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Testing a Novel Peptide-Infused Cream for Treatment of Rheumatoid Arthritis in DR4tg Mice

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Abstract

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that causes joint pain and damage. Studies have shown that inducing immune tolerance towards RA-specific proteins/peptides in RA mouse models can reduce arthritis severity and pro-inflammatory responses. The objectives of this study were to determine if a novel peptide cream treatment could modify RA-specific immune responses and reduce joint swelling in a humanized mouse model expressing the HLA-DRβ1*0401 allele (known as DR4tg mice), the strongest genetic risk-factor for RA. Hyaluronan-Phosphatidylethanolamine cream infused with synthetic peptides HomoCitJED and CitJED was applied to HomoCitJED-immunized DR4tg mice before or after arthritis induction. Knee joint swelling and T cell and B cell responses to HomoCitJED and CitJED were measured in this study, however there were no significant differences between peptide-infused cream treated mice and controls. Further optimization of the peptide-infused cream is required to successfully modify immune responses and treat arthritis in DR4tg mice.

Keywords

Rheumatoid arthritis, mouse model, autoantibodies, autoimmune disease, antigen-specific treatment

Summary for Lay Audience

Rheumatoid arthritis (RA) is a life-long autoimmune disease that causes joint swelling, damage and pain, affecting about 1% of the Canadian population. Current treatments for RA involve the use of drugs that do not stop the disease from developing. Instead, these treatments slow down the progression of RA by blocking basic immune responses, and only alleviate some of the pain and symptoms. Additionally, these treatments have severe side effects. Thus, there is a need for a new treatment that is able to treat RA without blocking immune function. In RA, abnormal immune responses to proteins, or small pieces of proteins called citrullinated and/or homocitrullinated peptides, have been shown to be involved in the development of the disease. The development of RA is also associated with the expression of the strongest genetic risk factor for RA called the Shared Epitope. We have previously shown that mice containing the Shared Epitope develop immune responses seen in RA patients when injected with homocitrullinated peptides. We believe that applying citrullinated and homocitrullinated peptides onto the skin of these mice using a new cream might be able to modify immune responses and treat RA. In this study, the cream was applied to mice that contain the Shared Epitope either before or after arthritis onset. We found that the application of this cream did not modify immune responses or reduce joint swelling in mice that received the cream treatment. Therefore, this new cream treatment requires adjustments in order for it to effectively treat RA.

Co-Authorship Statement

All experimental work was performed by the author. Garth Blacker assisted in the immunization of mice, as well as sera collection, during the early months of this project.

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1 Introduction

1.1 Overview

Rheumatoid arthritis (RA) is a chronic inflammatory autoimmune disease that affects 0.5-1% of the world's population (1,2). RA manifests as inflammation in the synovial joints, primarily of the wrist and hands, causing joint swelling and pain (3,4). The disease frequently results in cartilage damage and bone erosion, leading to immobility and irreversible joint destruction (3,4). Within 2-3 years of initial diagnosis, 20-30% of RA patients become work disabled, leading to an increase in economic burden (5). RA patients have a higher rate of mortality when compared to healthy individuals and experience higher rates of cardiovascular disease and other systemic complications (3,4).

Susceptibility to RA involves a combination of both environmental and genetic factors (4). Smoking is known to be an important environmental risk factor; the risk for developing RA is twice as high for smokers than non-smokers (6,7). Periodontal disease is another environmental risk factor, with multiple studies suggesting an association between periodontal disease severity and RA disease activity (8,9). Genetics also contributes to disease susceptibility. As with most autoimmune diseases, RA is more commonly seen in females, and sex may influence disease severity (10). Additionally, the expression of certain human leukocyte antigen (HLA) alleles, which encode major histocompatibility complexes (MHC), are believed to contribute to 30-50% of RA susceptibility (11). The expression of the Shared Epitope (SE), a consensus amino acid sequence encoded by these HLA alleles, is known to be the strongest genetic risk factor for RA (12).

While multiple risk factors have been identified, the exact cause of RA is currently unknown. However, the production of autoantibodies is believed to contribute to RA pathogenesis. Antibodies that bind citrullinated proteins/peptides (referred to as anti-citrullinated protein/peptide antibodies or ACPAs) are highly specific for RA (13,14). More recently, anti-homocitrullinated protein/peptide antibodies (AHCPAs) have been implicated in RA pathogenesis (15) and have also shown a high specificity towards the

disease (16). Adaptive immune responses to citrullinated and homocitrullinated proteins within the joint either propagate and/or initiate pro-inflammatory responses; this includes the infiltration of macrophages and neutrophils into the joint synovium, which are key players in bone erosion and cartilage damage in RA (4). The secretion of pro-inflammatory cytokines, such as TNF-α and IL-6, by activated leukocytes encourages the propagation of immune cell activity in the joint, causing a positive feedback loop which further encourages disease activity (4).

There is no cure for RA, and RA-specific treatments are lacking. Current treatments involve non-specific immunosuppressants, which aim to inhibit pro-inflammatory signals, thereby decreasing inflammation, pain, and slow down disease progression (3). However, only 40% of RA patients achieve sustained remission (17), and treatments usually require the use of multiple immunosuppressants in order to be effective (18). Additionally, these treatments are accompanied with severe side effects, such as increased risk of opportunistic infections and illnesses (19,20). Multiple studies have been performed on animal models for RA to develop a treatment that targets RA-specific pathways (21); however, none have been approved for use in humans. Therefore, there is a need for a novel treatment for RA that targets specific pathways involved in its pathogenesis.

1.2 Genetic Risk Factor: The HLA-DRβ1 Gene

Over the past three decades, various genes have been implicated in RA susceptibility (22). However, genes within the HLA locus have been shown to have the greatest contribution to RA susceptibility (11,23), and their association with RA has long been established. Statsny was the first to describe an association between an HLA gene and RA in 1976 (24). In 1987, Gregerson *et al*. described the molecular basis for this association: certain HLA-DRβ1 alleles, which encode the beta chain of MHC class II molecules, were found to contain a consensus amino acid sequence called the Shared Epitope (25). They hypothesized that the SE participates in RA pathogenesis by facilitating the presentation of autoantigens to autoreactive T cells, thereby activating RA-specific immune responses (25).

The presentation of antigenic peptides is performed by MHC class II molecules located on antigen presenting cells (APCs), such as dendritic cells, macrophages and B cells. MHC class II molecules are encoded by HLA genes found within the human HLA complex on chromosome 6 (25). There are three major regions of the HLA complex that encode MHC class II molecules: the DP, DQ and DR regions (25). Each region contains at least one functional alpha chain and one functional beta chain gene, with their gene products binding non-covalently to form membrane-bound heterodimers (25). The DR region (also denoted as HLA-DR) contains two functional beta chains genes, denotated HLA-DRβ1 and HLA-DRβIII, both of which are highly polymorphic (25). The gene for the alpha chain (HLA-DRα) lacks polymorphisms, and its gene product non-covalently binds to both HLA-DRβ1 and HLA-DRβIII gene products to form MHC class II molecules (25).

The HLA-DRβI gene in particular contains several allelic variations (26). For RA, the strongest genetic association is seen in HLA-DRβI alleles that contain the SE and include the HLA-DRβI*04, *01, *13 and *10 alleles (26,27). The HLA-DRβI*0401 allele (also referred to as HLA-DR4) is known to confer the highest risk for RA (26,27). RA patients expressing the HLA-DRβI*0401 also have a higher risk of producing ACPAs (28). It is believed that this increase in risk for developing RA is due to structural adaptations in the peptide-binding pocket caused by the SE (27).

The SE is a consensus amino acid sequence made of five amino acid residues in positions 70-74 of the HLA-DRβI gene, located within one of the alpha-helical walls of the peptide binding groove (25,26). A study performed by Tezenas du Montcel *et al*., where RA patients were genotyped for the HLA-DRβ1 gene, noted that positions 70 and 71 modulate the risk for RA, while positions 72-74 are always Arg (R)-Ala (A)-Ala (A) in SE-positive HLA-DRβI alleles (26). For positions 70 and 71, different amino acids confer different risks; the highest risk occurring when Gln (Q) and Lys (K) are found in positions 70 and 71, respectively (26). Thus, the sequence QKRAA has the highest risk for RA (which is found in the HLA-DRβI*0401 allele) (26). Studies examining the structure of SE-positive HLA-DRβI alleles have shown that Gln70 and Lys71 of the SE directly interacts with the T cell receptor (29) and antigenic peptides (30), respectively, suggesting a possible

explanation for the increase in risk for RA. Additionally, the SE provides a positive charge to the peptide-binding groove, which has been shown to increase the binding affinity to citrulline-containing peptides due to the presence of an uncharged carbonyl side-chain groups in the citrulline amino acid (31,32). This increase in binding affinity between citrullinated peptides and the peptide-binding groove due to the SE has been suggested to facilitate the presentation of citrullinated peptides to autoreactive T cells (31,32). Mice that are transgenic for the HLA-DRβI*0401 allele have shown that immunization with a citrullinated protein results in the production of ACPAs, as well as the induction of arthritis (33). This does not occur when these SE-expressing mice are immunized with unmodified protein (33). Thus, the expression of the SE increases the binding affinity of citrullinated autoantigens in the peptide-binding pocket of MHC class II molecules through amino acid interactions and a positively charged peptide binding pocket, thereby facilitating the generation of RA-specific immune responses.

1.3 T Cell Responses Involved in RA Pathogenesis

The pathogenesis of RA is believed to be mediated by CD4+ T cell immune responses. The strong association between the expression of the SE, which facilitates autoantigen presentation to naïve CD4+ T cells, and RA susceptibility suggests that autoreactive CD4+ T cells contribute to the development of the disease. Studies have also shown that CD4+ T helper 1 (Th1) and T helper 17 (Th17) cells are involved in the pathogenesis of RA (4). Th1 cells have been shown to be the predominate T helper cell subtype in the joints of RA patients (34). Citrulline-specific Th1 cells have also been identified in the peripheral blood of RA patients using HLA-DRβI*0401 tetramers (35). An increase in the expression of IFN-γ, IL-12 and IL-18, cytokines involved in the differentiation of Th1 cells, have also been detected in the synovium of RA patients (36–38). Additionally, Th1 cells have been suggested to influence B cell class switching to IgG1 and IgG3 isotypes in humans (39–41). In RA patients, antibodies to type II collagen and citrullinated fibrin of the IgG1 and IgG3 subclasses have been identified (42,43), providing more support for the role of Th1 cells in RA pathogenesis.

The involvement of Th17 cells in RA pathogenesis is a more recent discovery. Th17 cells were identified in 2005 and are defined by their ability to secrete the cytokine IL-17A (44–46). The role of Th17 cells in RA pathogenesis was immediately suggested due to the discovery of CD4+ T helper cells that secreted IL-17A in the synovium of RA patients (47,48). Mouse models for RA have demonstrated that IL-17A is involved in the development of experimental inflammatory arthritis, as messenger RNA levels of IL-17A increased as the disease progressed, and blockage of IL-17A pathways supressed disease development (49). Multiple animal studies have further supported the importance of IL-17A and Th17 cells in the development of experimental inflammatory arthritis (50). However, the role of Th17 cells in RA patients is more controversial. IL-17A has been shown to be upregulated in the joint synovium of RA patients (51); however, others have shown that Th1 cells, but not Th17 cells, predominate in the joint of RA patients (34). Nonetheless, inducing immune responses that are known to counteract Th1 and Th17 responses, such as T helper 2 (Th2) and T regulatory responses respectively, could be used as a treatment for RA.

1.4 Autoantibodies in RA

One of the many roles of effector CD4+ T cells in RA is the activation of B cells, which are able to produce autoantibodies. The presence of autoantibodies has long been a key diagnostic factor for RA. The first autoantibodies associated with RA development were rheumatoid factor (RF) and have been included in the criteria for RA diagnosis since 1987 (52–54). RF targets the Fc portion of human IgG; however, RF is not specific to RA, and is detected in patients with other autoimmune diseases, such as Sjogren's syndrome and lupus, as well as healthy individuals and individuals with certain infectious diseases (55). Over the years, multiple autoantibodies have been implicated in RA pathogenies (56). However, the identification of anti-citrullinated protein/peptide antibodies (ACPAs) and anti-homocitrullinated protein/peptide antibodies (AHCPAs) has provided more insight towards RA pathogenesis and more accurate disease diagnosis.

The production of ACPAs are highly specific for RA (13,14), and include anti-cyclic citrullinated peptide (anti-CCP2) antibodies, which are commonly measured in clinics when diagnosing RA. Approximately 70% of RA patients are ACPA-positive (14,57). The

presence of ACAPs in the sera of individuals can pre-date clinical diagnosis by several years and predict RA development in high-risk populations (58,59). The production of ACPAs have also been associated with more severe disease outcomes (57,60). ACPAs target proteins/peptides within the joint that have undergone a post-translational modification called citrullination (61). Multiple citrullinated candidate autoantigens have been identified and include citrullinated fibrinogen (62–64), vimentin (63,65–67), type II collagen (68), and α-enolase (69).

Citrullination is the enzymatic conversion of the positively charged amino acid arginine to the neutrally charged non-essential amino acid citrulline and is performed by the peptidyl arginine deaminase (PAD) enzyme family (70). To date, five isoforms of PAD have been identified, and are expressed in multiple cell types and tissues (71,72). In RA, the isoforms PAD2 and PAD4 have been shown to be expressed in the synovium of RA patients, and thus are suspected to be the isoforms involved in the citrullination of proteins (73). Immune cells such as neutrophils, which play a key role in RA pathogenesis, are known to express PAD2 and PAD4 (74,75). Normally, PAD is found intracellularly and inactive; activation of PAD requires a concentration of calcium that can only be achieved when exposed to the extracellular space (72). This exposure can occur during cell death, when the plasma membrane is no longer intact (72), or when PAD is released from the cell via neutrophil extracellular traps, a process known as NETosis (72,76). Interestingly, the presence of citrullinated proteins was shown to not be specific to RA synovium, as it was also found in the synovial tissue of non-RA patients (77). The process of citrullination has also been associated with hair growth, skin keratinization, and myelin formation (74). Nonetheless, immune responses to citrullinated proteins have been shown to be specific to RA (14).

Several studies have implicated ACPAs in RA pathogenesis. The passive transfer of sera collected from RA patients (78) and purified IgG ACPAs (79) into FcγRIIB deficient mice, which lack the receptors responsible for inhibiting B cell activation (80), resulted in the development of inflammatory arthritis (78,79). Additionally, immunization with citrullinated fibrinogen in mice transgenic for the SE-positive HLA-DRβI*0401 allele led to the production of ACPAs and the induction of arthritis (33). It has been proposed that ACPAs form immune complexes in the joint with their respective antigens, resulting in the

activation of complement and the production of pro-inflammatory signals. This theory has been supported by the identification of immune complexes composed of ACPAs and citrullinated fibrinogen in the synovium of RA patients (81). *In vitro* studies using anti-CCP positive sera from RA patients have also shown that ACPAs are capable of activating the classical and alternative pathways of complement (82). Additionally, complement proteins such as C1 and C3b have been detected on the cartilage surface in RA patients (83,84) and the level of C1q in the serum of RA patients was shown to correlate with clinical disease activity (85), further implicating complement activation in RA pathogenesis.

More recently, AHCPAs, also known as anti-carbamylated protein antibodies (anti-CarP) have been detected in the sera of RA patients (86,87). AHCPAs are also highly specific for RA (16) and can be detected in RA patients that are ACPA-negative (87). As with ACPAs, AHCPAs can be detected before disease onset (88) and are associated with more severe radiological damage (87). AHCPAs target proteins/peptides that have undergone a post-translational modification called homocitrullination (also referred to as carbamylation) (61). Homocitrullination is a chemical modification that occurs in the presence of cyanate and results in the conversion of the positively charged amino acid lysine to the neutrally charged amino acid homocitrulline (89). Homocitrulline is structurally similar to citrulline, with the only difference being the addition of an extra carbon (89). Myeloperoxidase (MPO), an enzyme found abundantly in neutrophils, can stimulate homocitrullination by converting thiocyanate to cyanate in the presence of hydrogen peroxide (61). However, levels of cyanate within the body are normally too low for substantial homocitrullination to occur, and thus require conditions like smoking and/or uremia to increase cyanate concentrations (61,89). The physiological role of homocitrullination in the body is currently unknown (61).

The production of AHCPAs have also been implicated in RA pathogenesis. Turunen *et al*. were the first to suggest this after the immunization of rabbits with homocitrullinated albumin and/or type I collagen resulted in the production of high-affinity ACPAs (90). Mydel *et al*. also demonstrated that the immunization of mice with homocitrullination filaggrin-derived peptides can induce arthritis (15). More recently, Lac *et al*. immunized

mice transgenic for the HLA-DRβI*0401 allele with synthetic homocitrullinated peptides, resulting in the production of antibodies that target homocitrullinated and citrullinated peptides (91). In a separate study, Lac *et al*. showed that RA patients also have antibodies that bound to the same synthetic homocitrullinated and citrullinated peptides (92). *In silico* analysis demonstrated that the SE can also accommodate homocitrullinated peptides (86) and cross-reactivity between ACPAs and AHCPAs has also been observed (86,92), suggesting that AHCPAs may play a similar role to ACPAs in RA pathogenesis.

1.5 Mouse Models for RA

Many different mouse models have been developed in an attempt to further elucidate the pathways involved in RA pathogenesis and develop novel treatments. The most commonly used mouse model for RA is the collagen-induced arthritis (CIA) model. To induce arthritis, mice of various strains (including but not limited to: DBA/1, C57BL/6 and HLA-DRβI*0401 transgenic mouse strains) are immunized with complete Freud's adjuvant (CFA) and either bovine or chicken type II collagen (93,94). Type II collagen (CII) is a structural macromolecule found within the extracellular matrix of articular cartilage, and represents about 90-95% of the total collagen in the joint (95). Arthritis onset is seen within 21-28 days after the initial immunization and results in erosive polyarthritis within the joints of the ankles, displaying pathological features commonly seen in human RA, such as synovitis, pannus formation and cartilage damage (96). Rheumatoid factor and ACPAs have also been detected in immunized mice (97–99), however autoantibodies to CII are the main drivers of arthritis pathogenesis in the CIA mouse model (100). In RA, antibodies to CII have only been reported in 3-30% of patients (68,101), and it is believed that antibodies to citrullinated antigens, including citrullinated type II collagen, have a greater impact in RA pathogenesis (68,102).

Another commonly used mouse model for RA is the K/BxN serum-transfer arthritis mouse model. This model requires the use of K/BxN mice, which contain a transgenic T cell receptor that recognizes glucose-6-phosohate isomerase (G6PI), an enzyme that is ubiquitously expressed and involved in glycolysis (103). By 4-5 weeks of age, K/BxN mice spontaneously develop severe arthritis and autoantibodies to G6PI (104). The passive

transfer of serum from arthritic K/BxN mice into a different mouse strain, such as C57BL/6 mice, results in the development of polyarthritis within 2-3 days (104). While this mouse model has been shown to develop multiple pathological features seen in human RA (such as leukocyte infiltration, cartilage damage, synovitis and pannus formation in the joint), it lacks similarities in disease immunopathogenesis (103,104). The K/BxN antibody transfer mouse model relies solely on autoantibodies to cause disease (104); in RA, genetic and environmental factors, T cells and B cells also play a role in RA pathogenesis (4). RA is also a chronic disease, which is not recapitulated in this model, as the arthritis dissipates within 15-30 days of initial serum transfer (103). Additionally, the role of G6PI in RA pathogenesis remains controversial. Elevated levels of soluble G6PI in the sera and synovial tissues of RA patients have been reported (105,106). Anti-G6PI antibodies and G6PI immune complexes have also been identified in the sera of RA patients (106,107). A more recent study has suggested that G6PI could increase the proliferation of synovial fibroblasts and regulate hypoxia-induced angiogenesis, both of which contribute to the development of RA (105). However, studies have suggested that anti-G6PI antibodies are not specific to RA (108,109).

A mouse strain transgenic for the HLA-DRB1*0401 allele (referred to as DR4tg mice) has also been used for studies on RA. The DR4tg mice were initially developed by Ito *et al*. in 1996 to investigate autoimmune diseases associated with the expression of the HLA-DRB1*0401 allele (110). DR4tg mice express a human-mouse chimeric MHC class II molecule, which was created by inserting the second exon, where the SE is located, of the HLA-DRα and HLA-DRβ1*0401 genes into the mouse IE^d-α and IE^d-β genes, respectively (110). The IE genes in mice encode MHC class II molecules and bear high homology to the HLA-DR MHC class II molecules in humans (110). These mice were then backcrossed with mice that were deficient in endogenous MHC class II molecules ($IA\beta^-$/$IE\alpha^-$) to produce HLA-DRA-IEα/HLA-DRB1*0401-IEβ transgenic mice, or DR4tg mice (110).

Multiple studies have used DR4tg mice as a model for RA. Our group demonstrated that when DR4tg mice are immunized and boosted with citrullinated fibrinogen (CitFib), they develop antibody and T cell recall responses to citrullinated fibrinogen and spontaneously

developed joint swelling and arthritis (33,111). Additionally, Lac *et al*. demonstrated that the immunization of these mice with a synthetic homocitrullinated peptide, called HomoCitJED, resulted in the production of antibodies that can also be detected in RA patients (91,92). Thus, the DR4tg mice not only expresses the strongest genetic risk factor for RA, but can also mimic autoantigen disease pathways that are seen in RA patients, unlike the CIA and K/BxN serum-transfer mouse models. However, Lac *et al*. also found that DR4tg mice immunized with HomoCitJED did not spontaneously develop arthritis (91) as previously described in CitFib-immunized DR4tg mice (33). Instead, intra-articular injections of HomoCitJED into the knee joint of HomoCitJED-immunized DR4tg mice are needed to induced joint swelling, as demonstrated by a pilot study performed by our group (112). Overall, the use of DR4tg mice as a mouse model for RA can allow for studies that more accurately represent RA patients, and thus allow for easier translation to humans when developing RA-specific treatments in mice.

1.6 Mechanisms of T Cell Immune Tolerance

In general, autoimmune diseases develop due to an inability of immune cells to distinguish between self and non-self peptides. This occurs due to a lack of tolerance in B cells and/or T cells towards self-peptides. In RA, it is believed that CD4+ T cells drive disease pathogenesis due to a loss in tolerance that is specific to RA (27).

Immune tolerance is defined as the state of unresponsiveness to self-antigens (113). There are several mechanisms by which immune tolerance occurs in T cells. During T cell development, all T cells undergo the process of central tolerance, which aims to delete autoreactive T cells before they reach maturity (113). This process occurs in the thymus and begins when thymocytes interact with MHC class I or II molecules containing self-peptides, expressed by thymic epithelial cells (113). The binding affinity between the T cell receptor (TCR) and the MHC/self-peptide complexes will determine if the thymocytes are clonally deleted (a process referred to as negative selection), die due to neglect, or receive survival signals (a process referred to as positive selection) (113). Thymocytes that are positively selected for will then migrate to the medulla of the thymus and undergo another round of negative selection, this time referred to as central tolerance (113). Medullary thymic epithelial cells that express the autoimmune regulator (AIRE+)

transcription factor will present self-peptides that are found in other tissues in the body via MHC class I or II molecules (113). The binding affinity between the TCR and these MHC/self-peptide complexes will once again determine if the T cells receive apoptotic or survival signals (113). Thymocytes that receive survival signals will emigrate to the periphery and are then considered mature (but naïve) CD4+ or CD8+ T cells (113).

Once in the periphery, naïve T cells also undergo the process of peripheral tolerance, which aims to delete autoreactive T cells that escaped central tolerance (114,115). There are several mechanisms for peripheral tolerance, and can occur in several different tissues, including the skin and mucosal surfaces (116–118). In general, peripheral tolerance begins with immature dendritic cells (iDC), which constantly undergo phagocytosis and endocytosis, capturing apoptotic bodies and tissue specific proteins (115,118). Under homeostatic conditions (i.e. in the absence of inflammation), the iDCs remain immature upon ingestion of proteins, expressing low levels of co-stimulatory and MHC class II molecules (115,119). However, these iDCs are considered "antigen-loaded" and are able to migrate to lymph nodes, where they then interact with naïve CD4+ T cells (120,121). Due to a lack of stimulatory molecules from the iDCs, the naïve CD4+ T cells will undergo anergy, cell death, or will become antigen-specific CD4+CD25+FoxP3+ T regulatory cells (Tregs), which are capable of supressing pro-inflammatory responses (115,119). These immunomodulatory responses are mediated by anti-inflammatory cytokines such as TGF-β and IL-10, as well as the expression of molecules such as programmed-death ligand 1 (PD-L1) on tolerogenic dendritic cells, which regulate Treg differentiation and provide inhibitory signals to activated T cells (118,122).

In RA, it is believed that autoreactive CD4+ T cells evade central tolerance. During central tolerance AIRE+ medullary thymic epithelial cells express tissue specific proteins; however, proteins that undergo post-translational modifications may not be presented by these AIRE+ epithelial cells, as suggested by Raposo *et al*. (123). Thus, T cells that have a binding affinity to citrullinated/homocitrullinated proteins/peptides may not be deleted and are able migrate to the periphery.

There is also evidence that peripheral tolerance is broken in RA. Mature dendritic cells (DCs) are found in high concentrations in the joint synovial tissue and fluid of RA patients (124,125). These DCs secrete cytokines such as IL-1β, IL-6, IL-23 and IL-12, which are able to induce Th1 and Th17 responses (126). They also overexpress the transcription factor NF-κβ, which is responsible for maturation and pro-inflammatory responses in DCs (126). Additionally, RA patients have high levels of soluble programmed death ligand-1 (PD-L1), which causes a downregulation in the PD-1/PD-L1 inhibitory pathway used in mediating peripheral tolerance (127). Thus, the development of a treatment that is able to create antigen-specific tolerogenic DCs and induce immune tolerance towards autoantigens could potentially stop the positive-feedback loop of pro-inflammatory immune responses caused by DCs and treat RA.

1.7 General Treatments for RA

There is no cure for RA, and current treatments focus on disease management, which depend on anti-inflammatory drugs and immunosuppressant agents that lack specificity to RA disease-causing pathways, resulting in the development of severe adverse effects (18). Drugs commonly used in short durations are non-steroidal anti-inflammatory drugs (NSAIDs) and glucocorticoids (3,20). These drugs rapidly lessen pain and stiffness; however, they do not limit disease progression and thus are not used for long-term disease management (3). They are typically used when flare-ups occur and immediate improvement is necessary (3,20).

Conventional disease modifying anti-rheumatic drugs (cDMARDs) are the most commonly used drug for treatment of RA (20). They are synthetic drugs that are known to reduce joint swelling and pain, limit disease progression, and improve mobility/function (3). Each cDMARD has a different mechanism of action; in general, they inhibit pro-inflammatory immune responses that are not specific to RA pathogenesis (20). Methotrexate is the most commonly used cDMARD amongst RA patients and is usually combined with other cDMARDs, such as sulfasalazine and hydroxychloroquine, or with biological DMARDs, in order to increase the efficacy of treatment (3,20,128). The use of

cDMARDs is accompanied with adverse side effects, such as hepatotoxicity, gastrointestinal effects and oral ulcers (20,129).

Biological DMARDs (or bDMARDS) are also commonly used in the treatment for RA and consist of genetically engineered proteins that target immune system pathways (130). These include anti-TNF-α agents, anti-IL-1 and anti-IL-6 receptor blockers, which inhibit pro-inflammatory pathways, and anti-CD20 antibodies, which eliminates B cells (20,130). These treatments target the general pro-inflammatory immune responses that contribute to RA disease symptoms, and have been shown to be highly effective at improving physical function and quality of life in RA patients (131). Similar to conventional DMARDs, biological DMARDs aim to reduce joint swelling and pain and prevent the progression of the disease (20). However, bDMARDs are also accompanied with severe adverse effects, such as increased risk of tuberculosis and opportunistic infections, due to the general inhibition of pro-inflammatory responses that are required for defending against pathogens and other adverse events (20).

In general, treatments for RA have a high loss of effectiveness over time (132). Additionally, only 40% of RA patients achieve sustained remission (17). Thus, there is a need for a RA-specific treatment that is able to effectively manage disease progression and pain without having the severe side effects seen with non-specific immunosuppressants.

1.8 Treatments Using Mucosal Tolerance

Mucosal tolerance is defined as the suppression of immune responses to foreign antigens that have been previously exposed to the mucosa, through either the oral or nasal routes (133). It is best known for its ability to prevent immune responses to commensal bacteria and food particles (133); however, multiple studies have explored whether mucosal tolerance can also be used to treat autoimmune diseases. When antigens are introduced into the lumen of the gut and/or nasal cavity, they cross the mucosal surface into mucosal-associated lymphoid tissues (MALT) through a variety of mechanisms (116,134). Dendritic cells located within the MALT phagocytose antigens and migrate to the mesenteric lymph nodes where they initiate antigen-specific immune responses that result in immune tolerance (116). The dosage of the antigen, delivered either orally or

intranasally, can influence the mechanism of immune tolerance that occurs. Lower doses of antigens have been shown to induce T regulatory cells, whereas higher doses of antigens result in T cell anergy and cell death (116,135).

In 1986, Thompson *et al*. were the first to show that the induction of oral tolerance can treat experimental arthritis in mice (136). Using the CIA mouse model for RA, Thompson *et al*. showed that feeding type II collagen to mice before induction of CIA suppressed the incidence of arthritis (136). Since then, multiple studies have developed treatments for RA that induces oral or nasal tolerance towards an autoantigen. The majority of published studies used type II collagen (either protein or peptide) as the target autoantigen, along with the CIA mouse model for RA. The dose of the antigen, timing of delivery (before or after arthritis induction) and frequency of treatment varied between study (137–143). In general, all were shown to have success in preventing or reducing arthritis severity. Common outcomes included reduced arthritis score (138,140,142,144,145), decrease in anti-type II collagen IgG antibodies (137,140,142–145), decrease in pro-inflammatory cytokines such as IFN-γ (137,139,140), increase in anti-inflammatory cytokines such as IL-10 (141,145,146) and TGF-β (138,139,141,143), and a decrease in splenic T cell responses to type II collagen (138,139,146). A decrease in mRNA expression of TNF-α and IL-6 was also seen in the joint tissues of nasally treated CIA mice (139). Another study also reported a decrease in TNF-α mRNA expression, however it was observed in the draining lymph nodes of orally treated CIA mice (143). Due to the success of these treatments in mice, multiple clinical trials have been conducted to determine if the induction of mucosal tolerance towards an autoantigen can treat RA.

The majority of the clinical trials have studied the effects of oral administration of type II collagen on disease severity in RA patients. Type II collagen was provided in either a capsule or tablet (147,148), or solubilized in acetic acid (149–151) and was taken daily for either 12 (150,151) or 24 weeks (147–149). The daily dose of type II collagen varied for each study; the lowest was 20 μg/day (149), whereas the highest was 5mg/day (148).

Overall, each clinical trial showed an improvement in clinical responses when compared to controls and when examining individuals over time (147–151). However, the efficacy

of these treatments remain inconclusive due to several reasons. Firstly, the sample size was small, with the majority of the studies having 15-50 patients per treatment (148,149,151). Secondly, a few studies allowed for the use of NSAIDs during the clinical trial, which have been suggested to have negative gastrointestinal effects and could impact the absorption of the type II collagen and thus influence the efficacy of the treatment (147,152). Lastly, the effects of oral treatment with type II collagen were shown to not be better than conventional treatments such as methotrexate (147). Nonetheless, these clinical trials suggest that an antigen-specific treatment could be effective at reducing disease severity at the correct dosage and may even be beneficial if supplemented with conventional treatments; however, more clinical trials are required.

1.9 Treatments Using Cutaneous Tolerance

Another approach to inducing immune tolerance towards autoantigens is by introducing the proteins/peptides into the skin and eliciting cutaneous, or skin-induced, immune tolerance. The application of proteins/peptides onto the skin (referred to as epicutaneous immunization) allows for proteins/peptides to be taken up by Langerhans cells, a subset of tolerogenic dendritic cells located within the epidermis (114). Langerhans cells (LCs) are defined by their expression of langerin (CD207) and CD11c on the cell surface, and make up 3-5% of the dendritic cell population found within the skin (153). Multiple studies have demonstrated the importance of LCs in cutaneous tolerance (154–156).

The microenvironment of the skin also plays a key role in cutaneous tolerance. Keratinocytes are the epithelial cells that make up the epidermis and contribute to cutaneous immune responses (157). Disruption of the epidermal layers activates keratinocytes, causing them to secrete cytokines that promote LC migration and skew LCs to prime anti-inflammatory Th2 cells (158–160). Keratinocytes also contribute to immune tolerance by secreting TGF-β and supporting the generation and function of T regulatory cells (157). Surrounding the keratinocytes, within the extracellular matrix, is hyaluronan (HA), a large glycosaminoglycan that is found in high concentrations of the skin and has also been known to have immunological roles (161). HA is a known ligand for CD44, a receptor found on dendritic cells, T cells, B cells, macrophages and other leucocytes, and

has been suggested to regulate cell adhesion and trafficking (161). Studies have also shown that HA also promotes migration of LCs to skin-draining lymph nodes, as well as facilitate LC maturation in the presence of inflammation (161,162). Hyaluronan-Phosphatidylethanolamine (HA-PE) cream, developed by Symonette *et al*., was shown to increase the concentration of HA and keratinocyte proliferation (163). This HA-PE cream could therefore be used as an aid in inducing cutaneous tolerance towards proteins and/or peptides, specifically as a method of delivery, since it was also shown to penetrate into the deep layers of the epidermis (163).

Studies have shown that epicutaneous immunizations with proteins/peptides can induce cutaneous immune tolerance in mice and prevent the development of autoimmune diseases. Bynoe *et al*. demonstrated that the application of an autoantigenic myelin peptide to the skin of these mice resulted in the generation of T suppressor cells and prevented experimental allergic encephalitis (EAE) (164). Marcińska *et al*. demonstrated that the application of type II collagen protein to the skin of the CIA mouse model also inhibited collagen-induced arthritis through the generation of T suppressor cells (165,166). In the chronic CIA mouse model for RA, Strid *et al*. demonstrated that epicutaneous immunization with type II collagen prevented the onset and progression of inflammatory arthritis through an anti-inflammatory Th2 response (167).

Studies have also shown that generating tolerogenic dendritic cells towards RA autoantigens can treat inflammatory arthritis (Table 1.1). In a study performed by Gertel *et al*., a novel multiepitope peptide derived from RA-specific citrullinated autoantigens was subcutaneously injected into rats after arthritis induction (168). Rats that received this treatment saw a reduction in disease severity and an increase in T regulatory cells within the spleen (168). Several studies by Martin *et al*. also demonstrated that treatment with tolerogenic DCs could alleviate arthritis in RA mouse models (169,170). Tolerogenic DCs were generated from the bone marrow of mice and pulsed with an antigen such as type II collagen (169,170). These DCs were also treated with NF-κβ inhibitors to induce a tolerogenic phenotype, and were then injected into CIA mice after disease induction (169,170). Similar studies were also conducted by Stoop *et al*. and van Duivenvoorde *et al*., except reagents such as vitamin D_3 and dexamethasone (171) or tumor necrosis factor

Table 1.1: Summary of studies that utilize antigen-specific tolerogenic DCs as treatment in RA animal models.

Dose of Antigen	Antigen	Animal Model	Timing of Treatment	Method of Treatment	Reported Effects	Ref.
50 μg	bovine CII protein	CIA (mice)	3 weeks prior or 1 week post disease induction	solubilized and applied directly to skin	- decrease in T cell responses to CII - decrease in anti-CII total IgG, IgG2a - increase in anti-CII IgE - decrease IFN-γ - increase IL-4	167
100 μg	bovine CII protein	CIA (mice)	1 week prior to disease induction	soaked gauze pad applied to skin	- increase in Tαβ suppressor cells - decrease in anti-CII IgG2a and anti-CCP - decrease IFN-γ - increase IL-17A	166
300 μg	Cit-ME	adjuvant-induced arthritis (rats)	7 days after disease induction	s.c injection	- increase in splenic CD4+CD25+FoxP3+ Tregs - decrease in IL-17+CD4+ T cells - decrease in splenic IL-17 and IL-6 mRNA - increase in CD4+ T cell apoptosis via FAS receptor pathway	168
100 μM*	mBSA	antigen-induced arthritis (mice)	2, 4 or 6 days after disease induction	$5x10^5$ tolDCs s.c injection	- decrease in mBSA-specific T cell responses - IL-10 dependent suppression of arthritis - decrease in anti-mBSA IgG2b and 2c - increase in anti-mBSA IgG1 and IgA	169

10 μg/mL*	bovine CII protein	CIA (mice)	3, 7 and 11 days after disease onset	$2.5x10^6$ tolDCs i.v. injection	- decrease in T cell responses to CII - no change in anti-CII antibody levels - increase in IL-10 producing CD4+ T cells - decrease in IL-17 producing CD4+ T cells	171
2 μg/mL*	bovine CII peptide	CIA (mice)	3, 5 and 7 days prior to disease induction	$2.5x10^6$ tolDCs i.v. injection	- disease in arthritis severity (determined via histopathological analysis) - decrease in anti-CII IgG2a - decrease in splenocyte IFN-γ production	172
2.5 mg/mL **	mBSA	antigen-induced arthritis (mice)	7 days after disease induction	100 μL of liposomes s.c. injection	- reduction in arthritis severity	173

* = concentration of peptide added to tolergenic DCs (tolDCs)
** = concentration of antigen within liposome
CII = type II collagen
CIA = collagen induced arthritis
Cit-ME = multiepitope citrullinated peptide
mBSA = methylated bovine serum albumin
s.c. = sub-cutaneous
i.v. = intra-venous

(172) were used to induce the tolerogenic phenotype, respectively. Capini *et al*. also demonstrated that the injection of liposomes containing methylated bovine serum albumin and NF-κβ inhibitors could target DCs and induce antigen-specific tolerance (173). These methods of treatment ensures that the DCs are both tolerogenic and antigen loaded. Due to the success seen in mouse studies, a Phase I clinical trial with 34 participants assessing the safety of autologous tolerogenic DCs treated with NF-κβ inhibitors and pulsed with a mixture of citrullinated peptides (including aggrecan, vimentin, type II collagen and fibrinogen) as a treatment for RA occurred (174). This treatment was deemed safe and effective, as it was shown to reduce activated CD4+ T effector cells, pro-inflammatory cytokines and chemokines, and decrease disease activity (174). However, due to the small sample size and lack of controls (e.g. injection of unmodified DCs or tolerogenic DCs with irrelevant autoantigens) more clinical trials are required before the treatment can be used by RA patients.

1.10 Rationale, Hypothesis, and Objectives

Current treatments for RA involve non-specific immunosuppressants that inhibit general pro-inflammatory responses instead of targeting RA-specific immune responses (3,20). These treatments are also accompanied with severe side effects (20). Studies inducing immune tolerance towards RA autoantigens, in particular type II collagen, have shown success in mouse models for RA. However, clinical trials inducing oral tolerance towards type II collagen as a potential treatment have shown inconclusive results.

Immune responses to citrulline and homocitrulline are highly specific to RA (13,14), and are associated with the expression of the Shared Epitope, a consensus amino acid sequence found in the peptide binding pocket of MHC class II molecules (25). However, prior studies of new treatments for RA do not utilize mouse models that express the SE or display immune responses to both homocitrulline and citrulline. The induction of immune tolerance towards citrullinated or homocitrullinated proteins/peptides as a treatment for RA has yet to be explored in a clinically relevant mouse model such as the SE-expressing DR4tg mouse model for RA.

This study aims to develop a novel treatment for RA by delivering synthetic cyclic homocitrullinated (HomoCitJED) and citrullinated (CitJED) peptides into the skin of the DR4tg mouse model for RA. These peptides contain equal numbers of citrulline or homocitrulline residues on the same peptide backbone. To deliver these peptides into the skin, HA-PE cream was used since it is able to penetrate into the deep epidermal layers of the skin (163), where tolerogenic dendritic cells reside.

The hypothesis of this study is that epicutaneous delivery of HomoCitJED and CitJED in HA-PE cream will modify RA specific immune responses in DR4tg mice and prevent and/or treat arthritis. The effects of this cream treatment were investigated at two time points: after antibody responses have developed but before arthritis onset (pre-arthritis) and after antibody responses and arthritis have developed (post-arthritis). The objectives for each time point are:

1) Determine if the epicutaneous delivery of HomoCitJED and CitJED in HA-PE cream reduced joint swelling.
2) Characterize HomoCitJED and CitJED T cell and B cells responses in mice epicutaneously treated with HomoCitJED and CitJED infused HA-PE cream.

Chapter 2

2 Materials and Methods

2.1 Antigens

The antigens used in this study were synthetic cyclic peptides containing 18 amino acids with 9 residues of either citrulline (CitJED) or homocitrulline (HomoCitJED) on the same peptide backbone (86,91,92,175). Both CitJED and HomoCitJED were synthetized by Creative Peptides (Shirely, NY, USA).

2.2 Mice and Immunizations

Male and female C57BL/6 mice transgenic for the human HLA-DRβ1*0401 allele and deficient in endogenous MHC class II molecules (termed DR4tg mice) (33,110) were used in this study. Briefly, these mice express a chimeric MHC class II molecule which contains the antigen binding domain of the human HLA-DRα and HLA-DRβ1*0401 genes (110). The remaining domains are composed of the mouse IE-α and IE-β genes, which bare high homology with the HLA-DR MHC class II molecule (110). The Shared Epitope is located within the antigen binding domain of the HLA-DRβ1*0401 allele (25), and is therefore expressed in DR4tg mice. These mice were bred and maintained in a pathogen-free Animal Care and Veterinary Services barrier facility at the University of Western Ontario according to the Canadian Council of Animal Care guidelines. This study was approved by the Animal Care and Use Committee (The University of Western Ontario, London, ON). Male and female DR4tg mice between 8-12 weeks of age were subcutaneously (s.c.) immunized and boosted 21 days later with 100 μg of HomoCitJED suspended in PBS, as described previously by Lac *et al*. (91).

2.3 Arthritis Induction

HomoCitJED-immunized DR4tg mice received intra-articular (i.a.) injections of 25 μg of HomoCitJED suspended in PBS into the left knee joint on days 75, 82 and 89 post-immunization. The contralateral knee received an i.a. injection of PBS as a control. I.a injections of CitFib were previously shown to induce arthritis in naïve DR4tg mice that

received CitFib activated splenocytes (33). Since DR4tg mice do not develop spontaneous swelling after immunization with HomoCitJED, i.a. injections of HomoCitJED into the knee joint are necessary to induce joint swelling in HomoCitJED-immunized DR4tg mice (112). Day 75 was chosen for the beginning of i.a. injections since anti-HomoCitJED antibodies levels have previously been shown peak at day 70 post-immunization (91) and it is thought that immune complexes contribute to RA pathogenesis within the joint (81).

2.4 Cream Treatment

Hyaluronan-Phosphatidylethanolamine (HA-PE) cream (163) was generously provided by Dr. Eva Turley. On day of cream treatment, 25 μg of HomoCitJED and 25 μg of CitJED, dissolved in PBS, were mixed with 0.18 g of HA-PE cream (referred to as **peptide-infused cream**). Control mice received HA-PE cream mixed with a volume of PBS equal to that of the peptides (referred to as **PBS-infused cream**). Studies have shown that the topical application of protein/peptide doses between 10-50 μg on mouse models for autoimmune diseases, including rheumatoid arthritis, are capable of inducing immune tolerance and preventing disease onset and progression (164,167). Since two different peptides were to be used, equal amounts of each peptide for a total peptide dose of 50 μg were added to each mouse for each cream application. The volume of cream was determined based on preliminary trials. Twenty-four hours before the first cream application, a 1-inch by 1-inch circle was shaved onto the back of the mice using an electric razor, and depilatory cream was applied to remove remaining fur (Figure 2.1). On day of cream treatment, tape was used to remove the stratum corneum of the skin before application of the cream to both the shaved area on the back and the ears of each mouse. Strid *et al*. demonstrated that removal of the stratum corneum can alter Langerhans cell morphology and encourage anti-inflammatory Th2 responses (158), and previous studies have shown that the application of proteins/peptides to either the ears or the back of mice can prevent or stop disease activity (164,166,167). Mice received cream treatment at one of two timepoints: 1) before i.a. injections, starting at day 54 post-immunization (pre-arthritis onset), representing RA patients that have circulating antibodies but do not have disease symptoms, or 2) after i.a. injections, starting at day 100 post-immunization (post-arthritis onset), representing pre-RA patients that have both circulating antibodies and disease symptoms. Cream application

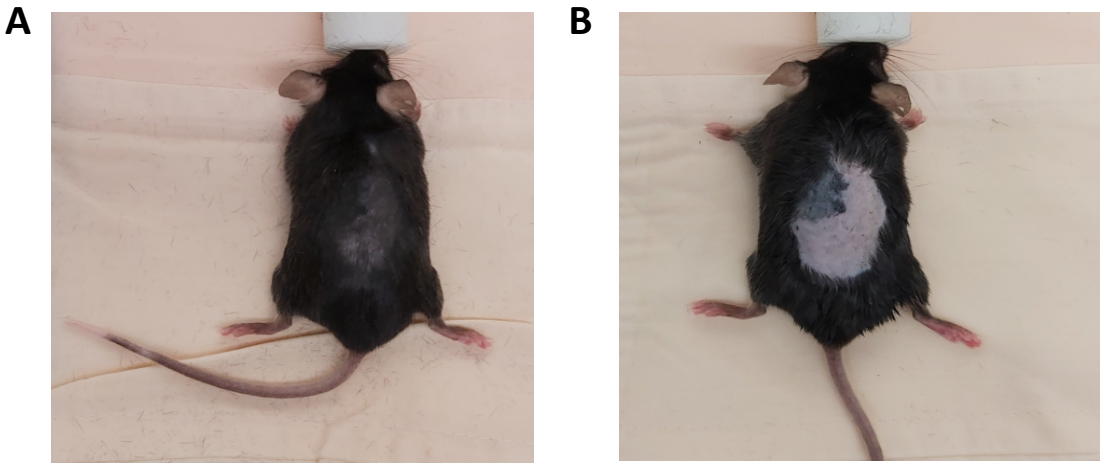

Figure 2.1: Preparation of a DR4tg mouse for HA-PE cream treatment. Twenty-four hours before initial cream treatment, HomoCitJED-immunized DR4tg mice were anesthetized with isoflurane. Using an electric razor, an approximately 1-inch by 1-inch circle was shaved onto the back (A). Afterwards, a depilatory cream was applied to the newly shaved area and removed after 20 seconds to remove remaining fur (B). The next day, tape was used to remove the stratum corneum from the ears and shaved backs, and peptide-infused or PBS-infused cream was applied and massaged into the skin until absorbed.

occurred for three consecutive days and once more two weeks after initial cream treatment, and was adapted from Strid *et al*. (167) (see Figure 2.2 for experimental timeline).

2.5 Joint Swelling Measurements

Swelling of the knee joints were monitored using digital calipers. A cut off of 0.2 mm change in knee width was used to indicate knee joint swelling and was calculated by adding 2 standard deviation (SD) to the average change in knee width from PBS i.a. injections in PBS-immunized DR4tg mice. Measurements were taken immediately prior to i.a. injection and three consecutive days thereafter until day 92 post-immunization for both pre- and post-arthritis cream treated mice. For mice that received cream treatment before i.a. injections (pre-arthritis), knee swelling was also monitored weekly starting at day 96 post-immunization, to determine if the peptide-infused HA-PE cream had an effect on knee swelling over time. For mice that received cream treatment after i.a. injections (post-arthritis), knee swelling was also measured on day of cream treatment (starting on day 100 post-immunization) plus three times a week until sacrifice, in order to monitor the effects of peptide-infused HA-PE cream.

2.6 Splenocyte Proliferation

Splenocyte proliferation was performed as previously described by Lac *et al*. (91). In brief, the spleens from cream-treated mice were aseptically harvested on day of sacrifice (day 137 post-immunization). Splenocytes were cultured in complete RPMI media (Gibco) at a concentration of 4 x 10^5 splenocytes/well and treated with 100 μg/mL of HomoCitJED, 100 μg/mL of CitJED, 0.5 μg/mL of Concanavalin A (ConA) or 10 ng/mL of phorbol myristate acetate (PMA) plus 100 ng/mL of ionomycin. Splenocytes cultured in media alone were used as a control. After 54 hours at 37°C and 5% CO_2, 100 μL of media was removed from each well and replaced with 100 μL of fresh media containing 1 μCi of ^{3}H-thymidine and incubated for an additional 18 hours. After the 18-hour incubation, splenocytes were harvested using a Harvester96 (Tomtec) and radioactivity was measured in counts per minutes (cpm) using a MicroBeta JET (Perkin Elmer). Proliferative responses to each antigen were performed in sextuplet. The six replicates were averaged and reported

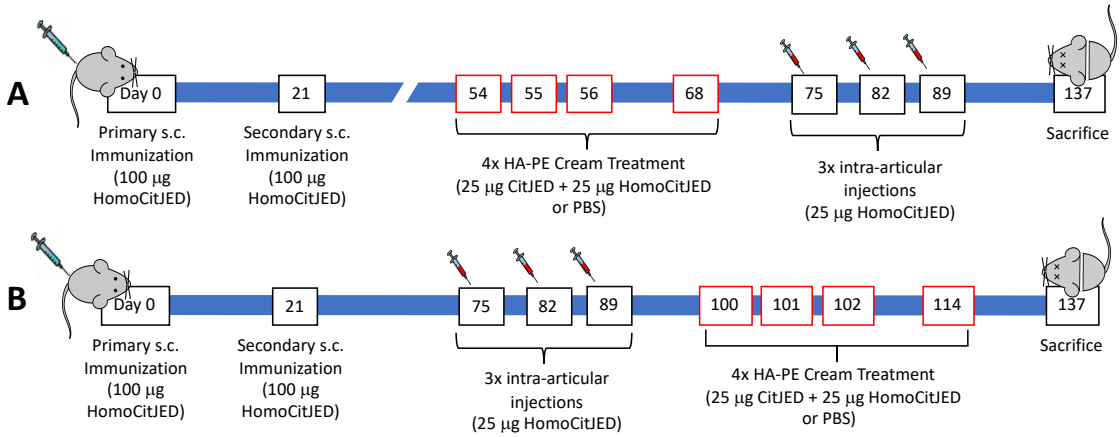

Figure 2.2: Schematic of experimental timeline. DR4tg mice were s.c. immunized and boosted 21 days later with HomoCitJED. On days 75, 82 and 89 post-immunization, all mice received intra-articular injections of HomoCitJED. Mice received either HomoCitJED + CitJED (peptide-infused) or PBS-infused HA-PE cream on (A) days 54, 55, 56 and 68 post-immunization or on (B) days 100, 101, 102 and 114 post-immunization. Mice were sacrificed on day 137 post immunization. s.c.= subcutaneous; HA-PE= Hyaluronan-Phosphatidylethanolamine.

as a Stimulation Index (SI; [cpm of samples with antigen – cpm of media only]/[cpm of samples with no antigen – cpm of media only]). A SI > 2.0 was used as a cut-off value for a positive proliferative response towards an antigen, since it is 2 SD above the average SI taken from wells containing media + cells from PBS-immunized DR4tg mice.

If a mouse did not have an SI > 5 to positive controls (ConA and/or PMA + ionomycin), the mouse was removed from the data. A total of 5 mice that received cream treatment post-arthritis onset (3 PBS-infused cream treated, 2 peptide-infused cream treated) were removed due to SI < 5 to positive controls. Additionally, mice were removed from the experiment if the average cpm from the media + cells and/or media only wells were not within +/- 2 SD of an average taken from previous experiments. A total of 3 mice that received cream treatment post-arthritis onset (2 PBS-infused cream treated, 1 peptide-infused cream treated) and 1 mouse that received cream treatment pre-arthritis onset (PBS-infused cream treated) were removed from the experiment due to the average cpm of the media + cells or media only wells not being within +/- 2 SD of an average taken from previous experiments.

2.7 Antibody Assays

Sera were collected from all mice at several timepoints and were screened for IgG, IgG1 and IgG2b anti-HomoCitJED antibodies and IgG anti-CitJED antibodies by direct antibody-binding ELISA. These ELISAs were performed at least in duplicate, with an intra-assay variation <20%. However, due to a lack of sera, intra-assay variation for eight sera samples were <40%, specifically for the anti-HomoCitJED IgG1 and IgG2b assays. Semi-quantitative standard curves were generated using a mouse serum sample known to be highly positive for the antibody being tested. The samples were read at an optical density (OD) of 450 nm and converted to Relative Units (RU)/mL using the generated standard curves.

2.7.1 IgG anti-HomoCitJED and IgG anti-CitJED

HomoCitJED and CitJED were dissolved in 7.5% HCl in sterile distilled water. The 7.5% HCl did not alter the structure or binding properties of CitJED (91). The protocol for IgG anti-HomoCitJED and IgG anti-CitJED direct antibody-binding ELISAs were adapted

from Lac *et al*. (91). Briefly, wells were coated with 20 μg/mL of HomoCitJED or 40 μg/mL of CitJED suspended in carbonate coating buffer and incubated overnight at 4°C. The next day, the wells were blocked with 0.1% bovine serum albumin (BSA) for 30 min at room temperature before adding 100 μl of serum diluted 1:100 to the wells in duplicate. Diluted serum samples were incubated at room temperature for 30 minutes. For antibody detection, 100 μL/well of biotin-conjugated goat anti-mouse IgG antibodies (Jackson; 1:3000 for HomoCitJED or 1:5000 for CitJED) and streptavidin horseradish peroxidase polymer (Abcam; 1:4000) were added and incubated at room temperature for 30 minutes. IgG levels were visualized by adding 100 μL/well of 3,3',5,5'-tetramethylbenzidine (TMB) for 10 minutes before adding 50 μL/well of 1M sulphuric acid. Plates were read at an OD of 450 nm and were converted to RU/mL using standard curve equations for anti-HomoCitJED total IgG ($RU/mL = 168.51*OD_{450}+3.6176$, $R^2=0.9986$) and for anti-CitJED total IgG ($RU/mL = 81.678*OD_{450}+3.8848$, $R^2=0.9630$). Serum samples with an $OD_{450} > 2.0$ were considered saturated and were serially diluted and re-tested before being converted to RU/mL. An OD_{450} of 0.1 was used as a cut-off for antibody positivity since it is the lower detection limit of the assay. Serum samples with an $OD_{450} <0.1$ or RU/mL lower than 20.32 RU/mL (anti-HomoCitJED IgG) or 12.05 RU/mL (anti-CitJED total IgG) were considered negative for the corresponding antibodies.

For mice that received cream treatment pre-arthritis onset, sera were measured for anti-HomoCitJED total IgG antibodies starting at day 42 post-immunization (before cream treatment) and every two weeks thereafter. Sera was not collected before day 42 post-immunization since anti-HomoCitJED antibody levels between days 0 to 100 post-immunization have been previously described in DR4tg mice immunized with HomoCitJED (91) and were expected to remain the same for both peptide-infused and PBS-infused cream treated mice until cream treatment occurred at day 54 post-immunization. Since antibody responses to CitJED can be detected in the DR4tg mice immunized with HomoCitJED (91), sera were screened for anti-CitJED total IgG antibodies. The CitJED antibody response has previously been shown to not be as strong as the HomoCitJED antibody response (91). Therefore, to save sera, only two timepoints were assessed for anti-CitJED total IgG antibodies: day 42 post-immunization (before

cream treatment) and day 84 post-immunization (six weeks later, after cream treatment is completed).

In this study, mice that received cream treatment post-arthritis onset received i.a. injections starting at day 75 and cream treatment starting at day 100 post-immunization. Antibody responses to HomoCitJED and CitJED have been previously described between days 0-100 post-immunization in DR4tg mice that were immunized with HomoCitJED (91). Therefore, for mice that received cream treatment post-arthritis onset, sera were collected starting at day 70 post-immunization and every two weeks thereafter, to determine if i.a. injections and/or cream treatment had an effect on anti-HomoCitJED total IgG responses. For antibody responses to CitJED, only two timepoints were assessed to save sera: day 70 post-immunization (before cream treatment) and day 112 post-immunization (six weeks later). While cream treatment was completed on day 114 post-immunization, day 112 post-immunization was chosen for mice that received cream treatment post-arthritis onset in order to maintain the same timeframe between the days CitJED antibodies were assessed in mice that received cream treatment pre-arthritis onset.

2.7.2 IgG1 and IgG2b anti-HomoCitJED

The ELISA protocol for anti-HomoCitJED IgG1 and IgG2b antibodies was performed as described above, but with the following modification: 100 µL/well of biotin-conjugated goat anti-mouse IgG1 antibodies (Jackson; 1:5000) or biotin-conjugated goat anti-mouse IgG2b antibodies (Jackson; 1:5000) were used for detection of anti-HomoCitJED IgG1 and IgG2b antibody levels, respectively. The OD_{450} readings were converted to RU/mL using standard curve equations for anti-HomoCitJED IgG1 (RU/mL = 187.87*OD_{450}-46.352, R^2=0.9857) and IgG2b (RU/mL = 222.66*OD_{450}-18.977, R^2=0.9948) antibodies. Serum samples with antibody levels equal to 0 RU/mL or 0.246 OD_{450} were considered negative for anti-HomoCitJED IgG1 antibodies, since an OD_{450} <0.246 would result in a negative RU/mL value. Serum samples with an OD_{450} <0.1 or an RU/mL lower than 3.29 RU/mL were considered negative for anti-HomoCitJED IgG2b antibodies, since an OD_{450} of 0.1 is the detection limit of the assay.

To save sera, anti-HomoCitJED IgG1 and IgG2b antibody levels were only measured at four timepoints. For mice that received cream treatment pre-arthritis onset, these timepoints were: day 42 (before cream treatment), day 70 (after cream treatment was completed and before i.a. injections), day 98 (after i.a. injections were completed) and day 137 post-immunization (day of sacrifice). For mice that received cream treatment post-arthritis onset, these timepoints were: day 70 (before i.a. injections), day 98 (after i.a. injections and before cream treatment), day 112 (two days before the last cream treatment) and day 137 post-immunization (day of sacrifice).

2.8 Statistical Analysis

GraphPad Prism 9.0 was used to generate graphs and perform statistical analysis. The area under the curve (AUC) was determined from the changes in knee width data collected from each individual mouse. An unpaired t-test was used to compare the AUC values between cream treatments. A one-way ANOVA was used to compare the SI for splenocyte proliferative responses to HomoCitJED and CitJED between cream treatments. A two-way ANOVA with repeated measures was used to compare anti-HomoCitJED total IgG, IgG1 and IgG2b, as well as anti-CitJED total IgG antibody levels between cream treatments at the indicated timepoints. In order to perform the two-way ANOVA with repeated measures, three mice that received cream treatment pre-arthritis onset were removed due to a lack of data at day 42 post-immunization.

Chapter 3

3 Results

3.1 Knee Joint Swelling in Pre- and Post-Arthritis Cream Treated DR4tg Mice

Joint swelling is a clinical symptom of rheumatoid arthritis and is commonly used as a measurement of disease severity (1) in RA patients and in RA mouse models (2). To determine if the application of the peptide-infused cream to mice pre- or post- arthritis onset had an effect on knee joint swelling, changes in knee width were measured using digital calipers. The area under the curve (AUC) was determined based on the change in knee width measured from HomoCitJED i.a. injected knees over the course of the experiment, starting at day 70 post-immunization (five days before the first i.a. injection). For mice that received cream treatment pre-arthritis onset, the mean AUC for PBS-infused cream treated mice was 3.690 cm^2, whereas the mean AUC for peptide-infused cream treated mice was 3.118 cm^2. There was no significant difference between the AUC for mice that received PBS-infused or peptide-infused cream treatments pre-arthritis onset ($p=0.3320$; Figure 3.1A). The peak change in knee width (occurring between days 90 and 131 post-immunization; after cream treatment and i.a. injections were completed) for mice that received PBS-infused cream treatment pre-arthritis onset ranged from 0.26 - 0.49 mm, whereas the peptide-infused cream treated mice had a peak change in knee width range of 0.18 - 0.41 mm (Appendix 1). A cut off of a 0.2 mm change in knee width was used as an indicator of swelling. Between days 90-131 post-immunization (after pre-arthritis cream treatment and i.a. injections were completed), 8/8 PBS-infused cream treated mice displayed a change in knee width > 0.2 mm at least once (Appendix 1). For mice that received peptide-infused cream treatment pre-arthritis onset, 6/8 mice displayed a change in knee width > 0.2mm at least once between days 90-131 post-immunization (Appendix 1).

For mice that received cream treatment post-arthritis onset, there was no significant difference in the change in knee width between PBS-infused (mean AUC = 5.317 cm^2) or peptide-infused (mean AUC = 5.788 cm^2) cream treated mice over the course of the

experiment (p=0.2800; Figure 3.1B). Mice that received PBS-infused cream treatment post-arthritis onset displayed a peak change in knee width range of 0.16 – 0.32 mm after the completion of cream treatment (i.e. after day 114 post-immunization). In comparison, the peptide-infused cream treated mice displayed a peak change in knee width range of 0.18 – 0.31 mm (Appendix 1). Between days 115-134 post-immunization (after i.a. injections and cream treatment were completed), 5/10 PBS-infused cream treated mice had a change in knee width > 0.2 mm on at least one day of measurement (Appendix 1). For mice that received the peptide-infused cream, 9/12 mice had a change in knee width > 0.2 mm at least once between days 90-134 post-immunization (Appendix 1).

All the mice in this study also received a PBS i.a. injection into the contralateral knee. Swelling was not detected in the PBS i.a. injected knee over the duration of the experiment (data not shown). There was also no significant differences in the change in knee width in the HomoCitJED i.a. injected knee between PBS-infused cream treated males and females or peptide-infused cream treated males and females, for both pre- and post-arthritis cream treated mice (data not shown). Overall, these results suggest that the epicutaneous application of the peptide-infused HA-PE cream pre- or post-arthritis does not have a significant effect on knee joint swelling.

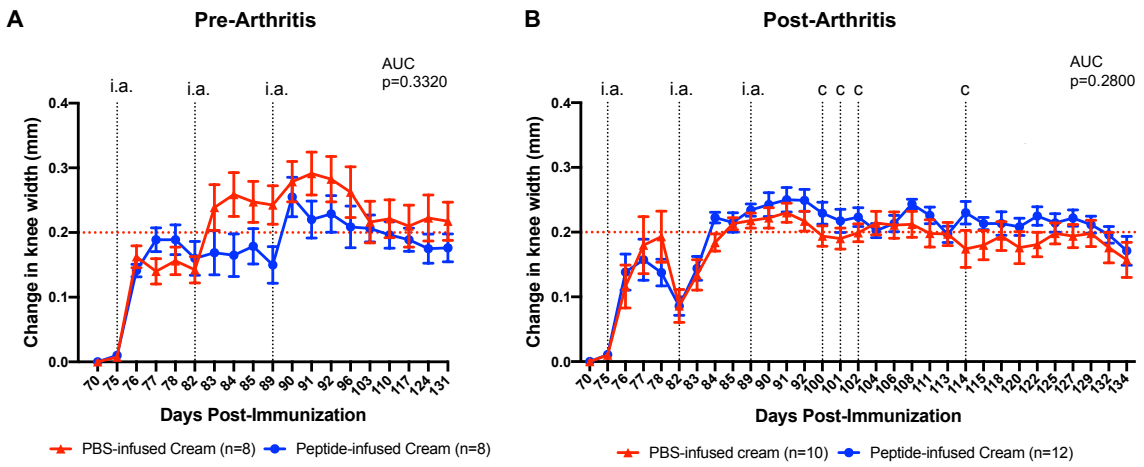

Figure 3.1: Knee joint swelling in DR4tg mice treated with peptide-infused HA-PE cream pre- or post-arthritis onset. HomoCitJED-immunized DR4tg mice were epicutaneously treated with either peptide-infused or PBS-infused HA-PE cream before HomoCitJED i.a. injections (pre-arthritis onset) (A) or after HomoCitJED i.a. injections (post-arthritis onset) (B). Knee joint swelling was measured using digital calipers on the days indicated post-immunization and was reported as mean change in knee width (mm SEM). The vertical black dashed line indicates the day in which i.a. injections or cream treatment occurred. The horizontal red dashed line at 0.2 mm change in knee width represents the cut-off for knee swelling. There were no significant differences between groups. i.a.= intra-articular; c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine; AUC = area under the curve statistical analysis.

3.2 Splenocyte Proliferative Responses to HomoCitJED and CitJED in Pre- and Post-Arthritis Cream Treated DR4tg Mice

The initiation of T cell responses to proteins/peptides containing homocitrulline or citrulline has been implicated in RA pathogenesis (3). It has been previously shown that DR4tg mice immunized with HomoCitJED develop T cell recall responses to both HomoCitJED and CitJED (4). A change in T cell recall responses to either HomoCitJED or CitJED could suggest that the peptide-infused cream treatment was able to induce antigen-specific immune tolerance. To determine if the peptide-infused HA-PE cream had an effect on T cell recall responses, splenocyte proliferative responses to CitJED and HomoCitJED in mice treated with peptide-infused or PBS-infused HA-PE cream treatment either pre- or post-arthritis onset were measured on day of sacrifice (day 137 post-immunization). For mice that received cream treatment pre-arthritis onset, 1/7 and 2/7 PBS-infused cream treated mice had a stimulation index (SI) > 2 for HomoCitJED (mean SI =1.843 SEM 0.4720) and CitJED (mean SI = 1.814 SEM 0.5175), respectively (Figure 3.2A). The one PBS-cream treated mouse that had a SI > 2 for HomoCitJED also had a SI > 2 for CitJED (referred to as mouse 14 or M14; Appendix 2). For peptide-infused cream treated mice, 0/8 and 1/8 mice had a SI > 2 for HomoCitJED (mean SI = 1.213 SEM 0.0833) and CitJED (mean SI = 1.225 SEM 0.1612), respectively (Figure 3.2A). There was no significant difference in proliferative responses to HomoCitJED and CitJED between peptide-infused and PBS-infused cream treated mice (p=0.9523; Figure 3.2A).

For mice that received cream-treatment post-arthritis onset, there was no significant difference in proliferative responses to HomoCitJED and CitJED between peptide-infused and PBS-infused cream treated mice (p=0.4122; Figure 3.2B). Only one mouse (referred to as mouse 19 or M19) out of the five PBS-infused cream treated mice displayed a SI > 2 to HomoCitJED (mean SI = 1.460 SEM 0.2441) and CitJED (mean SI = 1.540 SEM 0.3544), respectively (Figure 3.2B, Appendix 2). For the peptide-infused cream treated mice, 2/9 and 3/9 mice had a SI > 2 for HomoCitJED (mean SI = 1.556 SEM 0.1994) and CitJED (mean SI = 2.256 SEM 0.4522), respectively (Figure 3.2B). The two peptide-infused cream treated mice that had a SI > 2 for HomoCitJED also had a SI >2 for CitJED

(M30 and M38; Appendix 2). These results suggest that the application of the peptide-infused HA-PE cream pre- or post-arthritis onset did not significantly affect splenocyte proliferative responses at day 137 post-immunization.

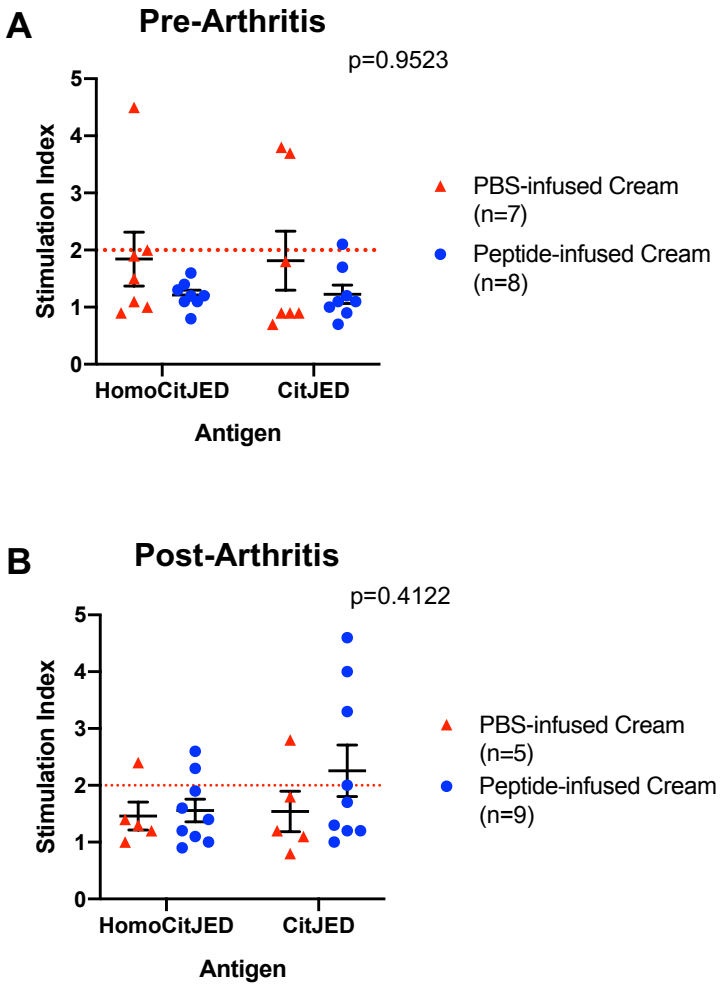

Figure 3.2: Splenocyte proliferative responses in HomoCitJED-immunized DR4tg mice treated with peptide-infused HA-PE cream. On day of sacrifice (day 137 post-immunization), splenocytes from HomoCitJED-immunized DR4tg mice that received cream treatment either pre-arthritis (A) or post-arthritis (B) onset were cultured in the presence of 100 μg of HomoCitJED or CitJED. Splenocyte proliferation was determined using ^{3}H-thymidine incorporation and is shown as a mean stimulation index (SI SEM). Stimulation indices greater than 2.0 (red dashed line) were considered positive proliferative responses. There is no significant differences between groups, determined by two-way ANOVA. Each symbol represents one mouse. HA-PE = Hyaluronan-Phosphatidylethanolamine.

3.3 Antibodies to HomoCitJED and CitJED in Pre-Arthritis Cream Treated DR4tg Mice

The production of antibodies is highly characteristic of rheumatoid arthritis (13,14). Antibodies to HomoCitJED and CitJED have been previously described in RA patients (92) and DR4tg mice immunized with HomoCitJED (91). Modification of HomoCitJED and CitJED antibody responses could indicate changes in pro-inflammatory immune responses due to the peptide-infused cream treatment. Sera from HomoCitJED-immunized DR4tg mice that received PBS-infused or peptide-infused cream treatment pre-arthritis onset (before i.a. injections) were assessed for total IgG antibodies to HomoCitJED via direct ELISA biweekly, starting at day 42 post-immunization. There was no significant difference in anti-HomoCitJED total IgG antibody levels between PBS-infused and peptide-infused cream treated mice ($p=0.3753$; Figure 3.3A). All 7/7 PBS-infused cream treated mice tested positive for anti-HomoCitJED total IgG antibodies for all days post-immunization tested (Figure 3.3A). For mice that received the peptide-infused cream treatment pre-arthritis onset, 6/7 were positive for anti-HomoCitJED total IgG antibodies for all days post-immunization tested (Figure 3.3A). One mouse (M1) tested positive for anti-HomoCitJED total IgG antibodies from days 42-126 post-immunization; however, by day 137 post-immunization HomoCitJED total IgG antibodies were no longer detected (Appendix 3). The average RU/mL for mice that received PBS-infused cream treatment pre-arthritis onset ranged from 1433.39 – 7318.17 RU/mL, with the lowest average seen at day 42 post-immunization and the highest average seen at day 137 post-immunization (Figure 3.3A). Mice that received peptide-infused cream treatment had an average RU/mL that ranged from 495.99 – 2023.79 RU/mL with the lowest average seen on day 42 post-immunization and the highest seen on day 112 post-immunization (Figure 3.3A).

The trends in antibody levels over time also varied by mouse and did not relate to the type of cream treatment the mouse received. For mice that received PBS-infused cream treatment pre-arthritis onset, 3/8 mice had antibody levels increasing over time, 1/8 had decreasing antibody levels over time, and 4/8 had antibody levels that peaked between days 70-112 post-immunization (Appendix 3). For peptide-infused cream treated mice, 2/8

displayed an increasing trend, 2/8 displayed a decreasing trend, and 4/8 had a peak antibody level between days 70-112 post-immunization (Appendix 3).

Since CitJED is included in the peptide-infused cream and DR4tg mice immunized with HomoCitJED have also been shown to develop antibodies towards CitJED (91), sera was screened for anti-CitJED total IgG antibodies to determine if the peptide-infused cream treatment had an effect on CitJED antibody levels, if present. There was no significant difference between anti-CitJED total IgG antibody levels in PBS-infused and peptide-infused cream treated mice pre-arthritis onset ($p=0.1993$; Figure 3.3B). Only 1/7 PBS-infused cream treated mice tested positive for anti-CitJED total IgG antibodies and this mouse only tested positive at day 84 post-immunization (Figure 3.3B). This mouse (referred to as M14) also displayed a positive splenocyte proliferative response to CitJED, with an SI > 2 (Appendix 2). M6 and M16 also displayed a SI >2 to CitJED (Appendix 2); however, these mice were negative for anti-CitJED total IgG antibodies. Mice that received peptide-infused cream treatment all tested negative for anti-CitJED total IgG antibodies at the timepoints indicated (Figure 3.3B).

Previous studies using epicutaneous immunizations of proteins or peptides to treat arthritis in RA mouse models have shown a decrease in pro-inflammatory IgG2a antibodies (166,167). Since the IgG1 and IgG2a/b subclasses are known to play a pivotal role in anti- and pro-inflammatory responses, respectively (176), anti-HomoCitJED IgG1 and IgG2b antibody levels were assessed in mice that received cream treatment pre-arthritis onset. There were no significant differences in anti-HomoCitJED IgG1 antibody levels between peptide-infused and PBS-infused cream treated mice pre-arthritis onset ($p=0.3089$; Figure 3.3C). For PBS-infused and peptide-infused cream treated mice, 6/7 and 5/6 mice were positive for anti-HomoCitJED IgG1 antibodies at all timepoints tested, respectively (Appendix 4). The average RU/mL for mice that received PBS-infused cream treatment ranged from 285.14 – 831.92 RU/mL, with the lowest average seen on day 42 post-immunization and the highest seen on day 137 post-immunization (Figure 3.3C). For mice that received peptide-infused cream treatment pre-arthritis onset, the average RU/mL ranged from 120.85 – 409.22 RU/mL, with the lowest average seen on day 42 post-immunization and the highest average seen on day 98 post-immunization (Figure 3.3C).

The trends in IgG1 antibody levels varied by mouse. For mice that received PBS-infused cream treatment, 3/7 displayed increasing IgG1 antibody levels over time, 3/7 mice peaked at days 70 or 98 post-immunization, and 1/7 mice fluctuated between negative and positive detection of IgG1 antibodies (Appendix 4). None of the PBS-infused cream treated mice displayed a decreasing trend in IgG1 antibody levels. For peptide-infused cream treated mice, 1/6 mice displayed increasing IgG1 antibody levels over time, 2/6 mice displayed decreasing levels over time, and 3/6 mice peaked at either day 70 or day 98 post-immunization (Appendix 4).

There were also no significant differences in anti-HomoCitJED IgG2b antibody levels between PBS-infused and peptide-infused cream treated mice pre-arthritis onset ($p=0.4483$; Figure 3.3D). For PBS-infused and peptide-infused cream treated mice, 6/7 and 2/6 mice were for positive anti-HomoCitJED IgG2b antibodies at all days post-immunization tested, respectively (Appendix 5). The average RU/mL for mice that received PBS-infused cream treatment was 164.51 – 1170.40 RU/mL (Figure 3.3D). The lowest average RU/mL was seen on day 42, whereas the highest average was seen on day 137 post-immunization for PBS-infused cream treated mice (Figure 3.3D). For mice that received the peptide-infused cream treatment, the average RU/mL ranged from 9.83 – 108.80 RU/mL (Figure 3.3D). The lowest average RU/mL for the peptide-infused cream treated mice was also seen at day 42 post-immunization; however, the highest average was seen at day 98 post-immunization, which is earlier than the controls and is similar to IgG1 antibody levels in the peptide-infused cream treated mice (Figure 3.3C,D). While not significant, anti-HomoCitJED IgG2b antibody levels were lower compared to controls at all time points tested, including before cream treatment occurred (Figure 3.3D).

As with HomoCitJED total IgG and IgG1, the trend for IgG2b antibody levels varied by mouse. For PBS-infused cream treated mice, 4/7 displayed increasing IgG2b antibody levels over time and 3/7 displayed a decreasing trend in IgG2b antibody levels over time (Appendix 5). For the peptide-infused cream treated mice, 4/6 mice displayed an IgG2b antibody trend where antibody levels peaked at days 70 or 98 post-immunization (Appendix 5). The remaining two peptide-infused cream treated mice were negative for all time points tested.

Examination of each individual mouse showed that there was no relationship between anti-HomoCitJED total IgG, IgG1 and IgG2b antibody levels, the amount of knee swelling measured and splenocyte proliferative responses to HomoCitJED. For example, peptide-infused cream treated mouse 7 (M7) showed an increasing total IgG trend, resulting in the highest anti-HomoCitJED total IgG antibody level in this study (66,094 RU/mL at day 137 post-immunization) (Appendix 3); however, knee swelling for M7 decreased over time after the last i.a. injection (Appendix 1). Additionally, a positive splenocyte proliferative response to HomoCitJED did not relate to higher anti-HomoCitJED total IgG antibody levels at day 137 post-immunization or greater amounts of knee swelling. Anti-HomoCitJED IgG1 and IgG2b antibody levels also did not relate to any of the other outcomes measured in this study. The change in anti-HomoCitJED total IgG, IgG1 and IgG2b antibody levels were also calculated before and after cream treatment, however there were no significant differences between PBS-infused and peptide-infused cream treatments (data not shown). Additionally, there was no relationship between IgG1 and IgG2b antibody levels (i.e. some mice had increasing antibody levels of both IgG1 and IgG2b, some had decreasing of both, and some had fluctuating antibody levels over time) (Appendix 5, 6). There were also no significant differences between male and female mice of the same treatment for all antibodies tested (data not shown). Overall, these data suggests that the application of the peptide-infused cream pre-arthritis onset does not have an effect on anti-HomoCitJED total IgG, IgG1 and IgG2b antibody levels.

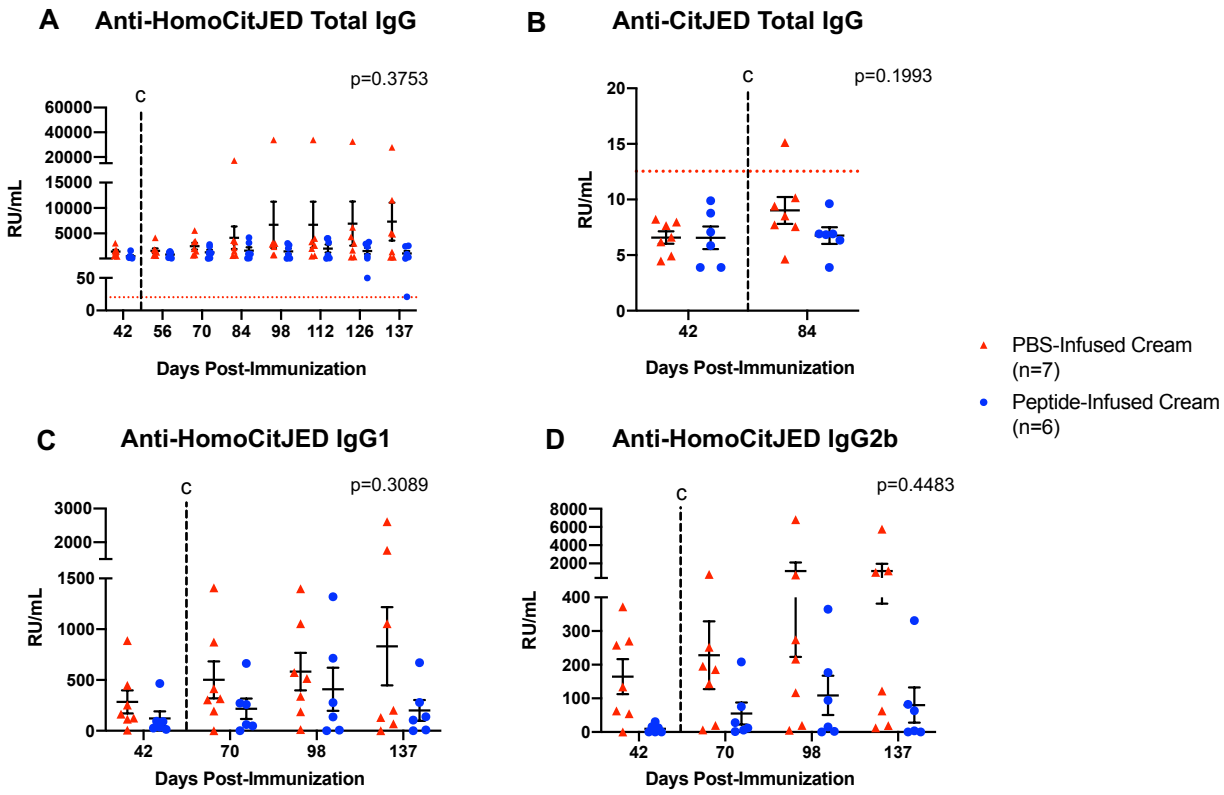

Figure 3.3: Antibody responses in HomoCitJED-immunized DR4tg mice treated with peptide-infused HA-PE cream pre-arthritis onset. Sera were collected starting at day 42 post-immunization in HomoCitJED-immunized DR4tg mice that received either peptide-infused or PBS-infused HA-PE cream treatment pre-arthritis onset. Sera were measured for anti-HomoCitJED total IgG (A), anti-CitJED total IgG (B), anti-HomoCitJED IgG1 (C) and anti-HomoCitJED IgG2b (D) antibodies by direct ELISA at the time points indicated post-immunization. Values are displayed as mean RU (Relative Units)/mL SEM. The vertical black dashed line indicates the beginning of cream treatment (day 54 post-immunization). RU/mL values above the cut off for positivity are indicated by the horizontal red dashed line. The RU/mL cut off values were 20.32 for anti-HomoCitJED total IgG, 12.05 for anti-CitJED, 0.00 for anti-HomoCitJED IgG1 and 3.29 for anti-HomoCitJED IgG2b. There were no significant differences in antibody levels between cream treatments, determined by two-way ANOVA with repeated measures. Each symbol represents one individual mouse. c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine.

3.4 Antibodies to HomoCitJED and CitJED in Post-Arthritis Cream Treated DR4tg Mice

Sera from mice that received cream treatment post-arthritis onset were measured for anti-HomoCitJED total IgG to determine if peptide-infused cream treatment after i.a. injections could modify antibody levels. There were no significant differences in anti-HomoCitJED total IgG antibody levels between mice that received PBS-infused and peptide-infused cream treatment post-arthritis onset ($p=0.3427$; Figure 3.4A). For PBS-infused cream treated mice, 9/10 mice were positive for anti-HomoCitJED total IgG antibodies for all days post-immunization tested (Figure 3.4A, Appendix 3). For peptide-infused cream treated mice, all 12/12 mice were positive for anti-HomoCitJED total IgG antibodies for all days post-immunization tested (Figure 3.4A, Appendix 3). For PBS-infused cream treated mice, the average RU/mL ranged from 3176.98 – 6189.87 RU/mL, with the lowest average seen on day 70 and the highest seen on day 137 post-immunization (Figure 3.4A). Mice that received peptide-infused cream treatment post-arthritis onset displayed an average RU/mL range of 1741.38 – 2192.81 RU/mL for anti-HomoCitJED total IgG antibodies (Figure 3.4A). The lowest average was seen on day 126 post-immunization, while the highest average was seen on day 98 post-immunization (Figure 3.4A).

The trends in anti-HomoCitJED total IgG antibody levels displayed by each mouse varied. Increasing total IgG antibody levels over time were seen in 2/10 PBS-infused cream treated mice, while 6/10 PBS-infused cream treated mice displayed decreasing total IgG antibody levels over the course of the experiment, and 2/10 PBS-infused cream treated mice displayed increasing antibody levels which peaked at day 98 or 112 post-immunization before decreasing (Appendix 3). For peptide-infused cream treated mice, 3/13 mice displayed increasing anti-HomoCitJED total IgG antibody levels over time, 7/12 displayed decreasing total IgG antibody levels over time, and 2/12 mice peaked at day 112 or 126 post-immunization before decreasing until day of sacrifice (Appendix 3).

Sera were also screened for anti-CitJED total IgG antibodies in PBS-infused and peptide-infused cream treated mice post-arthritis onset. There was no significant difference in anti-CitJED total IgG antibody levels between PBS-infused and peptide-infused cream treated mice ($p=0.0511$, Figure 3.4B). Only 1/10 PBS-infused cream treated mice were positive

for anti-CitJED total IgG antibodies at day 70 post-immunization (Figure 3.4B). By day 112 post-immunization, the mouse no longer displayed detectable CitJED total IgG antibodies (Figure 3.4B). Out of 12 peptide-infused cream treated mice, zero mice tested positive for anti-CitJED total IgG antibodies on day 112 post-immunization (Figure 3.4B). Mice that displayed splenocyte proliferative responses to CitJED > 2 SI were negative for anti-CitJED total IgG antibodies at both timepoints tested (Appendix 2, 3)

Anti-HomoCitJED IgG1 and IgG2b antibody responses were also assessed in mice that received PBS-infused or peptide-infused cream treatment post-arthritis onset. There were no significant differences in anti-HomoCitJED IgG1 antibody levels between PBS-infused and peptide-infused cream treated mice ($p=0.6452$; Figure 3.4C). For mice that received PBS-infused cream treatment, 8/10 mice were positive for IgG1 antibodies at all days post-immunization assessed (Figure 3.4C, Appendix 4). There was one mouse (M33) which was negative for all antibodies tested (total IgG, IgG1 and IgG2b). There was one PBS-infused cream treated mouse (M21) that was positive for IgG1 antibodies at day 42 post-immunization but was negative for IgG1 antibodies by day 137 post-immunization (Appendix 4). For peptide-infused cream treated mice, 9/12 mice were positive for anti-HomoCitJED IgG1 antibodies at all timepoints tested (Figure 3.4C, Appendix 4). The remaining three peptide-infused cream treated mice were positive for IgG1 antibodies on day 42 post-immunization but decreased over time, so that IgG1 antibodies were no longer detected on day 137 post-immunization (Appendix 4). The average RU/mL for IgG1 antibodies ranged from 661.58 – 1246.83 RU/mL for mice that received PBS-infused cream treatment post-arthritis onset (Figure 3.4C). The lowest average was seen on day 70 post-immunization, whereas the highest average was seen on day 137 post-immunization (Figure 3.4C). Mice that received peptide-infused cream had an average IgG1 RU/mL range of 317.98 – 433.83 RU/mL, where the lowest average was seen on day 137 post-immunization, and the highest average was seen on day 112 post-immunization (Figure 3.4C).

Trends in anti-HomoCitJED IgG1 antibody levels varied by mouse. Excluding the one PBS-infused cream treated mouse that was always negative (M33), 1/9 PBS-infused cream treated mice displayed an increase in IgG1 antibody levels over time, 5/9 mice displayed a

decreasing trend and 3/9 mice had antibody levels peak at day 98 or 112 post-immunization (Appendix 4). For peptide-infused cream treated mice, 3/12 mice displayed an increase in IgG1 antibody levels over time, 5/12 mice displayed a decrease in IgG1 antibody levels over time, and 4/12 mice peaked on day 98 or 112 post-immunization (Appendix 4).

There were also no significant differences in anti-HomoCitJED IgG2b antibody levels between PBS-infused and peptide-infused cream treated mice post-arthritis onset ($p=0.2073$; Figure 3.4D). There were 8/10 PBS-infused and 10/12 peptide-infused cream treated mice that were positive for anti-HomoCitJED IgG2b antibodies at all timepoints assessed (Appendix 5). Mice that received PBS-infused cream displayed an average RU/mL range of 159.60 – 618.77 RU/mL, with the lowest average seen on day 70 post-immunization and the highest average seen on day 137 post-immunization (Figure 3.4D). For mice that received peptide-infused cream, the average RU/mL ranged from 149.63 – 202.04 RU/mL (Figure 3.4D). Unlike the controls, peptide-infused cream treated mice displayed the lowest average RU/mL on day 137 post-immunization and the highest average RU/mL on day 70 post-immunization (Figure 3.4D).

As with the HomoCitJED total IgG and IgG1 antibodies, the trend in IgG2b HomoCitJED antibody levels over time varied by mouse. Mice that received PBS-infused cream treatment displayed an increasing trend in IgG2b antibody levels in 2/9 mice, a decreasing trend in antibody levels in 4/9 mice, and a peak in antibody levels at day 98 or 112 post-immunization in 3/9 mice (Appendix 5). For the peptide-infused cream treatment, 3/11 mice displayed increasing IgG2b antibody levels over time, 6/11 mice displayed decreasing IgG2b antibody levels over time, and 2/11 mice had the antibody levels peak at day 98 or 112 post-immunization (Appendix 5).

Examination of each individual mouse showed that there was no relationship between anti-HomoCitJED total IgG, IgG1, IgG2b antibody levels, the amount of knee swelling that occurred, and splenocyte proliferative responses to HomoCitJED at day 137 post-immunization. The lack of a relationship between antibody levels and knee swelling is highlighted by M33, which was negative for all antibodies tested, however displayed a change in knee width > 0.2 mm several times throughout the course of the experiment

(Appendix 1, 3-5). Mice that had HomoCitJED splenocyte proliferative responses with a SI > 2 did not show any relationship to the antibody data (i.e. lower or higher antibody levels at day 137 post-immunization). The change in antibody levels before and after cream treatment were also examined; however, there was no significant difference between PBS-infused and peptide-infused cream treatments (data not shown). Additionally, there was no significant differences between male and female mice of the same treatment for all antibodies tested (data not shown). Overall, these data suggest that the epicutaneous application of the peptide-infused cream post-arthritis onset did not have an effect on anti-HomoCitJED total IgG, IgG1 or IgG2b antibody levels.

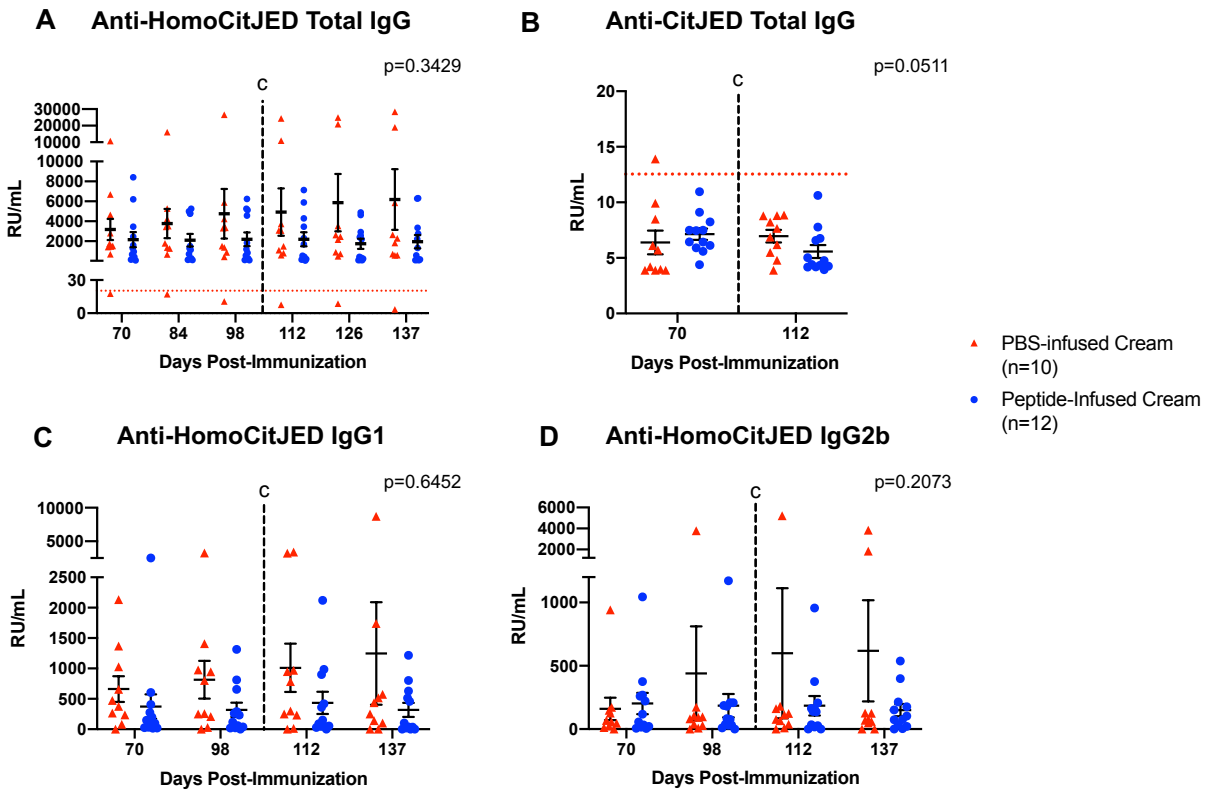

Figure 3.4: Antibody responses in HomoCitJED-immunized DR4tg mice treated with peptide-infused HA-PE cream post-arthritis onset. Sera were collected starting at day 70 post-immunization in HomoCitJED-immunized DR4tg mice that received either peptide-infused or PBS-infused HA-PE cream treatment post-arthritis onset. Sera were measured for anti-HomoCitJED total IgG (A), anti-CitJED total IgG (B), anti-HomoCitJED IgG1 (C) and anti-HomoCitJED IgG2b (D) antibodies by direct ELISA at the time points indicated post-immunization. Values are displayed as mean RU (Relative Units)/mL. The vertical black dashed line represents the beginning of cream treatment (day 100 post-immunization). RU/mL values above the cut off for positivity are indicated by the red dashed line. The RU/mL cut off values were 20.32 for anti-HomoCitJED total IgG, 12.05 for anti-CitJED and 0.00 for anti-HomoCitJED IgG1 and 3.29 for anti-HomoCitJED IgG2b. There were no significant differences in antibody levels between cream treatments, determined by two-way ANOVA with repeated measures. Each symbol represents one individual mouse. c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine.

Chapter 4

4 Discussion

4.1 Summary

Immune responses to citrulline and homocitrulline amino acids are specific to RA (13,14,86,87). However, current treatments involve immunosuppressants that lack specificity to immune responses involved in RA pathogenesis (18). The development of a cream infused with HomoCitJED and CitJED peptides could be used as a potential treatment for RA, as it could induce immune tolerance towards the homocitrulline and citrulline amino acids and modify RA-specific immune responses. However, the major findings of this study were that the epicutaneous application of the peptide-infused HA-PE cream to HomoCitJED-immunized DR4tg mice either pre- or post-arthritis onset did not have an effect on joint swelling or HomoCitJED- and CitJED-specific T cell and B cell responses when compared to controls.

4.2 Joint Swelling in RA Mouse Models

This study monitored the changes in knee swelling in HomoCitJED immunized DR4tg mice that received peptide-infused HA-PE cream treatment either pre- or post-arthritis onset. Swelling of the joints is a common hallmark of RA (3,4). As such, mouse models for RA also experience joint swelling, and the degree of swelling is used in the scoring of arthritis severity in mice (93). Previous studies have shown that epicutaneous immunization with type II collagen before or after arthritis induction in the CIA mouse model for RA reduces joint swelling and arthritis severity (165–167). Our group has also shown that epicutaneous application of the peptide-infused HA-PE cream can prevent joint swelling when applied before HomoCitJED immunization and i.a. injections, thereby demonstrating that this novel peptide-infused cream treatment has the ability to reduce joint swelling (177). However, in this study, the epicutaneous application of the peptide-infused HA-PE cream after HomoCitJED immunizations did not reduce knee joint swelling when compared to mice that received PBS-infused HA-PE cream treatment.

There are several explanations as to why the peptide-infused cream did not have an effect on knee joint swelling. Studies in CIA mice have shown that inducing immune tolerance using type II collagen, either by epicutaneous immunizations or injection of antigen-pulsed tolerogenic dendritic cells, reduces not only joint swelling and arthritis severity, but also T cell responses to type II collagen (165–167,171). Our group has previously suggested that arthritis development and joint swelling in DR4tg mice may be mediated by T cell responses, since a reduction in arthritis severity was accompanied by a reduction in T cell, but not B cell responses (33,178). In this study, there were no significant differences in T cell responses to HomoCitJED or CitJED between cream treatments; thus, a lack in differences in T cell responses between cream treatments could explain why there was no difference in knee swelling between peptide-infused and PBS-infused cream treated mice.

Furthermore, it is currently unclear whether antibody responses in HomoCitJED-immunized DR4tg mice contribute to knee joint swelling. In the CIA mouse model, immunization with native and citrullinated type II collagen results in antibodies and inflammatory arthritis (100,179). When CIA mice receive epicutaneous immunizations with type II collagen, a reduction in arthritis severity and joint swelling was accompanied by a reduction in anti-type II collagen antibodies (166,167). This is an agreement with the preliminary study performed by our group, which saw a reduction in anti-HomoCitJED IgG antibodies along with the prevention of knee joint swelling in DR4tg mice that received the peptide-infused cream treatment prior to HomoCitJED immunizations (177). However, a reduction in both antibody levels and knee joint swelling were not observed in this study. More specifically, knee joint swelling was shown to be independent of anti-HomoCitJED total IgG responses in this study. While this is an unexpected observation for this study, it is not surprising, as other studies have also demonstrated that a reduction in joint swelling is independent of antibody responses. Injection of type II collagen pulsed tolerogenic dendritic cells into CIA mice after arthritis induction reduced arthritis severity but did not alter anti-type II collagen antibody levels (171). In DR4tg mice immunized with human citrullinated fibrinogen (CitFib), treatment with CTLA-4Ig, a soluble fusion protein that limits T cell activation, reduced arthritis severity and primary T cell responses, but anti-CitFib antibody levels remained unchanged (178).

While it is possible that knee joint swelling was not affected by the cream treatment because it did not modify anti-HomoCitJED antibody levels, it is also possible that antibody responses do not influence knee swelling at all. Knee swelling occurs due to pro-inflammatory signals which result in leukocyte infiltration, macrophage activation and synovial hyperplasia (4); the formation of antibody immune complexes is just one mechanism in which pro-inflammatory signals are initiated/propagated in RA (4). An improvement in arthritis severity without a change in antibody levels in response to a treatment could be due to a decrease in pro-inflammatory cytokines and/or an increase in anti-inflammatory cytokines. Nonetheless, studies that examine the pathogenicity of anti-HomoCitJED antibodies in DR4tg mice are needed to further understand the mechanism of knee joint swelling and the overall efficacy of the peptide-infused cream treatment.

An issue with peptide concentration could also explain why knee joint swelling was not affected by the peptide-infused cream treatment either pre- or post-arthritis onset in HomoCitJED-immunized DR4tg mice. A study published by Bynoe *et al*. demonstrated that the epicutaneous immunization of an autoantigenic peptide to a mouse model for multiple sclerosis prior to disease induction can reduce disease severity; however only at a specific peptide concentration (164). Peptide concentrations ranging between 0.1 μg to 1 mg were tested (164). Interestingly, Bynoe *et al*. found that 10 μg was the most effective at reducing disease severity (164). This is just one of many studies that have demonstrated the importance of protein/peptide concentration when inducing mucosal or cutaneous tolerance as a treatment method for inflammatory arthritis (148,149,166).

In this current study, 25 μg of HomoCitJED and 25 μg of CitJED were added to the HA-PE cream, for a total of 50 μg of peptide epicutaneously applied on each day of treatment. Our study is unique in that both HomoCitJED and CitJED peptides were used, since RA patients and HomoCitJED-immunized DR4tg mice display immune responses to both HomoCitJED and CitJED peptides (91,92). Although our preliminary study demonstrated that the application of the peptide-infused cream before HomoCitJED immunization prevented knee swelling in DR4tg mice (177), it is possible that higher or lower concentrations of the peptides are needed in order to modify the pre-existing immune responses caused by the HomoCitJED immunizations, as seen in this study. A study

performed by Marcińska *et al*. demonstrated that epicutaneous immunization with type II collagen after CIA induction in mice reduced arthritis severity; however, 100 μg of type II collagen protein was used (166). Gertel *et al*. injected 300 μg of a multiepitope citrullinated peptide into a rat model for RA and saw a reduction in arthritis severity (168). Our treatment procedure was adapted from Strid *et al*, who also used a dosage of 50 μg, but instead used type II collagen protein (167). The variation in dosage between these studies suggests that optimization is required in order to effectively induce immune tolerance and treat experimental arthritis using the peptide-infused HA-PE cream.

An unexpected observation of this study is the degree of knee swelling measured in the PBS-infused cream treated mice. Each individual mouse received an i.a. injection of HomoCitJED into one knee and a PBS injection into the contralateral knee. Knee swelling did not occur in the PBS injected knee, indicating that the swelling observed in the HomoCitJED injected knee was not due to the injection itself, but rather due to HomoCitJED-specific immune responses. However, the knee swelling measured on the HomoCitJED injected knee in PBS-infused cream treated mice on average did not reach the swelling cut off. Our group has previously shown that DR4tg mice immunized with HomoCitJED can reach a change in knee width between 0.4-0.8 mm in the HomoCitJED i.a. injected knee, well above the 0.2 mm cut off (112). In our preliminary study, where peptide-infused and PBS-infused cream was applied to DR4tg mice before HomoCitJED immunization and i.a. injections, the mean knee swelling in mice that received PBS-infused cream ranged from 0.3-0.5 mm after the last HomoCitJED i.a. injection and until the end of the experiment (177). The knee swelling of the PBS-infused cream treated mice in this study was therefore expected to reach the same change in knee width as observed in our preliminary study. It is unclear why the PBS-infused cream treated mice in this study did not demonstrate a change in knee width as previously observed. However, it should be noted that this study is the first to measure knee joint swelling when the PBS-infused cream is applied starting at day 54 or day 100 post-immunization, and thus it is possible that the HA-PE cream itself could have an effect on knee joint swelling. Nonetheless, the lack of swelling in the controls makes interpreting the efficacy of the peptide-infused cream treatment on knee joint swelling difficult. It is possible, however, that the peptide-infused

cream was able to modify or prevent the development of pathological features of RA within the joint (such as leukocyte infiltration or cartilage damage). Assessing arthritis severity by histopathological examination would provide more insight regarding the effectiveness of the peptide-infused cream treatment compared to controls.

4.3 T Cell Responses in Epicutaneous Immunized Mice

Autoreactive T cells play an important role in RA pathogenesis (27). In this study, splenocyte proliferative responses to HomoCitJED and CitJED were measured on day 137 post-immunization in mice that received cream pre- or post- arthritis onset, to determine if cream treatment was able to modify HomoCitJED- and CitJED-specific T cell responses. In studies performed by various groups, a decrease in T cell responses to type II collagen was observed on day of sacrifice in CIA mice that were epicutaneously immunized with type II collagen before and after arthritis induction (166,167). In this current study, the application of the peptide-infused cream pre- or post-arthritis onset did not have a significant effect on splenocyte proliferative responses to HomoCitJED or CitJED when compared to PBS-infused cream treated mice. While this contradicts what has been previously seen in the CIA model, it is in agreement with what our group has previously described in our preliminary study, where the peptide-infused cream was applied prior to HomoCitJED immunizations and i.a. injections in DR4tg mice (177).

T cell responses to HomoCitJED and CitJED in HomoCitJED immunized DR4tg mice have previously been characterized by our group. Lac *et al*. found that T cell responses to HomoCitJED peaked between day 70 and day 100 post-immunization, while T cell responses to CitJED peaked at day 100 post-immunization (91). It is possible that changes to HomoCitJED- and CitJED-specific T cell responses due to the peptide-infused cream occurred earlier on in this experiment and were not detected on day 137 post-immunization. Thus, a limitation to this study is that T cell responses were not assessed at earlier time points. It should also be noted that Lac *et al*. did not assess T cell responses at day 137 post-immunization (91); this is the first time T cell responses to HomoCitJED and CitJED are being reported at this late timepoint in HomoCitJED-immunized DR4tg mice. Therefore, T cell recall responses on day 137 post-immunization to HomoCitJED and CitJED in HomoCitJED-immunized DR4tg mice that did not receive any type of cream

treatment is unknown. Overall, assessment of earlier T cell responses to HomoCitJED and CitJED are required to determine the efficacy of the peptide-infused cream on modifying HomoCitJED- and CitJED-specific T cell responses.

A lack of differences in T cell responses observed at this late time point may also be due to T cell anergy or T cell exhaustion. T cell anergy is a primary mechanism for immune tolerance and is referred to as a state of unresponsiveness; if restimulated with an antigen, a lack of proliferative response would be observed (116,180). In this study, mice that received peptide-infused and PBS-infused cream treatment pre-arthritis onset mostly showed undetectable proliferative responses to both HomoCitJED and CitJED. This could be due to T cell anergy but could also be due to T cell exhaustion. When T cells are exposed to antigens for long periods of times, such as chronic infections or diseases, they can lose their ability to function properly as effector CD4+ or CD8+ T cells (181). This includes a dysregulation in cytokine production (181,182). Eventually, these exhausted antigen-specific T cells undergo physical deletion (181). When restimulated *ex vivo* with an antigen, proliferative responses would not be seen. Thus, T cell anergy and/or exhaustion should be assessed in future experiments to provide more insight regarding T cell responses to HomoCitJED and CitJED at day 137 post-immunization.

In mice that received cream treatment post-arthritis onset, there was no significant differences in T cell responses to HomoCitJED and CitJED between cream treatments. However, 3/9 peptide-infused cream treated mice did show a positive proliferative response to CitJED, while only 1/5 PBS-infused cream treated mice had a positive proliferative response. A limitation to this study is that the sample size for PBS-infused cream treated mice is low. This means that definitive conclusions regarding T cell responses when cream was applied post-arthritis are hard to make with the data presented in this study. It is possible that with a larger sample size for the control mice, a difference in T cell responses to CitJED between cream treatments post-arthritis onset could have occurred.

Another limitation to this study is that T helper cell subsets were not assessed, and thus limits the ability to understand the type of T cell responses that may be occurring in the

peptide-infused cream treated mice. Multiple studies examining the induction of peripheral tolerance as a method of treatment for autoimmune diseases have demonstrated the importance of T cell responses in effective treatments. The induction of T suppressor cells by epicutaneous immunization with type II collagen in CIA mice (166) or an autoantigenic peptide in an experimental allergic encephalitis (EAE) mouse model for multiple sclerosis (164) was shown to reduce disease severity. A study performed by Gertle *et al*. injected a citrullinated multiepitope peptide into adjuvant-induced arthritis rats, which was assumed to induce immune tolerance towards the peptide and resulted in the production of CD4+CD25+FoxP3+ T regulatory cells (168). In contrast, Strid *et al*. found that epicutaneous immunization with type II collagen in the CIA mouse model resulted in a Th2 response, identified by cytokine profile and the production of IgE antibodies, and specifically noted that there were no differences in CD4+CD25+FoxP3+ T regulatory cells in their study (167). Collectively, these studies demonstrated that inducing peripheral immune tolerance towards an antigen is able to modify antigen-specific T cell responses in mouse models for autoimmune diseases; however, the type of T cell response that occurs varies. Thus, to further understand the T cell responses that may be occurring in the mice in this study, experiments that examine cytokine profile or are able to identify T cell subtypes, such as ELISpot or flow cytometry, are required. This would provide insight in regard to not only the mechanism of the peptide-infused cream treatment, but also the pathogenesis of arthritis in HomoCitJED-immunized DR4tg mice and could assist in the optimization and development of the cream treatment.

4.4 B Cell Responses in Epicutaneous Immunized Mice

The production of antibodies is a characteristic trait for RA (13,14). As such, antibody responses to HomoCitJED and CitJED were assessed in mice that received cream treatment either pre- or post-arthritis onset. Studies have shown that epicutaneous immunization with type II collagen before or after CIA induction resulted in a reduction in anti-type II collagen total IgG antibodies (166,167). Antibody isotypes for IgG were also measured in some studies and found that epicutaneous immunization resulted in a reduction in the pro-inflammatory IgG2a isotype, while the anti-inflammatory IgG1 isotype remain unchanged (166,167). In a preliminary study, our group demonstrated that the epicutaneous

application of the peptide-infused cream prior to HomoCitJED immunization significantly reduced anti-HomoCitJED total IgG responses over the course of the experiment (177). In contrast, anti-HomoCitJED total IgG antibody levels were not significantly different between cream treatments in this study.

Assessment of total IgG antibody levels can provide insight regarding the overall effect of treatments, such as the peptide-infused cream treatment, on B cell responses. By examining IgG1 and IgG2a/b antibody levels, suggestions can be made regarding the types of T cell responses that are occurring. IgG2a/b are pro-inflammatory antibodies that are produced in Th1 pro-inflammatory responses in mice (176). IgG2a and IgG2b are considered to have similar functions; however, their production is based on different polarizing cytokines secreted by Th1 cells (176). IgG1 is an antibody produced during anti-inflammatory responses in mice and are produced by B cells after exposure to IL-4, a cytokine mainly secreted by Th2 cells (176). In this study, there were no significant differences between cream treatments for both anti-HomoCitJED IgG1 and IgG2b antibody levels. While IgG1 antibodies have been reported to remain unchanged in studies that examine peripheral tolerance as a method of treatment for experiment arthritis, a decrease in IgG2a antibodies is also typically observed in these studies (166,167). A decrease in IgG2a antibodies could be due to the presence of anti-inflammatory T cells, such as T suppressor cells, which were reported by Marcińska *et al*. (166), or Th2 cells, which were reported by Strid *et al*. (167). These anti-inflammatory T cells are capable of regulating pro-inflammatory responses, such as B cell responses, by a process known as bystander suppression, which regulates pro-inflammatory mechanisms via the secretion of anti-inflammatory cytokines (166,167). However, in this study, the generation of T suppressor cells or other anti-inflammatory T cell subsets were not assessed, and thus conclusions cannot be made regarding why IgG2b antibody levels remained unchanged in this study.

Studies have also shown that epicutaneous immunization results in the production of IgE antibodies (167,183) and are indicative of an anti-inflammatory Th2 response. B cell class switching to IgE antibody production occurs in the presence of the anti-inflammatory IL-4 cytokine (184). In this study, IgE responses, as well as serum cytokine levels, were not examined and thus limits the ability to determine what type of T cell and B cell responses

were occurring and whether the application of peptide-infused cream pre- or post-arthritis onset was able to modify HomoCitJED-specific immune responses.

CitJED total IgG responses were also assessed in this study; however, at the timepoints tested, there were no detectable CitJED total IgG antibodies. Our group has previously shown that HomoCitJED-immunized DR4tg mice develop CitJED antibody responses between days 30 and 70 post-immunization (91). From this finding, our group suggested that the development of these antibodies were due to epitope spreading, since the DR4tg mice were only immunized with HomoCitJED (91). It was therefore surprising that CitJED responses were not detected in this study. However, since only two timepoints were tested for pre-arthritis and post-arthritis cream treated mice, it is possible that CitJED antibody responses occurred at timepoints that were not assessed.

4.5 Possible Effects of the HA-PE Cream on Peripheral Tolerance

This study is unique in that it utilizes HA-PE cream as a vehicle for delivery of the HomoCitJED and CitJED peptides, since the cream is able to penetrate into the deep epidermal layers (163). Our group has previously shown that HA-PE cream can deliver CitJED into the deep epidermal layers and prolong its duration (unpublished data). This is beneficial, as it could increase the probability of tolerogenic dendritic cells, such as Langerhans cells, phagocytosing the peptides.

In the original study published by Symonette *et al*., they demonstrated that the HA-PE cream does not initiate local or systemic innate immune responses (163). However, the effects of the HA-PE cream on adaptive immune responses has not been examined. One of the effects of the HA-PE cream is that it is able to increase the production of hyaluronan (HA), which has been shown to promote migration of Langerhans cells (LC) to skin-draining lymph nodes, as well as facilitate LC maturation in the presence of inflammation (161,162). The HA-PE cream is also able to increase proliferation of keratinocytes (163), which are also capable of influencing LC migration and maturation in the presence of inflammation (157). Thus, it is possible that the HA-PE cream had an effect on LC maturation and migration through these mechanisms. A study performed by Strid *et al*.

demonstrated that the disruption of the skin using tape causes LC maturation, visualized by changes in morphology and expression of maturation markers (158). When an antigen was applied, the LC underwent migration to the lymph nodes and initiated a Th2 response (158). This mechanism of action was later shown to prevent the progression and onset of arthritis in CIA mice using type II collagen as the antigen (167). By disrupting the skin and using the HA-PE cream to deliver the peptides used in this study, we further increase the possibility of LC maturation and migration that was previously described by Strid *et al*. However, in this study, there were no significant differences between PBS or peptide-infused cream treatment in all the outcomes measured. Based on the data in this study, it is difficult to say exactly how the HA-PE cream itself is influencing LC maturation and migration and thus how it influences peripheral tolerance. Studies examining how disruption of the skin and the application of the HA-PE cream influences LC maturation and migration in DR4tg mice are needed.

4.6 Strengths and Limitations

A strength of this study is the use of the synthetic peptides HomoCitJED and CitJED. These peptides were designed to develop and detect immune responses to multiple homocitrullinated and citrullinated antigens. This is more representative of RA patients as reactivity to multiple homocitrullinated and citrullinated antigens have been described (92,102). Both of these peptides are used in the HA-PE cream treatment, meaning that this cream treatment could be beneficial for RA patients that are ACPA-positive, AHCPA-positive, or positive for both ACPA and AHCPA antibodies.

Another strength of this study is the use of the HA-PE cream as a method of peptide delivery into the skin. The HA-PE cream not only effectively delivers the peptides into the skin and prolongs their duration (unpublished data), but also provides a treatment that can be easily translated to humans, as it is not invasive and does not require ingestion for the treatment to work.

To assess the effects of the peptide-infused cream treatment, HomoCitJED-immunized SE-containing DR4tg mice were used. These mice express the strongest genetic risk factor for RA (12). The production of ACPAs have been shown to be associated with the SE in RA

(28). The production of HomoCitJED immune responses in DR4tg mice were shown to occur earlier on and at a greater magnitude when compared to the background mice, C57Bl/6 (91). Only HomoCitJED-immunized DR4tg mice developed CitJED immune responses (91). Additionally, the HomoCitJED and CitJED antibody responses described previously in HomoCitJED-immunized DR4tg mice have also been described in RA patients (91,92). Therefore, the DR4tg mouse model for RA provides a clinically relevant mouse model that captures the genetic risk factor, antibody responses and joint swelling of RA, allowing for easier translation between mice and humans.

In our study, the DR4tg mice are bred and maintained in a pathogen-free facility until day of sacrifice. Since this study examines immune responses, keeping the DR4tg mice in a pathogen-free facility prevents the mice from exposure of unwanted pathogens, ensuring that changes in the immune response are due to the peptide-infused cream treatment instead of an infection. However, maintaining the mice in a pathogen-free facility could also influence the development of arthritis, since it can alter the composition of the gut microbiota (185). Recent studies have suggested that the gut microbiome may play an important role in RA pathogenesis (185,186). Additionally, some mouse models do not develop arthritis when maintained in a pathogen-free facility (186) Thus, it is possible that the gut microbiome of the mice used in this study had an influence on disease pathogenesis.

The HomoCitJED-immunized DR4tg mouse model also lacks sex-related differences. As with most autoimmune diseases, RA is more commonly seen in females (10). In HomoCitJED-immunized DR4tg mice, the only sex differences previously described by our group were a higher ratio of IL1α and IL-5 in females (187). In this study, there were no differences between sex for all outcomes measured. A study by Taneja *et al*. examined the sex differences in various mouse strains using the CIA mouse model for RA (94). They discovered that the DR4tg mouse strain used in this study, which has a functional knockdown of endogenous MHC class II genes, did not display any sex differences in arthritis incidence (94). However, DR4tg mice that were a complete knockout for all endogenous MHC class II genes, displayed sex-related differences in the incidence of CIA, as well as an increase in arthritis severity compared to the DR4tg mice used in this study (94).

4.7 Future Directions and Significance

Our group has previously shown that the peptide-infused cream has the ability to modify immune responses and prevent knee swelling when applied prior to the development of RA-specific immune responses and arthritis in DR4tg mice (173) and acts as a proof of principle for this current study. Although the application of the peptide-infused cream after the development of RA-specific immune responses but before or after arthritis induction did not have an effect on the outcomes measured in this study, it did provide insight towards the next steps for optimizing the peptide-infused cream treatment. Since RA patients would only seek treatment after disease symptoms have developed, it is vital that the peptide-infused cream is effective when applied after RA-specific immune responses and/or early signs of arthritis are present.

Future directions to be taken by our group include assessment of anti-HomoCitJED IgE antibody levels and serum cytokine levels, in order to better understand the type of T cell and B cell responses that are occurring in the peptide-infused and PBS-infused cream treated mice. Since the disruption of the skin and timing of the epicutaneous cream treatment was adapted from Strid *et al*., it would be hypothesized that IgE and Th2 related cytokines would be elevated.

Additionally, histopathological analysis will need to be examined to determine if the peptide-infused cream had an impact on pathological features of arthritis in the knee joints. It would be expected that if the peptide-infused cream treatment was effective, then a reduction in leukocyte infiltration, synovial hyperplasia and cartilage damage would be seen. Future studies should also assess pain severity in the mice throughout the experiment, as it could provide insight regarding the effectiveness of the cream on arthritis severity.

Furthermore, the effects of the HA-PE cream on peripheral tolerance and adaptive immune responses should be examined. In particular, studies should be undertaken that examine whether the application of HA-PE cream after disruption of the stratum corneum is able to induce LC maturation and migration in our DR4tg mouse model, as previously described by Strid *et al*. (158).

Additionally, demonstrating the effects of the peptide-infused cream on different RA mouse models, such as the CIA mouse model, would further highlight the efficacy of the treatment and indicate that the cream is able to modify a multitude of immune responses that are seen in RA patients. Using mice that display sex-related differences in arthritis severity, such as the DR4tg mice with a complete knockout of endogenous MHC class II genes, could provide more insight regarding the effectiveness of the peptide-infused cream on female vs male patients.

This study describes a novel antigen-specific treatment for RA. The epicutaneous delivery of HomoCitJED and CitJED using HA-PE allows for a treatment for RA that targets homocitrullinated and citrullinated immune responses seen in RA patients while avoiding the severe side effects associated with conventional non-specific immunosuppressants. By using the humanized DR4tg mouse model for RA, this study is able to mimic the pathogenesis of ACPA-positive RA, allowing for the development of a treatment that can be easily translated to RA patients. Understanding the mechanism of how this peptide-infused cream treatment is able to induce immune tolerance and treat arthritis could provide insight towards the dysregulation of peripheral tolerance experience by RA patients. Furthermore, the development of this novel peptide therapy for RA could provide a foundation for the development of peptide therapies using HA-PE cream in other autoimmune diseases.

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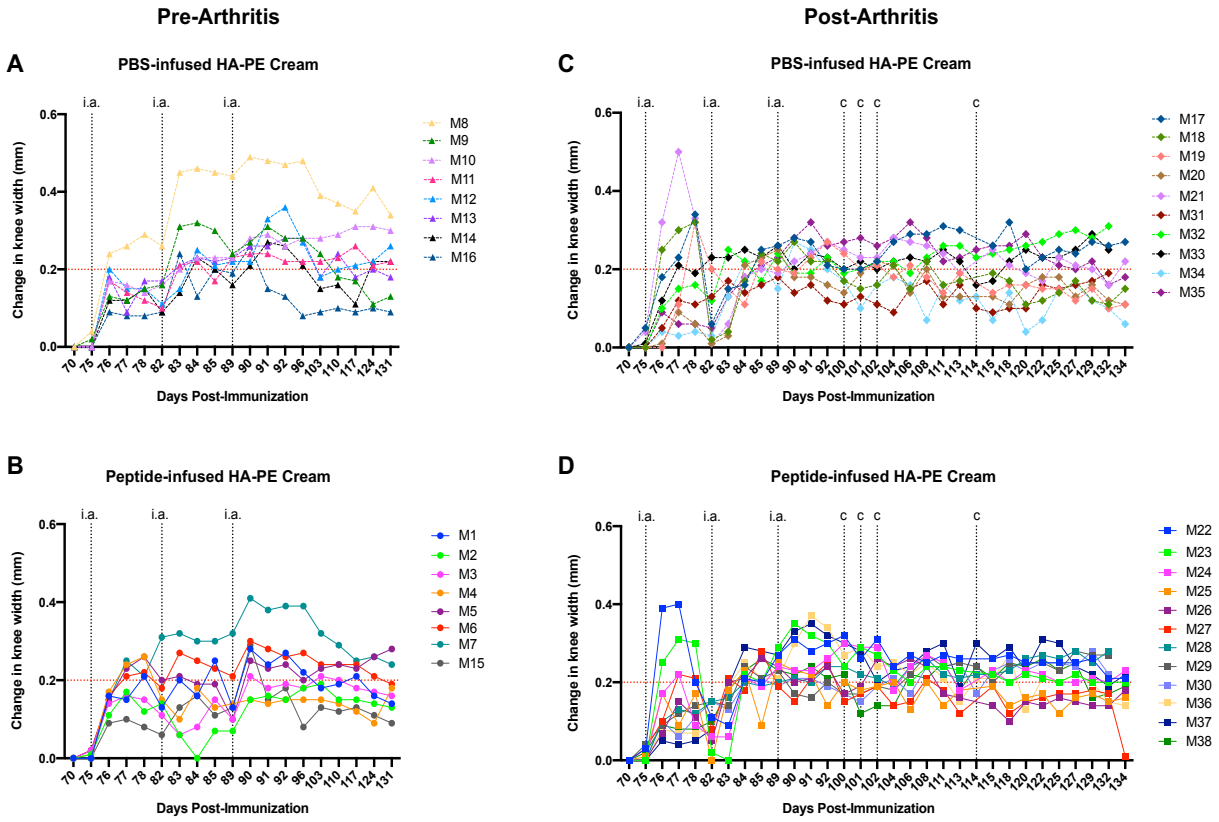
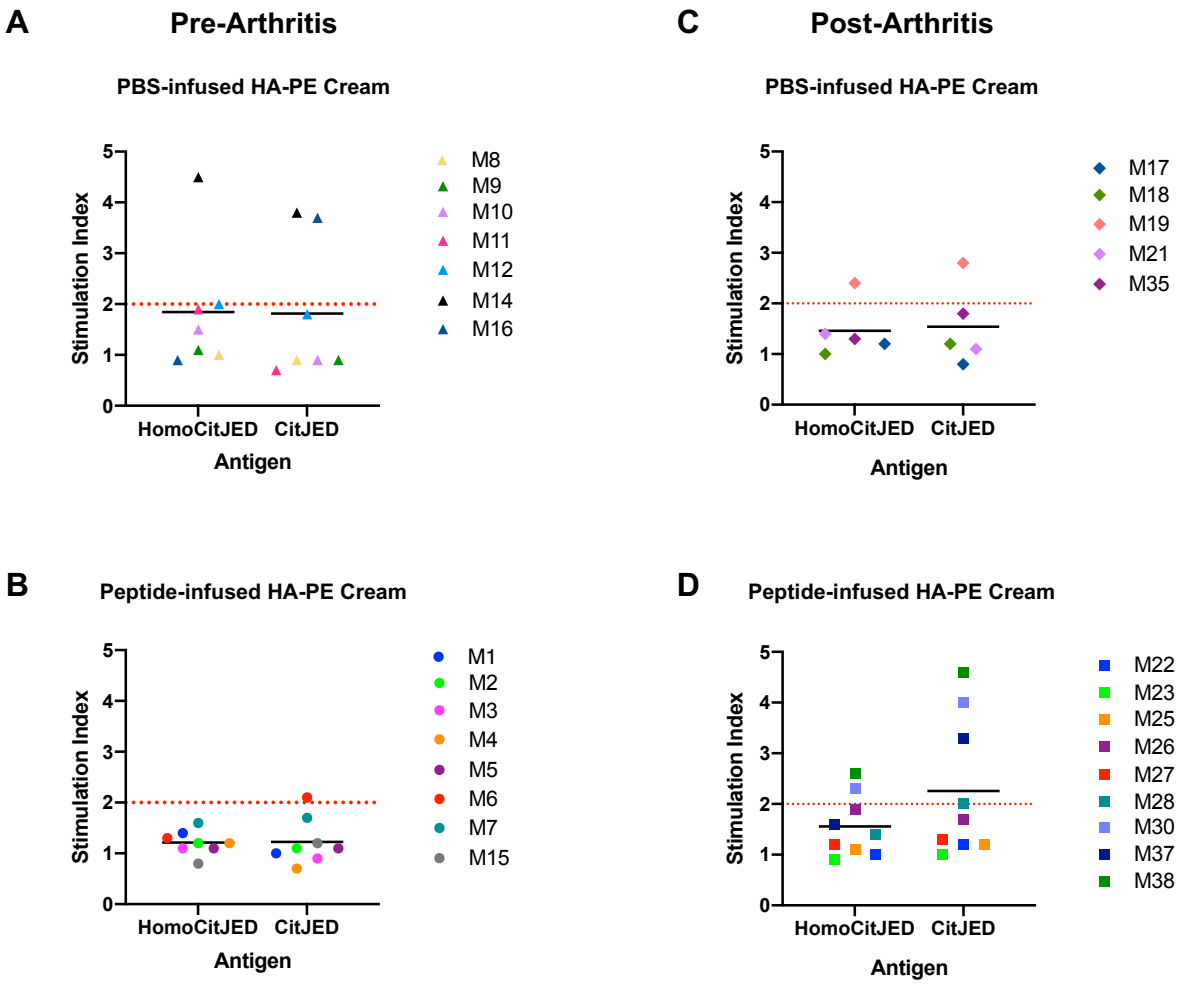
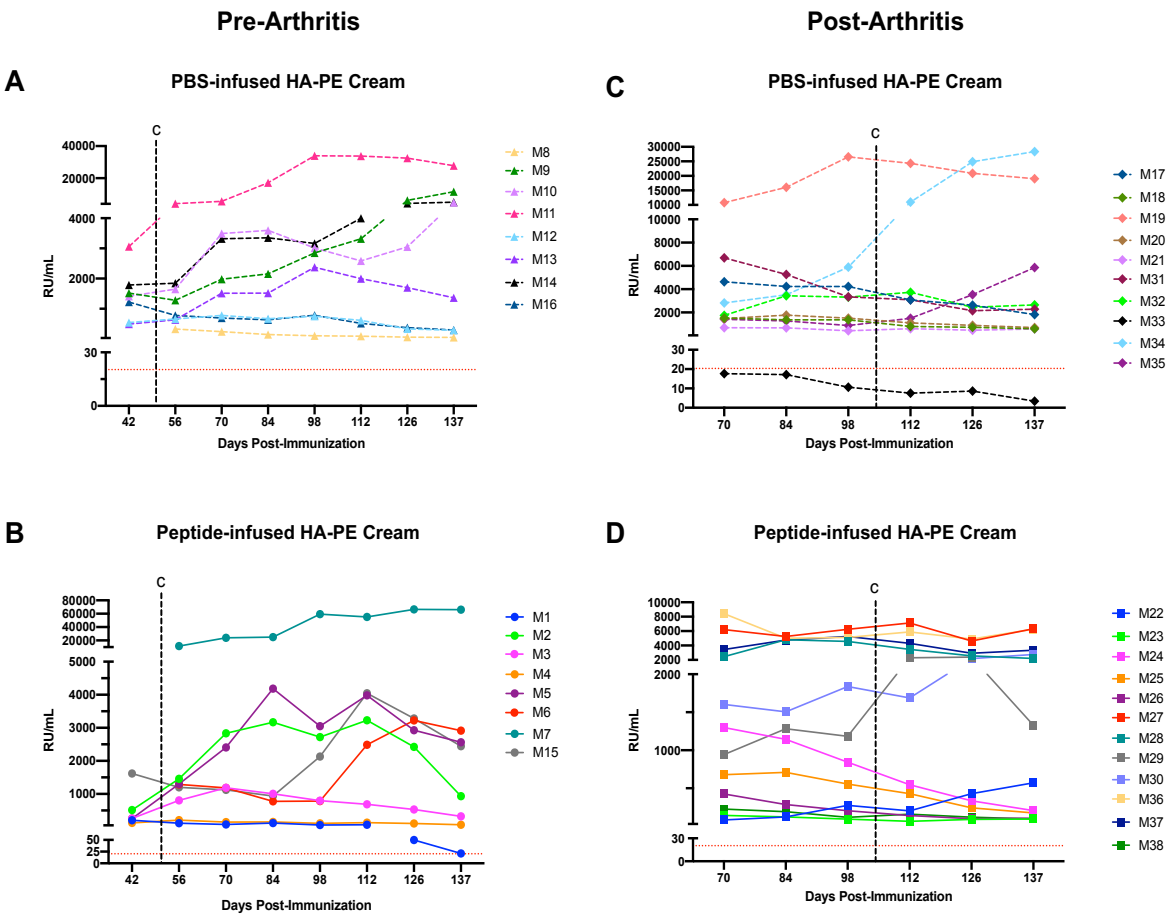
Appendices



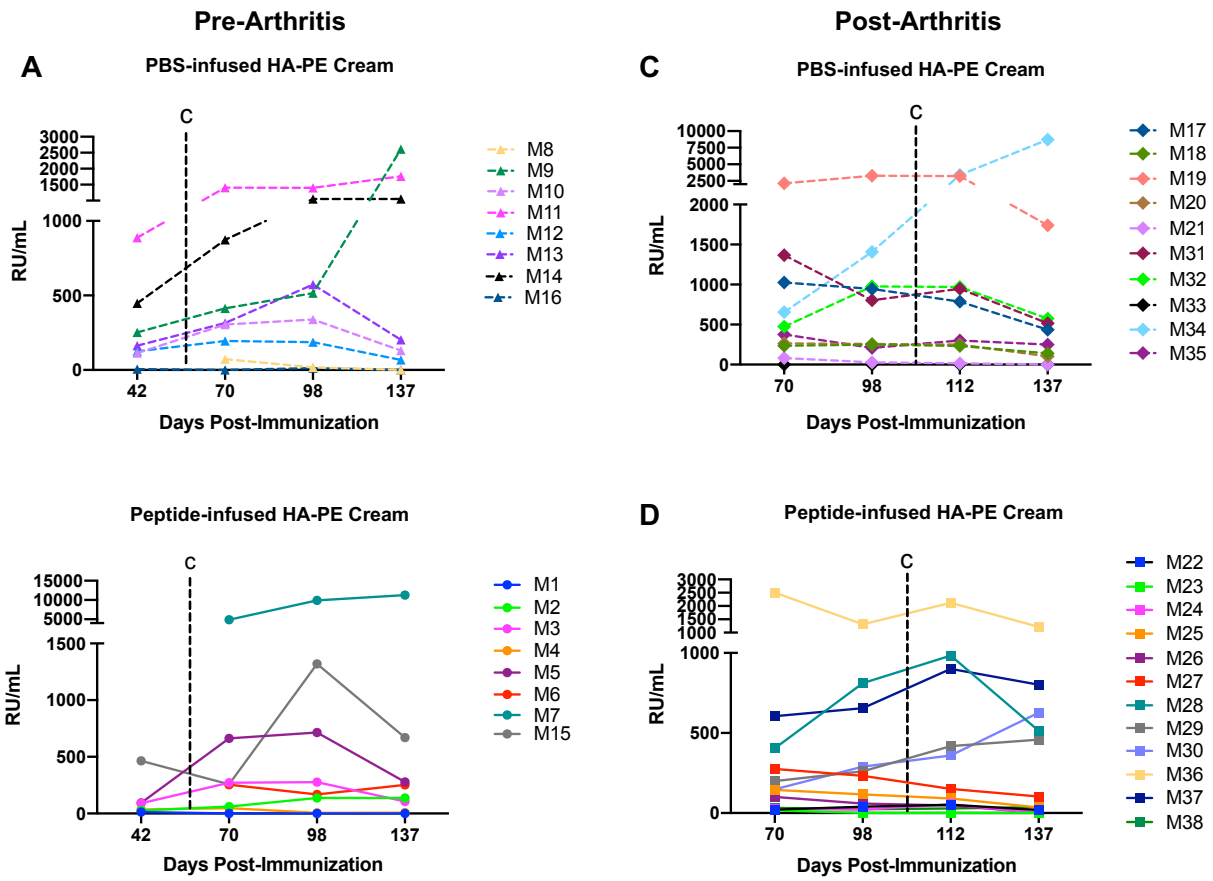
Appendix 1: Knee joint swelling for individual HA-PE cream treated DR4tg mice pre- and post- arthritis onset. HomoCitJED-immunized DR4tg mice were epicutaneously treated with either PBS-infused or peptide-infused HA-PE cream before HomoCitJED i.a. injections (pre-arthritis onset) (A and B) or after HomoCitJED i.a. injections (post-arthritis onset) (C and D). Knee joint swelling was measured using digital calipers on the days indicated post-immunization and is reported as a change in knee width (mm). The vertical black dashed line indicates the day in which i.a. injections occurred. The horizontal red dashed line at 0.2 mm change in knee width represents the cut-off for knee swelling. i.a.= intra-articular; c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine; M# = mouse identification number.



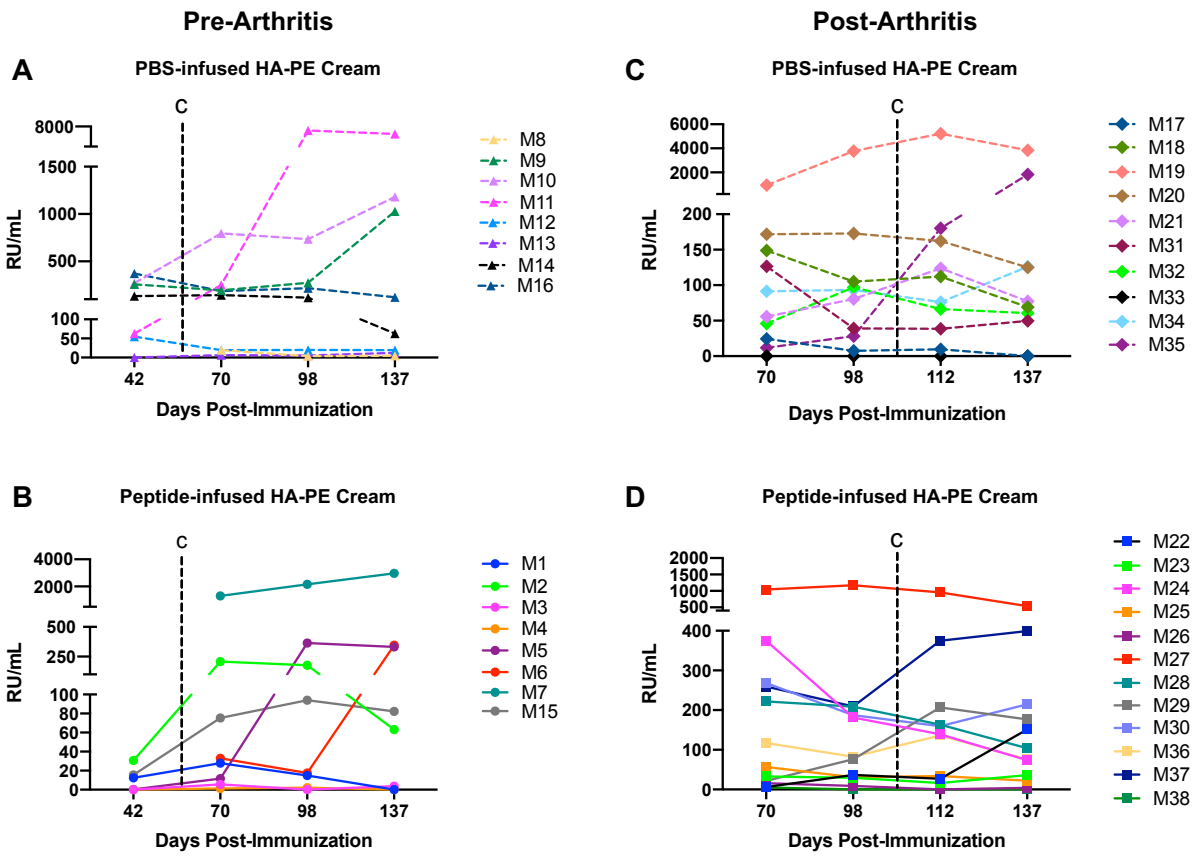
Appendix 2: Splenocyte proliferative responses for individual HomoCitJED-immunized DR4tg mice cream treated pre- or post- arthritis onset. On day of sacrifice (day 137 post-immunization), splenocytes from HomoCitJED-immunized DR4tg mice that received either PBS-infused or peptide-infused HA-PE cream treatment either pre-arthritis (A and B) or post-arthritis (C and D) onset were cultured in the presence of 100 µg of HomoCitJED or CitJED. Splenocyte proliferation was determined using ^{3}H-thymidine incorporation and is shown as a mean stimulation index. Stimulation indices greater than 2.0 (red dashed line) were considered positive proliferative responses. HA-PE = Hyaluronan-Phosphatidylethanolamine; M# = mouse identification number.



Appendix 3: Anti-HomoCitJED total IgG antibody levels for individual HomoCitJED-immunized DR4tg mice cream treated pre- or post- arthritis onset. Sera were measured for anti-HomoCitJED total IgG antibody levels in HomoCitJED-immunized DR4tg mice that received either PBS-infused or peptide-infused HA-PE cream treatment pre-arthritis (A and B) or post-arthritis (C and D). Sera were assessed via direct ELISA at the timepoints indicated post-immunization. Values are displayed as mean RU (Relative Units)/mL. The vertical black dashed line represents the beginning of cream treatment (day 54 or 100 post-immunization). RU/mL values above the cut off for positivity are indicated by the horizontal red dashed line. The RU/ml cut off for anti-HomoCitJED total IgG was 20.32 RU/mL. c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine; M# = mouse identification number.



Appendix 4: Anti-HomoCitJED IgG1 antibody levels for individual HomoCitJED-immunized DR4tg mice cream treated pre- or post- arthritis onset. Sera were measured for anti-HomoCitJED IgG1 antibody levels in HomoCitJED-immunized DR4tg mice that received either PBS-infused or peptide-infused HA-PE cream treatment pre-arthritis (A and B) or post-arthritis (C and D). Values are displayed as mean RU (Relative Units)/mL. The vertical black dashed line represents the beginning of cream treatment (day 54 or 100 post-immunization). The RU/mL cut off for anti-HomoCitJED IgG1 positivity was 0.00 RU/mL. c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine; M# = mouse identification number.



Appendix 5: Anti-HomoCitJED IgG2b antibody levels for individual HomoCitJED-immunized DR4tg mice cream treated pre- or post- arthritis onset. Sera were measured for anti-HomoCitJED IgG2b antibody levels in HomoCitJED-immunized DR4tg mice that received either PBS-infused or peptide-infused HA-PE cream treatment pre-arthritis (A and B) or post-arthritis (C and D). Mice that received cream treatment pre-arthritis onset but lacked sera for day 42 post-immunization are included in A and B. Sera were assessed via direct ELISA at the timepoints indicated post-immunization. Values are displayed as mean RU (Relative Units)/mL. The vertical black dashed line represents the beginning of cream treatment (day 54 or 100 post-immunization). The RU/mL cut off for anti-HomoCitJED IgG2b positivity was 3.29 RU/mL. c = cream treatment; HA-PE = Hyaluronan-Phosphatidylethanolamine; M# = mouse identification number.

eSirius3G Notification -- 2018-131 New Protocol Approved

Western

AUP Number: 2018-131
PI Name: Barra, Lillian
AUP Title: The Role of Citrullination and the Shared Epitope in the accelerated atherosclerosis of Rheumatoid Arthritis
Approval Date: 11/01/2018

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2018-131:1: entitled " The Role of Citrullination and the Shared Epitope in the accelerated atherosclerosis of Rheumatoid Arthritis"
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:

a) Western's Senate MAPPs 7.12, 7.10, and 7.15
http://www.uwo.ca/univsec/policies_procedures/research.html

b) University Council on Animal Care Policies and related Animal Care Committee procedures

http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm

2) As per UCAC's Animal Use Protocols Policy,

a) this AUP accurately represents intended animal use;

b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;

c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and

d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.

e)

http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will
a) be made familiar with and have direct access to this AUP;
b) complete all required CCAC mandatory training (training@uwo.ca); and
c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
a) Practice will align with approved AUP elements;
b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
c) UCAC policies and related ACC procedures will be followed, including but not limited to:
i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to
hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

Appendix 6: Ethical approval for the use of animal subjects.

Curriculum Vitae

Name: Alisha J. Moynahan

Post-secondary Education and Degrees:

The University of Western Ontario
London, Ontario, Canada
2019 - Present M.S.c in Microbiology & Immunology

University of Waterloo
Waterloo, Ontario, Canada
2015 - 2019 B.S.c in Honours Biology, Specialization in Animal Biology

Honours and Awards:

Western Graduate Research Scholarship (WGRS)
The University of Western Ontario, London, Ontario
Sept 2019-Apr 2021

Dr. Robert George Everitt Murray Graduate Scholarship in Microbiology & Immunology
The University of Western Ontario, London, Ontario
Sept 2020

Best Oral Presentation at the University of Western Ontario/McMaster University Rheumatology Research Day
Ingersoll, Ontario
Nov 2019

Dr. Frederick W. Luney Graduate Entrance Scholarship in Microbiology & Immunology
The University of Western Ontario, London, Ontario
Sept 2019

Lawson Internal Research Fund Scholarship
London Health Sciences, London, Ontario
July 2019

Dean's Honours List
University of Waterloo, Waterloo, Ontario
Sept 2015-April 2019

University of Waterloo President's Scholarship of Distinction
University of Waterloo, Waterloo, Ontario
Sept 2015

Related Work Experience:

Research Assistant, Laboratory of Dr. Brian Dixon
University of Waterloo
Waterloo, Ontario
May 2019-Aug 2019

Undergraduate Teaching Assistant, Department of Chemistry
University of Waterloo
Waterloo, Ontario
Jan 2018-Apr 2019

Summer Student, Research and Development/Bulk Departments
Hiram Walker and Sons Ltd.
Windsor, Ontario
May 2016-Aug 2016; May 2017-Aug 2017

Leadership Experience:

Infection and Immunity Research Forum (IIRF) Committee Member
Department of Microbiology & Immunology
The University of Western Ontario, London, Ontario
Jan 2020-Oct 2020

Let's Talk Science Volunteer
University of Waterloo, Waterloo, Ontario
Oct 2017-Apr 2019

Presentations:

Moynahan A, Blackler G, Turley E, Bell DA, Cairns E, Barra L. Testing a novel peptide therapy for treatment of rheumatoid arthritis in DR4tg mice (oral presentation).
Infection and Immunity Research Forum
The University of Western Ontario, London, Ontario
Oct 2020

Moynahan A, Blackler G, Saunders S, Song Y, Bell DA, Cairns E, Barra L. Pre-clinical trial of a novel peptide therapy for rheumatoid arthritis (oral presentation).
The University of Western Ontario/McMaster University Rheumatology Research Day
Ingersoll, Ontario
Nov 2019

Moynahan A, Frenette AP, Rodriguez-Ramos T, Dixon B. The development of a protein-level assay for detection and quantification of tumor necrosis factor alpha in rainbow trout (*Oncorhynchus mykiss*) (poster presentation).
North American Comparative Immunology Workshop
University of Waterloo, Waterloo, Ontario
Jun 2019

Moynahan A, Frenette AP, Rodriguez-Ramos T, Dixon B. The development of a protein-level assay for detection and quantification of tumor necrosis factor alpha in rainbow trout (*Oncorhynchus mykiss*) (oral presentation).
11th Ontario-Quebec Undergraduate Immunology Conference
University of Toronto, Toronto, Ontario
May 2019

Frenette AP, Rodriguez-Ramos T, Moynahan A, Guo H, Semple SL, Zanuzzo F, Soulliere C, Sever L, Iwanczyk J, Rix J, Gamperl AK, Dixon B. The development of cytokine protein assays for assessing immune function in salmonids (oral presentation).
Canadian Society of Zoologist Meeting
University of Windsor, Windsor, Ontario
May 2019