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Immune responses to homocitrulline- and citrulline-containing peptides in rheumatoid arthritis

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Abstract

Anti-citrulline immune responses contribute to rheumatoid arthritis (RA) development, and are linked with the expression of HLA-DR molecules that encode the Shared Epitope (SE). Immune responses to homocitrulline, a structural analog of citrulline, have been recently detected in RA, but their role in RA is unknown.

The objectives of this study were to examine the specificity of anti-homocitrulline antibodies for RA, the dependence of anti-homocitrulline responses on SE-expression, and the cross-reactivity of responses to homocitrulline and citrulline. These objectives were addressed in RA patients and in SE-expressing DR4tg mice using homocitrulline- and citrulline-rich peptides.

Anti-homocitrulline antibodies occurred specifically in RA. The SE was associated with the development of immune responses to homocitrulline and cross-reactivity to citrulline in homocitrullinated peptide-immunized mice. Anti-homocitrulline and anti-citrulline antibodies were cross-reactive in RA patients and DR4tg mice, and the responses to homocitrulline- and citrulline-containing peptides were immunologically related.

Keywords

Rheumatoid arthritis, anti-homocitrulline antibodies, anti-citrulline antibodies, cross-reactivity, Shared Epitope, human patients, mouse model

Co-Authorship Statement

In regards to the work presented in Chapter 2:

P Lac performed experiments on sera from RA patients and healthy subjects, analyzed data and participated in the writing of the manuscript. M Racapé contributed the data on sera from systemic lupus erythematosus patients and patients with psoriatic arthritis shown in figure 2.1. L Barra, DA Bell and E Cairns are co-senior authors and contributed equally to this research. They secured funding, designed and supervised experiments, analyzed data, and contributed to the writing of the manuscript.

In regards to the work presented in Chapter 3:

P Lac performed experiments and analyzed data, contributed the figures, and wrote the initial draft of the manuscript. S Saunders contributed to the data shown in Figure 3.2 (B,D) and assisted with the mouse experiments over the course of these studies. E Tutunea-Fatan participated in collecting the data shown in Figure 3.2 (B,D) and aided P Lac in collecting data for Figure 3.4. L Barra, DA Bell and E Cairns acquired the funding for the study, designed the experiments and analyzed data. L Barra and E Cairns wrote the final version of the paper.

Acknowledgments

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List of Abbreviations

^{3}H-thymidine	Tritiated thymidine
ACPA	Anti-citrullinated protein/peptide antibodies (another term for anti-citrulline antibodies; general term for anti-citrulline antibodies of various specificities)
ACR	American College of Rheumatology
AHCPA	Anti-homocitrullinated protein/peptide antibodies (another term for anti-homocitrulline antibodies; general term for anti-homocitrulline antibodies of various specificities)
ANOVA	Analysis of variance
Anti-CarP	Anti-carbamylated protein antibodies
Anti-CarP-FCS	Anti-carbamylated fetal calf serum antibodies
Anti-CCP	Anti-cyclic citrullinated peptide antibodies
Anti-CitFib	Anti-citrullinated fibrinogen antibodies
Anti-HomoCitFib	Anti-homocitrullinated fibrinogen antibodies
Anti-Fib	Anti-fibrinogen antibodies
APC	Antigen presenting cell
AU	Arbitrary units
B6	C57Bl/6
BSA	Bovine serum albumin
CarP-FCS	Carbamylated fetal calf serum
CASPAR	Classification criteria for psoriatic arthritis
CCP	Cyclic citrullinated peptides
CCP2	Cyclic citrullinated peptides, second generation
CI	Confidence interval
CitFib	Citrullinated fibrinogen
CO_2	Carbon dioxide

Cpm	counts per minute
DNA	Deoxyribonucleic acid
DR4tg	HLA-DR4-IE transgenic
ELISA	Enzyme-linked immunosorbent assay
FCS	Fetal calf serum
Fib	Fibrinogen
H2	Histocompatibility-2 (murine major histocompatibility complex)
H_2SO_4	Sulfuric acid
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HLA-DR	Human leukocyte antigen DR antigen
HLA-DR4	Human leukocyte antigen DR4 serotype
HLA-DRB1	Gene encoding for the human leukocyte antigen DR antigen beta chain
HomoCitFib	Homocitrullinated fibrinogen
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL	Interleukin
MHC	Major histocompatibility complex
OD	Optical density
PAD	Peptidylarginine deiminase
PAD2	Peptidylarginine deiminase type 2
PAD4	Peptidylarginine deiminase type 4
PADI4	Gene encoding for Peptidylarginine deiminase type 4
PBS	Phosphate-buffered saline
PsA	Psoriatic arthritis

PTPN22	Gene encoding for protein tyrosine phosphatase, non-receptor type 22
RA	Rheumatoid arthritis
REB	Research ethics board
RF	Rheumatoid factor
RPMI	Roswell Park Memorial Institute
RT	Room temperature
SE	Shared Epitope
SEM	Standard error of the mean
SI	Stimulation index
SJHC	St. Joseph's Health Care London
SLE	Systemic lupus erythematosus
TNFα	Tumor necrosis factor alpha

Chapter 1

1 Rheumatoid arthritis

1.1 Overview

Rheumatoid arthritis (RA) is one the most prevalent chronic inflammatory diseases and is estimated to affect up to 1% of the population worldwide (1,2). RA is an autoimmune disorder which can often result in irreversible damage to bone and cartilage tissue. This leads to a progressive loss of joint function and often results in disability in RA patients. RA is a debilitating disease associated with a decreased quality of life, high economic burden (2,3), and an increased rate of mortality compared to the general population (4,5).

RA is a complex disorder and susceptibility to disease has been reported to be affected by various factors. In RA, similar to other autoimmune diseases, there is a gender bias. The prevalence of RA is greater in women, and gender may also affect the disease course (6). Susceptibility to RA is influenced by environmental exposures (7), and especially with smoking which has been associated with a heightened incidence of RA (8). Genetics also modify the risk of RA (9) and genes, such as those coding for major histocompatibility antigens (especially Shared Epitope (SE)-encoded HLA-DRB1) can predispose individuals to develop RA.

RA is systemic in nature, and has been shown to affect various organ systems. The joints, skin, cardiovascular system and lungs are a few examples of the tissues that can be affected by RA (10). The mechanism by which RA affects these various tissues is not well understood. Joint inflammation, which is the primary manifestation of RA, often involves the large-scale infiltration of leukocytes into the joint tissue and pro-inflammatory changes to the environment of the joint (11). These changes are mediated by cells of both the innate and adaptive immune system; various immune cells such as dendritic cells, macrophages, neutrophils, T and B cells are present in the synovial tissue of RA patients (12–14). Amongst the changes that occur in the synovial tissue are increased expression of inflammatory cytokines including TNFα, IL-6, IL-17, and type I

interferons (15) as well as matrix metalloproteinases (16), which contribute to the degradation of bone and cartilage in RA. Additionally, phenotypic changes occur in joint-resident cell types including fibroblast-like synoviocytes which normally play a role in maintaining the synovial fluid (17), and osteoclasts which function in bone resorption, thereby promoting the dysfunction and degradation of the joint (11).

The etiology of RA is currently unknown and thus, targeted treatments for RA are unavailable. Instead, strategies for the treatment of RA employ disease-modifying antirheumatic drugs, glucocorticoids and biologics (18). Some of these therapeutics, methotrexate and cortisol for example, are immunosuppressive and have inhibitory effects on the immune system. Other therapeutics have been developed that target inflammatory cytokines such as TNFα (19), immune cells including B (20) and T (21) cells, as well as immune signaling pathways (22). These current treatment options for RA have broad effects on immune system functions and as a result, are accompanied with risks of side-effects including increased risk of infection and cardiovascular disease (23–25). To more specifically treat RA, further study of the mechanisms underlying the disease is required.

The disease process in RA is often associated with autoantibodies which predicts disease and are believed to participate in RA pathogenesis. The importance of autoantibodies in RA is underscored by the classification of patients as seropositive or seronegative depending on the expression of autoantibodies; seropositive and seronegative RA patients often differ in disease patterns (ie. disease severity and outcome). In RA, one of the major autoantibodies is rheumatoid factor (RF), an autoantibody which targets IgG and is diagnostic for RA. Anti-citrulline antibodies, which are antibodies that bind to antigens containing citrulline, are another class of antibodies that are frequently found in RA patients (26–29). Anti-citrulline antibodies are also a diagnostic biomarker for RA and are measured clinically as anti-cyclic citrullinated peptide antibodies (anti-CCP). Several other autoantibody systems have been identified in RA patients, including autoantibodies reactive to proteins that have been modified post-translationally by acetylation and by formation of malondialdehyde-acetaldehyde adducts (30,31). Recently, anti-homocitrulline antibodies have been detected in RA patients and have garnered much

interest as a potential biomarker for disease. Whether anti-homocitrulline antibodies have a role in RA pathogenesis remains to be determined.

1.2 Risk factors for RA

RA is a multifactorial disorder and the triggers that initiate the disease are poorly understood. A study of monozygotic twins by MacGregor *et al.* estimated the overall heritability of RA (seropositive and seronegative disease) to be 60% (9), indicating that genetic risk factors play a substantial role in RA development. Environmental exposures have been reported to be important contributors to RA risk (7,32). The susceptibility to RA is believed to be an interaction between both genetic and environmental risk factors. As previously mentioned, RA patients with and without autoantibodies are phenotypically different; the complicated nature of RA development is further highlighted by the findings that both genetic (33–35) and environmental (32) risk factors differ between anti-CCP-positive and anti-CCP-negative RA.

1.2.1 Smoking

The most characterized environmental risk factor for RA is smoking. Meta-analyses by Sugiyama *et al.* (8) and Di Giuseppe *et al.* (36) have provided evidence that smoking increases the incidence of RA. Moreover, the risk of RA is increased for years even after smoking cessation (37). The mechanisms by which smoking contributes to RA development is not clear, though several possibilities have been previously described (38). Smoking induces a pro-inflammatory state that may induce oxidative stress, cellular apoptosis and changes in cytokine expression, among many other effects (38). In particular, smoking has been linked to increased TNFα activity (39) which is known to mediate RA pathogenesis.

Notably, smoking has been linked to anti-CCP-positive, but not anti-CCP-negative RA. Studies by Kallberg *et al.* (40) and Linn-Rasker *et al.* (41) have found associations between smoking and expression of anti-CCP (as well as SE-encoded HLA-DRB1), which emphasizes the contribution of smoking to the development of anti-citrulline immune responses. This is supported by evidence that smoking promotes the

citrullination of self-proteins in the lungs by upregulating the expression of an enzyme (peptidylarginine deiminase) that catalyzes protein citrullination (42,43), as well as the finding of citrullinated proteins in lung tissue (44). Smoking has also been linked to protein carbamylation, which could result in the generation of homocitrulline-containing antigens (45). These antigens may help in triggering the development of anti-homocitrulline antibodies and may also be targets for the autoimmune response. However, Jiang *et al.* did not find an association between anti-homocitrulline antibodies and smoking in RA patients that were negative for anti-CCP (46).

1.2.2   Periodontal disease

Periodontal disease is a common inflammatory disease that has been associated with anti-CCP-positive RA. Both of these diseases share some similar risk factors including smoking and expression of HLA-DR4 alleles (47). Compared to the general population, patients with RA are at a higher risk of developing periodontal disease and vice-versa (48). Moreover, RA patients are more likely to develop more severe periodontal disease (48).

Porphyromonas gingivalis is a bacteria that is often found in dental plaque. *P. gingivalis* has been identified as a major pathogen in periodontal disease and antibodies against this pathogen have also been detected in patients with RA (49,50). Moreover, Kinloch *et al.* demonstrated that α-enolase from *P. gingivalis* is arthritogenic in a mouse model of RA (DR4tg mice) (51). *P. gingivalis* expresses several virulence factors that enable the pathogen to survive and colonize in host tissue. Among these are a class of extracellular cysteine proteases known as gingipains, which can subvert the host immune response and promote inflammation (52). *P. gingivalis* also expresses a peptidylarginine deiminase which is capable of citrullinating host proteins (53–55), and thereby may initiate anti-citrulline immune responses in RA-susceptible individuals that express SE-encoded HLA-DR molecules.

1.2.3   Shared Epitope

The expression of certain class II MHC molecules play an important role in susceptibility to RA. Genes coding for HLA-DR have been known risk factors since the work of Stastny (56,57). He identified that RA patients shared a common MHC class II haplotype (57). In 1987, Gregersen *et al.* from the Winchester lab posed the Shared Epitope hypothesis to explain the association of several HLA-DRB1 alleles with RA (58). They demonstrated that RA-associated alleles of HLA-DRB1 (including *0401, *0404, *0405, *0408, *0413, *0101, *0102, *1402, and *1001) encode for a conserved amino acid consensus sequence: QKRAA, QRRAA, or RRRAA (58). The SE is encoded at positions 70-74, located in the third hypervariable region of the N-terminal domain of the HLA-DRβ chain (58) and forms the P4 peptide-anchoring pocket of HLA-DR (58,59). Alleles encoding for SE-encoded HLA-DR molecules are highly prevalent in RA patients, are reported to be expressed in approximately 75% of RA patients (60–62) and have also been associated with more severe disease (63).

The SE is believed to participate in the pathogenesis of RA. One mechanism by which the SE is believed to contribute to RA development is through the presentation of citrullinated peptides by antigen presenting cells that express SE-positive HLA-DR molecules, leading to the initiation of anti-citrulline immune responses. The evidence that SE-encoded HLA-DR binds to citrullinated peptides was first reported by Hill *et al* (64). They demonstrated that a citrullinated peptide derived from vimentin bound to SE-positive HLA-DRB1 molecules encoded by the alleles *0101, *0401 and *0404, but not to several SE-negative HLA-DRB1 molecules (64). This was subsequently confirmed by the work of Scally *et al.* who reported on crystal structures of HLA-DRB1*0401 complexed to four citrullinated epitopes derived from vimentin and aggrecan (59). Moreover, Huizinga *et al.* found the SE to be a risk factor for anti-CCP-positive RA, but is not associated with anti-CCP-negative disease (65), in keeping with the original findings by Hill *et al* (64). Additionally, in a mouse model of RA that is transgenic for human HLA-DR4 (DR4tg mice), Hill *et al.* showed that immunization with citrullinated fibrinogen induced citrullinated fibrinogen-reactive T and B cell responses as well as inflammatory arthritis (66).

Besides the involvement in the anti-citrulline immune response, the SE has been shown to also act as a signal transduction ligand. Ling *et al.*, from the Holoshitz lab, showed that the SE acts as a ligand that increased production of nitric oxide, resulting in higher oxidative stress (67,68). This was later identified to be mediated through the binding of the SE to cell surface calreticulin (69,70). This signalling event has been shown to have various consequences that may contribute to RA pathogenesis. Almeida *et al.* from the same group, demonstrated that SE-activated signalling in dendritic cells induced a Th17-polarizing response (71), which is associated with autoimmunity. In another study by this group, Holoshitz *et al.* reported that SE-mediated signalling is able to induce osteoclastogenesis and promote the degradation of bone tissue (72). Recently, they also showed that the SE is associated with spontaneous skeletal bone damage in DR4tg mice (73). The observations of Holoshitz and colleagues suggest that the SE may be involved in the development of arthritis independent of its role in antigen presentation.

1.2.4   Other genetic risk factors

In addition to genes coding for HLA-DR with the SE, other genetic risk factors have also been associated with RA susceptibility. One of these non-HLA risk factors is PTPN22, which encodes for a tyrosine phosphatase that is expressed by leukocytes and functions in immune system signalling; a polymorphism of PTPN22 has been associated with an increased risk for developing anti-CCP-positive RA (34,74,75). PADI4, which encodes for an enzyme that participates in protein citrullination, is another risk locus for RA development (75,76) and has been associated with radiographic damage (77). Recently, a genome-wide association study performed by Okada *et al.* identified approximately 100 loci that modify the susceptibility to RA (78). Many of these loci contain genes related to immune system functions including T cell signalling, cytokines such as TNFα and IL-6, and also members of the Janus kinase signalling family (78). Nonetheless the SE is the most important risk factor for RA (79).

1.3   Autoantibodies in RA

The breakdown of immunological tolerance to endogenously expressed proteins is often the first step in the development of autoimmunity. In RA, the disease process results in the production of autoantibodies and the detection of these antibodies is therefore important for RA diagnosis. As noted (in 1.1), the presence of autoantibodies in patients is often a predictor for disease severity in RA. Thus screening for these antibodies is crucial for the early identification of RA, allowing for therapeutic intervention prior to the development of potentially irreversible tissue damage.   Further studies of autoantibody systems in RA is important to understand the mechanisms leading to the pathogenesis of RA and additionally, to provide clinically relevant information to aid in the treatment of this disease.

1.3.1   Rheumatoid factor

RFs were the first autoantibodies that were associated with RA and were initially described in the 1940's when Waaler (80) and Rose *et al.* (81) demonstrated that sera from RA patients could promote the agglutination of sheep red blood cells that were sensitized with rabbit IgG. RFs are autoantibodies (of several isotypes) that recognize the Fc portion of human IgG (82), and were included in the diagnostic criteria for RA in 1987 (83). RFs have been shown to be present in approximately 50-65% of patients with early RA (27,84) and in up to 90% of patients with longstanding disease (84,85). However, RFs are not specific for RA; RFs have been shown to be found in patients with other autoimmune diseases as well as in healthy individuals (30,84,86). Despite the evidence that RFs are not specific for RA, they have been associated with RA activity. RFs have been reported to predict the development of RA (87) and have been associated with radiographic progression (88,89).

1.3.2   Anti-citrulline antibodies

Anti-citrulline antibodies (a general term for anti-citrullinated protein/peptide antibodies; ACPA) are highly characteristic of and are specific for RA. Citrullination is a normal post-translational process that involves the enzymatic conversion of positively charged

arginine residues into non-charged citrulline residues (Figure 1.1A). The importance of this arginine-to-citrulline modification for the binding of peptides to SE-encoded HLA-DR molecules was first reported in the aforementioned study of the SE by Hill *et al*. (64) and was corroborated by the study of Scally *et al* (59). The process of citrullination is mediated by the peptidylarginine deiminase (PAD) enzyme family, of which five isozymes are expressed in humans (90). PAD enzymes are expressed ubiquitously and can be found in many different cell and tissue types. PAD2 and PAD4 in particular are expressed by cells of the immune system (90) and have been demonstrated to be in RA synovial tissue (91–93). It is unknown whether these two PAD isozymes contribute equally or if one of them is more important in the generation of citrullinated antigens in RA. The antigen(s) responsible for driving inflammation in RA remains unknown, though several candidate citrullinated autoantigens have been identified in RA joints including fibrinogen (94), vimentin (95,96), type II collagen (97) and α-enolase (92,98).

Schellekens *et al*. were the first to identify that citrulline was the target of anti-citrulline antibodies (99). Prior to this report, these RA-associated autoantibodies were described as anti-perinuclear factor (100), anti-keratin antibodies (101) and anti-filaggrin antibodies (102). As previously noted, anti-citrulline antibodies are commonly measured clinically using cyclic citrullinated peptide(s) (CCP) and have been included as a diagnostic marker for RA alongside RF (103). These antibodies are detected in approximately 70% of RA patients and are almost 100% specific for RA (26,104,105). Further, anti-CCP have been shown to precede the onset of arthritic symptoms as well as predict RA development (29,106,107). Anti-citrulline antibodies are also believed to be arthritogenic because RA patient sera (108) and purified human IgG anti-citrulline antibodies (109) have been reported to induce arthritis in young FcγRIIB-deficient mice.

1.3.3   Anti-homocitrulline antibodies

Anti-homocitrulline antibodies (a general term for anti-homocitrullinated protein/peptide antibodies; AHCPA), also known as anti-carbamylated protein antibodies (anti-CarP), are a family of antibodies that were reported to be associated with RA in 2010. Turunen *et al*. showed that the immunization of rabbits with homocitrullinated albumin and type I
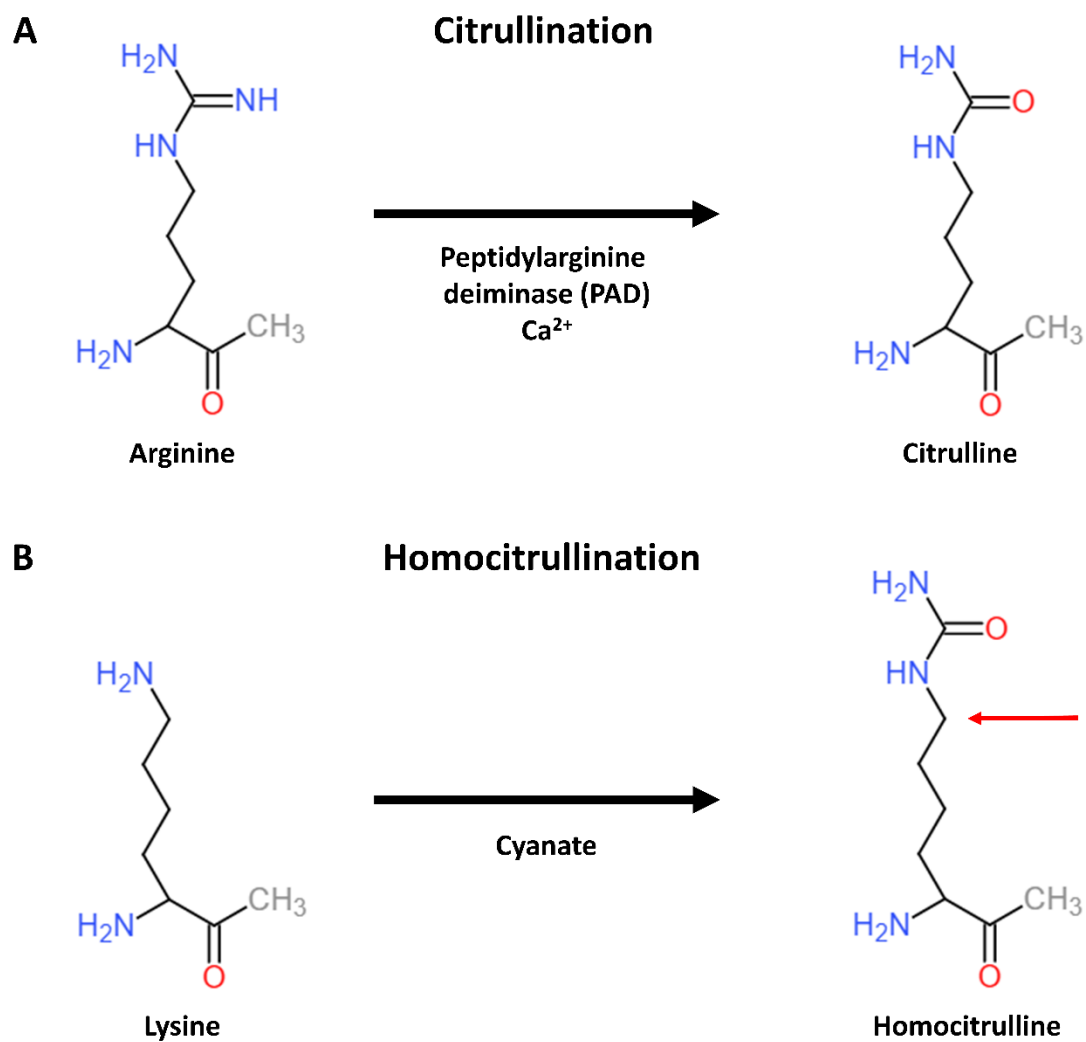
Figure 1.1. Citrullination and homocitrullination. The post-translational conversion of arginine to citrulline (citrullination; A) and the conversion of lysine to homocitrulline (homocitrullination; B). The chemical structures of homocitrulline and citrulline are similar. The difference between these residues is that homocitrulline possesses an additional carbon in its side chain (red arrow).

collagen induced RA-associated autoantibodies (110). Also, evidence that homocitrulline may play a role in RA pathogenesis was reported by Mydel *et al.*, who demonstrated that homocitrulline-containing peptides derived from filaggrin induced inflammatory arthritis in mice (111). The susceptibility to arthritis in these mice varied depending on the strain, suggesting that the susceptibility to arthritis is influenced by the MHC class II haplotype (111).

Anti-homocitrulline antibodies are frequently detected in RA patients and have garnered interest as a potential biomarker for RA. However, the anti-homocitrulline immune response remains poorly understood in RA. The genetic factors (eg. HLA-DR) that contribute to this response have not been characterized. The *in vivo* antigenic target of these antibodies is unknown and as a consequence, different systems of homocitrullinated antigens are currently being used to screen for these antibodies (mentioned in further detail below).

1.3.3.1　Homocitrulline

Anti-homocitrulline antibodies target proteins/peptides that have undergone a post-translation process known as homocitrullination (also called carbamylation). In this process, the ε-amino group of a peptidyl-lysine residue is modified to generate homocitrulline (45) (Figure 1.1B). Homocitrulline shares a similar chemical structure with citrulline. Both homocitrulline and citrulline are polar, neutrally-charged amino acids and both of these residues possess an ureido functional group. The only difference between citrulline and homocitrulline is that the homocitrulline side chain is extended by an additional carbon.

The process of homocitrullination is mediated chemically by cyanate, which can be generated in the body through the spontaneous degradation of urea; cyanate and urea exist in equilibrium in solution (45). Thus, low levels of cyanate are present under physiological conditions. Thiocyanate is a metabolite that is elevated in the serum of smokers (112), and the oxidation of thiocyanate is also able to produce cyanate (45). The oxidation of thiocyanate has been associated with the activity of myeloperoxidase (45), which is an enzyme that is abundantly expressed in neutrophils and monocytes. In a

mouse model of peritonitis (inflammation of the peritoneum), Wang *et al.* reported higher levels of homocitrullinated proteins in wild-type mice compared to myeloperoxidase-deficient mice (45) and there is also evidence that myeloperoxidase and homocitrulline are found in the synovial tissue of RA patients (93). These observations demonstrate an association between inflammation, homocitrullination and RA.

1.3.3.2 Anti-carbamylated fetal calf serum antibodies

Anti-homocitrulline antibodies were first detected in RA patients in 2011 by Shi *et al.* who screened for antibodies reactive to carbamylated fetal calf serum (anti-CarP-FCS) (113). Fetal calf serum is a mixture of proteins of bovine origin, thus CarP-FCS likely does not correspond to *in vivo* homocitrullinated antigens in RA patients. However, CarP-FCS remains a commonly used antigen for the detection of anti-homocitrulline antibodies.

The prevalence of anti-CarP-FCS in RA patients is estimated to be 30-45% (46,113–115). These antibodies are mainly found in patients who are also positive for anti-CCP, but can also be detected in a small proportion of anti-CCP-negative RA patients (46,113). Similar to RF and anti-CCP, anti-CarP-FCS can be present in individuals prior to the onset of RA (116,117), and are also predictive of more severe disease outcomes (113,118). Anti-CarP-FCS have been shown to also be expressed in other inflammatory arthritides and connective tissue diseases (114,119–122). This has led to the notion that anti-CarP-FCS may be a biomarker of inflammation rather than being specific for RA, as proposed by Reed *et al* (123). However, the expression of anti-CarP-FCS in other inflammatory (non-rheumatologic) conditions is yet to be examined.

1.3.3.3 Anti-homocitrullinated fibrinogen antibodies

Homocitrullinated fibrinogen as a candidate protein antigen for anti-homocitrulline antibodies was first studied by Scinocca *et al.* (124), based on the evidence that citrullinated forms of fibrinogen were located in RA synovial tissue (94) and are the target of anti-citrulline antibodies in RA patients (28,94,125). Scinocca *et al*. reported that human fibrinogen is accessible to homocitrullination; 89 of 103 lysines (in

fibrinogen) were able to undergo homocitrullination *in vitro* (124). Anti-homocitrullinated fibrinogen antibodies have been shown to exist as several isotypes in RA patients. IgG anti-homocitrullinated fibrinogen antibodies were reported to be the predominant isotype, having been found in approximately 30-45% of RA patients (124,126) as well as in up to 20% of first-degree relatives of RA patients (126). The prevalence of IgM and IgA anti-homocitrullinated fibrinogen antibodies were noted to be lower, and were present in 20% and 7% of RA patients respectively (126). In addition, Scinocca *et al.* reported that anti-homocitrullinated fibrinogen antibodies were highly specific for RA as these antibodies were not detected in patients with systemic lupus erythematosus nor in patients with psoriatic arthritis (124).

1.3.3.4 Antibodies to other homocitrulline-containing antigens

Since anti-homocitrullinated antibodies have been described, several other homocitrullinated peptide antigens have also been used for antibody screening. So far, homocitrulline-containing peptides derived from proteins that are commonly found in the synovial tissue of RA patients have been examined, including vimentin (127), collagen (128), and α-enolase (123). The frequency of IgG antibodies to these homocitrullinated peptides in RA patients is approximately 20-35% (123,127,128).

1.4 Immunological relatedness of citrulline and homocitrulline

Citrulline and homocitrulline are structurally similar amino acids and the idea that these amino acids are immunologically related was first brought up by Turunen *et al.* in 2010. They were the first to show that a commonly employed commercial antibody (also known as the Senshu antibody) for the detection of citrulline, also recognized homocitrulline (110). They also found anti-citrulline antibodies following the immunization of rabbits with homocitrullinated proteins (110). In contrast, Mydel *et al.* found that antibodies to homocitrulline and citrulline in mice were specific for the homocitrullinated or citrullinated peptide used for immunization (111).

Human studies have shown that anti-homocitrulline antibodies and anti-citrulline antibodies are often co-expressed in RA (46,113,114,123). RA patients frequently express the SE (60–62), which has been shown to accommodate citrulline and homocitrulline similarly (124). This event could initiate citrulline-specific, homocitrulline-specific or cross-reactive immune responses in RA.

1.4.1   Anti-citrulline and anti-homocitrulline antibody cross-reactivity in RA patients

Several studies have examined the cross-reactivity of anti-citrulline and anti-homocitrulline antibodies in RA patients (113,123,124,127–129), with varying degrees of cross-reactivity between these antibodies being reported. The differences in the observed cross-reactivity between anti-citrulline and anti-homocitrulline antibodies have led to the two opposing views that these antibodies constitute a single family of antibodies that are reactive to both citrulline and homocitrulline (cross-reactive), or that anti-citrulline and anti-homocitrulline antibodies are distinct antibody systems (non-cross-reactive).

The first studies on the cross-reactivity of anti-citrulline and anti-homocitrulline antibodies in RA patients were conducted by Shi *et al*., using citrullinated and homocitrullinated forms of FCS (113) and later, fibrinogen (129). Two studies that were conducted by Shi *et al.* found differing levels of cross-reactivity between anti-citrulline and anti-homocitrulline antibodies, depending on the antigens that they used (113,129). In one study, they reported that anti-CCP and anti-CarP-FCS were not cross-reactive to citrullinated and homocitrullinated FCS (113). In another study, they used homocitrullinated fibrinogen and they showed that antibodies to this antigen were cross-reactive in some RA patients because the binding of these antibodies to homocitrullinated fibrinogen could be inhibited by citrullinated fibrinogen (129). Using the same antigen (fibrinogen) and method, Scinocca *et al.* also showed cross-reactivity of anti-homocitrullinated fibrinogen antibodies, but in the majority of RA patients that they examined (124).

A limitation of these studies however, was the use of modified (homocitrullinated or citrullinated) proteins as a target of anti-citrulline and anti-homocitrulline antibodies. As

reported by Scinocca *et al.* (124), proteins such as fibrinogen differ in the number of arginine and lysine residues, and this could result in different numbers of citrulline or homocitrulline residues following each modification. Differences in the position of citrulline and homocitrulline in the protein sequence could also affect citrulline and homocitrulline cross-reactivity because the backbone surrounding these residues are likely to be different and this could influence antibody binding. Thus, more recent studies on the cross-reactivity of anti-citrulline and anti-homocitrulline antibodies have utilized citrulline- and homocitrulline-containing peptides (123,127,128) instead of proteins. Juarez *et al*. (127) and Turunen *et al.* (128) have found only partial overlap in the specificities of antibodies to citrullinated and homocitrullinated peptides of vimentin and collagen, whereas Reed *et al*. (123) showed significant cross-reactivity using a peptide of α-enolase. Thus, the evidence of the relatedness of anti-citrulline and anti-homocitrulline antibodies is controversial, and the identity of these antibodies as a single (cross-reactive) or discrete (non-cross-reactive) antibody families remains unresolved.

1.4.2   Anti-citrulline and anti-homocitrulline antibody cross-reactivity in animal models

Evidence of cross-reactive citrulline and homocitrulline immune responses in experimental animal models has been explored by only two groups. Turunen *et al.* examined the cross-reactivity of citrulline and homocitrulline immune responses in rabbits (110,130). They showed that rabbits that were immunized with homocitrullinated proteins (albumin and type I collagen) generated antibodies to the proteins used in immunization as well as antibodies to citrullinated antigens, including CCP, citrullinated vimentin and citrullinated telopeptides of type I and type II collagen (110,130). In some of these rabbits, the antibody binding to citrulline-containing peptides of type I collagen could be inhibited by similar peptides which contained homocitrulline residues instead of citrulline (110,130). Similarly, the immunization of rabbits with citrullinated peptides (derived from type I and type II collagen) generated antibodies to citrullinated and homocitrullinated peptides (130). Thus these studies by Turunen *et al* showed that anti-citrulline and anti-homocitrulline antibodies are cross-reactive.

Mydel *et al*. examined the cross-reactivity of anti-homocitrulline and anti-citrulline immune responses in mice (111). They showed that mice immunized with a homocitrullinated filaggrin peptide developed antibodies to this homocitrullinated peptide, but which did not cross-react with its citrullinated counterpart. These mice also developed arthritis when given intra-articular injections of citrullinated filaggrin peptide despite not having antibodies to this antigen. The mechanism underlying the induction of arthritis in these mice is not known.

1.5   Rationale, hypothesis and objectives

RA is associated with immune responses to citrulline and more recently, with anti-homocitrulline immune responses. Homocitrulline is structurally similar to citrulline, thus these amino acids may be recognized similarly by the immune system.

In RA patients, studies on the relatedness of anti-citrulline and anti-homocitrulline antibodies have reported discrepant findings on the cross-reactivity of these antibodies. The degree of cross-reactivity has been found to differ depending on the antigen of study (113,123,124,127–129). In experimental animal models, cross-reactive anti-citrulline and anti-homocitrulline immune responses have been shown in rabbits (110,130) but not in mice (111). Thus the relatedness of the anti-citrulline and anti-homocitrulline immune response remains controversial.

In addition, immune responses to citrulline have been associated with the SE, the strongest genetic risk factor for RA (79). SE-encoded HL-DR molecules have been reported to bind to citrulline-containing peptides, resulting in T and B cell responses to these antigens in DR4tg mice (66). It is unknown whether the SE plays a similar role in the development of anti-homocitrulline immune responses.

This study attempts to address some of these issues using RA patients and in a mouse model of RA (DR4tg mice). We examined the anti-homocitrulline and anti-citrulline immune response using a synthetic homocitrulline-rich peptide, HomoCitJED, and its citrulline-containing counterpart, CitJED. These peptides share the same number of homocitrulline or citrulline residues and share the same peptide backbone.

The hypothesis of this study is that the anti-homocitrulline and anti-citrulline immune responses are related and are specific for RA. Anti-homocitrulline and anti-citrulline antibodies are cross-reactive and their expression is dependent on the SE.

To test this hypothesis, the objectives of this study are to examine:

i) The frequency and specificity of antibodies to a homocitrulline-containing peptide (HomoCitJED) and its citrulline-containing counterpart (CitJED) in RA patients.

ii) The cross-reactivity of these antibodies to HomoCitJED and CitJED in RA patients.

iii) The development of T and B cell responses to HomoCitJED in (SE-positive) DR4tg mice and (SE-negative) wildtype B6 mice following immunization with HomoCitJED.

iv) The cross-reactivity of the immune responses in HomoCitJED-immunized DR4tg and B6 mice.

1.6   References

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Chapter 2

2 Relatedness of antibodies to peptides containing homocitrulline or citrulline in patients with rheumatoid arthritis

This chapter is under review for publication in The Journal of Rheumatology.

2.1 Introduction

Rheumatoid arthritis (RA) is an inflammatory autoimmune disorder that predominantly affects the joints and is specifically associated with anti-citrullinated protein/peptide antibodies (ACPA) (1–4). Recently, anti-homocitrullinated protein/peptide antibodies (AHCPA), also known as anti-carbamylated protein antibodies (anti-CarP) have been identified in RA patients (5–9). Both ACPA and AHCPA can occur prior to disease onset (6,10,11) and are predictive of more severe disease (8,12). ACPA have been demonstrated to be arthritogenic (13,14); the role of AHCPA in RA pathogenesis is unknown.

ACPA and AHCPA target citrullinated and homocitrullinated antigens, which arise from the post-translational modification of arginine and lysine respectively. Citrulline and homocitrulline have identical chemical structures, except homocitrulline has an additional carbon (15). Studies show that ACPA can detect proteins containing homocitrulline (5,16). However, the degree of cross-reactivity of ACPA for homocitrullinated proteins varied between the studies. These discrepancies may be due to proteins having different numbers and locations of citrulline and homocitrulline, as we have previously reported for fibrinogen (5), potentially resulting in different peptide antigens. Studies using citrulline and homocitrulline-containing peptides with the same backbone were inconclusive because the affinity of antibodies to citrullinated and homocitrullinated peptides were not investigated (17–19).

In this study, we used a synthetic citrulline-rich peptide, JED (referred to here as CitJED) and its homocitrullinated counterpart (HomoCitJED). These peptides have a high

proportion of citrulline and homocitrulline residues (9/18) rendering immune reactivity to the backbone unlikely. Although not derived from an endogenously expressed autoantigen, we showed that CitJED captures clinically relevant antibody reactivities from RA patients (anti-cyclic citrullinated peptide and anti-citrullinated fibrinogen antibodies) (5). Herein we employed CitJED and HomoCitJED to examine the expression, relatedness and relative affinity of antibodies to citrullinated and homocitrullinated peptides in RA patients.

2.2 Materials and methods

2.2.1 Study population

Serum samples were collected from healthy subjects, and from RA patients attending a tertiary care Rheumatology clinic at St. Joseph's Health Care London (SJHC; London, ON, Canada). All RA patients (n=137) satisfied the American College of Rheumatology 2010 criteria for RA (20) and all were treated with disease-modifying antirheumatic drugs. The swollen joint count in RA was determined by the treating rheumatologist who performed a 64 joint examination; only swollen joints attributed to active RA were included in the count. RA patients without joint swelling were considered to be in remission. Demographic, serological and clinical characteristics of RA patients are summarized in Table 2.1. Healthy subject serum samples (n=51) were collected from accompanying individuals who were unrelated to the RA patients, and from staff members at SJHC or The University of Western Ontario (London, ON, Canada). Healthy subjects were 65% female with a median age of 45 years (range 21-74). Healthy subjects did not have autoimmune disease, and were excluded from the study if they had a history of joint symptoms.

Systemic Lupus Erythematosus (SLE) and Psoriatic Arthritis (PsA) patients were previously described (5). SLE patient serum samples were collected from patients attending SJHC (n=37). All of these patients satisfied the American College of Rheumatology criteria for SLE (21). SLE patients were 89% female with a mean age of 53 years (range 24-81). Of the 37 SLE patients, 2 also had inflammatory arthritis and one

Table 2.1. Demographic, serological and clinical characteristics of RA patients

	RA Patients (n=137)
Demographic Features	
No. (%) Female	100 (73)
Age, median (range) years	59 (19-82)
Disease duration, median (range) years	4 (0.17-51)
IgG anti-CCP2	
No. positive (%)	87 (64)
Mean concentration, (range) (Units/mL)	121.3 (6.0-475.0)
Rheumatoid Factor	
No. positive (%)	83 (61)
Smoking History[a]	
No. (%) with smoking history	81 (61)
Joint Symptoms[b]	
No. (%) with joint swelling	57 (47)
Average No. of swollen joints	7.2

a   Data available for 132 RA patients

b   Data available for 121 RA patients; Joint swelling was determined by the treating rheumatologist and was associated with active RA disease activity

had secondary Sjögren's syndrome. PsA patient sera were collected from SJHC (n=10) or were provided by Dr. Dafna Gladman from the Centre for Prognosis Studies in the Rheumatic Diseases, Toronto Western Hospital (n=27), all of whom either met CASPAR criteria for PsA (22) or were diagnosed as having PsA by a rheumatologist in a specialized PsA clinic. PsA patients were 46% female with a mean age of 53 years (range 36-82). This study was approved by the Human Ethics Committee of The University of Western Ontario (London, ON, Canada) according to the Declaration of Helsinki (REB #09684E and #100868).

2.2.2   Antigens

The following peptide antigens were used for antibody assays: i) JED, referred to here as CitJED, a proprietary synthetic, cyclic peptide, 18 amino acids in length with 9 residues of citrulline (5,23,24); and ii) Homocitrullinated JED (HomoCitJED), identical to CitJED except that all 9 citrulline residues were replaced with homocitrulline. The sequences of both peptides were confirmed by mass spectrometry and the purity was greater than 85%, as provided by the supplier.

CitJED and HomoCitJED were synthesized by Creative Peptides (Shirley, NY, USA). CitJED and HomoCitJED were dissolved in 15% HCl in sterile distilled water for direct Enzyme-Linked Immunosorbent Assay (ELISA) and for inhibition ELISAs. HomoCitJED was dissolved in 15% HCl in sterile distilled water to increase its solubility. Although water soluble, CitJED was also dissolved in 15% HCl in order to compare the antibody-binding properties of CitJED and HomoCitJED. This treatment did not alter peptide structure as determined by mass spectrometry and ELISA (data available upon request).

2.2.3   Antibody assays

Human sera were tested for expression of IgG anti-CitJED and IgG anti-HomoCitJED using direct antibody-binding ELISA. The protocol was adapted from Hill *et al* (4). Wells of ELISA plates (Nunc Maxisorp, VWR) were coated overnight at 4˚C with 2 μg of peptide (20 μg/mL; CitJED or HomoCitJED) per well in carbonate coating buffer, pH

9.6. The wells were washed with wash buffer (PBS, 0.05% Tween) and blocked for 30 minutes at room temperature (RT) with 200 µl of blocking buffer (PBS, 0.1% BSA). Serum samples were diluted 1:100 or higher in diluent (PBS, 0.1% BSA, 0.05% Tween). 100 µl of diluted sera were added to duplicate wells and incubated for 30 minutes at RT. After washing, 100 µl of biotin-conjugated goat anti-human IgG (1:8000; Sigma) and streptavidin horseradish peroxidase polymer (1:4000; Abcam) were added for 30 minutes at RT. After washing again, 100 µl of 3,3',5,5'-Tetramethylbenzidine substrate (Sigma) was added to the wells and incubated for 10 minutes at RT. The reaction was stopped with 50 µl of 1M H_2SO_4 and the absorbance was determined at 450 nm.

Anti-CitJED and anti-HomoCitJED antibody concentrations in tested serum samples were determined by comparison to a reference serum sample, which was positive for both antibodies. These concentrations were quantified in Arbitrary Units/mL (AU/mL). The cut-off values for positive anti-CitJED and anti-HomoCitJED expression were 3.8 AU/mL and 25.3 AU/mL respectively. The cut-off values represent the mean antibody concentration of healthy subjects + 2 standard deviations. Anti-CitJED and anti-HomoCitJED concentrations are reported as an average of at least 3 repeat determinations (<20% inter-assay variation). The number of patients with antibody levels greater than twice the upper limit of normal were also reported, similar to ACR criteria (20). IgG anti-CCP2 and IgM Rheumatoid Factor (RF) concentrations were determined by Pathology and Laboratory Medicine, London Health Sciences Centre (London, ON, Canada) or by our laboratory using anti-CCP2 (Euroimmun) or RF (Inova Diagnostics) ELISA kits.

Cross-reactivity of anti-CitJED and anti-HomoCitJED was determined by inhibition ELISA. The protocol was adapted from Scinocca *et al* (5). Various amounts of soluble peptide in diluent were incubated with diluted RA patient sera for 1 hour at RT prior to addition to ELISA wells coated with CitJED or HomoCitJED and carried out as described for direct antibody-binding ELISA. Inhibition assays were repeated at least 2 times for each patient. Sera were selected for inhibition ELISA based on antibody concentration which were a minimum of 10 AU/mL for anti-CitJED and 70 AU/mL for anti-HomoCitJED. These minimum concentrations were required to obtain a dose-

dependent, reproducible inhibition. Anti-CitJED and anti-HomoCitJED were considered as cross-reactive if inhibition with the non-cognate peptide was equal or higher than 50% at any inhibiting peptide concentration.

2.2.4   Statistical analysis

Graphpad Prism 6.0 software was used for data analysis. Fisher's exact test was used for comparison of categorical values. The Kruskal-Wallis Test with Dunn's multiple comparison was used to compare expression of anti-CitJED and anti-HomoCitJED in RA patients to healthy subjects, SLE patients, and PsA patients ($p<0.005$ was considered significant). Correlation between anti-CitJED and anti-HomoCitJED expression was tested using Spearman's correlation. Two-way ANOVA with Bonferonni correction was used for statistical analysis of inhibition experiments ($p<0.01$ was considered significant).

2.3   Results

2.3.1   Occurrence of anti-CitJED and anti-HomoCitJED antibodies in RA patients, healthy subjects and patients with SLE or PsA

IgG antibodies reactive to CitJED and HomoCitJED were assessed in 137 RA patients, 51 healthy subjects, 37 SLE patients, and 37 PsA patients. Anti-CitJED levels were significantly higher in RA patients compared to other groups ($p<0.0001$; Figure 2.1A). Anti-CitJED was expressed in 69/137 (50%) of RA patients; 54/69 (78%) of these RA patients had anti-CitJED levels greater than twice the cut-off. Anti-CitJED was detected in 2/51 (4%) of healthy subjects, and was not detected in patients with SLE or PsA. Anti-HomoCitJED was also detected at elevated levels in RA patients compared to other groups ($p<0.0001$; Figure 2.1B). Of the RA patients, 78/137 (57%) were positive for serum anti-HomoCitJED with 49/78 (63%) of these anti-HomoCitJED positive RA patients having antibody levels greater than twice the cut-off.   In contrast, only 4/51 (8%) of healthy subjects had low levels of anti-HomoCitJED. Anti-HomoCitJED was not found in PsA, but was detected in 1 patient with SLE with a concentration of 162.6 AU/mL. This SLE patient was felt to have an overlap connective tissue disease with
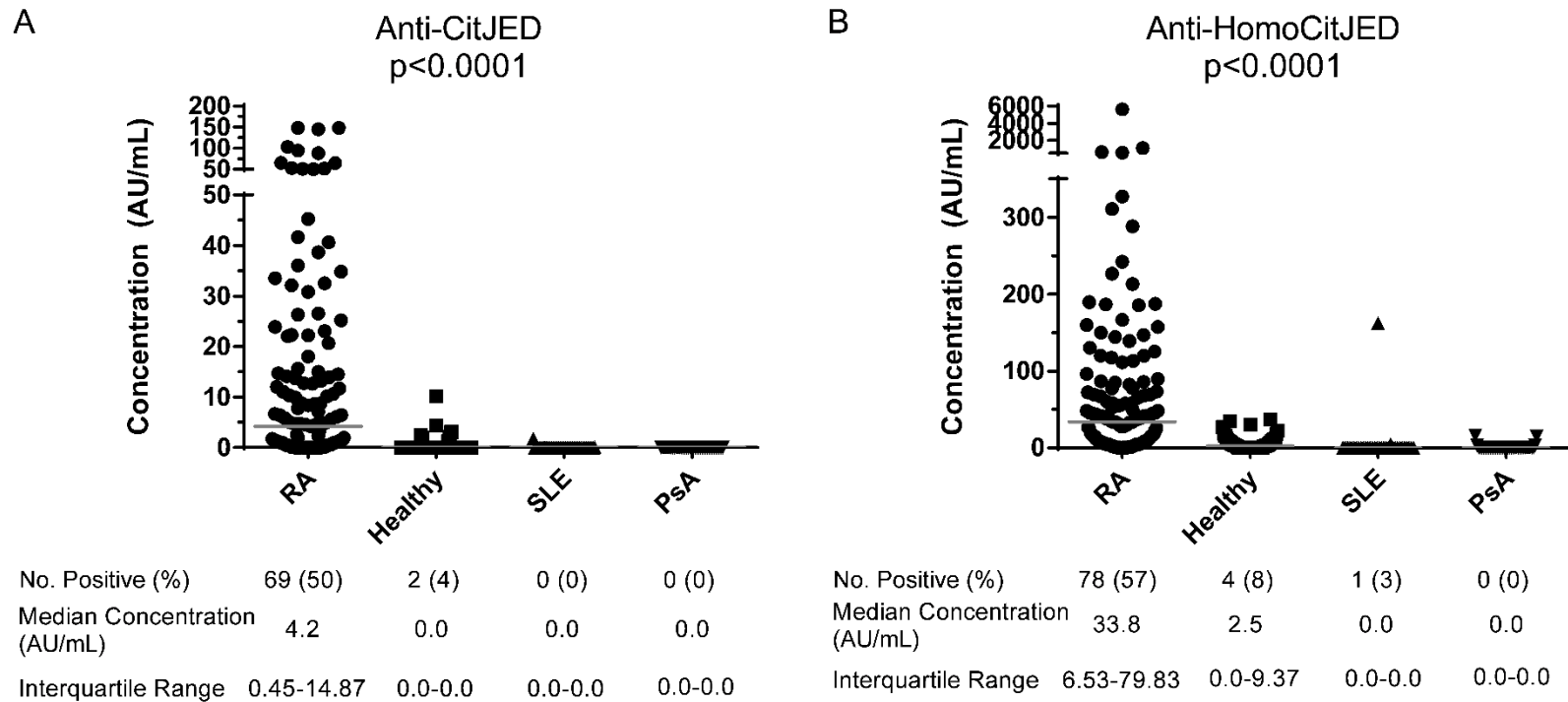
Figure 2.1. Serum anti-CitJED and anti-HomoCitJED levels are increased in RA patients. Serum anti-CitJED (**A**) and anti-HomoCitJED (**B**) were screened in RA patients (n=137), healthy subjects (n=51), SLE patients (n=37) and PsA patients (n=37). Cut-off values were 3.8 AU/mL for anti-CitJED and 25.3 AU/mL for anti-HomoCitJED. Grey bars represent the median concentration. Number and percentages of positive sera, median concentrations and the interquartile range are shown below the respective group.

features of both SLE and RA given that this patient had erosive inflammatory arthritis and was also anti-CCP2 positive. These results indicate that anti-CitJED and anti-HomoCitJED antibodies are frequently present at high concentrations in RA patients, and infrequently found in healthy subjects, SLE or PsA patients.

2.3.2 Co-expression of anti-CitJED and anti-HomoCitJED antibodies in RA patients

Forty-eight of 137 (35%) RA patients were negative for both anti-CitJED and anti-HomoCitJED, while 58/137 (42%) of RA patients were positive for both antibodies. Eleven of 137 (8%) and 20/137 (15%) of RA patients were single positive for anti-CitJED or anti-HomoCitJED, respectively. Thus, anti-CitJED and anti-HomoCitJED have a concordance of 77%. A strong correlation was found in the levels of serum anti-CitJED and anti-HomoCitJED (Spearman rs=0.6676, p<0.0001; Figure 2.2) and we observed an association between anti-CitJED and anti-HomoCitJED antibody expression with an odds ratio of 12.65 (95% CI: 5.52-29.00). The levels of anti-HomoCitJED in patients with anti-CitJED and anti-HomoCitJED were significantly greater compared to patients who had anti-HomoCitJED alone (p<0.001, data not shown).

The expression of anti-CitJED and anti-HomoCitJED in relation to anti-CCP2 is shown in Figure 2.3. Eighty-seven of 137 (64%) RA patients were positive for anti-CCP2. Of the anti-CCP2 positive RA patients, 53/87 (61%) expressed anti-CitJED and 58/87 (67%) were positive for anti-HomoCitJED. In the 50 RA patients who lacked anti-CCP2, 16/50 (32%) had anti-CitJED and 20/50 (40%) had anti-HomoCitJED. Only 6/137 (4%) of RA patients were positive for anti-HomoCitJED and negative for both anti-CitJED and anti-CCP2; Only 1 of these patients had anti-HomoCitJED levels greater than twice the cut-off. Thirty-six of 137 (26%) RA patients were negative for both anti-CCP2 and/or RF. In this latter subpopulation, 12/36 (33%) of RA patients expressed anti-CitJED and/or anti-HomoCitJED; 2/36 (6%) only expressed anti-CitJED, 2/36 (6%) only expressed anti-HomoCitJED and 8 expressed both antibodies).
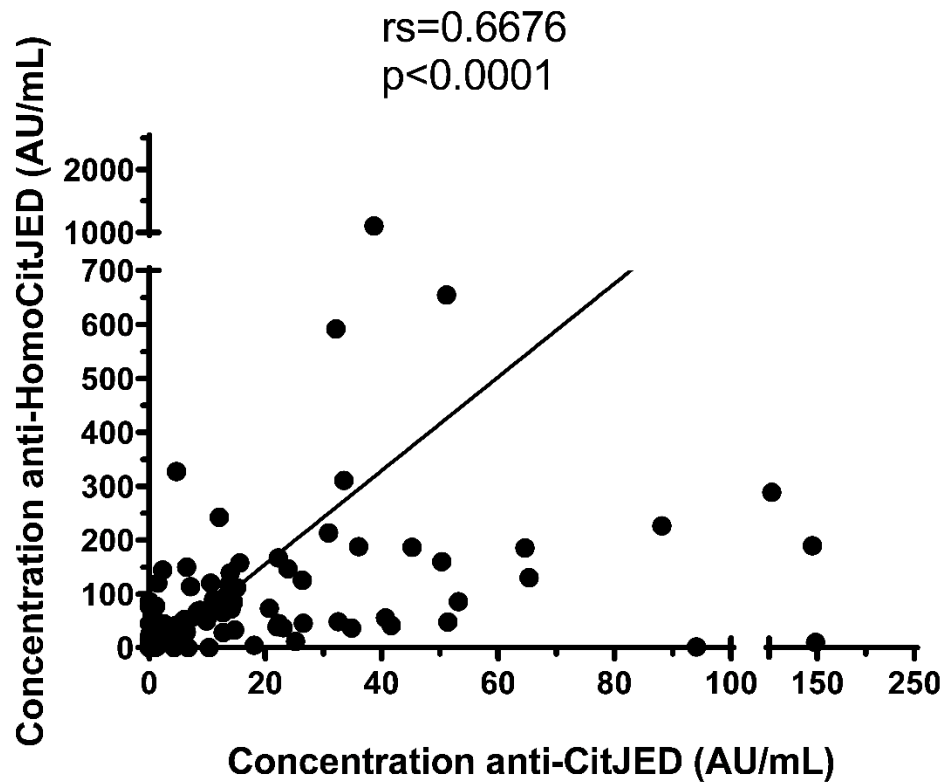
Figure 2.2. Serum anti-CitJED and anti-HomoCitJED levels are correlated in RA.
Correlation graph comparing serum anti-CitJED and anti-HomoCitJED concentrations of
RA patients (n=137). The levels of IgG anti-CitJED and anti-HomoCitJED antibodies
were compared by Spearman nonparametric correlation.

A   anti-CCP2 positive RA Patients (n=87)

B   anti-CCP2 negative RA Patients (n=50)

- CitJED-/ HomoCitJED-
- CitJED+/ HomoCitJED+
- CitJED+/ HomoCitJED-
- CitJED-/ HomoCitJED+
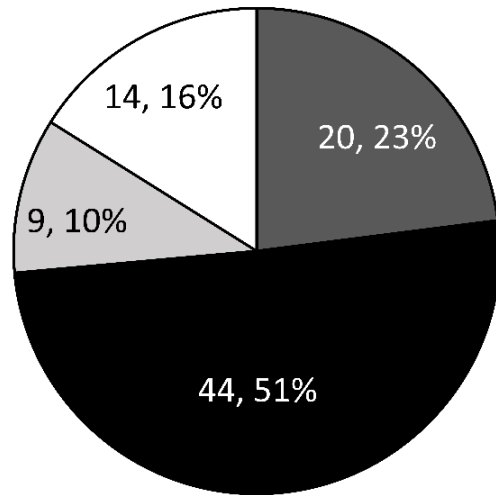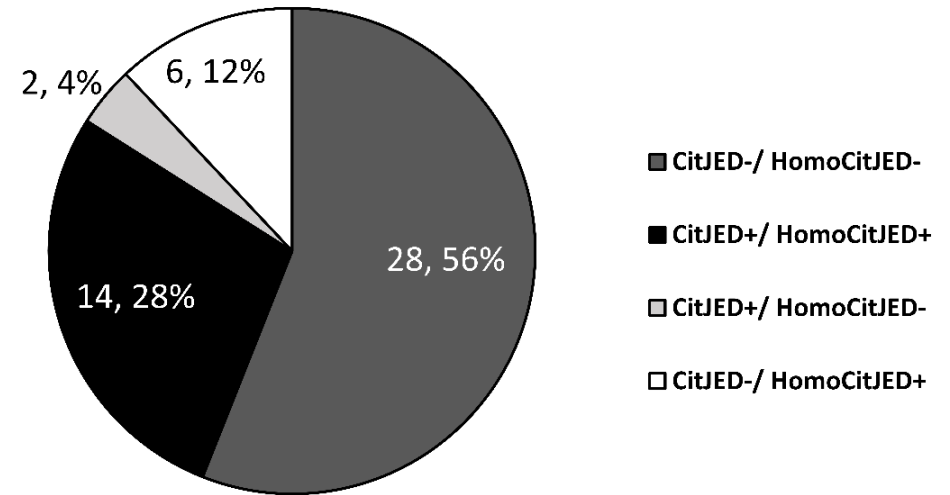
Figure 2.3. Anti-CitJED and anti-HomoCitJED are found in anti-CCP2 positive and anti-CCP2 negative RA patients. Frequency and percentage of anti-CitJED and anti-HomoCitJED subpopulations in anti-CCP2 positive RA patients (**A**) and anti-CCP2 negative RA patients (**B**).

2.3.3 Cross-reactivity of anti-CitJED and anti-HomoCitJED antibodies

To examine the cross-reactivity of anti-CitJED and anti-HomoCitJED, we performed inhibition ELISAs on RA sera (Figure 2.4) which met required anti-CitJED levels of 10 AU/mL and/or anti-HomoCitJED levels of 70 AU/mL as described in Materials and methods. We examined 15 anti-CCP2 positive and 2 anti-CCP2 negative RA patients that expressed both anti-CitJED and anti-HomoCitJED (referred to here as double positive) (Figure 2.4A, Figure 2.4B, Appendix 1, and Appendix 2) We also examined 5 patients that were anti-CitJED positive but anti-HomoCitJED negative (anti-CitJED single positive) (Figure 2.4C and Appendix 3) and 5 patients who were anti-HomoCitJED positive but anti-CitJED negative (anti-HomoCitJED single positive) (Figure 2.4D and Appendix 3). These single positive patients were also positive for anti-CCP2. None of the sera from single positive patients who were anti-CCP2 negative met the required antibody concentrations for the inhibition assay.

Anti-CitJED and anti-HomoCitJED from double positive and single positive RA patients were inhibited by both CitJED and HomoCitJED peptides (Figure 2.4). The binding affinity for CitJED and HomoCitJED peptides was examined by comparing the peptide concentrations of CitJED and HomoCitJED required to achieve 50% inhibition of antibody reactivity. The cognate peptide produced significantly greater inhibition of antibody reactivity ($p<0.0001$; Figure 2.4). In double positive and anti-CitJED single positive RA patients, inhibition exceeding 50% of anti-CitJED binding activity required 2 µg/mL of CitJED on average, compared to 100 µg/mL for HomoCitJED (Figure 2.4A and Figure 2.4C). Anti-CitJED from 2/5 anti-CitJED single positive patients showed little to no inhibition by HomoCitJED (Appendix 3). In double positive RA patients, inhibition of 50% of anti-HomoCitJED binding activity required 10 µg/mL of HomoCitJED compared to 50 µg/mL for CitJED (Figure 2.4B). In the group of anti-HomoCitJED single positive patients, 50% inhibition of anti-HomoCitJED activity required 50 µg/mL of HomoCitJED compared to 100 µg/mL of CitJED (Figure 2.4D). These results indicate that anti-CitJED and anti-HomoCitJED antibodies are cross-reactive, and have a higher inferred binding affinity to their respective cognate peptide.
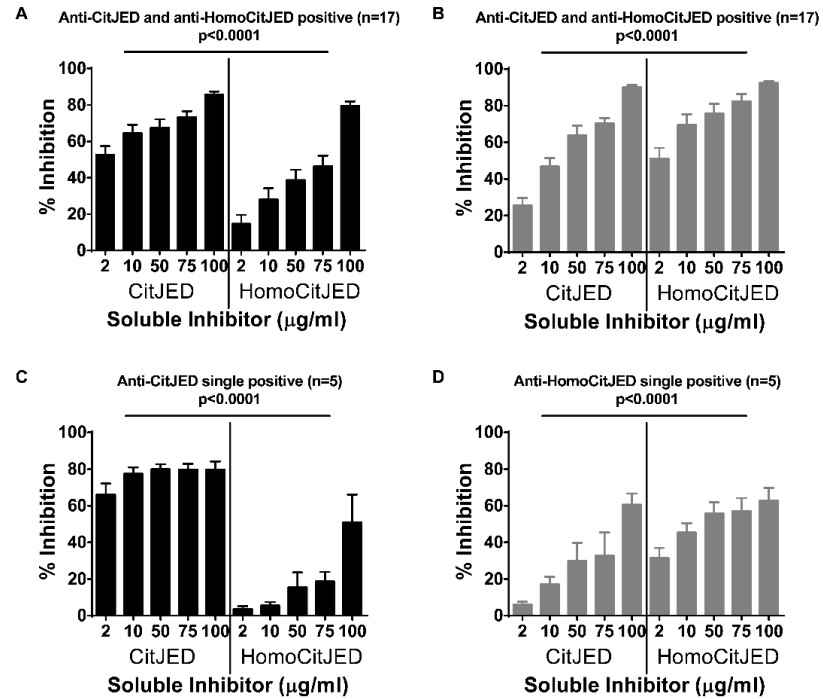
Figure 2.4. Anti-CitJED and anti-HomoCitJED from anti-CCP2 positive and anti-CCP2 negative RA patients are cross-reactive. Average inhibitions of anti-CitJED (**A**) and anti-HomoCitJED (**B**) from 15 anti-CCP2 positive and 2 anti-CCP2 negative RA patients who were positive for both anti-CitJED and anti-HomoCitJED. Average inhibitions of anti-CitJED from 5 anti-CitJED single-positive RA patients (**C**) and average inhibitions of anti-HomoCitJED from 5 anti-HomoCitJED single-positive RA patients (**D**). Anti-CitJED and anti-HomoCitJED single-positive RA patients had anti-CCP2. ELISA plates were coated with 20 µg/mL (2ug/well) of CitJED (**A,C**) or HomoCitJED (**B,D**). p<0.01 was considered significant by Two-way ANOVA.

2.4 Discussion

In this study, we used synthetic citrullinated and homocitrullinated peptides, containing equal numbers of citrulline or homocitrulline residues, to examine the expression pattern and cross-reactivity of ACPA (represented by anti-CitJED) and AHCPA (represented by anti-HomoCitJED) in RA patients. We found that antibodies reactive to CitJED, and HomoCitJED, were frequently expressed in the serum of RA patients. These antibodies were rarely found in healthy individuals and in patients with SLE or PsA. The expression of anti-CitJED and anti-HomoCitJED antibodies was 77% concordant. Additionally, we have demonstrated that anti-CitJED and anti-HomoCitJED are frequently cross-reactive in the patients studied and have higher affinity for the cognate peptide.

We detected IgG anti-HomoCitJED antibodies in 57% of RA patients, a higher proportion than what was reported for other antibodies targeting homocitrullinated proteins or peptides (19-45%) (8,17–19,25–27). Similarly, IgG anti-HomoCitJED was expressed in a significant proportion (28%) of anti-CCP2/RF negative RA patients. Antibodies to homocitrullinated proteins and peptides have been previously reported in anti-CCP2-negative RA patients to varying degrees (8,18,25–27). This variability may be attributed to several factors, including differences in the characteristics of the study populations (genetics, environmental exposures and disease activity). Consistent with a prior study, we did not observe a significant difference in IgG anti-HomoCitJED expression between RA patients with and without a history of smoking (data not shown) (25). Our RA population had longer disease duration compared to the other studies and therefore may have developed more AHCPA reactivity over time. We have also found that IgG anti-HomoCitJED were rarely found in healthy individuals and patients with SLE or PsA. The specificity of IgG anti-HomoCitJED for RA requires further study with other types of inflammatory conditions. Some studies have identified antibodies to carbamylated fetal calf serum (anti-CarP-FCS) in other inflammatory arthritides and connective tissue diseases (in up to 50% of patients) (26–30).

Prior studies did not examine the extent and affinity of antibody cross-reactivity of antibodies to citrullinated or homocitrullinated peptides, leading to the question of whether ACPA and AHCPA are part of a single antibody family or 2 separate antibody systems. We performed detailed inhibition experiments, allowing for analyses of the prevalence and affinity of cross-reactive ACPA and AHCPA. Using CitJED and HomoCitJED peptides which have equal numbers of citrulline and homocitrulline residues respectively, we observed that anti-CitJED and anti-HomoCitJED antibodies were inhibited by both peptides in the majority of RA patients (25/27 of RA patients examined by inhibition ELISA), including those who were positive for only one of these antibodies by direct ELISA. The difference in antibody peptide-binding activity observed between direct ELISA and inhibition ELISA could be caused by lower peptide concentrations used in direct ELISA, or possibly due to conformational differences between soluble peptides and plate-bound peptides. These results suggest significant cross-reactivity between ACPA and AHCPA and support the notion that these antibodies are part of the same antibody family, as proposed by Reed *et al* (19). Cross-reactivity to citrullinated and homocitrullinated antigens has also been demonstrated in experimental animals. Turunen *et al.* showed that immunization with homocitrullinated antigens led to the development of anti-citrulline reactivity in rabbits (15). Furthermore, Mydel *et al.* observed the development of erosive arthritis in mice, immunized with a homocitrullinated filaggrin peptide followed by the introduction of citrullinated filaggrin peptide into the knee (31).

In RA, it is not known whether citrullinated or homocitrullinated antigens (or both) are responsible for the initiation of autoantibody development. ACPA and AHCPA have been detected in patients years prior to disease onset (6,10,11,32). Furthermore, the anti-citrullinated protein antibody response has been demonstrated to undergo epitope spreading, resulting in an expansion of the ACPA repertoire over time (32). Because of the structural similarity between citrulline and homocitrulline, epitope spreading may lead to ACPA acquiring AHCPA specificities or vice versa. We observed that the degree of cross-reactivity varied between RA patients with the majority having higher affinity for the cognate antigen. This finding likely reflects the abundance of the cognate antigen, which drives B cell maturation towards the production of higher affinity antibodies to the

same antigen. We identified 2 patients that did not have cross-reactive antibodies who were both anti-CitJED positive, however, we did not determine whether these patients would later develop cross-reactivity to homocitrulline.

There are limitations to this study: measurements were performed at one time point, few patients had early disease and samples from pre-RA subjects were not available. Future longitudinal studies of early and pre-RA patients could provide a better understanding of the role of anti-homocitrulline and anti-citrulline antibodies in the initiation and progression of RA. Detailed clinical information was also not available; therefore, we could not assess the impact of the cross-reactivity and affinity of these antibodies on RA disease activity and severity.

In conclusion, antibodies to HomoCitJED and CitJED were frequently found in RA patients and were rare in other conditions. These findings demonstrate the immunologic relatedness of ACPA and AHCPA as detected by assays using CitJED and HomoCitJED peptides with equal numbers of citrulline and homocitrulline residues, and suggest that these antibodies are potentially derived from the same B cell population. Cross-reactive B cells may provide a mechanism by which AHCPA are involved in RA pathogenesis.

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Chapter 3

3 Immune responses to peptides containing homocitrulline or citrulline in the DR4-transgenic mouse model of rheumatoid arthritis

This chapter has been prepared for submission to The Journal of Autoimmunity.

3.1 Introduction

RA is characterized by the production of autoantibodies that target citrullinated proteins/peptides (ACPA). Clinically, the most commonly measured ACPA is anti-cyclic citrullinated peptide antibodies (anti-CCP2), which are highly specific for RA (1,2) and predictive of erosive joint disease (3,4). The susceptibility to ACPA-positive RA is significantly higher in individuals expressing HLA alleles (5,6) that encode a consensus sequence called the Shared Epitope (SE) (7). The SE binds to peptides containing citrulline with high affinity initiating citrulline-specific T and B cell responses (8,9). Target antigens for these immune responses are present in the joint, including citrullinated fibrinogen (10), α-enolase (11,12), vimentin (13,14) and collagen II (15). Evidence suggests that ACPA bind to these targets in the joint leading to complement activation and chronic inflammation (16). Experiments done in animal models further support the notion that T and B cell citrulline-specific immune responses are pathogenic (17–20).

It is known that some ACPA also bind peptides or proteins containing homocitrulline (21–24), an amino acid that is structurally related to citrulline and is also present in RA synovium (25,26). Antibodies to homocitrullinated antigens (also referred to as anti-carbamylated antibodies (anti-CarP)) have been detected in the sera of RA patients (21,27–30) and are associated with more severe disease (27,31). *In silico* evidence suggests that homocitrullinated peptides can be accommodated within the binding groove of the SE (21). However, the relationship between the SE and homocitrullinated peptides remains unclear. When immunized with homocitrullinated filaggrin-derived peptides and given an intra-articular injection of citrullinated peptide, different strains of mice

developed arthritis with varying frequency, suggesting that the MHC-H2 haplotype influences the development of arthritis (32). The objective of this study was to examine the role of the SE in the development of immune responses to a peptide containing homocitrulline using a humanized SE-expressing DR4-transgenic mouse model of RA.

3.2   Materials and methods

3.2.1   Antigens

The antigens used in this study were Citrullinated JED (CitJED) (21,33,34) and Homocitrullinated JED (HomoCitJED), which are synthetic cyclic peptides containing 18 amino acids with 9 residues of citrulline and homocitrulline, respectively. These peptides were synthesized by Creative Peptides (Shirley, NY, USA). Human fibrinogen (VWR) was left unmodified or was citrullinated or homocitrullinated for the antibody assays. Citrullination of fibrinogen was previously described by Hill *et al* (35) and homocitrullination was performed as per the method of Scinocca *et al* (21).

3.2.2   Mice and immunizations

DR4-IE transgenic mice deficient in endogenous MHC class II on the C57Bl/6 background (referred to as DR4tg) (17,36) were bred in house. C57Bl/6 mice (B6) mice were purchased from The Jackson Laboratory (Maine, USA). Both strains of mice were housed in pathogen-free conditions at the Animal Care and Veterinary Services barrier facility at the University of Western Ontario as per the Canadian Council on Animal Care guidelines. The study was approved by the Animal Care and Use Committee (The University of Western Ontario, London ON, Canada). Female and male DR4tg and B6 mice were immunized and boosted 21 days later with 100 μg of HomoCitJED suspended in PBS or PBS alone using the method of Hill *et al* (17).

3.2.3   Splenocyte proliferation

The splenocyte proliferation assay was adopted from Hill *et al* (17). In brief, splenocytes were harvested from mice sacrificed at various time points and cultured in complete RPMI media (Gibco) at a concentration of 4×10^5 splenocytes/well. Splenocytes were treated with 100 μg/mL of CitJED, HomoCitJED, or no peptide and incubated for 54

hours at 37°C, 5% CO_2. One μCi of ^{3}H-thymidine was added to each well and incubated for an additional 18 hours. Splenocytes were harvested using a Harvester96 (Tomtec) and radioactivity was measured with a MicroBeta JET (Perkin Elmer). Proliferation experiments were conducted at least in quadruplicate and replicate counts per minute (cpm) were averaged (<20% intra-assay variation). Proliferative responses are reported as a Stimulation Index (SI; cpm of samples with peptide/cpm of samples with media alone) +/- standard error of the mean. A cut-off value of 2.0 was considered a positive proliferative response.

3.2.4   Antibody assays

Sera from mice at various time points were screened for IgG anti-HomoCitJED, anti-CitJED, anti-citrullinated fibrinogen (anti-CitFib), anti-homocitrullinated fibrinogen (anti-HomoCitFib), anti-fibrinogen (anti-Fib) and anti-cyclic citrullinated peptide (anti-CCP2) antibodies by direct antibody-binding ELISA. HomoCitJED and CitJED were dissolved in 15% HCl in sterile distilled water. The 15% HCl did not alter the structure of CitJED or its binding properties (data available upon request). The protocol for anti-HomoCitJED and anti-CitJED direct antibody-binding ELISA was similar to Hill *et al* (35) with the following modifications: wells were coated with 20 μg/mL of HomoCitJED or 40 μg/mL of CitJED in carbonate coating buffer, and 100 μL/well of biotin-conjugated goat anti-mouse IgG (1:5000; Jackson) with streptavidin horseradish peroxidase polymer (1:4000; Abcam) were used for antibody detection. The ELISA protocol for anti-CitFib, anti-HomoCitFib and anti-Fib was also adapted from Hill *et al* (35) as described above, but 10 μg/mL of CitFib, HomoCitFib and Fib in carbonate coating buffer were used instead of the peptides. These ELISAs were performed twice for each serum sample and the average OD was reported (<20% inter-assay variation). The detection of anti-CCP2 antibodies in mouse sera was performed using an anti-CCP2 ELISA kit (Euroimmun). The manufacturer's protocol was followed with the exception that peroxidase-conjugated rabbit anti-mouse IgG (1:5000, Dako) was used. The optical density was determined at 450 nm. The cut-off value for anti-CCP2 was 0.38 OD equal to two standard deviations above the mean value for PBS-immunized B6 mice. The cut-off values for the other

antibodies were 0.1 OD, which is the lower detection limit of the ELISA (the mean values for these antibodies in PBS immunized mice were below 0.1).

The cross-reactivity of IgG anti-HomoCitJED, anti-CitJED, and anti-CCP2 antibodies was examined by inhibition ELISA. The protocol was adapted from Scinocca *et al* (21) with the following plate-bound antigens: CitJED, HomoCitJED and CCP2. Inhibitions were performed with soluble CitJED and HomoCitJED at various concentrations (2 to 100 μg/mL). The average percent inhibition of two repeat determinations is reported, except for CCP2 that was performed once due to insufficient quantity of sera. A minimum OD of 0.5 by direct antibody-binding ELISA was required to obtain a dose-dependent, reproducible inhibition.

3.2.5   Statistical analysis

Graphpad Prism 6.0 software was used for data analysis. The Mann-Whitney U test was used to compare SI or OD between DR4tg and B6 mice at the indicated time points ($p<0.05$ was considered significant). Two-way ANOVA with Bonferonni correction was used for statistical analysis of inhibition experiments ($p<0.01$ was considered significant).

3.3   Results

3.3.1   Splenic T cell proliferative responses

Splenocytes from DR4tg and B6 mice were assessed for proliferative responses to HomoCitJED and CitJED peptide by ^{3}H-thymidine incorporation assay. In DR4tg mice, HomoCitJED-specific splenocyte proliferative responses were observed as early as day 10 in 3/7 (43%) of mice post-immunization with HomoCitJED. These responses increased at later time points with 9/11 (82%) of mice having a significant proliferative response to HomoCitJED at day 100 (Figure 3.1A). We also monitored DR4tg mice for T cell proliferative responses to CitJED peptide (Figure 3.1B). Proliferation to CitJED was observed mainly in mice at day 100, with splenocytes from 8/9 (89%) of mice proliferating in response to CitJED stimulation. In DR4tg mice immunized with PBS, HomoCitJED-specific T cell proliferation was not detected, but CitJED-specific T cell proliferation was detected in 2/9 (22%) at 100 days; the proliferative responses to CitJED
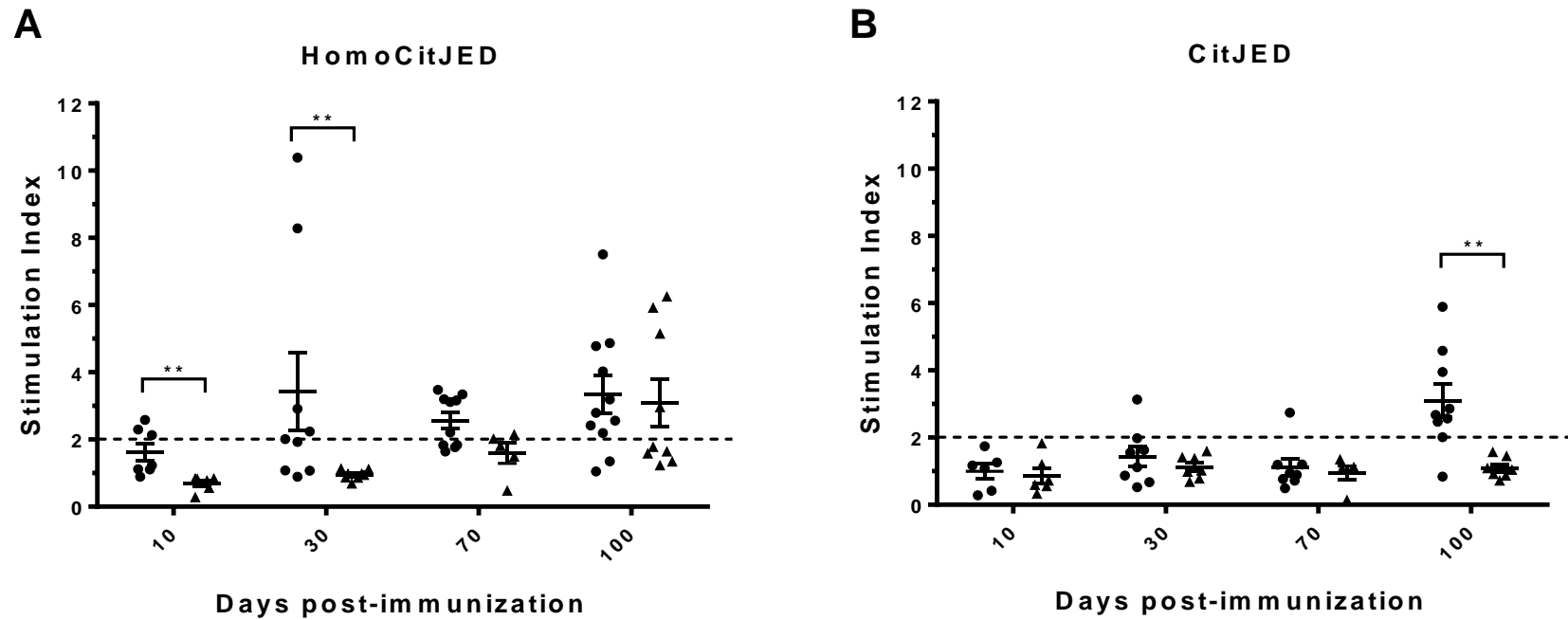
Figure 3.1. Splenocyte proliferative responses in DR4tg (●) and B6 (▲) mice. Splenocytes isolated from HomoCitJED-immunized mice were cultured in the presence of 100 μg/mL of HomoCitJED (**A**) or CitJED (**B**). Splenocyte proliferation, determined by ^{3}H-thymidine incorporation at the days indicated, is shown as a mean stimulation index (± the SEM). Stimulation indices greater than 2.0 (dashed line) were considered as a positive proliferative response. ** - $p<0.01$ by the Mann-Whitney U test. Each symbol represents one mouse; n=5-11.

were significantly lower in PBS-immunized DR4tg mice compared to HomoCitJED-immunized DR4tg mice (p<0.05; data not shown).

To determine whether T cell responses to HomoCitJED and CitJED were restricted to SE-expressing DR4tg mice, we also examined the splenocyte proliferative response in B6 mice which lack the SE. HomoCitJED-immunized B6 mice developed HomoCitJED-specific T cell responses, however these appeared only at day 100 in 4/9 (44%) of mice (Figure 3.1A). These mice did not have CitJED specific T cell responses (Figure 3.1B). Splenocytes from PBS-immunized B6 mice did not proliferate to either peptide (data not shown).

3.3.2 Antibodies to citrulline- and homocitrulline-containing antigens in DR4tg and B6 mice

Sera from DR4tg and B6 mice were screened for the presence of various antibodies targeting citrullinated and homocitrullinated proteins or peptides (Figure 3.2). In DR4tg mice immunized with HomoCitJED, anti-HomoCitJED antibodies were detected in a small proportion (6/22, 27%) of mice at day 10 post-immunization, followed by an increase in anti-HomoCitJED antibody expression at subsequent time points (Figure 3.2A). The peak of anti-HomoCitJED activity occurred at day 70, at which time 11/11 (100%) of DR4tg mice were positive for these antibodies. The expression of anti-HomoCitFib antibodies (Figure 3.2B) also occurred in a similar pattern over time (the concordance of anti-HomoCitJED and anti-HomoCitFib antibodies was 86%). Antibodies to citrullinated peptides were present at later time points (day 50 and 70 post-immunization) with 7/11 (64%) of mice expressing anti-CitJED and 8/11 (73%) expressing anti-CCP2 antibodies at day 70 (Figure 3.2C and Figure 3.2D). Moreover, anti-CitJED and anti-CCP2 were concordantly expressed in 88% of DR4tg mice. There were no detectable antibodies to CitFib and only 1 mouse had a low antibody response to Fib (data not shown). All DR4tg mice that had antibodies targeting citrullinated peptides also expressed anti-HomoCitJED antibodies.

In B6 mice immunized with HomoCitJED, antibody responses to HomoCitJED and HomoCitFib were observed at a later time point compared to DR4tg (day 100) in 4/9
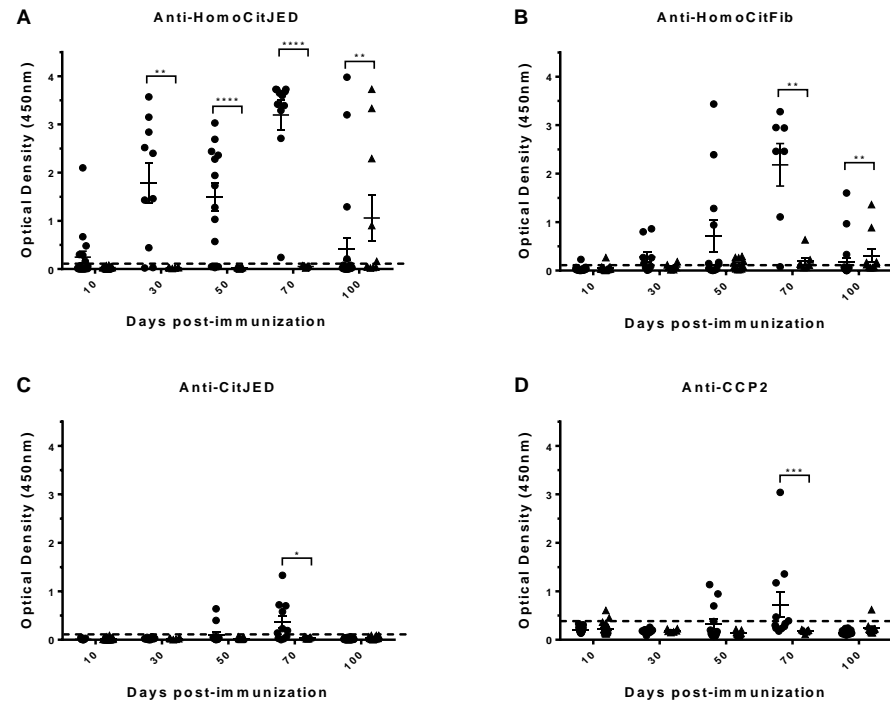
Figure 3.2. Antibody responses in DR4tg (●) and B6 (▲) mice. Sera from HomoCitJED-immunized mice were screened for the presence of anti-HomoCitJED (**A**), anti-homocitrullinated fibrinogen (**B**), anti-CitJED (**C**), and anti-CCP2 (**D**) antibodies at the time points indicated post-immunization. Antibody levels, determined by direct ELISA, are shown as the mean optical density (± the SEM). Optical densities above the cut-off value are indicated by the dashed line. The cut-off values were 0.38 for anti-CCP2 and 0.1 for the other antibodies. * - p<0.05, ** - p<0.01, *** - p<0.001, **** - p<0.0001 by the Mann-Whitney U test. Each symbol represents one mouse; n=6-22.

(44%) and 2/9 (22%) mice, respectively (concordance = 71%; Figure 3.2A and Figure 3.2B). These B6 mice did not respond to citrullinated antigens except for one mouse that had a low response (Figure 3.2D). PBS-immunized B6 or DR4tg mice did not have significant antibody responses to any of the antigens tested (data not shown).

3.3.3   Antibody cross-reactivity in DR4tg and B6 mice

To examine anti-CitJED and anti-HomoCitJED antibody cross-reactivity, antibodies from DR4tg mouse sera were inhibited with soluble CitJED and HomoCitJED peptides. We inhibited IgG anti-HomoCitJED from 8 DR4tg mice that had anti-HomoCitJED antibodies, but lacked anti-CitJED based on direct ELISA (Figure 3.3A). In these mice, anti-HomoCitJED antibodies were efficiently inhibited by cognate HomoCitJED peptide. However, two patterns emerged when these antibodies were inhibited with CitJED (Appendix 4). In 5/8 mice, anti-HomoCitJED were not inhibited by CitJED, which suggests that these antibodies were HomoCitJED-specific. In the other 3 mice, antibody reactivity to HomoCitJED was significantly inhibited by CitJED peptide, indicating that anti-HomoCitJED antibodies in these mice were cross-reactive. Antibodies from serum collected at various time points (days 10-70 post-immunization) were tested in the inhibition assays; antibody cross-reactivity did not appear to be influenced by the time at which it was measured (Appendix 4).

In DR4tg mice that expressed both anti-HomoCitJED and anti-CitJED antibodies by direct ELISA, anti-HomoCitJED antibodies were inhibited by HomoCitJED, but were not inhibited by CitJED (Figure 3.3B and Appendix 5A-C). However, anti-CitJED antibodies from the same three mice were inhibited by both HomoCitJED and CitJED peptide (Figure 3.3C and Appendix 5D-F). Anti-CitJED in these mice was inhibited more efficiently by the non-cognate peptide, HomoCitJED (p<0.0001).

We also examined the antigen specificity of anti-CCP2 antibodies in DR4tg mice (Figure 3.4). Similar to anti-CitJED, anti-CCP2 antibodies were always co-expressed with anti-HomoCitJED in DR4tg mice (n=4). Anti-CCP2 antibodies from 3/4 mice were efficiently
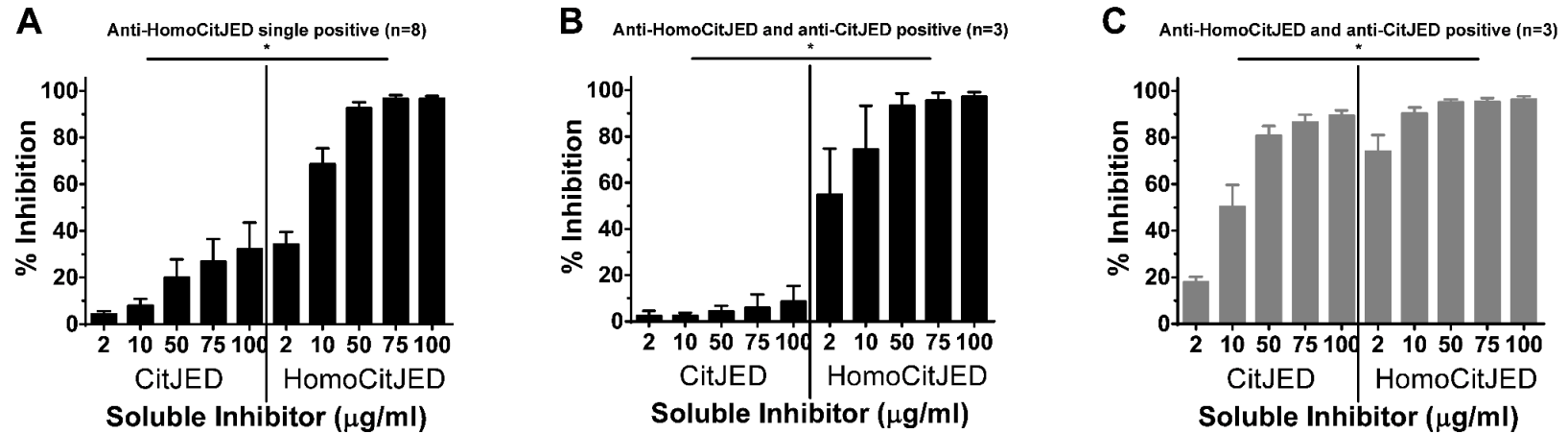
Figure 3.3. Inhibition of anti-HomoCitJED (black bars) and anti-CitJED (grey bars) antibodies. Sera from HomoCitJED-immunized DR4tg mice were used in inhibitions assays. As measured by direct ELISA, these mice expressed anti-HomoCitJED antibodies but not anti-CitJED antibodies (n=8; **A**) or were positive for both anti-HomoCitJED and anti-CitJED antibodies (n=3; **B, C**). The mean percent inhibition (pooled from inhibitions performed on n=3 or 8 mice) ± the SEM is shown. * - p<0.0001 by Two-way ANOVA with Bonferonni correction.

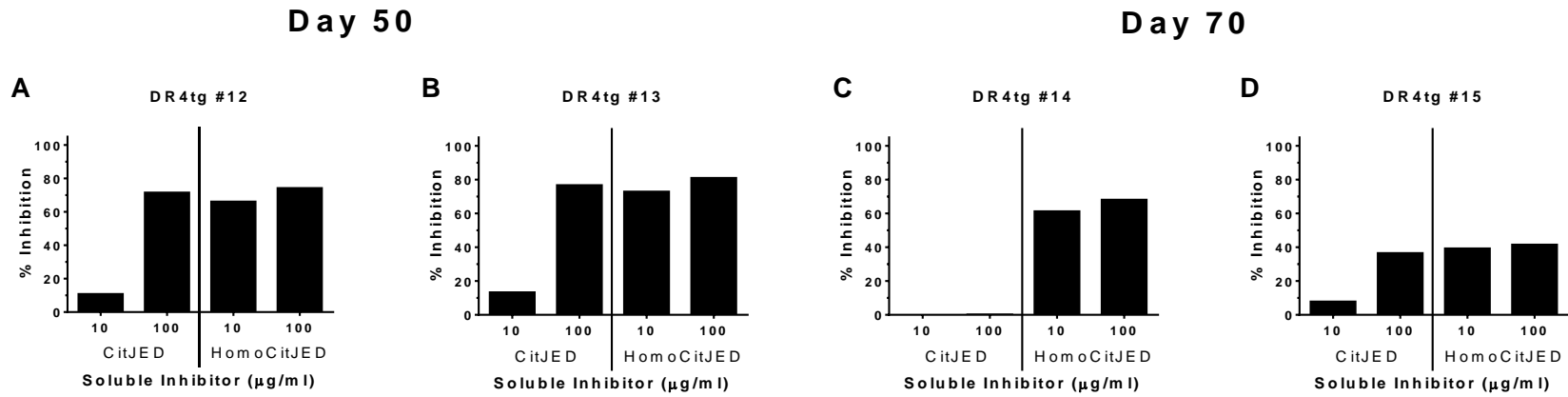**Figure 3.4. Inhibition of anti-CCP2 antibodies.** Sera from four HomoCitJED-immunized DR4tg mice (DR4tg #12-15; **A-D**) were used in inhibition assays at the indicated days post-immunization. As detected by direct ELISA, these mice expressed anti-CCP2 antibodies. The percent inhibition from one experiment per individual mouse serum is shown.

inhibited by HomoCitJED peptide (Figure 3.4 A-C). Two of these mice had anti-CCP2 antibodies that were also inhibited by CitJED (Figure 3.4A and Figure 3.4B). In one mouse, anti-CCP2 antibodies were not efficiently inhibited by either CitJED or HomoCitJED (Figure 3.4D).

To determine whether antibody cross-reactivity was affected by the SE, we also inhibited anti-HomoCitJED from four B6 mice (Figure 3.5). By direct ELISA, these mice expressed anti-HomoCitJED, but were negative for anti-CitJED and anti-CCP2 antibodies. In these mice, anti-HomoCitJED antibodies were inhibited by HomoCitJED peptide; there was no difference in the relative affinity of anti-HomoCitJED antibodies for HomoCitJED in B6 compared to DR4tg mice (Appendix 6). However, anti-HomocitJED antibodies from B6 mice were not inhibited by CitJED peptide. Thus, unlike antibodies from DR4tg mice, anti-HomoCitJED antibodies from B6 mice were not cross-reactive.

3.4 Discussion

In this study, we immunized DR4tg and B6 mice with a synthetic homocitrullinated peptide, HomoCitJED and examined the development of splenic T cell proliferative and antibody responses to homocitrullinated and citrullinated peptides and proteins. In DR4tg mice that expressed the SE and were immunized with HomoCitJED, we detected the early development of splenocyte proliferative and antibody responses to HomoCitJED. These mice also generated antibodies to HomoCitFib, an antigen shown to be present in the RA synovium (26). Anti-HomoCitJED antibodies from DR4tg mice also bound citrullinated antigens (CitJED and CCP2), supporting the notion that these antibodies are cross-reactive.

It is known that there is a strong association between the SE and anti-citrullinated protein/peptide antibodies (ACPA) in RA patients (5). Studies have shown that the SE binds citrullinated peptides with high affinity, initiating T and subsequently B cell responses (8,9). In DR4tg mice, but not B6 mice, immunization with citrullinated fibrinogen causes arthritis suggesting that the SE is necessary for arthritis development
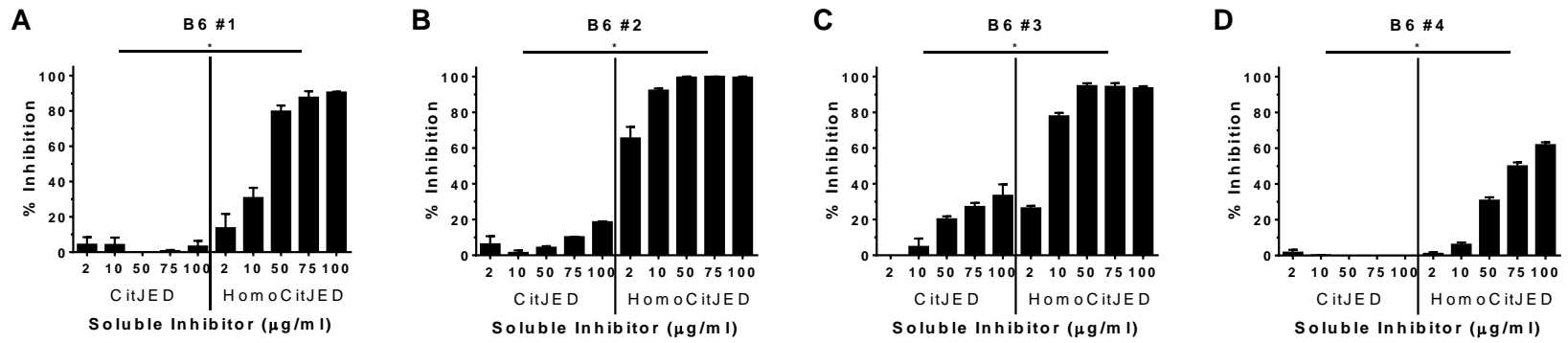
Figure 3.5. Inhibition of anti-HomoCitJED from B6 mice. Inhibition assays were performed on sera from four HomoCitJED-immunized B6 mice (B6 #1-4; **A-D**) at day 100 post-immunization. As measured by direct ELISA, these mice expressed anti-HomoCitJED antibodies but lacked both anti-CitJED and anti-CCP2 antibodies. The mean percent inhibition of 2 repeats per individual mouse serum ± the SEM is shown. * - p<0.0001 by Two-way ANOVA with Bonferonni correction.

(17,20). The chemical structure of homocitrulline is identical to citrulline except for one carbon atom; however, the affinity of homocitrullinated peptides for the SE and its effect on the immune response is unknown. Moreover, the association between the SE and anti-CarP was not found in a study of RA patients from Northern Europe (29). The association between the SE and other anti-homocitrullinated protein/peptide antibodies (AHCPA) in other populations has not been previously studied. Immune responses to homocitrullinated antigens occur in mice expressing different MHC-H2 alleles (32). Our study is the first to use mice that express the SE and not endogenous mouse MHC class II molecules to study responses to homocitrulline and although we show that some B6 mice that express MHC-H2b did develop responses to homocitrullinated antigens, the DR4tg mice had earlier responses. It is possible that I-A^b from B6 mice binds homocitrulline poorly. In contrast, DR4 may be able to bind homocitrulline with high affinity, similar to citrulline (8,9), in its P4 binding pocket via the SE, accelerating T and B cell responses to homocitrullinated peptides.

In addition to earlier T and B cell responses to homocitrullinated peptides and proteins, DR4tg mice immunized with HomoCitJED had sustained responses from day 10 to 100 post-immunization. Sustainability of an immune response may enhance epitope spreading (37), which is supported by our finding that DR4tg mice developed responses to citrullinated peptides and proteins at day 100 post-HomoCitJED immunization. The ACPA responses from these mice had a higher inferred affinity by inhibition assays for HomoCitJED than CitJED, suggesting that the ACPA arise from B cell responses to HomoCitJED. As in other studies showing that AHCPA are cross-reactive (21–24), some anti-HomoCitJED antibodies from DR4tg mice also bound CitJED. Notably, B6 mice immunized with HomoCitJED did not develop antibodies to citrullinated antigens and anti-HomoCitJED antibodies from these mice did not bind CitJED, providing further evidence for the strong relationship between SE and citrullinated peptides.

Our study has several other strengths. We used a homocitrullinated peptide and its citrullinated counterpart that had the same backbone sequence and identical location and number of modified amino acids, minimizing the effect of the backbone on the

assessment of the immune response. These synthetic peptides can act us surrogate antigens for an endogenously expressed modified protein (fibrinogen), known to be involved in the pathogenesis of RA (10,17,21,38). The animal model we used is relevant to human RA in that the mice express the strongest genetic risk factor for this disease (39). We describe the immune responses in these mice over an extended period of time. Detailed inhibition analyses were performed to examine the relative affinity of the anti-HomoCitJED and anti-CitJED antibodies from these mice. Unfortunately, we were unable to perform more extensive inhibition with other antigens due to insufficient sera volume. Another limitation is that we did not continue our experiments past day 100 post-immunization at which time the DR4tg mice immune responses started to decline, while in B6 mice, they were first detected.

In conclusion, this study revealed that HomoCitJED is immunogenic leading to T and B cell responses to homocitrullinated antigens in SE-expressing DR4tg mice, which later evolve into immune responses to citrullinated antigens. This evolution appears to be driven by epitope spreading rather than *de novo* generation of citrullinated antigens and relies on the SE. These mice did not develop arthritis, however, in other animal models, arthritic mice expressed antibodies to homocitrullinated antigens (40). Future work will assess the arthritogenic properties of anti-homocitrulline immune responses in the DR4tg mouse model.

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Chapter 4

4 Discussion

4.1 Summary

In this study, the objectives were to investigate the anti-homocitrulline as well as the anti-citrulline immune responses in RA patients and in a mouse model of RA (DR4tg and B6 mice). The major findings in chapter 2 were that anti-HomoCitJED and anti-CitJED antibodies were frequently and specifically detected in RA patients. These two antibodies were co-expressed in RA patients and were cross-reactive. In chapter 3, DR4tg and B6 mice were immunized with HomoCitJED and the immune responses to HomoCitJED were examined. Anti-homocitrulline immune responses were shown for the first time to be associated with the SE. These responses could also occur in the absence of the SE but the development of cross-reactive anti-citrulline responses only occurred in DR4tg mice.

4.2 Anti-homocitrulline antibodies are specifically associated with RA and are cross-reactive to citrulline

Anti-homocitrulline antibodies are a family of antibodies that have been recently associated with RA. However, some aspects of these antibodies, including the prevalence of these antibodies in RA patients, their specificity for disease, as well as their relationship with anti-citrulline antibodies are not well understood. The studies that have been performed thus far on anti-homocitrulline and anti-citrulline cross-reactivity are few in number and have used different homocitrulline-containing antigens with limited specificity (1–6). Thus, a major strength of this study was the use of a novel, homocitrulline-rich peptide (HomoCitJED) as a surrogate target of anti-homocitrulline antibodies in RA patients (ie. designed with the potential to detect anti-homocitrulline antibodies to numerous homocitrullinated antigens). CitJED, the citrulline-containing counterpart of HomoCitJED, was used for the detection of anti-citrulline antibodies. These peptides allowed for the examination of antibody reactivity (and cross-reactivity) to homocitrulline and citrulline.

The antibody responses to HomoCitJED and CitJED were evaluated for their specificity for RA. Another strength of this study design was the use of patients with other inflammatory conditions as the most appropriate controls. The presence of anti-HomoCitJED and anti-CitJED antibodies was examined in sera from patients with PsA, an inflammatory joint disorder that shares many clinical features with RA but are not associated with autoantibody expression (7). Additionally, sera from patients with SLE, an inflammatory autoimmune disorder that is associated with the development of autoantibodies to DNA and ribonucleoproteins (8), were also screened for antibody activity to HomoCitJED and CitJED.

Anti-CitJED antibodies were found to be specific for RA because they were not detected in healthy subjects, SLE patients, nor PsA patients. This is in agreement with the literature that other anti-citrulline antibodies to CCP (9–11), citrullinated fibrinogen (12) and mutated citrullinated vimentin (13) are highly specific for RA. Previously, Reed *et al*. suggested that anti-homocitrulline antibodies could be a biomarker of inflammation instead of being specifically associated with RA (6). However, this was not the case for anti-HomoCitJED as these antibodies were not detected in patients with other inflammatory conditions (PsA or SLE).

Anti-HomoCitJED antibodies were detected in a large proportion of RA patients (approximately 60%), and were found to be expressed alongside anti-CitJED in many of these patients. This is in agreement with the findings that the anti-CarP-FCS are frequently found in anti-CCP positive RA patients (1,14). Anti-HomoCitJED and anti-CitJED antibodies in patients that had both of these antibodies were inhibited by both HomoCitJED and CitJED peptides (cross-reactive). In addition, a small proportion of RA patients were positive for anti-HomoCitJED but not anti-CitJED (15%) or the converse (8%) as measured by direct antibody ELISA. However, anti-HomoCitJED and anti-CitJED from these patients were also inhibited by both HomoCitJED and CitJED. Thus, the evidence presented here indicates that anti-homocitrulline and anti-citrulline antibodies constitute a single, cross-reactive antibody population as proposed by Reed *et al* (6).

4.3 The SE is involved with anti-homocitrulline immune responses and cross-reactivity to citrulline

This study examined the development of anti-homocitrulline and anti-citrulline immune responses in DR4tg mice. This is a relevant model of RA because these mice express the SE, which is found in the majority of RA patients (15–17) and confers disease susceptibility (18). DR4tg mice have also been shown to develop arthritis when immunized with citrullinated fibrinogen (19), an antigen that has been found to be present in the joints of arthritic DR4tg mice (19) as well as in the synovial tissue of RA patients (20).

The T and B cell responses following immunization with HomoCitJED in mice with (DR4tg mice) and without the SE (B6 mice) were investigated to examine i) the development of anti-homocitrulline immune responses, ii) whether these immune responses were SE-dependent, and iii) the cross-reactivity of anti-homocitrulline responses to citrulline.

Anti-HomoCitJED T and B cell responses were observed in both DR4tg and B6 mice. Additionally, antibodies to homocitrullinated fibrinogen could be detected in both mouse strains and were found to be concordant with anti-HomoCitJED. The finding that anti-homocitrulline responses can occur in the absence of the SE is in agreement with previous work by Mydel *et al.* (21), and suggests that homocitrulline can be accommodated by some mouse MHC class II molecules, including I-A^b from B6 mice, resulting in anti-homocitrulline immune responses. Nonetheless, this study provides the first *in vivo* evidence that the immune response to homocitrulline is SE-associated.

A difference observed between DR4tg and B6 mice was that the immune responses in DR4tg mice were more frequent and occurred earlier. This may indicate that SE-encoded HLA-DR molecules are better binders of homocitrulline-containing peptides compared to those without the SE. Thus, antigen presentation in DR4tg mice may be more efficient in activating homocitrulline-specific T cells that help B cells to generate antibodies to this peptide.

The main difference between DR4tg mice and B6 mice was that immunization with HomoCitJED also led to the development of T cell responses to CitJED and antibodies to citrullinated peptides (CitJED and CCP) in (SE-positive) DR4tg mice. These immune responses occurred later compared to the anti-homocitrulline immune responses in DR4tg mice. Anti-citrulline antibodies were only found in mice that also had anti-HomoCitJED and were also cross-reactive to HomoCitJED. This shows that the anti-homocitrulline immune response precedes the anti-citrulline response in mice and the same may occur in RA patients. In contrast, anti-citrulline T and B cell responses were not detected in (SE-negative) B6 mice in any of the assays used in this study. This reinforces the idea that cross-reactivity between anti-homocitrulline and anti-citrulline immune responses is SE-dependent.

The anti-homocitrulline and anti-citrulline antibody responses in DR4tg mice shared similarities with the antibody responses that were observed in RA patients of this study. DR4tg mice expressed antibodies to HomoCitJED, CitJED and CCP following immunization with HomoCitJED, and similarly, these antibodies were also expressed by RA patients. DR4tg mice developed anti-homocitrullinated fibrinogen antibodies, which have also been reported to be present in the same cohort of RA patients as used here (3,22). Antibodies in both the DR4tg mice and RA patients were cross-reactive. Not surprisingly, due to the differences in genetic makeup, cross-reactivity was observed in a different proportion of RA patients compared to DR4tg mice. These observations, in addition to a previous study performed by the laboratory of Cairns and Bell (19), supports the use of the DR4tg mouse model for the study of mechanisms underlying the pathogenesis of RA.

4.4   Homocitrulline plays a role in RA pathogenesis

Autoimmunity involves the breakdown of immunological tolerance to self-tissues. In RA patients, there is a loss of tolerance to citrullinated proteins as well as to homocitrullinated proteins. This study provides evidence that homocitrulline is able to induce both anti-homocitrulline and cross-reactive anti-citrulline immune responses. This may be due to the structural similarity between homocitrulline and citrulline. The combination of molecular mimicry, which exists when antigens share similar sequences

(23,24), and B cell cross-reactivity to these related antigens (23) have previously been described as driving factors in B cell epitope spreading. Epitope spreading is the diversification of epitopes that are recognized by the immune system (23) and has been implicated in the development of autoimmune diseases including RA (23,25) and SLE (23,26).

In RA, epitope spreading has been shown to cause an expansion in the repertoire of anti-citrulline antibodies in patients prior to disease onset (25). The findings in this study suggest that epitope spreading can also occur between the anti-homocitrulline and anti-citrulline antibody responses and is dependent on the expression of the SE. Additionally, B cells express MHC class II molecules and studies have shown that B cells are able to activate antigen-specific T cells in mouse models of RA (27,28). Thus, cross-reactive B cells with SE-encoded HLA-DR may be able to present homocitrulline- or citrulline-containing epitopes to T cells. This could result in the propagation of immune responses to both homocitrulline and citrulline in RA patients and further exacerbate the autoimmune response in RA patients by promoting T cell activity.

Currently, the mechanism underlying RA pathogenesis is believed to involve the presentation of citrullinated peptides by antigen presenting cells that express SE-encoded HLA-DR molecules to citrulline-specific helper T cells, as initially described by Hill *et al* (29). These T cells subsequently activate citrulline-specific B cells, which then drive the production of anti-citrulline antibodies. Evidence from this study suggests that homocitrulline can play a similar role (Figure 4.1). Homocitrulline can be presented by SE-encoded HLA-DR molecules and result in cross-reactive anti-homocitrulline and anti-citrulline antibodies. These cross-reactive antibodies can then bind to either homocitrullinated or citrullinated antigens, which have been shown to be present in RA joint tissue (21,30,31), resulting in inflammation and RA development. Thus, it is possible that homocitrulline plays a role in RA pathogenesis by contributing to the breakdown of self-tolerance to citrulline and vice-versa. However it is not known whether homocitrulline or citrulline (or both) is responsible for RA development.
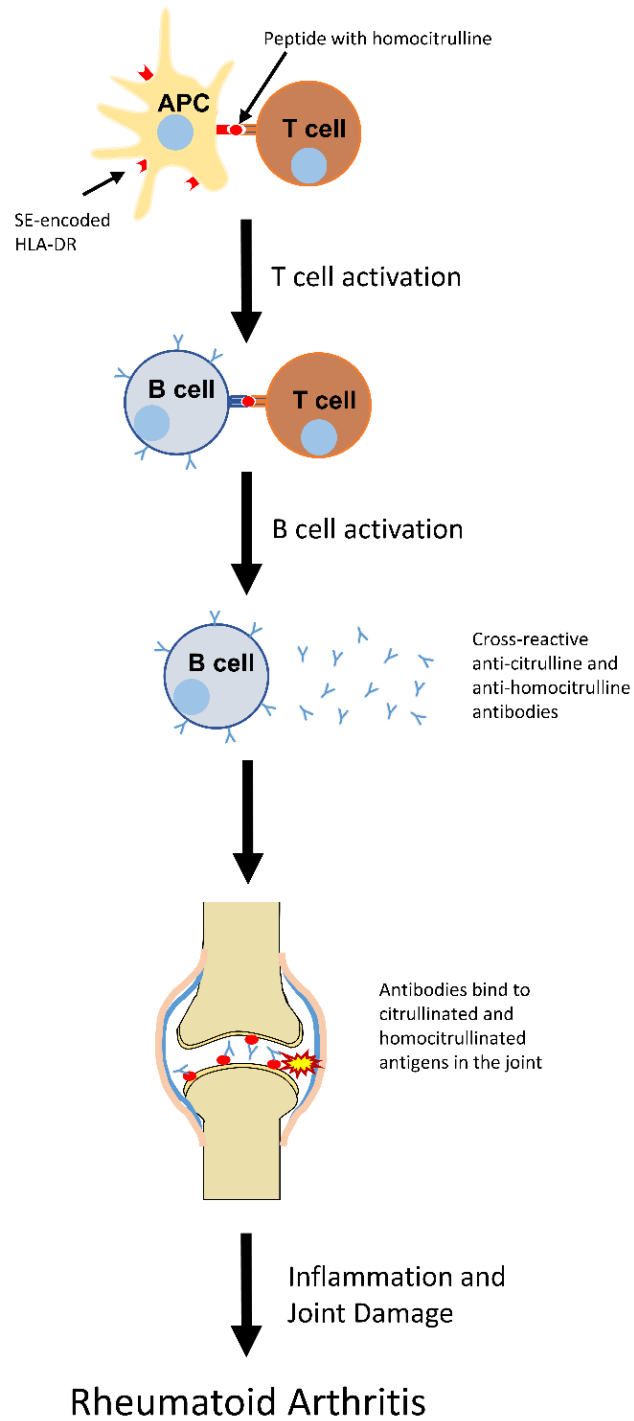
Figure 4.1. Immune responses to homocitrulline in RA. Homocitrulline-containing peptides are presented by SE-encoded HLA-DR on antigen presenting cells (APC) resulting in T and B cell activation. Activated B cells produce cross-reactive antibodies to homocitrulline and citrulline which mediate inflammatory responses in RA joint tissue.

4.5   Limitations and future directions

This study examined the expression and cross-reactivity of anti-HomoCitJED and anti-CitJED antibodies in RA patients with established (long-standing) disease. It is possible that the longer disease durations allowed for the RA patients to develop more anti-homocitrulline or anti-citrulline antibody activity over time (due to epitope spreading). To better understand the development of the anti-homocitrulline and anti-citrulline immune responses in RA patients, anti-HomoCitJED and anti-CitJED expression and cross-reactivity will need to be studied in patients with early disease and in patients prior to the onset of RA.

The studies in mice revealed that anti-homocitrulline antibodies can be generated in the absence of the SE, but cross-reactivity to citrulline was dependent on the expression of the SE. Whether this is the same in RA patients is unknown because the HLA haplotypes of the RA patients were not investigated. Genotyping of RA patients will provide further insight on the association of anti-homocitrulline and anti-citrulline responses with the SE.

Another limitation of this study was that the association between the SE and homocitrulline was demonstrated using functional assays (T and B cell responses to homocitrulline) in DR4tg mice. Further studies which examine whether homocitrulline-containing peptides can directly bind to SE-encoded HLA-DR molecules are required to confirm this finding.

Additionally, the mouse studies were limited by the duration of the study period. B6 mice were found to not develop cross-reactive immune responses to citrulline at any of the time points examined. However, the immune responses in B6 mice were detected at day 100 (the last day time point that was assessed). It is possible that the B6 mice would later develop reactivity to citrulline. Thus, studies over longer periods of time are required.

In the DR4tg mouse model of RA, immunization with HomoCitJED did not induce arthritis. A possible explanation for this finding, is that the DR4tg mice may not have expressed or expressed insufficient levels of homocitrulline (or citrulline) in their joints.

Future studies will involve the injection of homocitrullinated or citrullinated antigen into the knees of DR4tg mice, to provide a target for the immune response and to examine if this model can recapitulate the disease seen in RA patients.

Furthermore, this study examined the development of cross-reactive immune responses to a homocitrulline-containing antigen (HomoCitJED). It is unknown if cross-reactive immune responses to homocitrulline can develop from an anti-citrulline response. Thus future studies will examine the induction of anti-citrulline and anti-homocitrulline responses in mice immunized with CitJED.

4.6   Significance

RA is an inflammatory joint disorder with an unknown etiology, but which is characterized by antibodies to proteins modified by citrullination. RA patients also specifically express antibodies to a structural analog of citrulline, homocitrulline. Using the homocitrulline- and citrulline-rich peptides, HomoCitJED and CitJED, this study demonstrates that anti-citrulline and anti-homocitrulline antibodies are cross-reactive to these two amino acids. In addition, this study demonstrated the first *in* vivo evidence that the SE is associated with the initiation of immune responses to homocitrulline. Further, the development of cross-reactive anti-citrulline and anti-homocitrulline immune responses are dependent on the expression of SE-encoded HLA-DR. A novel mechanism is presented by which homocitrulline-containing antigens can induce a breakdown in immunological tolerance to citrullinated self-proteins. Cross-reactive B cells to citrulline and homocitrulline play a role in propagating autoimmune responses and contribute to RA pathogenesis. Thus homocitrulline may have an important role in RA development.

Presently, there is no antigen-specific treatment for RA. The current therapeutic strategies for RA include the use of immunosuppressants, cytokine inhibitors, and biologics that target components of the immune system and often have side-effects. Specific treatment options for RA are being explored. One approach is the use of peptide vaccines, including citrullinated peptide vaccines, to induce antigen-specific immune tolerance (32-34). HomoCitJED peptide may also be useful in this approach because this peptide could potentially block both anti-homocitrulline and anti-citrulline immune

responses in RA patients. Pilot studies using HomoCitJED peptide to induce tolerance are ongoing in the Cairns and Bell laboratory.

4.7 References

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Appendices
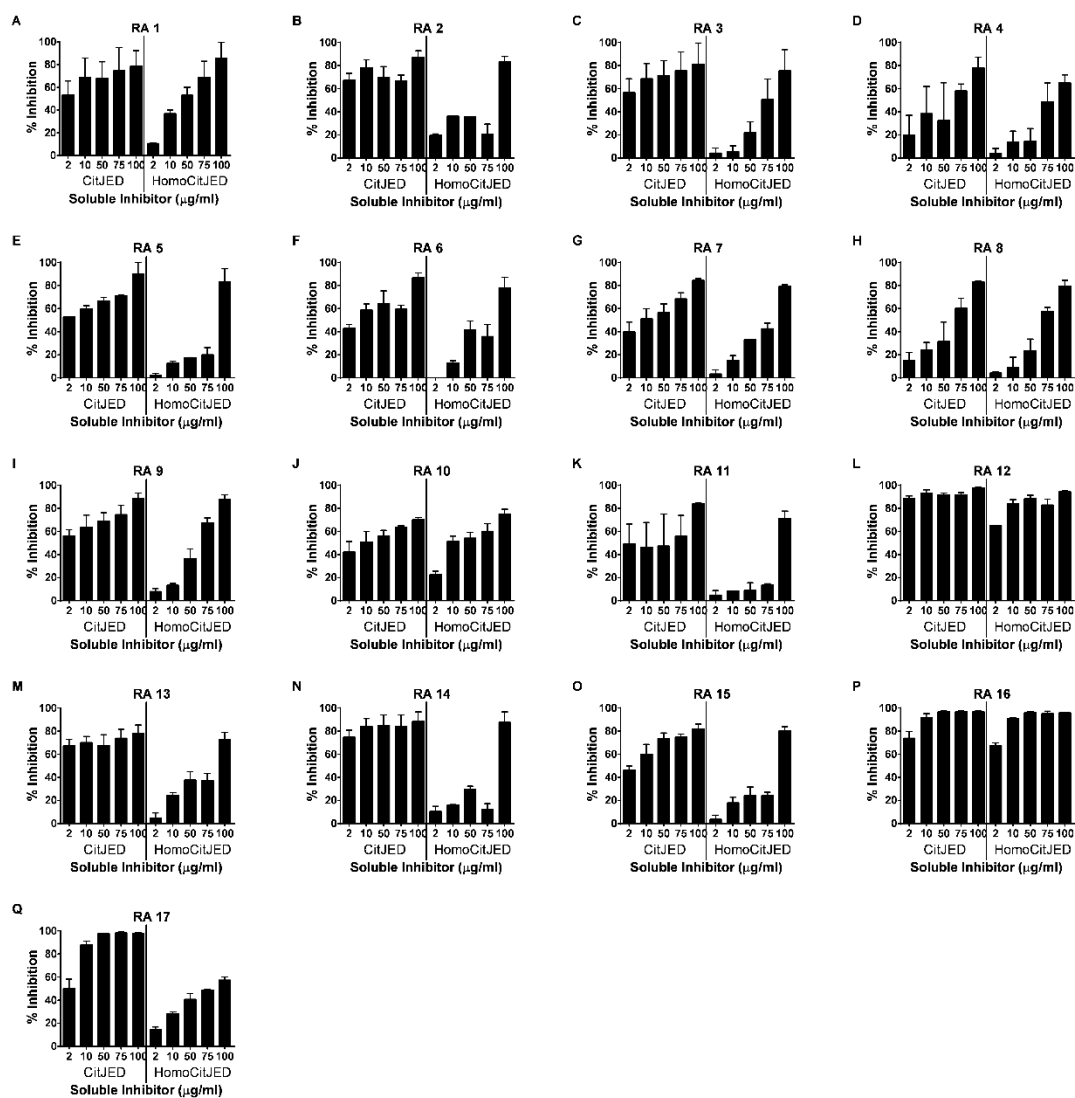
Appendix 1. Anti-CitJED inhibition by CitJED and HomoCitJED peptides. Anti-CitJED inhibition profiles for 15 anti-CCP2 positive (**A-O**) and 2 anti-CCP2 negative (**P-Q**) RA patients who expressed anti-CitJED and anti-HomoCitJED. Antibodies were inhibited by various concentrations of either soluble CitJED or HomoCitJED peptide.
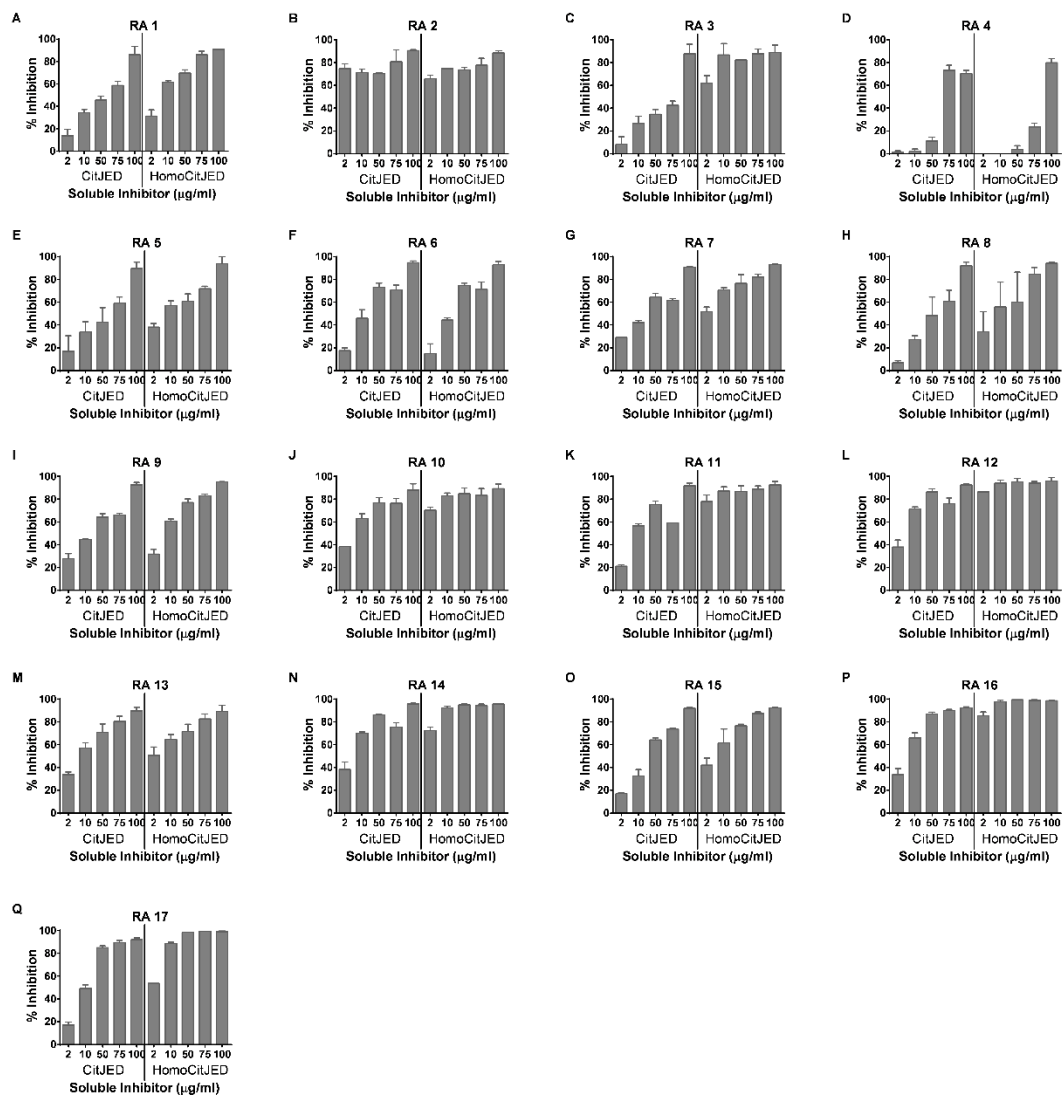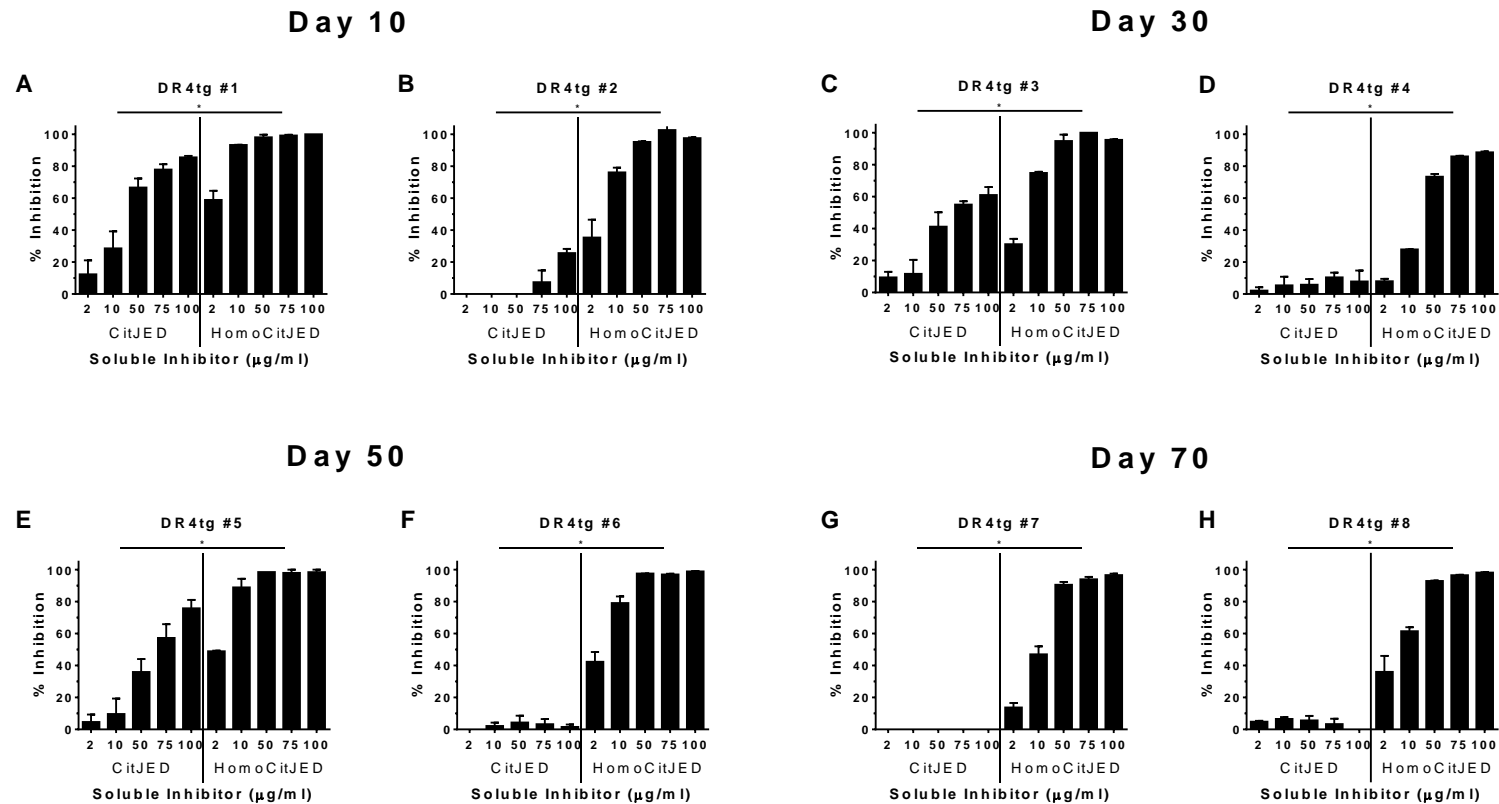
Appendix 2. Anti-HomoCitJED inhibition by CitJED and HomoCitJED peptides. Anti-HomoCitJED inhibition profiles for 15 anti-CCP2 positive (**A-O**) and 2 anti-CCP2 negative (**P-Q**) RA patients (same RA patients as in Appendix 1) who expressed anti-CitJED and anti-HomoCitJED. Antibodies were inhibited by various concentrations of either soluble CitJED or HomoCitJED peptide.
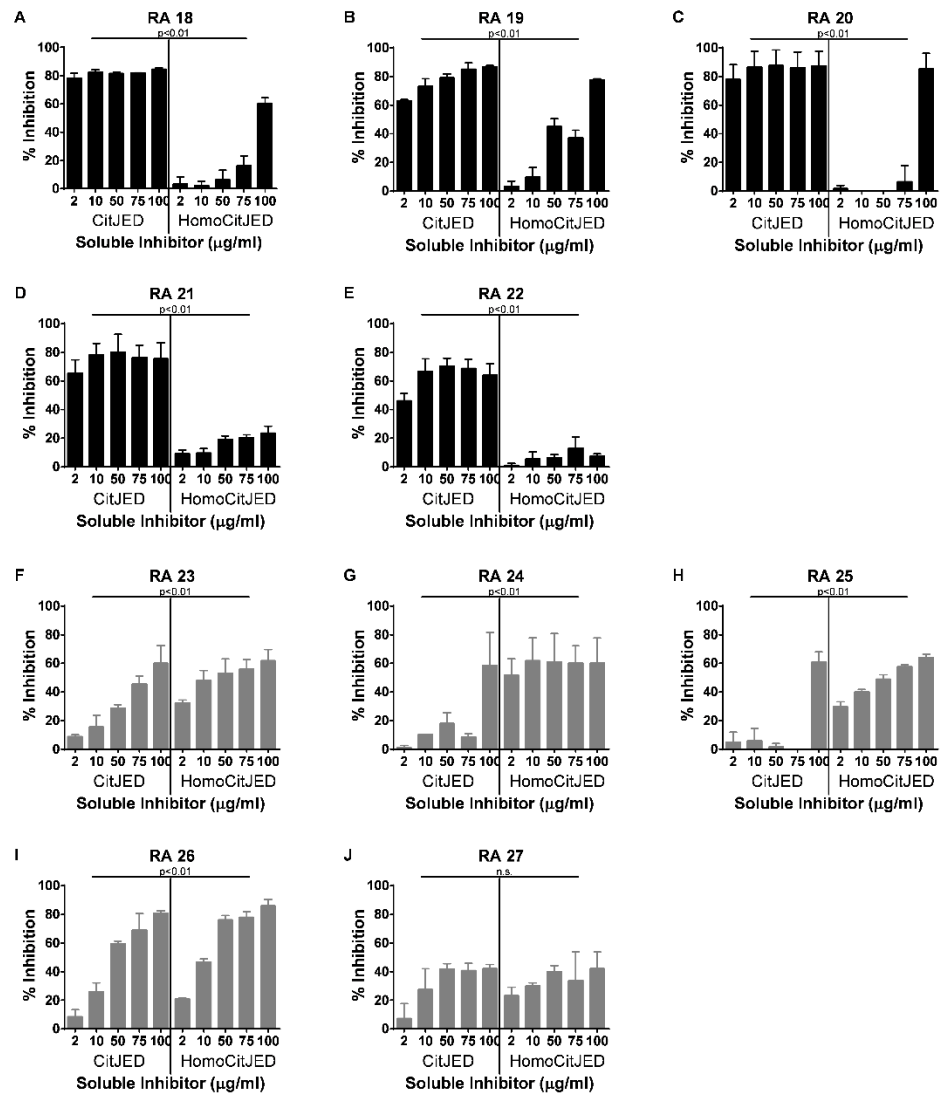
Appendix 3. Most anti-CitJED and anti-HomoCitJED from single positive RA patients are cross-reactive. (**A-E**) Inhibition of anti-CitJED from 5 anti-CitJED single positive RA patients. (**F-J**) Inhibition of anti-HomoCitJED from 5 anti-HomoCitJED single positive RA patients. All patients tested were positive for anti-CCP2; $p<0.01$ was considered significant by Two-way ANOVA. n.s. – not significant.

Appendix 4. Inhibition of anti-HomoCitJED antibodies. Sera from eight HomoCitJED-immunized DR4tg mice (DR4tg #1-8; **A-H**) were used in inhibitions assays at the various days post-immunization as indicated. As measured by direct ELISA, these mice expressed anti-HomoCitJED antibodies but not anti-CitJED antibodies. The mean percent inhibition of 2 repeats per individual mouse serum ± the SEM is shown. * - p<0.0001 by Two-way ANOVA with Bonferonni correction.
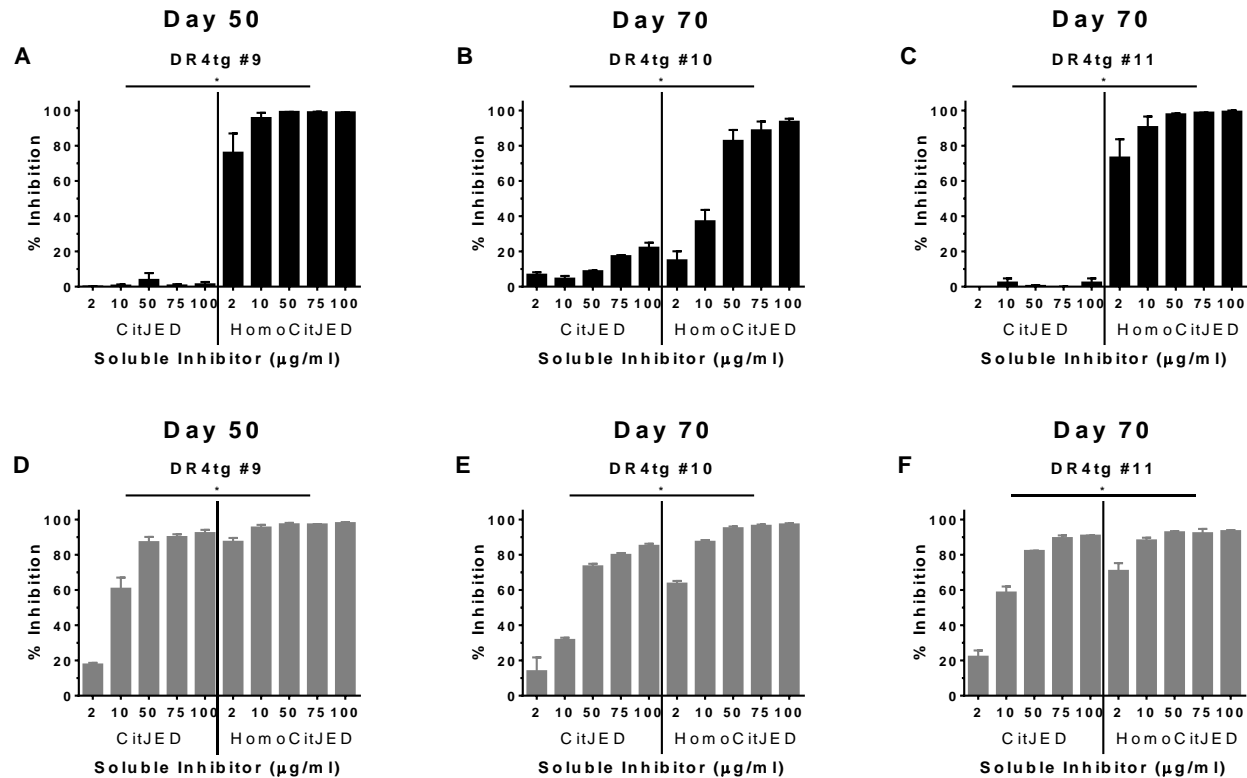
Appendix 5. Inhibition of anti-HomoCitJED (black bars) and anti-CitJED (grey bars) antibodies. Sera from three HomoCitJED-immunized DR4tg mice (DR4tg #9-11; **A-F**), were used in inhibitions assays at the various days post-immunization as indicated. As detected by direct ELISA, these mice were positive for both anti-HomoCitJED and anti-CitJED antibodies. The mean percent inhibition of 2 repeats per individual mouse serum ± the SEM is shown. * - p<0.0001 by Two-way ANOVA with Bonferonni correction.
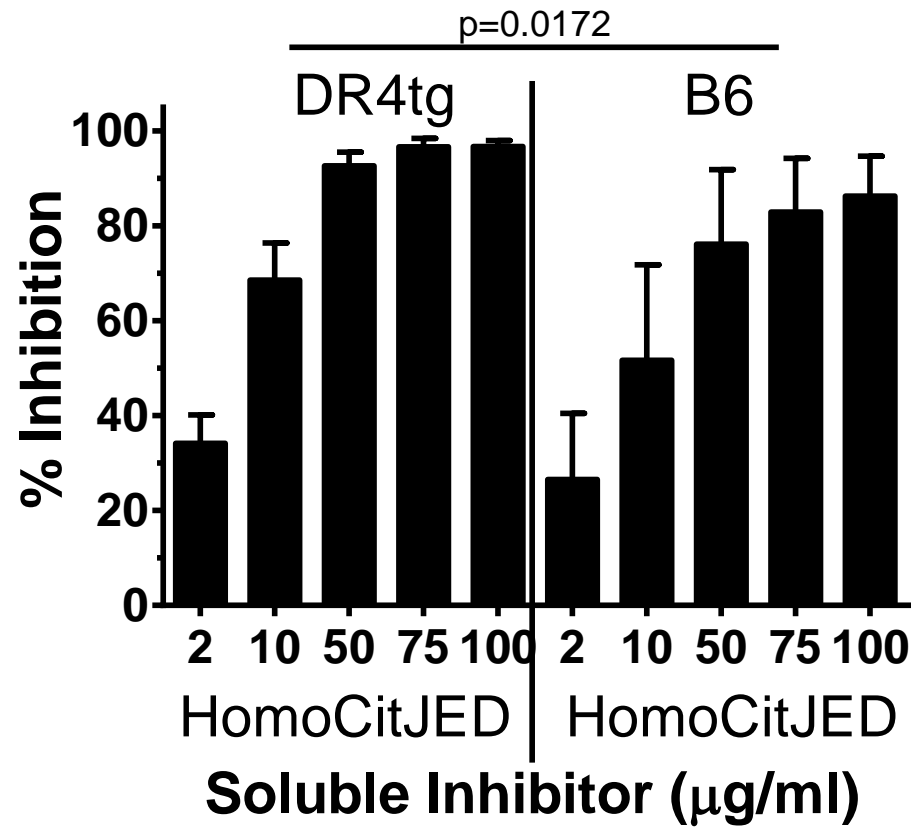
Appendix 6. Inhibition of anti-HomoCitJED antibodies with HomoCitJED peptide. Inhibition assays were performed on sera from HomoCitJED-immunized DR4tg and B6 mice. As measured by direct ELISA, these mice expressed anti-HomoCitJED antibodies but not anti-CitJED antibodies. The mean percent inhibition (pooled from inhibitions performed on n=4 or 8 mice) ± the SEM is shown. The p value was determined by Two-way ANOVA with Bonferonni correction.

Western Research

Western University Health Science Research Ethics Board
HSREB Annual Continuing Ethics Approval Notice

Date: April 20, 2017
Principal Investigator: Dr. Ewa Cairns
Department & Institution: Schulich School of Medicine and Dentistry\Medicine-Dept of,London Health Sciences Centre

Review Type: Delegated
HSREB File Number: 1087
Study Title: The role of DR4(*0401) (MHC class II molecules) in Rheumatoid Arthritis pathogenesis 09684E

HSREB Renewal Due Date & HSREB Expiry Date:
Renewal Due -2018/04/30
Expiry Date -2018/05/27

The Western University Health Science Research Ethics Board (HSREB) has reviewed the Continuing Ethics Review (CER) Form and is re-issuing approval for the above noted study.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice (ICH E6 R1), the Ontario Freedom of Information and Protection of Privacy Act (FIPPA, 1990), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair
EO: Erika Basile ___ Grace Kelly ___ Katelyn Harris___ Nicola Morphet ___ Karen Gopaul ___

Appendix 7. Ethical approval for the use of human samples.

From: eSiriusWebServer <esiriusadmin@uwo.ca> <esiriusadmin@uwo.ca>
Sent: Tuesday, May 2, 2017 10:24 AM
To: Ewa Cairns
Cc: Animal Care Committee
Subject: eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2009-101::7

Western

2009-101::7:
AUP Number: 2009-101
AUP Title: Induction and Prevention of Rheumatoid Arthritis in DRB1*0401 Transgenic Mice

Yearly Renewal Date: 05/01/2017
The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-101 has been approved, and will be approved for one year following the above review date.

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office.
    Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.
Submitted by: Schoelier, Marianne
on behalf of the Animal Use Subcommittee

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, ● London, Ontario ● CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 ● FL 519-661-2028
Email: auspc@uwo.ca ● http://www.uwo.ca/animal/website/

Appendix 8. Ethical approval for the use of animal subjects.

Curriculum Vitae

Name: Patrick Lac

Post-secondary   The University of Western Ontario
Education and   London, Ontario, Canada
Degrees: Sept 2014 - Present M.Sc. in Microbiology & Immunology

                         University of Waterloo
                         Waterloo, Ontario, Canada
                         Sept 2010 - Apr 2013 B.Sc. in Honours Biomedical Sciences

                         The University of Western Ontario
                         London, Ontario, Canada
                         Sept 2009 - Apr 2010 B.MSc.

Honours and   McMaster/Western Rheumatology Research Day poster
Awards: presentation award, second place
                         Cambridge, Ontario
                         June 2017

                         Frederick Banting & Charles Best Canada Graduate Scholarship -
                         Master's (CGS-M)
                         The University of Western Ontario, London, Ontario
                         Sept 2015 – Aug 2016

                         Western Graduate Research Scholarship (WGRS)
                         The University of Western Ontario, London, Ontario
                         Sept 2014-Aug 2016

                         McMaster/Western Rheumatology Research Day oral presentation
                         award, second place
                         Cambridge, Ontario
                         June 2016

                         Canadian Bone & Joint Conference oral presentation award for
                         Basic Science category
                         London, Ontario
                         April 2016

                         Ontario Graduate Scholarship (declined)
                         The University of Western Ontario, London, Ontario
                         Sept 2015

Dr. F.W. Luney Graduate Entrance Scholarship in Microbiology &
Immunology
The University of Western Ontario, London, Ontario
Sept 2014

Dean's Honours List
University of Waterloo, Waterloo, Ontario
Sept 2011 - Apr 2013

Related Work Experience:
Summer Research Worker, Laboratory of Dr. Ines Colmegna
McGill University
Montreal, Quebec, Canada
May 2014-Aug 2014

Leadership Experience:
Society of Graduate Students Councilor for the Department of
Microbiology & Immunology
The University of Western Ontario, London, Ontario
Sept 2015-Aug 2016

Society of Graduate Students Alternate Councilor for the
Department of Microbiology & Immunology
The University of Western Ontario, London, Ontario
Sept 2014-Aug 2015

Publications in Progress:
Lac P, Racapé M, Barra L, Bell DA, Cairns E. Relatedness of
antibodies to peptides containing homocitrulline or citrulline in
patients with rheumatoid arthritis. (Under review for publication in
The Journal of Rheumatology*).

Lac P, Saunders S, Tutunea-Fatan E, Barra L, Bell DA, Cairns E.
Immune responses to peptides containing homocitrulline or
citrulline in the DR4-transgenic mouse model of rheumatoid
arthritis. (In preparation for submission to The Journal of
Autoimmunity).

Presentations:
Lac P, Racapé M, Barra L, Bell DA, Cairns E. Relatedness of
antibodies to peptides containing homocitrulline or citrulline in
patients with Rheumatoid Arthritis (poster presentation).
McMaster/Western Rheumatology Research Day
Cambridge, Ontario
June 2017

Lac P, Saunders S, Tutunea-Fatan E, Barra L, Bell DA, Cairns E.
Anti-citrulline and anti-homocitrulline immune responses in a
mouse model of Rheumatoid Arthritis (oral presentation).
McMaster/Western Rheumatology Research Day

Cambridge, Ontario
June 2017

Lac P, Racapé M, Barra L, Bell DA, Cairns E. Relatedness of antibodies to peptides containing homocitrulline or citrulline in patients with Rheumatoid Arthritis (poster presentation).
Department of Medicine Research Day
London, Ontario
May 2017

Lac P, Barra L, Bell DA, Cairns E. Immune responses to homocitrulline and citrulline in patients with Rheumatoid Arthritis (poster presentation).
Infection and Immunity Research Forum
London, Ontario
September 2016

Lac P, Barra L, Bell DA, Cairns E. Immune responses to homocitrulline and citrulline in patients with Rheumatoid Arthritis (poster presentation).
Department of Medicine Research Day
London, Ontario
May 2016

Lac P, Barra L, Bell DA, Cairns E. Immune responses to homocitrulline and citrulline in a mouse model of Rheumatoid Arthritis (poster presentation).
Department of Medicine Research Day
London, Ontario
May 2016

Lac P, Barra L, Bell DA, Cairns E. Homocitrulline and citrulline cross-reactivity in rheumatoid arthritis (oral presentation).
Canadian Bone & Joint Conference
London, Ontario
April 2016

Lac P, Barra L, Bell DA, Cairns E. Homocitrulline and citrulline cross-reactivity in Rheumatoid Arthritis (poster presentation).
London Health Research Day
London, Ontario
March 2016

Lac P, Racapé M, Barra L, Bell DA, Cairns E. The role of homocitrulline in the pathogenesis of Rheumatoid Arthritis (poster presentation).
McMaster/Western Rheumatology Research Day
Woodstock, Ontario
June 2015

Lac P, Racapé M, Barra L, Bell DA, Cairns E. The role of homocitrulline in Rheumatoid Arthritis (poster presentation).
Department of Medicine Research Day
London, Ontario
May 2015