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Immune Responses to Homocitrullinated Protein/Peptide in Rheumatoid Arthritis

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A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science
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IMMUNE RESPONSES TO HOMOCITRULLINATED PROTEIN/PEPTIDE IN RHEUMATOID ARTHRITIS

(Spine Title: Anti-Homocitrulline Immune Responses in Rheumatoid Arthritis)

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by

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Schulich School of Medicine and Dentistry

A thesis submitted in partial fulfillment

of the requirements for the degree of Master of Science

The School of Graduate and Postdoctoral Studies

The University of Western Ontario

London, Ontario, Canada

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THE UNIVERSITY OF WESTERN ONTARIO
SCHOOL OF GRADUATE AND POSTDOCTORAL STUDIES

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Immune Responses to Homocitrullinated Protein/Peptide in Rheumatoid Arthritis

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Abstract

Rheumatoid Arthritis (RA) is an inflammatory autoimmune disease which causes joint destruction. RA pathogenesis involves citrullinated peptides binding to the shared epitope (SE) during autoantigen presentation, and subsequent Anti-Citrulline Antibody (ACA) production. Their target, citrulline, is very similar to homocitrulline.

The main objective of this study was to investigate anti-homocitrulline immune responses in RA. Specifically, it investigated if: i) Anti-Homocitrulline Antibodies (AHA) were RA specific by screening patients with various inflammatory rheumatic diseases and healthy individuals. ii) ACA also bound homocitrulline by affinity purification and characterization. iii) anti-homocitrulline immune responses involved the SE by computer modelling and immunization of mice.

Results showed that AHA were common in RA only, some ACA also bound homocitrulline, and the SE could accommodate homocitrulline but did not restrict anti-homocitrulline responses in mice. In conclusion, AHA are specific to RA and some ACA cross-react with homocitrullinated targets. The SE is not essential for anti-homocitrulline responses.

Keywords: Rheumatoid Arthritis, Homocitrulline, Citrulline, Autoantibodies,

Cross-reactivity, Shared Epitope

Co-authorship

Chapter 2.

Scinocca, M.J., Shaw, G., Joseph, R., Bell, D.A*., Barra, L.*, Cairns, E*. Homocitrulline: An antigen specific to rheumatoid arthritis and a target of anti-citrulline protein/peptide antibodies.

M.J. Scinocca- conducted all experiments, interpreted results, and wrote the paper. G. Shaw- designed molecular modelling mutation file, aided with molecular modelling and interpreted results. R. Joseph- aided with some experiments. *D.A. Bell, L. Barra, and E. Cairns are co-senior authors and contributed equally to this research. They co-supervised this research, designed experiments, interpreted results, and wrote the paper.

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List of Abbreviations

ACFA	Anti-Citrullinated Fibrinogen Antibodies
ACPA	Anti-Citrullinated Protein/Peptide Antibodies
AHFA	Anti-Homocitrullinated Fibrinogen Antibodies
AHPA	Anti-Homocitrullinated Protein/Peptide Antibodies
AMC	Anti-Modified Citrulline
CAD	Coronary Artery Disease
CCP2	Cyclic Citrullinated Peptide 2
CFA	Complete Freund's Adjuvant
CIA	Collagen Induced Arthritis
Da	Daltons
DTT	Dithiotreitol
ELISA	Enzyme-Linked ImmunoSorbant Assay
FBS	Fetal Bovine Serum
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IFA	Incomplete Freund's Adjuvant
Ig	Immunoglobulin

MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
MPO	Myeloperoxidase
NET	Neutrophil Extracellular Trap
OD	Optical Density
PAD	Peptidyl Arginine Deiminase
PAMP	Pattern-Associated Molecular Patterns
PBS	Phoshpate-Buffered Saline
PBST	Phoshpate-Buffered Saline with Tween
PsA	Psoriatic Arthritis
RA	Rheumatoid Arthritis
RMS	Root Mean Squared
SE	Shared Epitope
SLE	Systemic Lupus Erythematosus
Tg	Transgenic

Chapter 1:

Introduction

1.1 Immunology overview

The immune system is critical for the human body's development, maintenance, and protection from foreign entities. It is broadly separated into two different categories: innate and adaptive immunity. In the context of infection, the former plays an important role in prevention and the initial, general response. Alternatively, the adaptive immune system recognizes specific targets to promote elimination of pathogens as well as sustained protection from re-infection through immunological memory.

1.1.1 Innate immunity

The innate immune system is comprised of any cellular and humoral factors that act as a first line of defense to prevent or combat disease and infection. The major cells involved are neutrophils, macrophages (1), natural killer cells (2), and dendritic cells (3-4). The innate immune system does not recognize specific regions of invading pathogens or unhealthy tissue, but rather binds common structures known as Pathogen-Associated Molecular Patterns (PAMPs) (5) or Damage-Associated Molecular Patterns (DAMPs) (6). PAMPs and DAMPs are recognized by various proteins known as Pattern Recognition Receptors (PRRs) such as the Toll-Like Receptors (TLRs) (5). The binding of PAMPs/DAMPs to membrane bound or intracellular PRRs triggers the expression of inflammatory genes leading to the release of signaling proteins (cytokines) and cellular attractants (chemokines) (7). The subsequent induction of inflammation, observed clinically as heat, swelling, redness, and pain, occurs very quickly. Another mechanism for the induction of inflammation is through biochemical cascades involving soluble plasma proteins known as complement factors (8). Complement proteins are the major

humoral component of innate immunity and can aid the immune response by coating foreign invaders (9). The coating of complement proteins on foreign cell bodies can promote membrane instability leading to cell death and improve immune recognition (10).

During inflammation, one of the primary ways infectious agents are removed from the body after recognition is through phagocytosis. Phagocytosis is a mechanism by which particles are engulfed by the cell. Pathogens and damaged tissues are bound and surrounded by host cell membrane forming an intracellular vesicle known as a phagosome (11). The phagosome then binds to a lysosome which drops the pH of the compartment (12). This triggers destruction of the pathogen by hydrolytic enzymes (13) and reactive oxygen species (14). Phagocytosis is not only important for innate immunity but it is also important for the adaptive immune system, as it allows exposure to foreign peptides and proteins (immunogens) for which specific adaptive immune responses can be mounted against (15).

1.1.2 Adaptive immunity

In contrast to the innate immune system, the adaptive immune system takes time to initiate a response and the response is specific since it targets unique peptide/protein sequences and structures (epitopes). These responses can also provide long-term continued protection from re-exposure to the same entity through the generation of memory cells. Adaptive immunity is divided into two different components; T cell mediated cellular immunity, and B cell mediated humoral immunity.

1.1.2.1 T cells

T cells are divided into two different, major subsets, CD4+ and CD8+ (16). Each individual T cell expresses T cell antigen receptors with a unique variable region that is generated by the genetic recombination of genomic sequences during development (17). An individual has a large repertoire of T cells with different antigen specificities and through a process known as "clonal selection," (18) T cells which bind their cognate peptide become activated. T cell receptors recognize foreign peptides in the context of Major Histocompatibility Complex (MHC) molecules (19-21). In a process termed antigen presentation, peptides from degraded proteins are bound to MHC molecules and exported to the surface of cells to be recognized by T cells (22-23). CD8+ T cells recognize peptides that are bound to MHC class I molecules which are expressed by every nucleated cell in the body (24). Typically, intracellular proteins are digested and loaded onto MHC class I molecules through what is known as the "endogenous pathway." This allows CD8+ T cells to recognize and destroy infected or dysplastic host cells (25-26). CD4+ T cell antigen receptors, on the other hand, recognize peptides bound to MHC class II molecule (27-29). MHC class II molecules are found on specialized Antigen Presenting Cells (APCs), such as macrophages and dendritic cells. They bind to phagocytosed extracellular peptides generated through the "exogenous pathway" and present these peptides to CD4+ T cells which results in CD4+ T cell activation (30). Activated CD4+ T cells can then enhance killing by phagocytes (31) and promote B cell responses (32).

1.1.2.2 B cells

B cells are responsible for the humoral response of adaptive immunity. Like T cells, each B cell expresses B cell antigen receptors with a unique variable region (33)

that is generated by the genetic recombination of genomic sequence segments during development (34-36). Also similar to T cells, B cells undergo clonal selection in which cells of the diverse repertoire that encounter their cognate target are activated (37-38). B cell antigen receptors, however, bind to peptides or complete proteins without MHC restriction. The protein region that an antibody binds is known as an epitope or antigenic determinant. When a B cell encounters and binds its antigen, it becomes activated, proliferates (39), and further differentiates into a plasma cell that secretes a soluble form of its membrane B cell antigen receptor, known as immunoglobulins (Ig) or antibodies (40-41). Like the membrane receptor, each soluble Ig molecule is comprised of two identical variable regions that can bind antigen as well as a constant region (42-43). The constant region of antibodies can be bound by protein and specific cell receptors (44). There are five different classes of Ig which differ in respect to their constant regions and thus, effector molecule interactions. The five different isotypes are IgA, IgD, IgE, IgG, and IgM (45-47). Membrane bound B cell antigen receptors are IgM and IgD (48) but during B cell activation and proliferation, daughter cells can switch isotypes to IgA, IgE, or IgG while maintaining their variable region and binding specificity (49). IgG is the most abundant isotype circulating in the human body and, therefore, plays a major role in controlling infection. Antibody class switching typically requires signalling from activated CD4+ T cells (50-51) and the isotype of the antibody determines its potential effector functions and interactions.

Upon binding and coating of its target, secreted Ig can result in functional neutralization, improved immune recognition and phagocytosis, complement fixation, and the formation of antigen:antibody immune complexes (52). Antigen:antibody

binding relies upon numerous non-covalent bonds and the affinity by which an antibody can bind its target depends upon the net attractive and repulsive forces. Hydrogen bonds, salt bridges, hydrophobic forces and van der Waals interactions are all important for overall affinity and specificity. Thus, not only the chemical properties of the targeted amino acids such as charge are important, but also the structural conformation or shape of the targeted region (53-54). Although antibodies bind their target with high specificity and affinity, some are capable of binding multiple targets. Antibodies binding to targets other than the original immunogen are known as "cross-reactive." (55-56) Even when binding the original, immunogenic antigen, an individual's naive B cell repertoire does not usually include antibodies which can bind with high affinity. As an immune response progresses, however, antibody responses can undergo affinity maturation which increases antibody binding affinity through somatic hypermutation of B cell clone variable genes (57-58). High affinity recognition by antibodies helps to ensure efficient clearance of foreign pathogens.

1.2 Tolerance and autoimmunity

The immune system must be able to initiate immune responses against pathogens or non-self proteins while leaving healthy tissue unharmed. The ability to discriminate between foreign entities and native host proteins is important for both B and T cells and is known as tolerance. Self tolerance is divided into two major categories: central and peripheral. Central tolerance for B and T cells is achieved through negative selection during development in the bone marrow (59-60) and thymus (26, 61), respectively. During this process, cells which bind self targets with high affinity are eliminated by programmed cell death known as apoptosis (62-64). Some auto-reactive clones may

escape negative selection however, especially those that only weakly recognize self proteins (65-66).

Peripheral tolerance allows control of immune responses throughout the body after B and T cell development. Self reactive clones which were not negatively selected or which did not have their cognate self-antigen expressed in the thymus or bone marrow can be controlled by multiple mechanisms. T regulatory (Treg) cells can control localized immune responses in areas of inflammation (67-68). Also, tolerance can be induced simply by the amount of exposure to antigens. Self antigens are usually exposed to the immune system consistently at high levels while in the case of infection, immunogens are available in an acute pattern where availability peaks early. This distinction can promote an altered, inactive immune cell state known as anergy to prevent responses against self proteins (69).

When the ability of adaptive immune responses to distinguish self from non-self is compromised, and immunological tolerance is broken, autoimmune disease occurs. Autoimmunity can be triggered by infection/injury (70-72). The inflammation which ensues can cause the activation of self-reactive clones which escaped central tolerance (73). Infection can also trigger autoimmunity through a process known as molecular mimicry. If a bacterial or viral antigen is unique enough to trigger an immune response, but the target resembles a self protein, cross-reactivity can develop and progress into an autoimmune response (74-75).

Another mechanism which can trigger autoimmune responses is immune reaction to altered-self (76). This is exemplified by healthy responses to tumour cells. Cancerous

mutations lead to the expression of proteins that are different from native, self proteins and normal immune responses lead to the elimination of these cells due to their novel immunogenic epitopes. Instead of mutation, however, post-translational modification can induce autoimmunity in otherwise healthy tissue (77). The modification of native proteins due to enzymatic modification or chemical insult occurs throughout the body and proteins with longer half-lives are likely to accumulate more post-translational modifications due to their longer "lifespan." As with cancerous mutation, these protein modifications generate structurally and chemically unique areas which can be detected and targeted by the immune system (78). If the modification consistently generates adequate amounts of targets in tissues, and the individual has the genetic capacity to mount a response against the modified protein, an autoimmune response can be maintained.

1.3 Rheumatoid Arthritis (RA)

RA is an autoimmune disease characterized by inflammation of the joints leading to tissue destruction. RA affects approximately 1% of the world's population, occurs more frequently in women and is a serious cause of morbidity in Canada as well as worldwide. In the RA joint, inflammation and hyperplasia of the joint synovium can be observed with excess joint fluid causing swelling. There is also formation of abnormal tissue known as pannus and the infiltration of immune cells such as neutrophils, macrophages, B cells, and T cells. Progression of the disease can lead to loss of protective cartilage, bone erosion, and eventually loss of joint function. Inflammation in the lungs, eyes and heart, including increased atherosclerosis can also accompany RA. It is well documented that genetic and environmental factors are involved in the development of RA (79-82).

The major genetic risk factors are MHC class II genes encoding the Shared Epitope (SE). The PTPN22 gene which encodes a tyrosine kinase important for T and B cell responses is also linked to RA, however, it is associated with other autoimmune diseases as well (83-85) so it is likely involved in the general development of autoimmunity rather than the specific pathogenesis of RA. The autoimmune responses in RA involve T and B cells that lead to the production of autoantibodies.

Antibodies known to be associated with RA are Rheumatoid Factor (RF) and Anti-Citrullinated Protein/Peptide Antibodies (ACPA). In 2010 Mydel *et al*. demonstrated the presence of Anti-Homocitrullinated Protein/Peptide antibodies (AHPA) in RA (86). While RF and ACPA have been extensively studied, little is known about these recently discovered AHPA in RA.

1.3.1 Rheumatoid Factor (RF)

The first autoantibody identified in RA was RF, an antibody which binds to the constant region of human IgG (87). Therefore, RF can form immune complexes with IgG and also increase immune complex formation by binding and cross-linking pre-existing antigen:IgG antibody complexes. RF is most commonly identified as the IgM or IgA isotype and has been associated with increased risk of RA with a sensitivity of 60-80% (88-90). Its involvement in RA pathogenesis has been questioned, however, as RF is not unique to this disease. It can be detected in other autoimmune diseases such as Sjögren's syndrome and Systemic Lupus Erythematosus (SLE), and even in healthy individuals (91).

1.3.2 Anti-Citrullinated Protein/Peptide Antibodies (ACPA)

Anti-Citrullinated Protein/Peptide Antibodies (ACPA) are antibodies which bind to proteins or peptides that have undergone the post-translational modification known as citrullination (described in 1.3.2.1) Unlike RF, antibodies which target citrullinated antigens are highly specific for RA (>95%) (90). ACPA were originally identified as antibodies that bound to the protein filaggrin in epithelial tissue (92-93) which is not a RA-relevant antigen. It is now well documented that the same anti-filaggrin antibodies recognize citrullinated fibrinogen (94) which is a RA-relevant antigen because: i) antibodies to it are highly specific for RA (94-95); ii) half of ACPA positive RA patients have circulating citrullinated fibrinogen:IgG antibody complexes (96); iii) it is present in inflamed RA joints (97); and iv) immunization with it induces arthritis in DR4 Transgenic (Tg) mice expressing the SE (98). Human fibrinogen is a conserved, hexameric protein that is composed of a pair of the α, β, and γ chain (Figure 1.1) (99) and contains 79 arginines that potentially can be citrullinated.

Other identified targets of ACPA include: i) citrullinated collagen II - an extracellular matrix protein that is abundant in cartilage (100-101); ii) citrullinated α-enolase - an enzyme found in most tissues (102); and iii) citrullinated vimentin - an intracellular cytoskeletal protein (103). Citrullinated vimentin was originally described as Sa antigen (104-105). These other citrullinated targets are also found in the joints of RA patients (94, 102, 104-105).

ACPA, which comprise antibodies to these targets, are commonly detected using cyclic citrullinated peptide (CCP2), a surrogate citrullinated antigen that is not present in the body. The specificity of antibodies binding to CCP2 for RA is >95% and its sensitivity is 50-70% (90, 106-107). A citrullinated peptide, JED, with similar sensitivity

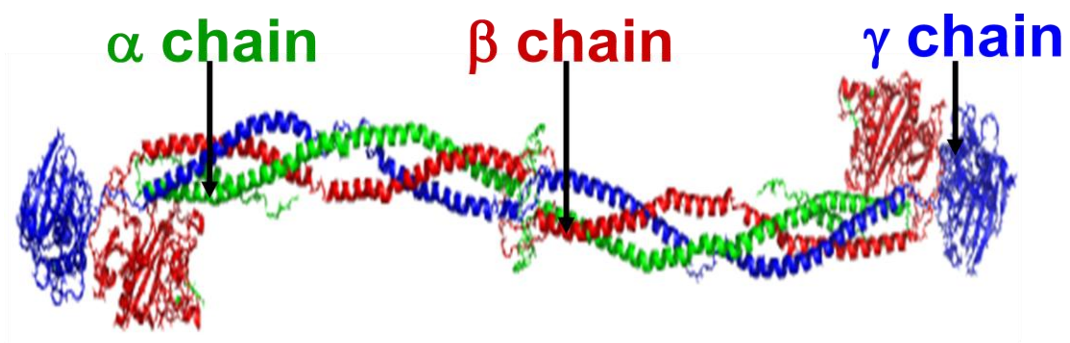


Figure 1.1 Human fibrinogen X-ray crystal structure. Ribbon diagram of the structure of human fibrinogen obtained by of the X-ray crystallography and viewed in PyMol (PDB structure 3GHG) (99).

and specificity for RA as CCP2 has been developed in this laboratory (108-109). JED is a proprietary synthetic, cyclic peptide that is 18 amino acids in length with C-terminal amidation. 9/18 residues are citrulline and 2/18 are cysteine to facilitate cyclization through disulphide bond formation. JED can capture anti-CCP2, anti-citrullinated fibrinogen, and anti-mutated citrullinated vimentin antibodies and thus, ACPA. Therefore, JED peptide is a critical reagent for ACPA purification and characterization of these antibodies (108).

1.3.2.1 Citrullination

ACPA targets are generated by citrullination which is the post-translational deimination of arginine and generates the amino acid citrulline. The deimination reaction is catalyzed by the Ca^{2+} dependant intracellular enzyme Peptidyl Arginine Deiminase (PAD) (110). During the reaction, an amine of arginine's guanidine functional group is converted to a carbonyl, generating the functional ureido group which is found on citrulline (Figure 1.2A). Thus, the modification effectively neutralizes the positive charge that arginine has at neutral pH, producing an uncharged but polar side-chain that has an increased affinity for the SE which is commonly found in RA.

There are five different PAD enzymes encoded by the human genome which suggests that citrullination is an important physiological process. It has been shown that citrullination plays an important role in Neutrophil Extracellular Trap (NET) release by neutralizing positively charged histones to facilitate DNA dissociation and also in ribosome assembly by competing with methylation (111-112). Supporting the role of PAD enzyme in RA, some PAD variants have been identified as genetic traits linked to

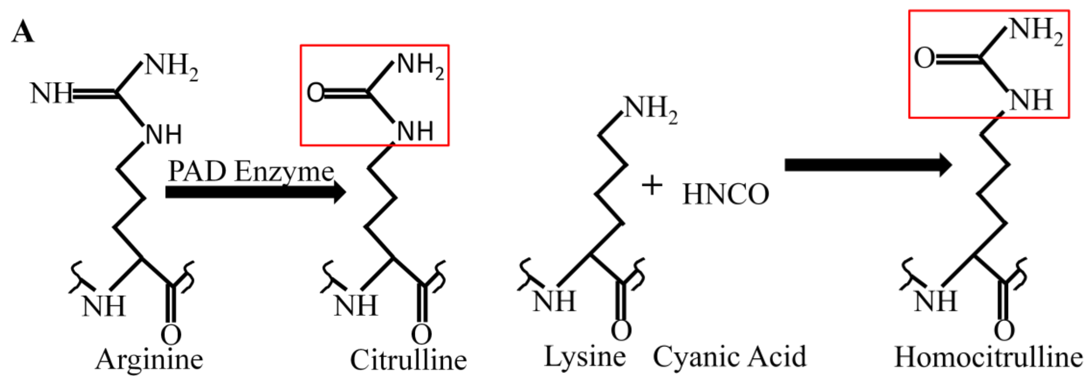


Figure 1.2 Citrullination and homocitrullination. Citrulline is generated enzymatically from arginine (A), and homocitrulline is generated chemically from lysine (B). The identical ureido groups are indicated with a red box.

RA. Citrullination has been shown to occur during the process of apoptosis (113-114) which involves influx of calcium that is necessary for PAD activation. Thus, intra-cellular proteins such as vimentin can be citrullinated during this process and exposed on the surface of apoptotic blebs. During apoptosis, PAD enzyme can also be released to citrullinate extracellular proteins in the surrounding tissue, such as fibrinogen and collagen. This process can occur in neutrophils and macrophages during inflammation (114-115). If citrullinated proteins/peptides are not cleared efficiently, these post-translationally modified proteins can be recognized by the immune system and trigger immune responses that lead to the production of ACPA. The target of ACPA, citrulline, has been shown to be increased in the RA joint as detected by both mass spectrometry and immunohistochemistry using Anti Modified Citrulline (AMC) antibodies.

1.3.2.2 ACPA and the SE

The major heritable risk factors for RA are genes encoding MHC class II molecules with the Shared Epitope (SE). Specifically, the SE is linked to the production of ACPA (79). The SE is a consensus amino acid sequence (glutamine/arginine, lysine/arginine, arginine, alanine, and alanine) located in the peptide binding groove of MHC class II molecules (116). The SE forms a positively charged P4 binding pocket of the MHC molecule. Hill *et al*. in the laboratory of Dr. Ewa Cairns showed that the SE has an increased affinity for peptides which have polar amino acids at this position compared to a positive charge (116). Peptides with a positively charged amino acid at this position are repelled due to the like charge, however, an amino acid such as citrulline binds to the SE expressed on MHC class II molecules with high affinity. This can

facilitate an immune response which leads to production of ACPA (116-117) (Figure 1.3).

1.3.2.3 ACPA and environmental factors

The role of environmental risk factors in the development of RA is not well understood. The primary risk factor which has been identified is smoking (79, 118). Smoking has been shown to promote citrullination in the lungs and was shown to be associated with ACPA production in RA (79, 119).

An alternative environmental risk factor for RA is infection. Exposure to Epstein-Barr virus (120), Hepatitis B and C viruses (121-122), and Human Parvovirus B19 (123) have been shown to be associated with RA. In addition to triggering inflammatory responses in the presence of auto-reactive T and B cell clones and/or citrulline, it is thought that these infections could contribute to the development of RA through antigenic mimicry. In such cases, pathogen epitopes which are targeted by the immune system resemble host proteins. Such an observation has been made in the case of periodontitis, which is linked to RA. In periodontitis, infection of the oral cavity by *Porphyromonas gingivalis* triggers inflammation and immune responses (102). *P. Gingivalis* is the only known bacterium to express a PAD enzyme. Therefore, infection can lead to exposure to citrullinated bacterial protein, specifically α-enolase (102). This can trigger an antibody response that is cross-reactive with the human form of citrullinated α-enolase, indirectly causing ACPA production leading to RA development.

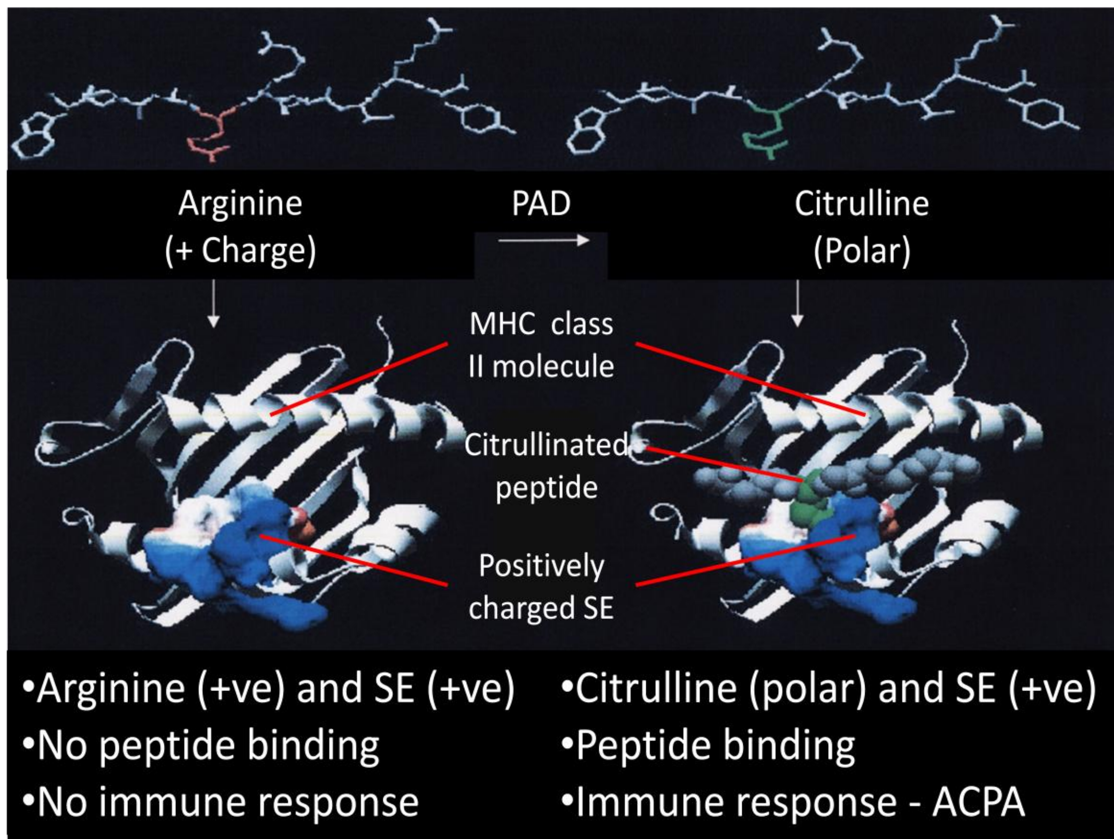


Figure 1.3 Citrullination promotes binding to the SE leading to ACPA production. Peptides with positively charged arginine at the P4 position are repelled by the positively charged SE (Left) and do not trigger an immune response. Conversion to a polar citrulline residue through citrullination increases affinity for the SE (Right), resulting in peptide binding and a subsequent immune response that leads to ACPA production. Figure was adapted from Hill *et al.* (117) (See Appendix 4)**.**

1.3.2.4 ACPA and the pathogenesis of RA

Studies in human RA and in experimental models of this disease provided evidence that the immune responses to citrullinated protein/peptides targets are involved in pathogenesis of RA. (98, 101-102, 124-127). ACPA can be detected in humans years before clinical RA onset (128-130), and their titres increase until disease initiation. Radiological and histological analyses show that the presence of high titres of ACPA predicts more erosive and severe disease (131). Petkova *et al.* demonstrated that passive transfer of human ACPA positive serum is able to cause a transient form of arthritis in autoimmune susceptible, FcγRIIb deficient mice (124). The laboratory of Dr. Ewa Cairns extended this observation further and directly showed that it is ACPA that cause disease in the same mouse strain (132). This was achieved by affinity purification of ACPA from the human serum using the JED peptide and injection of these human purified ACPA antibodies into FcγRIIb deficient mice. These mice express citrulline in their joints. FCγRIIb deficient mice who received ACPA developed transient arthritis but the mice that received IgG void of ACPA did not. The same human ACPA did not cause arthritis in B6 mice unless citrullinated protein target was injected into the joint. These experimental findings emphasize that arthritogenicity of human ACPA is highly dependent on the presence of citrullinated antigen in the joint.

Studies in experimental animal models of RA further support the notion that citrullination and immune responses to citrulline are important in pathogenesis of RA. It was reported that concomitant injection of a monoclonal antibody targeting citrullinated collagen II increased severity of the Collagen Induced Arthritis (CIA) model (101, 126-127). Further evidence was obtained by the development of a citrullinated fibrinogen

induced arthritis model in this laboratory (98). This model consists of humanized DR4 Tg mice that express chimeric MHC class II molecule with the SE. These mice have a C57BL/6 background but do not express any of their endogenous murine MHC class II (133). The chimeric MHC class II includes the peptide binding region of human origin with the SE, however the rest of the molecule is the mouse form of the protein (Figure 1.4). This means that the mice APCs can present peptides to T cells in a similar manner as RA patients who express the SE.

1.3.2.5 Current model for ACPA involvement in the pathogenesis of RA

Although the exact etiology of RA is unknown, current research findings allow the proposal of a model for the pathogenesis of RA. This model involves at least two events.

The first event is break in immunological tolerance. This occurs in periphery through citrullination and ACPA production in genetically susceptible subjects expressing the SE. In such subjects, infection or injury causes inflammation and cell death. This leads to PAD activation, and the subsequent citrullination of proteins generates peptides with novel epitopes. If these citrullinated antigens are not cleared efficiently, possibly due to defects in phagocytosis or merely an overwhelming amount of cell death, citrullinated antigens are taken up by APCs. The citrullinated peptides derived from these antigens are able to bind to the SE with high affinity are presented to citrulline-specific CD4+ T cells and cause their activation. As a consequence, activated citrulline-specific CD4+ T cells will help B cells to produce IgG ACPA. This first event is believed to demarcate pre-clinical RA which with time can develop into RA if the second event takes place.

The second event takes place in the joint and can be triggered by infection or physical

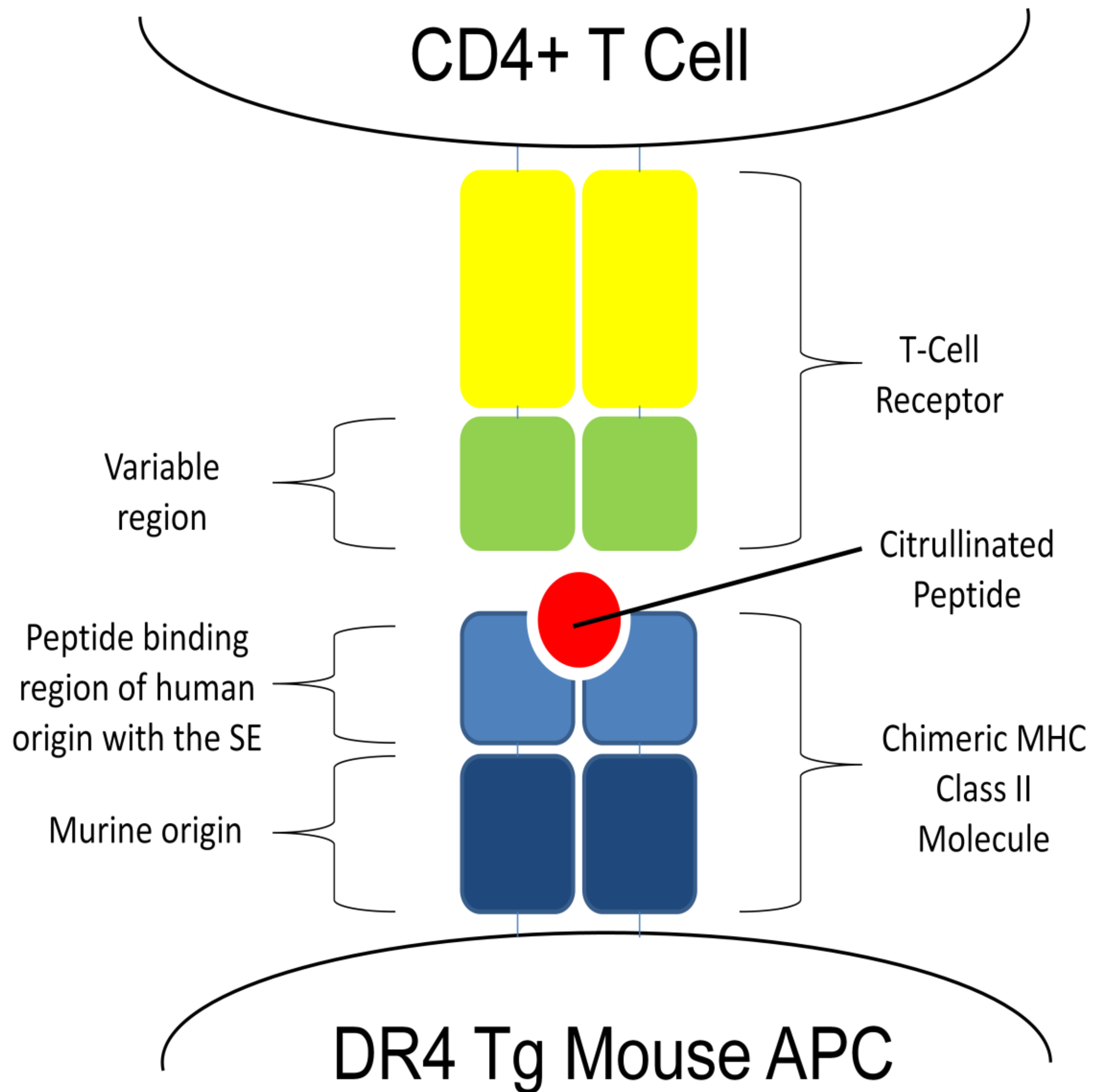


Figure 1.4 DR4 Tg mouse MHC class II peptide presentation. Citrullinated peptide is bound to the DR4 Tg mouse, chimeric MHC class II molecule with the SE. The peptide in the context of MHC molecule is recognized by a CD4+ T cell receptor.

injury causing acute inflammation resulting in the production of citrullinated protein targets. Again, with insufficient clearance of these citrullinated protein targets, ACPA can form antigen:antibody complexes which activate complement cascade. This cycle of ACPA causing inflammation which leads to increased citrullination of proteins in the joint can then exacerbate and perpetuate inflammatory disease leading to sustained, chronic inflammation and clinical RA (Figure 1.5). The strong association of RA with the SE, the presence of IgG and IgA ACPA isotypes, and the large number of CD4+ T cells in the RA joint, support the notion that citrullination and citrulline-specific immune responses play an important role in arthritogenesis

1.3.3 Anti-Homocitrullined Protein/Peptide Antibodies (AHPA)

Anti-Homocitrullinated Protein/Peptide Antibodies (AHPA) are antibodies which bind to proteins or peptides that have undergone the post-translational modification known as homocitrullination (described in 1.3.3.1). It has been demonstrated that RA patients can generate AHPA and homocitrullinated protein targets of these antibodies can be found in the RA synovium (86, 134), however, the sensitivity of these antibodies in RA has not been well characterized and their specificity to this disease is unknown.

1.3.3.1 Homocitrullination

The carbamylation of lysine's side-chain, a process known as homocitrullination, generates the amino acid homocitrulline (135). Homocitrulline is very similar to citrulline, both structurally and chemically as it has the exact same functional, ureido group (Figure 1.2B). The only major structural difference of the two amino acids is that the side-chain of homocitrulline is extended by one carbon making its total bond length

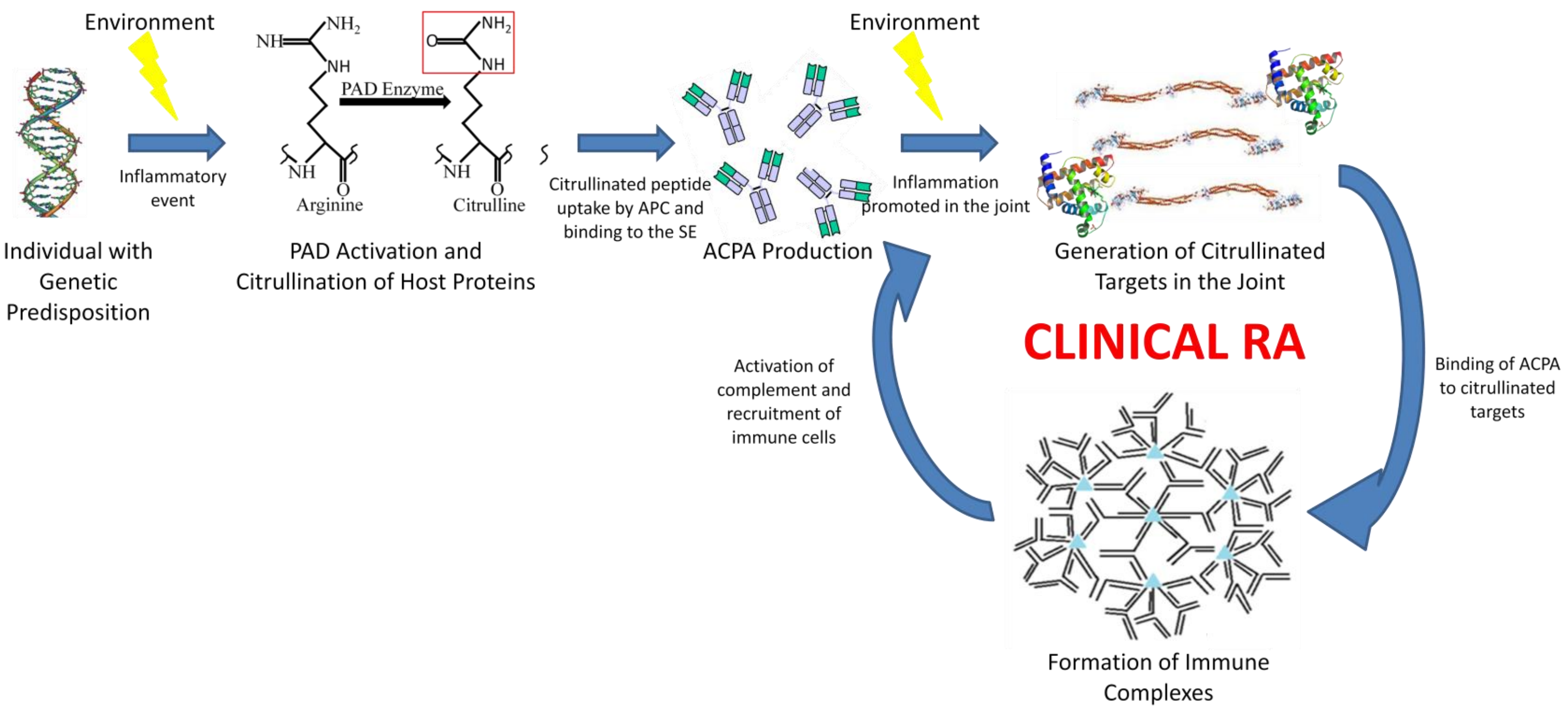


Figure 1.5 Current model for ACPA involvement in the pathogenesis of RA. All images were from public domain or produced originally.

approximately 1.56 Å longer from the backbone α-carbon to the terminal, ureido carbon after hydrochloride crystallization (136).

Unlike citrullination which is catalyzed enzymatically, homocitrullination is a chemical modification. Although the exact reaction mechanism seems unclear, homocitrullination can occur ubiquitously as long as the reactive metabolite, cyanate/cyanic acid, is present. Cyanate is generated in the body by at least two sources. The first of which is the spontaneous degradation of urea. Urea is ubiquitous in the body and always in equilibrium with cyanate. It is estimated that at physiological conditions, approximately 0.8% of the molar concentration of urea is in the cyanate form (137). Therefore, wherever there is urea, there is cyanate and the potential for homocitrullination. The second source is production by the enzyme MyeloPerOxidase (MPO). MPO is found primarily in neutrophil granules and is active at sites of inflammation. When the enzyme encounters thiocyante (its preferred substrate) it catalyzes conversion to cyanate (138). Thus, while increased levels of urea promote carbamylation systemically, MPO provides a mechanism for localized increase in cyanate levels at the sites of inflammation.

Early research into carbamylation focused on the modification of serum proteins during renal failure and the interference of carbamylation with protein identification and analysis. Like other protein modifications, carbamylation can lead to altered isoelectric points, protein structure, and enzymatic function. It has even been shown that carbamylation can cause altered hormone activity, and interfere with protein synthesis and insulin uptake (139). Carbamylation was previously implicated in the formation of cataracts and more recently, it was reported to be involved in the molecular pathogenesis

of atherosclerosis (138, 140). The chemical modification, carbamylation can clearly have a wide variety of effects on biological molecules and processes.

1.3.3.2 Homocitrullination in RA

Homocitrullination was originally introduced into RA research through the observation that the AMC reagent commonly used to detect citrulline also stained homocitrulline (141). This suggested that some of the previous *in situ* identification of citrulline in arthritic joint tissue may have been homocitrulline. The same study also demonstrated that homocitrulline could be immunogenic. They reported that rabbits immunized with homocitrullinated protein could produce AHPA and also suggested that in some rabbits these antibodies could bind citrullinated targets as well. Additional evidence for homocitrulline immunogenicity was provided by Mydel *et al.* who further demonstrated an arthritogenic role for anti-homocitrulline immune responses. They reported that immunization of some strains of mice with homocitrullinated peptide can cause erosive arthritis (86). Their study was also the first to report that human RA patients can have AHPA and homocitrulline in the joints and circulation. They found that levels of AHPA and circulating homocitrulline were greater in patients with erosive RA. Their observations were recently confirmed by Shi *et al* who also reported that AHPA correlated with joint damage but additionally found AHPA in ACPA-negative RA patients (134). This latter study by Shi and colleagues demonstrated that human RA patients had antibodies to carbamylated fibrinogen and demonstrated that these antibodies do not recognize citrullinated fibrinogen. Neither Mydel or Shi's study addressed the question of whether anti-homocitrulline antibodies are specific for human RA by testing AHPA in other inflammatory rheumatic conditions.

1.4 Rationale and hypothesis

Numerous studies have shown that citrullination is important in the pathogenesis of RA. The modification can alter protein structure to generate neo-epitopes that trigger autoimmune responses and generation of ACPA which target various citrullinated human proteins that are found in the RA joint. This laboratory has previously shown that the RA-associated SE plays an important role in this process. It can bind citrullinated peptides with high affinity leading to the activation of auto-reactive T cells and subsequent production of ACPA which are found specifically in RA patients and play a role in arthritogenesis.

Homocitrulline is related to citrulline both structurally and chemically. Since homocitrullination can alter protein structure, it will also be able to generate neo-epitopes on human proteins that are recognized by the immune system.

The hypothesis of this study is that homocitrullination is involved in RA. More specifically, it is hypothesized that homocitrulline will be able to bind to the SE, like citrulline can. This will result in activation of homocitrulline specific T cells and AHPA production. The APHA will be found in RA patients specifically. Some ACPA will be able to bind both citrullinated and homocitrullinated antigens.

1.4.1 Specific objectives

i) Homocitrullinate human fibrinogen and analyze sites of potential modification to identify potential autoantibody targets.

ii) Screen sera from RA, psoriatic arthritis and SLE patients as well as normal individuals for Anti-Homocitrullinated Fibrinogen Antibodies (AHFA) to investigate if these AHPA are specific to RA among inflammatory, rheumatic disease.

iii) Investigate the role of the SE in homocitrulline immunogenicity using molecular modelling and DR4 Tg mice expressing the SE. Since the SE is strongly linked to RA and it restricts the production of ACPA, the SE may also affect anti-homocitrulline immune responses and AHPA production in RA patients.

iv) Affinity purify ACPA with citrullinated peptide (JED) and test for "cross-reactivity" to homocitrullinated antigen. Since ACPA have previously been shown to be pathogenic, the existence of cross-reactive antibodies which bind both citrullinated and homocitrullinated antigens could add additional complexity to the current two-hit model. Citrulline/homocitrulline cross-reactivity may introduce novel mechanisms for break in immunological tolerance and/or the generation of autoantibody targets in the joint.

The studies presented in this thesis are significant because immune responses to homocitrulline may be involved in the pathogenesis of RA. A better understanding of the mechanisms underlying arthritogenesis will aid the development of antigen-specific therapeutic treatments and improved patient care.

1.5 References

1. Kaufmann SH. 2008. Immunology's foundation: the 100-year anniversary of the Nobel Prize to Paul Ehrlich and Elie Metchnikoff. *Nat Immunol* 9: 705-12

2. Kiessling R, Klein E, Pross H, Wigzell H. 1975. "Natural" killer cells in the mouse. II. Cytotoxic cells with specificity for mouse Moloney leukemia cells. Characteristics of the killer cell. *Eur J Immunol* 5: 117-21
3. Steinman RM, Cohn ZA. 1973. Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. *J Exp Med* 137: 1142-62
4. Steinman RM, Witmer MD. 1978. Lymphoid dendritic cells are potent stimulators of the primary mixed leukocyte reaction in mice. *Proc Natl Acad Sci U S A* 75: 5132-6
5. Janeway CA, Jr. 1989. Approaching the asymptote? Evolution and revolution in immunology. *Cold Spring Harb Symp Quant Biol* 54 Pt 1: 1-13
6. Matzinger P. 2002. The danger model: a renewed sense of self. *Science* 296: 301-5
7. Medzhitov R, Preston-Hurlburt P, Janeway CA, Jr. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* 388: 394-7
8. Matsushita M. 1996. The lectin pathway of the complement system. *Microbiol Immunol* 40: 887-93
9. Liszewski MK, Farries TC, Lublin DM, Rooney IA, Atkinson JP. 1996. Control of the complement system. *Adv Immunol* 61: 201-83
10. Dempsey PW, Allison ME, Akkaraju S, Goodnow CC, Fearon DT. 1996. C3d of complement as a molecular adjuvant: bridging innate and acquired immunity. *Science* 271: 348-50

11. Aderem A, Underhill DM. 1999. Mechanisms of phagocytosis in macrophages. *Annu Rev Immunol* 17: 593-623

12. Jacques YV, Bainton DF. 1978. Changes in pH within the phagocytic vacuoles of human neutrophils and monocytes. *Lab Invest* 39: 179-85

13. Bretz U, Baggiolini M. 1974. Biochemical and morphological characterization of azurophil and specific granules of human neutrophilic polymorphonuclear leukocytes. *J Cell Biol* 63: 251-69

14. Johnston RB, Jr., Keele BB, Jr., Misra HP, Lehmeyer JE, Webb LS, Baehner RL, RaJagopalan KV. 1975. The role of superoxide anion generation in phagocytic bactericidal activity. Studies with normal and chronic granulomatous disease leukocytes. *J Clin Invest* 55: 1357-72

15. Savina A, Amigorena S. 2007. Phagocytosis and antigen presentation in dendritic cells. *Immunol Rev* 219: 143-56

16. Cantor H, Asofsky R. 1972. Synergy among lymphoid cells mediating the graft-versus-host response. 3. Evidence for interaction between two types of thymus-derived cells. *J Exp Med* 135: 764-79

17. Davis MM, Bjorkman PJ. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature* 334: 395-402

18. Burnet FM. 1962. The immunological significance of the thymus: an extension of the clonal selection theory of immunity. *Australas Ann Med* 11: 79-91

19. Bevan MJ. 1975. The major histocompatibility complex determines susceptibility to cytotoxic T cells directed against minor histocompatibility antigens. *J Exp Med* 142: 1349-64

20. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. 1987. Structure of the human class I histocompatibility antigen, HLA-A2. *Nature* 329: 506-12

21. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. *Nature* 329: 512-8

22. Babbitt BP, Allen PM, Matsueda G, Haber E, Unanue ER. 1985. Binding of immunogenic peptides to Ia histocompatibility molecules. *Nature* 317: 359-61

23. Buus S, Colon S, Smith C, Freed JH, Miles C, Grey HM. 1986. Interaction between a "processed" ovalbumin peptide and Ia molecules. *Proc Natl Acad Sci U S A* 83: 3968-71

24. Norment AM, Salter RD, Parham P, Engelhard VH, Littman DR. 1988. Cell-cell adhesion mediated by CD8 and MHC class I molecules. *Nature* 336: 79-81

25. Zinkernagel RM, Doherty PC. 1974. Immunological surveillance against altered self components by sensitised T lymphocytes in lymphocytic choriomeningitis. *Nature* 251: 547-8

26. Zinkernagel RM, Callahan GN, Althage A, Cooper S, Klein PA, Klein J. 1978. On the thymus in the differentiation of "H-2 self-recognition" by T cells: evidence for dual recognition? *J Exp Med* 147: 882-96

27. Shevach EM, Green I, Paul WE. 1974. Alloantiserum-induced inhibition of immune response gene product function. II. Genetic analysis of target antigens. *J Exp Med* 139: 679-95

28. Paul WE, Shevach EM, Pickeral S, Thomas DW, Rosenthal AS. 1977. Independent populations of primed F1 guinea pig T lymphocytes respond to antigen-pulsed parental peritoneal exudate cells. *J Exp Med* 145: 618-30

29. Doyle C, Strominger JL. 1987. Interaction between CD4 and class II MHC molecules mediates cell adhesion. *Nature* 330: 256-9

30. Teyton L, O'Sullivan D, Dickson PW, Lotteau V, Sette A, Fink P, Peterson PA. 1990. Invariant chain distinguishes between the exogenous and endogenous antigen presentation pathways. *Nature* 348: 39-44

31. Raveh D, Kruskal BA, Farland J, Ezekowitz RA. 1998. Th1 and Th2 cytokines cooperate to stimulate mannose-receptor-mediated phagocytosis. *J Leukoc Biol* 64: 108-13

32. Baumgarth N. 2000. A two-phase model of B-cell activation. *Immunol Rev* 176: 171-80

33. Valbuena O, Marcu KB, Weigert M, Perry RP. 1978. Multiplicity of germline genes specifying a group of related mouse kappa chains with implications for the generation of immunoglobulin diversity. *Nature* 276: 780-4

34. Hozumi N, Tonegawa S. 1976. Evidence for somatic rearrangement of immunoglobulin genes coding for variable and constant regions. *Proc Natl Acad Sci U S A* 73: 3628-32

35. Brack C, Hirama M, Lenhard-Schuller R, Tonegawa S. 1978. A complete immunoglobulin gene is created by somatic recombination. *Cell* 15: 1-14

36. Early P, Huang H, Davis M, Calame K, Hood L. 1980. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. *Cell* 19: 981-92

37. Jerne NK. 1955. The Natural-Selection Theory of Antibody Formation. *Proc Natl Acad Sci U S A* 41: 849-57

38. Talmage DW. 1957. Allergy and immunology. *Annu Rev Med* 8: 239-56

39. Lamm ME. 1997. Interaction of antigens and antibodies at mucosal surfaces. *Annu Rev Microbiol* 51: 311-40

40. Benner R, Hijmans W, Haaijman JJ. 1981. The bone marrow: the major source of serum immunoglobulins, but still a neglected site of antibody formation. *Clin Exp Immunol* 46: 1-8

41. Kosco MH, Burton GF, Kapasi ZF, Szakal AK, Tew JG. 1989. Antibody-forming cell induction during an early phase of germinal centre development and its delay with ageing. *Immunology* 68: 312-8

42. Hilschmann N, Craig LC. 1965. Amino acid sequence studies with Bence-Jones proteins. *Proc Natl Acad Sci U S A* 53: 1403-9

43. Titani K, Whitley E, Jr., Avogardo L, Putnam FW. 1965. Immunoglobulin structure: partial amino acid sequence of a Bence Jones protein. *Science* 149: 1090-2

44. Boyden SV, Sorkin E. 1960. The adsorption of antigen by spleen cells previously treated with antiserum in vitro. *Immunology* 3: 272-83

45. Tomasi TB, Jr., Zigelbaum S. 1963. The Selective Occurence of Gamma-1a Globulins in Certain Body Fluids. *J Clin Invest* 42: 1552-60

46. Rowe DS, Fahey JL. 1965. A New Class of Human Immunoglobulins. Ii. Normal Serum Igd. *J Exp Med* 121: 185-99

47. Ishizaka K, Ishizaka T. 1966. Physicochemical properties of reaginic antibody. 1. Association of reaginic activity with an immunoglobulin other than gammaA- or gammaG-globulin. *J Allergy* 37: 169-85

48. Stern C, McConnell I. 1976. Immunoglobulins M and D as antigen-binding receptors on the same cell, with shared specificity. *Eur J Immunol* 6: 225-7

49. Stavnezer J. 1996. Antibody class switching. *Adv Immunol* 61: 79-146

50. Coffman RL, Seymour BW, Lebman DA, Hiraki DD, Christiansen JA, Shrader B, Cherwinski HM, Savelkoul HF, Finkelman FD, Bond MW, et al. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunol Rev* 102: 5-28

51. Schultz CL, Rothman P, Kuhn R, Kehry M, Muller W, Rajewsky K, Alt F, Coffman RL. 1992. T helper cell membranes promote IL-4-independent expression of germ-line C gamma 1 transcripts in B cells. *J Immunol* 149: 60-4

52. Ravetch JV, Kinet JP. 1991. Fc receptors. *Annu Rev Immunol* 9: 457-92

53. Chothia C, Janin J. 1975. Principles of protein-protein recognition. *Nature* 256: 705-8

54. Janin J, Chothia C. 1990. The structure of protein-protein recognition sites. *J Biol Chem* 265: 16027-30

55. Cameron DJ, Erlanger BF. 1977. Evidence for multispecificity of antibody molecules. *Nature* 268: 763-5

56. Mariuzza RA, Poljak RJ. 1993. The basics of binding: mechanisms of antigen recognition and mimicry by antibodies. *Curr Opin Immunol* 5: 50-5

57. Griffiths GM, Berek C, Kaartinen M, Milstein C. 1984. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. *Nature* 312: 271-5

58. Berek C, Milstein C. 1987. Mutation drift and repertoire shift in the maturation of the immune response. *Immunol Rev* 96: 23-41

59. Nossal GJ, Pike BL. 1975. Evidence for the clonal abortion theory of B-lymphocyte tolerance. *J Exp Med* 141: 904-17

60. Nemazee DA, Burki K. 1989. Clonal deletion of B lymphocytes in a transgenic mouse bearing anti-MHC class I antibody genes. *Nature* 337: 562-6

61. von Boehmer H. 1990. Developmental biology of T cells in T cell-receptor transgenic mice. *Annu Rev Immunol* 8: 531-56

62. Norvell A, Mandik L, Monroe JG. 1995. Engagement of the antigen-receptor on immature murine B lymphocytes results in death by apoptosis. *J Immunol* 154: 4404-13

63. Smith CA, Williams GT, Kingston R, Jenkinson EJ, Owen JJ. 1989. Antibodies to CD3/T-cell receptor complex induce death by apoptosis in immature T cells in thymic cultures. *Nature* 337: 181-4

64. MacDonald HR, Lees RK. 1990. Programmed death of autoreactive thymocytes. *Nature* 343: 642-4

65. Fairchild PJ, Wildgoose R, Atherton E, Webb S, Wraith DC. 1993. An autoantigenic T cell epitope forms unstable complexes with class II MHC: a novel route for escape from tolerance induction. *Int Immunol* 5: 1151-8

66. Liu GY, Fairchild PJ, Smith RM, Prowle JR, Kioussis D, Wraith DC. 1995. Low avidity recognition of self-antigen by T cells permits escape from central tolerance. *Immunity* 3: 407-15

67. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-64

68. Asano M, Toda M, Sakaguchi N, Sakaguchi S. 1996. Autoimmune disease as a consequence of developmental abnormality of a T cell subpopulation. *J Exp Med* 184: 387-96

69. Jenkins MK, Schwartz RH. 1987. Antigen presentation by chemically modified splenocytes induces antigen-specific T cell unresponsiveness in vitro and in vivo. *J Exp Med* 165: 302-19

70. Gilkeson GS, Pippen AM, Pisetsky DS. 1995. Induction of cross-reactive anti-dsDNA antibodies in preautoimmune NZB/NZW mice by immunization with bacterial DNA. *J Clin Invest* 95: 1398-402

71. Deng GM, Nilsson IM, Verdrengh M, Collins LV, Tarkowski A. 1999. Intra-articularly localized bacterial DNA containing CpG motifs induces arthritis. *Nat Med* 5: 702-5

72. Mizrachi Y, Ohry A, Aviel A, Rozin R, Brooks ME, Schwartz M. 1983. Systemic humoral factors participating in the course of spinal cord injury. *Paraplegia* 21: 287-93

73. Horwitz MS, Bradley LM, Harbertson J, Krahl T, Lee J, Sarvetnick N. 1998. Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. *Nat Med* 4: 781-5

74. Fujinami RS, Oldstone MB. 1985. Amino acid homology between the encephalitogenic site of myelin basic protein and virus: mechanism for autoimmunity. *Science* 230: 1043-5

75. Zhao ZS, Granucci F, Yeh L, Schaffer PA, Cantor H. 1998. Molecular mimicry by herpes simplex virus-type 1: autoimmune disease after viral infection. *Science* 279: 1344-7

76. Gordon EE. 1983. Altered oligosaccharides as the initiating autoantigen in rheumatoid arthritis. *Med Hypotheses* 10: 347-52

77. Hara I, Takechi Y, Houghton AN. 1995. Implicating a role for immune recognition of self in tumor rejection: passive immunization against the brown locus protein. *J Exp Med* 182: 1609-14

78. Anderton SM. 2004. Post-translational modifications of self antigens: implications for autoimmunity. *Curr Opin Immunol* 16: 753-8

79. Klareskog L, Malmstrom V, Lundberg K, Padyukov L, Alfredsson L. 2011. Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. *Semin Immunol* 23: 92-8

80. Oliver JE, Silman AJ. 2006. Risk factors for the development of rheumatoid arthritis. *Scand J Rheumatol* 35: 169-74

81. Coenen MJ, Gregersen PK. 2009. Rheumatoid arthritis: a view of the current genetic landscape. *Genes Immun* 10: 101-11

82. Padyukov L, Silva C, Stolt P, Alfredsson L, Klareskog L. 2004. A gene-environment interaction between smoking and shared epitope genes in HLA-DR provides a high risk of seropositive rheumatoid arthritis. *Arthritis Rheum* 50: 3085-92

83. Bottini N, Musumeci L, Alonso A, Rahmouni S, Nika K, Rostamkhani M, MacMurray J, Meloni GF, Lucarelli P, Pellecchia M, Eisenbarth GS, Comings D, Mustelin T. 2004. A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes. *Nat Genet* 36: 337-8

84. Velaga MR, Wilson V, Jennings CE, Owen CJ, Herington S, Donaldson PT, Ball SG, James RA, Quinton R, Perros P, Pearce SH. 2004. The codon 620 tryptophan allele of the lymphoid tyrosine phosphatase (LYP) gene is a major determinant of Graves' disease. *J Clin Endocrinol Metab* 89: 5862-5

85. Vandiedonck C, Capdevielle C, Giraud M, Krumeich S, Jais JP, Eymard B, Tranchant C, Gajdos P, Garchon HJ. 2006. Association of the PTPN22*R620W polymorphism with autoimmune myasthenia gravis. *Ann Neurol* 59: 404-7

86. Mydel P, Wang Z, Brisslert M, Hellvard A, Dahlberg LE, Hazen SL, Bokarewa M. 2010. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. *J Immunol* 184: 6882-90

87. Corper AL, Sohi MK, Bonagura VR, Steinitz M, Jefferis R, Feinstein A, Beale D, Taussig MJ, Sutton BJ. 1997. Structure of human IgM rheumatoid factor Fab bound to its autoantigen IgG Fc reveals a novel topology of antibody-antigen interaction. *Nat Struct Biol* 4: 374-81

88. Jonsson T, Thorsteinsson J, Valdimarsson H. 2000. Elevation of only one rheumatoid factor isotype is not associated with increased prevalence of rheumatoid arthritis--a population based study. *Scand J Rheumatol* 29: 190-1

89. Vallbracht I, Rieber J, Oppermann M, Forger F, Siebert U, Helmke K. 2004. Diagnostic and clinical value of anti-cyclic citrullinated peptide antibodies compared with rheumatoid factor isotypes in rheumatoid arthritis. *Ann Rheum Dis* 63: 1079-84

90. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ. 2000. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 43: 155-63

91. Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, Domenech I, Aydintug AO, Jedryka-Goral A, de Ramon E, et al. 1993. Systemic lupus erythematosus: clinical and immunologic patterns of disease expression in a cohort of 1,000 patients. The European Working Party on Systemic Lupus Erythematosus. *Medicine (Baltimore)* 72: 113-24

92. Young BJ, Mallya RK, Leslie RD, Clark CJ, Hamblin TJ. 1979. Anti-keratin antibodies in rheumatoid arthritis. *Br Med J* 2: 97-9

93. Nienhuis RL, Mandema E. 1964. A New Serum Factor in Patients with Rheumatoid Arthritis; the Antiperinuclear Factor. *Ann Rheum Dis* 23: 302-5

94. Masson-Bessière C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, Serre G. 2001. The major synovial targets of the rheumatoid arthritis-

specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J Immunol* 166: 4177-84

95. Hill JA, Al-Bishri J, Gladman DD, Cairns E, Bell DA. 2006. Serum autoantibodies that bind citrullinated fibrinogen are frequently found in patients with rheumatoid arthritis. *J Rheumatol* 33: 2115-9

96. Zhao X, Okeke NL, Sharpe O, Batliwalla FM, Lee AT, Ho PP, Tomooka BH, Gregersen PK, Robinson WH. 2008. Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. *Arthritis Res Ther* 10: R94

97. Raijmakers R, van Beers JJ, El-Azzouny M, Visser NF, Bozic B, Pruijn GJ, Heck AJ. 2012. Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis Res Ther* 14: R114

98. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, Lee DM, Hueber W, Robinson WH, Cairns E. 2008. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. *J Exp Med* 205: 967-79

99. Kollman JM, Pandi L, Sawaya MR, Riley M, Doolittle RF. 2009. Crystal structure of human fibrinogen. *Biochemistry* 48: 3877-86

100. Yoshida M, Tsuji M, Kurosaka D, Yasuda J, Ito Y, Nishizawa T, Yamada A. 2006. Autoimmunity to citrullinated type II collagen in rheumatoid arthritis. *Mod Rheumatol* 16: 276-81

101. Burkhardt H, Sehnert B, Bockermann R, Engström A, Kalden JR, Holmdahl R. 2005. Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. *Eur J Immunol* 35: 1643-52

102. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, Bell DA, Cairns E, Venables PJ. 2011. Immunization with Porphyromonas gingivalis enolase induces autoimmunity to mammalian alpha-enolase and arthritis in DR4-IE-transgenic mice. *Arthritis Rheum* 63: 3818-23

103. Tilleman K, Van Steendam K, Cantaert T, De Keyser F, Elewaut D, Deforce D. 2008. Synovial detection and autoantibody reactivity of processed citrullinated isoforms of vimentin in inflammatory arthritides. *Rheumatology (Oxford)* 47: 597-604

104. Vossenaar ER, Despres N, Lapointe E, van der Heijden A, Lora M, Senshu T, van Venrooij WJ, Menard HA. 2004. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther* 6: R142-50

105. Bang H, Egerer K, Gauliard A, Luthke K, Rudolph PE, Fredenhagen G, Berg W, Feist E, Burmester GR. 2007. Mutation and citrullination modifies vimentin to a novel autoantigen for rheumatoid arthritis. *Arthritis Rheum* 56: 2503-11

106. van Gaalen FA, Linn-Rasker SP, van Venrooij WJ, de Jong BA, Breedveld FC, Verweij CL, Toes RE, Huizinga TW. 2004. Autoantibodies to cyclic citrullinated peptides predict progression to rheumatoid arthritis in patients with undifferentiated arthritis: a prospective cohort study. *Arthritis Rheum* 50: 709-15

107. Avouac J, Gossec L, Dougados M. 2006. Diagnostic and predictive value of anti-cyclic citrullinated protein antibodies in rheumatoid arthritis: a systematic literature review. *Ann Rheum Dis* 65: 845-51

108. Ercan A, Cui J, Chatterton DE, Deane KD, Hazen MM, Brintnell W, O'Donnell CI, Derber LA, Weinblatt ME, Shadick NA, Bell DA, Cairns E, Solomon DH,

Holers VM, Rudd PM, Lee DM. 2010. Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis. *Arthritis Rheum* 62: 2239-48

109. Hill J, Cairns, E, Bell DA, inventors. Peptides associated with MHC class II molecules involved in autoimmune diseases. Patent Treaty Cooperation Application (WO 04/078098) filed 2004 Mar 5 to secure worldwide protection.

110. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. 2003. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays* 25: 1106-18

111. Wang Y, Li M, Stadler S, Correll S, Li P, Wang D, Hayama R, Leonelli L, Han H, Grigoryev SA, Allis CD, Coonrod SA. 2009. Histone hypercitrullination mediates chromatin decondensation and neutrophil extracellular trap formation. *J Cell Biol* 184: 205-13

112. Guo Q, Bedford MT, Fast W. 2011. Discovery of peptidylarginine deiminase-4 substrates by protein array: antagonistic citrullination and methylation of human ribosomal protein S2. *Mol Biosyst* 7: 2286-95

113. Hagiwara T, Nakashima K, Hirano H, Senshu T, Yamada M. 2002. Deimination of arginine residues in nucleophosmin/B23 and histones in HL-60 granulocytes. *Biochem Biophys Res Commun* 290: 979-83

114. Nakashima K, Hagiwara T, Yamada M. 2002. Nuclear localization of peptidylarginine deiminase V and histone deimination in granulocytes. *J Biol Chem* 277: 49562-8

115. Chang X, Yamada R, Suzuki A, Sawada T, Yoshino S, Tokuhiro S, Yamamoto K. 2005. Localization of peptidylarginine deiminase 4 (PADI4) and citrullinated protein in synovial tissue of rheumatoid arthritis. *Rheumatology (Oxford)* 44: 40-50

116. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. 2003. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J Immunol* 171: 538-41

117. Hill J, Cairns E, Bell DA. 2004. The joy of citrulline: new insights into the diagnosis, pathogenesis, and treatment of rheumatoid arthritis. *J Rheumatol* 31: 1471-3

118. Stolt P, Bengtsson C, Nordmark B, Lindblad S, Lundberg I, Klareskog L, Alfredsson L. 2003. Quantification of the influence of cigarette smoking on rheumatoid arthritis: results from a population based case-control study, using incident cases. *Ann Rheum Dis* 62: 835-41

119. Makrygiannakis D, Hermansson M, Ulfgren AK, Nicholas AP, Zendman AJ, Eklund A, Grunewald J, Skold CM, Klareskog L, Catrina AI. 2008. Smoking increases peptidylarginine deiminase 2 enzyme expression in human lungs and increases citrullination in BAL cells. *Ann Rheum Dis* 67: 1488-92

120. Toussirot E, Wendling D, Tiberghien P, Luka J, Roudier J. 2000. Decreased T cell precursor frequencies to Epstein-Barr virus glycoprotein Gp110 in peripheral blood correlate with disease activity and severity in patients with rheumatoid arthritis. *Ann Rheum Dis* 59: 533-8

121. Permin H, Aldershvile J, Nielsen JO. 1982. Hepatitis B virus infection in patients with rheumatic diseases. *Ann Rheum Dis* 41: 479-82

122. Lee YH, Ji JD, Yeon JE, Byun KS, Lee CH, Song GG. 1998. Cryoglobulinaemia and rheumatic manifestations in patients with hepatitis C virus infection. *Ann Rheum Dis* 57: 728-31

123. Naides SJ, Scharosch LL, Foto F, Howard EJ. 1990. Rheumatologic manifestations of human parvovirus B19 infection in adults. Initial two-year clinical experience. *Arthritis Rheum* 33: 1297-309

124. Petkova SB, Konstantinov KN, Sproule TJ, Lyons BL, Awwami MA, Roopenian DC. 2006. Human antibodies induce arthritis in mice deficient in the low-affinity inhibitory IgG receptor Fc gamma RIIB. *J Exp Med* 203: 275-80

125. Yue D, Brintnell W, Mannik LA, Christie DA, Haeryfar SM, Madrenas J, Chakrabarti S, Bell DA, Cairns E. 2010. CTLA-4Ig blocks the development and progression of citrullinated fibrinogen-induced arthritis in DR4-transgenic mice. *Arthritis Rheum* 62: 2941-52

126. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, Zendman AJ, Harris HE. 2005. Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. *Arthritis Res Ther* 7: R458-67

127. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, Holers VM. 2006. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. *J Clin Invest* 116: 961-73

128. Kurki P, Aho K, Palosuo T, Heliovaara M. 1992. Immunopathology of rheumatoid arthritis. Antikeratin antibodies precede the clinical disease. *Arthritis Rheum* 35: 914-7

129. Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 48: 2741-9

130. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, Habibuw MR, Vandenbroucke JP, Dijkmans BA. 2004. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum* 50: 380-6

131. Bukhari M, Thomson W, Naseem H, Bunn D, Silman A, Symmons D, Barton A. 2007. The performance of anti-cyclic citrullinated peptide antibodies in predicting the severity of radiologic damage in inflammatory polyarthritis: results from the Norfolk Arthritis Register. *Arthritis Rheum* 56: 2929-35

132. Brintnell W, Bell, DA, Cairns, E. 2009. The mechanisms underlying arthritogenicity of human anticitrulline antibodies [Abstract]. *Arthritis Rheum.* 58 (10): S433

133. Ito K, Bian HJ, Molina M, Han J, Magram J, Saar E, Belunis C, Bolin DR, Arceo R, Campbell R, Falcioni F, Vidovic D, Hammer J, Nagy ZA. 1996. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J Exp Med* 183: 2635-44

134. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, Levarht NE, van der Helm-van Mil AH, Cerami A, Huizinga TW, Toes RE, Trouw LA. 2011. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A* 108: 17372-7

135. Bobb D, Hofstee BH. 1971. Gel isoelectric focusing for following the successive carbamylations of amino groups in chymotrypsinogen A. *Anal Biochem* 40:209-17

136. Ashida D, Funakoshi, K., Tsukihara, T., Ueki, T. and Kakudo, M. 1972. The crystal structures of L-citrulline hydrochloride and L-homocitrulline hydrochloride. *Acta Crystallogr* B28: 1367

137. Marier JR, Rose D. 1964. Determination of Cyanate, and a Study of Its Accumulation in Aqueous Solutions of Urea. *Anal Biochem* 7: 304-14

138. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. 2007. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* 13: 1176-84

139. Jaisson S, Pietrement C, Gillery P. 2011. Carbamylation-derived products: bioactive compounds and potential biomarkers in chronic renal failure and atherosclerosis. *Clin Chem* 57: 1499-505

140. Harding JJ, Rixon KC. 1980. Carbamylation of lens proteins: a possible factor in cataractogenesis in some tropical countries. *Exp Eye Res* 31: 567-71

141. Turunen S, Koivula MK, Risteli L, Risteli J. 2010. Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. *Arthritis Rheum* 62: 3345-52

Chapter 2:

Homocitrulline: An antigen specific to rheumatoid arthritis and a target of anti-citrulline protein/peptide antibodies*

*Note: This chapter is an extended version of the paper which is to be submitted for peer review. Modifications have been made to provide a more comprehensive description of the methods and results in this study and to comply with Faculty of Graduate and Postdoctoral Studies formatting guidelines

2.1 Introduction

In autoimmune disease, the post-translational modification of native proteins can generate novel targets which provoke the immune system. The conversion of protein/peptide arginine to citrulline in Rheumatoid Arthritis (RA) is one example of this. In individuals with the Shared Epitope (SE), arginine to citrulline conversion increases peptide binding affinity to MHC class II molecules, specifically in the P4 binding pocket (1). The subsequent T cell immune response to citrullinated peptides, and production of Anti-Citrullinated Protein/Peptide Antibodies (ACPA), is highly specific for RA (>95%) (2). ACPA can occur years before onset (3-5) and some studies show that their presence correlates with disease severity (6). Taken together, it is not surprising that these citrulline-targeting antibodies have been implicated in RA pathogenesis and there is recent evidence to support such claims. More specifically, in some murine models: monoclonal antibodies targeting citrullinated collagen II can exacerbate disease in collagen-induced arthritis (7-9), immunization with citrullinated fibrinogen can cause arthritis (10), and passive transfer of human RA serum (11) or IgG ACPA can cause transient arthritis (12).

A recent study showed that Anti-Modified Citrulline (AMC) antibodies, which have been utilized to detect citrullinated proteins/peptides *in situ*, can also detect the amino acid homocitrulline (13). Homocitrulline is structurally and chemically very similar to citrulline, with both having the same functional group (ureido group); however, homocitrulline's side-chain is larger due to the presence of an additional carbon atom. Whereas citrulline is generated by the enzymatic modification of arginine residues by Peptidyl Arginine Deiminase (PAD) (14), homocitrulline is generated chemically by the

reaction of cyanate with an amine group of lysine residues (carbamylation) (15). The cyanate required for carbamylation can be found in the body from at least two sources: the spontaneous dissociation of urea (16), and production by the enzyme Myeloperoxidase (MPO) which is active at sites of inflammation. Enzymatic production of cyanate during inflammation can be enhanced by environmental influence through the increase of serum thiocyanate - MPO's preferred substrate, which is converted to cyanate (16-19). One such environmental factor is smoking, a well known risk factor for RA (20).

The initial evidence implicating a role for homocitrulline in RA was provided by Mydel *et al.* They showed that in some mice, immunization with homocitrullinated filaggrin peptides triggered an immune response that led to erosive arthritis and some RA patients had antibodies which targeted this homocitrullinated peptide (21). More recently, Shi *et al.* also reported that RA patients have antibodies which bind to homocitrullinated proteins (AHPA), but additionally observed that AHPA predicted joint damage (22). Sera from patients with other inflammatory rheumatic diseases were not tested. Therefore, it is unclear whether this response is specific to RA.

Since citrullination and ACPA production was first identified and linked to RA, there has been an interest in mapping protein sites which can be modified and targeted by antibodies. Citrullinated fibrinogen (or citrullinated peptides derived from fibrinogen) is a frequently identified target of ACPA (23) and is often found in the joints of RA patients (24-25). We therefore investigated whether homocitrullinated human fibrinogen is a target of IgG antibodies exclusively in RA, and whether some ACPA are able to bind

homocitrullinated proteins/peptides as well. We also examined whether the SE can be involved with homocitrulline immunogenicity.

2.2 Materials and methods

2.2.1 Patients

Sera were obtained from 84 RA patients (Table 2.1) attending St. Joseph's Health Centre Rheumatology Clinic (London, Ontario), as well as 37 Psoriatic Arthritis (PsA) patients, 37 Systemic Lupus Erythematosus (SLE) patients and 27 normal individuals. All RA patients fulfilled the American College of Rheumatology classification criteria for RA (26). This research study was approved by the Human Ethics Committee of Western University, London, ON, Canada.

2.2.2 Mice

DR4-IE Transgenic (Tg), murine MHC class II-deficient mice (27) were bred in a specific pathogen free animal facility at Western University. The corresponding Wild-Type (WT) background strain, C57BL/6 mice (referred to as B6 mice), were purchased from The Jackson Laboratory (Maine, USA). 8-12 week old mice were used for these experiments and housed in the Animal Care and Veterinary Services Barrier Facility at Western University under specific pathogen free conditions for the duration of study. All procedures were approved by the Animal Care and Use Committee.

Table 2.1 RA Patient Demographics.

	Mean	SD
Age (Years)	59.11	13.6
Disease Duration (Years)	9.4	10.14
Health Assessment Questionnaire	0.96	0.75
Swollen Joint Count	4.34	6.06
Gender (25% Male: 75% Female)	-	-

2.2.3 Mouse immunization

Mice were immunized subcutaneously in each inner flank with 50µg of antigen (or equivalent volume of Phosphate Buffered Saline (PBS)) emulsified with Complete Freund's Adjuvant (CFA) in 1v:1v ratio for a total of 100µg of protein in 100µL as previously described (10). CFA was prepared by supplementing Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich Co, Canada) with 4 mg/ml of dessicated *Mycobacterium tuberculosis* HA37 (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). On day 21 post-primary immunization mice received booster immunizations in an identical manner with IFA instead of CFA. Mice were sacrificed at various time points post-immunization to study their immune response to antigens. Spleens were harvested, blood was obtained by cardiac puncture and sera were collected after blood centrifugation for antibody analyses.

2.2.4 Antigens

The following proteins and peptides were used for immunization of mice, ACPA affinity purification, and/or antibody assays:

1. Human Fibrinogen (CalBiochem™) – Fibrinogen was used in either its unmodified, citrullinated or homocitrullinated form (see "modifications of fibrinogen" below).
2. JED - A proprietary synthetic citrullinated peptide developed by us and previously described (28-29).

   Sequence: S**C**CitCitYCitGCitCitSCitCitSCitCitR**C**S, cyclized with a **C**-**C** bond.

3. Homocitrullinated JED - Homocitrullinated JED was identical to JED with the exception that *all* citrulline residues were replaced with homocitrulline.

JED and homocitrullinated JED were synthesized at the Advanced Protein Technology Centre, Peptide Synthesis Facility at Sick Kids Hospital in Toronto, Ontario. Proteins and peptides were dissolved in sterile, distilled water or sterile PBS with the exception that homocitrullinated JED was dissolved in 7.5% concentrated HCl in sterile distilled water.

2.2.5 Modifications of fibrinogen

2.2.5.1 Fibrinogen citrullination

Human fibrinogen was citrullinated as previously described (23). Briefly, fibrinogen was incubated with 7U/mg rabbit skeletal PAD II (Sigma) for 3 h at 52°C in 0.1 M Tris-HCL (pH7.4), 10 mM $CaCl_2$ buffer. PAD was removed by spinning 15 mL volumes of the reaction mixture and PBS through a 100K Macrosept column (Pall Corporation) 3 times. Gel electrophoresis and mass spectrometry were preformed to confirm citrullination (see 2.2.5.3 below).

2.2.5.2 Fibrinogen homocitrullination

Human fibrinogen was homocitrullinated using treatment with 0.1 M KOCN in 0.15 M sodium phosphate buffer and incubation at 37°C for 24 h. 10 mg of fibrinogen was homocitrullinated in a 5 mL reaction. Excess KOCN was removed by spinning 15 mL volumes of the reaction mixture and PBS through a 100K Macrosept column (Pall Corporation) 3 times. Gel electrophoresis and mass spectrometry were preformed to confirm homocitrullination (see 2.2.5.3 below).

2.2.5.3 Fibrinogen digestion and analysis

The remaining concentrated, modified human fibrinogen was used alongside unmodified fibrinogen in 12% SDS gel electrophoresis with Coomassie blue staining. From the gel, protein bands representing each chain of fibrinogen were isolated using an Ettan Spot Picker (GE Healthcare) and submitted to Western University Functional Proteomics Facility (London, Ontario) for digestion. In-gel digestion was performed using a MassPREP automated digester station (PerkinElmer). Gel pieces were Coomassie destained using 50 mM ammonium bicarbonate and 50% acetonitrile, which was followed by protein reduction using 10 mM dithiotreitol (DTT), and alkylation using 55 mM iodoacetamide (IAA). Samples were then digested with approximately 5 ng/µL of sequencing grade porcine trypsin (Promega Corporation). After digestion, peptides were extracted using a solution of 1% formic acid and 2% acetonitrile and lyophilized.

For mass spectrometry analysis, samples were reconstituted in 20 µL of 0.1% formic acid in water and 10 µL of each sample was analyzed using a 70 min LC-MSMS method. Separation using LC (5-60% ACN 0.1% FA over 40 min gradient) was performed on a Waters nano Acquity UPLC, (Ultra Performance Liquid Chromatography) with a 75 µm x 250 mm, 1.7 µm, C18, reverse phase column (Waters). Ions were detected in ES (Electrospray) MS+ve ion mode using DDA (Data Dependent Acquisition), (Q-ToF Global; Waters). Mass spectrometry was analyzed using MASCOT server with the NCBInr 101910 database (Homo sapiens taxonomy specified) and the following acceptable modifications: Carbamidomethyl (C), Oxidation (M), Phospho (ST), Phospho (Y), and either Carbamyl (K) or Citrullination (R).

2.2.6 Molecular modelling

The available, published X-ray crystallographic structures of peptides bound to the SE positive MHC class II DR0401 molecule (30-32) were initially analyzed in pairs. For each pair of structures, the back bone atoms of the of the MHC class II DR0401 β -chain that contain the SE sequence were superimposed using the “Magic Fit” function of Swiss PDB Viewer. In the same program, variation in peptide orientation was measured by the average distance between backbone atoms, measured as a Root Mean Squared (RMS).

The crystal structure of collagen II peptide 261-273 bound to MHC class II DR0401 SE (PDB code 2SEB) (30) was used for further molecular modelling which was done by substituting the aspartic acid binding at the P4 position to various rotomers of lysine or arginine without changing the backbone atom orientation. A custom written file was used in PyMOL to modify the lysine/arginine residues further to homocitrulline/citrulline. The modified structures were then analyzed in SwissPDBViewer for steric clashes.

Modified fibrinogen (citrullinated or homocitrullinated) peptide binding to the SE was predicted using a program written by Hammer *et al.* (33) as previously described by us for the prediction of citrullinated peptides binding to the SE (1). This relied on the assumption that homocitrulline bound similarily to the SE as citrulline. Briefly, lysine residues (potential homocitrullination sites) were changed to glutamine because its side-chain functional group is most similar to homocitrulline (and citrulline). The program then compared each 9 residue long, continuous peptide to the known consensus binding motif for the MHC class II DR0401 molecule and assigned a score. All peptides with a score of 2.0 or higher were considered SE binders.

2.2.7 ACPA purification

ACPA was affinity purified using Hi-trap NHS-Activated (GE) column chromatography as per manufacturer's instructions and as previously described (28). Briefly, 3 mg of citrullinated JED peptide was linked to a 1 mL column. Five mL of serum from each patient was diluted separately with 20 mL of binding buffer (20 mM sodium phosphate buffer, pH 7.4) and run through the column. Anti-JED was eluted from the column using 10 mM sodium phosphate, 2.5 M $MgCL_2$ buffer and antibodies were transferred to PBS using a desalting column (GE) as per manufacturer's instructions. The third mL eluted from the desalting column consistently had the most IgG and was, therefore used for analyses.

2.2.8 Antibody assays

Human sera (and/or purified ACPA) were tested for the presence of antibodies that bound to JED, homocitrullinated JED, citrullinated fibrinogen, and homocitrullinated fibrinogen using Enzyme-Linked ImmunoSorbant Assay (ELISA) with 1:100 dilution except if high titres required further serial dilution for accurate quantification. Mouse sera were tested for anti-homocitrullinated fibrinogen antibodies at 1:30000 and for anti-JED antibodies at 1:100.

All ELISAs were performed at room temperature as previously described (10, 23). In brief, MaxiSorp Nunc 96-well plates (Thermo Scientific Inc.) were coated with 1 µg of protein or peptide/well in carbonate coating buffer (10 µg antigen/mL) overnight at 4°C. Plates were then washed with PBS, 0.05% Tween (PBST) and blocked with PBS, 0.1% BSA. After washing, human or mouse serum samples diluted in PBST 0.1% BSA were

incubated in duplicate on the plate for 30 minutes before being washed away with PBST. Biotin-conjugated goat anti–mouse IgG secondary antibodies (Sigma) or biotin-conjugated goat anti–human IgG secondary antibodies (Sigma) were diluted 1:10,000 in PBST, 0.1% BSA containing streptavidin/horseradish peroxidase diluted 1:4,000 (Sigma-Aldrich) and incubated in the wells for 30 min before being washed with PBST. 100 µL of TetraMethyl Benadine substrate (Sigma) was incubated in each well for 10 minutes before the reaction was stopped with 50 µL of 2M H_2SO_4. To quantify antibody concentrations in sera, Optical Density (OD) at 450 nm was read using a microplate reader (BIORAD). Antibody reactivity to BSA (Sigma) was subtracted from each duplicate sample.

Antibody inhibition was tested using the ELISA described above with the exception that various amounts of soluble antigen in PBST, 0.1% BSA were pre-incubated with diluted serum for one hour at room temperature prior to incubation on ELISA plate.

Antibodies to homocitrullinated fibrinogen and the synthetic peptides JED and homocitrullinated JED were quantified in Arbitrary Units/mL (AU/mL) by comparison to a reference serum sample for each antigen. Briefly, the 1:100 dilution of reference serum was assigned a concentration of 250 AU/mL and serial dilutions were used to produce a standard curve (Absorbance vs. Concentration). Absorbance of unknown normal and patient sera were then compared to the standard to determine concentration. The cut-off value for positive anti-JED was 3.5 AU/mL, for anti-homocitrullinated JED was 18.5 AU/mL and for anti-homocitrullinated fibrinogen was 3.6 AU/mL. Cut-off values were determined using the mean of normal sera reactivity + 2 standard deviations.

Anti-CCP2 antibodies were detected in human serum using a commercially available ELISA kit (Euroimmun AG).

Total IgG was measured in mg/mL using a commercially available ELISA kit (Cedarlane).

2.2.9 Proliferation assay

Spleens were harvested from mice sacrificed at different times post-immunization and their splenocyes were suspended in complete RPMI (1% Glutamax (Gibco), 1% penicillin/streptomycin (Gibco), 1% sodium pyruvate (Gibco), 1% non-essential amino acids (Gibco), 0.25% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Gibco)) supplemented with 5% Fetal Bovine Serum (FBS) (Gibco). 400,000 splenocytes per well were cultured in 96-well U-bottom plates (BD) with 50 μg/mL antigen for 72 hours at 37°C, 5% CO_2. 1 μCi of [^{3}H]thymidine (ICN Biomedicals, Irvine, CA, USA) was added to each well 18 hours prior to culture termination. Cells were harvested using a Tomtec Harvester 96 and immersed in scintillation fluid before radioactive counts were read in counts per minute (cpm) using a Wallac 1450 microbeta liquid scintillation counter. Spleen cell proliferation experiments were conducted in quadruplicate, and the results are presented as the mean stimulation index (cpm of experimental sample/cpm of control sample) ± the SEM.

2.2.10 Statistical analysis

Mann-Whitney U test was used to compare groups in Graphpad Prism V5 and a p value of <0.05 was considered significant.

2.3 Results

2.3.1 Occurrence of anti-homocitrullined fibrinogen antibodies in RA, patients with other inflammatory rheumatic diseases, and healthy individuals

We used *in vitro* carbamylated fibrinogen as a homocitrullinated antigen to determine the presence of Anti-Homocitrullinated Fibrinogen Antibodies (AHFA) in sera from RA (n=84, Table 2.1), SLE (n=37), and psoriatic arthritis (PsA) (n=37) patients and normal individuals (n=27) (Figure 2.1A). Serum antibody reactivity was significantly increased in RA only ($p<0.05$). Only 5% of SLE patients and 3% of PsA patients tested weakly positive for anti-homocitrullinated fibrinogen antibodies (AHFA). In our RA cohort that was 89% anti-CCP2 positive, reactivity to homocitrullinated fibrinogen was detected in 49% of patients. 69% of the RA patients also had Anti-Citrullinated Fibrinogen Antibodies (ACFA). Although AHFA were not found in any of the 10 RA patients without anti-CCP2, 6% of the RA patients had AHFA but did not have antibodies to the citrullinated form of fibrinogen (Table 2.2).

To confirm that the observed antibodies to homocitrullinated fibrinogen involved homocitrulline containing epitopes, serum from three different RA patients with AHFA (two of which were also Anti-Citrullinated Fibrinogen (ACFA) positive) were inhibited with either soluble homocitrullinated fibrinogen or soluble unmodified fibrinogen (Figure 2.1B). In all three cases, there was an increase of inhibition from 25-38% with unmodified fibrinogen up to 71-89% with the equivalent concentrations of homocitrullinated fibrinogen. This suggests that a large portion of the AHFA response (approximately 67%) is homocitrulline specific.

Table 2.2 Summary of human serology findings.

	RA (n=84)	**Normal (n=27)**	**SLE (n=37)**	**PsA (n=37)**
anti-CCP2 +	74 (88)	0 (0)	2 (5)	1 (3)
AHFA +	41 (49)	0 (0)	2 (5)	1 (3)
ACFA +	58 (69)	0 (0)	-	-
AHFA +, ACFA +	36 (43)	0 (0)	-	-
AHFA -, ACFA +	22 (26)	0 (0)	-	-
AHFA +, ACFA -	5 (6)	0 (0)	-	-

Values shown represent: number of individuals who tested positive (percentage)

SLE = Systemic Lupus Erythematosus

PsA = Psoriatic Arthritis

AHFA = Anti-Homocitrullinated Fibrinogen Antibodies;

ACFA = Anti-Citrullinated Fibrinogen Antibodes.

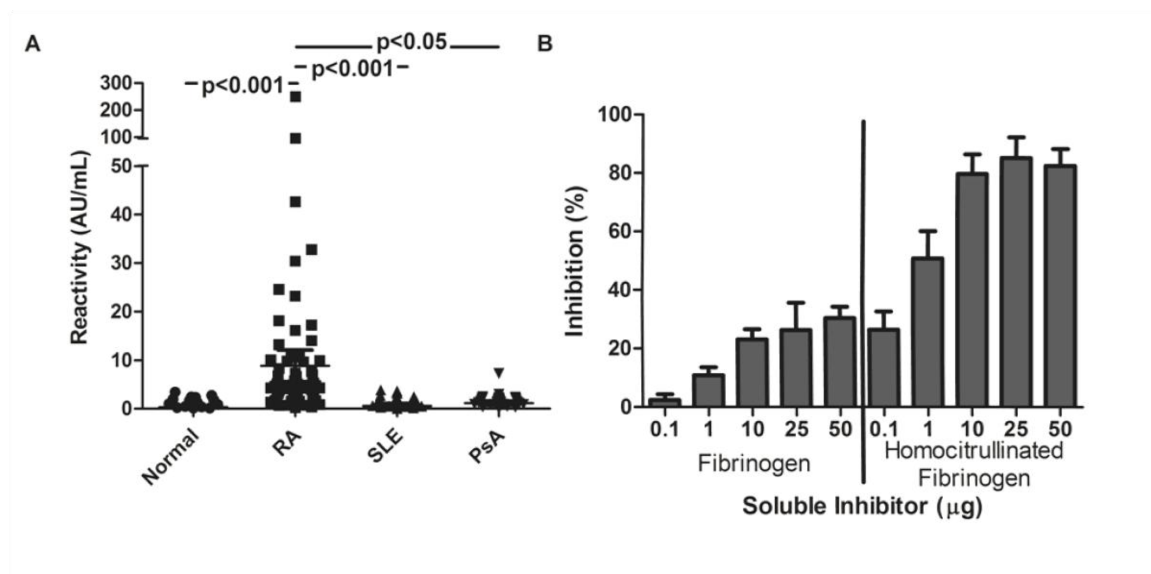


Figure 2.1 Antibodies that bind homocitrullinated fibrinogen are specific to RA. A) ELISA detecting serum antibodies that bind to homocitrullinated fibrinogen in normal subjects (n=27) and RA (n=84), SLE (n=37), and Psoriatic Arthritis (n=37) patients. **B)** ELISA demonstrating inhibition of anti-homocitrullinated fibrinogen antibodies from RA patients (N=3) with various quantities of either soluble unmodified fibrinogen or soluble homocitrullinated fibrinogen.

2.3.2 Affinity purified human ACPA can bind homocitrulline

We next investigated whether anti-citrulline antibodies were also able to bind homocitrullinated protein and peptide. ACPA was affinity purified from the sera of five different RA patients using a the citrullinated JED peptide and antibody reactivity with CCP2, citrullinated JED, citrullinated fibrinogen, homocitrullinated JED, and homocitrullinated fibrinogen was measured in AU/mg of IgG and compared to serum values to determine enrichment (Table 2.3). Enrichment (purified concentration/serum concentration) of JED reactivity was seen in all five purifications with an average 172 times increase in concentration (AU/mg of IgG). Co-enrichment of anti-CCP2 and anti-citrullinated fibrinogen antibody reactivity was also observed with an average 94 times and 182 times increase in concentration (RU/mg of IgG), respectively whereas a reduction of antibody reactivity to all citrullinated antigens was observed in the IgG flowthrough remaing after purification. This provides evidence that our procedure successfully affinity purified ACPA. Interestingly, purification of ACPA from all five RA patients also strongly enriched AHPA (average 150 times increase in anti-homocitrullinated JED concentration) while in the remaining flowthrough IgG that did not bind the citrullinated JED column, concentration of anti-homocitrullinated JED was consistently reduced. All three patients with strong serum anti-homocitrullinated fibrinogen antibodies also showed enrichment (average of 76 times) after citrullinated JED purification, however this cross-reactivity was not complete as some remaining flowthrough IgG fractions were also enriched for anti-homocitrullinated fibrinogen antibodies. This demonstrates that at least some antibodies that bind citrullinated peptide (ACPA) are also able to bind to homocitrullinated proteins and peptides as well.

Table 2.3 Cross-reactivity of human ACPA. Summary of RA sera, purified ACPA, and remaining IgG antibody binding properties (n=5).

	Serum					ACPA					IgG*				
	Anti-CCP2	**Anti-Cit JED**	**Anti-Cit Fib**	**Anti-Homocit JED**	**Anti-Homocit Fib**	**Anti-CCP2**	**Anti-Cit JED**	**Anti-Cit Fib**	**Anti-Homocit JED**	**Anti-Homocit Fib**	**Anti-CCP2**	**Anti-Cit JED**	**Anti-Cit Fib**	**Anti-Homocit JED**	**Anti-Homocit Fib**
1	54	5	3	40	3	5954	1286	599	7601	312	22	3	2	6	2
2	66	8	N/A	121	2	3831	1667	N/A	11016	198	39	2	N/A	78	4
3	136	7	4	49	3	3987	599	206	2880	36	49	3	3	19	5
4	26	3	3	26	N/A	6307	837	1178	9317	N/A	5	3	0	8	N/A
5	173	23	25	41	N/A	5465	798	1130	2242	N/A	17	4	2	6	N/A

***IgG refers to the IgG antibodies remaining in the ACPA purification flowthrough which did not bind to the JED column. Therefore, these antibody fractions should have reduced concentrations of anti-JED.**

CitJED = Citrullinated JED Peptide; Cit Fib = Citrullinated Fibrinogen;
Homocit JED = Homocitrullinated JED Peptide; Homocit Fib = Homocitrullinated Fibrinogen

Quantification ofantibodies in serum, affinity purified ACPA, and JED depleted IgG is reported in Units/mg of IgG.

2.3.3 Human fibrinogen is extensively accessible to both homocitrullination and citrullination

Since lysine can be found in different regions of proteins than arginine, we sought to investigate which sites of human fibrinogen could be homocitrullinated and potentially serve as a target of AHPA. Fibrinogen's primary structure includes 103 lysines as opposed to 80 arginines. Of the 100 lysines that were successfully analyzed by mass spectrometry, 89 of them (89%) were capable of modification to homocitrulline. Similar analysis of citrullination showed that 55 of the 71 (78%) of arginines which were analyzed were able to be modified *in vitro* (summarized in Table 2.4). Neither citrulline nor homocitrulline were detected with mass spectrometry in our sample of unmodified human fibrinogen (Representative mass spectra of homocitrullination and citrullination in Appendix 1 and Appendix 2, respectively; complete homocitrullination and citrullination sequence analysis in Appendix 3).

Using the modelling program developed by Hammer *et al.* (33) as previously described (1), with the assumption that homocitrulline could bind to MHC Class II molecules like citrulline, 35 peptides were predicted to bind to the SE after homocitrullination. Of these peptides, five could be citrullinated by PAD *in vitro* as well. Therefore, there are potentially antigenic regions which are subject to both citrullination and homocitrullination in close proximity.

2.3.4 The Shared Epitope can accommodate homocitrulline

To investigate whether the SE can be involved in the immune response to homocitrulline we attempted to model this interaction. To date, three different peptides bound to the

Table 2.4 Summary of mass spectrometry on modified fibrinogen.

Modification	**α**	**β**	**γ**	**Total**
Homocitrullination				
Lysines	38	31	34	103
Lysines analyzed by mass spectrometry	37	31	32	100
Homocitrullinated	33	29	27	89
Predicted to bind the SE*	12	13	10	35
Predicted binders also citrullinated	2	3	0	5
Citrullination				
Arginines	42	27	11	80
Arginines analyzed by mass spectrometry	35	27	9	71
Citrullinated	29	19	7	55

*Predicted using the algorithm generated by Hammer *et al.* (33)

MHC II DR0401 molecule have had structural data obtained through X-ray crystallography but none contained citrulline or homocitrulline (Table 2.5). The first is a peptide from human collagen II, the second is a peptide derived from the influenza HA antigen, and the third is a peptide from human Myelin Basic Protein (MBP). To help validate our mutational modelling strategy, we first investigated changes in peptide backbone orientation between existing structures. This helps to ensure that side-chain conformation (not backbone variation) was of primary importance in determining whether the SE can accommodate homocitrulline. When the backbone atoms of the HLA DR4 β-chain that contain the SE sequence are superimposed using the "Magic Fit" function of Swiss PDB Viewer, the backbone atoms of the 9 peptide amino acid residues binding the P1-P9 pockets (see Table 2.5) differed in structural orientation by an average difference of 0.71 Å, 1.18 Å, and 1.02 Å in structures 1 vs 2, 1 vs 3, and 2 vs 3, respectively (measured as a Root Mean Squared (RMS)). Similar analysis of only the P1-4 residues reveals lower overall variation with orientation differences of 0.72 Å, 0.66 Å, and 0.56 Å, respectively after superimposition of the same structure pairs. Therefore, there was little variation in peptide backbone orientation of the 9 amino acid residues which were most important for peptide binding. Additionally, the first four amino acids, (including the P4 position that interacts with the SE) had orientations which were most conserved, as the majority of differences in conformation were seen in the C-terminal portion of the peptides (Figure 2.2).

The P4 pocket that is known to be capable of accommodating citrulline is occupied by aspartic acid, glutamine and glutamic acid in structures 1-3, respectively. Separate files were generated that are capable of altering these residues to either citrulline

Table 2.5 Peptide structures used for molecular modelling. Summary of the peptides for which there are X-ray crystallographic structural data of binding to the SE. The 9 amino acid residues primarily involved in HLA binding specificity are underlined and the SE restricted amino acid which binds the "Position 4" (P4) pocket is bolded.

#	PDB Code	Source Protein	Location	Sequence	Structure Resolution
1	2SEB	Human Collagen II	1168-1179	AYMRA**D**AAAGGA	2.5 Å
2	1J8H	Influenza HA Antigen	306-318	PKYVK**Q**NTLKLAT	2.4 Å
3	3O6F	Human Myelin Basic Protein		FSWGA**E**GQRPGFGSGG	2.8 Å

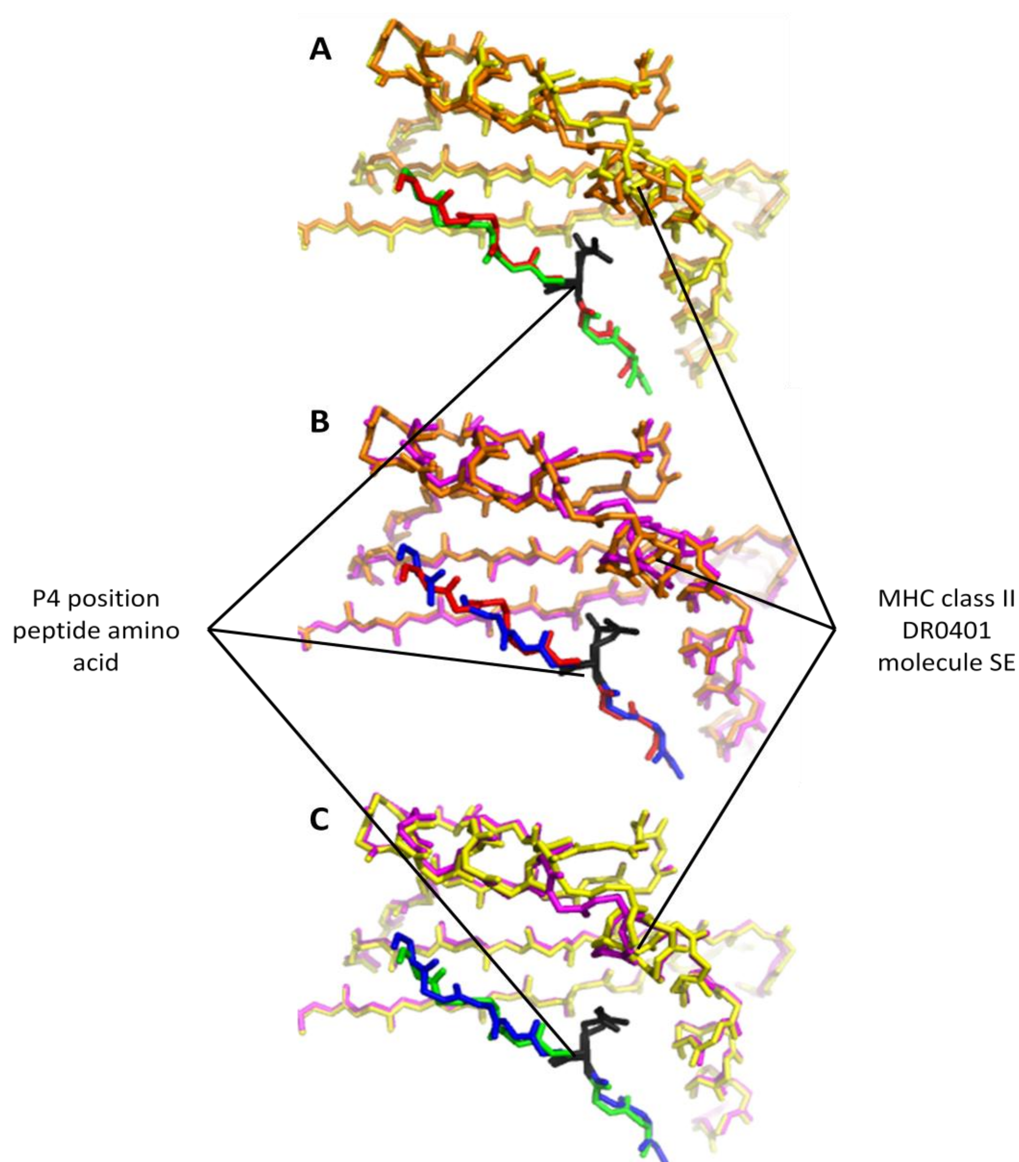


Figure 2.2 Pair-wise superimpositions of the existing structures of peptides bound to the SE. The three existing X-ray crystallographic structures of SE positive human MHC class II (DR0401) bound to different peptides superimposed in pairs. Peptide 1 is shown in red (MHC orange), Peptide 2 in green (MHC yellow) and Peptide 3 is shown in blue (MHC purple). After fitting the structured backbone atoms of the MHC class II β-chain, the differences in peptide orientations can be seen. The SE restricted P4 amino acid, including its side-chain, is shown in black and the peptide N-terminal is positioned at the lower right of each image.

or homocitrulline in PyMOL. Following this success, topology files were written so that the modified amino acids could be recognized and analyzed by the Swiss PDB Viewer program. Since collagen II is a well documented target of autoantibodies in RA, we used the 2SEB structure for further analysis.

Even with the additional files that were written, the modelling programs had an inadequate amount of data to allow manipulation of citrulline and homocitrulline side-chain torsion angles. Therefore, some clashes occurred after modification to either citrulline or homocitrulline and could not be alleviated. As an alternative approach, the P4 aspartic acid residue was changed to existing conformational rotomers of arginine or lysine, chosen manually on the basis of available space for modification. The P4 amino acid was then modified to citrulline or homocitrulline without changing the backbone atom position. This method utilizing 2SEB generated structures with homocitrulline and citrulline orientations that fit into the SE binding pocket SE without steric hindrance (Figure 2.3). Therefore, it is likely that the SE can accommodate homocitrulline.

2.3.5 The SE does not restrict the immune response to homocitrullinated fibrinogen

To investigate whether homocitrullination can cause the proliferation of immune cells and whether the SE restricts this process, we performed proliferation assays in which splenocytes from mice immunized with either unmodified or homocitrullinated human fibrinogen, or PBS alone were re-exposed *in vitro* to either unmodified or homocitrullinated human fibrinogen (Figure 2.4). Both DR4 Tg and B6 mice immunized with either unmodified or homocitrullinated fibrinogen had a proliferative response

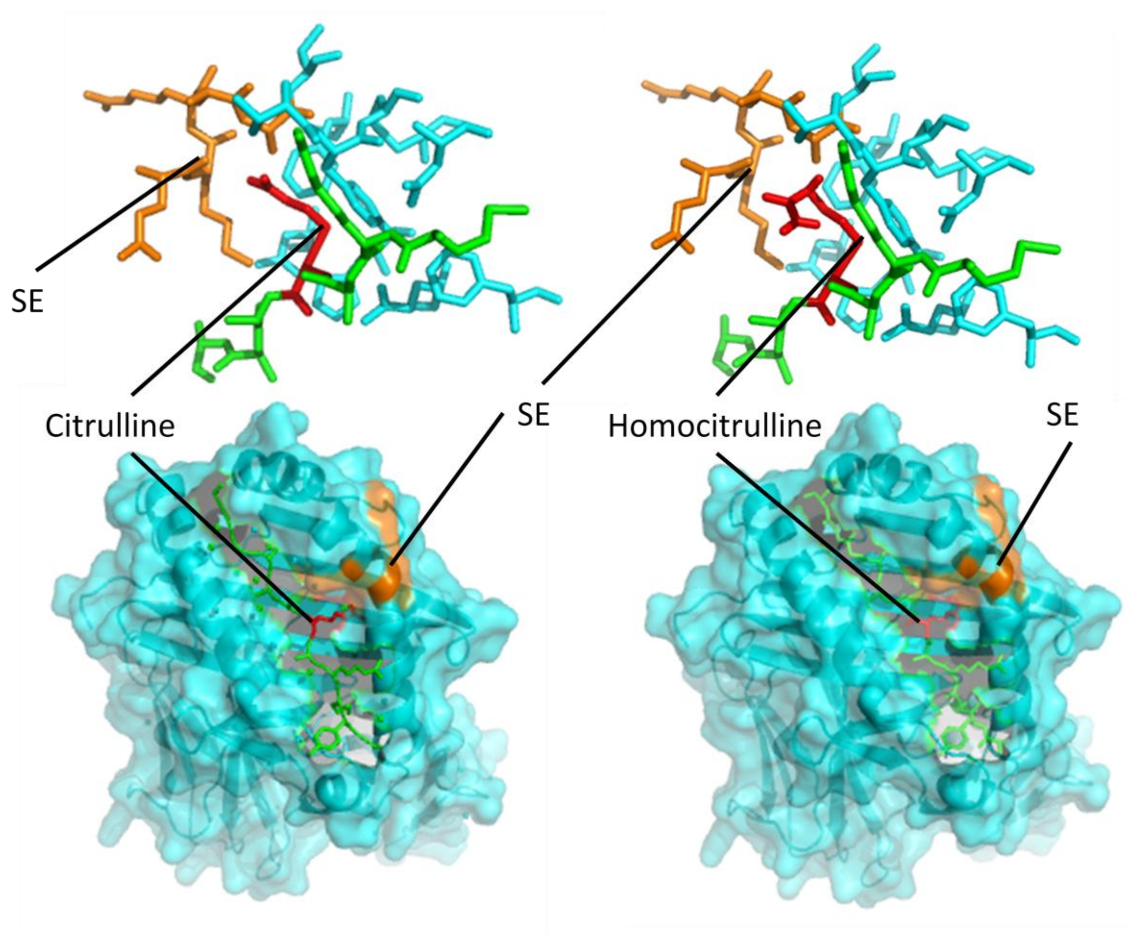


Figure 2.3 The SE can accommodate homocitrulline. Molecular modelling images of collagen peptide (261-273) binding MHC class II DR0401 (PDB structure 2SEB) after citrullination (left) or homocitrullination (right) of the P4 position. Collagen peptide is shown in green with homocitrulline colored red. Homocitrulline is shown in the P4 binding pocket (blue) with the SE colored orange. The MHC class II DR0401 SE accommodates homocitrulline without steric hindrance.

compared to mice immunized with PBS alone. In DR4 Tg mice 10 days post-immunization, there was a significantly greater response in mice immunized with and re-exposed to homocitrullinated fibrinogen compared to mice immunized with and re-exposed to unmodified fibrinogen. Also, the DR4 Tg mice 10 days post-immunization with unmodified fibrinogen had a significantly higher response to homocitrullinated fibrinogen compared to re-exposure to unmodified fibrinogen, however, after immunization with homocitrullinated fibrinogen, no groups of mice had a significantly greater response upon re-exposure to the homocitrullinated form of fibrinogen compared to the unmodified form. Therefore, we were unable to detect any homocitrulline specific T cell response in DR4 Tg or B6 mice. Although not statistically significant, each individual mouse (both DR4 Tg and B6) did have an increased proliferative response to homocitrullinated fibrinogen compared to unmodified fibrinogen after immunization with either form of the protein. Since B6 mice responded to homocitrullinated fibrinogen, the immune response to the protein modification appears to not be SE restricted in mice.

2.3.6 Anti-homocitrulline antibody responses in DR4 Tg and B6 mice

To determine if the mice immunized with homocitrullinated fibrinogen or unmodified fibrinogen generated antibody responses, and to study the nature of these responses, we tested the antibody reactivity of mouse sera to homocitrullinated fibrinogen and inhibited this response with either soluble unmodified or homocitrullinated fibrinogen. All mice immunized with fibrinogen produced IgG anti-fibrinogen antibodies compared to mice immunized with PBS alone ($p<0.001$) (Figure 2.5A). Subsequently, antibody reactivity was inhibited with both unmodified and homocitrullinated fibrinogen. In all mice, the homocitrullinated fibrinogen was able to inhibit all reactivity while, in

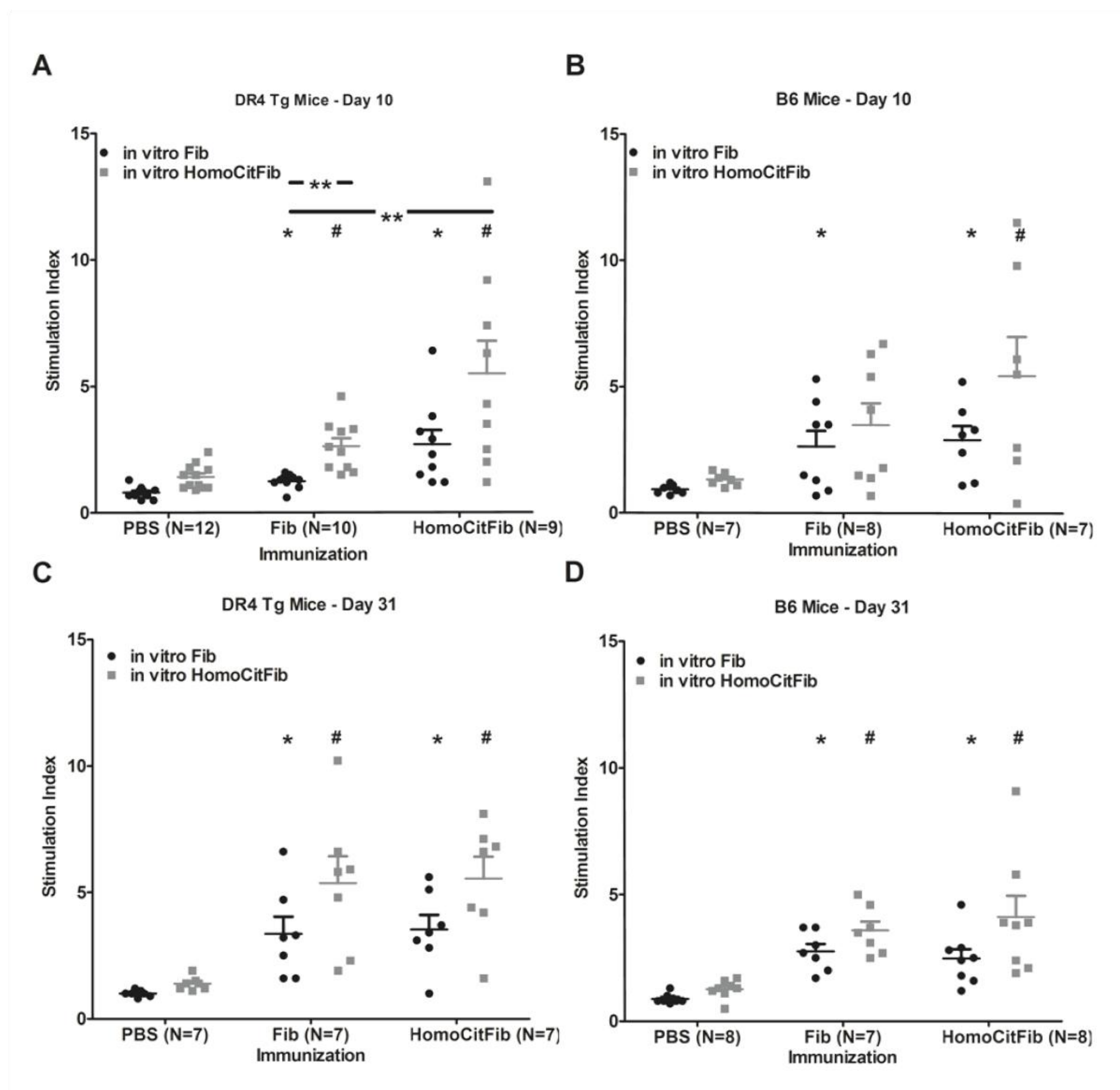


Figure 2.4. Proliferative responses of splenocytes from DR4 Tg and B6 mice. Results from proliferation assays performed on splenocytes from DR4 Tg mice 10 (A) or 31 days (C) post-immunization and from B6 mice 10 (B) or 31 days (D) post immunization after re-exposure to antigen *in vitro* (*= $p<0.05$ compared to PBS immunized, Fib exposed; #= $p<0.05$ compared to PBS immunized, HomoCitFib exposed; **= $p<0.01$).

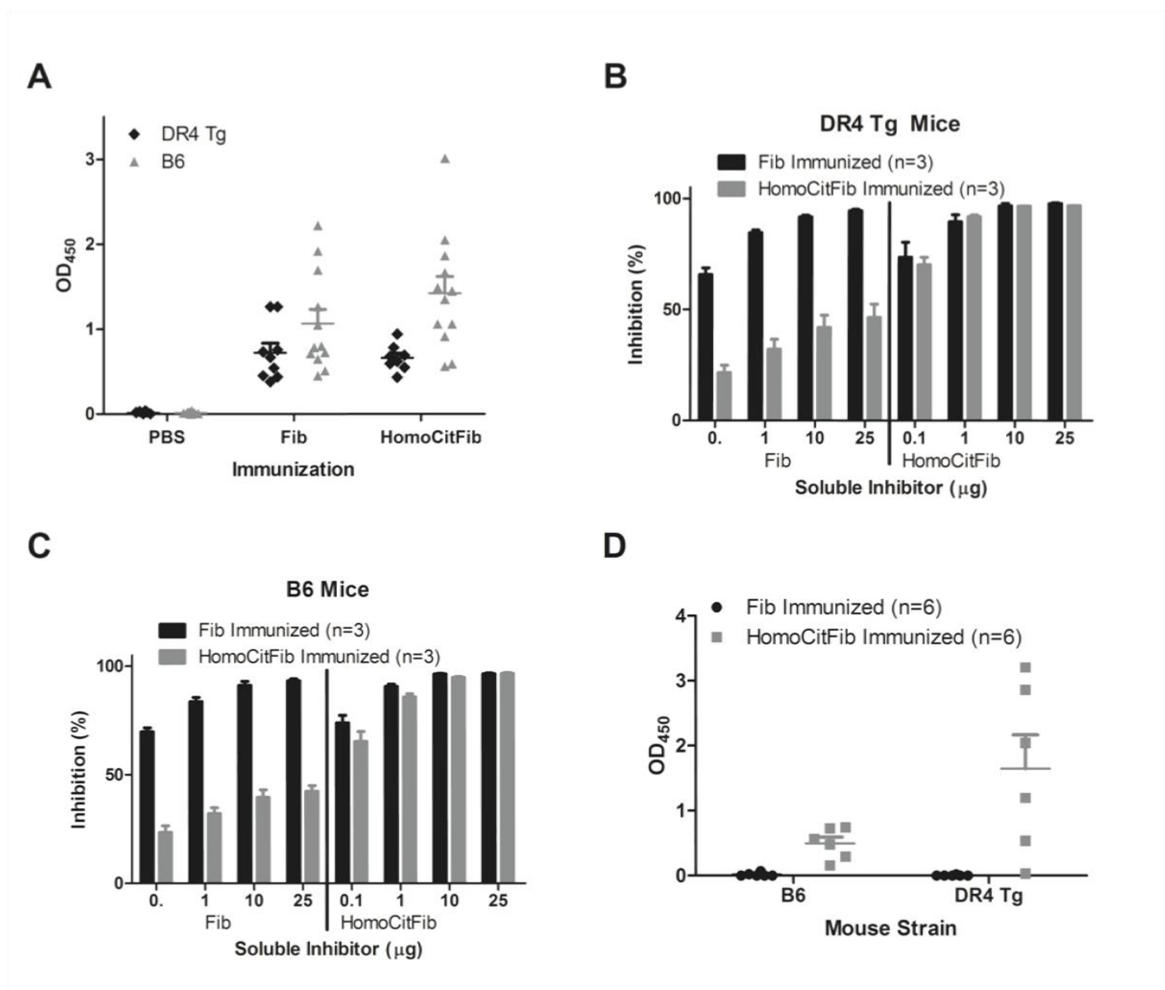


Figure 2.5 Antibody responses in DR4 Tg and B6 mice. A) ELISA detecting serum IgG AHFA responses in DR4 Tg and B6 immunized mice day 31 post immunization with PBS, unmodified fibrinogen, or homocitrullinated fibrinogen. All mice responded to protein vs. PBS immunization ($p<0.001$). **B&C)** ELISA showing inhibition of AHFA from immunized DR4 Tg (B) and B6 (C) mice with soluble unmodified fibrinogen or homocitrullinated fibrinogen. **D)** ELISA detecting serum anti-JED IgG ACPA in mice immunized with homocitrullinated fibrinogen 90 days post-immunization. All mice immunized with homocitrullinated fibrinogen had higher anti-JED IgG responses than mice immunized with unmodified fibrinogen ($p<0.01$).

DR4 Tg and B6 immunized with homocitrullinated fibrinogen, the unmodified fibrinogen could only inhibit 34-53%, indicating that both mice generated a homocitrulline specific antibody response (Figure 2.5B and C). Interestingly, when serum from both B6 and DR4 Tg mice immunized with homocitrullinated fibrinogen was tested 90 days post-immunization for antibodies to citrullinated JED peptide, anti-JED antibodies (ACPA) were detected. These were not observed with B6 and DR4 Tg mice immunized with unmodified fibrinogen (Figure 2.5D) or even DR4 Tg mice immunized with citrullinated fibrinogen (data not shown).

2.4 Discussion

This study showed that the protein fibrinogen is extensively accessible to homocitrullination and that homocitrullinated fibrinogen is a target of autoantibodies in RA, specifically. We also showed that homocitrullination can generate unique neo-epitopes on proteins and although homocitrulline is predicted to bind to the SE, the generation of AHPA is not restricted by the SE, unlike ACPA. Additionally, we demonstrated that the targets of ACPA are not limited to citrullinated sites as previously thought, but at least some human ACPA is able to bind homocitrullinated protein/peptide as well. Therefore, ``cross-reactive`` antibodies which can bind both citrulline and homocitrulline exist. These promiscuous antibodies could have implications on current models of RA pathogenesis since ACPA have been shown to be arthritogenic (10-12), but their relevant *in* vivo targets have not been identified and their affinity for homocitrulline has not been investigated.

To date, there are few studies describing protein homocitrullination in immunology (13, 17, 21-22). In agreement with previous work (21-22), we found that AHPA were produced in RA. Our study showed that a high proportion of RA patients had antibodies that bind homocitrullinated protein/peptide. Initial identification of AHPA in humans employed carbamylated total calf serum (22), or a short peptide derived from filaggrin (21). It is, however, important to study anti-homocitrulline responses against proteins which are found in the arthritic joint and, thus potentially relevant in disease pathogenesis. The investigation by Shi *et al.* demonstrated that RA patients have AHPA that target homocitrullinated fibrinogen (22). We also employed homocitrullinated fibrinogen since: i) antibodies to citrullinated fibrinogen are highly specific for RA (23-25); ii) half of ACPA positive RA patients have circulating citrullinated fibrinogen:IgG antibody complexes (34); iii) it is present in inflamed RA joints (24-25); and iv) immunization with the citrullinated form induces arthritis in DR4 Tg mice expressing the SE (10).

Our study showed that fibrinogen is also extensively accessible to homocitrullination and that there are substantially more sites for homocitrullination in this molecule compared to citrullination. In agreement with Shi *et al.*, we confirmed that RA patients do have antibodies which bind to homocitrullinated regions of fibrinogen (AHFA) (22). Additionally, we showed that AHPA were specific to RA as AHFA were not found in patients with other inflammatory rheumatic conditions. The fact that some RA patients in our study had antibodies targeting the homocitrullinated form of fibrinogen but not the citrullinated form supports the concept that homocitrullination can generate unique structural antigens on proteins, different from citrullination.

Although Shi *et al.* were unable to show cross-reactivity between citrullinated and homocitrullinated fibrinogen (22) we showed that some human ACPA can bind both citrulline and homocitrulline. There are possible explanations for the differences observed. Their initial study employed a single peptide in the N-terminal region of the fibrinogen beta-chain. The native N-terminal region of the fibrinogen beta-chain includes an arginine that we confirmed can be citrullinated, but Shi *et al.* also altered it to a lysine or homocitrulline at this position. Studying antibodies which bind a single peptide, however, entails studying a very limited number of, or even a single clonal specificity. Thus, this experiment demonstrated that some antibody clones are highly specific and are able to discriminate between citrulline and homocitrulline. Our study, on the other hand, investigated the large polyclonal response of patients. We cannot exclude the possibility that some affinity purified ACPA are not able to bind homocitrulline.

Shi *et al.* employed the use of whole fibrinogen and studied the inhibition of anti-homocitrullinated fibrinogen antibodies with citrullinated fibrinogen (and vice versa) (22). These studies show that there is minimal inhibition by the alternatively modified form of fibrinogen, however, these studies do not rule out the presence of cross-reactivity between anti-modified fibrinogen and other protein antigens. As we have shown, homocitrullination and citrullination generate distinct antigens on fibrinogen as they modify different amino acids which are often found in different regions of the protein. Additionally, antibodies do not bind single amino acids, but rather an area of protein with many amino acids. The majority of cross-reactive antibodies likely occur because protein regions that contain homocitrulline structurally resemble an alternative protein region that contains citrulline. Additionally, to cross-react, the antibodies which recognize the

protein structure must not exclusively discriminate between the two amino acid modifications (as was the case with the antibodies targeting the fibrinogen β-chain peptide studied by Shi *et al.*). Therefore, these inhibition studies conclude that there are no (or few) antigenic regions of homocitrullinated fibrinogen that resemble citrullinated fibrinogen regions (and vice versa). Thus, there is little cross reactivity between AHFA and citrullinated fibrinogen or ACFA and homocitrullinated fibrinogen. This is likely due to: i) a lack of structural similarity between homocitrullinated and citrullinated fibrinogen antigenic regions which is insufficient to facilitate cross-reactive binding; and/or ii) the generation of highly specific antibodies which discriminate between the modifications in areas that are similar. However, just as there are sites on the citrullinated filaggrin protein which structurally resemble citrullinated fibrinogen (25), there may be modified sites on other human proteins which would cross-react with fibrinogen antigens and allow the inhibition of AHFA or ACFA in Shi's experiments.

Another important difference between our study and that of Shi *et al.* is the amount of cyanate used to homocitrullinate fibrinogen. Since homocitrullination is a chemical process, not an enzymatic one, the reactant, cyanate, gets consumed in the process. Therefore, if the reaction is allowed to progress to completion, the amount of cyanate relative to fibrinogen will determine how many lysine residues can be modified and, therefore which antigens will be generated. Theoretically, if enough cyanate is present, all accessible lysines could be converted to homocitrulline. Mass spectrometry of our homocitrullinated fibrinogen preparations showed a heterogenous population of fibrinogen peptides with different combinations of peptidyl lysine/homocitrulline, representing a wide variety of antigens. We used 0.05 mol of KOCN/g of fibrinogen

while their study used twice as much. This could have reduced their ability to detect cross reactive antibody specificities which rely on the presence of lysine in their antigenic region.

The existence of cross-reactive antibodies was also suggested previously by Turunen *et al.*. (13) who reported that immunization of rabbits with homocitrullinated human albumin can trigger the generation of ACPA. We also observed this phenomenon, as immunization of both DR4 Tg and B6 mice with homocitrullinated fibrinogen, but not citrullinated or unmodified fibrinogen, triggered ACPA production. Neither study, however, investigated whether the homocitrulline induced ACPA that were generated were still able to bind to homocitrullinated targets as well, or if the immune response was citrulline specific. Interestingly, Mydel *et al.* reported more severe arthritis in mice which were immunized with homocitrullinated peptide prior to the introduction of a citrullinated target peptide in the joint. All these (cross-reactivity) data suggest that, like citrullination, homocitrullination may be involved in the pathogenesis of RA by triggering the generation of autoantibodies (including ACPA) and/or by generating targets for antibody responses in the arthritic joint.

Increased homocitrulline levels have been found in the serum and joints of RA patients by Mydel *et al*. (21). Additionally, the finding that AMC stains homocitrulline residues as well as citrulline (13) suggests that some previous *in situ* identification of citrulline in arthritic tissue may actually have been homocitrulline. It was also reported that RA patients with erosive disease, specifically, have increased circulating homocitrulline and higher levels of AHPA (21). We did not investigate the presence of homocitrullinated protein *in vivo* or the correlation between disease activity/severity and

AHPA status. AHPA has also been reported to be found in ACPA negative RA (22). This was not the case in our study, but only 10 ACPA negative RA patients were studied and AHPA was only detected with homocitrullinated fibrinogen.

Mydel *et al*. demonstrated a role for homocitrulline sensitized T cells and MHC class II dependency in their mouse arthritis model (21). We have previously shown that ACPA production triggered by citrullination (as opposed to homocitrullination) is restricted by the presence of the MHC class II SE (1) and our molecular modelling suggests that although homocitrulline is larger than citrulline, it can also bind to the SE. This computer modelling method did not consider conformational changes, or "induced fitting" which usually occurs through minor changes in local protein structure and result in improved binding. Further investigation of homocitrulline and the SE by immunization of DR4 Tg and B6 mice, however, indicated that IgG AHPA production was not SE restricted. We were unable to detect net homocitrulline specific T cell responses and the only differences detected after unmodified or homocitrullinated fibrinogen immunization were in DR4 Tg mice 10 days after immunization. These mice seemed to have a more robust response to homocitrullinated fibrinogen however, the underlying cause of both differences could be the consistent inability of DR4 Tg mice to respond to unmodified fibrinogen early after unmodified fibrinogen immunization. Additional studies which utilize homocitrullinated peptide will be required to confirm that T cell responses were homocitrulline specific and homocitrulline bound the P4 binding pocket, specifically.

We showed that AHPA were indeed specific to RA as they were not found in normal individuals or patients with other inflammatory rheumatic conditions such as

Psoriatic Arthritis and SLE. Since AHPA are not found in all individuals even though everyone experiences inflammation and homocitrullination, there may be factors, both genetic and environmental, which influence the anti-homocitrulline immune response. Perhaps there is a threshold of homocitrulline levels required to be immunogenic. Genetic variants of MPO have been investigated in studies of cancer but, to our knowledge, have not been studied in RA (35-36). Since some MPO variants have different levels of expression, they could either enhance or restrict the production of cyanate (and homocitrullination potential) during inflammation. Increased homocitrulline levels could also occur in people with carbamylation associated pathologies (17, 37-38) or specific environmental influences, since serum cyanate levels are increased both directly and indirectly by many different factors such as cigarette smoking, dietary intake, and air quality (17-18, 39). These factors could influence the level of cyanate in the internal environment.

It is unknown whether antibody responses binding to homocitrullinated and citrullinated targets in the joint are the sole cause and initiator of RA, however, literature on ACPA suggests antibodies targeting protein modifications can at least exacerbate disease (7, 10, 12, 40). Our study clearly demonstrates that homocitrullination provides a mechanism for the generation of neo-epitopes different from those produced by citrullination. We also showed that AHPA production is not restricted by the SE and anti-homocitrulline responses may constitute a pathway for SE-independent ACPA production. Furthermore, ACPA could be further classified by their binding properties to homocitrullinated antigens and/or by the origin of the immune response (whether triggered by citrullinated or homocitrullinated protein/peptide).

2.5 References

1. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. 2003. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J Immunol* 171: 538-41
2. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ. 2000. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 43: 155-63
3. Kurki P, Aho K, Palosuo T, Heliovaara M. 1992. Immunopathology of rheumatoid arthritis. Antikeratin antibodies precede the clinical disease. *Arthritis Rheum* 35: 914-7
4. Rantapää-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 48: 2741-9
5. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, Habibuw MR, Vandenbroucke JP, Dijkmans BA. 2004. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum* 50: 380-6
6. Bukhari M, Thomson W, Naseem H, Bunn D, Silman A, Symmons D, Barton A. 2007. The performance of anti-cyclic citrullinated peptide antibodies in predicting

the severity of radiologic damage in inflammatory polyarthritis: results from the Norfolk Arthritis Register. *Arthritis Rheum* 56: 2929-35

7. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, Holers VM. 2006. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. *J Clin Invest* 116: 961-73

8. Lundberg K, Nijenhuis S, Vossenaar ER, Palmblad K, van Venrooij WJ, Klareskog L, Zendman AJ, Harris HE. 2005. Citrullinated proteins have increased immunogenicity and arthritogenicity and their presence in arthritic joints correlates with disease severity. *Arthritis Res Ther* 7: R458-67

9. Burkhardt H, Sehnert B, Bockermann R, Engström A, Kalden JR, Holmdahl R. 2005. Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. *Eur J Immunol* 35: 1643-52

10. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, Lee DM, Hueber W, Robinson WH, Cairns E. 2008. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. *J Exp Med* 205: 967-79

11. Petkova SB, Konstantinov KN, Sproule TJ, Lyons BL, Awwami MA, Roopenian DC. 2006. Human antibodies induce arthritis in mice deficient in the low-affinity inhibitory IgG receptor Fc gamma RIIB. *J Exp Med* 203: 275-80

12. Brintnell W, Bell, DA, Cairns, E. 2009. The mechanisms underlying arthritogenicity of human anticitrulline antibodies [Abstract]. *Arthritis Rheum.* 58 (10): S433

13. Turunen S, Koivula MK, Risteli L, Risteli J. 2010. Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. *Arthritis Rheum* 62: 3345-52

14. Vossenaar ER, Zendman AJ, van Venrooij WJ, Pruijn GJ. 2003. PAD, a growing family of citrullinating enzymes: genes, features and involvement in disease. *Bioessays* 25: 1106-18

15. Bobb D, Hofstee BH. 1971. Gel isoelectric focusing for following the successive carbamylations of amino groups in chymotrypsinogen A. *Anal Biochem* 40: 209-17

16. Stark GR. 1965. Reactions of Cyanate with Functional Groups of Proteins. Ii. Formation, Decomposition, and Properties of N-Carbamylimidazole. *Biochemistry* 4: 588-95

17. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. 2007. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* 13: 1176-84

18. Olea F, Parras P. 1992. Determination of serum levels of dietary thiocyanate. *J Anal Toxicol* 16: 258-60

19. Husgafvel-Pursiainen K, Sorsa M, Engstrom K, Einisto P. 1987. Passive smoking at work: biochemical and biological measures of exposure to environmental tobacco smoke. *Int Arch Occup Environ Health* 59: 337-45

20. Klareskog L, Malmstrom V, Lundberg K, Padyukov L, Alfredsson L. 2011. Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. *Semin Immunol* 23: 92-8

21. Mydel P, Wang Z, Brisslert M, Hellvard A, Dahlberg LE, Hazen SL, Bokarewa M. 2010. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. *J Immunol* 184: 6882-90

22. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, Levarht NE, van der Helm-van Mil AH, Cerami A, Huizinga TW, Toes RE, Trouw LA. 2011. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A* 108: 17372-7

23. Hill JA, Al-Bishri J, Gladman DD, Cairns E, Bell DA. 2006. Serum autoantibodies that bind citrullinated fibrinogen are frequently found in patients with rheumatoid arthritis. *J Rheumatol* 33: 2115-9

24. Raijmakers R, van Beers JJ, El-Azzouny M, Visser NF, Bozic B, Pruijn GJ, Heck AJ. 2012. Elevated levels of fibrinogen-derived endogenous citrullinated peptides in synovial fluid of rheumatoid arthritis patients. *Arthritis Res Ther* 14: R114

25. Masson-Bessière C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, Serre G. 2001. The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J Immunol* 166: 4177-84

26. Arnett FC, Edworthy SM, Bloch DA, McShane DJ, Fries JF, Cooper NS, Healey LA, Kaplan SR, Liang MH, Luthra HS, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum* 31: 315-24

27. Ito K, Bian HJ, Molina M, Han J, Magram J, Saar E, Belunis C, Bolin DR, Arceo R, Campbell R, Falcioni F, Vidovic D, Hammer J, Nagy ZA. 1996. HLA-DR4-IE chimeric class II transgenic, murine class II-deficient mice are susceptible to experimental allergic encephalomyelitis. *J Exp Med* 183: 2635-44

28. Ercan A, Cui J, Chatterton DE, Deane KD, Hazen MM, Brintnell W, O'Donnell CI, Derber LA, Weinblatt ME, Shadick NA, Bell DA, Cairns E, Solomon DH, Holers VM, Rudd PM, Lee DM. 2010. Aberrant IgG galactosylation precedes disease onset, correlates with disease activity, and is prevalent in autoantibodies in rheumatoid arthritis. *Arthritis Rheum* 62: 2239-48

29. Hill J, Cairns, E, Bell DA, inventors. Peptides associated with MHC class II molecules involved in autoimmune diseases. US full patent application (US10/548258)filed 2004 Mar 5; published 2007 Dec 20. Canadian full patent application (CA2518187) filed 2005 Sept 20. Patent Treaty Cooperation Application (WO 04/078098) filed 2004 Mar 5 to secure worldwide protection. Europe (EP1603937) published 2005 Dec 14. Japan (JP2007524583) published 2007 Aug 30. Australia (AU4216925) issued 2010.

30. Dessen A, Lawrence CM, Cupo S, Zaller DM, Wiley DC. 1997. X-ray crystal structure of HLA-DR4 (DRA*0101, DRB1*0401) complexed with a peptide from human collagen II. *Immunity* 7: 473-81

31. Hennecke J, Wiley DC. 2002. Structure of a complex of the human alpha/beta T cell receptor (TCR) HA1.7, influenza hemagglutinin peptide, and major histocompatibility complex class II molecule, HLA-DR4 (DRA*0101 and

DRB1*0401): insight into TCR cross-restriction and alloreactivity. *J Exp Med* 195: 571-81

32. Yin Y, Li Y, Kerzic MC, Martin R, Mariuzza RA. 2011. Structure of a TCR with high affinity for self-antigen reveals basis for escape from negative selection. *EMBO J* 30: 1137-48

33. Hammer J, Bono E, Gallazzi F, Belunis C, Nagy Z, Sinigaglia F. 1994. Precise prediction of major histocompatibility complex class II-peptide interaction based on peptide side chain scanning. *J Exp Med* 180: 2353-8

34. Zhao X, Okeke NL, Sharpe O, Batliwalla FM, Lee AT, Ho PP, Tomooka BH, Gregersen PK, Robinson WH. 2008. Circulating immune complexes contain citrullinated fibrinogen in rheumatoid arthritis. *Arthritis Res Ther* 10: R94

35. Piedrafita FJ, Molander RB, Vansant G, Orlova EA, Pfahl M, Reynolds WF. 1996. An Alu element in the myeloperoxidase promoter contains a composite SP1-thyroid hormone-retinoic acid response element. *J Biol Chem* 271: 14412-20

36. Van Schooten FJ, Boots AW, Knaapen AM, Godschalk RW, Maas LM, Borm PJ, Drent M, Jacobs JA. 2004. Myeloperoxidase (MPO) -463G->A reduces MPO activity and DNA adduct levels in bronchoalveolar lavages of smokers. *Cancer Epidemiol Biomarkers Prev* 13: 828-33

37. Harding JJ, Rixon KC. 1980. Carbamylation of lens proteins: a possible factor in cataractogenesis in some tropical countries. *Exp Eye Res* 31: 567-71

38. Lapko VN, Smith DL, Smith JB. 2001. In vivo carbamylation and acetylation of water-soluble human lens alphaB-crystallin lysine 92. *Protein Sci* 10: 1130-6

39. Roberts JM, Veres PR, Cochran AK, Warneke C, Burling IR, Yokelson RJ, Lerner B, Gilman JB, Kuster WC, Fall R, de Gouw J. 2011. Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. *Proc Natl Acad Sci U S A* 108: 8966-71

40. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, Bell DA, Cairns E, Venables PJ. 2011. Immunization with Porphyromonas gingivalis enolase induces autoimmunity to mammalian alpha-enolase and arthritis in DR4-IE-transgenic mice. *Arthritis Rheum* 63: 3818-23

Chapter 3:

Conclusions and discussion

3.1 Study overview

Post-translational modification of self proteins generates neo-epitopes that can be recognized by the immune system. The identification of antibodies which target the modified amino acid, citrulline, and are highly specific for RA has provided new insights into disease pathogenesis (1-3). MHC class II genes were previously reported as the strongest genetic risk factor for RA (4) and have since been shown to contribute to arthritis by facilitating the immune response to citrullinated peptide (5). The subsequent production of anti-citrulline antibodies (ACPA) occurs prior to disease onset (6-8) and there is an accumulation of evidence that ACPA are arthritogenic (9-11). The recent finding that RA patients also have antibodies which target an alternative post-translationally modified amino acid, homocitrulline (12-13), has suggested that there may be additional mechanisms involved in RA development and prgression.

This research supported the hypothesis that homocitrullination and antibody responses targeting homocitrulline (AHPA) are also involved in RA. It demonstrated that, as with ACPA, AHPA are specific for RA as well. The protein fibrinogen, a known target of ACPA (14), was shown to also be extensively accessible to homocitrullination and a target of autoantibodies (AHFA) after such modification. Additionally, there are substantially more sites for homocitrullination compared to citrullination in the fibrinogen protein. Some RA patients had antibodies which targeted the homocitrullinated form of fibrinogen but not the citrullinated form (and vice versa). This, combined with the previous observations that citrullinated fibrinogen minimally inhibits AHFA and homocitrullinated fibrinogen minimally inhibits Anti-Citrullinated Fibrinogen Antibodies (ACFA) (13), supports the previous notion that ACPA and AHPA can form distinct groups of antibodies. Thus, homocitrullination can generate unique structural antigens on proteins, different from citrullination. Although ACPA and AHPA can form distinct

antibody groups, this study showed that there can also be overlap between immune responses against the two modifications. Specifically, at least some human ACPA is able to bind homocitrulline as well, but the extent of this cross-reactive overlap is unknown since this experiment does not quantify the proportion of the polyclonal ACPA response which was also able to bind homocitrulline. It remains unknown whether these cross-reactive antibodies are involved in the previously demonstrated arthritogenicity of ACPA (11).

In human RA, the SE has a very important role in the production of ACPA (5). Therefore, this study investigated if homocitrulline could also bind to the SE. Computer modelling suggested homocitrulline could be accommodated by the SE binding pocket. Again, this computer modelling method did not consider local conformational changes, or “induced fitting” which would usually occur to optimize binding. Homocitrullination and the SE were investigated further by immunizing transgenic mice which expressed the SE alongside their wild-type B6 counterpart. The only differences detected after unmodified or homocitrullinated fibrinogen immunization were in DR4 Tg mice 10 days after immunization. Although not statistically significant in any other group, all individual mice immunized with either form of fibrinogen did have a greater response upon re-exposure to homocitrullinated protein.

All mice immunized with fibrinogen (unmodified or homocitrullinated) did generate IgG antibodies which bound to homocitrullinated fibrinogen, however only mice immunized with homocitrullinated fibrinogen generated IgG antibodies that were homocitrulline specific. The antibodies produced in mice that were immunized with unmodified fibrinogen were likely due to differences between native mouse fibrinogen and the human form of the protein which was used for immunization, as the sequence homology between the mouse’s native fibrinogen and the human fibrinogen is 58%, 87%, and 84% for the alpha, beta, and gamma chains, respectively.

The proliferative responses and generation of AHPA in both DR4 Tg and B6 mice indicate that the response to homocitrulline is not SE restricted. In addition to generating anti-homocitrullinated fibrinogen antibodies, both DR4 Tg and B6 mice also generated ACPA (anti-JED) when immunized with homocitrullinated fibrinogen, but not when immunized with unmodified fibrinogen or even citrullinated fibrinogen. The generation of ACPA in mice after immunization with homocitrulline supports this study's finding of cross-reactive ACPA in human sera as well as Turunen *et al.*'s observation in rabbits (15). It also suggests a mechanism by which ACPA can be generated independently of the SE. Specifically, after exposure to homocitrullinated protein or peptide, an immune response which binds citrullinated epitopes can develop. Although there are similarities between citrullination and homocitrullination, there are important differences too.

3.2 Homocitrullination vs. Citrullination

Citrulline and homocitrulline are structurally quite similar as they have the same functional, ureido group with only an extra carbon atom that makes the homocitrulline side-chain longer (16). There are, however, major differences in the generation and consequences of the two modified amino acids.

As this study has shown, the modification of human proteins with either citrullination or homocitrullination can generate new antigens that are targeted by the immune system and promote break in immunological tolerance. In the case of citrullination, the main proteins which are identified *in vivo* and targeted by ACPA are citrullinated fibrinogen (14, 17), citrullinated collagen II (18), citrullinated vimentin (19-20), and citrullinated human α-enolase (21). Whereas citrullination sites can be limited by PAD enzyme specificity and accessibility, chemical

homocitrullination at physiological conditions is mainly restricted by isocyanate concentration and lysine solvent exposure, so a large percentage of protein lysines can usually be modified (22-24). Therefore, there are likely a large number of lysine residues in the human proteome which can be modified by homocitrullination and serve as potential immunological antigens.

Like the generation of citrulline, the process of homocitrullination is influenced environmentally. Numerous factors such as diet, air pollution and cigarette smoking can influence serum cyanate levels through multiple mechanisms (25-27) and can therefore, promote protein homocitrullination. Additionally, pathologies which involve carbamylation and have their own environmental risks (26, 28-29) could promote increased exposure to homocitrulline. Specifically, atherosclerosis could potentially trigger the production of AHPA because carbamylated protein accumulation is involved in inflammatory plaque development (26). Supporting this idea, coronary artery disease (CAD) due to atherosclerosis has been shown to be associated with RA (30), however, from current literature it is unclear whether CAD is strictly a complication (consequence) of RA or if it can be a factor contributing to RA development as well. Perhaps early, undetected atherosclerosis can play a causal role in RA pathogenesis by producing an environment that promotes increased exposure to homocitrulline and, thus, the generation of AHPA. CAD as a cause of AHPA production has not yet been demonstrated, however, as an alternative hypothesis is that CAD and RA merely often "co-exist" because homocitrullination is involved in both pathologies. In other words, because RA can be promoted by an environment that has increased carbamylation and this process also promotes atherogenesis, they are just often found together.

With citrullination, it has been shown that infection from the environment can cause exposure to citrulline leading to ACPA production (21). Bacterial protein α-enolase from

Porphyromonas gingivalis has been implicated as a target and initiator of the anti-citrulline response through cross-reactive molecular mimicry (21). Whereas the PAD enzyme responsible for citrullination is only known to be expressed in this one bacterium, any bacterium, or bacterial antigens which resemble human proteins, could potentially be exposed to urea/cyanate and be subsequently carbamylated. It has even been shown that carbamylation is critical for the enzymatic activity of bacterial Class D β-Lactamases, which confer antibiotic resistance to many common pathogens (31). Thus, any infection could not only promote inflammatory homocitrullination of self proteins (26), but also cause exposure to carbamylated bacterial antigens. This could potentially trigger additional molecular mimicry responses in individuals with an adequate genetic background, similar to the citrullination of bacterial α-enolase and subsequent production of ACPA in SE positive individuals.

Previous studies have identified genetic factors which are associated with, and predispose people to the development of RA. These factors include genes encoding MHC class II molecules with the SE, PTPN22 variants, and PAD variants and are primarily associated with ACPA positive RA (32). Genetic factors predisposing individuals to the development anti-homocitrulline responses, if any, still await identification. As noted previously, at least some variants of MPO with different levels of expression do exist and altered MPO expression could affect the amount of exposure to homocitrulline. No MHC genes, however have been linked to AHPA production. Even in the case of citrullination, the presence of identified heritable risk factors is not an absolute necessity, as there are RA patients with ACPA that do not express the SE. Since this study demonstrated that the SE is not essential to AHPA production, these exceptions to the common model of ACPA production could be explained by homocitrullination and citrulline/homocitrulline cross-reactivity.

3.3 AHPA/ACPA "cross-reactivity"

This study and the one by Turunen *et al*. observed the generation of anti-citrulline antibodies after immunization with homocitrullinated antigen (15). These cross-reactive antibodies occurred even in the absence of the SE and, thus, may provide an explanation for the presence of ACPA in SE-negative individuals. Although both of these studies were in animal models, the affinity purification of ACPA allowed for the identification of citrulline/homocitrulline cross-reactive antibodies in human RA patients.

Cross-reactive antibodies which can bind both citrullinated and homocitrullinated targets could have additional effects on both phases of RA development. It increases the potential for break in immunological tolerance since either modification could generate relevant neo-epitopes leading to AHPA/ACPA autoantibody responses. Secondly, it could also contribute to the initiation of joint inflammation since targets in the joint could be generated by both citrullination and homocitrullination. At the very least, since ACPA have been shown to be pathogenic and this study has shown at that some ACPA are AHPA, the pathogenic ACPA needs to be further characterized. These observations do suggest that some AHPA may also be involved in RA pathogenesis.

3.4 Homocitrullination in inflammation and RA pathogenesis

The identification of homocitrulline, AHPA, and cross-reactive antibodies can add additional complexity to the current model of RA pathogenesis involving ACPA. Immune responses to homocitrulline can be incorporated into the current model to develop a new, potential model for RA pathogenesis involving homocitrullination (Figure 3.1). To summarize, RA research thus far suggests an etiological model for RA by which immunological tolerance

can be broken through numerous means. This process which generates autoantibodies comprises what is known as "hit one." For example, immunogenic exposure to citrullination could occur in the lungs and would be increased by factors such as smoking however, smoking promotes homocitrullination as well. The previous observation that 12% of RA patients have antibodies that target MPO and anti-MPO antibodies are more common in patients with lung involvement (33), provides further evidence that homocitrullination is also a significant factor during inflammation of the lungs. Thus, a response to cigarette-induced citrullination or homocitrullination, infection with pathogens that are modified (as with *P. Gingivalis*), and/or exposure to other inflammation-induced (homo)citrullinated self proteins could all potentially trigger the production of ACPA and/or AHPA. ACPA/AHPA production, regardless of its origin, may then generate a volatile environment which favours the development of RA and awaits the second event, a "spark" to initiate disease.

It may only be a matter of time until an occurrence such as physical trauma or infection triggers inflammation in the joint, or "hit two." The inflammation would promote cell death, the subsequent release of PAD and MPO, and the accumulation of citrullinated and/or homocitrullinated targets in the joints. With adequate autoantibody responses (ACPA and/or AHPA) the generation of these targets in the joint may facilitate arthritogenesis by the formation of citrulline and/or homocitrulline immune complexes, recruitment of immune cells, activation of complement and the release of pro-inflammatory cytokines (34-35). Disease could then be perpetuated in a cycle whereby inflammation leads to the generation of additional targets which promotes more inflammation. Once such a cycle is sustained chronically, arthritogenesis can ensue leading to clinical RA.

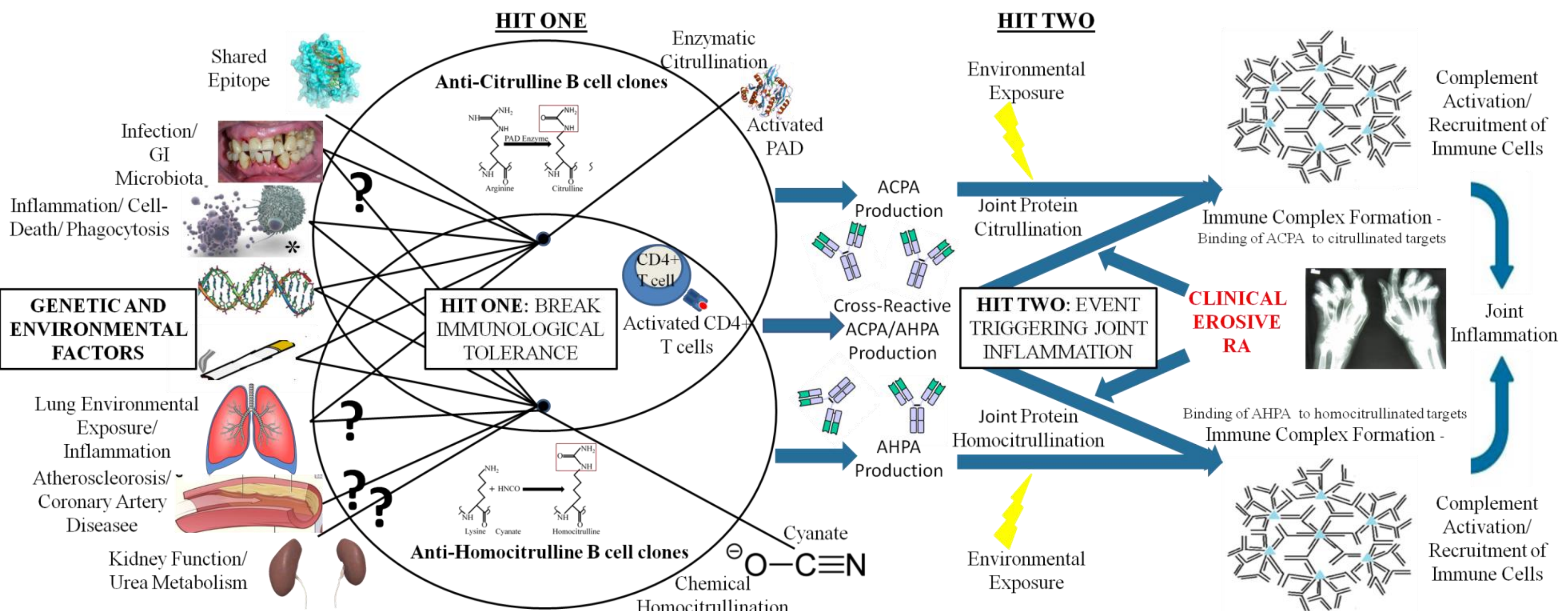


Figure 3.1 Potential model for ACPA and AHPA involvement in the pathogenesis of RA. * Image obtained from the National Library of Medicine. All other images were from public domain or produced originally. "?" indicates a possible link that lacks supporting direct, experimental evidence.

In summary, based on the findings of this study that AHPA occur specifically in RA and some cross-react with citrulline, the current model for RA pathogenesis is more complex than that which was previously proposed. This current model now includes immune responses to homocitrulline in addition to citrulline as well as possible factors that may be involved in the initiation and/or perpetuation of these responses. Many of the steps in this current model await validation in human RA and in experimental animal models for this disease.

3.5 Future Directions

There is much work which needs to be done do study the involvement of homocitrullination in RA and investigate a pathogenic role for AHPA. First of all, the relevant human *in vivo* targets of AHPA should be investigated, including those that resemble citrullinated antigenic regions and are, therefore, involved in cross-reactivity. Furthermore, if AHPA are pathogenic, it will need to be demonstrated that anti-homocitrulline immune responses precede the onset of RA, as with ACPA production. This could be done by retrospective screening pre-RA serum samples, and by screening sera from the sibling of RA patients (since RA siblings can share some genetic and environmental risk factors, they can represent a pre-RA state). Alternative isotypes of AHPA, such as IgA and IgM, should also be screened. Additionally, further screening for AHPA, including in currently "seronegative" patients, would benefit from screening with a surrogate homocitrullinated peptide that is representative of many homocitrullinated antigens, as CCP2 does with ACPA. Such studies are underway in this laboratory which is further screening the RA cohort utilizing the homocitrullinated JED peptide. Although the antibodies to the similar, citrullinated JED peptide have been well characterized, future screening with homocitrullinated JED should test patients with other diseases and also include testing RA patient antibody reactivity to a lysinated form of

the peptide. The homocitrullinated JED peptide should also be used to purify AHPA. This would allow us to study their targets and demonstrate a direct arthritogenic role for them in mice, as we have done with ACPA.

Experimental animal models have also been very useful for the study of AHPA. Mydel *et al.* previously developed a homocitrulline induced arthritis model and we should also try to induce disease in mice to test the arthritogenic properties of homocitrullinated fibrinogen, as well as the effect of the SE. Although both DR4 Tg and B6 mice in our studies generated similar antibody responses, preliminary characterization of these responses suggest important differences between their anti-JED antibody production, with respect to both timing and intensity (Figure 3.2). Therefore, it may be informative to perform studies in which blood is drawn from mice at various times throughout the course of immunization and response. This will help determine when the cross-reactive immune response develops in DR4 Tg mice, as even 31 days after the primary homocitrullinated fibrinogen immunization, anti-JED was not detected. Characterizing the difference between the immune responses of DR4 Tg and B6 mice may elucidate a role for the SE in anti-homocitrulline responses. Also, in Mydel and colleagues' arthritis model it was shown that B6 mice did not develop disease like other mice, but we show that they do generate AHPA. The absence of arthritis in B6 mice leads to a question that can be investigated in future experimental studies: is there merely insufficient production of targets in joints of these mice or are there specifics in the nature of the immune response which determine arthritogenicity?

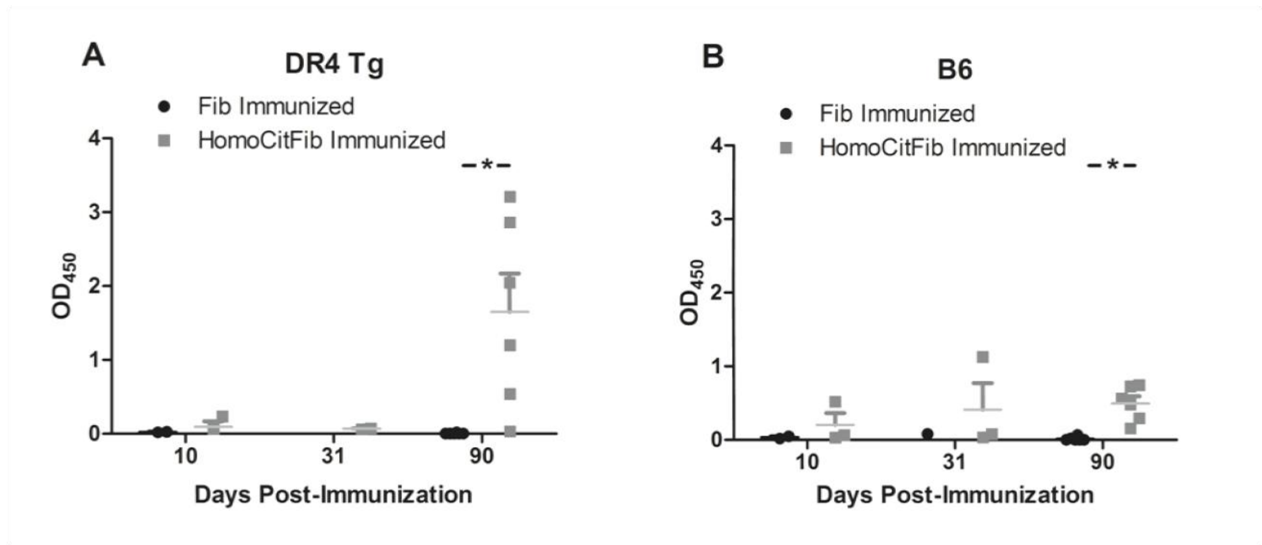


Figure 3.2 Development of ACPA in DR4 Tg and B6 mice immunized with homocitrullinated fibrinogen. ELISA on sera obtained from DR4 Tg and B6 mice after immunization with unmodified fibrinogen or homocitrullinated fibrinogen. Preliminary study comparing anti-JED antibody responses in the serum of DR4 Tg (A) and B6 (B) mice 10, 31, or 90 days post immunization with homocitrullinated fibrinogen. For day 90 tests, n=6, however, for days 10 and 31, n=3 or less. Therefore, statistical analyses were only performed on day 90 responses. * = $p<0.01$.

3.6 Conclusions

This study provides evidence that homocitrulline is involved in RA and it is clear that there may be many opportunities for homocitrullination to trigger break in immunological tolerance. It demonstrated that a high proportion of RA patients had antibodies to homocitrullinated fibrinogen and that these antibodies were specific to RA, as AHFA were not found in normal individuals or patients with other inflammatory rheumatic conditions such as Psoriatic Arthritis and SLE. Furthermore, this study demonstrates that homocitrullination provides a mechanism for the generation of neo-epitopes different from those produced by citrullination and responses to homocitrulline may constitute a pathway for SE-independent ACPA production. Molecular modelling was used to provide evidence that homocitrulline can bind to the SE and this prediction was supported using transgenic mice. Perhaps in the future, as the exponential increase in structural data deposition continues, modelling methods with non-traditional amino acids can be used to predict auto-epitopes/auto-antigens which are potentially immunogenic after post-translational modification.

It is unknown whether citrullination and/or homocitrullination are critical for the development of RA in some patients. The observation that human ACPA precedes disease, combined with animal research involving citrullination does suggest a pathogenic role for these autoantibodes. Considering current research on homocitrullination in RA, its similarities with citrullination, and its aforementioned strong link with environmental influence (including smoking, and its involvement in the known complication of RA, atherosclerosis (30, 36-37)), it is reasonable to anticipate that AHPA is also involved in RA pathogenesis. Furthermore, evidence implicates ACPA as arthritogenic and we have shown that at least some of these antibodies are AHPA as well. The existence of these cross-reactive antibodies demands further investigation

into the target specificity of auto-antibodies in RA. As previously mentioned, ACPA could be further classified regarding binding properties to homocitruline and/or the origin of the immune response (whether triggered by citrullination or homocitrullination). If antibody responses to citrulline and homocitrulline are indeed arthritogenic, important questions remain: which antibodies are pathogenic? Is it the specificity of the target antigen, the quantity and diversity of the response, and/or merely the binding affinity to available targets in the arthritic joint which are important in determining arthritogenicity and clinical disease progression. As Turunen *et al*. reported "homocitrulline as a confounder in citrulline detection" (15), this study suggests homocitrulline is also a confounder of anti-citrulline antibody responses in rheumatoid arthritis.

3.7 References

1. Schellekens GA, Visser H, de Jong BA, van den Hoogen FH, Hazes JM, Breedveld FC, van Venrooij WJ. 2000. The diagnostic properties of rheumatoid arthritis antibodies recognizing a cyclic citrullinated peptide. *Arthritis Rheum* 43: 155-63
2. Young BJ, Mallya RK, Leslie RD, Clark CJ, Hamblin TJ. 1979. Anti-keratin antibodies in rheumatoid arthritis. *Br Med J* 2: 97-9
3. Nienhuis RL, Mandema E. 1964. A New Serum Factor in Patients with Rheumatoid Arthritis; the Antiperinuclear Factor. *Ann Rheum Dis* 23: 302-5
4. Klareskog L, Malmstrom V, Lundberg K, Padyukov L, Alfredsson L. 2011. Smoking, citrullination and genetic variability in the immunopathogenesis of rheumatoid arthritis. *Semin Immunol* 23: 92-8

5. Hill JA, Southwood S, Sette A, Jevnikar AM, Bell DA, Cairns E. 2003. Cutting edge: the conversion of arginine to citrulline allows for a high-affinity peptide interaction with the rheumatoid arthritis-associated HLA-DRB1*0401 MHC class II molecule. *J Immunol* 171: 538-41

6. Kurki P, Aho K, Palosuo T, Heliovaara M. 1992. Immunopathology of rheumatoid arthritis. Antikeratin antibodies precede the clinical disease. *Arthritis Rheum* 35: 914-7

7. Rantapaa-Dahlqvist S, de Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ. 2003. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. *Arthritis Rheum* 48: 2741-9

8. Nielen MM, van Schaardenburg D, Reesink HW, van de Stadt RJ, van der Horst-Bruinsma IE, de Koning MH, Habibuw MR, Vandenbroucke JP, Dijkmans BA. 2004. Specific autoantibodies precede the symptoms of rheumatoid arthritis: a study of serial measurements in blood donors. *Arthritis Rheum* 50: 380-6

9. Hill JA, Bell DA, Brintnell W, Yue D, Wehrli B, Jevnikar AM, Lee DM, Hueber W, Robinson WH, Cairns E. 2008. Arthritis induced by posttranslationally modified (citrullinated) fibrinogen in DR4-IE transgenic mice. *J Exp Med* 205: 967-79

10. Kuhn KA, Kulik L, Tomooka B, Braschler KJ, Arend WP, Robinson WH, Holers VM. 2006. Antibodies against citrullinated proteins enhance tissue injury in experimental autoimmune arthritis. *J Clin Invest* 116: 961-73

11. Brintnell W, Bell, DA, Cairns, E. 2009. The mechanisms underlying arthritogenicity of human anticitrulline antibodies [Abstract]. *Arthritis Rheum.* 58 (10): S433

12. Mydel P, Wang Z, Brisslert M, Hellvard A, Dahlberg LE, Hazen SL, Bokarewa M. 2010. Carbamylation-dependent activation of T cells: a novel mechanism in the pathogenesis of autoimmune arthritis. *J Immunol* 184: 6882-90

13. Shi J, Knevel R, Suwannalai P, van der Linden MP, Janssen GM, van Veelen PA, Levarht NE, van der Helm-van Mil AH, Cerami A, Huizinga TW, Toes RE, Trouw LA. 2011. Autoantibodies recognizing carbamylated proteins are present in sera of patients with rheumatoid arthritis and predict joint damage. *Proc Natl Acad Sci U S A* 108: 17372-7

14. Hill JA, Al-Bishri J, Gladman DD, Cairns E, Bell DA. 2006. Serum autoantibodies that bind citrullinated fibrinogen are frequently found in patients with rheumatoid arthritis. *J Rheumatol* 33: 2115-9

15. Turunen S, Koivula MK, Risteli L, Risteli J. 2010. Anticitrulline antibodies can be caused by homocitrulline-containing proteins in rabbits. *Arthritis Rheum* 62: 3345-52

16. Ashida D, Funakoshi, K., Tsukihara, T., Ueki, T. and Kakudo, M. 1972. The crystal structures of L-citrulline hydrochloride and L-homocitrulline hydrochloride. *Acta Crystallogr* B28: 1367

17. Masson-Bessière C, Sebbag M, Girbal-Neuhauser E, Nogueira L, Vincent C, Senshu T, Serre G. 2001. The major synovial targets of the rheumatoid arthritis-specific antifilaggrin autoantibodies are deiminated forms of the alpha- and beta-chains of fibrin. *J Immunol* 166: 4177-84

18. Burkhardt H, Sehnert B, Bockermann R, Engström A, Kalden JR, Holmdahl R. 2005. Humoral immune response to citrullinated collagen type II determinants in early rheumatoid arthritis. *Eur J Immunol* 35: 1643-52

19. Bang H, Egerer K, Gauliard A, Luthke K, Rudolph PE, Fredenhagen G, Berg W, Feist E, Burmester GR. 2007. Mutation and citrullination modifies vimentin to a novel autoantigen for rheumatoid arthritis. *Arthritis Rheum* 56: 2503-11

20. Vossenaar ER, Despres N, Lapointe E, van der Heijden A, Lora M, Senshu T, van Venrooij WJ, Menard HA. 2004. Rheumatoid arthritis specific anti-Sa antibodies target citrullinated vimentin. *Arthritis Res Ther* 6: R142-50

21. Kinloch AJ, Alzabin S, Brintnell W, Wilson E, Barra L, Wegner N, Bell DA, Cairns E, Venables PJ. 2011. Immunization with Porphyromonas gingivalis enolase induces autoimmunity to mammalian alpha-enolase and arthritis in DR4-IE-transgenic mice. *Arthritis Rheum* 63: 3818-23

22. Stark G, Stein, WH, and Moore, S. 1960. Reactions of the Cyanate Present in Aqueous Urea with Amino Acids and Proteins. *The Journal of Biological Chemistry* 235

23. Mun KC, Golper TA. 2000. Impaired biological activity of erythropoietin by cyanate carbamylation. *Blood Purif* 18: 13-7

24. Stark GR. 1965. Reactions of cyanate with functional groups of proteins. 3. Reactions with amino and carboxyl groups. *Biochemistry* 4: 1030-6

25. Roberts JM, Veres PR, Cochran AK, Warneke C, Burling IR, Yokelson RJ, Lerner B, Gilman JB, Kuster WC, Fall R, de Gouw J. 2011. Isocyanic acid in the atmosphere and its possible link to smoke-related health effects. *Proc Natl Acad Sci U S A* 108: 8966-71

26. Wang Z, Nicholls SJ, Rodriguez ER, Kummu O, Horkko S, Barnard J, Reynolds WF, Topol EJ, DiDonato JA, Hazen SL. 2007. Protein carbamylation links inflammation, smoking, uremia and atherogenesis. *Nat Med* 13: 1176-84

27. Olea F, Parras P. 1992. Determination of serum levels of dietary thiocyanate. *J Anal Toxicol* 16: 258-60

28. Harding JJ, Rixon KC. 1980. Carbamylation of lens proteins: a possible factor in cataractogenesis in some tropical countries. *Exp Eye Res* 31: 567-71

29. Lapko VN, Smith DL, Smith JB. 2001. In vivo carbamylation and acetylation of water-soluble human lens alphaB-crystallin lysine 92. *Protein Sci* 10: 1130-6

30. Goodson N. 2002. Coronary artery disease and rheumatoid arthritis. *Curr Opin Rheumatol* 14: 115-20

31. Golemi D, Maveyraud L, Vakulenko S, Samama JP, Mobashery S. 2001. Critical involvement of a carbamylated lysine in catalytic function of class D beta-lactamases. *Proc Natl Acad Sci U S A* 98: 14280-5

32. Coenen MJ, Gregersen PK. 2009. Rheumatoid arthritis: a view of the current genetic landscape. *Genes Immun* 10: 101-11

33. Cambridge G, Williams M, Leaker B, Corbett M, Smith CR. 1994. Anti-myeloperoxidase antibodies in patients with rheumatoid arthritis: prevalence, clinical correlates, and IgG subclass. *Ann Rheum Dis* 53: 24-9

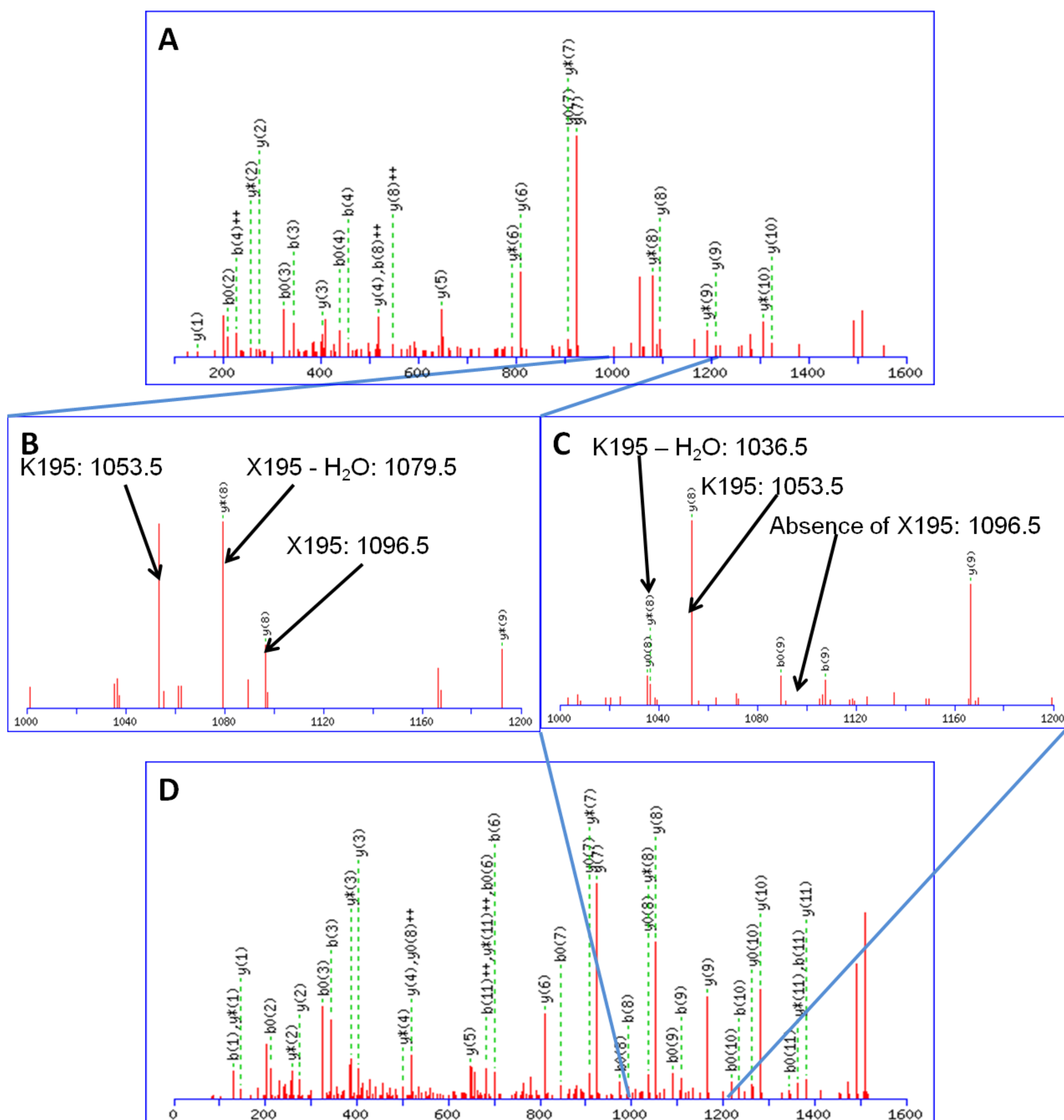
34. Trouw LA, Haisma EM, Levarht EW, van der Woude D, Ioan-Facsinay A, Daha MR, Huizinga TW, Toes RE. 2009. Anti-cyclic citrullinated peptide antibodies from rheumatoid arthritis patients activate complement via both the classical and alternative pathways. *Arthritis Rheum* 60: 1923-31

35. Sokolove J, Zhao X, Chandra PE, Robinson WH. 2011. Immune complexes containing citrullinated fibrinogen costimulate macrophages via Toll-like receptor 4 and Fcgamma receptor. *Arthritis Rheum* 63: 53-62

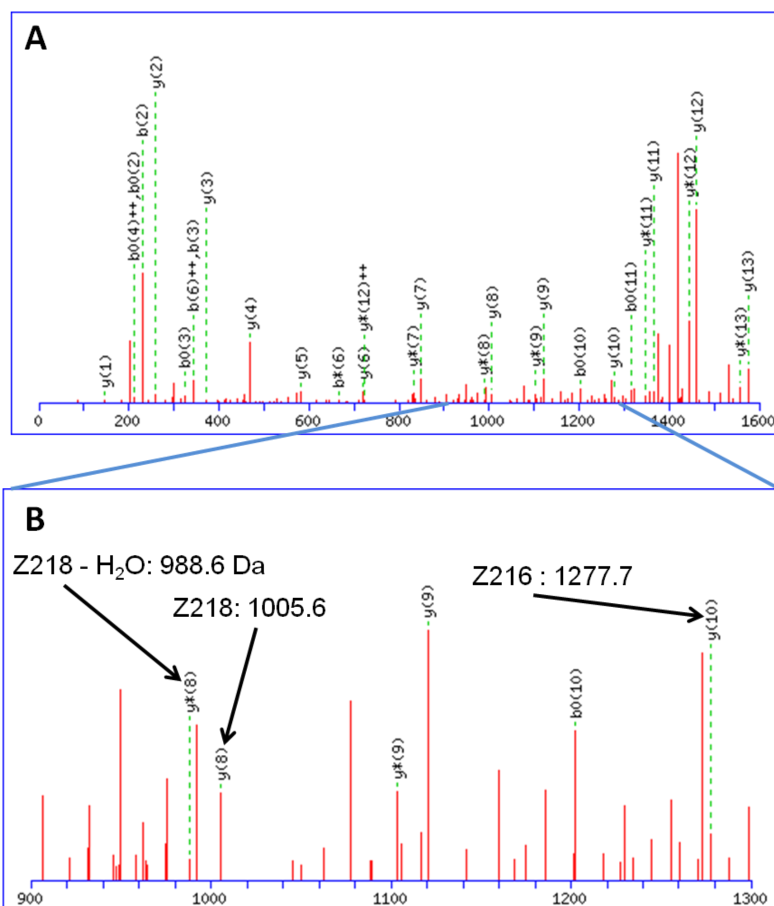
36. Chung CP, Oeser A, Raggi P, Gebretsadik T, Shintani AK, Sokka T, Pincus T, Avalos I, Stein CM. 2005. Increased coronary-artery atherosclerosis in rheumatoid arthritis: relationship to disease duration and cardiovascular risk factors. *Arthritis Rheum* 52: 3045-53

37. Dessein PH, Joffe BI, Veller MG, Stevens BA, Tobias M, Reddi K, Stanwix AE. 2005. Traditional and nontraditional cardiovascular risk factors are associated with atherosclerosis in rheumatoid arthritis. *J Rheumatol* 32: 435-42

Appendices



Appendix 1. Mass spectra indicating homocitrullination of human fibrinogen. Mass spectrometry (ESI-MS/MS) analysis confirming homocitrullination (observed as a mass shift of +43 Da) of the fibrinogen alpha-chain peptide 191-202 (native sequence: EVDLKDYEDQQK) at K195. KOCN treated peptide is shown in A with a magnified and labelled image shown in B while the KCl, negative control treated peptide is shown in D with a magnified and labelled image shown in C. X-axis represents mass to charge ratio (m/z). Lysine is indicated with "K" and homocitrulline is indicated with "X". Monoisotopic mass of native peptide is 1551.705 Da and monoisoptopic mass of modified peptide is 1508.6994 Da (Δ = 43 Da).



Appendix 2. Mass spectra indicating citrullination of human fibrinogen. Mass spectrometry (ESI-MS/MS) analysis confirming citrullination (observed as +1 Da mass shift) of the fibrinogen alpha-chain peptide 211-225 (native sequence: DLLPS<u>R</u>D<u>R</u>QHLPLIK) at R216 and R218. PAD enzyme treated peptide is shown in A with a magnified and labelled image shown in B. The untreated negative control peptide could not be detected because the primary structure causes peptides to be too small after tryptic digest. X-axis represents mass to charge ratio (m/z). Citrulline is indicated with "Z". Monoisotopic mass of native peptide is 1800.0370 Da and monoisoptopic mass of modified peptide is 1802.0050 (Δ = 2 Da).

Alpha-Chain

1 MFSMRIVCLVLSVVGTAWTADSGEGDFLAEGGGVZGPZVVEZHQSACXDS
51 DWPFCSDEDWNYKCPSGCZMXGLIDEVNQDFTNZINXLXNSLFEYQXNNX
101 DSHSLTTNIMEILZGDFSSANNZDNTYNZVSEDLZSZIEVLKRXVIEXVQ
151 HIQLLQXNVRAQLVDMXRLEVDIDIKIZSCZGSCSZALAZEVDLXDYEDQ
201 QXQLEQVIAXDLLPSZDZQHLPLIXMXPVPDLVPGNFXSQLQXVPPXEWXA
251 LTDMPQMRMELERPGGNEITRGGSTSYGTGSETESPRNPSSAGSWNSGSS
301 GPGSTGNRNPGSSGTGGTATWKPGSSGPGSTGSWNSGSSGTGSTGNQNPG
351 SPRPGSTGTWNPGSSERGSAGHWTSESSVSGSTGQWHSESGSFRPDSPGS
401 GNAZPNNPDWGTFEEVSGNVSPGTZZEYHTEXLVTSXGDXELZTGXEXVT
451 SGSTTTTZZSCSXTVTXTVIGPDGHXEVTXEVVTSEDGSDCPEAMDLGTL
501 SGIGTLDGFZHZHPDEAAFFDTASTGKTFPGFFSPMLGEFVSETESZGSE
551 SGIFTNTXESSSHHPGIAEFPSZGXSSSYSXQFTSSTSYNZGDSTFESXS
601 YXMADEAGSEADHEGTHSTXZGHAKSZPVRGIHT

Beta-Chain

1 QGVNDNEEGFFSAZGHZPLDXXZEEAPSLZPAPPPISGGGYZAZPAXAAA
51 TQXXVERXAPDAGGCLHADPDLGVLCPTGCQLQEALLQQEZPIZNSVDEL
101 NNNVEAVSQTSSSSFQYMYLLXDLWQXZQXQVXDNENVVNEYSSELEXHQ
151 LYIDETVNSNIPTNLZVLZSILENLZSKIQXLESDVSAQMEYCRTPCTVS
201 CNIPVVSGXECEEIIZXGGETSEMYLIQPDSSVXPYZVYCDMNTENGGWT
251 VIQNZQDGSVDFGZXWDPYXQGFGNVATNTDGXNYCGLPGEYWLGNDXIS
301 QLTRMGPTELLIEMEDWXGDXVXAHYGGFTVQNEANXYQISVNXYRGTAG
351 NALMDGASQLMGENRTMTIHNGMFFSTYDZDNDGWLTSDPZXQCSXEDGG
401 GWWYNRCHAANPNGZYYWGGQYTWDMAKHGTDDGVVWMNWXGSWYSMRXM
451 SMXIRPFFPQQ

Gamma-Chain

1 MSWSLHPRNLILYFYALLFLSSTCVAYVATRDNCCILDEZFGSYCPTTCG
51 IADFLSTYQTXVDKDLQSLEDILHQVENKTSEVXQLIXAIQLTYNPDESS
101 XPNMIDAATLXSRXMLEEIMXYEASILTHDSSIZYLQEIYNSNNQXIVNL
151 XEKVAQLEAQCQEPCXDTVQIHDITGXDCQDIANXGAXQSGLYFIXPLXA
201 NQQFLVYCEIDGSGNGWTVFQXZLDGSVDFXXNWIQYXEGFGHLSPTGTT
251 EFWLGNEKIHLISTQSAIPYALZVELEDWNGZTSTADYAMFXVGPEADXY
301 ZLTYAYFAGGDAGDAFDGFDFGDDPSDKFFTSHNGMQFSTWDNDNDKFEG
351 NCAEQDGSGWWMNKCHAGHLNGVYYQGGTYSKASTPNGYDNGIIWATWKT
401 ZWYSMXXTTMXIIPFNZLTIGEGQQHHLGGAXQAGDV

Appendix 3. ***In vitro*** **homocitrullination and citrullination sites on human.fibrinogen as determined by mass spectrometry.** Amino acid sequence of fibrinogen. Black **X** represents sites where homocitrulline was detected and black **Z** represents sites where citrulline was detected. Pale colored R or K represents Arg or Lys for which no data was obtained. Underlining represents homocitrullinated peptides which are predicted to bind to the SE.

Subject: RE: Permission to Use Copyrighted Material in a Master's Thesis
To: 'Mathias Scinocca'
image001.jpg (2kB)

Date: 08/15/12 02:57 PM
From: Violet Turalba

RE: HILL J, et al: The Joy of Citrulline: New Insights into the Diagnosis, Pathogenesis, and Treatment of Rheumatoid Arthritis ***J Rheumatol 2004;31(8); 1470-1473. Figure 1A page 1472***

Dear Mathias Scinocca,

A one-time permission is granted to use figure 1A in the above articles for thesis purposes, you may use this permission at your convenience. Please print a copy of this e-mail as proof of your permission, should you need original signed copy please provide preferred mailing address. We further ask that you to seek (lead) author permission as well, this will make the author aware of the use of their material outside The Journal.

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Yours truly,

Violet Turalba

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Appendix 4. Permission to use copyrighted material. (Figure 1.3 in this thesis)

Use of Human Participants - Ethics Approval Notice

Research Western

Principal Investigator: Dr. Ewa Cairns
Review Number: 09684E
Review Level: Delegated
Approved Local Adult Participants: 178
Approved Local Minor Participants: 0
Protocol Title: The role of DR4(*0401) (MHC class II molecules) in Rheumatoid Arthritis pathogenesis
Department & Institution: Medicine-Dept of, London Health Sciences Centre
Sponsor:
Ethics Approval Date: April 28, 2011 **Expiry Date:** October 31, 2013
Documents Reviewed & Approved & Documents Received for Information:

Document Name	Comments	Version Date
Change in Study Personnel	Dr. Ewa Cairns has taken over the responsibility of PI from Dr. David Bell. Dr. Lillian Barra has been added as a Co-PI.	
Revised Letter of Information & Consent		
Other	Questionnaire has been added.	
Revised Study End Date	The study end date was revised to October 31, 2013 to allow for study completion.	

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research

Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

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

Signature

Ethics Officer to Contact for Further Information

Janice Sutherland (jsutherl@uwo.ca)	**Elizabeth Wambolt (ewambolt@uwo.ca)**	**Grace Kelly (grace.kelly@uwo.ca)**

This is an official document. Please retain the original in your files.

The University of Western Ontario
Office of Research Ethics
Room 5150, Support Services Building • London, Ontario • CANADA - N6A 3K7
PH: 519-661-3036 • F: 519-850-2466 • ethics@uwo.ca • www.uwo.ca/research/ethics

Appendix 5. Ethical Approval for use of human subjects.

Subject:eSirius Notification - Annual Protocol Renewal APPROVED by the AUS 2009-101::2
Date:7/9/2012 3:31 PM
From:eSirius WebServer <esiriusadmin >
To:ecairns
CC:auspc, <auspc >

2009-101::2:

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

Approval Date: 02/24/2010

The YEARLY RENEWAL to Animal Use Protocol (AUP) 2009-101 has been approved.

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: Kinchlea, Will D
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: auspam@uwo.ca • http://www.uwo.ca/animal/website/

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

Appendix 7. Curriculum Vitae.

Name: Mathias Joseph Scinocca

Post-Secondary Education:

MSc Candidate
The University of Western Ontario, London, ON
Master of Science in Microbiology and Immunology (Department of Microbiology and Immunology)
SEPT 2010 – PRESENT

HBMSc
The University of Western Ontario, London, ON
Honors Specialization in the Biochemistry of Infection and Immunity (Department of Microbiology and Immunology/Department of Biochemistry)
Major in Genetics (Department of Biology)
SEPT 2005 – APR 2010

Honours and Awards:

Frederick Banting & Charles Best Graduate Scholarship
The University of Western Ontario,
London, ON
SEPT 2011 –PRESENT

Western Graduate Research Scholarship (WGRS)
The University of Western Ontario,
London, ON
SEPT 2010 - PRESENT

Graduate Student Research Award (GTRA)
The University of Western Ontario,
London, ON
Nov 2011

Departmental Graduate Student Travel Award
The University of Western Ontario,
London, ON
NOV 2011

Rheumatology Research Day Oral Presentation First Prize
London, ON
OCT 2011

Ontario Graduate Scholarship (OGS)
The University of Western Ontario,
London, ON
SEPT 2010 – SEPT 2011

Departmental Graduate Student Entrance Award
The University of Western Ontario,
London, ON
SEPT 2010

Biochemistry 4420 Course Award
The University of Western Ontario,
London, ON
JUNE 2005

Deans Honors List
The University of Western Ontario,
London, ON
SEPT 2005 - APRIL 2010

Work Experience:

Graduate Teaching Assistant
The University of Western Ontario,
London, ON
Microbiology and Immunology 2100A Lab Course
SEPT 2011 – DEC 2011

Immunology Laboratory Researcher
Laboratory of Dr. Ewa Cairns
The University of Western Ontario,
London, ON
MAY 2010 – AUG 2010

4th Year Honours Thesis
Laboratory of Dr. John McCormick
The University of Western Ontario,
London, ON
SEPT 2009 – APRIL 2010

Plant Genetics Researcher
Laboratory of Dr. Vojislava Grbic
The University of Western Ontario,
London, ON
MAY 2009 – SEPT 2009

Publications Abstracts:

Scinocca M, Bell D, Pope J, Cairns E, Barra L. Rheumatoid Arthritis Patients Have Anti-Homocitrullinated Fibrinogen Antibodies. Arthritis Rheum. 2011 Nov; 63(10).

Barra L, Wilson E, Scinocca M, Summers K, Cairns E, Bell D. Anti-Citrullinated Protein Antibodies (ACPA) in Unaffected Siblings of ACPA-Positive Rheumatoid Arthritis Patients. Arthritis Rheum. 2010 Nov; 62(10).

Oral Presentations:

Scinocca M, Bell D, Shaw G, Pope J, Cairns E, Barra L. Rheumatoid Arthritis Patients Have Anti-Homocitrullinated Fibrinogen Antibodies. Rheumatology Research Day, St. Joseph's Hospital, London, ON, Oct 27 2011.

Barra L, Scinocca M, Wilson E, Bell D, Cairns E, El-Gabalawy H. Pathogenicity of Anti-Citrullinated Protein/Peptide Antibodies from Unaffected First Degree Relatives of Rheumatoid Arthritis Patients in a Population of North American Natives. Mexican-Canadian Congress of Rheumatology/ Canadian Rheumatology Association Meeting, Mexico, Feb 2011.

Scinocca M, Barra L, Wilson E, Cairns E, Bell D. Pathogenesis and Prevalence of Anti-Citrullinated Protein/Peptide Antibodies (ACPA) in Unaffected Siblings of ACPA Positive Rheumatoid Arthritis Patients. Infection and Immunity Research Forum, University of Western Ontario, London, ON, Nov 5 2010.

Poster Presentations:

Scinocca M, Shaw G, Pope J, Bell D, Barra L, Cairns E. Homocitrulline: A Novel Target in Rheumatoid Arthritis. Department of Medicine Research Day, London, ON, May 2012.

Scinocca M, Bell D, Shaw G, Pope J, Cairns E, Barra L. Rheumatoid Arthritis Patients Have Anti-Homocitrullinated Fibrinogen Antibodies. American College of Rheumatology Conference, Chicago, IL, Nov 5-9 2011.

Scinocca M, Bell D, Shaw G, Pope J, Cairns E, Barra L. Rheumatoid Arthritis Patients Have Anti-Homocitrullinated Fibrinogen Antibodies. Infection and Immunity Research Forum, University of Western Ontario, London, ON, Nov 4 2011.

Scinocca M, Wilson E, El-Gabalawy H, Bell D, Cairns E, Barra L. Arthritogenicity of Anti-Citrullinated Protein/Peptide Antibodies from Healthy First Degree Relatives of Rheumatoid Arthritis Patients. Department of Medicine Research Day, London, ON, May 2011.

Barra L, Wilson E, Scinocca M, Summers K, Cairns E, Bell D. Anti-Citrullinated Protein Antibodies (ACPA) in Unaffected Siblings of ACPA-+ Rheumatoid Arthritis Patients. American College of Rheumatology Meeting, Atlanta GA, Nov 2010.

Barra L, Scinocca M, Wilson E, Summers K, Cairns E, Bell D. Pathogenesis and Prevalence of Anti-Citrullinated Protein Antibodies (ACPA) in Unaffected Siblings of ACPA-Positive Rheumatoid Arthritis Patients. Mexican-Canadian Congress of Rheumatology/ Canadian Rheumatology Association Meeting, February 2011.

Papers:

Scinocca MJ, Shaw G, Joseph R, Bell DA, Barra L., Cairns E. Homocitrulline: An antigen specific to rheumatoid arthritis and a target of anti-citrulline protein/peptide antibodies. (To be submitted to Arthritis and Rheumatism August 2012)

Barra L, Scinocca M, Saunders S, Bhayana R, Rohekar S, Racape M, Summers K, Coles R, Cairns E, Bell D. Anti-Citrullinated Protein Antibodies (ACPA) in Unaffected First Degree Relatives of Rheumatoid Arthritis Patients. (Under review by Arthritis and Rheumatism).