes (p=0.9), but addition of αIL-6 antibody to tri-cultures was able to restore *Col2b* gene expression (p=0.9) to a level not different from control co-cultures, suggesting that there are separate

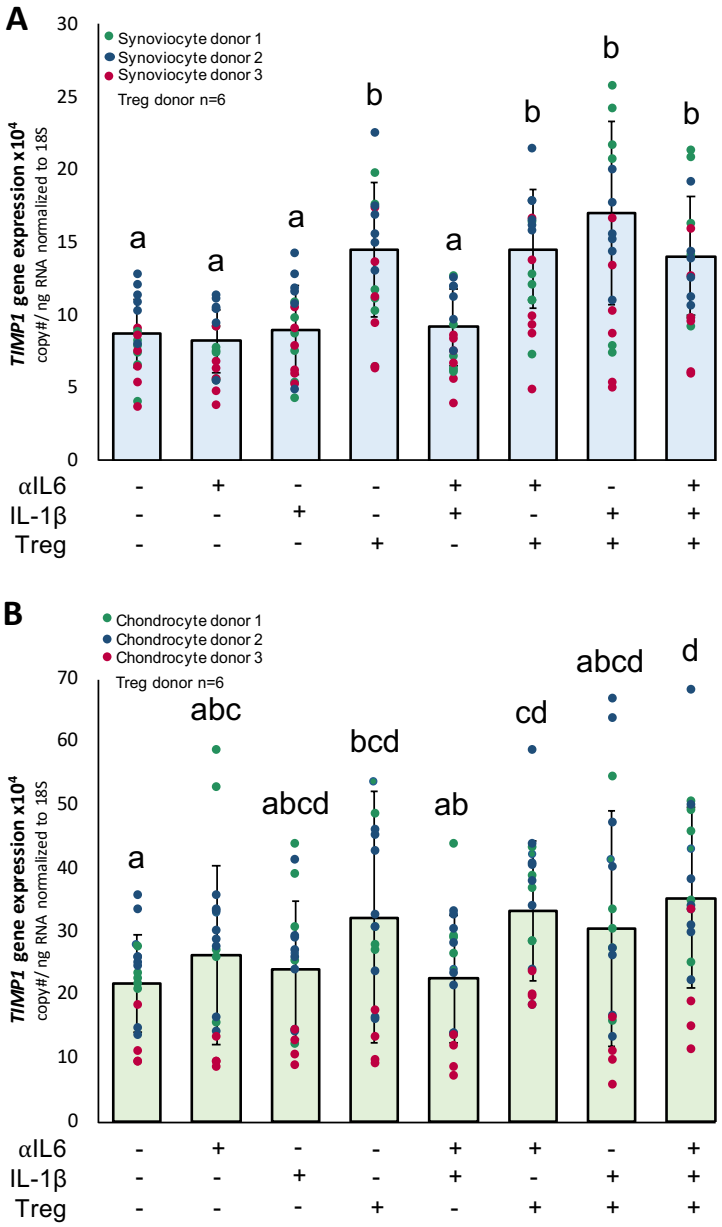

Figure 3.3. Tregs increase *TIMP1* gene expression in synoviocytes and chondrocytes and is unaffected by αIL-6 treatment. **A)** Tregs increased gene expression of *TIMP1* in synoviocytes regardless of IL-1β stimulation or treatment with αIL-6. **B)** Tregs similarly increased *TIMP1* gene expression in chondrocytes, except when chondrocytes had been stimulated with IL-1β. GLM with synoviocyte/chondrocyte donor as random effect followed by Tukey's post-hoc with, groups that do not share a letter are statistically different, $p<0.05$.

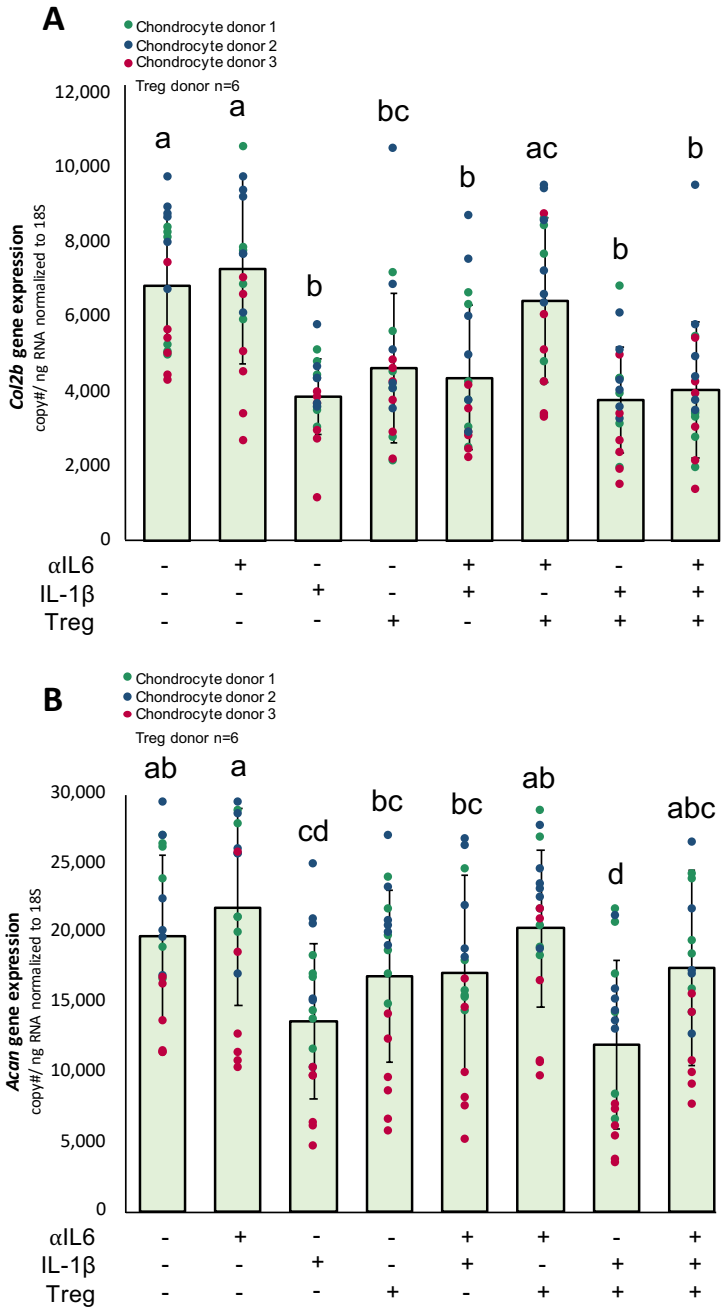

Figure 3.4. Treatment with αIL-6 does not rescue *Col2b* gene expression in chondrocyte stimulated with IL-1β but does rescue *Acan* expression alone and in combination with Tregs. **A)** Treatment of control tri-cultures with αIL-6 antibodies rescues reduction of *Col2b* gene expression by Tregs, but not in tri-cultures stimulated with IL-1β. **B)** Treatment with αIL-6 antibodies of co-cultures and tri-cultures stimulated with IL-1β does rescue *Acan* gene expression in chondrocytes. GLM chondrocyte donor as random effect followed by Tukey's post-hoc with, groups that do not share a letter are statistically different, $p<0.05$.

mechanisms by which IL-1β and Tregs reduce *Col2b* gene expression in chondrocytes, with the Treg mechanism being linked to IL-6 protein expression. It was therefore unexpected that treatment of IL-1β-stimulated tri-cultures with αIL-6 antibody did not rescue *Col2b* gene expression when compared to IL-1β-stimulated tri-cultures ($p=0.9$) and IL-1β-stimulated co-cultures ($p=0.9$).

Acan gene expression in co-culture chondrocytes was also reduced by nearly half following stimulation with IL-1β (**Figure 3.4B**; $p=0.0002$). Gene expression was also significantly reduced in IL-1β-stimulated tri-cultures ($p<0.0001$) compared to controls but was partially rescued by addition of αIL-6 antibody. Although expression was not different than in IL-1β-stimulated co-cultures ($p=0.07$), it was also not different than controls ($p=0.6$). This suggests that IL-6 is a major factor in failure of Tregs to rescue *Acan* expression following IL-1β stimulation in the acute phase response. Treg donor did not affect *Col2b* or *Acan* gene expression, chondrocyte donor ($p<0.0001$) did have a significant effect.

IL-6 secretion and neutralization - Addition of IL-1β (**Figure 3.5A**; $p<0.0001$) or Tregs ($p<0.0001$) increased IL-6 in conditioned media, but stimulation with IL-1β increased IL-6 by nearly twice that of Tregs ($p<0.0001$). Treatment of IL-1β stimulated co-cultures or control tri-cultures with αIL-6 antibody neutralized more than half of the IL-6 in conditioned media samples and reduced free IL-6 in control tri-cultures to that of control co-cultures ($p=0.9$). The effects of IL-1β and Tregs on IL-6 concentrations in conditioned media was additive and was increased 10-fold compared to co-culture controls ($p<0.0001$), but nearly half was neutralized by addition of αIL-6 antibody, reducing IL-6 to a concentration not different to that induced by Tregs ($p=0.3$). Conditioned media from Tregs alone indicated that Tregs secreted very little IL-6 following activation and enrichment and were not significant contributors to IL-6 concentration in tri-cultures. Treg donor did not affect IL6 concentration, but synoviocyte/chondrocyte donor did have a significant effect ($p<0.0001$).

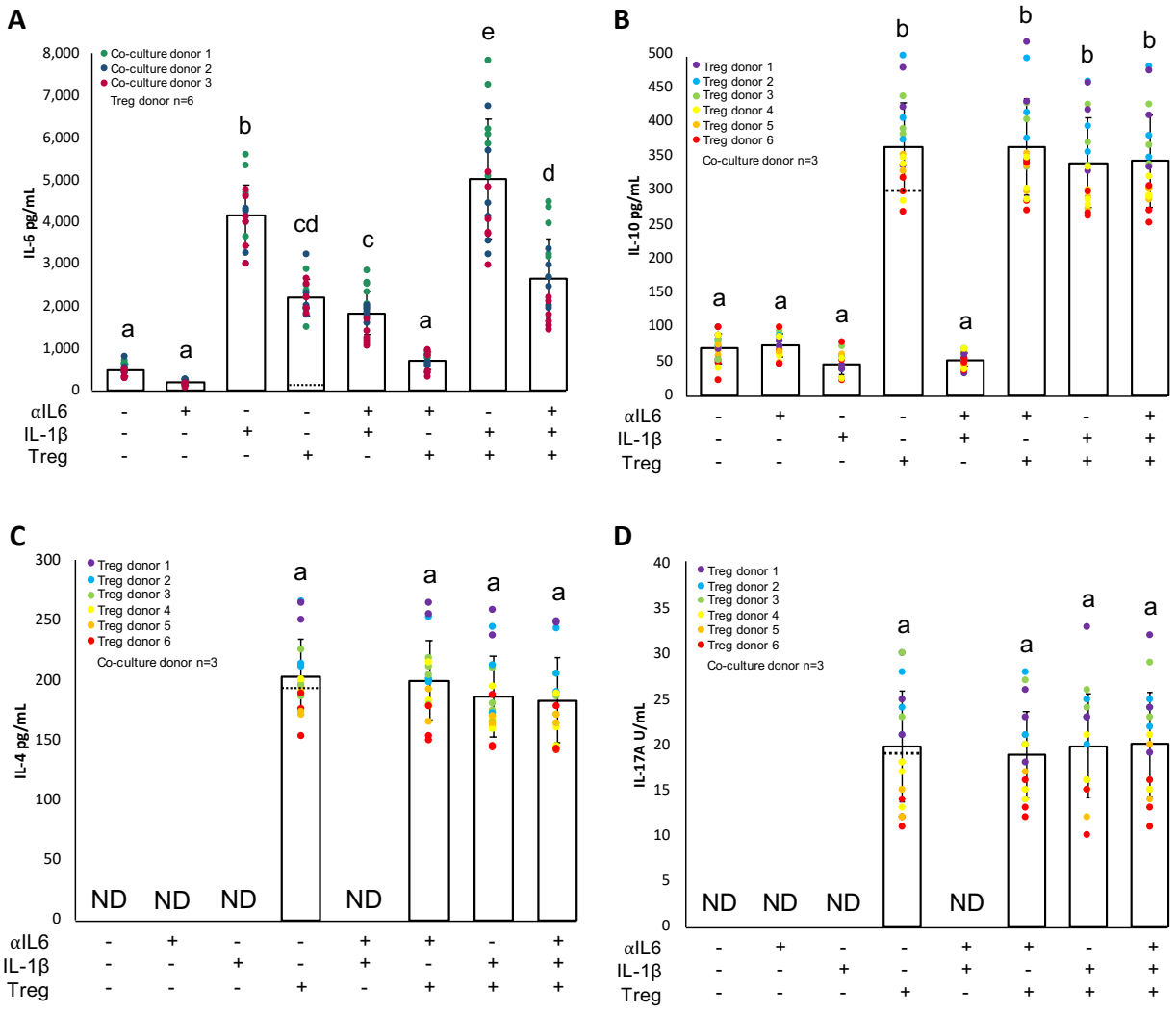

Figure 3.5 Cytokine concentrations in conditioned media samples following co- and tri-culture. **A)** αIL-6 antibodies neutralize approximately 50% of IL-6 in conditioned media from synoviocytes and chondrocytes. **B)** Synoviocytes and chondrocytes produce some IL-10, but most IL-10 in conditioned media is secreted by Tregs, regardless of inflammation or presence of αIL-6 antibodies. **C)** IL-4 was produced by Tregs, and concentration was unaffected by culture conditions. **D)** IL-17A was secreted by cells within the Treg population prior to tri-culture, and its secretion was not affected by tri-culture conditions. Dashed lines represent cytokine concentrations in conditioned media from Tregs alone, prior to tri-culture. ND = not detected. For IL-6: GLM with synoviocyte/chondrocyte donor as random effect followed by Tukey's post-hoc with, groups that do not share a letter are statistically different, $p<0.05$. For IL-10, IL-4, and IL-17A: GLM with Treg donor as random effect followed by Tukey's post-hoc, groups that do not share a letter are statistically different, $p<0.05$.

Cytokines IL-10, IL-4, and IL-17A – Function of Tregs was confirmed by IL-10 in conditioned media (**Figure 3.5B;** 315±72 pg/mL), and Tregs were confirmed as the main producers of IL-10 in tri-cultures where concentration was not affected by addition of IL-1β ($p=0.6$) or αIL-6 ($p=0.9$) alone or in combination ($p=0.7$). Tregs alone also secreted IL-4 (**Figure 3.5C**; 202±52 pg/mL) and IL-17A (**Figure 3.5D**; 20±6 U/mL), and IL-4 and IL-17A were not detected in co-cultures in the absence of Tregs. Concentration of IL-4 and IL-17A in tri-cultures were unaffected by presence of IL-1β ($p>0.2$) and αIL-6 antibody ($p>0.1$) alone or in combination ($p>0.1$). Synoviocyte/chondrocyte donor did not affect either IL-4, IL-10, or IL-17A concentration in conditioned media, however Treg donor did have a significant affect ($p<0.0001$).

Tregs phenotype and function following tri-culture - At culture end, Tregs were isolated and Foxp3 expression was reassessed. Pre-culture Foxp3 expression was 72±6% and was significantly decreased in tri-culture control (**Table 1;** 57±7%; $p<0.0001$), tri-culture with αIL-6 antibody (61±6%; $p<0.0001$), IL-1β-stimulated tri-culture (55±7%; $p<0.0001$) and tri-cultures stimulated with IL-1β and treated with αIL-6 (59±5%; $p<0.0001$) following tri-culture. Tregs recovered from tri-culture treated with αIL-6 antibody had a higher population of $Foxp3^+$ cells than Tregs recovered from IL-1β-stimulated tri-cultures ($p=0.01$). Synoviocyte/chondrocyte donor did not affect Foxp3 expression in Tregs, however Treg donor did have a significant affect ($p=0.01$).

Discussion

There is missed opportunity in the translation of existing and approved immunotherapies for the treatment of OA which could be due to choice of inappropriate timing or target. To truly intervene in OA progression, intervention must occur during early disease progression, before joint destruction has of this

occurred.[28,29] The purpose study was therefore to determine if neutralization of IL-6 would enable Treg to mitigate matrix catabolism and inflammation in an *in vitro* model of early OA.

Neutralization of IL-6 and addition of Tregs following stimulation of synoviocytes and chondrocytes with IL-1β aided in reducing inflammation and restoring metabolic balance but did not fully reduce gene expression of catabolic factors or rescue gene expression of structural proteins within chondrocytes. The secretion of pro-inflammatory cytokines, such as IL-17A, by the Treg population may have been a contributing factor. Conditioned media samples collected from activated and enriched Tregs alone revealed that secretion of IL-17A occurred prior to tri-culture and was not likely the result of phenotype instability due to the inflammatory environment. Although the reduction in $Foxp3^+$ Tregs recovered from all tri-cultures could be interpreted as phenotype instability of Foxp3, this may have occurred due to T cells within the Treg population adhering to synovial fibroblasts, which can occur when T cells are activated.[30] This would also indicate that addition of αIL-6 antibody to tri-cultures did not maintain Foxp3 expression in Tregs, but rather reduced adhesion of Tregs to synovial fibroblasts, increasing recovery of $Foxp3^+$ T cells. Furthermore, unchanged secretion of IL-10 and IL-4 by Tregs in tri-culture was indicative of phenotype stability.

Treatment of IL-1β-stimulated co-cultures with αIL-6 antibody reduced *MMP13* gene expression in both synoviocytes and chondrocytes, mitigating the synergistic effects of IL-1β and IL-6 on its gene expression. Addition of Tregs further reduced *MMP13* gene expression in synoviocytes to that of control levels, but this did not occur in chondrocytes. One possible mechanism behind these results is that IL-10 is capable of downregulating gene expression of MMPs in fibroblasts,[31] but does not do so in chondrocytes.[32] This may indicate that Treg secretome, even when IL-6 is neutralized, is not ideal for chondroprotection. However, induction of *TIMP1* gene expression by 4/4 and 3/4 synoviocyte and chondrocyte groups in tri-cultures, respectively, in combination with reduction of *MMP13* gene

expression following αIL-6 treatment provides an opportunity to prevent matrix catabolism early in disease progression and prevent joint degeneration.

Reduction of *Il6* gene expression in IL-1β-stimulated chondrocytes and synoviocytes following treatment with αIL-6 antibody was unexpected, but exogenous IL-6 has been shown to increase *Il6* gene expression in fibroblasts through the STAT4 pathway, creating a positive feedback loop.[20,22] Therefore, neutralization of IL-6 protein works directly and indirectly to decrease the inflammatory effect of IL-6. Furthermore, αIL-6 antibody reduced tri-culture synoviocyte *Il6* expression to control levels, mitigating a pro-inflammatory effect of Tregs on synoviocytes, which may aid in restoration of joint homeostasis by Tregs in a longer timeframe.

The failure of αIL-6 antibody to rescue *Col2b* was surprising given that IL-6 suppresses collagen type II synthesis in chondrocytes.[33] One explanation for this discrepancy may be that IL-6 and IL-1β work through different signaling pathways. IL-6 suppression of collagen type II synthesis is mediated by the JAK/STAT pathway,[33] whereas IL-1β-induced suppression of collagen type II synthesis is mediated through NF-κB.[34] Failure of Tregs alone to restore *Col2b* expression was expected because IL-10 does not restore collagen type II expression in chondrocytes following stimulation with TNF-α.[32] In contrast, αIL-6 antibody alone and in combination with Tregs did restore *Acan* gene expression in chondrocytes following stimulation with IL-4 andL-10 can restore *Acan* gene expression in chondrocytes following stimulation[32] and blocking of the JAK/STAT pathway through neutralization of IL-6 may be sufficient to restore *Acan* synthesis in chondrocytes.

Addition of αIL-6 antibody neutralized only about half of the IL-6 present within the conditioned media, which may be a result of the use of a polyclonal antibody that was not specifically raised for neutralization properties. This is a limitation of the study as it does not fully address the mechanistic

question of whether IL-6 neutralization enhances anti-inflammatory functions of Tregs in an *in vitro* model of OA. Prior to further investigation of αIL-6 antibody in equine models of OA, a targeted, neutralizing monoclonal antibody is needed to prevent off-target effects and to increase efficacy of IL-6 neutralization. Alternatively, small interfering RNAs could be used to fully silence expression of IL-6 by chondrocytes and synoviocytes. As a further limitation, it is likely that residual synovial macrophages were present in tri-cultures, and interactions between T cells and macrophages of non-matched horses may lead to induction of inflammation within tri-cultures that would not occur with matched samples. In future studies, the use of matched samples may be warranted to avoid these adverse effects. Alternatively, the use of Treg secretome instead of Tregs themselves may be ideal to reduce inflammation in synovial fibroblasts and chondrocytes while simultaneously promoting M2 phenotype in macrophages.

Overall, restoration of joint model homeostasis by Tregs was more successful in combination with treatment of αIL-6 antibodies and use of αIL-6 antibodies to treat early OA should therefore continue to be investigated either as a monotherapy, or in combination with treatment using exogenous IL-10 or IL-4. Synoviocyte/chondrocyte donor had a significant effect on gene expression analysis and IL-6 concentration in conditioned media, but Treg donor did not. This indicates that αIL-6 therapy would need to be applied on a patient-specific basis, when there is evidence of elevated IL-6 in synovial fluid and IL-6 neutralization would be most effective in mitigation of inflammation and OA.

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Author contributions

LEK designed the study, performed data collection, analyzed the data, and drafted the manuscript. EDTW and LAF contributed to study design, data interpretation, and manuscript preparation. All authors approved the final version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Table S3.1. Equine-specific Taqman® primers and probes used in gene expression analysis with a Viia 7 Real-Time PCR System (Applied Biosystems, Foster City, CA).

Gene	Abbreviation	Function	Sense	Anti-sense	Probe
18S ribosomal RNA	*18S*	Ribosome structural unit	GGCGTCCCCCAACTTCTT	AGGGCATCACAGACCTGTTATTG	TGGCGTTCAGCCACCCGAGATT
Collagenase 3	*MMP13*	Matrix metalloproteinase	TGAAGACCCGAACCCTAAACAT	GAAGACTGGTGATGGCATCAAG	CAAAACACCAGACAAATGCGATCCTTCCTTA
Interleukin 6	*Il6*	Inflammatory cytokine	AGTAACCACCCCTGACCCAACT	TGTTGTGTTCTTCAGCCACTCA	CCTGCTGGCTAAGCTGCATTCACAGA
Tissue inhibitor of metalloproteinases 1	*TIMP1*	Inhibitor of matrix metalloproteinases	TGGAGAGCCTCTGCGGATAC	CCGGCGATGAGAAACTCTTC	CCACAGGTCGGAGAACCGCAGC
Collagen 2 isoform b	*Col2b*	Cartilage extracellular matrix	CGCTGTCCTTCGGTGTCA	CTTGATGTCTCCAGGTTCTCCTT	TCCGGCAGCCAGGACCGAA
Aggrecan	*Acan*	Cartilage extracellular matrix	GATGCCACTGCCACAAAACA	GGGTTTCACTGTGAGGATCACA	CCGAGGGTGAAGCTCGAGGCAA

CHAPTER 4

PHENOTYPE SWITCHING OF REGULATORY T CELLS TO T HELPER 17-LIKE REGULATORY T CELLS IN NATURALLY OCCURING POSTTRAUMATIC OSTEOARTHRITIS

Laura E. Keller[1], Elia D. Tait Wojno[2], Lisa A. Fortier[1]

[1]Cornell University, College of Veterinary Medicine, Department of Clinical Sciences

[2]University of Washington, Department of Immunology

Abstract

Objective: This study aimed to characterize Regulatory T (Treg) cell and T Helper 17 (Th17) cells populations in synovial fluid from equine clinical patients with posttraumatic osteoarthritis (PTOA). We hypothesized that a dynamic Treg:Th17 imbalance would be associated with disease progression, suggesting multiple opportunities for immunomodulatory therapy.

Methods: Synovial fluid was aspirated from the joints of equine clinical patients undergoing arthroscopic surgery for PTOA resulting from intra-articular fragmentation. Joints were classified as mild or moderate PTOA. Synovial fluid was also obtained from unoperated (non-op) horses with normal cartilage at euthanasia. Peripheral blood was obtained from horses with normal cartilage, and mild PTOA, and moderate PTOA. Synovial fluid and peripheral blood cells were analyzed by flow cytometry and native synovial fluid was analyzed by single and multiplex ELISA.

Results: Detection of IL-10, IL-17A, IL-6, CCL2, and CCL5 by ELISA showed that concentrations were not different between groups. Most lymphocytes in synovial fluid were $CD3^+$ T cells, which increased in moderate PTOA. $CD4^+$ T helper cell populations were decreased in moderate PTOA while $CD14^+$ macrophages were increased in moderate PTOA. Less than 5% of $CD3^+$ T cells found within the joint were $Foxp3^+$ Tregs, but a higher percentage of joint Tregs secreted IL-10 than their peripheral blood counterparts. T Regulatory 1 cells that secreted IL-10 but did not express Foxp3 were present in all joints. Th17 cells and Th17-like Tregs were increased in moderate PTOA.

Conclusions: Most lymphocytes in synovial fluid from normal and PTOA patients were $CD3^+$ T cells, suggesting that T cells are important players in the joint and thus potential targets for immunotherapy. $Foxp3^+$ Tregs were present in normal joints, indicating tissue residence, but small and unchanged populations of Tregs suggests failure to either home or maintain these populations within damaged joints.

IL-17A contributes to catabolism and joint destruction and Th17 cells are likely contributors to joint destruction in moderate PTOA. Conversion of Treg to Th17-like Tregs suggests that the exposure of joint Tregs to the milieu of pro-inflammatory cytokines within the inflamed joint causes Treg dysfunction.

Introduction

Posttraumatic osteoarthritis (PTOA) develops following traumatic injury or mechanical instability in a joint. Most patients that develop PTOA have low-grade infiltration of immune cells, including T cells and macrophages, into the synovium and synovial fluid of the affected joint.[1] In mouse models of PTOA, T cells, including pro-inflammatory T Helper 17 (Th17) cells, infiltrate the joint within one week after injury,[2] and within one-month post-injury, immunosuppressive Regulatory T cells (Treg)[3] home to the joint. Th17 cells release pro-inflammatory and pro-catabolic cytokines, including IL-17A and IL-22[4] while Tregs control immune response and resolution through secretion of anti-inflammatory cytokines including IL-10 and TGF-β1 and through cell-to-cell interactions mediated by immune checkpoints including CTLA-4 and PD-L1.[5] Studies suggest that an imbalance of infiltrating T cells to favor Th17 over Treg phenotypes contributes to chronic joint destruction in PTOA.[2,6–8]

Th17 cells are named for their hallmark production of the pro-inflammatory cytokine IL-17A. IL-17A leads to joint catabolism through increased synthesis of MMP1 and MMP13 in chondrocytes and synoviocytes.[9,10] In an anterior cruciate ligament transection mouse model of PTOA, IL-17A expression was increased in $CD4^+$ T cells in bulk, digested joints within one week after injury.[2] The deleterious effects of IL-17A were further suggested by improved Osteoarthritis Research Society International (OARSI) Scores following intraarticular administration of IL-17A-neutralizing antibodies. In human patients with varying grades of knee OA, IL-17A was increased in synovial fluid compared to being below the lower limit of detection in unmatched controls and was positively correlated with OA pain.[11] IL-17A has been similarly detected in the synovial fluid of PTOA patients with both high and low energy acute tibial plateau fractures[8] as well as wrist and primary knee PTOA.[7] Furthermore, IL-17A gene and protein expression were increased in inflamed synovium as defined by macroscopic hypervascularization and hypertrophic and hyperemic villi further indicating a role to Th17 cells in the pathogenesis of PTOA.[12]

T regulatory (Treg) cells act in opposition to Th17 cells and play an immunomodulatory role through production of anti-inflammatory cytokines including IL-10, IL-35, and TFG-β1. Tregs also suppress activity of cells in both the innate (monocytes/macrophages)[13] and adaptive (T cells) immune systems[14]. The chondroprotective role of IL-10 is supported by *ex vivo* and *in vivo* animal model studies. Bovine cartilage explants treated with IL-10 and then subjected to unconfined axial compression had reduced glycosaminoglycan release and decreased *MMP3*, *MMP13*, and *ADAMSTS4* gene expression compared to controls subject to loading but not treated with IL-10.[15] Histological scores were improved in a rabbit model of PTOA at 21 days post-injury following intraarticular injection of synoviocytes overexpressing IL-10 at 5 days post-injury.[16] In human subjects with tibial plateau fractures, IL-10 was increased in synovial fluid within 24 hours following injury[8] and within 48 hours - 15 days following anterior cruciate ligament tear[17].

In these same patient cohorts, IL-6 was increased an average of 500-fold in injured compared to uninjured knees[8] and was increased in acute (0-15 days) compared to late sub-acute and chronic (>15 days),[17] but was still higher than reported concentrations for normal joints. And although typically associated with autoimmune arthritis, IL-23 was secreted by both inflamed and non-inflamed synovium from patients with end-stage knee OA.[12] This type of pro-inflammatory microenvironment with increased concentrations of IL-6 and IL-23 can suppress the Treg master transcription factor Foxp3 and drive activation of Th17 master transcription factor RORγt, resulting in Th17-like Tregs that maintain Foxp3 expression while secreting IL-17A.[18] Although not yet described in PTOA, this Treg phenotype plasticity is present in autoimmune arthritis[19,20] and in mice with collagen-induced arthritis[21].

These studies suggest that Treg:Th17 imbalance could contribute to PTOA progression and joint destruction. There is a significant knowledge gap in understanding the importance of the Th17/Treg ratio

and plasticity of phenotype and metabolic function in early OA. This is important because to truly intervene in disease progression, therapies to mitigate OA must be applied early, before damage is irreversible. To address these knowledge gaps, synovial fluid samples were collected from horses with naturally occurring PTOA and from matched or unmatched normal joints. The horse is an established model for PTOA with cartilage thickness and biomechanics approximating that of humans.[22–24] Here, we tested the hypothesis that a dynamic Treg:Th17 imbalance is present in PTOA and provides multiple opportunities for immunomodulatory therapy.

Materials and Methods

An overview of the study design is presented in Figure 1. All procedures were approved by the Institutional Animal Care and Use Committee.

Sample collection and stratification - Synovial fluid samples were obtained from client horses undergoing arthroscopic surgery for naturally occurring PTOA resulting from intra-articular fracture fragmentation (**Figure 4.1a**). Joints were classified by the operating surgeon (LAF) as mild PTOA (n=10) if cartilage outside of the fracture plane was visibly and palpably normal; moderate PTOA (n=11) if there was chondromalacia or surface fibrillation within the joint; no joint was considered as advanced PTOA with full thickness cartilage loss. Matched normal, contralateral joint synovial fluid (non-op normal) was obtained from mild (n=5), and moderate (n=1) PTOA cases. Synovial fluid (n=33) was also obtained from joints of horses that were euthanized for reasons unrelated to this study, and with normal appearing articular cartilage at necropsy (normal). Peripheral blood was obtained from horses with mild (n=4) and moderate (n=4) PTOA and healthy horses (n=10) for isolation of peripheral blood mononuclear cells.

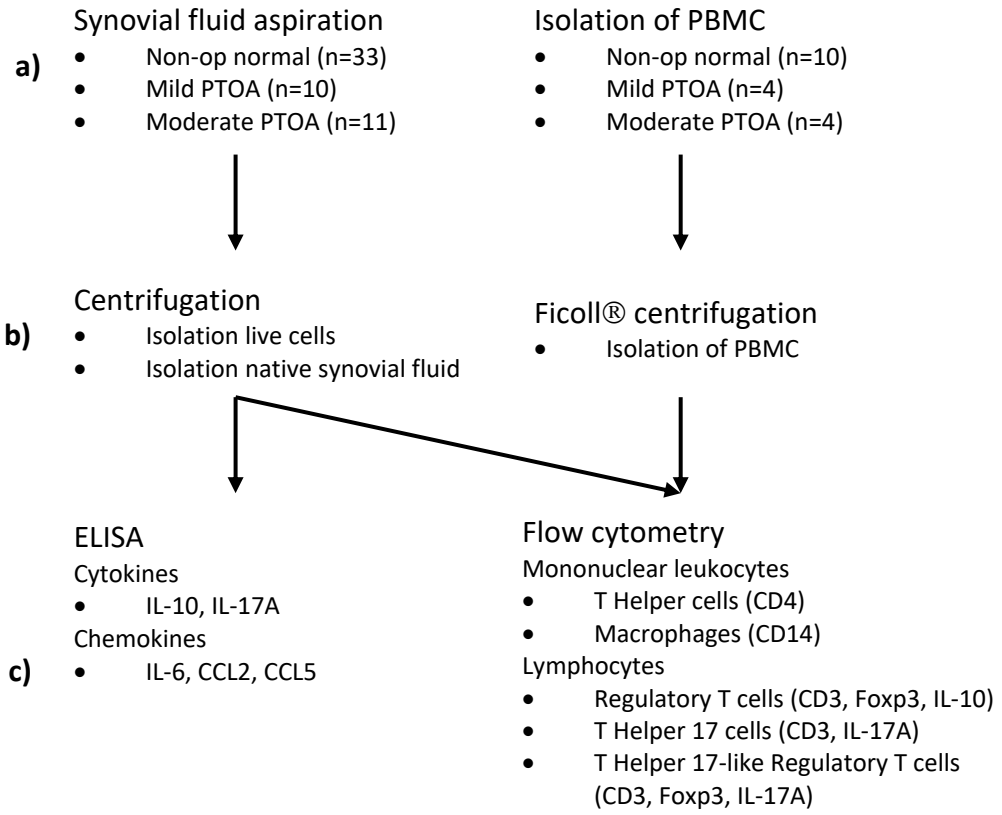

Figure 4.1 *Study design overview* **a)** Synovial fluid was aspirated from equine clinical patients undergoing arthroscopic surgery for PTOA resulting from intra-articular fragmentation. Joints were classified as mild (n=10) or moderate PTOA (n=11). Synovial fluid was also obtained from unoperated (non-op) horses with normal cartilage at euthanasia (n=33). Peripheral blood was obtained from non-op normal horses (n=10), and horses with mild PTOA (n=4) and moderate PTOA (n=4). **b)** Synovial fluid was centrifuged to obtain live cells and cell-free supernatant. Peripheral blood was centrifuged over Ficoll® to isolate peripheral blood mononuclear cells (PBMC). **c)** Native synovial fluid was analyzed by single and multiplex ELISA and synovial fluid cells and PBMC were analyzed by flow cytometry.

Sample preparation - Synovial fluid samples were centrifuged at 1,800 *xg* for 15 minutes to isolate viable cells and retain a cell-free supernatant (**Figure 4.1b**). Supernatants were stored at -80°C for subsequent chemokine and cytokine analysis (**Figure 4.1c**). The cell pellet was washed twice with 1mL of modified DMEM containing 10% fetal bovine serum, 0.1 mM 2-mercaptoethanol, penicillin (100 U/mL), streptomycin (100 μg/mL), sodium pyruvate (1 mM/mL), and MEM non-essential amino acids. Peripheral blood mononuclear cells were isolated using Ficoll®-Paque PLUS density gradient centrifugation according to the manufacturer directions (GE Healthcare, Chicago, IL) and resuspended in modified DMEM.

Flow cytometry to identify cell populations and measurement of intra-cellular cytokines - Cells isolated from synovial fluid and peripheral blood were analyzed by flow cytometry to assess population dynamics of T cells and macrophages. To identify $CD4^+$ T helper cells and $CD14^+$ macrophages, cells from synovial fluid and peripheral blood were labeled with mouse anti-equine CD4 (Washington State University, Pullman, WA)[25] conjugated to Alexa Fluor 488 (Invitrogen, Waltham, MA) and mouse anti-equine CD14 (Wagner Laboratory, Ithaca, NY)[26] conjugated to Alexa Fluor 647 (Invitrogen) at room temperature for 30 minutes, then fixed prior to flow cytometric analysis.

To identify Tregs (CD3, Foxp3, IL-10) and Th17 cells (CD3, IL-17A) within the T cell population, leukocytes from synovial fluid and peripheral blood were stimulated for 4-6 hours with phorbol 12-myristate 13-acetate (PMA) (25 ng/mL) and ionomycin (1 μM) to stimulate cytokine expression and in the presence of 1:1000 brefeldin A (Invitrogen) to prevent cytokine secretion. To analyze expression of CD3, intracellular cytokines (IL-10, IL-17A) and Foxp3, cells were then fixed, permeabilized and stained using the Foxp3 Transcription Factor Staining Buffer Set (Invitrogen) per the manufacturer's instructions. Cells were stained with rat anti-mouse Foxp3 PE (Invitrogen), rat anti-human CD3 Pacific Blue

(Thermofisher), mouse anti-equine IL-10 (Wagner Laboratory)[27] conjugated to Alexa Fluor 488, and mouse anti-equine IL-17A (Wagner Laboratory)[28] conjugated to Alexa Fluor 647.

Analysis for T helper cells (CD4) and macrophages (CD14) was performed by gating for mononuclear cells (**Figure S4.1**), while analysis for Tregs (CD3, Foxp3, IL-10), Th17 cells (CD3, IL-17A), and Th17-like Tregs (CD3, Foxp3, IL-17A) was performed by gating for lymphocytes. Both initial gating strategies were followed by a forward scatter doublet exclusion, then side scatter double exclusion. Gates for all fluorescent markers were determined by unstained and fluorescence-minus-one controls. Flow analysis was performed using a BD FACSymphony A5 Cell Analyzer (BD, Franklin Lakes, NJ) and analyzed with Flowjo software (TreeStar, Inc, Ashland, OR).

Cytokine and chemokine analysis in synovial fluid - Cytokines (IL-4, IL-10 and IL-17A) and chemokines (CCL2, CCL5) were measured using an equine specific multiplex ELISA as previously described.[29] Concentration of IL-6 in synovial fluid was separately measured according to manufacturer directions (R&D Systems, Minneapolis, MN).

Statistical analysis - Cell populations in synovial fluid and peripheral blood, and cytokine/chemokine concentrations in synovial fluid were analyzed using a generalized linear model with horse as a random effect. Tukey's post-hoc was used with p values ≤ 0.05 were considered significant. Statistical analyses were performed using JMP Pro 15 (SAS Institute, Cary, NC).

Results

Detected chemokines in native synovial fluid were unchanged throughout PTOA progression -
Many of the cytokines analyzed remained below the limit of detection and did not correlate with disease stage. Neither IL-6 ($p>0.15$), CCL2 ($p>0.4$), nor CCL5 ($p>0.9$) were significantly different between

Table 4.1 *There was detectable CCL2 within all groups, but IL-6 and CCL5 may wax and wane as PTOA progresses*. Concentrations of IL-6, CCL2, and CCL5 were variable between groups and between joints. GLM followed by Tukey's HSD post-hoc.

				***p* values**		
	Non-op	**Mild PTOA**	**Moderate PTOA**	**Non-op: Mild**	**Non-op: Moderate**	**Mild: Moderate**
IL-6 (pg/mL)	404 ±633 n=10	ND	2843±4211 n=3	N/A	0.35	N/A
CCL2 (pg/mL)	7322±9211 n=36	452±422 n=9	6308±13543 n=8	0.14	0.92	0.98
CCL5 (pg/mL)	74±100 n=7	100±75 n=4	ND	0.91	N/A	N/A

groups (**Table 1**). IL-6 in mild PTOA and CCL5 in moderate PTOA were detected in fewer than three samples, and therefore not used for statistical analysis. Only one non-op normal sample had detectable IL-10 (504 pg/mL), and three mild PTOA samples had detectable IL-10 (208±166 pg/mL). Only one moderate PTOA sample had detectable IL-17A (14 U/mL).

T cell and macrophage populations in synovial fluid increased as PTOA progressed without systemic changes in peripheral blood cell populations - $CD3^+$ T cells were the predominant lymphocyte in synovial fluid. In all joints, more than 80% of lymphocytes in synovial fluid were $CD3^+$ T cells (**Figure 4.2a and 4.2b**), which increased to 90% in moderate PTOA compared to non-op normal synovial fluid ($p=0.02$). $CD3^+$ T cells were increased in moderate PTOA synovial fluid compared to peripheral blood ($*p=0.001$), and in mild PTOA synovial fluid compared to peripheral blood ($*p=0.04$). The $CD4^+$ T cells and $CD14^+$ macrophage populations made up approximately 28% of the mononuclear cell population in non-op normal joints (**Figure 4.3a**), and decreased by nearly one half in moderate PTOA, down to 16% ($p=0.01$). $CD4^+$ T cells were a lower percentage in all joints compared to peripheral blood, where $CD4^+$ T cells comprised about 50% of the mononuclear cell population ($*p<0.009$). $CD14^+$ macrophages followed an inverse trend to $CD4^+$ T cells, where they made up about one fifth of the population in non-op normal and mild joints (**Figure 4.3b and 4.3c**) and were increased in moderate PTOA by an average of more than two-fold when compared to mild ($p=0.001$) and non-op normal ($p<0.0001$). CD14+ macrophages were increased in all joints by more than four-fold when compared to peripheral blood ($*p=0.02$). The was no effect of PTOA on peripheral blood populations of $CD3^+$ T cells ($*p>0.97$), $CD4^+$ T cells ($*p>0.6$), or $CD14^+$ macrophages ($*p>0.3$; data not shown). Therefore, non-op normal peripheral blood samples were used for comparisons.

$Foxp3^+$ Regulatory T cell populations were in low abundance and unchanged during PTOA – In all joint groups, less than 6% of $CD3^+$ T cells in the synovial fluid expressed Foxp3 (**Figure 4.4a and 4.4b**), and

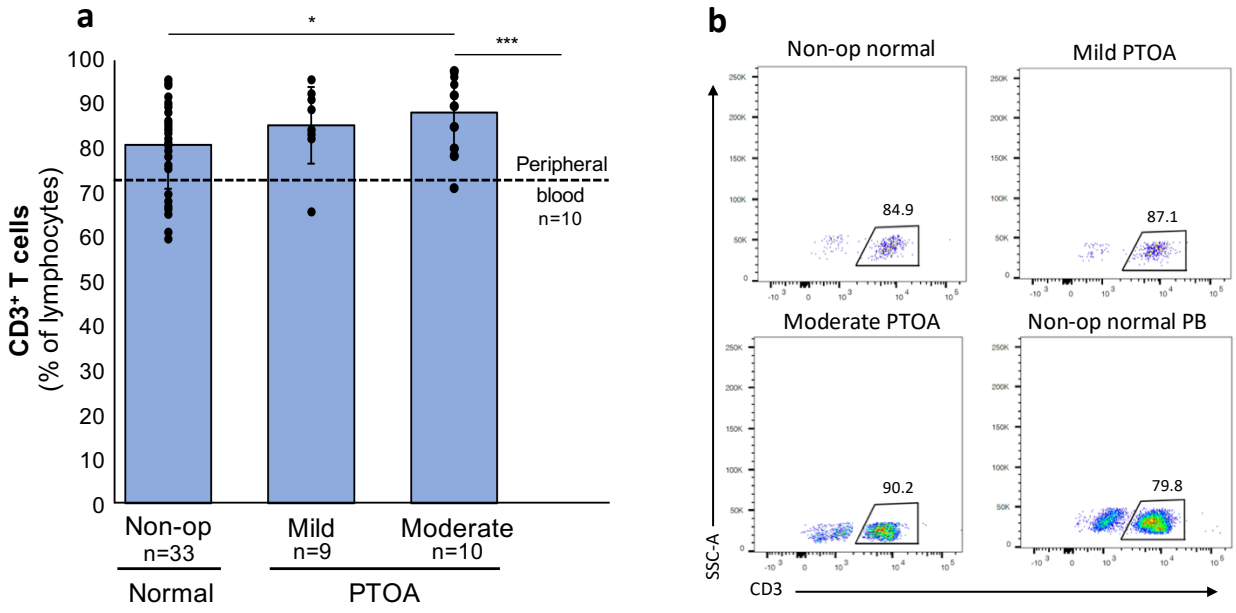

Figure 4.2. *CD3$^+$ T cell populations increased with disease progression* **a)** CD3$^+$ T cells were increased in moderate PTOA compared to non-op normal and peripheral blood **b)** Flow cytometry identification of CD3$^+$ T cells. GLM followed by Tukey's HSD post-hoc, *p<0.05, ***p<0.0001.

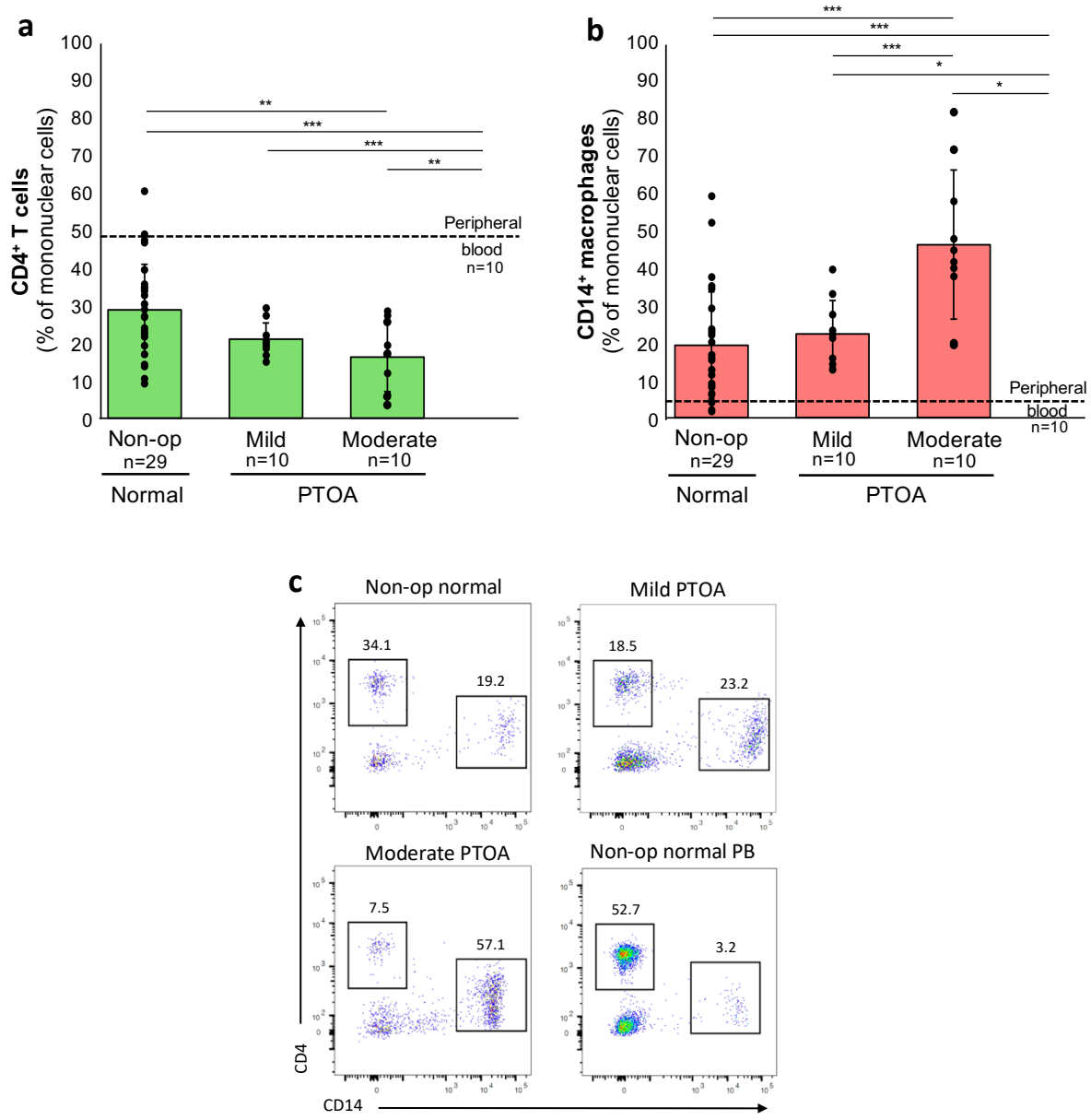

Figure 4.3. *$CD4^+$ T cell and $CD14^+$ macrophage populations shared an inverse relationship as disease progressed.* **a)** $CD4^+$ T cell populations decreased as PTOA progressed **b)** In contrast, $CD14^+$ macrophages were increased in all joints compared to peripheral blood. **c)** Flow cytometry identification of $CD4^+$ T cells and $CD14^+$ macrophages. GLM followed by Tukey's HSD post-hoc, *p<0.05, **p<0.001, ***p<0.0001.

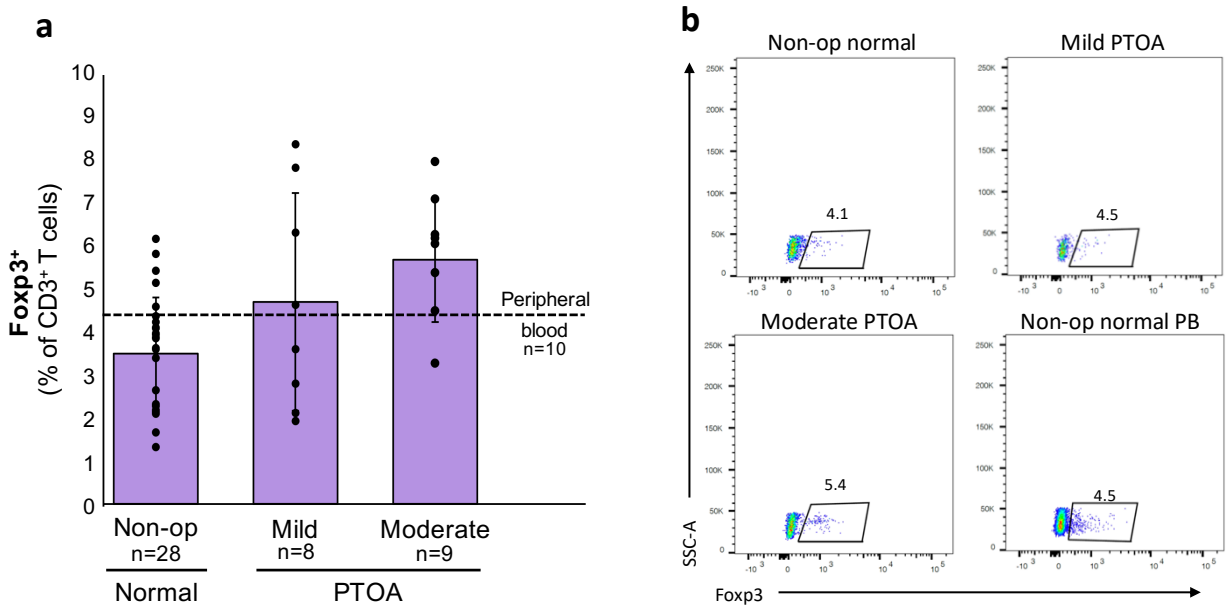

Figure 4.4. *Few immunosuppressive T cells were present in normal and PTOA joints.* **a)** Treg populations remained stable as PTOA progressed and were not different than percentages found in peripheral blood. **b)** Flow cytometry identification of $Foxp3^+$ cells within the $CD3^+$ T cell population. GLM followed by Tukey's HSD post-hoc, *p<0.05, **p<0.001, ***p<0.0001.

Treg populations were not different between groups ($p>0.2$). Secretion of IL-10 by Tregs was not different between joint groups upon PMA/ionomycin stimulation (**Figure 4.5a**; $p>0.5$). More Tregs in both non-op ($p<0.0001$) and mild PTOA ($p=0.005$), but not moderate PTOA ($p=0.06$) secreted IL-10 than in peripheral blood. Interestingly, most of the IL-10-secreting T cells in synovial fluid did so independently of Foxp3 expression (**Figure** 4.**5b and 4.5c**), indicating that there are multiple anti-inflammatory T cell populations active within all joints.

T Helper 17 cells and T Helper 17-like Regulatory T cells increased in synovial fluid as PTOA progressed - In non-op normal and mild PTOA joints, IL-17A-secreting Th17 cells accounted for less than 1% and 2% of the T cell populations, respectively (**Figure 4.6**). Despite the presence of the anti-inflammatory populations mentioned above, Th17 cells were increased by more than seven-fold in moderate PTOA compared to non-op normal ($p<0.0001$), and mild PTOA ($p<0.0001$). Th17 cells were also significantly increased in moderate PTOA joints compared to peripheral blood (*$p<0.0001$). Continuous exposure of Tregs to pro-inflammatory cytokines such as IL-6 and IL-1β can result in Treg phenotype plasticity, resulting in differentiation of Tregs to Th17-like Tregs that stably express Foxp3, but also secrete IL-17A.[18] In non-op normal and mild PTOA joints, less than 1% of Tregs secreted IL-17A (**Figure 4.7a and 4.7b**). However, there was an eight-fold increase of Th17-like Tregs in moderate PTOA compared to mild PTOA ($p<0.0001$) and non-op normal joints ($p<0.0001$). There was also a four-fold increase in Th17-like Tregs in moderate PTOA joints compared to non-op normal peripheral blood (*$p<0.0001$). Moreover, there was also an increase in Th17-like Tregs in the blood of horses with moderate PTOA ($p=0.02$) compared to non-op normal, suggesting systemic changes in Treg populations as PTOA progresses (data not shown).

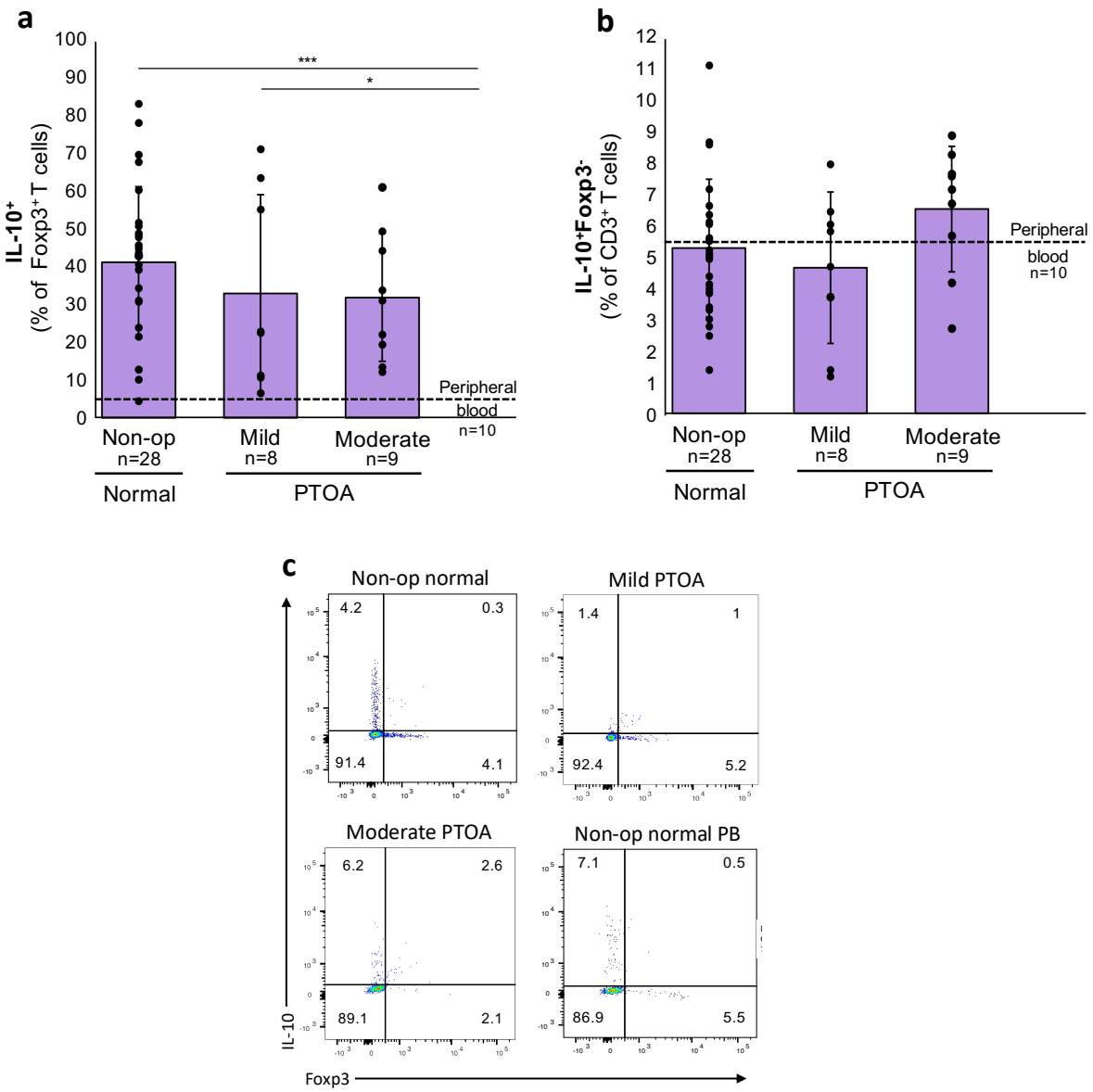

Figure 4.5. *Foxp3$^+$ Tregs within the joint actively secreted immunosuppressive IL-10, but most IL-10-secreting cells did not express Foxp3*. **a)** A higher percentage of Tregs in non-op normal and mild PTOA joints secreted IL-10 following stimulation than in peripheral blood, but Tregs in moderate PTOA were not different than any other groups. **b)** The majority of IL-10$^+$ T cells in joints did not express Foxp3, suggesting there are multiple anti-inflammatory T cell subtypes. **c)** Flow cytometry identification of IL-10$^+$ and Foxp3$^+$ cells within the CD3$^+$ T cell population. GLM followed by Tukey's HSD post-hoc, *p<0.05, **p<0.001, ***p<0.0001.

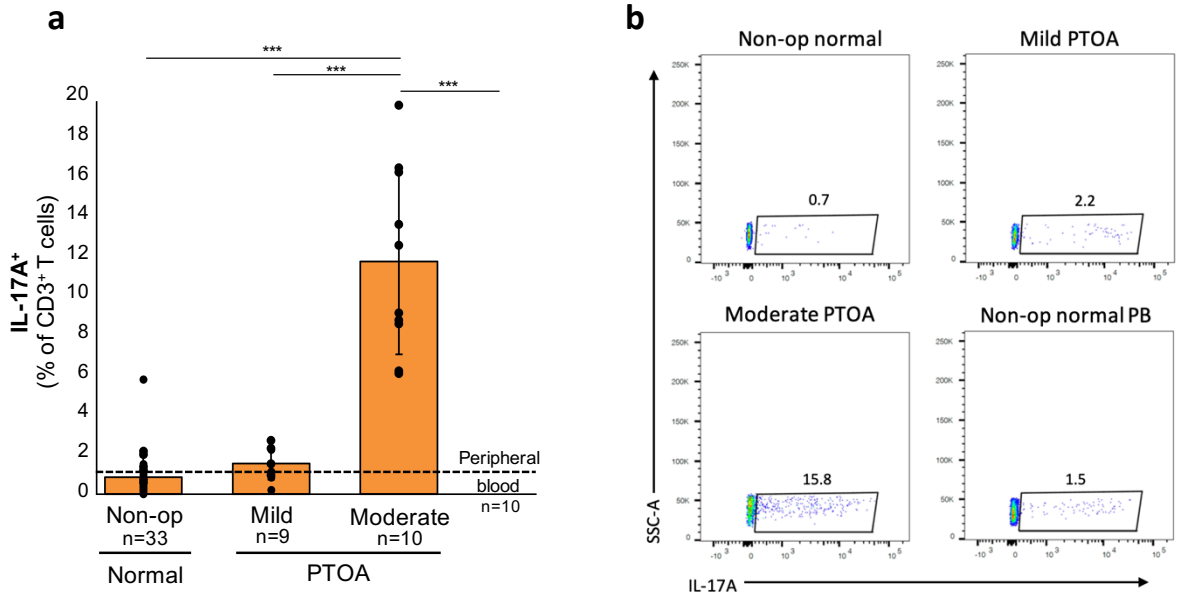

Figure 4.6. *Inflammation predominated in moderate PTOA.* **a)** Th17 cells increased in moderate PTOA compared to normal non-op, mild PTOA joints, and peripheral blood. **b)** Flow cytometry identification of IL-17A$^+$ cells within the CD3$^+$ T cell population. GLM followed by Tukey's HSD post-hoc, *p<0.05, **p<0.001, ***p<0.0001.

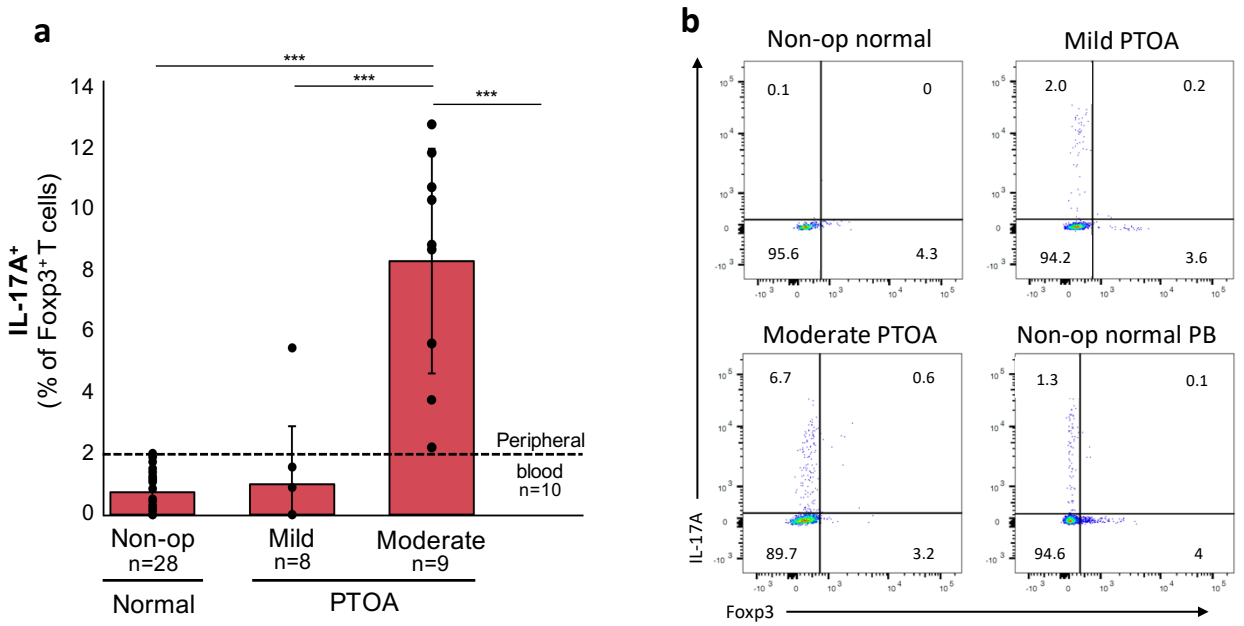

Figure 4.7. *Foxp3$^+$ Tregs appeared to have undergone phenotypic plasticity during progression of PTOA.* **a)** In moderate PTOA, more Tregs secreted IL-17A than Tregs in mild PTOA and peripheral blood, suggesting the inflammatory moderate PTOA environment promoted Tregs to switch to Th17-like Tregs. **b)** Flow cytometry identification of IL-17A$^+$ and Foxp3$^+$ cells within the CD3$^+$ T cell population. GLM followed by Tukey's HSD post-hoc, *p<0.05, **p<0.001, ***p<0.0001.

Discussion

There is increasing evidence from human clinical patients and animal models that immune cells within the joint play a pivotal role in maintaining homeostasis, as well as mediating disease progression following traumatic joint injury. In this study, utilization of the equine model has allowed for the first time, a study of Treg and Th17 population dynamics in the synovial fluid and peripheral blood of both healthy patients and those with naturally occurring PTOA. Our results showed that $CD3^+$ T cells represented most of the lymphocyte population in healthy and PTOA joints, that Tregs were present prior to and following traumatic injury to the joint, and that Th17 cells accumulate in joints with moderate PTOA. Our data also indicate that Tregs undergo a pathogenic phenotypic switch to Th17-like Tregs in moderate PTOA, likely further contributing to inflammation and catabolism within the joint.

In the present study, not only did $CD3^+$ T cells make up the largest proportion of the lymphocyte population as PTOA progressed previous studies have revealed that T cells are major contributors to the immune cell population within the joint.[6,30] Moreover, we found $CD14^+$ macrophages to be in nearly equal populations to $CD4^+$ T cell in healthy joints, accounting for about one-fifth and one-fourth of the mononuclear cell population, respectively. This is in contrast to other studies where macrophages are found to be the most abundant immune cell type in synovial fluid.[32,33] This may be explained by differences in methods of cell isolation and identification. Gómez-Aristizábal et al. centrifuged synovial fluid at 12,000 *xg*, whereas we have found that rates as low as 5,000 *xg* result in a compact cell pellet that cannot be fully resuspended, and Menarim et al., used total nucleated cell count to determine macrophage counts within synovial fluid.

$Foxp3^+$ Tregs have not previously been identified in normal synovial fluid. Their presence suggests that synovial tissues harbor a population of tissue-resident Tregs that maintain homeostasis and control immune responses similar to skeletal muscle[31] and adipose tissue[32]. Tissue-resident Tregs are transcriptionally and functionally distinct from their circulating and lymphoid tissue counterparts, and it is therefore likely that Tregs within synovial tissues comprise a distinct and unique population of tissue-

resident Tregs.[33,34] This is supported by the finding that more Tregs from normal and mild PTOA synovial fluid secreted IL-10 in response to PMA/ionomycin stimulation than did those from peripheral blood. A decrease in IL-10 expression by peripheral blood Tregs was observed Li et al.[35] in patients with mild and severe knee OA. Although there was no difference in Treg ability to secrete IL-10 between groups of equine patients, this may have been due to Li et al. using $CD4^{+}CD25^{hi}$ to sort for Tregs, whereas in the present study Foxp3 was used as the Treg marker. Of note, the majority of IL-10-secreting T cells did not express Foxp3, suggesting that there is a population of T Regulatory 1 cells also present in normal and PTOA joints to aid in suppression of tissue inflammation, though a broader flow cytometry panel, including CD49b and LAG3, would be needed to further classify this subset of cells.[36]

Th17 cells and their hallmark cytokine, IL-17A are more typically associated with autoimmune diseases such as rheumatoid [37] and psoriatic arthritis ,[38] IL-17A has been found in the synovial membrane and fluid of patients with OA [6,11,39] and a mouse model of PTOA.[2] IL-17A is known to induce the release of catabolic factors, including MMP1 and MMP13, from both chondrocytes and synoviocytes.[9] To the authors' knowledge, the presence of Th17 cells has not previously been reported in normal synovial fluid. However, tissue-resident Th17 cells in visceral adipose tissue that maintain tissue homeostasis have been described, and suggest a similar role for Th17 cells in normal synovial fluid in the absence of trauma or inflammation.[40] The six-fold increase in the percent of Th17 cells in moderate PTOA compared to mild PTOA indicates that PTOA severity drives homing of Th17 cells to the joint, their proliferation within the joint, or pathogenic conversion of naïve or effector T cells to a Th17 phenotype within the joint.

This is the first report of Th17-like Tregs in PTOA. The pathogenic polarization of Tregs to Th17-like Tregs has been previously reported in autoimmune diseases including rheumatoid arthritis[19,21,41] and juvenile idiopathic arthritis.[20] Not only were Th17-like Tregs significantly increased in the synovial fluid of joints with moderate PTOA compared to mild PTOA and normal joints, which was reflective of local inflammation, but Th17-like Tregs were also increased in the peripheral blood of horses with moderate PTOA, indicating that inflammation is also systemic. Increased concentration of IL-6 in moderate PTOA likely contributed to this phenotypic plasticity. In human patients, IL-6 has been

detected in the synovial fluid of patients with OA [6] and those with anterior cruciate ligament injury, who are at risk of developing PTOA.[42] This increase in IL-6 likely contributes to the conversion to a Th17-like phenotype by Tregs.[18]

There are several limitations to this current work. Although CD4 is typically used in flow cytometry to define Treg and Th17 populations, our group found that CD4 on equine T cells became unstable following stimulation with PMA/ionomycin and was no longer detectable by flow cytometry. We found that CD3 was a considerably more reliable marker for flow cytometric analysis. We did not use magnetic sorting to isolate $CD4^+$ T cells in advance of stimulation to avoid excess loss of cells, especially from non-op normal samples, which tended to be less cellular than PTOA samples. In future studies, more refined analyses of T cell populations within the synovial fluid, such as RNA-seq, are warranted to gain further insight into the mechanisms behind failure of Tregs to maintain homeostasis and prevent disease progression, as well as those driving pathogenic conversion of Tregs to Th17-like Tregs.

The goal of this study was to investigate T cell populations within the synovial fluid to provide potential targets for immunotherapeutic intervention to prevent disease progression. We found that the majority of lymphocytes in normal and PTOA joints are $CD3^+$ T cells, and that they are enriched compared to peripheral blood suggesting that PTOA is not a systemic immune disease. Although $Foxp3^+$ Tregs are present within the joint following injury, they do not appear able to prevent PTOA progression. The marked increase in Th17 cells and Th17-like Tregs in moderate PTOA implicates that there should be further investigation into IL-17A as a target for immunotherapy. Anti-IL-17A antibodies have met with success in reducing radiographic disease progression in the treatment of rheumatoid[43] and psoriatic arthritis,[44] which similarly present with higher numbers of Th17 cells in synovial fluid. Anti-IL-17A for treatment of OA is patented, but to the authors' knowledge there are no reports of its application in pre-clinical animal models or patients. Irrespective of IL-17A, our data suggest that persistent T cell populations within in the joint, and a fluctuating Treg:Th17 imbalance in the time course of disease provides multiple opportunities for immunotherapeutic intervention aimed at restoring joint homeostasis,

and that appropriate timing and targets of immunotherapies in the treatment of PTOA are critical to improve patient clinical outcomes.

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Author contributions

LEK designed the study, performed data collection, analyzed the data, and drafted the manuscript. EDTW and LAF contributed to study design, data interpretation, and manuscript preparation. All authors approved the final version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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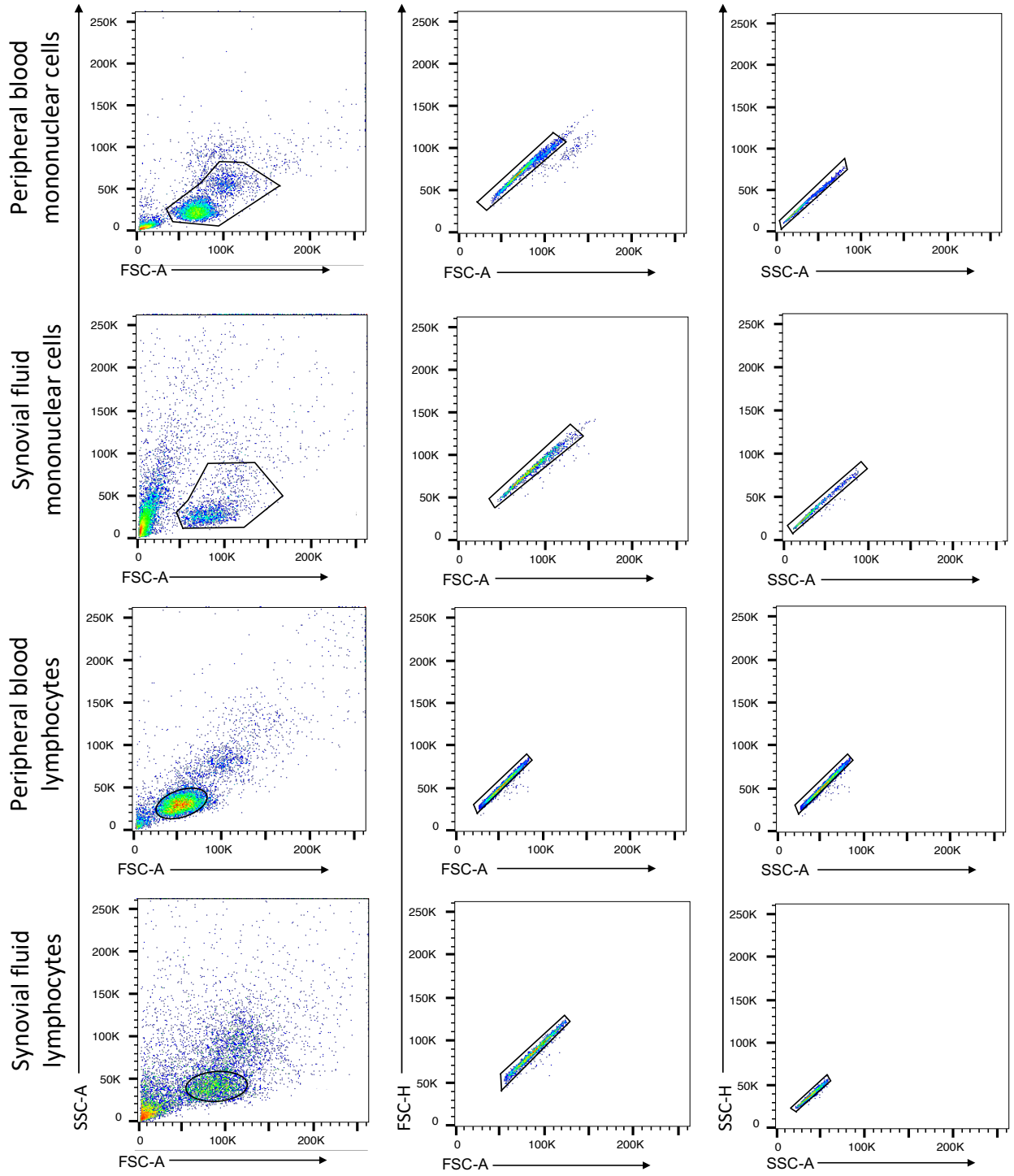

Figure S4.1. *Flow cytometry gating strategies and doublet exclusion* **a)** Peripheral blood mononuclear cells **b)** Synovial fluid mononuclear cells **c)** Peripheral blood lymphocytes **d)** Synovial fluid lymphocytes.

CHAPTER 5

PROTEOMICS REVEALS MULTIPLE INFLAMMATORY PATHWAYS AND NOVEL PROTEINS IN PATIENTS WITH PERSISTENT INFLAMMATION DEFINED BY HIGH INTERLEUKIN-6 SYNOVIAL FLUID CONCENTRTAION FOUR WEEKS AFTER ANTERIOR CRUCIATE LIGAMENT RECONSTRUCTION

Laura E. Keller[1], Cale A. Jacobs[2], Christian Lattermann[3], Emily R. Hunt[3], Sheng Zhang[4], Qin Fu[4], Lisa A. Fortier[1]

[1]Cornell University, College of Veterinary Medicine, Department of Clinical Sciences

[2]Department of Orthopedic Surgery and Sports Medicine, University of Kentucky

[3]Brigham and Women's Hospital, Harvard Medical School

[4]Cornell University, Biological Resource Center

Abstract

Objective: An unbiased, bottom-up proteomics approach was used to discover novel targets for therapeutics in relation to dysregulation in the orchestration of inflammatory pathways implicated in persistent joint inflammation after joint trauma.

Methods: Synovial fluid was aspirated from patients at 1-week and 4-weeks post ACL reconstruction and IL-6 concentrations were quantified by ELISA. Patients were segregated into IL-6^{low} and IL-6^{high} groups based on IL-6 concentrations in synovial fluid at 4-weeks postop and proteins in synovial fluid were analyzed using qualitative, bottom-up proteomics. Abundance ratios were calculated for IL-6^{high} and IL-6^{low} groups as 1-week postop: 4-weeks postop.

Results: A total of 292 proteins were detected in synovial fluid, of which 57 met criteria for further assessment. Proteins associated with complement pathways, neutrophil activation, cartilage catabolism and mitochondrial dysfunction were increased in the IL-6^{high} compared to IL-6^{low} group.

Conclusions: Direct and upstream targeting of proteins and pathways that are dysregulated early following joint trauma provides multiple existing and novel targets and pathways for disease-modifying therapeutics in the mitigation of PTOA.

Introduction

Posttraumatic osteoarthritis (PTOA) is the consequence of mechanical instability or joint trauma. Patients with anterior cruciate ligament (ACL) injury are at high risk of developing PTOA, with 50-60% of patients having radiographic changes consistent with osteoarthritis (OA) as early as 5 years, and clinical symptoms of OA within 10 years following injury.[1,2] Response to ACL reconstruction, rehabilitation, and treatment with anti-inflammatories has been met with varied results in the prevention of PTOA.[3,4] Traditional indices such as the extent of joint damage or patient pain have not been consistent predictors of success for the patient. Increasingly, the focus is on personalized medicine approaches to treatment, but predicative biomarkers need to first be identified.

Insights into why some patients recover while others with the same apparent magnitude of injury do not, come from studies in polytrauma patients where patients were identified as diverging into two subgroups during recovery which have been variably termed susceptible/non-responders and resistant/responders. These two groups could be identified based on their systemic inflammatory response in the acute stages following trauma, and define those who recover uneventfully, and those who suffer from persistent inflammation.[5,6] These outcomes have been tied to temporal coordination of inflammatory responses, and patients who show early establishment and resolution of inflammation tend to have better outcomes.[7,8]

Similarly, there is increasing focus on the inflammatory response within the joint following injury, and how the orchestration of this response is related to, and predictive of patient outcomes.[9,10] Increased IL-6 within the joint has been detected in the acute and chronic phases of PTOA after ACL injury or patients with partial meniscectomy and is associated with progression of OA.[11,12] IL-6 signaling activates JAK proteins leading to downstream activation of STAT family proteins, YAP-NOTCH pathways, PI3K-Akt signaling, MAPK cascade, and activation of NF-κB.[13,14] Subsequently, IL-6 signaling leads to an inflammatory cascade,[15] as well as to further release of IL-6 in a positive feedback loop.[16] Continued

elevation of IL-6 has also been indicated as a biomarker of chronic inflammation in multiple other diseases including rheumatoid arthritis[17] and several types of cancer,[18,19] IL-6 could therefore be considered as a biomarker to identify ACL injury patients with persistent joint inflammation/non-responders and those responder patients whose joint inflammation resolves after ACL repair.

The purpose of this study was to identify proteins which are dysregulated early in the inflammatory process that may be targets for therapy in the prevention of PTOA. We hypothesized that these dysregulated pathways would be associated with expression of IL-6 and could be identified using an unbiased, bottom-up proteomics approach.

Materials and Methods

Patients – Patients with primary anterior cruciate ligament (ACL) injury consented to enrollment in an institutional review board-approved randomized clinical trial assessing the use of hyaluronate injection vs placebo 1-week post reconstruction (clinicaltrials.gov: NCT03429140). Patients were enrolled within the first 10 days following ACL injury. To be included in this study, patients had to have an isolated ACL tear with no concurrent posterior crucial ligament (PCL) injury, and could not have a grade 3 medial or lateral collateral ligament injury. Patients were between the ages of 14 and 32, and were skeletally mature with closed knee growth plates verified via radiograph. They had to have no history of previous surgery on the ipsilateral or contralateral knee and their ACL injury had to occur during sports activity. Exclusion criteria included the ACL injury occurring more than 10 days prior to enrollment, previous ipsilateral or contralateral knee surgery, intra-articular cortisone injection into either knee within 3 months of injury, and a history of any inflammatory disease.

Study design – The current study is a secondary analysis of the previously mentioned randomized trial. One week post-operatively, patients received a knee aspiration, were randomized, and received an

injection of either hyaluronate (Gel-One, ZimmerBiomet, Warsaw, IN) or saline. Four-week post-reconstruction, all patients received a fourth and final knee aspiration. Synovial fluid was aspirated, aliquoted, and stored at -80°C for further analysis.

Biomarker assay – The synovial fluid biomarker for IL-6 representing joint inflammation was assessed using a commercially available enzyme-linked immunosorbent assay (ELISA, Meso Scale Discovery, Rockville, MD). The assay was run in duplicate, any samples outside the limits of detection or quantifications were rerun and intra-assay coefficients of variance were less than 9.5 for all plates. The IL-6 ELISA was completed per manufacturer guidelines. Based on IL-6 concentrations in synovial fluid at 4-weeks post-reconstruction, patients were placed into either an IL-6 low ($IL\text{-}6^{low}$) group (<316 pg/mL, n=6) or IL-6 high ($IL\text{-}6^{high}$) group (>316 pg/mL, n=10) to define persistent inflammation within the joint.

Bottom-up proteomics – The aim of this study was to use an unbiased approach to identify proteins and pathways involved in persistent inflammations as potential targets for therapeutic intervention. Therefore, the proteomic study was designed to identify proteins that were differentially regulated between $IL\text{-}6^{low}$ and $IL\text{-}6^{high}$ groups. Absolute protein quantification was not performed, and abundance ratios were used to compare groups.

In-gel trypsin digestion of SDS gel bands **-** Fifty micrograms of proteins for each sample from 16 pairs of samples were loaded and separated on 10% BisTris SDS gel under MES buffer. A gel band covering 15-45 kD for each sample was excised, cut into ~1 mm cubes and subjected to in-gel digestion followed by extraction of the tryptic peptide as reported previously.[20] The excised gel pieces were washed consecutively in 200 µL distilled water, 100 mM ammonium bicarbonate (Ambic)/acetonitrile (1:1) and acetonitrile. The gel pieces were reduced with 70 µL of 10 mM DTT in 100 mM Ambic for 1 hr at 56 °C, alkylated with 100 µL of 55 mM Iodoacetamide in 100 mM Ambic at room temperature in the dark for 60 mins. After wash steps as described above, the gel slices were dried and rehydrated with 50 µL trypsin in

50 mM Ambic, 10% ACN (20 ng/µL) at 37 °C for 16 hrs. The digested peptides were extracted twice with 70 µl of 50% acetonitrile, 5% FA and once with 70 µl of 90% acetonitrile, 5% FA. Extracts from each sample were combined, filtered by a 0.22-um spinning unit, and lyophilized.

Protein Identification by nano LC/MS/MS Analysis - The in-gel tryptic digests were reconstituted in 25 µL of 0.5% FA containing 125 fmol tryptic digest of yeast enolase for nanoLC-ESI-MS/MS analysis, which was carried out using an Orbitrap Fusion™ Tribrid™ (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with a nanospray Flex Ion Source, and coupled with a Dionex UltiMate3000RSLCnano system (Thermo, Sunnyvale, CA).[21,22] The gel extracted peptide samples (10 µL) were injected onto a PepMap C-18 RP nano trapping column (5 µm, 100 µm i.d x 20 mm) at 20 µL/min flow rate for rapid sample loading and then separated on a PepMap C-18 RP nano column (2 µm, 75 µm x 25 cm) at 35 °C. The tryptic peptides were eluted in a 120 min gradient of 5% to 35% acetonitrile (ACN) in 0.1% formic acid at 300 nL/min., followed by a 7 min ramping to 90% ACN-0.1% FA and an 8 min hold at 90% ACN-0.1% FA. The column was re-equilibrated with 0.1% FA for 25 min prior to the next run. The Orbitrap Fusion is operated in positive ion mode with spray voltage set at 1.6 kV and source temperature at 275°C. External calibration for FT, IT and quadrupole mass analyzers was performed. In data-dependent acquisition (DDA) analysis, the instrument was operated using FT mass analyzer in MS scan to select precursor ions followed by 3 second "Top Speed" data-dependent CID ion trap MS/MS scans at 1.6 m/z quadrupole isolation for precursor peptides with multiple charged ions above a threshold ion count of 10,000 and normalized collision energy of 30%. MS survey scans at a resolving power of 120,000 (fwhm at m/z 200), for the mass range of m/z 375-1575. Dynamic exclusion parameters were set at 40 s of exclusion duration with ±10 ppm exclusion mass width. All data were acquired under Xcalibur 4.3 operation software (Thermo-Fisher Scientific).

Data analysis - The DDA raw files for CID MS/MS were subjected to database searches using Proteome Discoverer (PD) 2.4 software (Thermo Fisher Scientific, Bremen, Germany) with the Sequest HT

algorithm. Processing workflow for precursor-based quantification. The PD 2.4 processing workflow containing an additional node of Minora Feature Detector for precursor ion-based quantification was used for protein identification and protein relatively quantitation analysis between samples. The database search was conducted against a *Homo sapiens* database containing 81,785 sequences downloaded from NCBI. Two-missed trypsin cleavage sites were allowed. The peptide precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.6 Da. Variable modification of methionine oxidation, deamidation of asparagines/glutamine and fixed modification of cysteine carbamidomethylation, were set for the database search. Identified peptides were further filtered for maximum 1% FDR using the Percolator algorithm in PD 2.4 along with additional peptide confidence set to high and peptide mass accuracy ≤5 ppm. The final protein IDs contained protein groups that were filtered with at least 2 peptides per protein. Relative quantitation of identified proteins between the paired samples for each of the 16 individuals was determined by the Label Free Quantitation (LFQ) workflow in PD 2.4. The precursor abundance intensity for each peptide identified by MS/MS in each sample were automatically determined and their unique plus razor peptides for each protein in each sample were summed, normalized against yeast enolase protein, and used for calculating the protein abundance by PD 2.4 software. Protein ratios were calculated based on pairwise ratio for the two data points of samples. Results were further analyzed using Protein Analysis Through Evolutionary Relationships (PANTHER)[23] and STRING software[24].

Protein selection – Albumin, structural and redundant proteins were not included in further analysis. Proteins of interest were identified as having an absolute difference of abundance ratio between IL-6^{low} and IL-6^{high} groups of greater than 0.8, and proteins with an absolute difference of abundance ratio of less than 0.8 but were differentially up- or down-regulated between IL-6^{low} and IL-6^{high} groups. Select additional proteins of interest with an absolute difference of abundance ratio of less than 0.8 were also identified because of their role in immune/inflammatory pathways.

Results

Liquid chromatography-mass spectrophotometry analysis of synovial fluid post-ACL injury - To identify novel targets in synovial fluid that may contribute to persistence of articular inflammation following ACL injury, liquid chromatography-mass spectrophotometry (LC-MS/MS) was used in an unbiased approach. Pilot studies indicated revealed heavy contamination of albumin at >50 kDa and potential albumin degradation product at <15 kDa; especially in patients within the IL-6^{high} group. Therefore, only proteins within the 15-45 kDa range were analyzed.

A total of 291 proteins were confidently identified in synovial fluid samples The complete list can be found in **Table S5.1**. Pathway analysis by PANTHER revealed that the proteins detected were associated with 19 protein classes (**Figure 5.1**). The majority (59%) fell under three classifications: metabolite interconversion enzymes (20%), protein modifying enzymes (21%), protein-binding activity modulators (18%). Metabolite interconversion enzymes included proteins related to mitochondrial function and glycolysis. Protein modifying enzymes included proteases and proteins related to the complement system. Protein-binding activity modulators also included proteins of the complement system, as well as protease inhibitors. Extracellular matrix proteins (9%) were in fourth highest abundance.

Proteins in synovial fluid differentially regulated in persistent inflammation - From the full list of 291 proteins that were identified, 57 proteins met the criteria for further assessment (**Table 5.1**). Of these proteins, 44 were in higher abundance in the IL-6^{high} group compared to IL-6^{low} group. Pathway analysis by PANTHER of this refined list reduced the number of protein classes from 19 to 11 (**Figure 5.2)**. Metabolite interconversion enzymes (20%), protein modifying enzymes (25%), and protein-binding enzymes (18%) still represented the majority of proteins identified. Proteins related to defense/immunity (10%) then became the fourth most abundant. Analysis of predicted protein-protein interactions by

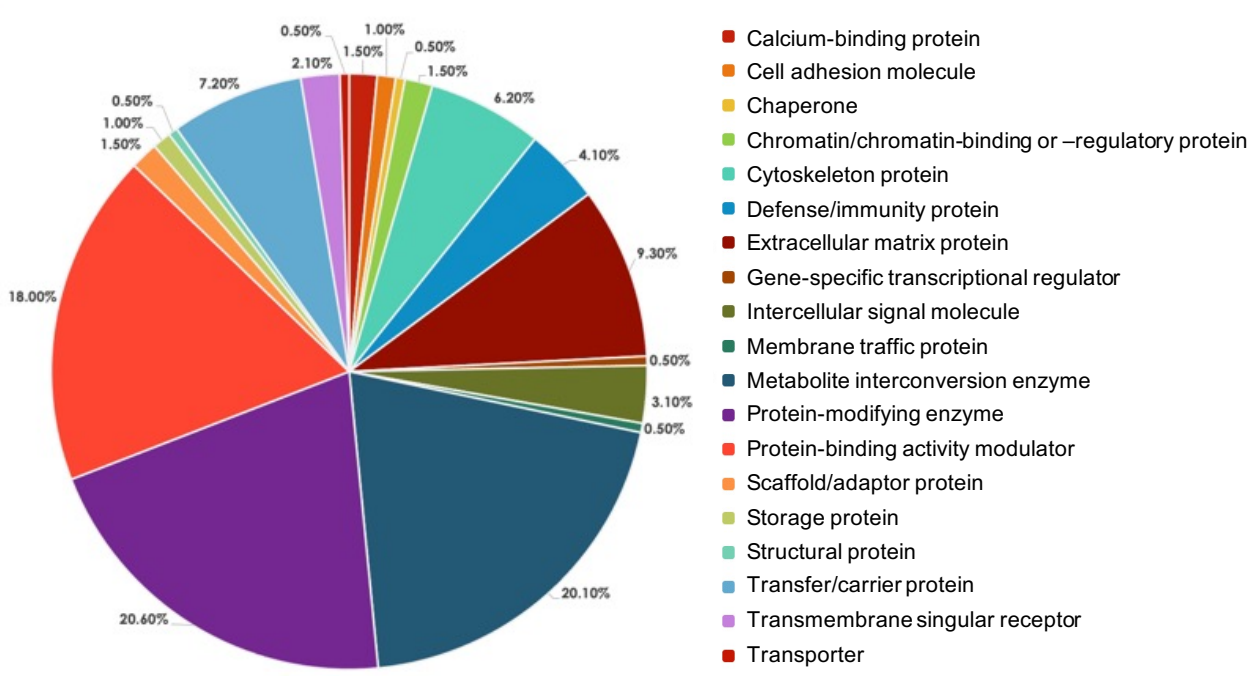

Figure 5.1. PANTHER analysis revealed 19 classes of proteins present in the synovial fluid following anterior cruciate ligament (ACL) injury. Proteins of high abundance in synovial fluid are largely involved in protein modification through enzymatic or protein-binding functions.

Table 5.1. Proteins of interest that had the greatest absolute difference in abundance ratio or were differentially regulated between IL-6^{high} and IL-6^{low} groups at 4- and 1-week post-operation.

Function	Protein	Abundance ratio ‖week 4 : week 1‖	
		IL-6^{high}	IL-6^{low}
Complement	Cathepsin G	2.65	0.01
	Complement C1r subcomponent-like protein	1.83	0.65
	Complement C2	1.35	0.77
	Complement C3	1.24	0.63
	Complement C4	1.54	0.75
	Complement C5	1.48	0.59
	Complement C6	1.22	0.69
	Complement C7	1.21	0.98
	Complement C8	1.45	0.69
	Complement C9	0.98	0.53
	Complement factor B	1.11	0.65
	Complement factor D	0.38	1.19
	Complement factor H	0.93	0.68
	Complement factor H-related protein 5	1.11	0.55
	Mannan-binding lectin serine protease 2	1.13	0.80
Granulocyte/ Macrophage	Annexin A1	8.61	0.80
	Annexin A3	6.65	0.79
	Azurocidin	3.37	0.01
	Cathelicidin antimicrobial peptide	2.62	0.01
	CD16a	0.35	2.56
	Endothelial protein C receptor	1.26	0.96
	Galectin-3-binding protein	1.50	0.01
	Lactotransferrin	2.39	0.71
	Leukocyte elastase inhibitor	2.16	0.22
	Lipopolysaccharide-binding protein	0.81	0.39
	Myeloblastin	4.97	0.01
	Myeloid cell nuclear differentiation antigen	7.93	0.01
	Myeloperoxidase	1.39	0.89
	Pentraxin-related protein PTX3	0.01	0.84
	Neutrophil gelatinase-associated lipocalin	4.38	0.47
T cell/ B cell	CD5 antigen-like	1.10	0.93
	Coronin-1A	0.71	0.01
	HLA class I histocompatibility antigen	100	1.18
	Immunoglobulin heavy variable 4-38-2-like	1.15	0.87
Enzyme	Carboxypeptidase N catalytic chain	1.18	0.45
	Creatine kinase M-type	1.95	0.79
	Delta-aminolevulinic acid dehydratase	0.01	0.98
	Fructose-bisphosphate aldolase C	1.07	0.01
	Glucose-6-phosphate isomerase	1.29	0.01
	Glutathione S-transferase P	6.36	0.31
	Matrix metalloproteinase 1	1.41	0.54
	Matrix metalloproteinase 3	2.44	1.29

	Selenium-binding protein 1	2.27	0.73
	Superoxide dismutase, mitochondrial	0.92	0.01
	Transketolase	2.01	0.87
Growth factor	Fibroblast growth factor-binding protein 2	0.28	1.17
	Insulin-like growth factor-binding protein 6	0.61	3.68
	Osteoglycin (mimecan)	100	1.05
ECM	Transforming growth factor-β-induced protein ig-h3	0.85	1.07
	Chondroadherin	0.16	0.55
	Periostin	2.75	7.00
Chaperone	Heat shock protein 70	0.90	2.56
Intracellular	POTE ankyrin domain family member E	8.95	0.97
Metabolism	Purine nucleoside phosphorylase	3.66	1.03
Transcription	Procollagen C-endopeptidase enhancer 1	0.88	1.03

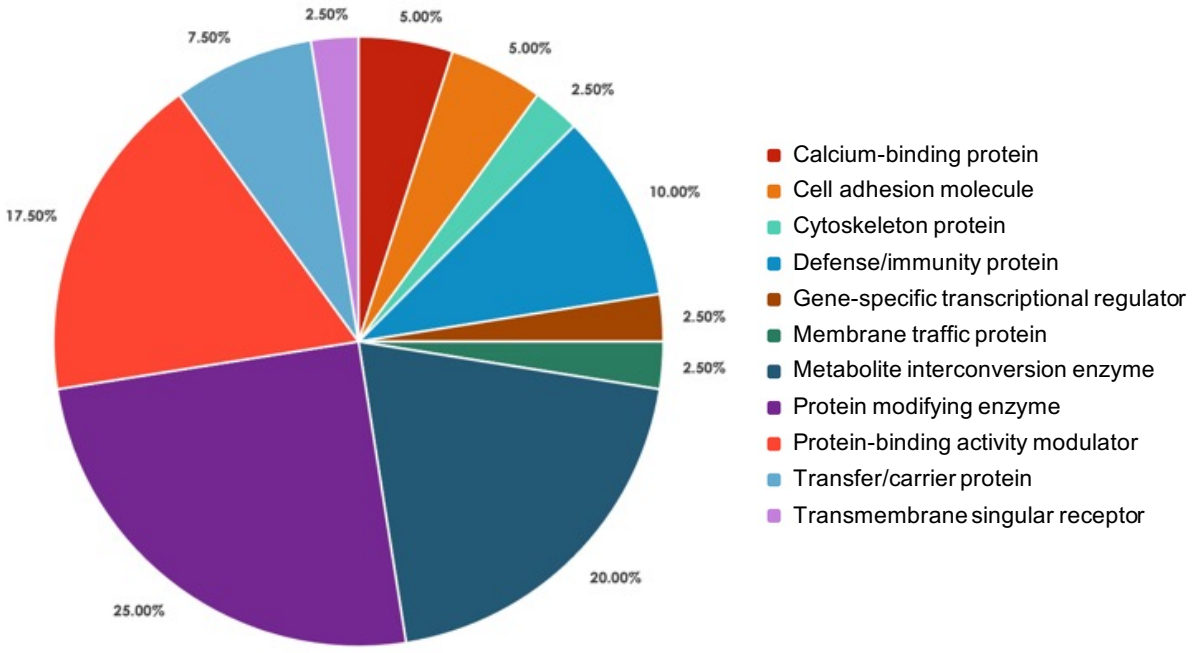

Figure 5.2. PANTHER analysis of proteins of interest reduces number protein classes to 11. The dominant protein classes following reduction remain as metabolite interconversion enzymes, protein modifying enzymes, and protein-binding activity modulators. The percent of proteins that are related to defense/immunity more than doubled following protein list refinement.

STRING showed that these proteins are highly related, with a total of 168 edges, which are protein-protein associations (**Figure 5.3**; PPI enrichment p-value<0.0001).

Proteins from all activation pathways of the complement system were represented, and 14/15 complement pathway proteins were in higher abundance in the IL-6^{high} group compared to the IL-6^{low} group. Furthermore, 12/14 of these proteins had a positive abundance ratio and thus were elevated in synovial fluid at 4 weeks postop. Proteins associated with granulocytes and macrophages were also identified, and 13/15 proteins had a higher abundance ratio in the IL-6^{high} group compared to the IL-6^{low} group. Many of these proteins are associated with neutrophil granules, which may contribute to persistence of joint inflammation in these patients. CD16A, which is expressed by macrophages, natural killer cells and mast cells, and pentraxin-related protein PTX3, an acute phase response protein,[25] were in higher abundance in the IL-6^{low} group. All proteins associated with T and B cells were in higher abundance in the IL-6^{high}. Multiple enzymes were in higher abundance in the IL-6^{high} group compared to IL-6^{low}. Of note, matrix metalloproteinase 1 (MMP1) and matrix metalloproteinase 3 (MMP3) were in higher abundance in the IL-6^{high} group, and MMP1 was in lower abundance in the IL-6^{low} group. Multiple enzymes are associated with glycolysis, ATP generation and mitochondrial function.

All growth factors and associated proteins had a value of greater than 1 in the IL-6^{low} group, and only osteoglycin,[26] which induces ectopic bone formation, had a higher abundance ratio in the IL-6^{high} group. Interestingly, POTE ankyrin domain family member E (POTE E) within the IL-6^{high} group had the highest abundance ratio below 100. This protein is associated with poor cancer prognosis,[27,28] but, to the authors' knowledge, has not previously been described within the context of the joint.

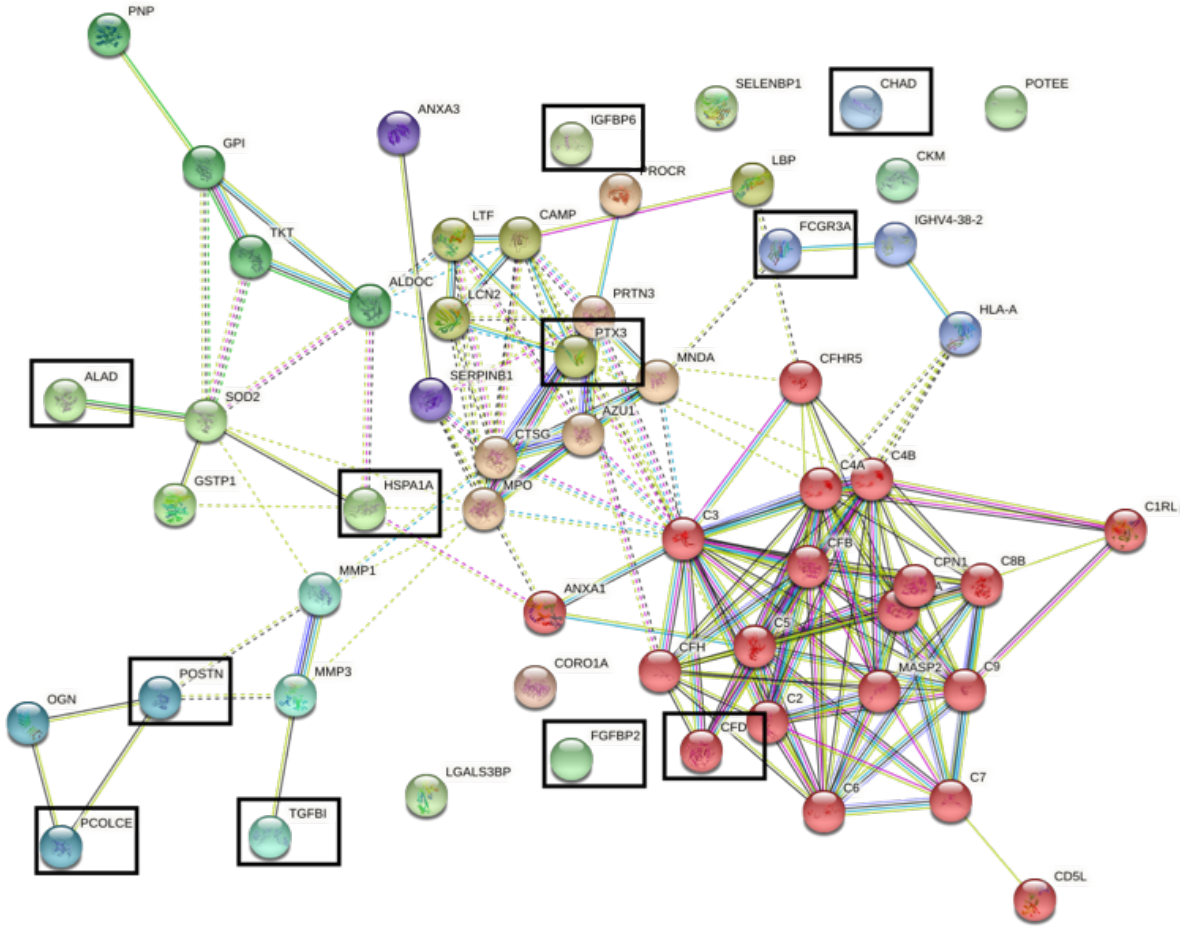

Figure 5.3. Analysis of protein-protein interactions by STRING. Proteins involved in the complement pathway are highly clustered (red) and share a high number of protein-protein interactions. Another smaller cluster represents macrophage/neutrophil associated proteins and pathways (peach, purple, chartreuse). Boxed proteins had higher abundance in the IL-6^{low} group compared to the IL-6^{high} group.

Discussion

The results of this study reveal that multiple protein classes are differentially regulated between patients with high concentrations of IL-6 in the synovial fluid at 4 weeks post ACL reconstructive surgery, and presumed persistent inflammation, and patients with low IL-6 and early resolution of inflammation. Patients in the IL-6^{high} group had an increase in abundance of proteins associated with complement pathways, granulocytes and macrophages, enzymes, growth factors, and several other protein classes, which offer therapeutic targets early in PTOA in the prevention of persistent inflammation following injury.

All three pathways of the complement system, classical, alternative, and lectin, have been recognized as components of the inflammatory response in PTOA, and contribute to the release of leukocyte chemoattractants.[29] While the complement system also contributes to the clearance of dead and dying cells, aiding in repair of damaged tissue, its persistence within the joint contributes to hypertrophy of chondrocytes and their transdifferentiation into osteoblasts, contributing to joint destruction.[30] This highlights the complement system as a pathway of inflammatory dysregulation, as multiple complement inhibitors, including eculizumab, Berinert®, and Cinryze®, have already been approved by the FDA for diseases such as paroxysmal nocturnal hemoglobinuria, neuromyelitis optica, and angioedema.[31]

Multiple proteins associated with neutrophil differentiation and maintenance and granulocyte degranulation had an abundance of greater than 1 in the IL-6^{high} group. Azurocidin,[32] lactotransferrin,[33] lipopolysaccharide-binding protein,[34] myeloblastin,[35] myeloperoxidase,[36] and neutrophil gelatinase-associated lipocalin (NGAL)[37] are all associated with neutrophil granules, respiratory burst, and secretion. These results indicate that activation of neutrophils within the joint contribute to inflammatory dysfunction. Therapies that target the recruitment and activation of neutrophils may therefore be used to

correct inflammatory dysregulation within the joint following injury. Otilimab, a monoclonal antibody that neutralizes granulocyte-macrophage colony stimulating factor (GM-CSF), is currently in development for the treatment of rheumatoid arthritis and was shown to improved patient pain score and physical function in a phase 2b study.[38] GM-CSF is involved in neutrophil recruitment and activation within the joint and release of NGAL, and may therefore be an upstream target for PTOA development.[39]

Although many of the markers associated with neutrophils/granulocytes and macrophages had an abundance ration of greater than 1 only in the IL-6^{high} group, CD16a, a marker for pro-inflammatory macrophages, had an abundance ratio of less than 1 in the IL-6^{high} group and more than 2.5 in the IL-6^{low} group. $CD14^{+}CD16a^{+}$ macrophages not only are enriched in knee OA synovial fluid compared to circulating populations, but correlated with concentrations of CCL2,[40] a pro-inflammatory cytokine, and have been shown to contribute to fibrosis in inflammatory bowel disease.[41] CD16a is also expressed on natural killer cells, and is shed through the action of ADAM17,[42] which is expressed by chondrocytes[43]. This may explain why abundance ratio of CD16a does not correlate with that of CD14 (data not shown) in the IL-6^{low} group and could indicate that there is an increase in natural killer cell activity in the IL-6^{low} group.

Proteins associated with T and B cells of the adaptive immune system generally had a higher absolute value in the IL-6^{high} group. CD5 antigen-like (CD5L) is a regulator of macrophage apoptosis and lipid synthesis[44] and is associated with disease severity in patients with RA[45]. It is also a repressor of pathogenic T helper 17 cells,[46] which are shown to be present in the joints of mice within 4 weeks post ACL transection[47]. The positive abundance ratio of CD5L in the IL-6^{high} group may therefore be tied to dysregulation of lipid metabolism within the immune system, contributing to inflammation.

Unsurprisingly, MMP1 and MMP3 had abundance ratios of greater than 1 in the IL-6^{high} group, which were also higher than in the IL-6^{low} group. Although not detected in this proteomic analysis, both IL-6 and

TNF-α are present in PTOA synovial fluid and induce expression of MMP1 and MMP3 in fibroblasts. Treatment of RA patients using etanercept (αTNF-α) or tocilizumab (αIL-6R) decreases serum MMP1 and MMP3, and indicate that these therapies may be used in the upstream targeting of MMP1- and MMP3-inducing cytokines.[48,49]

The two mitochondrial enzymes, creatine kinase M-type and superoxide dismutase, had higher abundance ratios in the IL-6^{high} group, with creatine kinase m-type having an abundance ratio of nearly 2. As these proteins are localized to the mitochondria, their detection within the synovial fluid could indicate mitochondrial dysfunction,[50,51] which is a known consequence of cartilage damage[52]. Therapies targeting mitochondrial structural stability,[53] and pathways related to mitochondrial dysfunction, including PI3K/Akt,[54] AMPK/SIRT2/SOD2,[55] have been evaluated *in vitro* and *in vivo* for preventing PTOA progression.

Not only was the finding of POTE E unexpected, but also that it was dysregulated between the high and low groups. POTE E is a newly recognized protein normally expressed within testes, ovary, and placenta, but is also highly expressed in multiple cancer types including prostate cancer,[56] lung cancer,[28] colon cancer,[27] and more. POTE E activates PI3K/AKT/GSK-3β/β-catenin signaling, which is related to cellular homeostasis, inflammation, metabolism, cell survival, and apoptosis, and is associated with OA development through promoting the release of inflammatory cytokines by synoviocytes, including IL-1β, IL-6, and TNF-α.[57] The elevation of POTE E in the IL-6^{high} group makes it a potential and novel target for the mitigation of inflammation in PTOA.

There are several limitations to this study. While bottom-up proteomics provides an unbiased approach for the discovery of therapeutic targets in PTOA, contamination of synovial fluid with albumin, keratins and fibrinogens suppressed detection of lower abundance proteins,[58] including interleukins, such as IL-6,

which are key to the orchestration of inflammation resolution[5,59]. Bottom-up proteomics also provides qualitative data rather than quantitative, and so comparisons between groups are limited. An alternative approach would be a targeted, multiplex ELISA with computational modeling and analysis.[6] Analysis of synovial fluid provides identification of proteins, but not their source. Pairing proteomic analysis with flow cytometric analysis or single cell RNA sequencing of cells present within the synovial fluid would provide a more comprehensive view on cellular targets for therapeutics in the mitigation of persistent inflammation leading to PTOA following traumatic joint injury.

In summary, patients segregated based on IL-6 concentrations in synovial fluid 4-weeks post-ACL reconstruction demonstrated differential regulation of multiple inflammatory pathways. This provides multiple opportunities to further investigate novel targets not previously identified in PTOA, as well as take advantage of therapeutics already approved by the FDA for use in other diseases. Furthermore, these findings highlight the need for patient-specific therapies that target the correct molecule at the right time in order to stop PTOA progression prior to the inevitability of joint destruction.

Author contributions

LEK, CAJ, CL, ERH, and LAF designed the study, analyzed the data, and drafted the manuscript. SZ and QF performed data collection and data analysis. All authors approved the final version of the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Table S5.1. All proteins detected in synovial fluid at 1 weeks and 4 weeks post anterior cruciate ligament reconstruction surgery by liquid chromatography/mass spectrophotometry.

Protein	Abundance Ratio	
	IL-6^{high}	IL-6^{low}
14-3-3 protein beta/alpha [Homo sapiens]	0.01	1.68
14-3-3 protein epsilon [Homo sapiens]	0.139	1.95
14-3-3 protein zeta/delta isoform X1 [Homo sapiens]	0.557	3.277
6-phosphogluconate dehydrogenase, decarboxylating isoform 1 [Homo sapiens]		
72 kDa type IV collagenase isoform 1 preproprotein [Homo sapiens]		
actin, alpha skeletal muscle [Homo sapiens]	0.85	1.258
actin, cytoplasmic 1 [Homo sapiens]	0.539	1.974
adenosylhomocysteinase isoform X1 [Homo sapiens]	0.01	100
adiponectin precursor [Homo sapiens]	1.035	1.672
afamin precursor [Homo sapiens]	1.108	1.463
aggrecan core protein isoform 3 precursor [Homo sapiens]	0.36	2.109
albumin preproprotein [Homo sapiens]	1.093	1.869
alpha-1-acid glycoprotein 1 precursor [Homo sapiens]	0.716	1.801
alpha-1-acid glycoprotein 2 precursor [Homo sapiens]	0.719	1.928
alpha-1-antichymotrypsin precursor [Homo sapiens]	1	1.831
alpha-1-antitrypsin precursor [Homo sapiens]	0.933	1.734
alpha-1B-glycoprotein precursor [Homo sapiens]	1.124	1.863
alpha-2-antiplasmin isoform X2 [Homo sapiens]	1.28	2.105
alpha-2-HS-glycoprotein isoform 1 preproprotein [Homo sapiens]	0.915	2.436
alpha-2-macroglobulin isoform X1 [Homo sapiens]	1.188	1.48
alpha-actinin-1 isoform X1 [Homo sapiens]	2.324	100
alpha-enolase isoform 1 [Homo sapiens]	0.655	5.395
angiotensinogen precursor [Homo sapiens]	1.064	1.577
ankyrin-1 isoform X1 [Homo sapiens]	0.01	1.654
annexin A1 isoform X1 [Homo sapiens]	8.608	0.818
annexin A3 [Homo sapiens]	6.645	1.506
antithrombin-III isoform 3 precursor [Homo sapiens]	1.127	1.687
apolipoprotein A-I isoform 1 preproprotein [Homo sapiens]	0.895	1.544
apolipoprotein A-II preproprotein [Homo sapiens]	0.541	2.25
apolipoprotein A-IV precursor [Homo sapiens]	0.838	1.172
apolipoprotein B-100 precursor [Homo sapiens]	1.146	1.374
apolipoprotein D precursor [Homo sapiens]	0.67	1.457
apolipoprotein E isoform a precursor [Homo sapiens]	0.525	1.364
apolipoprotein L1 isoform b precursor [Homo sapiens]	0.904	1.54

apolipoprotein M isoform 1 [Homo sapiens]	0.982	1.105
azurocidin preproprotein [Homo sapiens]	3.366	3.383
band 3 anion transport protein [Homo sapiens]	0.101	2.042
basement membrane-specific heparan sulfate proteoglycan core protein isoform X1 [Homo sapiens]		0.01
beta-2-glycoprotein 1 precursor [Homo sapiens]	0.346	9.35
beta-actin-like protein 2 [Homo sapiens]	0.01	1.539
beta-Ala-His dipeptidase precursor [Homo sapiens]	0.829	1.755
biotinidase isoform 1 precursor [Homo sapiens]	0.734	2.908
bisphosphoglycerate mutase [Homo sapiens]	0.067	1.974
C-reactive protein isoform 1 precursor [Homo sapiens]	0.376	4.114
C4b-binding protein alpha chain isoform X1 [Homo sapiens]	0.872	1.61
C4b-binding protein beta chain isoform 1 precursor [Homo sapiens]	0.567	0.657
carbonic anhydrase 1 isoform a [Homo sapiens]	0.029	3.92
carbonic anhydrase 2 isoform 1 [Homo sapiens]	0.152	2.846
carbonic anhydrase 3 [Homo sapiens]	0.01	1.798
carboxypeptidase B2 isoform 1 preproprotein [Homo sapiens]	0.938	2.823
carboxypeptidase N catalytic chain precursor [Homo sapiens]	1.175	2.168
carboxypeptidase N subunit 2 precursor [Homo sapiens]	1.179	1.901
cartilage acidic protein 1 isoform X1 [Homo sapiens]	0.851	1.611
cartilage intermediate layer protein 1 isoform X1 [Homo sapiens]	0.01	1.029
cartilage intermediate layer protein 2 precursor [Homo sapiens]	0.591	1.834
cartilage oligomeric matrix protein precursor [Homo sapiens]	0.336	1.734
catalase [Homo sapiens]	0.295	4.276
cathelicidin antimicrobial peptide preproprotein [Homo sapiens]	2.621	1.819
cathepsin B isoform 1 preproprotein [Homo sapiens]	0.314	1.541
cathepsin G isoform X1 [Homo sapiens]	2.646	0.812
CD5 antigen-like isoform 1 precursor [Homo sapiens]	1.104	1.69
ceruloplasmin precursor [Homo sapiens]	1.271	1.284
chitinase-3-like protein 1 precursor [Homo sapiens]	0.339	1.64
chitinase-3-like protein 2 isoform a precursor [Homo sapiens]	0.165	1.297
chondroadherin isoform X1 [Homo sapiens]	0.165	2.236
clusterin preproprotein [Homo sapiens]	1.027	1.182
coagulation factor IX isoform 1 preproprotein [Homo sapiens]	0.96	1.569
coagulation factor XII preproprotein [Homo sapiens]	1.476	1.465
coagulation factor XIII B chain isoform X1 [Homo sapiens]	0.934	1.397
cofilin-1 [Homo sapiens]	0.905	1.969
collagen alpha-1(I) chain preproprotein [Homo sapiens]	1.43	1.897
collagen alpha-1(III) chain preproprotein [Homo sapiens]	1.924	1.359
collagen alpha-1(V) chain isoform 1 preproprotein [Homo sapiens]	6.854	0.45
collagen alpha-1(VI) chain precursor [Homo sapiens]	1.56	1.454
collagen alpha-1(XVIII) chain isoform 3 preproprotein [Homo sapiens]	100	0.01

collagen alpha-2(I) chain precursor [Homo sapiens]	0.987	1.816
collagen alpha-2(V) chain preproprotein [Homo sapiens]	2.578	2.353
collagen alpha-3(VI) chain isoform 1 precursor [Homo sapiens]	1.176	1.84
collectin-11 isoform f precursor [Homo sapiens]	0.522	3.276
complement C1q subcomponent subunit A precursor [Homo sapiens]	0.997	1.075
complement C1q subcomponent subunit B isoform 1 precursor [Homo sapiens]	0.961	0.912
complement C1q subcomponent subunit C isoform 1 precursor [Homo sapiens]	0.964	1.011
complement C1r subcomponent isoform 2 preproprotein [Homo sapiens]	0.961	1.683
complement C1r subcomponent-like protein isoform 1 precursor [Homo sapiens]	1.829	1.397
complement C1s subcomponent isoform 1 preproprotein [Homo sapiens]	0.806	1.341
complement C2 isoform 1 preproprotein [Homo sapiens]	1.351	1.723
complement C3 preproprotein [Homo sapiens]	1.24	1.579
complement C4-A isoform 1 preproprotein [Homo sapiens]	1.535	1.444
complement C4-B preproprotein [Homo sapiens]	1.421	1.455
complement C5 isoform 2 [Homo sapiens]	1.481	1.821
complement component C6 isoform X1 [Homo sapiens]	1.22	1.479
complement component C7 precursor [Homo sapiens]	1.206	1.362
complement component C8 alpha chain preproprotein [Homo sapiens]	1.446	1.762
complement component C8 beta chain isoform 1 preproprotein [Homo sapiens]	1.377	1.707
complement component C8 gamma chain precursor [Homo sapiens]	0.767	1.106
complement component C9 preproprotein [Homo sapiens]	0.978	2.312
complement factor B preproprotein [Homo sapiens]	1.109	1.549
complement factor D isoform 2 precursor [Homo sapiens]	0.381	0.954
complement factor H isoform a precursor [Homo sapiens]	0.929	1.553
complement factor H-related protein 1 isoform 1 precursor [Homo sapiens]	0.685	1.769
complement factor H-related protein 2 isoform 1 precursor [Homo sapiens]	0.713	2.887
complement factor H-related protein 5 isoform X1 [Homo sapiens]	1.106	2.47
complement factor I isoform 1 preproprotein [Homo sapiens]	0.913	1.447
coronin-1A isoform X1 [Homo sapiens]	0.709	2.43
corticosteroid-binding globulin precursor [Homo sapiens]	1.216	1.461
creatine kinase M-type [Homo sapiens]	1.946	1.786
delta-aminolevulinic acid dehydratase isoform X2 [Homo sapiens]	0.01	2.524
EGF-containing fibulin-like extracellular matrix protein 1 isoform X2 [Homo sapiens]	0.992	2.06
endothelial protein C receptor precursor [Homo sapiens]	1.255	0.967
extracellular matrix protein 1 isoform 3 precursor [Homo sapiens]	1.206	1.016
extracellular superoxide dismutase [Cu-Zn] preproprotein [Homo sapiens]	0.516	1.574
F-actin-capping protein subunit alpha-1 isoform X1 [Homo sapiens]	2.483	2.131
F-actin-capping protein subunit beta isoform X1 [Homo sapiens]	0.01	1.547

ferritin heavy chain [Homo sapiens]	0.484	3.272
ferritin light chain isoform X1 [Homo sapiens]	0.206	1.717
fibrinogen alpha chain isoform alpha-E preproprotein [Homo sapiens]	0.949	1.773
fibrinogen beta chain isoform 1 preproprotein [Homo sapiens]	0.878	2.082
fibrinogen gamma chain isoform gamma-B precursor [Homo sapiens]	1.206	2.283
fibroblast growth factor-binding protein 2 precursor [Homo sapiens]	0.281	1.054
fibronectin isoform 13 precursor [Homo sapiens]	0.804	1.879
fibulin-1 isoform C precursor [Homo sapiens]	0.925	1.675
fibulin-1 isoform D precursor [Homo sapiens]	1.048	1.958
ficolin-2 isoform a precursor [Homo sapiens]	0.611	1.122
ficolin-3 isoform 1 precursor [Homo sapiens]	0.94	1.276
flavin reductase (NADPH) [Homo sapiens]	0.01	1.92
fructose-bisphosphate aldolase A isoform 2 [Homo sapiens]	0.308	1.896
fructose-bisphosphate aldolase C isoform X1 [Homo sapiens]	1.072	1.767
galectin-3 isoform 3 [Homo sapiens]	0.01	0.365
galectin-3-binding protein precursor [Homo sapiens]	1.5	0.461
gelsolin isoform a precursor [Homo sapiens]	1.02	1.732
glucose-6-phosphate isomerase isoform X1 [Homo sapiens]	1.288	2.606
glutathione peroxidase 3 isoform 2 precursor [Homo sapiens]	0.807	1.555
glutathione S-transferase omega-1 isoform 1 [Homo sapiens]	0.394	2.454
glutathione S-transferase P [Homo sapiens]	6.363	1.903
glyceraldehyde-3-phosphate dehydrogenase isoform 1 [Homo sapiens]	0.157	2.117
glycophorin-A isoform 1 precursor [Homo sapiens]		0.01
GTP-binding nuclear protein Ran isoform X1 [Homo sapiens]		0.01
guanine nucleotide-binding protein G(i) subunit alpha-2 isoform 1 [Homo sapiens]	1.213	3.897
haptoglobin isoform 1 preproprotein [Homo sapiens]	3.844	1.204
haptoglobin-related protein isoform X1 [Homo sapiens]	1.007	1.624
heat shock 70 kDa protein 1A [Homo sapiens]	0.897	2.49
hemoglobin subunit alpha [Homo sapiens]	0.012	6.019
hemoglobin subunit beta [Homo sapiens]	0.013	7.757
hemoglobin subunit delta [Homo sapiens]	0.016	7.671
hemoglobin subunit gamma-2 [Homo sapiens]	0.01	4.591
hemopexin precursor [Homo sapiens]	4.198	1.192
heparin cofactor 2 precursor [Homo sapiens]	1.146	1.535
histidine-rich glycoprotein isoform X1 [Homo sapiens]	1.169	1.79
histone H2A type 1 [Homo sapiens]	0.994	3.347
histone H2B type 1-D isoform X1 [Homo sapiens]	3.16	3.17
histone H4 [Homo sapiens]	2.175	1.884
HLA class I histocompatibility antigen, A alpha chain A*01:01:01:01 precursor [Homo sapiens]	100	0.01
hornerin [Homo sapiens]		

hyaluronan and proteoglycan link protein 1 isoform X1 [Homo sapiens]	0.01	0.529
hyaluronan-binding protein 2 isoform 1 preproprotein [Homo sapiens]	0.601	1.638
hypoxanthine-guanine phosphoribosyltransferase [Homo sapiens]	0.01	0.806
IgGFc-binding protein precursor [Homo sapiens]	0.991	1.946
immunoglobulin heavy variable 4-38-2-like [Homo sapiens]	1.149	1.815
immunoglobulin J chain precursor [Homo sapiens]	0.889	1.495
immunoglobulin lambda-like polypeptide 5 isoform 1 [Homo sapiens]	1.067	1.068
insulin-like growth factor-binding protein 3 isoform a precursor [Homo sapiens]	1.19	0.854
insulin-like growth factor-binding protein 6 precursor [Homo sapiens]	0.607	2.531
insulin-like growth factor-binding protein complex acid labile subunit isoform 1 precursor [Homo sapiens]	0.988	1.246
inter-alpha-trypsin inhibitor heavy chain H1 isoform a preproprotein [Homo sapiens]	1.062	1.659
inter-alpha-trypsin inhibitor heavy chain H2 precursor [Homo sapiens]	1.113	1.32
inter-alpha-trypsin inhibitor heavy chain H3 isoform X1 [Homo sapiens]	0.998	1.601
inter-alpha-trypsin inhibitor heavy chain H4 isoform 1 precursor [Homo sapiens]	1.021	1.541
interstitial collagenase isoform 1 preproprotein [Homo sapiens]	1.407	3.923
kallistatin isoform 1 [Homo sapiens]	1.035	1.543
keratin, type I cytoskeletal 10 isoform 2 [Homo sapiens]	0.971	1.076
keratin, type I cytoskeletal 14 [Homo sapiens]	0.549	1.319
keratin, type I cytoskeletal 16 [Homo sapiens]		0.01
keratin, type I cytoskeletal 17 [Homo sapiens]	0.413	0.176
keratin, type I cytoskeletal 9 [Homo sapiens]	0.582	1.478
keratin, type II cytoskeletal 1 [Homo sapiens]	0.774	1.264
keratin, type II cytoskeletal 1b [Homo sapiens]		
keratin, type II cytoskeletal 2 epidermal [Homo sapiens]	0.968	1.026
keratin, type II cytoskeletal 4 [Homo sapiens]		0.01
keratin, type II cytoskeletal 5 [Homo sapiens]	0.01	0.389
keratin, type II cytoskeletal 6B [Homo sapiens]	0.01	1.072
keratin, type II cytoskeletal 75 [Homo sapiens]	0.323	0.779
kininogen-1 isoform 1 precursor [Homo sapiens]	1.018	1.964
kininogen-1 isoform 2 precursor [Homo sapiens]	1.296	1.824
L-lactate dehydrogenase A chain isoform 3 [Homo sapiens]	0.539	1.131
L-lactate dehydrogenase B chain isoform X1 [Homo sapiens]	0.18	2.478
lactotransferrin isoform 1 preproprotein [Homo sapiens]	2.393	2.843
leucine-rich alpha-2-glycoprotein precursor [Homo sapiens]	0.835	2.81
leukocyte elastase inhibitor isoform X1 [Homo sapiens]	2.16	2.041
lipopolysaccharide-binding protein precursor [Homo sapiens]	0.813	1.282
low affinity immunoglobulin gamma Fc region receptor III-A isoform b [Homo sapiens]	0.348	2.799
lumican precursor [Homo sapiens]	0.94	2.34

malate dehydrogenase, peroxisomal isoform MDH1x [Homo sapiens]	0.01	1.284
mannan-binding lectin serine protease 2 isoform 1 preproprotein [Homo sapiens]	1.131	1.349
mannose-binding protein C precursor [Homo sapiens]	0.761	1.523
metalloproteinase inhibitor 1 precursor [Homo sapiens]	0.252	2.139
methanethiol oxidase isoform 3 [Homo sapiens]	2.277	2.858
mimecan isoform 1 [Homo sapiens]	100	0.01
moesin isoform X1 [Homo sapiens]	1.474	1.987
monocyte differentiation antigen CD14 precursor [Homo sapiens]	0.703	2.974
myeloblastin precursor [Homo sapiens]	4.971	1.211
myeloid cell nuclear differentiation antigen [Homo sapiens]	7.925	1.17
myeloperoxidase precursor [Homo sapiens]	1.394	2.615
myosin-11 isoform SM1B [Homo sapiens]	100	
N-acetylmuramoyl-L-alanine amidase isoform 2 precursor [Homo sapiens]	1.033	1.522
neutrophil gelatinase-associated lipocalin precursor [Homo sapiens]	4.383	1.408
Parkinson disease protein 7 [Homo sapiens]	0.264	1.401
pentraxin-related protein PTX3 precursor [Homo sapiens]	0.01	2.764
peptidoglycan recognition protein 1 precursor [Homo sapiens]	5.843	1.379
peptidyl-prolyl cis-trans isomerase A isoform 1 [Homo sapiens]	0.848	4.119
periostin isoform 5 precursor [Homo sapiens]	2.748	2.609
peroxiredoxin-1 [Homo sapiens]	0.088	1.857
peroxiredoxin-2 [Homo sapiens]	0.023	5.543
peroxiredoxin-6 [Homo sapiens]	0.068	2.339
phosphatidylethanolamine-binding protein 1 [Homo sapiens]	0.01	1.557
phosphoglycerate kinase 1 [Homo sapiens]	0.946	1.793
phosphoglycerate mutase 1 isoform 1 [Homo sapiens]	1.176	1.549
phospholipid transfer protein isoform a precursor [Homo sapiens]	0.658	1.627
pigment epithelium-derived factor isoform 1 precursor [Homo sapiens]	0.942	1.93
plasma kallikrein isoform X1 [Homo sapiens]	0.838	2.372
plasma protease C1 inhibitor precursor [Homo sapiens]	0.865	2.047
plasma serine protease inhibitor preproprotein [Homo sapiens]	1.113	2.202
plasminogen isoform 1 precursor [Homo sapiens]	1.061	1.556
plastin-2 isoform X1 [Homo sapiens]	1.298	1.57
POTE ankyrin domain family member E isoform X3 [Homo sapiens]	8.951	0.875
pregnancy zone protein isoform X1 [Homo sapiens]	0.857	1.031
procollagen C-endopeptidase enhancer 1 precursor [Homo sapiens]	0.882	2.194
prostaglandin-H2 D-isomerase precursor [Homo sapiens]	1.425	1.161
proteasome activator complex subunit 1 isoform 2 [Homo sapiens]	0.745	1.383
proteasome subunit alpha type-1 isoform 1 [Homo sapiens]		
proteasome subunit alpha type-2 [Homo sapiens]	0.564	1.376
proteasome subunit alpha type-5 isoform 1 [Homo sapiens]		
proteasome subunit alpha type-6 isoform a [Homo sapiens]	0.01	1.032

proteasome subunit alpha type-7 [Homo sapiens]	0.01	2.359
proteasome subunit beta type-1 [Homo sapiens]	0.301	3.096
proteasome subunit beta type-2 isoform 1 [Homo sapiens]		
proteasome subunit beta type-3 [Homo sapiens]	0.01	2.207
proteasome subunit beta type-5 isoform 1 [Homo sapiens]	0.158	4.124
protein 4.1 isoform X1 [Homo sapiens]	0.01	2.624
protein AMBP preproprotein [Homo sapiens]	1.018	1.309
protein S100-A9 [Homo sapiens]	0.43	3.25
proteoglycan 4 isoform X1 [Homo sapiens]	0.186	1.564
prothrombin preproprotein [Homo sapiens]	1.171	1.193
purine nucleoside phosphorylase [Homo sapiens]	3.658	1.388
pyruvate kinase PKM isoform X1 [Homo sapiens]	1.07	3.671
rab GDP dissociation inhibitor beta isoform 1 [Homo sapiens]	0.01	2.071
ras-related protein Rab-8A [Homo sapiens]	2.861	100
retinal dehydrogenase 1 [Homo sapiens]	0.01	3.405
retinol-binding protein 4 isoform a precursor [Homo sapiens]	1.113	1.471
rho GDP-dissociation inhibitor 2 [Homo sapiens]	2.349	1.171
ribose-5-phosphate isomerase [Homo sapiens]	0.01	1.17
SAA2-SAA4 protein precursor [Homo sapiens]	0.901	2.149
scavenger receptor cysteine-rich type 1 protein M130 isoform a precursor [Homo sapiens]		
serine protease HTRA1 precursor [Homo sapiens]	0.14	3.488
serotransferrin isoform 1 precursor [Homo sapiens]	1.146	0.971
serum amyloid A-1 protein preproprotein [Homo sapiens]	1.079	100
serum amyloid A-2 protein isoform a preproprotein [Homo sapiens]		
serum amyloid P-component precursor [Homo sapiens]	1.024	0.959
serum paraoxonase/arylesterase 1 precursor [Homo sapiens]	1.085	1.872
serum paraoxonase/lactonase 3 [Homo sapiens]		
sex hormone-binding globulin isoform 1 precursor [Homo sapiens]	1.366	2.235
SPARC isoform 3 precursor [Homo sapiens]	0.456	1.132
spectrin alpha chain, erythrocytic 1 isoform X1 [Homo sapiens]	0.01	1.424
spectrin beta chain, erythrocytic isoform a [Homo sapiens]	0.01	3.141
stomatin isoform a [Homo sapiens]	0.01	1.683
stromelysin-1 preproprotein [Homo sapiens]	2.439	2.204
sulfhydryl oxidase 1 isoform a precursor [Homo sapiens]		
superoxide dismutase [Cu-Zn] [Homo sapiens]	0.01	2.509
superoxide dismutase [Mn], mitochondrial isoform A precursor [Homo sapiens]	0.918	0.899
synaptic vesicle membrane protein VAT-1 homolog [Homo sapiens]	0.01	100
tenascin isoform X1 [Homo sapiens]	1.923	2.125
tetranectin isoform 1precursor [Homo sapiens]	1.352	1.116
thrombospondin-1 precursor [Homo sapiens]	0.224	1.204

thrombospondin-4 isoform a precursor [Homo sapiens]		
thyroxine-binding globulin isoform X1 [Homo sapiens]	1.471	1.261
transaldolase [Homo sapiens]	0.01	0.562
transforming growth factor-beta-induced protein ig-h3 precursor [Homo sapiens]	0.845	2.22
transketolase isoform X1 [Homo sapiens]	2.014	2.213
transthyretin precursor [Homo sapiens]	1.124	0.989
triosephosphate isomerase isoform 2 [Homo sapiens]	0.73	3.286
trypsin-1 isoform X1 [Homo sapiens]		
tubulin alpha-1C chain isoform a [Homo sapiens]	100	
tumor necrosis factor-inducible gene 6 protein precursor [Homo sapiens]	0.794	3.165
ubiquitin-conjugating enzyme E2 N [Homo sapiens]	0.01	100
V-set and immunoglobulin domain-containing protein 4 isoform 1 precursor [Homo sapiens]	0.681	1.298
versican core protein isoform 1 precursor [Homo sapiens]	0.241	2.238
vimentin [Homo sapiens]	1.81	1.975
vitamin D-binding protein isoform 3 precursor [Homo sapiens]	0.562	2.956
vitamin K-dependent protein C isoform X1 [Homo sapiens]	2.778	0.779
vitamin K-dependent protein S isoform 1 precursor [Homo sapiens]		
vitronectin precursor [Homo sapiens]	1.081	1.696
zinc-alpha-2-glycoprotein precursor [Homo sapiens]	0.827	1.302

General discussion

Thesis summary

The objective of this thesis was to provide novel information regarding the role of Tregs and Th17 cells in joint homeostasis to yield new insights into OA as an immune-mediated disease, and to provide targets for OA immunomodulatory therapy. Although OA had long been considered a non-inflammatory form of arthritis resulting from "wear and tear" of articular cartilage,[1] there has been a renewed interest in inflammatory[2,3] and immune[4,5] pathways involved in OA pathogenesis. However, many studies investigating immune involvement in OA development rely on samples from human patients in end-stage disease,[6–8] when joint destruction has already occurred, and targets for disease modification may no longer be present,[9,10] or on small animal models that suffer from small sample volume and biomechanics that do not approximate that of humans[11,12]. Furthermore, there remains a knowledge gap in understanding how immune cell phenotype and function contributes to initiation and propagation of OA. Our novel tri-culture system and availability of synovial fluid samples from equine and human patients with PTOA allowed us to elucidate novel mechanisms behind immune contributions to OA and to provide novel targets for immunotherapeutics in OA mitigation.

Overall, the results of this work support the roles of T cells and orchestration of inflammation in joint homeostasis, and initiation and progression of OA. Failure of Tregs to mitigate inflammation in Chapters 2 and 3 results in OA progression, and subsequent increase of Th17 cells into the joint in Chapter 4, further inducing joint catabolism. Poor orchestration of inflammatory response associated with elevated and persistent IL-6 in synovial fluid in Chapter 5 is marked by dysregulation of pathways associated with the complement system, neutrophil activation and degranulation, and enzymes associated with joint catabolism and mitochondrial dysfunction. Our results suggest that IL-6 is a major contributor to OA progression and is associated with dysregulation of homeostasis within the joint following trauma. Furthermore, positive feedback of IL-6 secretion induced by IL-6 stimulation in chondrocytes and synoviocytes[13] may be a contributor to Treg phenotype plasticity and conversion to Th17-like Tregs,

which maintain Foxp3 expression but secrete IL-17A[14]. IL-17A in turn promotes production of IL-6 by synovial fibroblasts.[15] Overall, this work presents multiple immunotherapeutic targets for the mitigation of OA early in disease, with IL-6 being an ideal first candidate. Future studies can build on this work, advancing our knowledge of immune and inflammatory pathways in the initiation, mitigation, and progression of OA.

Strengths of the work presented in this thesis

A major strength of this work it its use of the equine model to elucidate the roles of Treg and Th17 cell phenotype and function in the development of OA. The horse is an established model for OA in humans,[10,16] and similar to humans, horses are athletes who develop spontaneous OA[10]. Thus, insights gained from research performed using an equine model can not only be applied back to equine patients but are a direct steppingstone to human clinical studies for drugs and treatments that target OA.[17] Furthermore, the equine model has multiple advantages over both rodent models and other large animal models of OA. Although rodent models are low cost and can be genetically manipulated, there are dissimilarities in cartilage structure and loading compared to the human joint.[11] Additionally, drugs that show efficacy in rodent models are not always translatable to humans with similar success.[18] Similar to horses, dogs are subject to development of naturally occurring OA and are a widely used preclinical model of knee OA[19], but their popularity as a companion animal species draws scrutiny from the public in terms of their use for biomedical research. While sheep and goats better approximate human joint size than dogs, the majority of sheep and goat OA models involve joint destabilization that can lead to rapid disease progression, which is not particularly reflective of mild or moderate PTOA, making it difficult to study earlier stages of OA progression.[20] In comparison to other animal models of OA, cartilage thickness and biomechanical loading of joints in horses most closely approximates that of humans.[10,12] Additionally, there are multiple types of horse experimental models including intraarticular injection of chemicals, destabilization, trauma, osteochondral fragmentation and exercise, and disuse, enabling researchers to address a variety of questions related to OA pathobiology and potential treatments.[10]

In this particular work, equine joint size not only facilitates collection of a sufficient number of cells to allow for establishment of a novel tri-culture system, but also repeated use of chondrocyte/synoviocyte donor and Treg donor cells to account for biological variation between animals in aim 1. The tri-culture system enabled us to tease apart contributions of each specific cell type in early OA, and in future studies, can be applied to not only to additional T cell subsets, but also to additional immune cell subsets. Moreover, the repeated use of donor allowed us to determine that effects of IL-1β and IL-6 neutralization were dependent upon the donor, suggesting that an individualized medicine approach will be necessary for the use of αIL-6 neutralizing antibody and other immunotherapeutics in the treatment of OA.

In aim 2, the use of an equine model allowed for collection and analysis of critical non-op normal samples, which are simply not available from human patients. This enabled us to, for the first time, identify Treg and Th17 populations with the synovial fluid of healthy joints, leading to the discovery that these T cell subsets are present in the joint prior to injury. The research in this aim revealed that Treg phenotype plasticity is present in moderate PTOA. The use of equine patients allowed for collection of samples from the matched, normal, contralateral joint. Furthermore, analysis of samples from naturally occurring PTOA gives greater insight into potential targets for immunomodulatory therapies compared to small animal model of PTOA such as ACL transection and destabilization of the medial meniscus, which often result in rapid joint degeneration.[21] Additionally, equine joint size allowed for collection of sufficient synovial fluid volume to analyze cell populations. We also developed a novel protocol for isolation of synovial fluid cells without loss of viability, while also creating cell free supernatant for analysis of native synovial fluid samples. Importantly, this research confirms that the equine model is relevant for the study of immune cell dynamics in joint homeostasis and PTOA progression and provides an opportunity for future in-depth study into immune contributions to joint heath.

Limitations of the work presented in this thesis

Although the equine model is a powerful tool to study mechanics of OA initiation and progression, it does come with several limitations. Despite the fact that there is an ever-increasing availability of reagents to study equine immunology, they are still lacking, especially in comparison to availability of reagents for work in mice and humans. This is highlighted in the tri-culture model where an additional antibody, CD127, is typically used in the sorting of purified Tregs,[22] and kits with antibody cocktails are available commercially. Moreover, kits are also available for activation and expansion of purified Tregs, providing a more homogeneous population from which to study. In the same course, the α-equine IL-6 antibody used for IL-6 neutralization was not developed for the neutralization of IL-6, and thus was inefficient at doing so.

Furthermore, the limited availability of equine-specific and cross-reactive antibodies also had an impact on the ability to define T cell phenotype by flow cytometry. $CD3^+$ T cell and $CD4^+$ T cell populations were not evaluated simultaneously and thus there are insufficient explanations for why $CD3^+$ T cells in synovial fluid increased during PTOA progression, but $CD4^+$ T cells decreased. $CD8^+$ T cells are present in PTOA synovial fluid,[23] and therefore an antibody panel including CD3, CD4 and CD8 would have been advantageous for the characterization of infiltrating T cell populations. Antibodies targeting the Th17 master transcription factor, RORγt, and/or IFN-γ would also be valuable in distinguishing Th17 vs Th1/Th17 driven responses within the joint following trauma, as this would provide more specific insight in the future into what immunotherapeutics would be most appropriate to reduce inflammatory response and mitigate disease progression.

ELISAs have previously been used to quantify cytokines and chemokines in native synovial fluid,[24,25] but analysis of cytokines and chemokines in synovial fluid by multiplex and singleplex ELISA in this study resulted in most samples falling below the limit of detection. Cytokine/chemokine epitopes may have been more available for antibody binding if samples had been diluted in PBS[26] or digested with hyaluronidase[27] prior to analysis.

Although use of samples from patients with acute ACL injury gives insight into mechanisms involved in the development of PTOA, upon gross evaluation, most samples at 1-week postop from the IL-6^{high} groups had notable blood contamination and infiltration of erythrocytes. Mouse models of blood-induced cartilage exist, and it should therefore be taken into consideration that IL-6 concentrations correlated with blood-induced inflammation rather than as a biomarker for persistent inflammation.13,59 A major limitation to this study was that, while bottom-up proteomics offers an un-biased approach, the abundance of albumin in the samples outcompeted proteins of low abundance in the samples, including cytokines and chemokines, which are major orchestrators of inflammation. Depletion of albumin was not considered since albumin is a carrier protein, and its depletion might have also removed low abundance proteins, further limiting the pool of detected proteins.60

Future directions

This thesis provides the foundation for characterizing T cell populations and dynamics and dysregulation of inflammatory response orchestration in relation to OA initiation and progression and can thus be used to begin additional work to further out knowledge of the role of the immune system and inflammation in OA development. The use of equine and human samples from early OA will inform the development and implication of novel therapeutics to mitigate OA progression.

Interleukin-6 as an immunotherapeutic target in mitigation of OA

Beyond the scope of this thesis, further studies to test IL-6/IL-6R neutralization *in vivo* should be performed. These studies should take into consideration the stage of disease for timing of treatments as the data of this thesis suggests that multiple windows of opportunity exist for intervention. In parallel, a serum or urine biomarker for disease state should be investigated to provide this personalized medicine approach to OA immunotherapy.

Monoclonal antibodies targeting the IL-6 (siltuximab) and its receptor (tocilizumab) are already approved by the FDA as safe and effective for the treatment of a variety of diseases, including

rheumatoid[31–33] and systemic juvenile idiopathic arthritis[34,35]. IL-6 is involved in multiple intracellular signaling cascades, including the JAK/STAT, MAPK, and PI3K-protein kinase B/Akt pathways.[36] In chondrocytes, this induces production of matrix metalloproteinases (MMPs)[37,38] and A Disintegrin and Metalloproteinase with Thrombospondin motifs (ADAMTS)[39], which contribute to cartilage matrix degradation. IL-6 signaling pathways contribute to synovial hyperplasia and fibrosis[40], angiogenesis[41], secretion of pro-inflammatory cytokines such as monocyte chemotactic protein-1 and IL-8[42], and expression of cellular adhesion molecules[42].

As described in Chapter 3, neutralization of IL-6 alone in our tri-culture model reduced gene expression of both *MMP13* and *Il6* in synoviocytes and chondrocytes without deleterious off-target effects. Furthermore, in a mouse model of OA, IL-6 receptor neutralization following destabilization of the medial meniscus reduces severity of OA lesions, osteophyte formation, and synovitis score.[43] The next logical step is to assess the efficacy of IL-6R neutralization in a large animal model of OA, such as equine[16,44] or porcine[45,46]. In using an equine model, the first step would be development of an equine-specific IL-6R neutralizing monoclonal antibody. As discussed in Chapter 3, use of a polyclonal antibody that was not originally developed for neutralization led to deleterious effects on chondrocytes and synoviocytes prior to reaching a dose of complete IL-6 neutralization. Mild-moderate PTOA would then be induced in horses by arthroscopic surgery[44] and would be followed by intraarticular injection of αIL-6R antibody. Outcome measures would include 1) Histological evaluation of osteochondral blocks to assess articular surface and subchondral bone changes, and synovial membrane to assess synovitis and infiltration of immune cells; 2) Assessment of synovial fluid biomarkers of early PTOA and inflammation including MMPs and pro-inflammatory cytokines by ELISA; 3) Characterization of immune cell phenotype within the synovial fluid and synovial membrane by flow cytometric analysis; 4) Evaluation of expression of genes related to OA and IL-6 signaling pathways by RT-qPCR. Success with this preclinical model would enable clinical testing of αIL-6R for the use in equine veterinary patients and, ultimately, clinical trials of already available αIL-R therapeutics in human patients with early OA.

Temporal and phenotypic characterization of T cell dysfunction in OA progression

As shown in this thesis, multiple subsets of T cells, including Treg and Th17 cells, are present within the joint prior to and following injury, providing important implications for targeted immunotherapeutic intervention in the treatment of OA. However, the temporal progression and phenotypic repertoire of T cells within the joint is not currently well characterized. This may in part be due to the historical characterization of OA as "non-inflammatory" arthritis, with RA as the "inflammatory" arthritis. However, valuable insights can be gained from studies that compare these two diseases. Zhang et al. revealed that, similar to RA, T cells in late stage OA express hallmarks of inflammaging, which is characterized by progressive dysregulation of immune function, including expression of age-associated granzyme K (GZMK) and CCR7.[47] T cells that express GZMK simultaneously express markers associated with exhaustion and tissue homing, causing these cells to be phenotypically similar to exhausted T cells.[48] Furthermore, $GZMK^+$ T cells promote a senescence-associated secretory phenotype in fibroblasts,[48] which causes fibroblasts to secrete a variety of cytokines and chemokines including IL-6, IL-8, monocyte chemoattractant proteins (MCPs) and macrophage inflammatory proteins (MIPs), further promoting inflammation within the joint. CCR7 is expressed by central memory T cells, which have experienced antigen and do not carry out immediate effector functions, but act as a pool of memory stem cells ready to divide into effector cells upon antigen-specific stimulation.[49,50] Similar expression patterns are seen in dysfunctional T cells associated with cancer and chronic infection, raising the question if further investigation of T cell phenotype and function in OA should be more reflective of these diseases, rather than RA, which is an autoimmune disease.[51] The immunoprofile of OA likely reflects that of cancer and chronic infection, which can be characterized by the presence of naïve, functional, and early and late dysfunctional T cells.[51] Further exploration of these concepts within the context of OA using a combination of single-cell RNA sequencing and single-cell transposase-accessible chromatin sequencing in normal joints and during different stages of disease progression, including the parameters of timing and severity of disease, will fill the important knowledge gap of when and how T cell dysfunction occurs within the joint, and how this contributes to OA initiation

and progression. This information will yield novel insights into how T cells drive OA pathogenesis that can provide alternate immunotherapeutic approaches to mitigating OA progression.

Regulatory T cells as a novel immunotherapeutic target in the mitigation of OA

This thesis presents several novel targets that have the potential to be used as immunotherapeutics in the mitigation of OA progression. Based on our work in chapters 2 and 3 and others, it is clear that Tregs are capable of secreting chondroprotective[28,29] and immunosuppressive44,45 cytokines that can serve to both protect cartilage from catabolism and cause phenotypic switch of pro-inflammatory M1 macrophages within the synovial fluid and synovium to an anti-inflammatory M2 phenotype46,47 in order to enhance repair within the joint. Further investigation of Treg mechanisms of joint protection may have important implications for the development of immunotherapeutics.

Although we begin to investigate the effects of Treg cell-to-cell interactions and secreted factors on inflamed chondrocytes and synoviocytes, Tregs alone are not able to mitigate the effects of IL-1□-induced inflammation and catabolism, and αIL-6 neutralizing antibody is additionally required to facilitate protection. The increase in *Il6* gene expression by synoviocytes but not chondrocytes following incubation with Tregs suggests that a cell-contact dependent mechanism caused an increase in *Il6* gene expression. Suspected interactions between non-MHC-matched synovial macrophages and residual pro-inflammatory effector T cells within the Treg population may have been the cause of *Il6* gene expression increase. The next step to this initial set of experiments would be to apply Treg secretome alone, in the absence of Tregs themselves, to IL-1β-stimulated co-cultures.

The secretome of a cell includes secreted proteins such as cytokines, chemokines, shed receptors, and microRNAs that are involved in cell-to-cell communication. The mesenchymal stromal cell (MSC) secretome is currently under investigation for its immunomodulatory46,48,49 and regenerative50,51 capabilities and provides promise as an effective cell-free therapeutic option. However, reproducibility of the effects achieved in individual studies with MSC secretome is difficult to achieve.52 Tregs also play

critical roles in tissue repair,53–55 wound healing,56 and stem cell differentiation57. The Treg secretome includes immunosuppressive and anti-inflammatory cytokines including IL-10, IL-35 and TGF-β, but overall composition of Treg secretome is modified by both the tissue microenvironment and pathological conditions within the tissue,58 implying that not only is there likely a joint-specific Treg secretome, but also one that aids in mitigation and prevention of OA following joint trauma. Currently, synovial tissue Tregs are poorly characterized, and the secretome has not been studied. However, Treg secretome may offer an alternate to MSC secretome by suppressing inflammation within the joint and facilitating repair.

Monoclonal and polyclonal Tregs are currently under investigation for use in the alleviation of autoimmune diseases and prevention of graft vs host disease in tissue transplant patients.59 In regard to OA patients, it is known that T cells that react and expand in response to joint antigens are present within the circulation60,61 and within the affected joint62,63 of these patients. These T cells contribute to inflammation and joint pathogenesis and manage to escape regulation of self-tolerance by Tregs within the circulation and joint. There are several potential cell-based therapeutic options for these patients, including antigen-specific polyclonal Tregs expanded *in vitro*, and genetically engineered Tregs that express specific T cell receptors or chimeric antigen receptors59,64. Tregs suppress effector cells with which they share antigen, and it would therefore be critical to first establish the repertoire of self-antigens that effector T cells within the body are recognizing. But clinical trials and animal models have been successful in the mitigation of allograft rejection and autoimmunity with no cytotoxicity or adverse events.64 As the above Treg-centered therapeutic techniques are further investigated and become more widely available for a variety of pathologies, they offer promise in the resolution of joint inflammation and mitigation of OA.

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