Exploring the pathogenesis of Juvenile Idiopathic Arthritis-uveitis using rat models of uveitis and arthritis.

A thesis submitted to the College of Graduate and Postdoctoral Studies In partial fulfillment of the requirements for Masters of Science in Veterinary Ophthalmology, Small Animal Clinical Sciences, Western College of Veterinary Medicine, University of Saskatchewan

By

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Abstract

Purpose: The etiology and pathogenesis of uveitis associated with Juvenile Idiopathic Arthritis (JIA) are poorly understood. The purpose of this rat-based research is to explore the potential of a shared collagen based self-antigen within the joint and eye in the rat as a factor involved in the etiopathogenesis of arthritis and associated uveitis. As young age and female sex are risk factors for uveitis in children with JIA, their influence on experimental autoimmune anterior uveitis in the rat was evaluated by ophthalmic evaluation with biomicroscopic, light microscopic ocular examinations, and pro-inflammatory vitreous cytokine profiles.

Methods: Adult and juvenile male and female Lewis rats were inoculated intradermally with either: intact type I collagen derived from bovine skin, type II collagen, or derivatives of type I collagen including melanin associated antigen (MAA) *or* soluble MAA which was digested in *Staphylococcal* V8 protease, *Streptococcus* streptokinase C, or matrix metalloproteinase (MMP)-1. Inoculations were repeated up to three times at intervals of 1 or 4 weeks. Biomicroscopic and indirect ophthalmic examinations were completed in live rats at baseline and biomicroscopic examinations were repeated three time per week throughout the study period by a masked Diplomate of the American College of Veterinary Ophthalmologists (ACVO). At the end of the observation period globes were enucleated and vitreous was aspirated. Histopathology slides of the globes were reviewed by a masked Diplomate ACVO. Rats treated with insoluble MAA had clinical uveitis scores, ocular histopathological scores, and cytokine analysis compared between age and sex groups and control animals. An array of 27 cytokines were quantified with a multiplex bead-based immunoassay on vitreous from rats treated with MMP-1 digested type I collagen derived from bovine skin, or type II collagen derived from bovine cartillage, and rats treated with insoluble melanin associated antigen. Immunohistochemical labels for CD43 and CD45RC were compared between solubilized MAA groups and control animals.

Results: None of the rats inoculated with any form of type I collagen derived from bovine skin, or type II collagen derived from bovine cartilage, developed uveitis that could be detected clinically or light microscopically. 28/44 rats inoculated with intact type II collagen developed arthritis. Vitreous cytokine levels did not differ between any treatment group and controls. All rats inoculated with insoluble MAA developed uveitis on biomicroscopic and light microscopic examination and no differences were identified between age and sex groups. Uveitis was present

in 3/12 *Staphylococcus aureus* V8 protease MAA inoculated rats and 2/12 streptokinase C solubilized MAA inoculated rats.

Conclusions: None of the digested or intact forms of type I collagen derived from bovine skin, or type II collagen derived from bovine cartilage, resulted in uveitis in the Lewis rat. While type II collagen induced arthritis, digestion of type II collagen renders it non-pathogenic. *Streptococcus* streptokinase C and *Staphylococcus aureus* V8 protease digested MAA induced uveitis in some rats when inoculations are repeated three times. Insoluble MAA induced uveitis in all rats and no difference in disease incidence, severity, or onset was observed between sexes or age groups in rats inoculated with MAA.

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List of Abbreviations

ANA: Anti-nuclear antibody

CIA: Collagen induced arthritis

EAAU: Experimental autoimmune anterior uveitis

EGF : Epidermal growth factor

G-CSF : Granulocyte-colony stimulating factor

GM-CSF : Granulocyte-macrophage colony-stimulating factor

GRO : Human growth-regulated oncogene

IL: Interleukin

IMAA: Intact melanin associated antigen

INF: Interferon

IP-10 : Interferon-gamma induced protein

JIA: Juvenile Idiopathic Arthritis

KC : Keratinocyte chemoattractant

kDA: kilo Dalton

LIX : Lipopolysaccharide-inducible CXC chemokine

MAA: Melanin associated antigen

MCP-1 : Monocyte chemoattractant protein

MIP : Macrophage inflammatory protein

MHC: Major histocompatibility complex

MMP: Matrix metalloproteinase

RANTES : Regulated upon activation, normal T cell expressed, and secreted

SD: Standard deviation

Th: T-helper

Tregs: Regulatory T cells

TNF: Tumor necrosis factor

sMAA: Soluble melanin associated antigen

VEGF : Vascular endothelial growth factor

1. Literature Review

1.1 Juvenile Idiopathic Arthritis

JIA is a heterogeneous group of autoimmune arthritides of unknown etiology lasting longer than six weeks and having onset in children less than 16 years of age[1]. It is the most common form of arthritis in children in the western world[2]. In most cases the disease remains active for years and persists into adulthood[3,4]. JIA is likely influenced by interacting genetic and environmental factors[5-9].

There are seven JIA categories[1]

1. Systemic JIA: Arthritis in one or more joints with or preceded by fever of at least two weeks duration that has been documented to be daily for at least three days and accompanied by one or more of the following: evanescent erythematous rash, generalized lymph node enlargement, hepatomegaly and/or splenomegaly, and serositis
2. Oligoarticular: Arthritis affecting one to four joints during the first six months of disease. Includes a persistent form in which no more than four joints are involved during the course of the disease or an extended form in which more than four joints become involved after the first six months.
3. Rheumatoid factor-negative polyarthritis: Arthritis affecting five or more joints during the first six months of disease with a negative test for rheumatoid factor.
4. Rheumatoid factor-positive polyarthritis: Arthritis affecting five or more joints during the first six months of disease with a positive test for rheumatoid factor as documented on at least two occasions at least three months apart.
5. Psoriatic: Arthritis and psoriasis or arthritis and at least two of the following: Dactylitis, nail pitting or onycholysis, psoriasis in a first degree relative
6. Enthesitis-related arthritis: Arthritis and enthesitis or arthritis and two or more of the following:

   •Sacroiliac joint tenderness

- Inflammatory spinal pain
- Human leukocyte antigen (HLA)-B27
- Positive family history of anterior uveitis with pain, a spondyloarthropathy, or inflammatory bowel disease
- Anterior uveitis associated with pain, redness, or photophobia

7. Undifferentiated arthritis: Arthritis that fulfills criteria in no category or in two or more of the above categories.

1.2 JIA-uveitis

Uveitis, which is inflammation in the uvea, is the most common debilitating extra-articular manifestation of JIA[10]. Between 9.2% and 13.1% of children with JIA develop uveitis[11-13]. JIA-uveitis makes up between 15 and 67% of all pediatric uveitis presenting to tertiary referral centers [14-18]. Uveitis associated with JIA is an important cause of vision loss and blindness in developing countries[19-22].

1.2.1 Clinical Presentation and Outcome

JIA-uveitis can be acute, subacute, chronic or recurrent and its location within the eye can be anterior, intermediate or pan-uveal. The most common form is a chronic anterior uveitis occurring in 68% of children with JIA-uveitis[23]. Acute anterior, recurrent anterior and panuveitis also occur with JIA albeit less frequently than chronic anterior uveitis, with their incidence occurring in 16.2,12, and 3.5% of cases respectively[23]. The chronic asymptomatic uveitis is typical of children with the oligoarticular subtype of JIA while the acute, symptomatic form is characteristic of the enthesitis-related subtype of JIA. The chronic anterior uveitis is most common in females, and children diagnosed at a young age. Males are more likely to have acute symptomatic uveitis[23]. The uveitis is more often bilateral (60.6%) but can be unilateral[23]. It is most commonly asymptomatic, insidious in onset, and many children are too young to

communicate or discern changes in visual acuity. This clinically silent nature of the disease may result in advanced pathology at the time of diagnosis[23]. Regular screening for uveitis in children with JIA is recommended to allow detection and prevent vision threatening complications.

A significant improvement in uveitis control has been observed over the past decades[24-25]. In one study 30% of children had inactive uveitis in 2002, while 65% were inactive in 2013[24]. Advances in treatments have been credited for these successes. If uncontrolled clinical signs of uveitis can extend into adulthood and results in significant morbidity[26-29]. Despite progress in early detection and treatment of JIA-uveitis, vision-threatening complications have been reported in up to 60% of patients in some studies [28]. These vision disabling complications include band keratopathy, maculopathy (macular edema, macular cysts, and epiretinal membranes), glaucomatous optic neuropathy, and cataracts [28,26]. Uveitis can precede development of arthritis in 3-7% of children[30]. Discordant patterns of disease between the eye and the joint have been observed in 70% of patients suggesting differences in pathogenesis of recurrence and initiation of arthritis and uveitis[31].

1.2.2 Risk Factors

Risk factors for development of JIA-uveitis have been established and include HLA alleles, female sex, young age of onset, a positive test for anti-nuclear antibodies (ANA), and oligoarticular JIA[10,32-33].

There is substantial evidence for a genetic component of JIA. However, the multiple categories of JIA, its heterogeneous nature, and wide inclusion criteria for diagnosis of JIA, complicate genetic studies. JIA has not demonstrated Mendelian or monogenic patterns of inheritance and the genetic basis for JIA and JIA uveitis is likely very complex and considered modest[9]. Despite its complexity, multiple factors demonstrate the role of genetics. Concordance rates amongst monozygotic twins (25-40%) are higher than the overall population risk of 1 in 1000[34]. Aggregation of clinical features between affected sibling pairs and twin pairs also supports the role of genetics[35-36]. Genetic analysis has identified JIA susceptibility loci in both human leukocyte antigen and non-human leukocyte antigens. Susceptibility for development of

oligoarticular JIA has the most defined HLA associations including DRB1:01, DRB1:08, DRB1:11, DRB1:13, DPB1:02 and DQB1:04[37]. HLA DRB1*04 and DRB1*07 may be protective against development of oligoarticular JIA[38]. Human leukocyte antigen alleles HLA-DRB1:11 and HLA-DRB1:13 increase the risk of uveitis development in children with JIA[39].

Female sex is a risk factor for the development of uveitis in JIA patients. More females than males develop uveitis[19, 23, 40] with up to 79.6% of children that develop uveitis being female[13]. In addition to differences in prevalence between sexes, the disease onset and manifestations are also different. The risk of developing uveitis in girls with JIA is highest in girls aged 1-2 years old, whereas uveitis risk is not considered age-dependent in boys who are typically older than girls at the time of both JIA and uveitis diagnosis[23]. Only 15% of females present with uveitis as the initial manifestation of their disease, compared to 44% of males[41, 42]. Males are more likely to have acute symptomatic uveitis and a shorter interval between diagnosis of arthritis and uveitis. Males with uveitis are more likely to have enthesitis-related arthritis or psoriatic JIA, compared to females who were more likely to have oligoarticular JIA[23].

Disease severity and complication rates are also reported to vary between sexes, with males developing more severe disease and having a higher complication rate[10, 13,14,41,43]. It has not been determined if the increased likelihood of uveitis in girls is related to intrinsic biological differences between sexes, or if it can be attributed to the predominance of females with oligoarticular arthritis and positive ANA titer[23]. Autoimmune diseases in general are more prevalent amongst females with women making up 78% of those affected[44-46]. Basic immune responses differ between males and females. Women primarily respond to infection, trauma, and vaccination with classical Th2 mechanisms and increased antibody production. Men develop a stronger Th1 response which results in increased severity of inflammation[47-52]. Estrogen interaction with the immune system is complex and it exerts both suppressive and pro-inflammatory roles[51-53].

A positive ANA titer is also a known risk factor for uveitis development. In a Canadian study 76% of children with JIA-uveitis were ANA positive[54]. A positive ANA test present at the onset of uveitis is likely to portend arthritis if arthritis is not already present[55-56]. ANA positivity is not predictive of uveitis severity, relapses or outcomes[57]. The antigenic specificity of ANAs in children with JIA, and the role of ANA in the pathophysiology of JIA are unknown[58].

Young age at onset of arthritis is a risk factor for uveitis development. Children with an age of onset of JIA ≤4 years are at increased likelihood for developing uveitis. Several HLA associations have been correlated with onset age. HLA class II associations have demonstrated specific age windows of susceptibility to JIA subtypes[59]. HLA alleles associated with uveitis risk, HLA–DR11 and HLA–DR13, are more often observed in patients with disease onset before the age of 7 years[59]. An increase in the number of HLA risk alleles predisposes to earlier development of JIA[59]. Uveitis activity has demonstrated biphasic disease activity with a quiet phase around the age of 9 years followed by increased severity during early teenage years[42].

The oligoarticular form of JIA is a risk factor for the development of uveitis. In a Canadian study of children with JIA-uveitis 55% had oligoarticular arthritis, 22% had rheumatoid factor negative polyarthritis, 11% had undifferentiated arthritis, 3% had systemic JIA, and 1% had enthesis-related arthritis[54]. Oligoarthritis and rheumatoid factor negative polyarthritis subtypes are significantly associated with uveitis development[54]. Young onset age and ANA positivity are typical of oligoarthritis and therefore while oligoarthritis may be associated with uveitis development it may actually have a higher incidence related to factors other than ANA positivity such as sex, onset age, and JIA category[54].

1.2.3 Ocular Pathology

Few ocular tissues from children with JIA-uveitis are available for histopathologic analysis. Iridectomy samples of children with JIA-uveitis undergoing trabeculectomy demonstrated a slight predominance of CD4+ cells rather than CD8+ cells, and plasma cells and histiocytes were the cell type present most consistently[60]. These samples were obtained from eyes with minimal inflammation and the histologic picture may have been influenced by their treatment[60]. An enucleated globe from a patient with end stage JIA-uveitis identified B cells and plasma cells as the most abundant inflammatory infiltrate. Rare CD4+ and few CD8+ cells were present. Similar to the iridectomy sample discussed above, this histopathology may also have been influenced by the patient's intense immunosuppressive therapy including anti-tumor necrosis factor (TNF)-α treatment[61].

1.2.4 Pathophysiology

The underlying pathophysiology linking uveitis and arthritis in JIA-uveitis is unknown. It is believed to be a multifactorial autoimmune disorder occurring due to a combination of genetic, environmental, and infectious influences [5, 6, 8, 35]. It has been proposed that an immune response occurs targeting known intraocular antigens like S-arrestin, retinol-binding protein 3, and tyrosinase related proteins [62-65]. T-helper cell (Th)1, Th17, regulatory T cells (Tregs), and pro-inflammatory cytokines contribute to JIA-uveitis pathophysiology, however their roles remain to be fully elucidated[66-72].

1.3 Autoimmunity

Autoimmune disease is a dysregulation of the innate and adaptive immune system that leads the body to attack its own tissues. It occurs due to a loss of immune self-tolerance[73-74].

The normal function of the immune response is to eliminate a pathogen, minimize damage from the pathogen and prevent reinfection with the same pathogen. For this to occur, multiple steps occur to ensure the appropriate immune response is generated and to avoid aberrant self-targeting. The immune system operates through two interacting branches; the innate system which is the immediate defense, and the adaptive which is a delayed but antigen-specific response. The two branches of the immune system are bridged through antigen presenting cells[75]. These cells continually collect molecules from pathogens, as well as molecules from self, due to normal cellular apoptosis and necrosis. The antigen presenting cells combine the collected molecules with major histocompatibility complex (MHC) proteins and display them on their surface as an MHC-antigen complex[75]. The antigen presenting cells express their MHC-antigen complex locally and also travel to lymph nodes and present their MHC-antigen complexes to peripheral T-helper lymphocytes. If the T helper cell has already been exposed to ongoing inflammation, or an antigen-specific immune response, it will have an antibody or a T cell receptor on its surface that binds to the MHC-antigen complex[75]. A further safeguard must be met for the adaptive immune response to occur which is either simultaneous binding of

coactivation molecules on the antigen presenting cell, or the presence of co-activating cytokines in the local environment. The type of co-activating molecule determines the type of immune response[75].

Complement is a major component of innate immunity and also plays a role in antigen processing and presentation, T-cell proliferation and differentiation, B-cell activation,[76-77] and systemic tolerance induced by the introduction of antigen into an immune-privileged site such as the anterior chamber of the eye[78].

Cytokines are a group of small secreted proteins that act as chemical signals between cells[79]. Their physiologic role in inflammation and pathologic role in systemic inflammatory states are being increasingly recognized[79]. They can be produced in a cascade, act synergistically, or antagonistically with other cytokines. For example, anti-inflammatory cytokines control the pro-inflammatory cytokine response. T cells and macrophages are known to produce pro-inflammatory cytokines to upregulate inflammation[79]. In the normal physiologic state, pro-inflammatory cytokines are maintained in equilibrium with anti-inflammatory cytokines. Inflammation shifts this equilibrium, and without inflammation, an immune response does not occur. When inflammation is present various cytokines activate antigen presenting cells so that they express surface based co-activation factors. Finally, co-activation factors together with T helper-antigen presenting cell complex leads to activation of the immune response[79].

In addition to the requirement of T helper cell binding to the MHC-antigen in the presence of co-activation factors and inflammation, a further safeguard is in place to prevent aberrant activation of the immune response; thymic or central tolerance induction [80]. This process deletes T cells that are reactive to self but the process is not absolute and self-reactive T cells can be found peripherally. These cells do not typically lead to the activation of an immune response because of the peripheral requirement for activation cofactors and T helper cell binding to antigen presenting cells with antigen MHC complexes[81].

Triggering of autoimmunity has been suggested to occur through molecular mimicry, bystander activation, and epitope spreading. Molecular mimicry occurs when a molecule is similar enough to cross react with a self-antigen but different enough to break immune tolerance[82]. This mechanism was initially invoked to explain persistent viral infection and suggested that the MHC and viruses encoded similar antigens which allows the host to regard the virus as self[82-83].

Shared epitopes between host and virus were demonstrated in 4% of a panel of 600 antibodies and several antibodies reacted with antigens in more than one organ[84]. Molecular mimicry has been demonstrated in a rat model of experimental autoimmune uveitis between retinal S-antigen peptide PDSAg and class I HLA B27PD amino acids 125-138[82, 85-88]. Epitope spreading occurs in the face of inflammation and tissue destruction where previously unrecognized epitopes become targeted by the immune response, and is characterized by recurrent relapses with recruitment of newly reactive T cells[89-90]. Epitope spreading has been recognized in Equine Recurrent Uveitis and has been implicated in the remitting-relapsing character of the disease[91]. Bystander activation occurs when a pathogen stimulates cytokines which lead to activation of unrelated pre-primed autoreactive T cells[92-93].

1.4 Cytokines as mediators of pathology

Cytokines are considered the major mediators of joint damage in chronic arthritis. Several studies have evaluated cytokine expression in serum or synovial fluid of children with JIA and have found variable cytokine profiles[94-100].TNF-α, interleukin (IL) -6 and IL -8 are elevated in some JIA subtypes relative to other types or controls. TNF-α, IL -1β, IL -6 and IL -17 levels are elevated in JIA patients with active disease when compared to those with inactive disease. Serum profiles of children with JIA and uveal tract inflammation have demonstrated increased concentrations of pro-inflammatory TNF-α and IL-17 cytokines along with reduced IFN-γ and increased IL-10 levels when compared to children with JIA and no signs of uveal tract inflammation[101].

Aqueous humor cytokine levels in children with JIA-uveitis demonstrate significantly higher levels of IL -2, IL -6, IL -13, IL -18, IFN-γ, TNF-α, sICAM-1, RANTES and IP-10 compared to control children without JIA or uveitis[102]. IL-8 and IL-10 levels in aqueous humor were significantly increased over control levels in a group of children with uveitis of varying etiology including JIA. No differences were found between cytokine levels in aqueous humor samples of patients with different types of JIA[102]. Another study comparing cytokines between children with JIA-uveitis and other childhood uveitidies found children with JIA-associated uveitis and those

with idiopathic uveitis not associated with JIA had distinct profiles of intraocular soluble mediators. Aqueous humor levels of IFNλ1 were specifically decreased in patients with JIA-associated uveitis compared to children with idiopathic uveitis[103].

Even aqueous humor samples collected from children with clinically inactive uveitis demonstrated concentrations of IL-8, TGFβ-1, TGFβ-2, TGFβ-3, serum amyloid A, and TNF-α that were significantly elevated compared to controls[104]. Despite having clinically inactive JIA-uveitis and receiving intensive anti-inflammatory treatment, these cytokine levels suggest that clinically inactive disease remains immunologically active[104]. Subclinical cytokine activity could serve as an early warning system to predict disease flares and allow for early intervention and more clearly defined treatment endpoints[104].

Variation in aqueous cytokine profiles between children and adolescents and adults have been reported. Sijssens et al (2008) found children and adolescents had different aqueous levels of Il-1, IL-2, IL-4, IL-6, IL-10, IL-12 p-70, IL-13, IL-18, IFN-γ, TNF-α, sICAM-1, soluble vascular cell adhesion molecule 1, and Eotaxin compared to adults[105]. IL6 was the only cytokine significantly higher in adults whereas the remainder of them were significantly lower in adolescents and children. IP-10, RANTES, and IL-8 levels did not differ between age groups[105].

Gene associations coding for cytokines or their receptor have been described in children with oligoarticular JIA/polyarticular RF-negative JIA[106]. Tumor necrosis factor alpha gene encodes for pro-inflammatory cytokine TNFα and studies have identified associations between polymorphisms in the TNFA gene and JIA[107-112]. A particular allele, TNF G-30A has been identified repeatedly and in a meta-analysis[113]. There have also been reports of varied responses to therapy dependent on TNFA genotypes[114]. Polymorphisims in the macrophage migration inhibitory factor gene has also been associated with JIA [115-118] and a specific MIF -173 C allele has been identified that is associated with relapse[117]. The gene ILRA encodes the interleukin receptor alpha and has demonstrated associations with JIA[119]. CCR5 is a chemokine that promotes joint inflammation and a 32 base pair deletion in its gene has demonstrated a protective role against JIA[120-121].

Inhibition of specific cytokines in the course of JIA is credited for the improvement in outcomes observed in affected children over the recent decades. These medications include TNFα inhibitors and monoclonal antibodies targeting IL-2 or IL-6 receptors[122].

1.5 Animal Models

Animal models provide a valuable avenue for exploration of triggers, risk factors, pathophysiology, and treatments for human disease. Animal models can clarify pathogenesis by limiting variables, allowing study during early phases and providing longitudinal analysis of disease manifestation.

A suggested pathogenesis for JIA-uveitis involves cross reactivity with antigens between the eye and the joint. Collagen breakdown in either the eye or the joint via either an endogenous protease or exogenous protease may result in exposure of a collagen-related autoantigen. Currently, there is no animal model for JIA-uveitis but there is an established model for autoimmune anterior uveitis and an established model for autoimmune arthritis. Both of these models generate an autoimmune response to collagen. Further investigation is required to determine if there is a shared collagen-based antigen between the eyes and joint that could be involved in the pathogenesis of arthritis-uveitis.

Experimental models of either autoimmune uveitis or autoimmune arthritis can be initiated through inoculation with heterologous and homologous collagens[123-125]. Collagens are a family of fibrous proteins produced by many different cell types present throughout the body in many tissues. Over 25 different types of collagen have been recognized. All collagen molecules share a similar biochemical structure characterized by triple helical configuration, glycine in every third amino acid position, and an abundance of proline/hydroxyproline and lysine/hydroxylysine[126] . Although collagens are highly conserved proteins, tissue-specific structural differences have been noted[127-129].

1.5.1 Collagen Induced Arthritis

Collagen-induced arthritis (CIA) is a form of experimental autoimmune arthritis that can be induced in genetically susceptible rats or mice following injection of bovine intact type II collagen. It is the most commonly studied autoimmune model of rheumatoid arthritis[130].

Susceptibility to CIA is linked to major histocompatibility complex II genes and the immunopathogenesis involves both a T-cell and B-cell specific response to type II collagen[131].The arthritis develops in the tarsal and metatarsal joints 21-28 days after immunization[130]. Pathological features include synovial hyperplasia, mononuclear cell infiltration, and cartilage degradation[130]. The arthritis course and outcome vary depending on the age of animal inoculated[132]. Estrogen has been shown to protect against CIA in DBA/1LacJ mice and Lewis rats, and male mice are more susceptible to CIA than female[133-135]. Uveitis has been reported in 10% of female Sprague-Dawley rats inoculated with type II collagen[136]. Half of the affected rats had developed a concurrent arthritis while the other half had not[136]. Uveitis has also been documented in 1/15 rats receiving spleen cells from rats inoculated with type II collagen[137]. Further investigations of uveitis in rats inoculated with type II collagen are warranted to improve our understanding of the link between the eye and joint in children with JIA-uveitis.

1.5.2 Animal Models of Uveitis

Posterior Uveitis Models

Experimental autoimmune uveitis (EAU) induces a predominantly posterior uveitis with destruction of the retina. EAU is induced by inoculation with soluble retinal proteins, either interphotoreceptor binding protein[138], S-antigen[139], rhodopsin[140], or transducin[141]. The predominantly posterior nature of EAU does not reflect the clinical characteristics of autoimmune anterior uveitis in people or juvenile arthritis/uveitis.

Experimental Autoimmune Anterior Uveitis

Experimental autoimmune anterior uveitis (EAAU) is an animal model for human anterior uveitis. EAAU manifests as a severe bilateral predominantly anterior uveitis in nearly all rats approximately 11-15.5 days post inoculation[124,142-144]. Light microscopic examination of affected rats reveal a marked lymphocytic iritis and cyclitis [123, 124,142-146]. CD4+ T cells predominate throughout the course of EAAU and a small number of CD8 T cells and macrophages have been

observed[143]. Antigen specific CD4+ T cells can adoptively transfer disease into naive syngeneic recipients[123,143-144] whereas serum cannot[143-144].

The antigen in melanin associated antigen (MAA) has been identified as a 22 kDa fragment of the α-2 chain of type 1 collagen found in the anterior uvea[124].This experimental autoimmune uveitis model has been demonstrated using both heterologous (bovine) and homologous uveal type I collagen in the Lewis rat. Type I collagen is the most abundant collagen in the body and a key structural component of bones, tendons and ligaments[147]. Almost 50 different molecules that interact with type I collagen have been recognized[147]. Binding sites on type I collagen include sequences for integrins[148-153] ,IL-2[154], *Staphylococcus aureus* cell surface molecules[155], *Staphylococcus aureus* matrix binding proteins[156], MMP-1[157] and many others[147]. Immunohistochemistry of naive rats has demonstrated the presence of endogenous MAA in the normal iris and ciliary body[124].

To isolate the antigenic 22 kDa fragment of uveal type I collagen, MAA is digested by V8 protease, a proteolytic enzyme secreted by *Staphylococcus aureus*. It is possible that other endogenous and exogenous proteases could play a role in collagen degradation in either the eye or the joint resulting in exposure of an antigenic peptide. For example, MAA digestion with MMP-1 results in a fragment of type 1 collagen between 20 and 25 kDa[124], but the antigenicity of this fragment has not been evaluated. This suggests that MMP-1 may also play a role in the pathogenesis of JIA by acting endogenously to expose a shared pathogenic peptide between the joint and the eye.

The digested MAA, referred to as “soluble MAA”, has demonstrated antigenicity only when administered in conjunction with complete Freund's adjuvant[142, 144]. In its intact form prior to digestion it is also antigenic, even without the use of adjuvant. In the intact form, the MAA backbone has been speculated to act as an adjuvant[142, 144].

Without the use of adjuvant, soluble MAA has not induced uveitis. Complete Freund's adjuvant alone induces uveitis and arthritis[136, 158] confounding results when it is used in conjunction with soluble MAA. When CFA is injected at a dose of 0.25mg-1mg, more than 90% of Sprague

Dawley and Lewis rats develop histologic evidence of uveitis, while only 20-28% demonstrate uveitis on biomicroscopic examination[136]. Complete Freund's adjuvant contains inactive mycobacteria known to stimulate cell-mediated immunity, tumor necrosis factor dysregulation, and uveitis[136, 137,158-159]. Incomplete Freund's adjuvant provides a water-oil emulsion to facilitate inoculation administration without immune stimulation.

The majority of MAA studies have been completed in 6-8 week old male rats [123-124,142-144]. EAAU has not been evaluated in adult rats, and 5-6 week old female rats have been evaluated in only one study[144]. No comparisons between adult and juvenile, nor male and female rats have been reported. It is unknown why differences in uveitis manifestation occur between male and female children with uveitis. In a model of uveitis that uses interphotoreceptor retinoid binding protein peptide, estrogen enhanced uveitis in females, correlating with the ocular levels of Th1 (IFN- γ) and Th2 (IL-10) cytokine messengers[160].

Both humoral and cellular immune responses occur in EAAU. However, transfer of disease only occurs through CD4+ T cell transfer, and not through transfer of serum[123]. The expression of cytokines, chemokines, and adhesion molecules necessary for the development of EAAU requires availability and activation of complement. Interference in the availability of complement by systemic depletion leads to the suppression of disease[146]. Complement regulatory proteins also play an active role in the resolution of the disease by down regulating complement activation[161].

Cellular adhesion molecules are upregulated in EAAU. Intercellular adhesion molecule-1 (ICAM-1), a surface glycoprotein that binds leukocyte integrins in the face of inflammation, is upregulated prior to detectable lymphocytic infiltration and throughout the course of EAAU[143]. It has been proposed that uveal expression of ICAM-1 may facilitate the adherence and migration of immune cells into the uvea. MHC II cell surface antigen is recognized by antigen presenting cells, is important in T-cell induction[162], and is upregulated during EAAU [143]. Lymphocyte function-associated antigen 1 (LFA-1) is an integrin found on lymphocytes and plays a key role in leukocyte emigration and cytotoxic T-cell mediated immune response. It is also upregulated in EAAU [143].

Woon *et al* (1998) analyzed mRNA in the uvea of 5-6 week old male rats with EAAU and found TNFα gene expression levels paralleled the course of disease and that no significant changes

occurred in gene expression of IL -2,-4,-6, or -10 levels[163]. Another study evaluating the role of complement in EAAU, revealed IFNγ and IL-10 protein levels were elevated during EAAU and complement depleted rats had significantly reduced levels of these cytokine[146]. Tolerance to EAAU has been demonstrated though intravenous injection of MAA, and is mediated through the generation of T regulatory cells (Treg) which suppress T cell proliferative responses. Rats that developed tolerance had elevated levels of both IL-10 and transforming growth factor-2, whereas levels of TNF, IFNγ, and IL-2 were decreased. The tolerance was reversed by replenishing the rats with recombinant IL-2, leading the authors to conclude that tolerance was caused by reduced IL-2 levels[164].

1.6 Role of Bacterial Infections

Molecular mimicry occurs when self-reactivity is triggered by cross-reactivity between a self-protein and an exogenous protein that bears the same or similar amino acid sequence. Bacteria may serve as an exogenous protein that can trigger a molecular mimicry response in a vulnerable individual[165]. *Streptococcal* infection is implicated in flares or worsening of chronic disease in some children with JIA[166]. **β** *Hemolytic streptococcus*, similar to *Staphylococcus aureus* also produces a protease, streptokinase. Streptokinase has been associated with the development of uveitis when administered intravenously as a thrombolytic agent in humans[167]. Post *Streptococcal* reactive arthritis is known to occur in humans following pharyngeal and/or tonsillar infection with **β** hemolytic *Streptococcus* and a concurrent uveitis has been reported[168]. Uveitis accompanying arthritis has been observed in rats injected with various bacterial cell wall components including *Streptococcal* cell wall fragments[169], and the inactivated mycobacterium in complete Freund's adjuvant[158].

Infection with strains of bacteria that produce proteases like *Streptococcus* or *Staphylococcus* result in collagen breakdown in either the eye or joint and expose a shared antigen. The initial breakdown and exposure of the antigen may be facilitated by an infectious cause like *Staphylococcal* proteases or streptokinase or be mediated through endogenous proteases like MMP-1.

1.7 Role of Matrix Metalloproteinase

MMPs are a group of zinc-dependent extracellular enzymes that play a key role in normal and pathological tissue remodeling. Collagenases, a class of MMPs, are capable of degrading intact collagen type II, one of the main components of the articular cartilage[170-171]. MMP expression is influenced by cytokine expression[172-175]. Elevated serum and synovial fluid levels of MMP-3 are found in patients with active polyarticular and oligoarticular JIA [176-177] and they have been implicated in pathologic tissue degradation in rheumatoid arthritis and osteoarthritis. Elevated levels of MMPs are also found in humans with uveitis and rabbits with LPS- induced uveitis and are considered fundamental in the tissue destructive and repair processes[178]. Increased concentrations of MMP-2, MMP-3, and MMP-9 were observed in the aqueous of children with inactive JIA-uveitis compared to aqueous from eyes of children without inflammatory disease[104]. This study demonstrates that even children with clinically inactive disease likely have undetected ongoing pathology.

Single nucleotide polymorphisms coding for aberrant overexpression of MMP-1 and -13 in the face of vacant estrogen receptors have been identified[179]; vacant estrogen receptors are present in post-menopausal and pre-menarchal females. As discussed above, young (pre-menarchal) females are over represented in the JIA-uveitis population. A role for MMP over expression in these patients has not been investigated. MAA digested with MMP-1 results in a fragment of type 1 collagen between 20 and 25 kDa[124]. The uveitogenicity of this fragments has not been evaluated. It is possible that MMP-1, like V8 protease, could endogenously expose a shared pathogenic peptide between the joint and the eye.

1.8 Research Objectives

There are three chapters in the body of this thesis that encapsulate the objectives of this research.

Since young onset age and female sex are two risk factors for the development of uveitis in children with JIA, the first manuscript of this thesis examines the influence of age and sex on EAAU ocular findings and pro-inflammatory cytokine levels.

The second manuscript describes our exploration of a common collagen trigger for autoimmune arthritis and uveitis in the Lewis rat. A fragment of type I collagen from bovine skin and type II collagen from cartilage were evaluated for antigenicity in an intact form as well as digested. Three enzymes were used for collagen digestion; streptokinase, V8 protease, and MMP-1.

The final manuscript in this thesis evaluates the pathogenicity of MAA digested by streptokinase, V8 protease and MMP-1 without the use of complete Freund's adjuvant.

1.9 Chapter 1 References

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2. Influence of age and sex on ocular findings and vitreous cytokine profiles in rats inoculated with intact melanin associated antigen

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2.1 Abstract

Purpose: Experimental autoimmune anterior uveitis is an established animal model for autoimmune anterior uveitis. Young age and female sex are risk factors for uveitis in children with JIA. The objectives of this research were to determine if differences occur in experimental autoimmune anterior uveitis between adult and juvenile and male and female rats by evaluation with biomicroscopic and light microscopic ocular examinations, and pro-inflammatory vitreous cytokine profiles. **Methods:** Lewis rats were inoculated with insoluble MAA. Biomicroscopic examinations were completed three times a week by a masked examiner. At the termination of the experiment, the rats were euthanized and the eyes were enucleated. Vitreous was collected and assayed by bead-based multiplex immunoassay for levels of 27 inflammatory cytokines. Globes were fixed, sectioned, processed, mounted on slides, and stained with hematoxylin and eosin. Clinical uveitis scores, ocular histopathological scores, and cytokine analysis were compared between age and sex groups and control animals.

Results: Rats immunized with insoluble MAA developed fibrin in their anterior chambers, dilation of iridal vessels and miosis. Light microscopic examination revealed a lymphocytic anterior uveitis in 16/16 rats and choroiditis in 12/16 rats. The average time to onset of uveitis, peak clinical score, and vitreal cytokine levels did not differ significantly among the differing age and sex groups.

Conclusions: No difference in disease incidence, severity, or onset was observed between sexes or age groups in rats inoculated with MAA.

Keywords: Autoimmune Uveitis, Cytokines, Inflammation, Melanin Associated Antigen, Uveitis

2.2 Introduction

Chronic uveitis is among the most common debilitating extra-articular manifestation of JIA. Among children with JIA reported prevalence rates of uveitis range between 9.3 to 38% [1-7]. A combination of genetic, environmental and infectious factors are speculated to play a role in the pathophysiology of uveitis associated with JIA[8-11]. There is evidence supporting autoimmune and pro-inflammatory processes underlying JIA-uveitis pathogenesis. As examples, T-helper cell (Th)1, Th17, regulatory T cells (Tregs), and pro-inflammatory cytokines contribute to JIA-uveitis pathophysiology[12-18].

Female sex and young age at onset, are two risk factors for the development of JIA-uveitis[2, 19]. Females comprise approximately 80% of children with JIA who develop uveitis[20]; the risk of developing uveitis in girls with JIA is highest in those aged 1-2 years[21]. Uveitis risk is not age dependent in boys with JIA. Despite uveitis being more prevalent in females, only 15% of females with JIA present with uveitis as the initial manifestation of their disease, compared to 44% of males[22-23]. Disease severity and complication rates were also reported to vary between sexes; males develop more severe disease and have a higher complication rate [19-20, 22, 24-25].

Experimental autoimmune uveitis (EAAU) is induced following injection of uveal type I collagen harvested from the bovine eye[26-30]. It manifests as a severe bilateral uveitis approximately 11-15.5 days post [26-30]. Light microscopic examination of affected rats demonstrate a marked lymphocytic iritis and cyclitis [26-32]. The antigen in EAAU has been identified as a 22 kDa fragment found in the α2 chain of type 1 collagen. Most studies of EAAU have used 6-8 week old male rats [26-31]. EAAU serves as a valuable animal model for elucidating pathophysiology of autoimmune anterior uveitis. The EAAU model of uveitis is a suitable model for JIA-uveitis as it is a predominantly anterior uveitis. The main limitation of EAAU and all current rodent models of uveitis is its self-limiting course.

The objective of this study was to ascertain if rat EAAU had similar clinical and histologic characteristics to human JIA-uveitis. Since young age onset and female sex are risk factors for development of uveitis in JIA, the objectives of this study were to compare the biomicroscopic, light microscopic, and vitreous inflammatory cytokine manifestations of EAAU in four age groups and both sexes.

2.3 Materials and Methods

2.3.1 Animals

Pathogen free male and female adult (175 day old) and juvenile (28 day old) Lewis rats were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada). All rats were provided a seven day acclimation period prior to initiation of the experiment. All procedures were approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20150069). Rats were housed in groups of two or three with the treatment and control animals intermixed. Lighting was provided in a 12-hour light:12-hour dark cycle. Rats were provided *ad libitum* feed and water. After the acclimation period, ophthalmic examinations were completed and the rats were inoculated with their first injection; this was considered day one of the experiment. Only rats with normal clinical and ophthalmologic examinations were enrolled.

2.3.2 Ophthalmic Examinations

Ophthalmic examinations were completed by a masked Diplomate ACVO. Baseline examinations were completed on awake manually restrained rats and included an un-dilated and a dilated bio-microscopic anterior segment examination (Kowa SL-17 portable slit lamp, Kowa Optimed Inc., Vermont Avenue, Torrance, California, USA). Following the instillation of 0.5% tropicamide (Alcon, Que, Canada) indirect ophthalmoscopy (Heine Omega 200; Heine Instruments Canada, Kitchener) was completed. Following the baseline ocular examinations, rat inoculation protocols were completed and the ocular examinations were repeated three times per week beginning 14 days following initial inoculations until the rats were euthanized between day 27 and 29 post primary inoculation. The masked ophthalmologist assigned a uveitis score of 0-4 for each eye based on a previously described scale (Bora 1995).

2.3.3 Melanin Associated Antigen Preparation

Bovine globes were obtained from a local abattoir immediately following slaughter and frozen at -20 C until needed. Then the globes were thawed in room temperature water. The corneas, lens

and vitreous were removed initially and then the iris and ciliary body were dissected and placed in sterile tubes with sterile technique and the tissues frozen to -20 C until MAA extraction.

Dissected iris and ciliary body from ten bovine globes were thawed and 2 to 3 mL of 1X PBS, pH 7.2 was added dependent on viscosity of the mixture. The mixture was homogenized using a tissue homogenizer. The homogenized sample was filtered through a cheese cloth into a 50 mL conical tube (Fisher Scientific, Mississauga, Ontario, Canada). The sample was centrifuged (Sorvall ST 16R centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 4500 x g for 15 minutes at 4°C and the supernatant was removed from the pellet and discarded. The pellet was washed 3 times with 1X PBS and re-suspended in 10 mL of 1X PBS containing 2% Triton X-100 (Sigma Aldrich, Oakville, Ontario, Canada). The sample was incubated at room temperature for 3 hours at 200 rpm on an orbital shaker (Thermo Scientific Max Q200 orbital shaker, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sample was centrifuged again at 4500 x g for 15 minutes at 4°C. The supernatant was removed. The pellet was washed three times with 1X PBS and re-suspended in 10mL 1X PBS containing 2% SDS (Sigma Aldrich, Oakville, Ontario, Canada). This sample was incubated at 37°C for 3 hours at 200 rpm. Following incubation the sample was centrifuged at 4500 x g for 15 minutes at room temperature. The supernatant was collected and its protein concentration determined. The pellet containing the MAA was washed three times with 1X PBS, the PBS was removed and the pellet was stored at -20°C.

To prepare insoluble MAA for inoculation 1X PBS was added to the MAA pellet and agitated until fully suspended. The protein concentration of the insoluble MAA was determined using Bio-Rad DC protein assay.

2.3.4 Experimental Animal Groups

Four 36 day old males, four 36 day old females, four 182 day old males and four 182 day old females were included in the treatment group. Rats were anesthetized with isoflurane gas (Halocarbon Products Corporation, River Edge, NJ, USA) by mask and inoculated with 400 µg insoluble MAA and an equal volume of incomplete Freund's adjuvant administered

intradermally in two aliquots at the base of the tail (n=4) or in the foot pad (n=12) once on day one of the experiment. Ocular examinations were initiated on day 12 following inoculation and repeated three times per week until euthanasia on day 27-29 post inoculation.

A negative control group consisting of two 36 day old males, two 36 day old females, one 182 day old male and two 182 day old females were intermixed amongst the treatment groups and were examined and euthanized with treatment rats between days 27 and 29 of the experiment.

2.3.5 Euthanasia and Sample Collection

All rats received a lethal intraperitoneal injection of pentobarbital sodium (80 mg/kg; 54 mg/mL; Euthanyl Forte, Bimeda-MTC, Cambridge, Ontario, Canada). Once the palpebral reflex was absent trans-conjunctival enucleations were completed. Vitreous was obtained through a paracentesis adjacent to the optic nerve using a 30 GA needle and 1.0 mL syringe. Vitreous samples were stored at -80^{o}C until analysis.

Following vitreous collection globes were submerged in periodate-lysine-paraformaldehyde (PLP) fixative (Whiteland et al 1995). Following 24 hours of fixation, globes were dehydrated sequentially with 70% ethanol (45 minutes), 90% ethanol (45 minutes), and 100% ethanol (twice for 30 minutes each). This was followed by immersion in Histo-clear (National Diagnostics, USA) (twice for 30 minutes). Eyes were embedded under vacuum at 54 degrees for 30 minutes then processed in paraffin (Whiteland et al 1995). Paraffin blocks were sectioned into 6 µm sections which were floated on a water bath at 40^{o}C prior to transfer onto glass slides. Slides were dried and routinely stained using hematoxylin and eosin and were reviewed by a masked Diplomate ACVO.

2.3.6 Cytokine Analysis

Vitreous samples were analyzed using a MILLIPLEX MAP Rat Cytokine/Chemokine Magnetic Bead panel (Millipore, St. Charles, MO, USA) for multiplexed quantification of 27 rat cytokines, chemokines, and growth factors was used. The multiplexing analysis was performed using the

Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta, Canada). The 27-plex consisted of G-CSF, Eotaxin, GM-CSF, IL-1α, Leptin, MIP-1α, IL-4, IL-1β, IL-2, IL-6, EGF, IL-13, IL-10, IL-12 (p70), IFNy, IL-5, IL-17A, IL-18, MCP-1, IP-10, GRO/KC, VEGF, Fractalkine, LIX, MIP-2, TNFα, and RANTES. The assay sensitivities of these markers range from 0.3 – 30.7 pg/mL. A cubic spline regression was applied against the standard curve of fluorescent intensities to extrapolate concentration values. Low end of the standard curve values were extrapolated using a point-to-point semi-log regression as previously described (Masi et al 2017).

2.3.7 Statistical analysis

For all statistical comparisons, data was examined for normality and equality of variance. Data distribution was visualized and a Shapiro-Wilk test used to evaluate normality. Variances were directly compared. Where parametric data assumptions were not met, the Kruskal-Wallis test was used for evaluating differences between more than two groups and the Wilcoxon rank sum test was used to compare two groups. The level of significance was set to 0.05. For cytokine analyses two comparisons were completed. The first analysis compared observed cytokine levels of each cytokine in all IMMA treated rats to control rats. To account for multiple comparisons an individual comparison significance level was calculated using a Bonferroni correction. The individual comparison significance level used was 0.0019 (0.05/27; 27 cytokines). The second analysis was between the age and sex group. A Shapiro-Wilk test indicated unequal variance and the sample size was too small to determine distribution. A Kruskal-Wallis equity of populations rank test was completed with the level of significance set to 0.05.

All statistical analyses were completed using a commercial statistics package (STATA 15, StataCorp. 2017. *Stata Statistical Software: Release 15*. College Station, TX: StataCorp LLC).

2.4 Results

2.4.1 Control Groups:

Uveitis or other ocular abnormalities were not detected in control rats clinically or with histologic examination.

2.4.2 IMAA Treatment Group:

Biomicroscopic Examination:

All 16 rats inoculated with insoluble MAA developed uveitis on biomicrooscopic examination. The uveitis was characterized by dilation and engorgement of the iridial vessels, fibrin in the anterior chamber, miosis and dyscoria. Uveitis was bilateral in 15/16 rats and unilateral in one adult male rat. The average exam score on each exam date with 95% confidence intervals are shown in Figure 3.1. Peak uveitis occurred in all treatment groups on day 21. Uveitis severity decreased in all treatment groups following day 21; however, uveitis persisted in all at the final examination. Average onset of uveitis post-inoculation was 16.25 days in juvenile females, 16.75 days in juvenile males, 17.5 days in adult females and 20 days in adult males (Table 3.1). A Kruskal- Wallis analysis revealed no significant differences (p=0.4054) in uveitis onset. Average onset of uveitis in adult rats was 18.75 days, while average onset in all juvenile rats was 16.86 days. This difference was not significant (p=0.2439). Average onset of uveitis in male rats was 18.75 days, while average onset in female rats was 17.21 days. This difference was not significant (p=0.2439). Uveitis was rated severe (4/4) in each eye of all rats by day 21 post-inoculation except in two adult males and one juvenile male. One adult male only developed unilateral uveitis at day 26 and was graded 0.5. A second adult male and the juvenile male both developed grade 4/4 in one eye and a grade 3/4 in the fellow eye. All three of these rats were in the foot pad injection group.

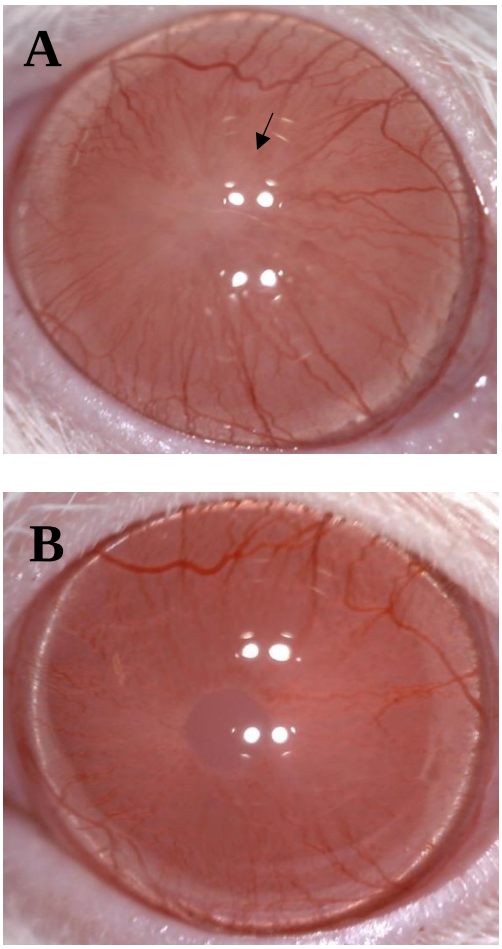


Figure 2- 1. A) Melanin associated antigen inoculated rats developed fibrin in their anterior chamber (arrow), miosis, pupillary occlusion and engorgement of iridial vessels. B) Naïve rat with normal anterior segment.

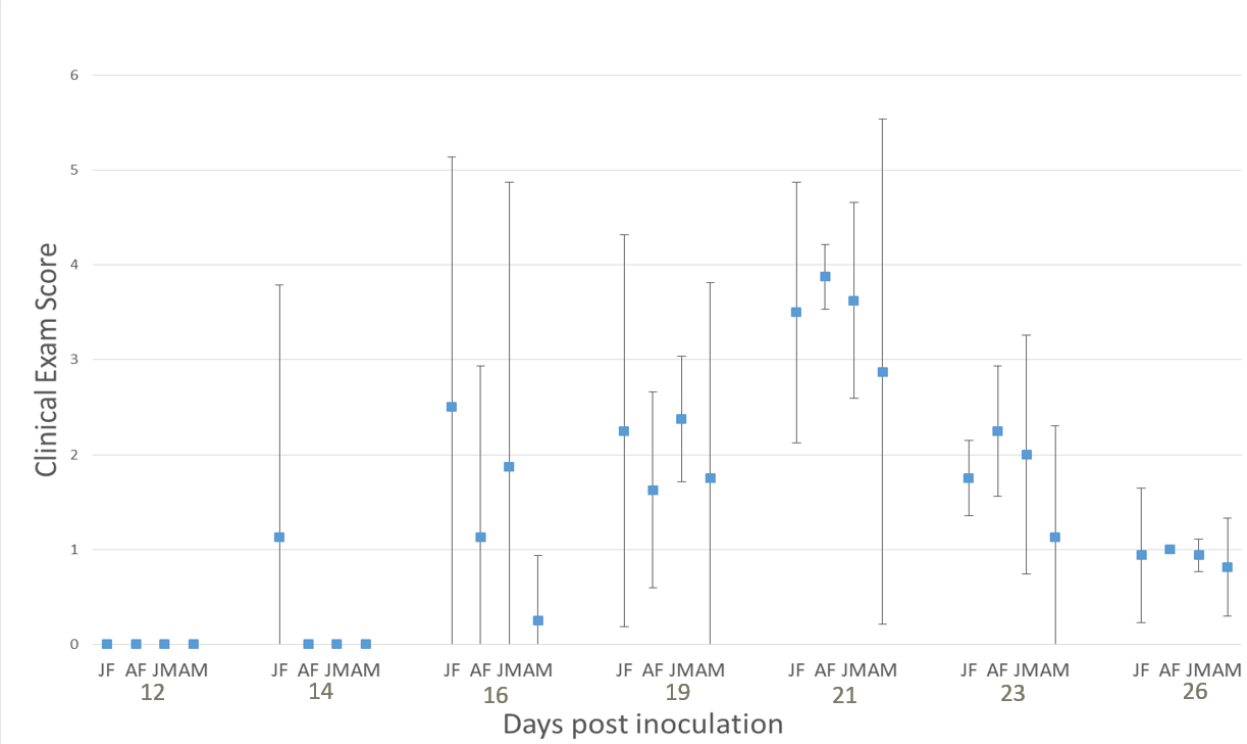


Figure 2- 2. The average clinical sore of each group with 95% confidence intervals between day 12 post inoculation and day 26. JF= juvenile female, AF=adult female, JM=juvenile male, AM=adult male.

Table 2-1: Average days post-inoculation for onset of uveitis in each group, and compared between sexes and age groups.

Group	Average Onset (days)	*p*
Juvenile Female	16.25	0.4054
Juvenile Male	16.75	
Adult Female	17.5	
Adult Male	20	
Adults	18.75	0.2439
Juvenile	16.86	
Males	18.75	0.2439
Females	17.21	

Light Microscopic Examination

All rats in the IMAA treatment group were confirmed histologically with severe lymphocytic iriditis and cyclitis (Figure 3B). A lymphocytic choroiditis was present in 14/16 rats (Figure 3D). One adult male rat had a unilateral choroiditis, while another adult male did not have choroidal inflammation in either eye. Control rats did not have inflammatory infiltrate in their iris, ciliary body, or choroid (Figure 3A and C).

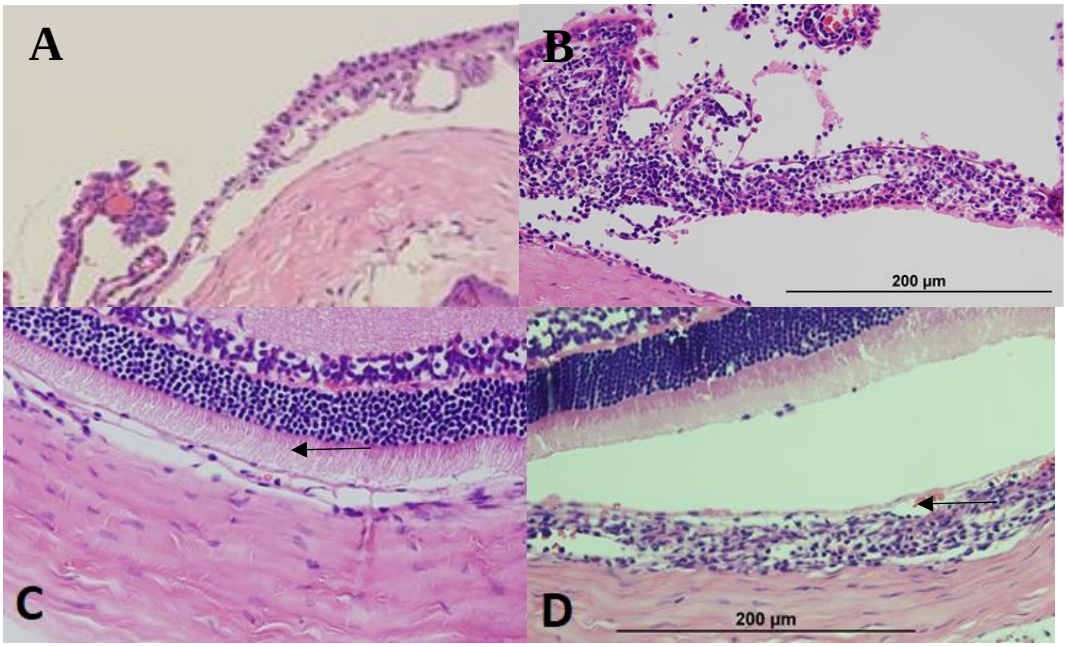
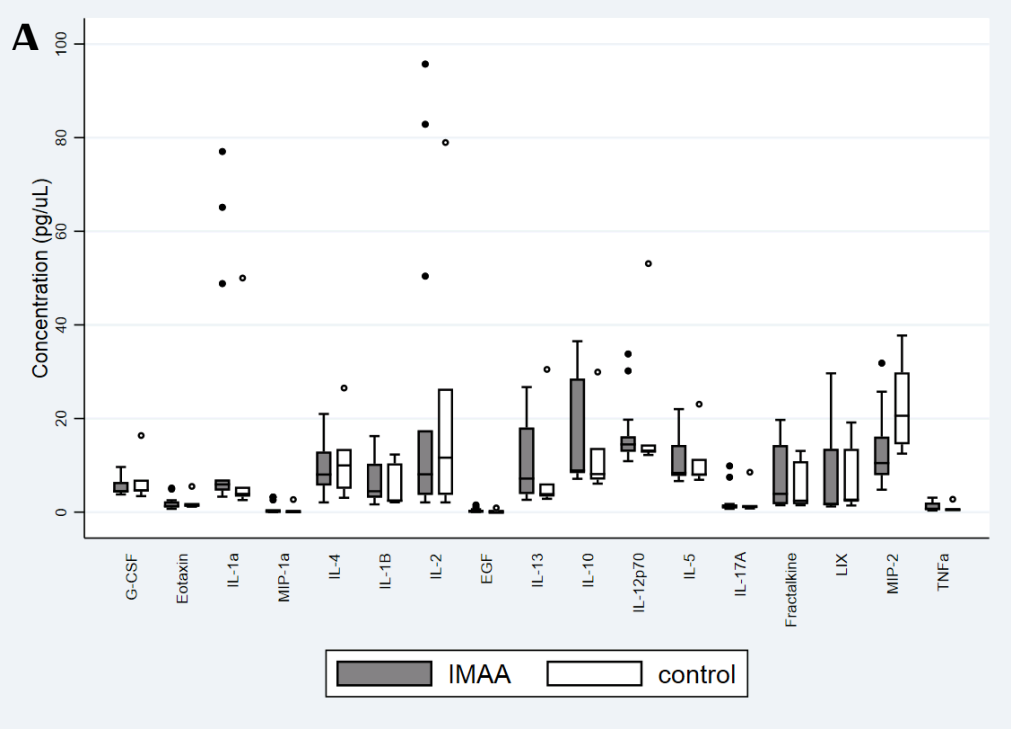


Figure 2- 3. Light microscopy of normal control iris and ciliary body (A) and choroid (C) compared to iris and ciliary body (B) and choroid (D) of a rat inoculated with intact melanin associated antigen. The black arrow points to the choroid. The intact melanin associated antigen inoculated rat has a marked lymphocytic choroidal infiltration.

Cytokines

Concentrations for 27 pro-inflammatory cytokines in the vitreous of IMAA treated rats compared to control rats are presented in Table 3.2 and Figure 3.4 A and B. The box and whisker charts Figure 3.4 A and B represent cytokines with values between 0 and 100 pg/µL (A), and 0 and 1500 pg/µL (B). There were no statistical differences between treatment and control levels when corrected for multiple comparisons (Table 3.2).

The cytokine levels for each age/sex group are presented in Figure 3.5 A and B. Figure 3.5 A and B represent cytokines with values between 0 and 100 pg/µL (A), and 0 and 1000 pg/µL (B). Despite the lack of power with the small sample size, for completeness statistical comparisons were made at the level of the group to evaluate for any differences related to age of sex. There was no statistically significant difference between groups for any of the 27 cytokines.



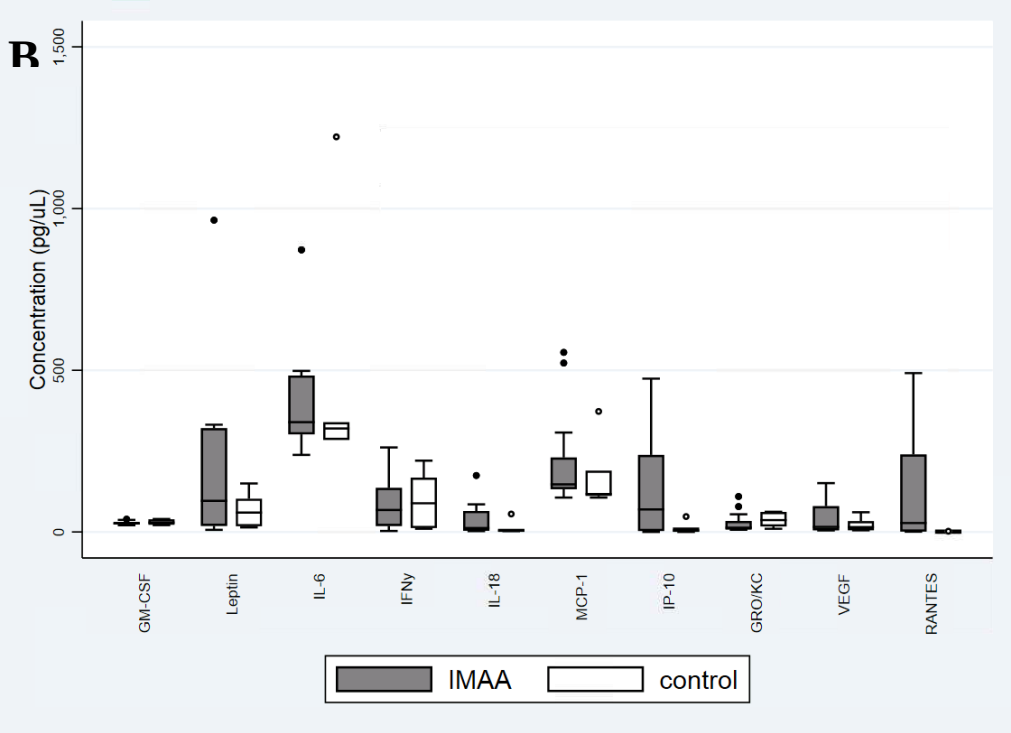


Figure 2- 4 A and B. Concentration of pro-inflammatory cytokines in the vitreous of control (white) and intact melanin associated antigen (grey) inoculated rats with values between 0 and 100 pg/µL (A) and 0 and 1500 pg/µL (B). Outliers are indicated by dots.

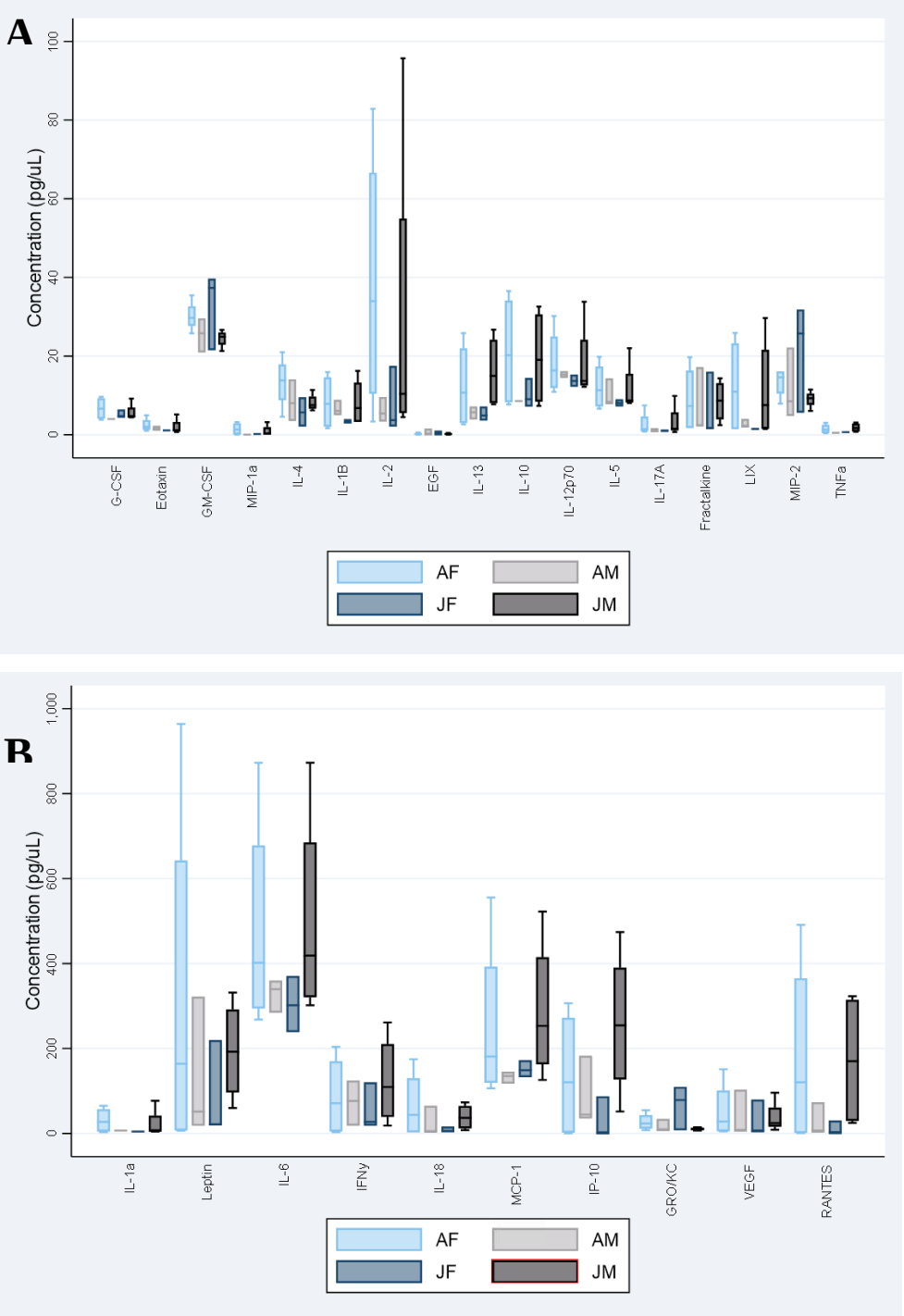


Figure 2- 5 A and B. Concentration of pro-inflammatory cytokines with values between 0 and 100 pg/µL (A) and 0 and 1000 pg/uL (B) in the vitreous of juvenile male (dark grey), juvenile female (dark blue), adult male (light grey), and adult female (light blue) intact melanin associated antigen inoculated rats.

Table 2-2: Vitreous cytokine means, standard deviations, and p values for controls and IMAA treated rats. Wilcoxon rank sum analysis revealed three significant values however to account for multiple comparisons using a Bonferroni correction an individual

	IMAA Mean (pg/uL)		Control mean (pg/uL)		
Cytokine	mean (pg/uL)	SD	mean (pg/uL)	SD	*P*
MIP2	13.43	8.15	22.87	9.21	0.0252
RANTES	109.75	160.88	1.17	0.55	0.0274
IL1a	17.92	25.43	10.42	17.48	0.0432
IP-10	138.12	149.44	10.75	16.95	0.0570
GROKC	28.34	31.78	38.43	21.62	0.1172
IL18	36.94	49.73	11.55	19.51	0.1453
MCP1	216.52	146.70	164.52	96.37	0.1787
IL10	15.62	11.22	11.57	8.48	0.1789
TNFa	1.18	1.03	0.81	0.87	0.2174
IL13	10.48	8.65	7.83	10.05	0.2177
IL1B	6.88	5.13	5.21	4.32	0.2623
MIP1a	0.74	1.25	0.44	1.00	0.2730
Eotaxin	1.88	1.43	2.05	1.54	0.3113
EGF	0.34	0.46	0.18	0.33	0.3296
Leptin	194.79	256.04	65.22	49.47	0.3908
Fractalkine	7.93	6.97	5.11	4.83	0.4321
LIX	7.72	10.23	6.53	6.94	0.4325
GMCSF	28.10	5.93	30.89	7.58	0.4781
IL12p70	16.74	6.85	18.89	15.10	0.5219
IL6	417.97	205.88	438.88	345.84	0.5484
GCSF	5.49	2.14	6.44	4.51	0.5749
VEGF	40.76	47.83	21.37	20.66	0.6815
IL5	10.81	5.71	10.51	4.95	0.7077
IL4	9.20	5.16	11.00	7.70	0.7088
IL2	22.63	30.94	20.57	26.93	0.9107
IL17A	2.22	2.80	2.19	2.81	0.9402
INFy	88.15	81.51	88.42	82.50	0.9701

2.5 Discussion

Insoluble MAA inoculations in this study demonstrated a similar incidence, onset, peak as with previous studies[26-32]. This is the first experiment to compare manifestations of the uveitis between adult and juvenile and male and female rats. Juvenile female rats had the earliest onset of disease. The onset between the age/sex ranged from 16.25 days to 20 days which was similar to other reports at 15 +/- 3 days[26-32]. Statistical significance was not achieved as these comparisons lacked the necessary power to determine statistical differences of modest magnitude.

This was the first report of vitreous cytokine levels in experimental autoimmune anterior uveitis. Woon et al analyzed mRNA in uveal tissue from rats with EAAU and found TNFα gene expression paralleled the course of disease[33]. They also found no change in expression of IL-2,-4,-6, or -10 consistent with the results in our study[33]. The change in TNF expression observed by Woon *et al* was not observed in our study and may be explained by our collection of vitreous in the late stages when the uveitis was resolving, or differences between cytokine gene expression in the uvea and cytokine immune assay in the vitreous. In addition to the cytokines evaluated by Woon *et al* we evaluated granulocyte-colony stimulating factor, eotaxin, granulocyte-macrophage colony stimulating factor 2, IL 1α, leptin, macrophage inflammatory protein 1a, IL 1β, epidermal growth factor, IL 13, IL 12p70, IL 5, IL 17a, IL 18, monocyte chemoattractant protein-1,interferon gamma induced protein 10,GRO/KC, vascular endothelial growth factor, fractalkine, LIX, macrophage inflammatory protein 2, regulated on activation t cell expressed and secreted.

Fang *et al* evaluated mRNA expression of CX3C chemokine, fractalkine, and its receptor CX3CR1 in the iris and ciliary body in rats inoculated with MAA. Fractalkine regulates adhesive and chemoattractant leukocyte functions and is preferentially expressed on Th1 cells[34]. Fang *et al* found fractalkine levels were elevated nine days following inoculation and preceding disease onset[34]. Expression of fractalkine receptor, CX3CR1, peaked with the disease onset at day 14. Following day 14 its levels were no longer statistically different from control rats[34]. We did not see any difference in fractalkine between inoculated rats and controls in our study, likely due to the later harvest of vitreous. Another key difference between studies was Fang's use of complete Freund's adjuvant.

A limitation of our study was the single time point utilized for tissue and vitreous harvesting. This prevented observation of the dynamic inflammatory process throughout the course of EAAU. Cytokine levels at varying time points would be valuable to establish a sequence of inflammatory events and the components that play a role in the signaling cascade in EAAU rather than a single point of reference in the late stage of disease.

RANTES expressed and secreted was elevated, albeit non significantly, in IMMA inoculated rats compared to controls. RANTES is a mononuclear chemoattractant capable of influencing migration of CD4 T cells to inflammatory sites. It is constitutively expressed in resting T cells and following activation recruits more T lymphocytes to the site of inflammation. Fang *et al* found RANTES aqueous concentrations significantly elevated between day 11 and 30 post inoculation[35]. The peak of approximately 4000pg/ml occurred on day 14, with a statistically significant elevation over controls persisting to day 30[35]. These levels were higher than what was observed in our study of 100pg/ml. Differences may be due to their use of complete Freud's adjuvant at the time of MAA inoculation as well as differences in aqueous vs vitreous concentrations. Fang also found elevations in MCP-1, MIP-1, IL-8, and IP-10 mRNA levels[35]. These elevations were all seen up until either day 14 or 18, then levels declined to concentrations that we not different from controls[35]. In our study both IP-10 and RANTES levels varied between age and sex group however statistical significance was not reached. The limited number of subjects per treatment group in our study in combination with the large number of factors evaluated made reaching statistical significance unlikely with only modest differences between groups.

For the first time, this study demonstrated the antigenicity of IMAA when injected at the base of the tail. This site has not been reported in the past for IMAA injections but is commonly used for bovine type II collagen in collagen induced arthritis. This location is superior to the traditional sMAA site of the foot pad especially when lower limbs are concurrently under evaluation for arthritis.

Conclusions

Juvenile female rats had an earlier but non-significant onset of disease. Vitreous cytokine values are described for juvenile male, juvenile female, adult male, and adult female rats inoculated with intact MAA.

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3. Pathogenicity of type I and II collagen solubilized using matrix metalloproteinase-1 or *staphylococcal* V8 protease in the Lewis rat.

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3.1 Abstract

Objective: The pathogenesis and etiology of JIA uveitis is poorly understood. The purpose of this research is to explore the potential of a shared collagen based self-antigen existing between the joint and eye in the pathogenesis of JIA-uveitis.

Methods: Adult and juvenile male and female Lewis rats were inoculated intradermally with either: intact *Staphylococcal* V8 protease solubilized or matrix metalloproteinase (MMP)-1 solubilized type I collagen derived from bovine skin, or II collagen derived from bovine cartilage. Inoculations were repeated up to two times at intervals of 1 or 4 weeks. Biomicroscopic and indirect ophthalmoscopic examinations were completed in live rats at baseline and biomicroscopic examinations were repeated three time per week throughout the study period by a masked Diplomate of the American College of Veterinary Ophthalmologists (ACVO). At the end of the observation period globes were enucleated and vitreous was aspirated. Histopathology slides of the globes were reviewed by a masked Diplomate ACVO. An array of 27 cytokines were quantified with a multiplex bead-based immunoassay on vitreous from rats treated with MMP-1 digested type I or type II collagen.

Results: None of the rats in this study developed uveitis that could be detected clinically or light microscopically. 28/44 rats inoculated with intact type II collagen developed arthritis. Rats inoculated with either *Staphylococcal* V8 protease solubilized, or MMP-1 solubilized type II collagen did not develop arthritis. Vitreous cytokine levels did not differ between treatment groups and controls.

Conclusions: Intact or MMP-1, *Staphylococcus* V8 protease digestions of type I or type II collagen did not result in uveitis in the Lewis rat. Digestion of type II collagen renders it non-pathogenic.

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3.2 Introduction

JIA is the most common form of chronic arthritis in children and uveitis is the most common debilitating extra-articular manifestation of JIA[1]. Little is known about the underlying pathophysiology linking the ocular and joint inflammation[2-5].

Currently, there are limited reports of animal models for JIA-uveitis. Uveitis and arthritis have been reported in female Sprague-Dawley rats inoculated with type II collagen[6]. Type II collagen inoculation is a well-established rodent model of arthritis used for investigating arthritis pathogenesis, and potential therapies[7]. Uveitis has also been documented in a 1/15 rats receiving spleen cells from rats inoculated with type II collagen[8].

Collagens are typically highly conserved proteins, although some tissue-specific structural differences have been identified[9-10]. Type I collagen is the most abundant collagen in the body and is the main structural component of bones, tendons and ligaments[11]. Almost 50 different molecules that interact with type I collagen have been recognized, including sequences for integrins[12-17], Il-2[18], *Staphylococcus aureus* cell surface molecules[19], *Staphylococcus aureus* matrix binding proteins[20], MMPs[21] and many others[11].

Experimental autoimmune uveitis (EAAU) is a severe bilateral uveitis in the Lewis rat that occurs in response to inoculation with a 22 kDa α chain fragment of type I collagen[22-26]. The antigen is harvested from bovine uvea, and its soluble form is isolated following digestion with *Staphylococcus aureus* V8 protease. The role of V8 protease in development of the antigenic 22 kDa fragment of type I collagen may implicate protease involvement in exposure of the antigenic peptide *in vivo*.

MMPs are a group of zinc dependent extracellular enzymes that play a key role in normal and pathological tissue remodeling. Collagenases, a class of MMPs, are capable of degrading intact collagen type II, one of the main components of the articular cartilage[27-28]. Elevated serum and synovial fluid levels of MMP-3 are found in patients with active polyarticular and oligoarticular JIA[29-30] and they have been implicated in pathologic tissue degradation in rheumatoid arthritis, and osteoarthritis. Increased concentrations of MMP-2, MMP-3, and MMP-9 have been observed in the aqueous of children with inactive JIA-uveitis compared to aqueous from eyes of children without inflammatory disease[31]. Uveal tissue digested with MMP-1 results in fragments of type 1

collagen between 20 and 25 kDa[26]; this is similar in size to the 22 kDa fragment known to cause uveitis in EAAU[22-26].

Type I collagen from bovine skin, Achilles tendon, and rat tail have been tested in their intact form as well as following digestion with V8 protease and were not uveitogenic[25]. Type I collagen derived from bovine skin, and type II collagen derived from bovine cartilage, digested with MMP-1 have not been evaluated for uveitogenicity.

The purpose of this study was to explore a common collagen trigger for autoimmune arthritis and uveitis in the Lewis rat. Commercially available purified type I collagen isolated from bovine skin (Chondrex, Redmond, VA, USA) and bovine type II collagen derived from fetal bovine articular cartilage (mdBioproducts, St Paul, MN) were evaluated in an intact form, and following digestion with either V8 protease or MMP-1.

Materials and Methods

3.2.1 Animals

Pathogen free male and female adult (175 day old) and juvenile (28 day old) Lewis rats were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada). All rats were provided a seven day acclimation period prior to initiation of the experiment. All procedures were approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20150069). Rats were housed in groups of two or three with the treatment and control animals intermixed. Lighting was provided in a 12 hour light: 12 hour dark cycle. Rats were provided *ad libitum* feed and water. After the seven day acclimation period ophthalmic examinations were completed and rats were inoculated with their first injection; this was considered to be day one of the experiment.

3.2.2 Ophthalmic Examinations

Ophthalmic examinations were completed by a masked Diplomate ACVO on awake, manually restrained rats. These examinations included an un-dilated and a dilated biomicroscopic anterior

segment examination (Kowa SL-17 portable slit lamp, Kowa Optimed Inc., Vermont Avenue, Torrance, California, USA). Following the instillation of 0.5% tropicamide (Alcon, Que, Canada) indirect ophthalmoscopy (Heine Omega 200; Heine Instruments Canada, Kitchener) was completed. Rats were excluded from the experiment if any intraocular inflammation or ophthalmic abnormalities were detected.

Following baseline ocular examinations, rat inoculation protocols were initiated and rats were re-examined three times per week beginning 14 days following initial inoculation and repeated until euthanasia. At repeat examinations uveitis was assigned a score of 0-4 based on previously described scales[32]. When a uveitis score of >0 was assigned, posterior segment examination was repeated. At each examination, examiner, uveitis score, flare score, and ocular abnormalities were recorded.

3.2.3 Reconstitution of Type I and Type II collagen:

Lyophilized Type I and Type II collagen (Chondrex, Redmond, VA, USA) were reconstituted with 0.05M Acetic Acid to 2.0 mg/ml and left overnight at 4°C with constant stirring.

3.2.4 Staphylococcus aureus V8 digestions

2.5 mL of Type I or Type II collagen were added to a conical tube containing 7.5 mL of 8M urea (EMD Millipore, Etobicoke, Ontario, Canada), 1.125mL of 1M potassium phosphate buffer, 1 mL of 1,500 unit Endoproteinase Glu-C from *Staphylococcus aureus* V8 (Sigma Aldrich, Oakville, Ontario, Canada) and 2.875 mL of double distilled water (Milli-Q Advantage A10 System, EMD Millipore, Etobicoke, Ontario, Canada) to yield final concentrations of 4M urea, 75mM potassium phosphate buffer and 100 units of Endoproteinase Glu-C from *Staphylococcus aureus* V8. The sample was incubated on an orbital shaker at 37° at 200 rpm for 30 minutes. Following incubation, the tubes were placed in a beaker of boiling water for two minutes to inactivate the V8 protease. The sample was centrifuged at 4,500 x g for 20 minutes at 4°C. The supernatant was collected and dialyzed against double distilled water (Milli-Q, EMD Millipore, Etobicoke, Ontario, Canada) using 3.5K MWCO Dialysis Membrane (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 48 hours with water changes every 2 hours for the first 10

hours and every 4 hours for the remainder of time. The sample was transferred to a 50 mL conical tube (Fisher Scientific, Mississauga, Ontario, Canada) and frozen at -80°C. Once frozen, the sample was lyophilized (FreeZone Plus 6 Liter Cascade Console Freeze Dryer, Labconco, Kansas City, Missouri, USA) for approximately 48 hours or until lyophilized. The lyophilized sample was stored at -20°C until ready for use. Prior to use, the lyophilized sample was dissolved in 1 mL of 1X PBS, pH 7.2 and protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, California, USA).

3.2.5 Matrix Metalloproteinase-1 digestions

Prior to use, the MMP-1 (EMD Millipore, Etobicoke, Ontario, Canada) was activated with trypsin (Trypsin from Bovine Pancreas, EMD Millipore, Etobicoke, Ontario, Canada) at a ratio of 10:1 MMP-1 to trypsin for 10 minutes at 25°C. The trypsin was inactivated with a 10-fold excess of soybean trypsin inhibitor (EMD Millipore, Etobicoke, Ontario, Canada). The 2.5 mL of type I or type II collagen was added to a conical tube containing 7.5 mL of 8M urea (EMD Millipore, Etobicoke, Ontario, Canada), 1.125mL of 1M potassium phosphate buffer, 0.60 mL of 50 µg/mL MMP-1 (EMD Millipore, Etobicoke, Ontario, Canada) and 3.275 mL of double distilled water (Milli-Q EMD Millipore, Etobicoke, Ontario, Canada) to yield final concentrations of 4M urea, 75mM potassium phosphate buffer and 2 µg/mL of MMP-1. The sample was incubated on an orbital shaker at 25°C at 200 rpm for 30 minutes. Following incubation, the reaction was stopped with 50 mM EDTA. The sample was centrifuged at 4,500 x g for 20 minutes at 4°C. Dialysis and lyophilization of the sample was completed.

3.2.6 Treatments

Rats were anesthetized using isoflurane gas (Halocarbon Products Corporation, River Edge, NJ, USA) provided through a flow by mask. The type I collagen used in the inoculations was commercially available purified type I collagen isolated from bovine skin (Chondrex, Redmond, VA, USA). Type II collagen was commercially available bovine type II collagen derived from fetal bovine articular cartilage (mdBioproducts, St Paul, MN). The dose for all intact and

digested inoculations was 400µg. The inoculations were administered in an equal volume of incomplete Freund's adjuvant (Sigma, Oakville, Ontario, Canada) in two aliquots at the base of the tail.

3.2.7 Control Animals:

Two control groups were used. A negative control group consisting of 8 naive rats and a second group of 8 rats inoculated with only incomplete Freund's adjuvant once at the beginning of the experiment were intermixed amongst all treatment groups and euthanized alongside treated rats.

3.2.8 Single inoculations

The following inoculations were administered to 2-5 rats of each age and sex cohort once at the beginning of the experiment: Intact type I collagen, intact type II collagen, V8 digested type I collagen, MMP-1 digested type I collagen, and MMP-1 digested type II collagen.

3.2.9 Repeated inoculations: short interval

The following inoculations were administered to 1-4 rats of each age and sex cohort at the beginning of the experiment and again 7 days later: Intact type II collagen, V8 digested type I collagen, V8 digested type II collagen.

3.2.10 Repeated inoculations: long interval

The following inoculations were administered to 2-4 rats of each age and sex cohort at the beginning of the experiment and again 28 days later: Intact type II collagen

3.2.11 Euthanasia and Tissue Collection

Rats were euthanized between day 33 and 57. All rats received a lethal intraperitoneal injection of pentobarbital sodium (80 mg/kg; 54 mg/mL; Euthanyl Forte, Bimeda-MTC, Cambridge, Ontario, Canada). Sub-conjunctival enucleation was completed and globes were immediately submerged in periodate-lysine-paraformaldehyde (PLP) fixative[33]. Following 24 hours of

fixation globes were processed using previously described methods[33]. Paraffin blocks were sectioned into 6 µm sections which were floated on a water bath at 40^0C prior to transfer onto a glass slide. Slides were dried and stained using hematoxylin and eosin stain. Histopathology slides were reviewed by a masked Diplomate ACVO and diagnosed and graded for uveitis by the previously cited grading scheme or designated as normal.

3.2.12 Cytokine Analysis

Vitreous samples collected from rats treated with MMP-1 digested Type I and MMP-1 digested Type II collagen were analyzed for cytokine/chemokine profiling. In this study, Luminex xMAP technology for multiplexed quantification of 27 cytokines, chemokines, and growth factors for rats were utilized. The multiplexing analysis was performed using the Luminex™ 100 system (Luminex, Austin, TX, USA) by Eve Technologies Corp. (Calgary, Alberta, Canada). Twenty-seven markers were simultaneously measured in the samples using a MILLIPLEX Rat Cytokine/Chemokine 27-plex kit (Millipore, St. Charles, MO, USA) according to the manufacturer's protocol. The 27-plex consisted of granulocyte-colony stimulating factor (G-CSF), Eotaxin, granulocyte-macrophage colony stimulating factor (GM-CSF), IL-1α, Leptin, macrophage inflammatory protein 1a (MIP-1α), IL-4, IL-1β, IL-2, IL-6, epidermal growth factor (EGF), IL-13, IL-10, IL-12 (p70), IFNy, IL-5, IL-17A, IL-18, monocyte chemoattractant protein-1 (MCP-1), interferon gamma induced protein 10 (IP-10), human growth-regulated oncogene/keratinocyte chemoattractant (GRO/KC), vascular endothelial growth factor (VEGF), Fractalkine, Lipopolysaccharide-inducible CXC chemokine (LIX), macrophage inflammatory protein 2 (MIP-2), tumor necrosis factor α (TNFα), and regulated on activation T cell expressed and secreted (RANTES). The assay sensitivities of these markers range from 0.3 – 30.7 pg/mL for the 27-plex.

3.3 Results

3.3.1 Ophthalmic evaluations

None of the rats in these experiments developed uveitis based on biomicroscopic and histologic examinations.

3.3.2 Joint Evaluations

The overall incidence of arthritis in intact type II collagen treated rats was 28/44. Of the rats inoculated with one injection, two injections 7 days apart, or two injections 28 days apart 5/12, 8/16, and 15/16 developed arthritis. The rate of arthritis at the cohort level per inoculation schedule is provided in table 1.

Rats inoculated with either V8 protease or MMP-1 digested type II collagen did not develop arthritis.

None of the rats inoculated with either digested or intact type I collagen developed arthritis.

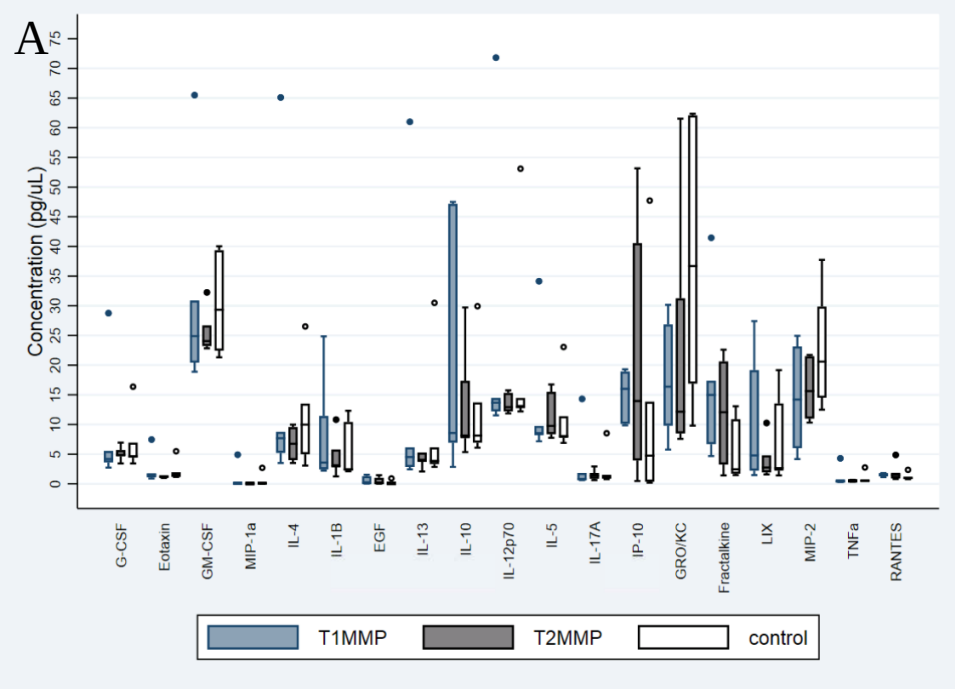
Naïve and IFA inoculated rats did not develop arthritis.

Table 3-1: Arthritis and uveitis outcomes for each treatment group receiving intact type II collagen listed by the age and sex grouping.

Inoculation Schedule	**Total**	**Arthritis**	**Uveitis**
One Inoculation			
Adult Male	3	3	0
Juvenile Male	3	0	0
Adult Female	3	2	0
Juvenile Female	3	0	0
Two Inoculations; 1 week apart			
Adult Male	4	2	0
Juvenile Male	4	1	0
Adult Female	4	4	0
Juvenile Female	4	1	0
Two Inoculations; 4 weeks apart			
Adult Male	4	4	0
Juvenile Male	4	3	0
Adult Female	4	4	0
Juvenile Female	4	4	0

3.3.3 Cytokine Evaluation

The vitreous cytokine profiles of rats inoculated with MMP-1 digested type I or II collagen did not differ from controls (Figure 3-1 A and B).



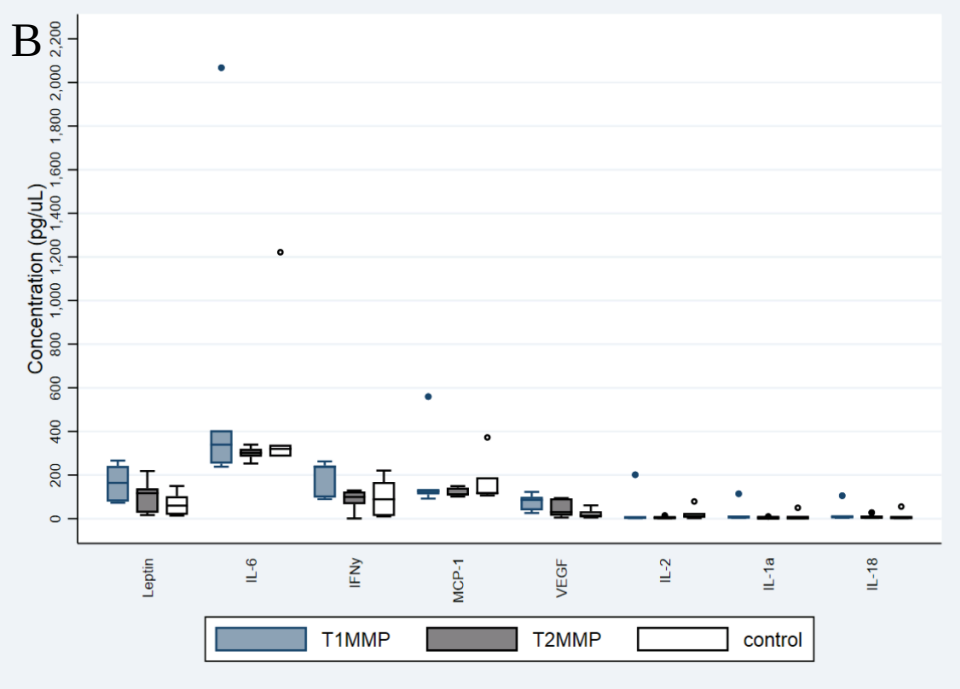


Figure 3- 1 A and B. Vitreal cytokine concentrations of 27 cytokines from the vitreous of rats inoculated with either type I or type II collagen digested in matrix metalloproteinase 1 compared to naive rats. The dots represent outliers. Abbreviations: T1MMP: type 1 collagen digested with matrixmetalloproteinase-1, T2MMP: type 2 collagen digested with matrix metalloproteinase-1.

3.4 Discussion

Bovine type I or type II collagen administered intact or following digestion with MMP-1 or V8 protease did not induce uveitis in this investigation. As expected, intact type II collagen inoculation resulted in arthritis in 28/44 rats. None of these rats developed uveitis. This was in contrast to a report by Petty *et al*[6] in which clinical uveitis was reported in 4/40 female Sprague-Dawley rats inoculated. Two of these rats had concurrent arthritis while two demonstrated uveitis as their only pathology[6]. Uveitis has also been documented in a 1/15 rats receiving spleen cells from rats inoculated with type II collagen[8]. The difference may be related to differences in genetic susceptibility between Lewis and Sprague-Dawley rats, dose, concurrent arthritis, or injection schedule. Petty *et al* administered 200 ug on day 1 and day 7 in the hind foot pad[6], while we administered 400 ug at the base of the tail either once, twice a week apart or twice four weeks apart.

Rats treated with type II collagen digested in either V8 protease or MMP-1 did not develop arthritis or uveitis. Possible explanations for the loss of antigenicity of type II collagen following digestion is loss of the antigenic epitope on the collagen or the single injection did not provide sufficient antigenic stimulation. Of the rats that were inoculated with only one injection of intact type II collagen 5/12 developed arthritis, compared to 8/16 receiving two injections one week apart or 15/16 receiving two injections one week apart. If the former speculation for loss of antigenicity is correct, then MMP-1 would have a protective role *in vivo*.

A single injection of intact, V8 protease digested or MMP-1 digested bovine type I collagen failed to induce uveitis. A previous report[25] found intact type I collagen isolated from bovine skin, Achilles tendon, and rat tail was not pathogenic, nor was V8 protease treated skin derived type I collagen. The study also evaluated MAA, an agent known to cause uveitis, and found deglycosylation of glycoproteins rendered it non-pathogenic. These authors concluded that the antigenic properties of type 1 collagen α-2 chain were specific to uveal collagen post-translational carbohydrate moieties[25].

This was the first investigation of the pathogenicity of MMP-1 digested collagen. MMP-1 digestions of both type I and II collagen derived from bovine skin and cartilage respectively, did

not induce uveitis. Furthermore, digestion of type II collagen with MMP-1 rendered it incapable of inducing arthritis.

3.5 Chapter 3 References

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4. Pathogenicity of Melanin Associated Antigen Digested with *staphylococcal* v8 protease, Streptokinase, and Matrix Metalloproteinase-1

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4.1 Abstract

Purpose: Experimental autoimmune anterior uveitis is induced with a 22 kDa fragment of type I collagen located on the α2 chain. It is isolated from MAA using *Staphylococcal* V8 protease. It is known to induce uveitis when administered in conjunction with complete Freund adjuvant (CFA). The purpose of this study was to determine the pathogenicity of MAA following digestion with either *Staphylococcal* v8 protease, streptokinase C, or matrix metalloproteinase-1 (MMP-1) administered without the use of CFA.

Methods: Lewis rats were inoculated with MAA that was solubilized with either *Staphylococcus aureus* V8 protease, *Streptococcus* streptokinase C, or MMP-1. Injections were repeated three times one week apart. Biomicroscopic examinations were completed three times per week until the termination of the experiment. Clinical uveitis scores and immunohistochemical labels for CD43 and CD45RC were compared between treatment groups and control animals.

Results: Uveitis was present in 3/12 *Staphylococcus aureus* V8 protease inoculated rats. All three were confirmed clinically and two were confirmed with light microscopy. Uveitis was present in 2/12 streptokinase C solubilized MAA inoculated rats. One rat confirmed clinically, and one was confirmed on light microscopy. None of the rats inoculated with MMP-1 digested MAA developed uveitis clinically or with light microscopy.

Conclusions: *Streptococcus* streptokinase C and *Staphylococcus aureus* V8 protease digested MAA induced uveitis in some rats when inoculations are repeated three times without the use of CFA.

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4.2 Introduction

Autoimmune uveitis is a common potentially blinding ocular inflammatory diseases. It affects between 17 and 52 per 100 000 people per year and it is implicated in 25% of legal blindness in the developing world[1]. Uveitis can be a result of inflammation isolated to the eye or can occur as a manifestation of a multi-system disease. The etiopathogenesis of autoimmune uveitis is not clearly understood but multiple factors including infectious triggers and genetic vulnerability have been implicated[2-5]. Loss of self-tolerance in autoimmune disease can occur through molecular mimicry, where infectious determinants mimic host antigens and trigger self -reactive T cell clones that destroy host tissue[6]. The normal immune privilege of the eye prevents peripheral autoreactive lymphocytes from acquiring tolerance to normal ocular tissues. When the blood-ocular barrier is compromised T lymphocytes may become primed to recognize ocular antigens[7].

Experimental autoimmune anterior uveitis (EAAU) is a rodent model of anterior uveitis induced with inoculation of soluble MAA[8-12]. MAA is derived from uveal tissue, typically bovine-derived, that has been digested with *Staphylococcal aureus* V8 protease[8-12]. *Staphylococcus aureus* V8 protease digested rat (autologous) MAA has been shown to induce uveitis[12]. The antigen in EAAU has been identified as a 22kDa fragment found in the α2 chain of type 1 collagen and immunohistochemistry of naive rats has demonstrated the presence of endogenous MAA in the normal iris and ciliary body[12].

EAAU manifests as severe bilateral anterior uveitis approximately 11-15.5 days post inoculation in nearly all rats inoculated[8-12]. The uveitis is mediated through an antigen specific CD4 + T cell response[8-15]. Light microscopic examination of immunohistochemistry slides of affected rats demonstrate a marked CD4+ T cell infiltration of the anterior uvea[8-15]. Soluble MAA has only induced uveitis when administered with complete Freund's adjuvant, which contains inactive mycobacteria known to stimulate cell-mediated immunity, tumor necrosis factor dysregulation, and uveitis[16-19]. Incomplete Freund's adjuvant provides a water-oil emulsion to facilitate inoculation administration without immunostimulation.

β Hemolytic *Streptococcus*, similar to *Staphylococcus aureus*, also produces a protease, streptokinase C. Streptokinase alone has been associated with the development of uveitis when

administered intravenously as a thrombolytic agent in humans[20]. People with post-streptococcal reactive arthritis following pharyngeal and/or tonsillar infection with **β** hemolytic *Streptococcus* have also been reported to developed uveitis[21]. Uveitis accompanying arthritis has also been observed in rats injected with various bacterial cell wall components including *Streptococcal* cell wall fragments[22]. To our knowledge the uveal pathogenicity of Streptokinase C digested MAA has not been evaluated in animal models.

Matrix metalloproteinases (MMPs) are endogenous proteases capable of lysing type I, II, III and X collagens. They are present in normal human iris, ciliary epithelium, uveoscleral outflow pathway and corneal endothelium[23]. Digestion of MAA with MMP-1 *in vitro* results in several collagen fragments between 20 and 25 kDa, which are similar to the molecular mass of MAA of 22 kDa[12] suggesting MMP-1 may play a role in MAA generation *in vivo*[12]. In rats with EAAU mRNA levels of MMP-1 increase significantly at day 11 post immunization and peak coinciding with peak uveitis at day 19 and then decline[12].

The objective of this study was to explore possible mechanisms of MAA exposure *in vivo* by evaluating the pathogenicity of MAA following proteolysis with V8 protease, streptokinase C, and MMP-1, without the use of complete Freund's adjuvant, in juvenile female Lewis rats.

4.3 Materials and Methods

4.3.1 Animals

Pathogen free female juvenile (28 day old) Lewis rats were obtained from Charles River Laboratories (Sherbrooke, Quebec, Canada). All rats were provided a seven day acclimation period prior to initiation of the experiment. All procedures were approved by the University of Saskatchewan Animal Research Ethics Board (protocol 20150069). Rats were housed in groups of two or three with the treatment and control animals intermixed. Lighting was provided in a 12 hour light: 12 hour dark cycle. Rats were provided ad libitum feed and water. After the acclimation period ophthalmic examinations were completed and the rats were inoculated with their first injection; this was considered to be day one of the experiment.

4.3.2 Ophthalmic Examinations

Ophthalmic examinations were completed by a Diplomate ACVO masked to the treatment groups. Baseline examinations were completed on awake manually restrained rats and included an un-dilated and a dilated bio microscopic anterior segment examination (Kowa SL-17 portable slit lamp, Kowa Optimed Inc., Vermont Avenue, Torrance, California, USA). Following the instillation of 0.5% tropicamide (Alcon, Que, Canada) indirect ophthalmoscopy (Heine Omega 200; Heine Instruments Canada, Kitchener) was completed. Rats were excluded from the experiment if there were detected ophthalmic abnormalities.

Following baseline examination, rat inoculation protocols were completed and rats were re-examined three times per week beginning 14 days following initial inoculation and repeated until euthanasia. At repeat examinations the masked examiner assigned a score of 0-4 for both the uveitis and flare based on previously described scales[24]. When a uveitis score of >0 was assigned the posterior segment examination was repeated at each examination thereafter. For each examination the examiner, uveitis score, flare score, ocular abnormalities were recorded and each eye was photographed. Rats were euthanized on days 26, 28, 36 or 42 post primary inoculation.

4.3.3 Melanin Associated Antigen Preparation

Iris-Ciliary Body Dissection for MAA

Bovine globes were obtained from a local abattoir immediately following slaughter and frozen until use. Globes were thawed in room temperature water. The corneas, lens and vitreous were removed. Using sterile instruments the iris and ciliary body were dissected and placed in sterile tubes and frozen at -20 until MAA extraction.

MAA Extraction

Dissected iris and ciliary body from ten bovine globes were thawed and 2 to 3 mL of 1X PBS, pH 7.2 was added dependent on viscosity of the mixture. The mixture was homogenized using a tissue homogenizer. The homogenized sample was filtered through a cheese cloth into a 50 mL conical tube (Fisher Scientific, Mississauga, Ontario, Canada). The sample was centrifuged (Sorvall ST 16R centrifuge, Thermo Fisher Scientific, Waltham, Massachusetts, USA) at 4500 x g for 15 minutes at 4°C and the supernatant was removed from the pellet and discarded. The pellet was washed 3 times with 1X PBS and re-suspended in 10 mL of 1X PBS containing 2% Triton X-100 (Sigma Aldrich, Oakville, Ontario, Canada). The sample was incubated at room temperature for 3 hours at 200 rpm on an orbital shaker (Thermo Scientific Max Q200 orbital shaker, Thermo Fisher Scientific, Waltham, Massachusetts, USA). The sample was centrifuged again at 4500 x g for 15 minutes at 4°C. The supernatant was discarded. The pellet was washed three times with 1X PBS and re-suspended in 10mL 1X PBS containing 2% SDS (Sigma Aldrich, Oakville, Ontario, Canada). This sample was incubated at 37°C for 3 hours at 200 rpm. Following incubation the sample was centrifuged at 4500 x g for 15 minutes at room temperature. The supernatant was discarded. The pellet containing the MAA was washed three times with 1X PBS, the PBS was removed and the pellet was stored at -20°C.

Staphylococcus aureus V8 digestion of MAA

The pellet containing the MAA (500 to 900 mg) was re-suspended with 10 mL of 8M urea (EMD Millipore, Etobicoke, Ontario, Canada), 1.5mL of 1M potassium phosphate buffer, 4 mL of 500 unit Endoproteinase Glu-C from *Staphylococcus aureus* V8 (Sigma Aldrich, Oakville, Ontario, Canada) and 4.5 mL of double distilled water (Milli-Q Advantage A10 System, EMD

Millipore, Etobicoke, Ontario, Canada) to yield final concentrations of 4M urea, 75mM potassium phosphate buffer and 100 units of Endoproteinase Glu-C from *Staphylococcus aureus* V8. The sample was incubated at 37° at 200 rpm for 30 minutes. Following incubation, the tubes were placed in a beaker of boiling water for two minutes to inactivate the V8 protease. The sample was centrifuged at 4,500 x g for 20 minutes at 4°C. The supernatant containing the soluble MAA was collected and dialyzed against double distilled water (Milli-Q, EMD Millipore, Etobicoke, Ontario, Canada) using 3.5K MWCO Dialysis Membrane (Thermo Fisher Scientific, Waltham, Massachusetts, USA) for 48 hours with water changes every 2 hours for the first 10 hours and every 4 hours for the remainder of time. The sample was transferred to a 50 mL conical tube (Fisher Scientific, Mississauga, Ontario, Canada) and frozen at -80°C. Once frozen solid, the sample was lyophilized (FreeZone Plus 6 Liter Cascade Console Freeze Dryer, Labconco, Kansas City, Missouri, USA) for approximately 48 hours or until lyophilized completely. The lyophilized MAA was stored at -20°C until ready for use. Prior to use, the lyophilized MAA was dissolved in 1 mL of 1X PBS, pH 7.2 and protein concentration was determined using Bio-Rad DC Protein Assay (Bio-Rad Laboratories, Hercules, California, USA)

Streptokinase C digestion of MAA

The pellet containing the MAA (500 to 900 mg) was re-suspended with 10 mL of 8M urea (EMD Millipore, Etobicoke, Ontario, Canada), 1.5mL of 1M potassium phosphate buffer, 0.2 mL of 10,000 unit Streptokinase C (Sigma Aldrich, Oakville, Ontario, Canada) and 8.3 mL of double distilled water (Milli-Q EMD Millipore, Etobicoke, Ontario, Canada) to yield final concentrations of 4M urea, 75mM potassium phosphate buffer and 100 units of Streptokinase C enzyme. The digestion reaction was done in the same manner as the *Staphylococcal aureus* V8 protease digestions described above.

Matrix Metalloproteinase-1 digestion of MAA

Prior to use, the MMP-1 enzyme (EMD Millipore, Etobicoke, Ontario, Canada) was activated with trypsin (Trypsin from Bovine Pancreas, EMD Millipore, Etobicoke, Ontario, Canada) at a ratio of 10:1 MMP-1 to trypsin for 10 minutes at 25°C. The trypsin was inactivated with a 10-fold excess of soybean trypsin inhibitor (EMD Millipore, Etobicoke, Ontario, Canada). The pellet containing the MAA (500 to 900 mg) was re-suspended with 10 mL of 8M urea (EMD

Millipore, Etobicoke, Ontario, Canada), 1.5mL of 1M potassium phosphate buffer, 0.225 mL of 50 µg/mL MMP-1 (EMD Millipore, Etobicoke, Ontario, Canada) and 7.9 mL of double distilled water (Milli-Q EMD Millipore, Etobicoke, Ontario, Canada) to yield final concentrations of 4M urea, 75mM potassium phosphate buffer and 2 µg/mL of MMP-1. The sample was incubated at 25°C at 200 rpm for 30 minutes. Following incubation, the reaction was stopped with 50 mM EDTA. The sample was centrifuged at 4,500 x g for 20 minutes at 4°C. The dialysis and lyophilization of the sample was completed as described above.

4.3.4 Control Animals

A negative control group consisting of 4 naive rats were intermixed amongst treatment groups and euthanized alongside treatment rats between day 26 and 42 of the experiment.

4.3.5 Experimental Inoculations

Prior to all inoculations rats were anesthetized using isoflurane gas (Halocarbon Products Corporation, River Edge, NJ, USA) provided through a flow by mask. The injection site was cleaned with an alcohol wipe and injections were completed as described below.
Three groups of twelve 36 day old females were inoculated with either *Staphylococcal* V8 protease digested MAA, streptokinase C digested MAA or matrix-metalloproteinase-digested MAA. Inoculations contained 400 µg solubilized MAA and an equal volume of incomplete Freund's adjuvant and were administered intradermally at the base of the tail divided over two sites. The inoculations were administered on the first day and repeated on days 7 and 14. Ocular examinations were initiated on day 14 and repeated three times per week until euthanasia. Rats were euthanized between day 26 and 42.

4.3.6 Euthanasia and Tissue Collection

All rats received a lethal intraperitoneal injection of pentobarbital sodium (80 mg/kg; 54 mg/mL; Euthanyl Forte, Bimeda-MTC, Cambridge, Ontario, Canada). Once the palpebral reflex was absent, transconjunctival enucleations were completed and globes were immediately submerged in periodate-lysine-paraformaldehyde (PLP) fixative[25]. Following 24 hours of fixation, globes were dehydrated in sequential 70% ethanol (45 minutes), 90% ethanol (45 minutes), and 100%

ethanol (twice for 30 minutes each). This was followed by immersion in Histo-clear (National Diagnostics, USA) twice for 30 minutes each immersion. Eyes were embedded under vacuum at 54°C for 30 minutes then routinely processed in conventional paraffin[25]. Paraffin blocks were sectioned into 6 µm sections which were floated on a water bath at 40°C prior to transfer onto glass slides. Slides were dried, hematoxylin and eosin stained, and examined with light microscopy by a masked Diplomate ACVO.

Sections from naive rats (n=4), V8MAA (n=12), SKMAA (n=12), and MMP-1 MAA (n=12) treated rats were evaluated using mouse anti-rat CD43 (clone W3/13) and CD45RC (clone OX-33) primary antibodies and secondary anti-mouse antibody. Rat lymph node served as positive control tissue.

4.4 Results

4.4.1 V8 Digested MAA

Three of 12 juvenile female rats inoculated with V8 protease digested MAA developed uveitis detected clinically (Figure 1.) and confirmed with light microscopic examination of immunohistochemically labelled slides in 2/3 affected rats (Figure 2.). The uveitis was detected ophthalmoscopically in two rats on day 17, was initially graded as 4/4 and persisted until euthanasia. The third rat developed unilateral uveitis on the 30th day, which resolved by the 33rd day and could not be detected with histologic examination of either eye after euthanasia. Uveitis was confirmed in the two rats affected until euthanasia with CD43 and CD45RC positive labelled cells in their iris and ciliary body (Figure. 1). The remaining nine rats in this treatment group, which were normal clinically, did not have light microscopic or immunohistochemical evidence of uveitis.

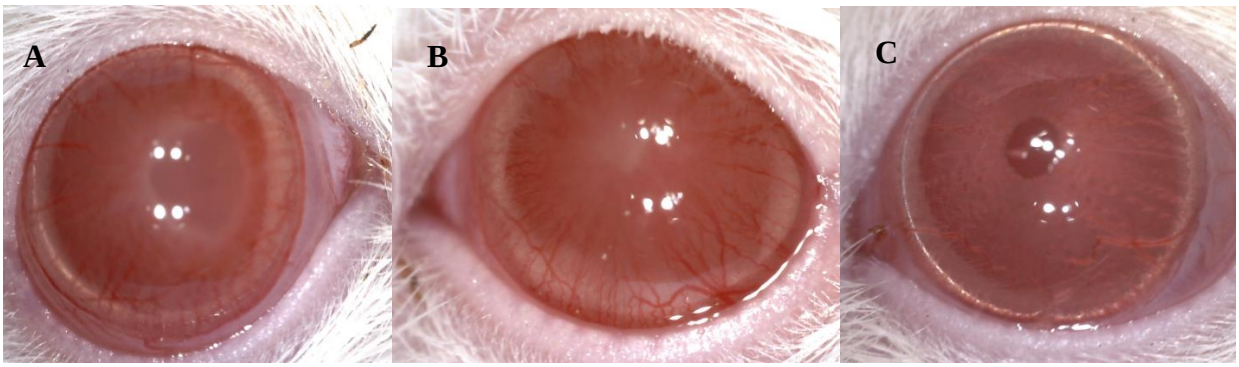

Figure 4- 1. Clinical images of a rat inoculated with V8MAA demonstrating severe uveitis in the right (A) and left (B) eyes. The eye in A demonstrates dyscoria and iridial hyperemia. The eye in image B demonstrates miosis, iridial hyperemia and obstruction of the pupil with aqueous flare and fibrin. C represents a control eye.

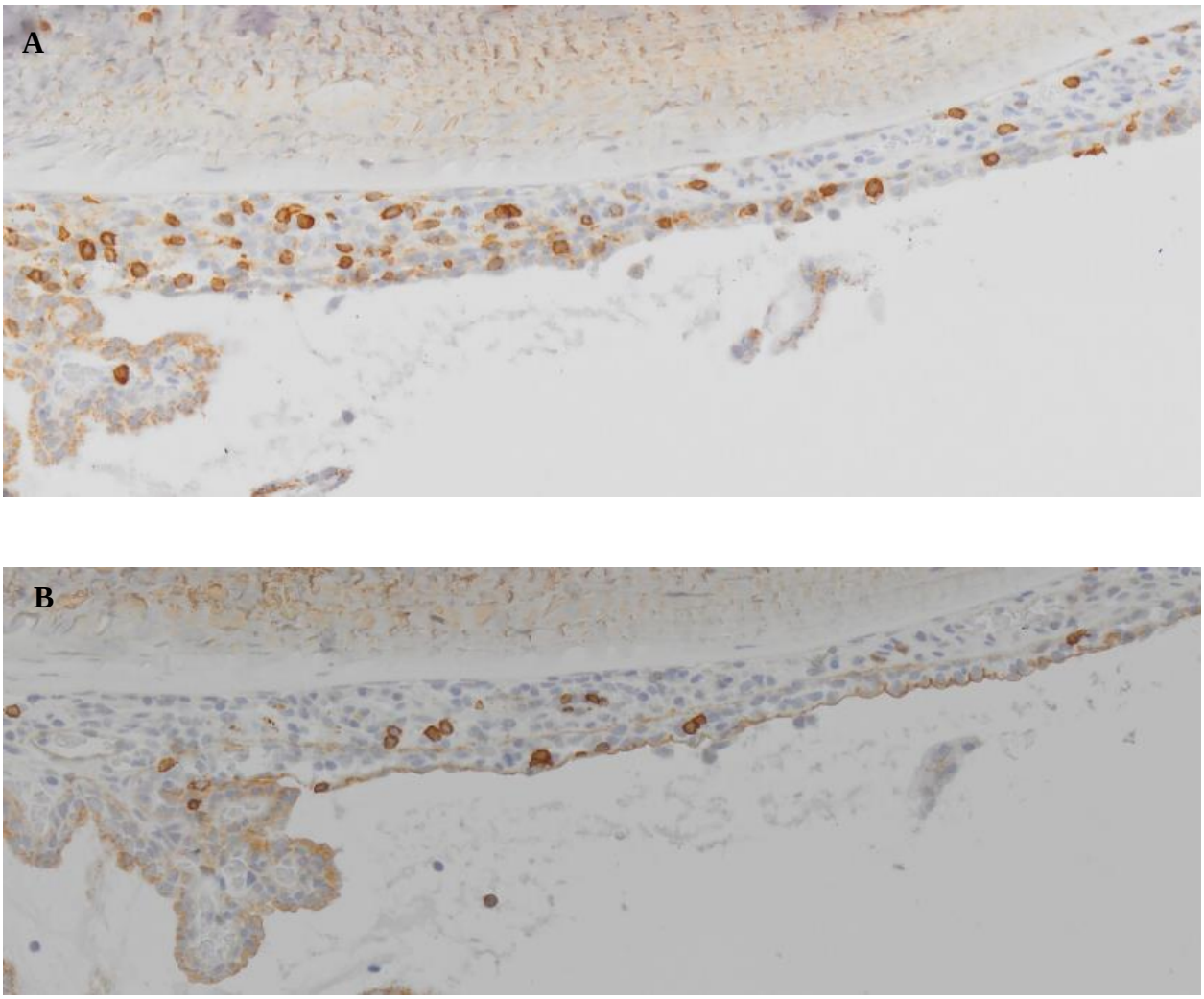

Figure 4- 2. Uveitis in a V8 protease MAA inoculated rat labelled with CD43 (A) and CD45RC (B) immunohistochemistry. The uveitis was graded 1/4 the day prior to euthanasia. A brown labelling dye highlights CD43 (A) positive cells and CD45RC (B) cells. Slight background staining of the ciliary epithelium and posterior iris epithelium is observed. (80X)

4.4.2 Streptokinase C Digested MAA

Two of 12 juvenile female rats inoculated with streptokinase C soluble MAA developed uveitis which was evident on biomicroscopic examination (Figure 3.). One rat was affected bilaterally on day 22 and clinical resolution occurred by day 34. This rat was euthanized on day 36 and did not have detectable CD45RC and CD43 cells within the iris and ciliary body. The second rat developed uveitis unilaterally on day 33 post inoculation, was graded 1/4 and resolution occurred by day 35. This rat was also negative for both CD45RC and CD43 cells within the iris and ciliary body. A third rat did not develop uveitis that could be detected clinically, however, immunohistochemical examination revealed CD45RC and CD43 cells present in the ciliary body (Figure 4).

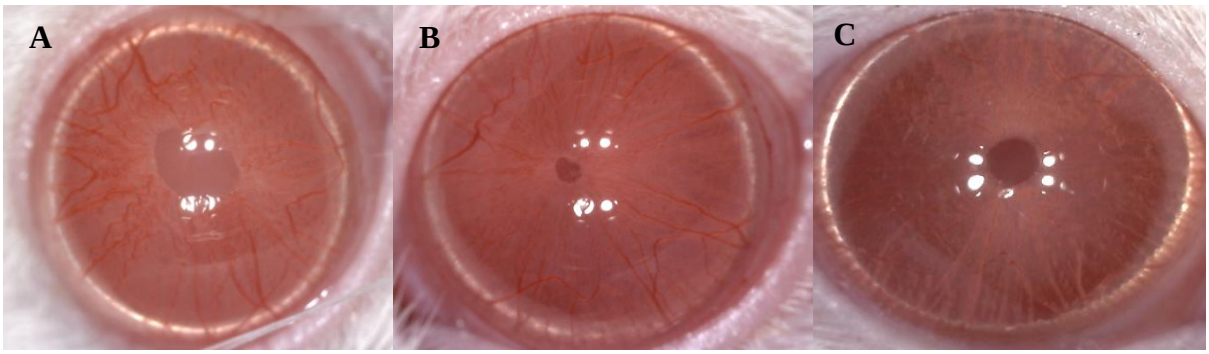

Figure 4- 3. Clinical photos of a SK MAA inoculated rat demonstrating grade 1/4 uveitis OD with dyscoria and mild iris hyperemia (A), and grade 2/4 uveitis OS demonstrating miosis and mild iris hyperemia (B). Image C is an unaffected control eye.

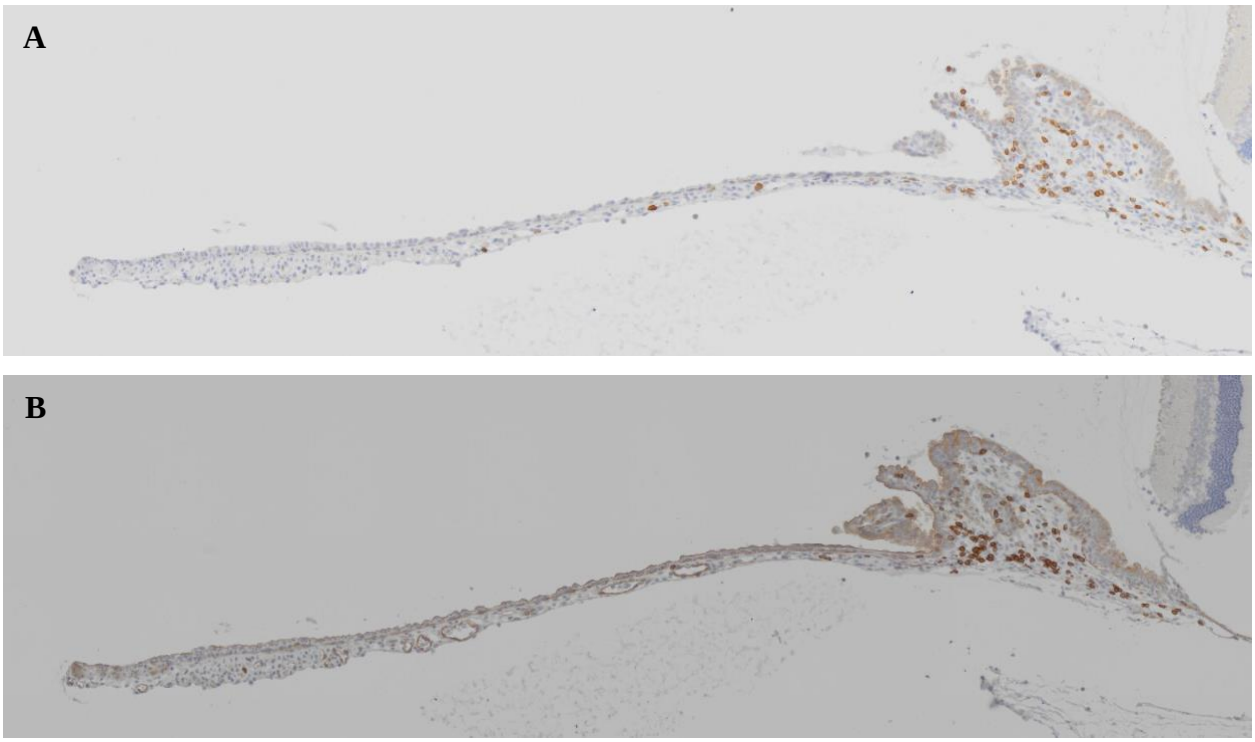

Figure 4- 4. Cyclitis in a streptokinase C MAA inoculated rat labelled with CD43 (A) and CD45RC (B) immunohistochemistry. The brown stain highlight CD43 (A) and CD45RC (B) cells. Cyclitis and iritis had not been detected with biomicroscopic examination. 40X

4.4.3 Matrix Metalloproteinase-1 Digested MAA

There was no clinical evidence of uveitis in any of the rats inoculated with the MMP-1 digested MAA. Similarly, none of the MMP-1 MAA treated rats had CD43 or CD45RC labelled cells in their iris and ciliary body.

4.4.4 Control Groups:

Uveitis was not detected in control rats by either clinical or with immunohistochemical examinations with CD43 and CD45RC labelling.

4.5 Discussion

Complete Freund's adjuvant contains inactive mycobacteria known to stimulate cell-mediated immunity, tumor necrosis factor dysregulation, and uveitis[16-19]. Incomplete Freund's adjuvant provides a water-oil emulsion to facilitate inoculation administration without immune stimulation. This study demonstrated that three inoculations of either streptokinase C or V8 protease digested MAA induced uveitis when administered with incomplete Freund's adjuvant. This is contrary to previous reports in which soluble MAA was not pathogenic as a sole agent and required use of complete Freund's adjuvant or inoculation in its intact form where the MAA backbone was speculated to act as an adjuvant[8,10]. Complete Freund's adjuvant alone causes uveitis and arthritis[16-17] confounding results when it is used in conjunction with soluble MAA. The added use of complete Freund's adjuvant adds additional variables including emulsion stability and adjuvant ratio to inoculum. There were three main differences in experimental design between this study and earlier reports which did not find digested MAA antigenic. First, we increased the dose of *Staphylococcal aureus* V8 protease digested MAA from 100 µg to 400 µg, the injection site was changed to the tail base from the footpad, and lastly, inoculations were repeated every seven days for three treatments. It is not known if one or all of the changes in design accounted for the difference in pathogenicity. Matta *et al* demonstrated IV administration of MAA in previously sensitized rats conferred dose dependent protection, with rats receiving higher doses demonstrating complete tolerance[14].

Streptokinase C digestion of MAA was evaluated for the first time and proved to induce uveitis in our study. Further studies would be required to verify this observation and ascertain the role of streptokinase C in uveitis etiopathogenesis. It is possible that streptokinase C digests uveal collagen in the same or similar location exposing the 22 KDa fragment of type I collagen that is known to induce uveitis.

Data from this study supports a possible role of *Streptococcus* and associated streptokinase C and *Staphylococcus* and associated V8 protease as a triggers for autoimmune uveitis. It is plausible that an infection with either agent could result in breakdown of endogenous collagen and exposure of endogenous MAA. Intravenous streptokinase C has been associated with the

development of uveitis in humans[20], while *Streptococcus* has demonstrated a role in uveitis and arthritis in both humans and rats[21-22].

To the authors' knowledge this was the first study to evaluate for the potential pathogenicity of MMP-1 digested MAA. Uveitis was not observed clinically nor with CD45RC and CD43 immunohistochemistry. MMP-1 cleaves MAA into a fragment that is between 20 and 25 KDa which is similar to the 22 kDa fragment of type 1 alpha II chain of MAA known to be antigenic[12]. It is been speculated that MMP-1 could play a role in exposure of MAA in vivo leading to the development of uveitis[12]. The results of our investigation did not support this theory and more studies are required to determine if MMP-1 plays a role in antigen exposure in autoimmune uveitis.

Conclusions

Streptokinase and V8 protease solubilized MAA induce uveitis without the use of Complete Freund's Adjuvant, while MMP-1 solubilized MAA did not.

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5. Conclusions

Examining the influence of sex on EAAU onset we found juvenile female rats had the earliest onset of disease. Although these results were not significantly different the trend mirrors observations in JIA uveitis where females have an earlier onset of uveitis. Girls with JIA developing uveitis are on average 2.9 years old, where boys are 8.1 years old[1]. The onset of uveitis we observed between the age/sex groups in rats ranged from 16.25 days to 20.00 days which was similar to other reports in juvenile male rats[2-6].Statistical significance was not achieved for the differences in onset between sex and age groups, as these experiments lacked the necessary power to determine statistical differences of modest magnitude.

In comparing the biomicroscopic manifestations of uveitis between age and sex groups we found that severity at peak disease did not differ between age or sex groups. All but one adult male rat developed a severe bilateral uveitis manifesting with marked miosis, fibrin in the anterior chamber, iridial hyperemia and conjunctival injection. The adult male only developed a unilateral mild uveitis at the end of the study. With only four rats per age/sex group, the significance of the variability in this single rat's disease could reflect a true difference in disease manifestation in this age and sex group or it could just be an outlier that coincidentally reflects that age of onset pattern between males and females with JIA uveitis. Future work including larger group sizes to allow enough statistical power to confirm or deny the trends observed in our work are needed.

Changes in vitreous cytokines were observed, both between treated rats with uveitis and controls, and between different age and sex groups. Due to the discovery nature of this study, we had a limited number of rats in each age/sex group, and a high number of comparisons (27 cytokines) this made statistical significance unlikely. The results provide descriptive information and will allow us to narrow our target group of inflammatory cytokines for future work. Cytokines with differences that should be evaluated further included MIP2, RANTES, IL-1a. IP-10, GROKC, IL-18, MCP1, IL-10, TNFα, and IL-13. These were the cytokines with the 10 lowest p values. In aqueous humor of children with JIA-uveitis IL -2, IL -6, IL -13, IL -18, IFN-γ, TNF-α, sICAM-1, RANTES and IP-10 have been reported to be elevated[7]. All of these except IL-13 and cICAM-1 were evaluated in our study which found no significance between rats with uveitis and controls. Another study on children with JIA-uveitis evaluating aqueous in children with inactive

disease found IL-8, TGFβ-1, TGFβ-2, TGFβ-3, serum amyloid A, and TNF-α levels to be increased in JIA-uveitis affected children[8]. While the inactive disease profile is likely more reflective of the stage of uveitis in the rats at the time of vitreous collection we did not evaluate IL-8, TGFβ-1, TGFβ-2, TGFβ-3, or serum amyloid A and we did not see a difference in TNF-α levels. Future work evaluating cytokines in rats with uveitis should include IL-8, TGFβ-1, TGFβ-2, TGFβ-3, and serum amyloid A to determine if a similar profile is seen.

Harvest of vitreous instead of aqueous was done in our study as our collection point was later in the disease. Aqueous humor turnover is more rapid and therefore was less likely to show cytokine changes.

Inhibition of specific cytokines in the course of JIA is credited for the improvement in outcomes observed in affected children over the recent decades. These medications include TNFα inhibitors and monoclonal antibodies targeting IL-2 or IL-6 receptors[9]. We did not observe a difference between IMMA rats with uveitis and controls in the levels of IL-2, IL-6, or TNFα. This may be reflective of our late harvest of vitreous or be due to differences in species or disease processes.

Previous studies evaluating cytokines in EAAU harvested rats sequentially throughout the disease process, and most cytokine elevations typically occurred between day 9 and 18 post inoculation[10-12]. In contrast, we harvested our rats at a single time-point on days 27-29. The later harvest was intended to allow adequate time for development and evaluation of joint pathology. Future work should include a sequential harvesting schedule evaluating for age/sex differences in the cytokine cascade throughout the course of disease.

Inoculation of rats intradermally at the base of the tail was found to produce equivalent uveitis to rats inoculated using the traditional technique in the foot pad. Either injection location is straight forward and easy to perform. In studies requiring evaluation of joints, the tail base injection site should be used.

During the harvest of eyes, rats were also exsanguinated. We plan to evaluate their serum cytokine profiles. It will be valuable to determine if the aqueous cytokine profiles mirror those of the serum and if there are systemic differences between age and sex groups. Future work with this group of rats includes evaluation of their joints. Unpublished work by members of our group

identified arthritis on micro computer tomography in rats inoculated with soluble MAA and complete Freund's adjuvant. While on physical examination the rats in the current experiment did not appear to have any evidence of arthritis, we plan to complete micro computer tomography and these results will be part of a later study.

For the first time we demonstrated that three inoculations of either streptokinase C or V8 protease digested MAA induced uveitis with incomplete Freund's adjuvant rather than complete Freud's adjuvant. Complete Freund's adjuvant, contains inactive mycobacteria known to stimulate cell-mediated immunity, tumor necrosis factor dysregulation, and uveitis[13-16]. Incomplete Freund's adjuvant provides a water-oil emulsion to facilitate inoculation administration without immunostimulation. Previous reports have found soluble MAA has not been capable of inducing uveitis without complete Freund's adjuvant[17-18]. We do not know if our increased dose, tail base injection site, or repeated of inoculations all accounted for the difference in pathogenicity of if only one factor contributed. We plan to image the joints of these rats using micro computer tomography to evaluate for arthritic change. In a pilot study members of our group identified arthritic changes in rats inoculated with soluble MAA and complete Freud's adjuvant. Comparison of these rats induced without the use of complete Freund's adjuvant will allow us to understand the separate roles of soluble MAA and complete Freund's adjuvant in the arthritis we had observed. It should also be considered that the soluble MAA could have been incompletely digested or contaminated with IMAA.

Streptokinase C digestion of MAA was evaluated for the first time and proven to induce uveitis in our study. This corroborates a possible role of *Streptococcus* and associated streptokinase C, and *Staphylococcus* and associated V8 protease as a triggers for autoimmune uveitis. It is plausible that an infection with either agent could result in breakdown of endogenous collagen and exposure of endogenous MAA. Intravenous streptokinase C has been associated with the development of uveitis in humans[19], while *Streptococcus* has demonstrated a role in uveitis and arthritis in both humans and rats[20-21]. We do not know the mechanism of pathogenicity of the streptokinase C digested uveitis, it is possible that streptokinase C digests uveal collagen in the same or similar location to V8 protease exposing the known 22 KDa fragment of ocular type I collagen that is antigenic.

Uveitis was not observed in rats inoculated with MMP-1 digested MAA. MMP-1 cleaves MAA into a fragment that is between 20 and 25 KDa which is similar to the 22 kDa fragment of type 1 alpha II chain of MAA known to be antigenic[5]. The lack of antigenicity of MMP-1 digested MAA did not support the theory that MMP-1 could play a role in exposure of MAA *in viv*o. As elevated serum and synovial fluid levels of MMP-3 are found in patients with active polyarticular and oligoarticular JIA[22-23] and increased concentrations of MMP-2, MMP-3, and MMP-9 were observed in the aqueous of children with inactive JIA-uveitis[8] future work should include evaluation of these proteases.

A common collagen trigger for autoimmune arthritis and uveitis in the Lewis rat was not found in our experiment. Neither commercial type I nor II collagen in their intact form or digested form resulted in uveitis. As expected, intact type II collagen inoculation resulted in arthritis in 28/44 rats, however, none of these rats developed uveitis. This was in contrast to previous work by Petty *et al*[14] in which clinical uveitis was reported in 4/40 female Sprague-Dawley rats inoculated. The difference may be related to differences in genetic susceptibility between Lewis and Sprague-Dawley rats, or differences in experimental design. Inclusion of Sprague-Dawley rats in treatment groups would be useful in confirming the previous findings by Petty *et al*[14] and evaluating the pathogenesis of shared inflammatory response between the joint and eye.

Rats treated with type II collagen digested in either V8 protease or MMP-1 did not develop arthritis. The reason for the loss of antigenicity with collagen digestion is unknown, however it is likely the antigenic epitope became damaged in the digestion process. These results make a direct role of V8 protease or MMP-1 in the exposure of a pathogenic antigen *in vivo* less likely as they in-fact diminished the arthritic antigenicity of type II collagen, the opposite effect to what was hypothesized. This reduced antigenicity somewhat parallels the results with MAA, where intact MAA induces uveitis in 100% of rats inoculated while the digested forms in our study had reduced disease incidence. We speculate that the digestion process may damage the antigenic epitope. Determining the epitope targeted in both MAA and type II collagen induced disease would advance our understanding of the trigger initiating uveitis or arthritis in the rat, which may have translational value to humans. Work in experimental autoimmune uveitis in rats has demonstrated molecular mimicry between retinal S-antigen peptide PDSAg and class I HLA B27PD amino acids 125-138[24-28].

Despite use of over 200 animals in this project a major limitation of this work was our small group sizes. The discovery nature of the work resulted in many groups of animals inoculated and failing to developing disease. This was predominantly the case with type I collagen derived from bovine skin, as it was not pathogenic intact or digested. The negative results speak to the decreased likelihood that type I collagen derived from bovine skin, plays a role in either the development of uveitis or arthritis.

Use of an animal model has inherent limitations as rats are not humans and experimental disease is not equivalent to naturally occurring morbidities. Determining where the differences occur between animal models and naturally occurring human disease plays a valuable role in developing our understanding of the disease and evaluating targets for treatment. Use of animal models allows for more extensive evaluation of pathology as more invasive tissue harvest is possible.

In conclusion, our animal model work does not suggest a role of type I collagen derived from bovine skin, in uveitis or arthritis. Consistent with previous studies we were able to induce EAAU with intact MAA, a uveal form of type I collagen. Differences in uveitis manifestations were observed between age and sex groups that should be further evaluated using experimental designs with adequate power to determine their significance. Streptokinase C was identified as a new agent capable of exposing the antigenic sequence of soluble MAA and continued work in this area is needed to determine its role in JIA-uveitis. Uveitis was induced without the use of complete Freund's adjuvant and we await micro computer tomography results of the joints to determine if MAA could be a common trigger of arthritis and uveitis**.**

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