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The Role of T cell Costimulation in the Development and Progression of Citrulline-induced Arthritis

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Abstract

MHC Class II molecules (e.g. DR4) expressing the Rheumatoid Arthritis (RA) shared epitope are strongly linked to the development of anti-citrulline (Cit) immune responses and the pathogenesis of RA. We have established a Cit-induced arthritis model (DR4 tg mice) where injection of citrullinated human fibrinogen (CithFib) induces anti-Cit immune responses and arthritis.

The main objective of this study was to assess the role of T cells in this Cit-induced arthritis mouse model using CTLA4-Ig, an agent that blocks T cell activation. To accomplish this, DR4 tg mice were immunized with CithFib to induce arthritis. At the disease onset or peak, DR4 tg mice were treated with CTLA4-Ig or control human IgG1 (hIgG1) or left untreated. Arthritis progression was monitored for 30 days, and then anti-Cit immune responses and the arthritogenicity of splenic lymphocytes from these mice were examined. The latter were done using adoptive lymphocyte transfers from CTLA4-Ig-treated mice or controls via intraperitoneal injection into naïve DR4 tg mice. The recipient mice also received intraarticular injection of CithFib or vehicle.

The results demonstrated that CTLA4-Ig but not hIgG1 treatment of arthritic DR4 tg mice significantly reduced ankle swelling and pathological joint damage in these mice. CTLA4-Ig, but not hIgG1 treatment suppressed T cell proliferative responses to Cit. Unlike splenic lymphocytes from untreated arthritic mice, lymphocytes transferred from CTLA4-Ig-treated arthritic mice did not cause arthritis in recipient mice. The conclusion from this study is that Cit-specific T cells play a direct role in the development and progression of arthritis in this Cit-induced model for human RA.

Keywords: Rheumatoid arthritis, Shared epitope, T cells, Citrulline, Costimulation blockade

Co-authorship

Chapter 2.

Yue, D., Brintnell, W., Mannik, L.A., Chistie, D.A., Haeryfar, S.M., Madrenas, J., Chakrabarti, S., Bell, D.A.*, Cairns, E.* CTLA4-Ig blocks the development and progression of citrulline-induced arthritis.

D. Yue- designed and conducted all experiments and wrote the paper. *W. Brintnell*- aided in some experiments. *L.A. Mannik*- performed the Treg and *i*NKT cell staining. *D. Christie*- Performed ELISA IL-2 quantitation. *S.M. Haeryfar*- interpreted results. *J. Madrenas*- interpreted results. *S. Chakrabarti*- interpreted results. **D.A. Bell.* 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.

Dedication

To my family, whose unwavering support has been invaluable.

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List of Abbreviations

ACA.....Anti-citrulline antibodies
AMC..... Anti-modified citrulline
APC.....Antigen-presenting cells
APC.....Allophycocyanin
AP..... Alkaline phosphatase
BCR..... B cell antigen receptor
CCP_2..... Cyclic citrullinated peptide 2
CFA..... Complete Freund's adjuvant
CIA..... Collagen-induced arthritis
CII..... Collagen type II
Cit..... Citrulline
CithFib..... Citrullinated human fibrinogen
CitmFib.....Citrullinated mouse fibrinogen
CFSE..... Carboxyfluorescein succinimidyl ester
Cpm..... Counts per minute
CTLA4.....Cytotoxic T lymphocyte antigen 4
CTLA4-Ig..... Cytotoxic T lymphocyte antigen 4 - immunoglobulin
DC..... Dendritic cell
DR4 tg mice.....HLA-DRβ1*0401 transgenic mice
EAE..... Experimental autoimmune encephalomyelitis
FBS..... Fetal bovine serum
FTIC..... Fluorescein isothiocyanate
G6PI..... Glucose-6-phosphate isomerase
H&E..... Hematoxylin and eosin
hFib..... Human fibrinogen
hIgG1..... Human immunoglobulin G1
ia..... Intraarticular
IDO..... Indoleamine 2,3-dioxygenase
IFA..... Incomplete Freund's adjuvant
IFN..... Interferon
IL..... Interleukin
***i*NKT cell**.....Invariant natural killer T cell
ip..... Intraperitoneal
mFib..... Mouse fibrinogen
MHC..... Major histocompatibilty complex
NK cell..... Natural killer cell
nTreg cell..... Naturally occurring T regulatory cell
OD..... Optical density
OVA..... Ovalbumin
PAMP..... Pathogen-associated molecular pattern
PAD.....Peptidyl arginine deiminase
PBS..... Phosphate buffered saline
PE..... Phycoerythrin

PRR..... Pattern recognition receptor
RA..... Rheumatoid arthritis
RF..... Rheumatoid factor
RT..... Room temperature
SE..... Shared epitope
SHRP..... Streptavidin horseradish peroxidase
SI..... Stimulation index
TCR..... T cell antigen receptor
TGF..... Transforming growth factor
Th cell..... T helper cell
TLR..... Toll-like receptor
TMB..... 3,3',5,5' tetramethylbenzidine
TNF..... Tumor necrosis factor
Treg cell..... T regulatory cell
WB..... Wash buffer

CHAPTER 1

INTRODUCTION

1.1 INNATE IMMUNITY

The host immune system is made up of both the innate and adaptive immune systems, which cooperate together to provide protection from infections. Innate immunity plays a critical role in the initial defense against infection. However the adaptive immune response possesses the ability to eliminate infections as well as provide specific and continued protection against re-infection (1).

The main innate immune cells are neutrophils, natural killer (NK) cells, macrophages, and dendritic cells (DCs). One of the mechanisms of defense provided by the innate immune system is phagocytosis. This process entails the engulfing of foreign particles such as microorganisms, which are then broken down in intracellular vesicles containing degradative enzymes and antimicrobial substances (2). Although neutrophils, macrophages and DCs are all phagocytic and capable of degrading foreign particles to varying extents, DCs are also specialized in another function. Upon phagocytosis or antigen uptake, they mature and through the presentation of antigens are capable of activating naïve T cells in the adaptive immune system. Due to this ability, DCs and macrophages to a lesser degree, are also known as antigen-presenting cells (APCs) and also play a crucial role in initiating an adaptive immune response (3).

During an infection, there are often inflammatory reactions due to recognition of specific patterns present on pathogens known as pathogen-associated molecular patterns (PAMPs) (4). Recognition of PAMPs, such as bacterial cell wall components, is mediated through receptors called pattern recognition receptors (PRRs). PRRs include toll-like receptors (TLRs) which are expressed by cells of the immune system, including but not restricted to, DCs and macrophages. PAMP recognition can induce phagocytosis

resulting in degradation of engulfed antigens, as well as the secretion of proteins known as cytokines and chemokines (5, 6). Cytokines affect the behavior of cells carrying the correct receptors, while chemokines can act to recruit immune cells such as neutrophils to the site of infection or inflammation. The recruitment of innate cells during an inflammatory response is also crucial for the activation of the adaptive immune system (7).

The innate immune system has an additional mechanism to trigger inflammation and phagocytosis at the site of infection. A group of plasma proteins known as complement can become activated by bacterial surface antigens as well as by autoantigens. Complement activation can lead to a cascade of reactions that ultimately coats the pathogen with complement fragments. This coating in turn targets pathogens for phagocytosis and degradation by cells that carry complement receptors such as macrophages (8). Furthermore, the complement components can assemble to form the membrane-attack complex, a pore in the lipid bilayer membrane which is thought to destroy pathogens by disrupting membrane integrity (9).

The adaptive immune system is capable of mounting a response against almost all pathogens that one might encounter in a lifetime. This is made possible through a huge repertoire of specific antigen receptors on adaptive immune cells that can recognize millions of antigens (9). Upon recognition of a foreign antigen, an adaptive immune response can be mounted. Adaptive immune responses are mediated mainly by T cells and B cells, each with unique roles in defense and protection.

1.2 T CELLS IN ADAPTIVE IMMUNITY

T cells possess a wide variety of specificities, but this recognition is dependent on presentation of antigens by specific cells. Furthermore, there are two main categories of T cells, the CD8 and CD4 T cells. CD8 T cells can destroy pathogen-infected cells whereas CD4 T cells can help to activate other immune cells. T cells originate from the bone marrow, mature in the thymus, and following maturation enter the circulation to provide protection from foreign pathogens (10).

1.2.1 T cell activation

T cells require APCs to provide the two necessary signals for optimal activation. APCs include dendritic cells, macrophages, and B cells, which are specialized cells that present antigens to T cells via major histocompatibility complex (MHC) molecules. The two main categories of T cells include the CD4 and CD8 T cells which recognize antigen presented on MHC class II and MHC class I molecules, respectively (11). The specific recognition of peptide:MHC complexes by the T cell antigen receptor (TCR) provides the first signal for T cell activation (Figure 1). The second required signal for T cell activation is also provided by activated APCs. In addition to phagocytosis of a pathogen, PAMP recognition by APCs also causes the upregulation of costimulatory molecules. Costimulatory molecules such as CD80 and CD86 can then bind the CD28 receptor on T cells and provide the second signal for optimal T cell activation (12) (Figure 1). Without costimulation, T cells can receive an inactivating signal leading to anergy, a state of functional unresponsiveness (13).

CD28 is a glycoprotein that is constitutively expressed on approximately 80% of

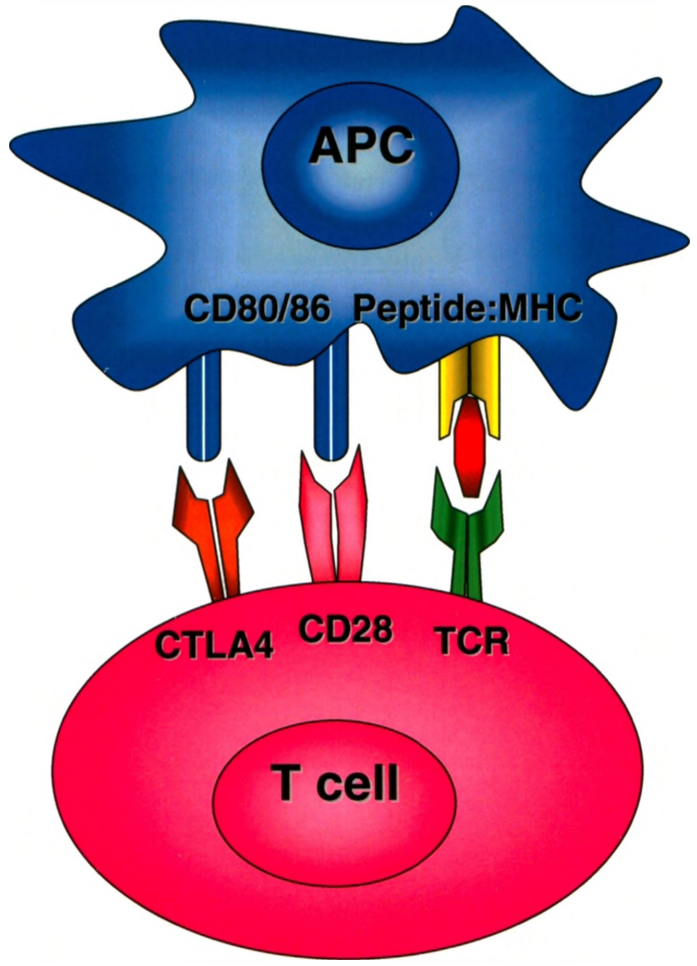


Figure 1. T cell activation and regulation. T cells require two signals for optimal activation. Binding of the foreign peptide:MHC complex (yellow and red) by the T cell antigen receptor (TCR) (green) provides the first required signal for activation. The second signal required for effective activation is the costimulatory signal which is delivered when CD28 (pink) on the T cell binds CD80/86 (blue) on the antigen-presenting cell (APC). CTLA4 (orange) on activated T cells binds with greater affinity than CD28 to CD80/86 on APCs and provides an inhibitory signal to T cells, thus regulating the immune response. This schematic is adapted from a previous representation (9).

human T cells and close to 100% of murine T cells (14, 15). It normally exists as a homodimer that is upregulated upon activation (16) (Figure 1). Similarly, cytotoxic T lymphocyte antigen 4 (CTLA4) is a transmembrane homodimeric glycoprotein and like CD28 binds to CD80/CD86, but with approximately 20 fold greater affinity (17) (Figure 1). CTLA4 delivers an inhibitory signal to T cells and thus along with its greater affinity for both CD80 and CD86 molecules, it normally functions to limit T cell responses following activation (17, 18). In contrast to CD28, CTLA4 is normally only expressed following the activation of T cells and expression on the surface of T cells peaks at 24-48 hours following activation (19). Interestingly, CTLA4 has also been found to be constitutively expressed on CD4+CD25+ T regulatory (Treg) cells (20) with evidence suggesting that CTLA4 expression is crucial for Treg cell homeostasis as well as its ability to directly suppress other T cells (21, 22). Furthermore, CTLA4 expression on Treg cells has been shown to be able to stimulate DCs to secrete indolamine 2,3 di-oxygenase (IDO), which is an additional mechanism to downregulate and thus limit T cell-mediated immune responses (23).

The ligands for CD28 and CTLA4 are the CD80 and CD86 molecules which are mainly found on APCs including B cells, macrophages, and DCs (24, 25) (Figure 1). CD80 and CD86 differ in their kinetics of expression. CD86 is expressed constitutively at low levels and is rapidly upregulated upon APC activation (within the first six hours after activation), whereas CD80 is only found to be expressed later following activation (after 48 hours) (24). This difference in temporal expression suggests that CD86 may predominantly bind with CD28, while CD80 which is expressed later following T cell activation would primarily interact with CTLA4 (24). Interestingly, beyond expression on

APCs, CD80 and CD86 can also be found on effector T cells where they were recently shown to be required for T cell susceptibility to suppression by Treg cells (21, 26).

Upon activation, T cells undergo clonal expansion whereby the antigen-specific T cell clone proliferates and differentiates into an effector cell. Due to clonal expansion and differentiation, there are a large number of effector cells, however they have a limited lifespan and the majority soon undergo apoptosis. A small proportion however becomes memory cells that persist and carry the same specificity, thus providing a faster and more effective response to subsequent antigen exposure (9).

1.2.2 T cell subsets

Activation of T cells via the recognition of a peptide antigen presented in the context of a MHC molecule along with costimulation leads to differentiation into effector cells. These effector T cells in turn are less dependent on costimulation (27). CD8 T cells, as mentioned previously, recognize antigen presented in the context of MHC class I molecules. When activated, they differentiate into cytotoxic effector T cells that can recognize and kill infected cells, cancer cells, as well as cells that are either damaged or dysfunctional (9).

CD4 T cells, in contrast, can differentiate into multiple different effector T cells. Activated CD4 T cells can differentiate into T helper (Th) 1 cells, Th2 cells, Th17 cells as well as Treg cells. Th1 cells are especially important for activating macrophages that in turn can destroy extracellular pathogens as well as those that persist in macrophage vesicles (28). Though Th1 cells can also activate B cells, Th2 cells primarily function to stimulate B cells to produce specific neutralizing antibodies that are crucial for pathogen

clearance (29). The Th17 cell subset was most recently described and is important for defense against extracellular pathogens by stimulating and recruiting neutrophils to sites of infection during the early adaptive immune response (30, 31). The final CD4 subset is the Treg cell, which in contrast to the previous three CD4 T cell subsets, functions to suppress T cell responses instead of helping to activate them. CD4 Treg cells are thus mainly involved in limiting immune responses and preventing autoimmune disease (9).

These effector subsets can be distinguished from one another through the secreted cytokine profile of the activated T cells. Briefly, Th1 cells produce interferon (IFN)γ and lymphotoxin (TNF-β), which help to activate macrophages, while Th2 cells characteristically produce interleukin (IL)-4, IL-5, IL-9 and IL-13, which help to activate B cells as well as IL-10 which inhibits macrophages (32, 33). The Th17 cell subset characteristically secretes IL-17, IL-6, and tumor necrosis factor (TNF)α, all of which help to recruit neutrophils to sites of infection (30, 31). T reg cells produce transforming growth factor (TGF)β and IL-10 both of which function to inhibit T cell responses.

Another important subset of T cells that are involved in immune responses are the invariant natural killer T (*i*NKT) cells. *i*NKT cells characteristically co-express NK cell markers such as NK1.1 along with a semi-invariant T cell receptor that recognizes glycolipid antigens in the context of CD1d, an MHC class I-like molecule (34, 35). An important property of *i*NKT cells is their ability to rapidly produce large amounts of immunomodulatory Th1- and Th2-type cytokines such as IFNγ and IL-4, respectively (36). As a result, they have been implicated in the regulation of autoimmune diseases such as type 1 diabetes, multiple sclerosis, and RA (37-39).

In summary, different T cell subsets play important roles in our immune system. CD8 effector T cells play an important role in cell-mediated immunity to a variety of infected or dysfunctional cells. CD4 Th1 cells activate macrophages, while Th2 are essential for activating B cells, and Th17 cells are effective in recruiting neutrophils, all of which are crucial for pathogen clearance. Finally, T reg cells and *i*NKT cells can function to regulate immune responses and as such are important in preventing autoimmunity.

1.3 B CELLS IN ADAPTIVE IMMUNITY

B cells also possess a wide range of specificities for antigens through their B cell antigen receptor (BCR). Activation of B cells usually requires both antigen recognition and T cell help. B cell activation then leads to differentiation into plasma cells, which secrete large amounts of antibodies, the soluble form of the BCR. Antibodies function in three main ways, namely neutralization, opsonization, and complement activation. Neutralization occurs when antibodies bind to pathogen or toxin and thus prevent them from adhering or entering target cells (9). Opsonization occurs in the coating of a pathogen's surface with antibody, and facilitates phagocytosis by Fc receptors that recognize the Fc region of the antibody (9). Finally, coating of a pathogen by antibodies can also activate the complement system, which is part of the innate immune system (40).

The BCR recognizes and binds native proteins, glycoproteins, and polysaccharides as well as intact viruses and bacteria. Upon its recognition, an antigen can be internalized and degraded intracellularly and presented on MHC class II molecules (41). When an activated CD4 Th cell recognizes a presented peptide derived from the

same internalized antigen, this is known as linked recognition and leads to T cell help (42). T cell help is required for B cell activation and is mediated through both the secretion of IL-4 and the binding of CD40 ligand on activated T cells to CD40 on B cells (43).

There are five main classes of antibodies, IgM, IgG, IgA, IgD and IgE. Each antibody class performs different effector functions ranging from the ability to activate the complement system, to properties allowing for the crossing of the placental barrier (44). All naïve B cells have surface expression of IgM and IgD. During the primary humoral immune response, IgM antibodies are the first to be produced. However, following the primary phase of an immune response, antibody production can switch to the other classes. Though IgM is predominant in primary responses, in subsequent secondary immune responses, IgG and IgA class antibodies are dominant (45). Class switching is thus a crucial process, and is also dependent on T cell help. Furthermore, IgG and IgA antibodies can be further divided into subclasses, IgG1-4 and IgA1-2 in humans, while in mice IgG antibodies are divided into IgG1-3. These subclasses also differ in their ability to elicit different functions such as complement activation and thus are associated with specific types of immune responses such as a Th1- vs. a Th2-type responses (46).

1.4 AUTOIMMUNITY

The immune system works effectively in providing specific defense against foreign pathogens while being tolerant to self tissues. This crucial distinction is achieved by multiple mechanisms and results in a state of self-tolerance. Autoimmunity arises

when a breakdown in the mechanisms of self tolerance occurs. This can result in our immune system mounting a response against self tissues or antigens which can then lead to autoimmune diseases.

1.4.1 Central tolerance

Self tolerance can be divided into central and peripheral tolerance. Central tolerance is achieved by the process of negative selection during the early stages of lymphocyte development in the thymus or bone marrow. Strong recognition of self antigens at this stage by immature lymphocytes leads to negative signals causing inactivation or apoptosis, a form of programmed cell death, thus achieving a state of self tolerance (47, 48). Despite the process of negative selection, weakly self reactive lymphocytes may still escape selection and enter the periphery and thus carry the potential to be activated by self antigens (9).

1.4.2 Peripheral tolerance

Peripheral tolerance is important for tolerance to self antigens not expressed in the thymus or bone marrow. Anergy is a mechanism that results in the functional unresponsiveness to an antigen. As described previously, this can occur when costimulatory signals are not provided by APCs during presentation of antigen to antigen-specific T cells (13). Another way to distinguish self from non-self is by the amount of antigen exposure. Self tissue antigens are abundant and provide strong and constant signals in contrast to pathogen-associated antigens that are sudden and usually increase rapidly with an infection. Strong and constant signals from self antigens can induce

tolerance or tolerize lymphocytes (49). Treg cells can also maintain peripheral tolerance by suppressing self-reactive lymphocytes of a variety of specificities so long as they are in the same tissue site. Finally, the natural self-limiting nature of immune responses can also regulate the potential for an autoimmune response through apoptosis of reactive lymphocytes, which normally occurs following an immune response (49).

1.4.3 Break in immunological tolerance

Autoimmunity arises when failures in mechanisms of self tolerance occur and thus lead to sustained activation against self antigens and the subsequent destruction of endogenous tissues. In the process of central tolerance, lymphocytes with low affinity for self antigens may escape negative selection and are termed ignorant. These ignorant cells however can later become self-reactive if the stimulus is strong enough. For example, during an infection, a naive T cell with low affinity for a self antigen can become activated by a DC that is presenting self antigen while simultaneously expressing high levels costimulatory signals due to the presence of an infection (50).

Another mechanism that can lead to an ignorant lymphocyte becoming activated and autoaggressive is when a lymphocyte is specific for an autoantigen that is also a ligand for a TLR. TLRs normally recognize PAMPs, but some of these patterns are not exclusive to pathogens including unmethylated CpG molecules in DNA. CpG is normally enriched in bacteria and DNA viruses, whereas mammalian cells only have enriched CpG during the process of apoptosis. In a situation where there is a large amount of cell death or if clearance of apoptotic fragments is ineffective, CpG fragments could be taken up, processed and bound by its respective TLR (TLR9) inducing expression of costimulatory

molecules on APCs. This then could lead to activation of self reactive T and B cells (9). A possible outcome due to such failures to negatively select or control self reactive T and B cells is the development of autoimmune diseases.

1.5 RHEUMATOID ARTHRITIS (RA)

Rheumatoid arthritis (RA) is a debilitating systemic autoimmune disease characterized by chronic synovial inflammation and pannus formation. Synovial inflammation is characterized by hyperplasia of the synovial membrane as well as infiltration by inflammatory cells such as neutrophils, macrophages and T cells. Pannus formation occurs during synovial hyperplasia and mediates the invasion of the bone and cartilage which can lead to damage in the affected peripheral joints (Figure 2).

RA is a prevalent chronic disease that affects 1% of the population and is three times more prevalent in women than men (51). It is an important cause of morbidity, disability and health care utilization costs in Canada and the world. In addition to its direct outcomes, RA is associated with a number of serious comorbidities that can lead to premature mortality. The development of RA is linked to an increased risk of cardiovascular disease as well as osteoporosis among other associated conditions which can lead to a reduced quality of life (52, 53). In Canada alone, the expenditure on arthritis and rheumatism is estimated to be at 6 billion Canadian Dollars annually (54).

1.5.1 Etiology of RA: Environmental factors

Though the precise cause of RA has not been established, current evidence clearly

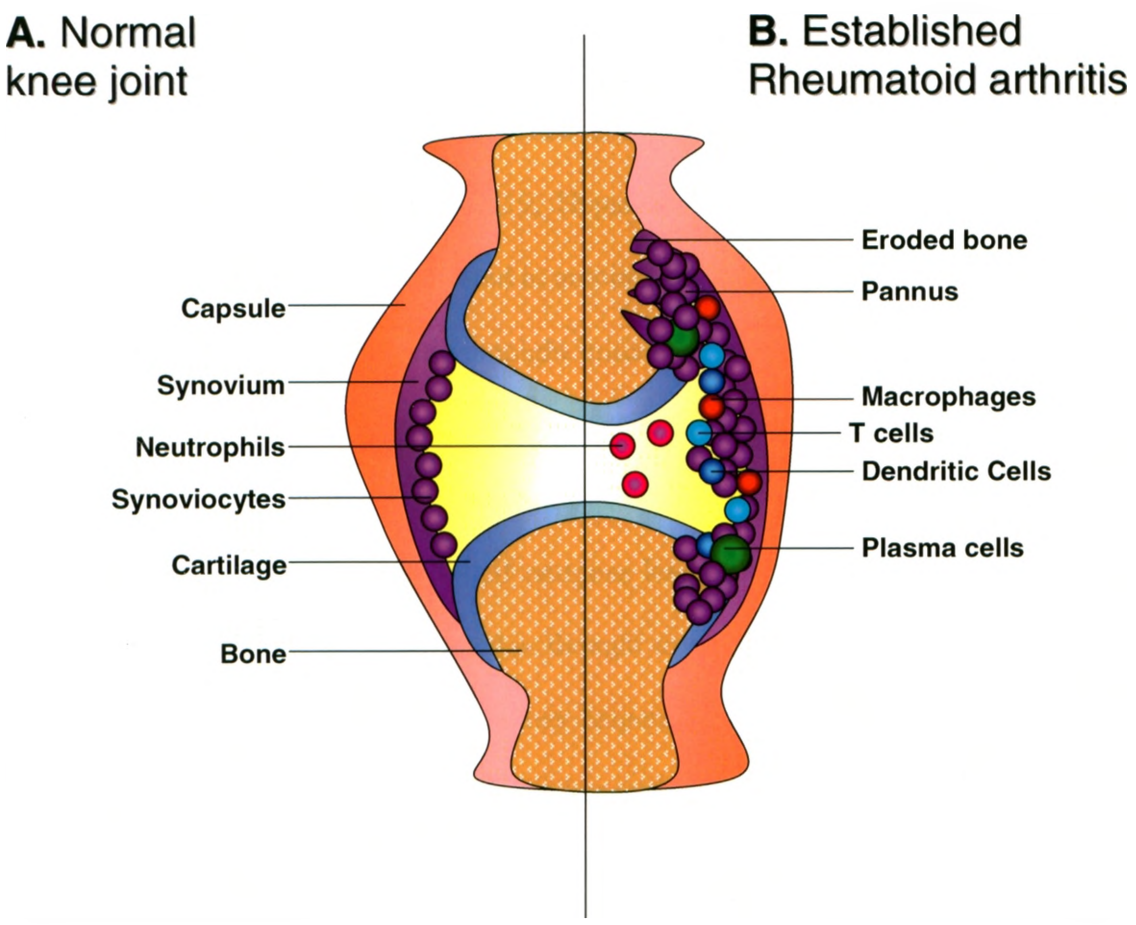


Figure 2. Representation of an arthritic vs. a normal joints. A) A normal joint with neither synovial hyperplasia, nor cartilage and bone erosions. B) An arthritic joint is characterized by chronic inflammation in the synovial tissue (purple) and this also involves the infiltration of inflammatory cells. Pannus formation occurs when the synovial tissue proliferates, and this in turn mediates the invasion and erosion of the bone (beige) and cartilage (blue). This schematic is adapted from a previous representation (55).

suggests a complex genetic component, along with a largely unknown contribution by environmental factors (56, 57).

A number of environmental factors have been associated with the RA risk including a number of different viral infections as well as smoking (58). Infections with pathogens such as Human Parvovirus B19, Hepatitis B virus, Hepatitis C virus, and Epstein-Barr virus have all been implicated in autoimmune RA (59-62).

It is thought that these infections might initiate an autoimmune response through mechanisms such as molecular mimicry and epitope spreading. Molecular mimicry is thought to occur when an immune response that is mounted against a pathogen also recognizes and thus cross-reacts with self antigens leading to a break in self tolerance (63). Epitope spreading on the other hand is thought to play a role in cases of persistent infection which can lead to an inflammatory response causing tissue destruction and release of self antigens. Autoreactive cells can recognize these released self antigens and receive the required costimulatory signals from APCs activated due to the persistent infection (64, 65). The activation of autoreactive cells thus results in the spreading of the immune response to target new autoantigen epitopes. However, despite a wide range of associations, there has not been a consistent infectious agent that explains the influence of the environment in RA.

Smoking has also long been implicated as an environmental risk factor for RA. However recently it was shown that the risk due to smoking correlated primarily with RA patients carrying an RA-specific autoantibody with certain HLA-DRβ1 MHC class II alleles (66). These associations suggest the notion that environmental factors may only be important for individuals with a given genetic predisposition.

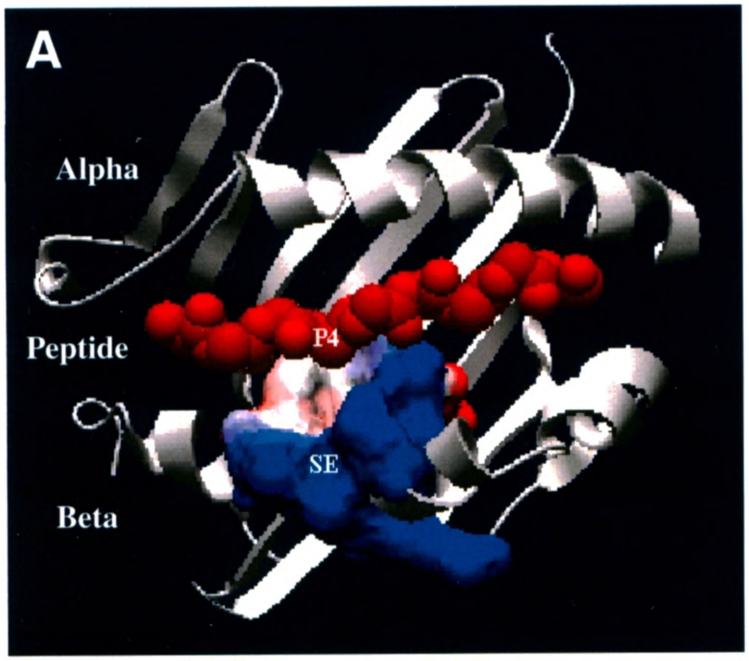
1.5.2 Etiology of RA: Genetic factors

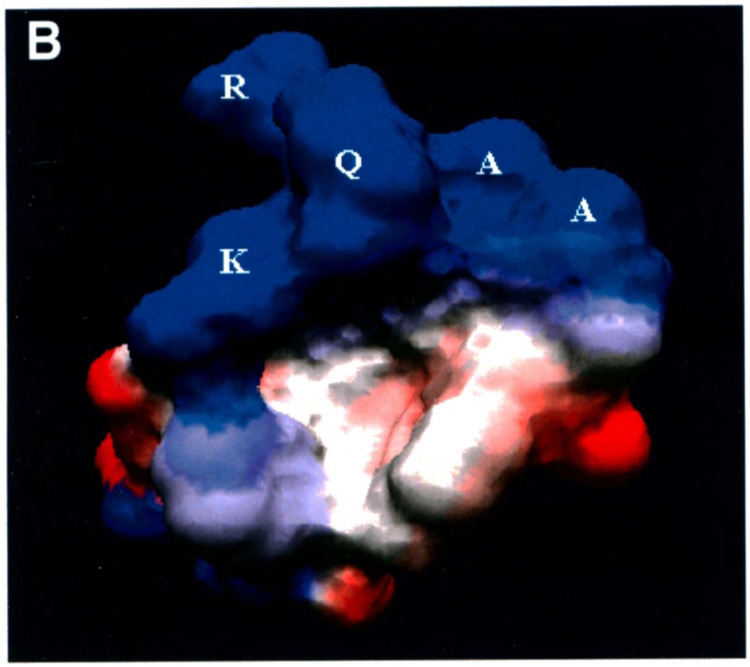
The genetic contribution to RA has been estimated at approximately 50% (67). Genes including PTPN22, TRAF1-C5 region, STAT4, and PADI4 are thought to be associated with RA. PTPN22 is an intracellular phosphatase that possesses the strongest association with RA among the non-MHC associated genes (57). Interestingly, PTPN22 is also associated with other autoimmune diseases including type 1 diabetes (68), Grave's disease (69), and Myasthenia Gravis (70) suggesting its general involvement in the autoimmune process rather than specifically to RA. Though there are a number of genetic associations with RA, MHC class II gene associations make up the single largest risk factor for RA (71-73).

DRβ1 is located on human chromosome 6. The original description of MHC class II gene associations with RA began with associations among a number of distinct HLA-DRβ1 alleles (74-76). This pattern of associations led to the idea of the shared epitope (SE) hypothesis and was based on the observation that there was a consensus amino acid motif (QK/RRAA) in the third hypervariable region of the DRβ1 molecules associated with RA (77) (Figure 3A). This motif makes up the P4 peptide binding pocket of MHC class II molecules and due to the positively charged K/R in the pocket it has a high affinity for negatively charged or uncharged polar residues (78) (Figure 3B).

Given the very strong associations of RA with various DRβ1 alleles, it seemed likely that these molecules were directly involved in pathogenesis at some level. The proposed shared epitope hypothesis predicted that RA-associated DRβ1 molecules would bind the same peptide(s) and thus facilitate the development of autoreactive T cells involved in the pathogenesis of RA. This hypothesis was most strongly supported with

Figure 3. Representation of the MHC class II peptide binding groove expressing the RA shared epitope (SE). A) the alpha and beta chains of the MHC class II molecule form the groove where antigenic peptides can bind. The SE is indicated in blue with the peptide in red. B) The P4 binding pocket that forms the amino acid consensus sequence of the SE (Q-glutamine, K-lysine, R-arginine, A-alanine, A-alanine) which carries a positive charge due to the K/R. This schematic is adapted from a previous representation (79).


A
Alpha
Peptide
P4
SE
Beta


B
R
Q
A
A
K

the recent finding that the HLA-DRβ1 associations were observed almost exclusively in RA patients who developed an autoimmune response to peptides containing citrulline, a post translationally modified arginine residue (80, 81). This provided support for the idea that the SE alleles were acting to directly influence a potentially pathogenic immune response.

1.5.3 Pathogenesis of RA

There is considerable evidence for the involvement of T cells in the development and progression of RA (82). T cells make up 30-50% of cells in the RA synovium, of which the majority are CD4 T cells (83). The strong association between MHC class II genes and RA also suggest the involvement of CD4 T cells in an immune response influenced by the peptides preferentially presented by SE-expressing RA patients. As well, the presence of activated CD4 T cells has been documented in the synovium during the early pathogenic stages of RA (84). Production of pro-inflammatory cytokines such as TNFα and IL-1 can be stimulated by a variety of cells including monocytes and macrophages (85). The level of TNFα is increased in the sera and the synovial fluid of RA patients (86, 87). Within the joint, TNFα, along with RANKL on activated T cells, can promote the resorption of bone and cartilage inducing osteoclast differentiation as well as inhibit the synthesis of collagen and proteoglycans (88-90). It is believed that the combination of these processes could contribute to maintaining an inflammatory response, which leads to the characteristic cartilage and bone destruction found in RA. It has however been difficult to study the role of T cells in RA, in large part due to their hypoproliferative state (91).

RA was initially classified as an autoimmune disease in part due to the presence of rheumatoid factor (RF), autoreactive antibodies that bind to immunoglobulin G (IgG) molecules (92), and to its strong association with MHC class II molecules (77). Autoantibodies have also been suggested to be involved in mediating RA. One of the most studied autoantibodies is rheumatoid factor (RF), which is used as a diagnostic marker for RA by the American College of Rheumatology (93). Furthermore, the presence of IgM and IgA RF has been associated with increased risk of developing RA (94, 95). However because RF is also found in a number of other autoimmune diseases, including Systemic Lupus Erythematosus (96), Sjögren's syndrome (97), and in healthy individuals as well, its relevance is controversial.

1.5.4 The immune response to citrulline

The interest in citrulline (Cit) began with the discovery that previously characterized autoantibodies in RA that targeted perinuclear factor, keratin, and fillagrin all had a common specificity for Cit (98, 99). Shortly afterwards, it was found that autoantibodies recognizing Cit were highly specific to RA (100). This observation sparked a number of other observations that led to the proposed involvement of the immune response to Cit in the pathogenesis of RA. First, as our lab has shown this response is strongly linked to and restricted by MHC class II genes (HLA-DRβ1) expressing the SE (78), the most common risk factor for RA (78, 80). Second, the presence of anti-Cit antibodies (ACA) is highly specific for RA, can precede clinical onset of RA by several years, and predicts a significantly more severe disease course

(101-105). Third, citrullinated proteins, such as citrullinated human fibrinogen (CithFib) and citrullinated vimentin are present in RA joints (106, 107) and are also targeted in this highly disease specific immune response (108, 109).

Cit results from the post-translational modification of peptide-bound arginine through the process of deimination (Figure 4) (110). This process is performed through the calcium dependent intracellular enzyme, peptidyl arginine deiminase (PAD) and results in the reduction of a positively charged arginine side chain to the polar uncharged side chain of a Cit residue (110). The significance of this modification was demonstrated when it was shown that the expression of the SE favoured the presentation of Cit peptides as compared to the argnine version of those peptides (78). This could in turn favour an anti-Cit immune response and provided an explanation for the association of RA to both the SE and to an anti-Cit immune response.

Though these studies provide important diagnostic and predictive usefulness, the underlying mechanism of RA remains unknown, in large part due to the limitations on human studies. Animal models allow for investigation into specific aspects of disease, that might involve potential risk or harm that would not be considered ethical in humans, such as the testing of novel therapies. Furthermore, the genetic and environmental diversity of any given human subject poses additional obstacles in understanding and/or interpreting the role of a specific area of interest such as the mechanistic contribution of a disease associated gene. As a result, animal models, though not perfect, can be useful tools for investigating the pathogenic mechanisms of underlying RA as well as for developing and evaluating new RA therapies.

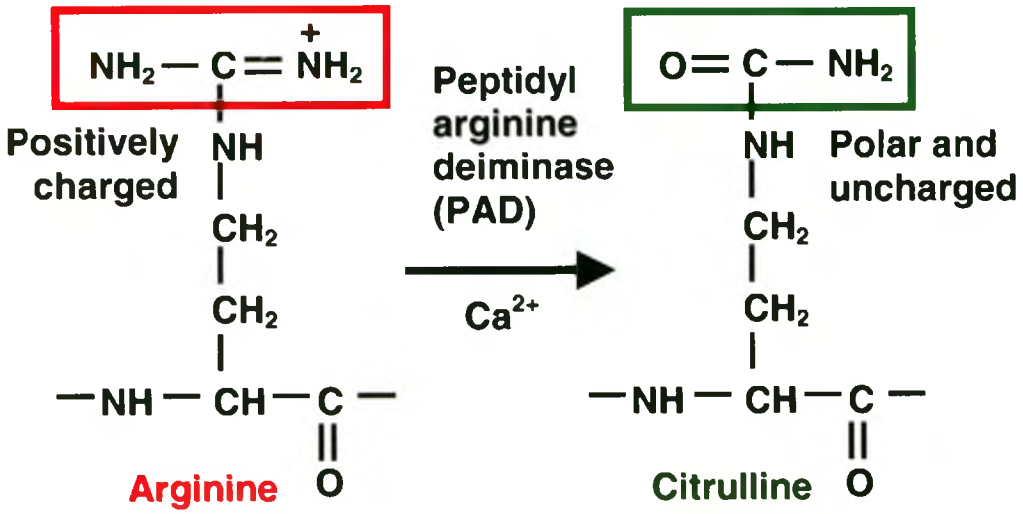


Figure 4. Citrulline results from the post-translational modification of arginine residues. The conversion of peptide bound arginine to citrulline is mediated by the calcium dependent enzyme peptidylarginine deiminase. Deimination results in the loss of a positive charge on the side chain of the arginine residue. This schematic was adapted from a previous representation (111).

1.5.5 Animal models of RA

There are a number of animal models for RA, including the KBxN arthritis model and the collagen induced arthritis (CIA) (112, 113).

The CIA model is commonly used as it shares some similarities to human RA such as persistent joint inflammation and inflammatory cell infiltration (112). However there are also important differences including the increased incidence and severity of the disease in males, which is in contrast with the human disease, where females are more frequently affected. Arthritis in this model is induced in susceptible DBA/1 and B10.RIII mice by immunization with heterologous type two collagen (CII) emulsified in complete Freud's adjuvant (CFA) (112). Importantly in this model, it was shown that autoantibodies specific to CII, a molecule found in articular cartilage, could cause joint inflammation on its own (114, 115). Interestingly, the use of antibodies to eliminate T cells prevented disease, suggesting a vital role for T cells (116). Furthermore, due to the inflammatory infiltration and accompanying bone and cartilage degeneration, CIA has also been useful in assessing treatments such as anti-TNF or anti-IL-1 therapies (117). However the relevance of CIA is questionable as a study of T cells in humans found that 12% of RA patients and 35% of controls had reactivity to CII (118).

Another model, though less commonly used, is the K/BxN model of arthritis. Arthritis in this model develops spontaneously and glucose-6-phosphate isomerase (G6PI), a ubiquitously expressed enzyme is targeted by autoantibodies (113). Similar to CIA, in this model, autoantibodies via serum could transfer a chronic erosive arthritis and could do so into a number of different backgrounds of mice (119). This suggests that the role of T cells in this model appear to be limited to providing help for enhanced

autoantibody production. Studies with these mice have provided valuable insight into how autoantibodies to an abundantly expressed antigen, could cause arthritis specifically in the joint. This was shown to be due to the formation of immune complexes on the surface of cartilage where G6PI is expressed (120). It is thought that these complexes in turn induce inflammation by activation of the complement system (121). However, similar to CIA, the prevalence of anti-G6PI antibodies in RA varies among studies (122-124) and thus its involvement in RA has yet to be proven.

1.5.6 Cit-induced model of human RA

Our laboratory has previously developed a Cit-induced model of human RA with mice transgenic for the human SE (DR4 tg mice) (125). These mice have a C57BL/6 background, which is normally resistant to arthritis induction (112). The DR4 tg mice do not express any endogenous MHC class II molecules. Rather, they only express a chimeric MHC class II molecule that is composed of the human α1 and β1 chain segments, which make up the antigen binding domain recognized by the mouse TCR. The α2 and β2 chain segments on the other hand are of mouse origin and form the CD4 binding domain (126) (Figure 5). Therefore these DR4 tg mice present peptides in the same manner as individuals expressing the human MHC class II molecule carrying the SE.

Immunization of these mice with CithFib, an autoantigen found in the joints of human RA patients, induces a chronic arthritis and a T cell and B cell immune response to citrulline (125). Arthritis development in this model is typically observed in approximately 40% of mice and is restricted by the expression of the human SE and by

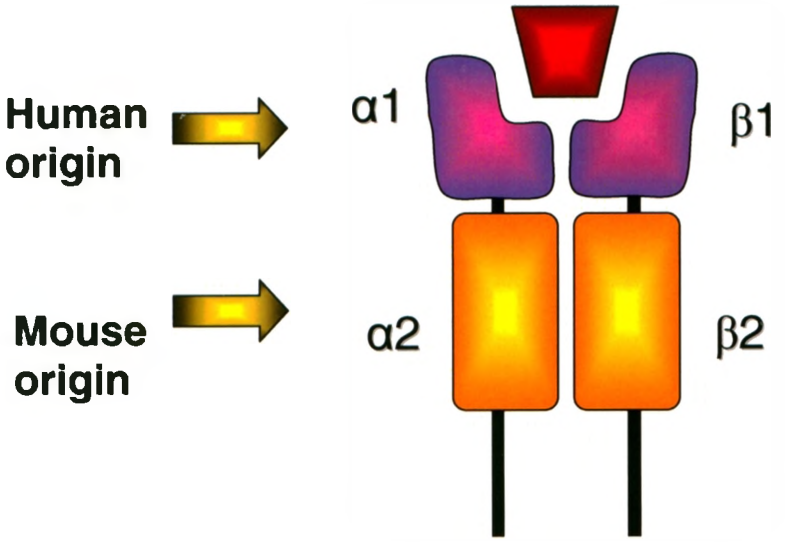


Figure 5. Schematic representation of the chimeric MHC class II molecule expressed in DR4 tg mice. The chimeric MHC class II molecule is composed of a peptide binding groove (alpha 1 and beta 1) that is of human origin (purple), while the remainder of the molecule (alpha 2 and beta 2) is of endogenous origin (yellow) and is responsible for interacting with murine CD4 molecules. This schematic was adapted from a previous representation (111).

citrullination of fibrinogen. This was demonstrated with the observations that wild type C57BL/6 mice did not develop arthritis and unmodified hFib was not able to induce arthritis (125). It was intriguing that despite immunized DR4 tg mice developing a vigorous T and B cell immune response to Cit, this response did not appear to distinguish between arthritic and non-arthritic mice. The T cell response also suggested a Th1 type response was initiated as shown by strong production of IFNγ with undetectable levels of IL-4 production (125). Adoptive transfer of Cit-activated T cells was performed to investigate the role of T cells in mediating disease in this model. It was found that these Cit-activated T cells transferred a transient arthritis and were predominantly CD4+ T cells (125). These findings further suggest the potential importance of the T cell immune response as a key mediator in Cit-induced arthritis.

1.5.7 Targeting T cell activation through costimulation blockade

Following the discovery of CD28 and its importance in costimulation, CTLA4, a molecule with many similarities to CD28 was identified (127). To characterize the effects of CTLA4, a soluble fusion protein was created consisting of the human extracellular domain of CTLA4 fused to the Fc portion of the human IgG1 molecule (17) (Figure 6A). Since mouse and human CTLA4 were highly conserved with an overall shared identity of 71% (128) this human CTLA4-immunoglobulin (CTLA4-Ig) fusion protein was successfully used in a number of murine models (129-133). CTLA4-Ig was found to dominate binding to CD80/86 molecules, thus effectively blocking the CD28-CD80/86 costimulatory interactions (17). It was quickly realized that despite its similarities to CD28, CTLA4 appeared to be a negative regulator of T cell activation (18, 134).

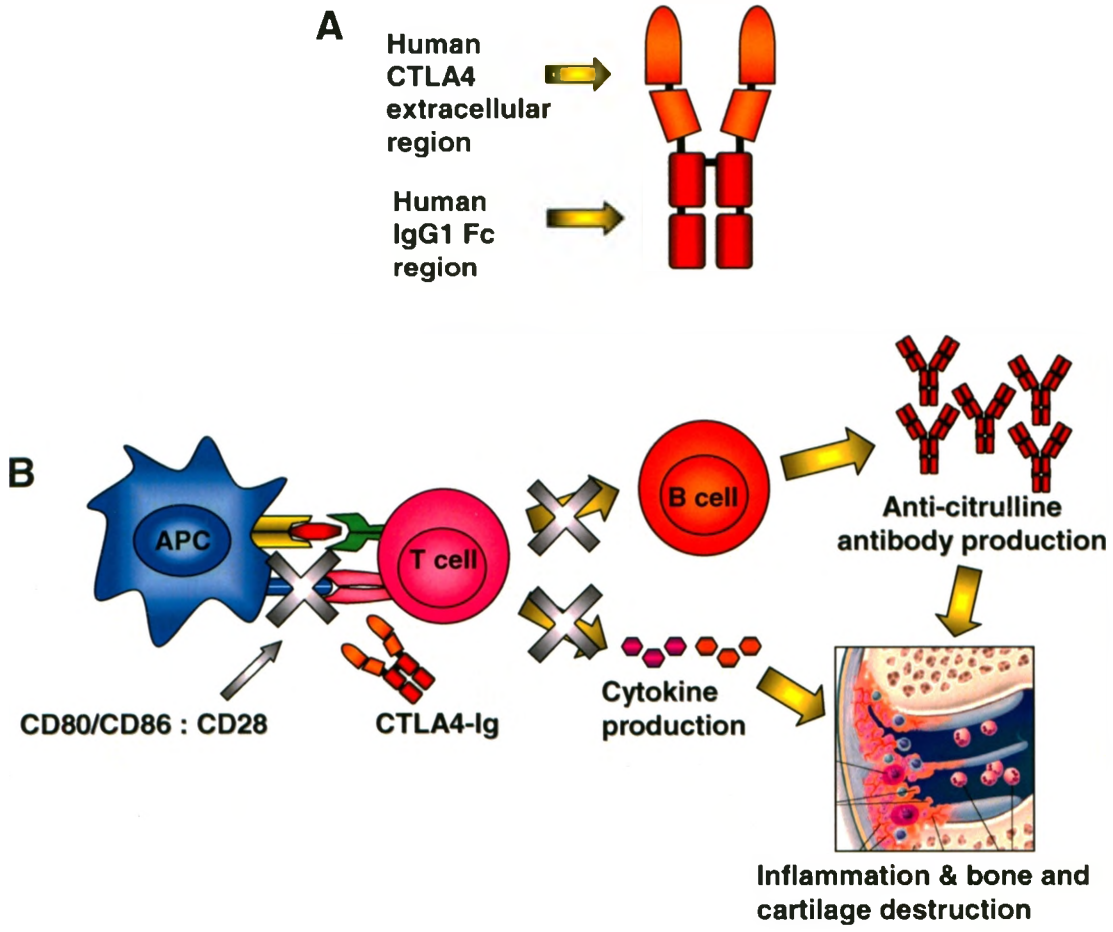


Figure 6. Targeting T cell activation by costimulation blockade. A) CTLA4-Ig is a fusion protein of the human CTLA4 extracellular domain and the human IgG1 Fc region. It binds with greater affinity than CD28 to CD80/86, thus blocking costimulation to T cells. B) T cell activation is a key initial step in the anti-citrulline immune response that leads to arthritis in DR4 tg mice. CTLA4-Ig blocks T cell activation and should therefore block the development and progression of citrulline-induced arthritis. This schematic is adapted from a previous representation (135, 136).

Early on in the studies of CTLA4-Ig as a suppressor of T cell activation, this reagent was found to induce tolerance experimentally to skin, cardiac tissue, as well as islets in transplantation models (137-139). Additionally, it was successfully used in the treatment of various experimental autoimmune diseases in mice, including experimental autoimmune encephalomyelitis (EAE) and lupus (130, 140). Furthermore, CTLA4-Ig was used to completely prevent CIA when administered concurrently with collagen immunization (141) and most recently, has been approved for treatment of human RA (142).

Through the many studies with CTLA4-Ig, a number of mechanisms of action have been suggested. CTLA4-Ig first of all blocks the engagement of CD28 by CD80/86 and thus can suppress the early stages of T cell activation (17). Furthermore, there is evidence that through reverse signaling, CTLA4-Ig can induce DCs expressing CD80/86 molecules to produce IDO, which breaks down tryptophan and inhibits T cell activation (23). Finally, there has also been evidence suggesting that CTLA4-Ig treatment can in certain cases modulate the immune response from a Th1- to a Th2-type response (130), and vice versa (129).

1.6 RATIONALE AND HYPOTHESIS

1.6.1 Rationale

Anti-Cit antibodies have been shown to predate as well as correlate with the severity of RA. However, the role of the overall immune response to Cit in mediating disease is still largely unknown. T cells have long been known to be involved in the progression of RA. However the role of the T cell immune response in the highly specific anti-Cit immune

response has not been investigated. The observations in our Cit-induced model of arthritis provide relevant comparisons for insight into human RA. An immune response to CithFib, a human autoantigen consistently targeted in human RA patients, along with a chronic erosive arthritis is induced in these DR4 tg mice. Furthermore, these mice express MHC class II molecules with the human SE, the largest genetic risk factor for human RA. The important pathogenic role of T cells in this immune response to Cit is supported by the adoptive transfer of arthritis with Cit-activated CD4 T cells. The use of CTLA4-Ig in our DR4 tg mouse model of human RA provides a unique opportunity to investigate the arthritogenic influence of the T cell immune response to Cit in the development and progression of Cit-induced arthritis.

1.6.2 Hypothesis

It is hypothesized that the activation of T cells by Cit is the initial step leading to an immune response to Cit which is arthritogenic in DR4 tg mice carrying the human SE. Blocking the activation of these T cells with CTLA4-Ig should prevent the progression of arthritis in this model by suppressing the immune response to Cit (Figure 6B).

1.6.3 Specific Goals

i. Determine whether *in vivo* CTLA4-Ig treatment can suppress the development and progression of Cit-induced arthritis in DR4 tg mice

ii. Determine whether *in vitro* CTLA4-Ig treatment can inhibit Cit-induced T cell activation

iii. Determine the effects of *in vivo* CTLA4-Ig treatment on the immune response to Cit in DR4 tg mice.

The first goal was achieved by treating arthritic DR4 tg mice with CTLA4-Ig at onset and peak of the disease. As well, mice were given preventative treatment with CTLA4-Ig before the development of arthritis (at the time of primary immunization and secondary boost).

The second goal was achieved by obtaining cells from CithFib immunized DR4 tg mice and stimulating them *in vitro* while also treating cells with either CTLA4-Ig or the control, human IgG1 (hIgG1).

The third goal was accomplished by assessing a number of measures. First, arthritis was monitored by measuring joint swelling during and after treatment. Following monitoring, joints from arthritic mice from different treatment groups were collected to assess histopathology. Adoptive transfers of splenic lymphocytes were also performed to assess the effect of treatment on the arthritogenic properties of the cells. Splenocytes and sera from treated and untreated arthritic mice were also obtained to assess T cell proliferative responses and for multiple antibody analyses, respectively.

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CHAPTER 2

CTLA4-Ig blocks the development and progression of citrulline-induced arthritis.*

*Note: This chapter is an extended version of the paper submitted for peer review. Modifications have been made to comply with formatting guidelines from the Faculty of Graduate and Postdoctoral Studies.

2.1 INTRODUCTION

Rheumatoid arthritis (RA) is a systemic autoimmune disease characterized by a chronic inflammatory response in the peripheral synovial joints that leads to cartilage damage and bone erosion. Though the cause of RA is not known, genetic factors are believed to be involved. The consensus amino acid motif in the P4 peptide binding pocket of MHC class II molecules known as the Shared Epitope (SE) (QK/RRAA) is expressed in a high proportion of RA patients and is a major risk factor for the disease (1, 2). Peptides containing citrulline, a modified arginine residue, bind with high affinity to MHC class II molecules expressing the SE thus favouring immune responses to citrullinated antigens (3).

The immune response to citrullinated antigens has been implicated in the pathogenesis of RA for a number of reasons. First, as we and others have previously shown, this response is strongly linked to and restricted by MHC class II genes (HLA-DRβ1) expressing the SE (3-6). Second, this immune response is highly specific for RA (7, 8), predicts a significantly more severe disease course (4, 9), and can precede the development of RA by several years (10-12). Third, several citrullinated proteins, including fibrinogen (13, 14), vimentin (15-17), and alpha enolase (18, 19) are present in RA joints and are also targeted in this immune response (20-23). Fourth, work in our laboratory demonstrates that CithFib induces arthritis in mice that are transgenic for the human DR4 expressing the SE (DR4 tg mice) (24). Thus, citrulline-induced arthritis provides a unique and useful mouse model of RA in which to investigate the importance of the immune response to citrullinated antigens in arthritis development and progression.

Immunization of these mice with CithFib induces T and B cell immune responses to an array of citrullinated antigens and arthritis in approximately one third of these mice (24). A puzzling observation, however, is that both arthritic and non-arthritic DR4 tg mice demonstrate vigorous T and B cell immune responses to citrullinated antigens following immunization.

Activation of CD4 T cells has been linked to the early pathogenic stages of RA (25). Upon infiltrating the synovium, these cells can release pro-inflammatory cytokines and aid in the activation of antigen-specific B cells (26). This can then maintain the inflammatory response leading to the cartilage and bone destruction characteristic of this disorder.

Costimulation plays an important role in the activation of CD4 T cells. The CD28-CD80/CD86 pathway is the classical costimulatory pathway, which can enhance and optimize T cell activation and cytokine production. CTLA4 is a receptor that, like CD28, can bind to CD80/CD86; however it is a negative regulatory receptor expressed upon T cell activation (27). Since CTLA4 binds to CD80/CD86 with greater affinity and avidity than CD28 (28), it normally functions to limit T cell responses following activation (29). The soluble form of this receptor, CTLA4-Ig, has been used to inhibit T cell activation by competing with CD28 for binding to CD80/CD86 (29). CTLA4-Ig is a fusion protein that consists of the extracellular domain of human CTLA4 linked to the Fc region of human IgG1 (28). It has been used experimentally to suppress graft rejection (30) and murine autoimmune diseases including experimental autoimmune encephalomyelitis (EAE) and lupus (31, 32). CTLA4-Ig was also shown to prevent

collagen-induced arthritis (CIA) when administered concurrently with collagen immunization (33) and has been approved for treatment of human RA (34).

We reasoned that administration of CTLA4-Ig to block citrulline-induced T cell activation should prevent arthritis in the DR4 tg mice if T cell activation is critical for arthritis development in this model. We thus investigated the effects of CTLA4-Ig on the clinical and pathological signs of citrulline-induced arthritis, on the ability of lymphocytes to transfer disease, as well as on lymphocyte activation.

Here we show that CTLA4-Ig can block citrulline-induced T cell responses and can both prevent and halt the progression of citrulline-induced arthritis in DR4 tg mice. This provides evidence that CD80/CD86-mediated costimulation of citrulline-specific T cells is required for the arthritogenicity of these cells in this model of human RA, and is an important target for the development of new therapeutics (35).

2.2 MATERIALS AND METHODS

2.2.1 Animals. HLA-DRβ1*0401 transgenic mice (henceforth referred to as DR4 tg mice) were bred at The John P. Robarts Research Institute's animal facility (London, ON, Canada). These tg mice express a chimeric MHC class II molecule composed of human antigen-binding domains (α1 and β1), while the α2 and β2 regions of the molecule are of murine origin (36). Both male and female mice were used for studies (6-8 weeks old). Mice were kept in an exclusion barrier facility at the Animal Care and Veterinary Services, University of Western Ontario, London, ON, Canada. All work with these mice

was performed according to the guidelines established by the Canadian Council on Animal Care (Appendix 1).

2.2.2 Antigens. The antigens, human fibrinogen (hFib) and mouse fibrinogen (mFib) were purchased from Calbiochem (San Diego, CA, USA), and Innovative Research (Southfield, Michigan, US), respectively. The citrullinated versions of hFib (CithFib) and mFib (CitmFib) were generated using peptidyl arginine deiminase (Sigma-Aldrich, Canada) as previously described (20). Deimination was confirmed by mobility shift on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by Western blot using the anti-modified citrulline kit (AMC) (Upstate, California) (Figure 7).

2.2.3 Immunizations and treatments. DR4 tg mice were injected subcutaneously (sc) into two sites in the posterior abdomen with a total of 100 μg of antigen (CithFib or hFib) emulsified with Complete Freund's Adjuvant (CFA) (Sigma) in 1v:1v ratio as previously described (24). CFA consisted of 4 mg/ml heat-killed *H37RA Mycobacterium tuberculosis* (Difco Laboratories, Detroit, MI, USA) suspended in Incomplete Freund's Adjuvant (IFA). On day 21 post-injection, mice received sc injections again at two sites with a total of 100 μg of CithFib emulsified with 1v:1v ratio IFA. In preventive treatment experiments, a total of seven doses of 100 μg CTLA4-Ig (Bristol Myers Squibb, New Jersey), given every other day, was administered with CithFib immunization at both the time of the primary immunization on day 0, and at the time of the secondary boost on day 21. The dose of CTLA4-Ig was determined based on literature with *in vivo* CTLA4-Ig use in mice (29, 33). In the remaining experiments, starting on day 0 (primary response), day 21 (secondary response), day 50 (the onset of Cit-induced arthritis) or day 70 (the peak of Cit-induced arthritis), mice were treated with a total of seven doses of

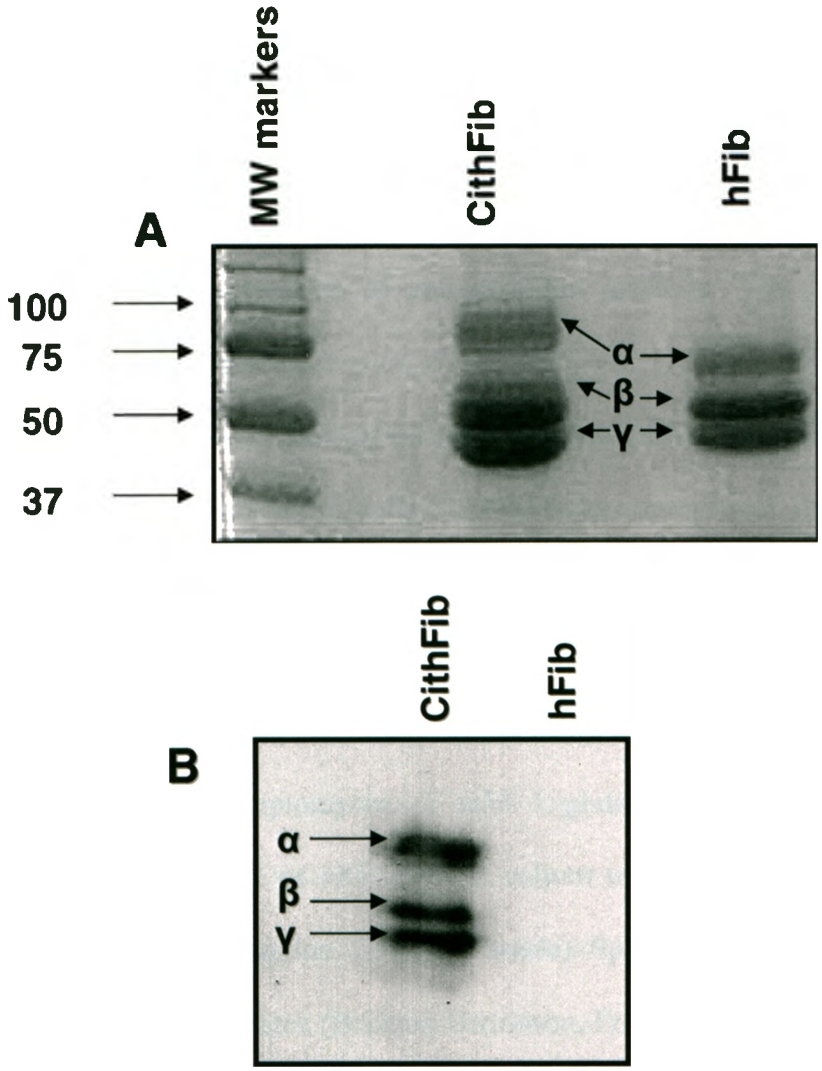


Figure 7. Confirmation of citrullination. Deimination was confirmed by A) mobility shift of α and β chains of hFib on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by B) western blot using the AMC kit showing citrullinated α, β and γ chains of CithFib.

either 100 μg of CTLA4-Ig or human IgG1 (hIgG1) control antibody (Sigma) every other day, or alternatively left untreated. After completion of treatment and following a 30-day monitoring period, mice were sacrificed (approximately on day 100). Splenocytes from different treatment groups of DR4 tg mice were harvested and used to test their T cell proliferative responses and for lymphocyte adoptive transfers. Sera from the sacrificed mice were collected for the detection of anti-citrulline antibody. The joints were harvested for pathological assessment.

2.2.4 T cell proliferation assay. The spleens were removed from DR4 tg mice on day 10 or 31 after the initial immunization with CithFib. Splenocytes were isolated after osmotic lysis of erythrocytes with 0.84 % NH_4Cl (Sigma). After several washes in phosphate buffered saline (PBS), and RPMI-1640, the splenocytes were re-suspended in RPMI-1640 culture medium supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 50 mM 2-β mercaptoethanol (complete RPMI or cRPMI). All culture medium supplements were purchased from Gibco BRL (Burlington, Ontario, Canada). Splenocytes were cultured in 96-well U-bottom tissue culture plates (Beckton Dickinson, Franklin Lakes, NJ, USA) at a concentration of 4×10^5 cells/well in 200 μl cRPMI (Gibco). The cells were cultured in the absence (background proliferation) or presence of various antigens at 50 μg/ml (antigen-specific proliferation) for 4 days at 37°C in a humidified incubator with 5% CO_2. In the *in vitro* treatment experiments, CTLA4-Ig or hIgG1 was added at 20 μg/ml, which was first determined to be optimal by a titration experiment of both CTLA4-Ig and hIgG1 control. Eighteen hours before the termination of the cultures, half the media of each well was supplemented with 5 μCi/ml of [^{3}H] thymidine (ICN Aurora, Ohio, USA).

At the end of the culture, the cells were harvested onto glass fibre filters (PerkinElmer, Woodbridge, Ontario, Canada). Radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer, Woodbridge, Ontario, Canada). All experiments were done in triplicate and data are expressed as average stimulation index (SI) which represents a ratio of decay counts per minute (cpm) of experimental samples to cpm of control samples.

2.2.5 IL-2 cytokine detection in supernatants. Supernatants were removed from cultures after 72 h for cytokine quantification by ELISA. Anti-mouse IL-2 (Pharmingen) was diluted 1:250 (2 μg/ml) in coating buffer (0.1M $NaHCO_3$) and added to a 96 well flat bottom ELISA plate (Nunc). Plates were incubated overnight at 4°C. The following day, wells were washed three times with washing buffer (WB) containing 0.1% BSA, 0.05% Tween 20 (BDH, Chicoutami, Quebec, Canada) in PBS. Non-specific binding was avoided by adding 200 μl of blocking solution (10% fetal calf serum in 1 x PBS) for 2 hours at room temperature. Excess blocking solution was removed by three washes with WB. One hundred μl of IL-2 standards (1000 pg/ml stepwise to 15.625 pg/ml) and 100 μl of experimental supernatants diluted 1:2 with diluent were added. Plates were incubated overnight at 4°C. Plates were then washed six times with WB. and was incubated for 2 hours at RT. One hundred μl of biotinylated anti-IL-2 antibody (Pharmingen) and alkaline phosphatase (AP)-extravidin (Pharmingen) diluted 1:1000 was added and incubated at room temperature for two hours. Detection antibody was removed by six washes with WB followed by two washes with distilled water. One hundred μl of Sigma FAST tablet (AP substrate) was added and incubated for 30 minutes in dark at room temperature. The plates were read at 405 nm using a Benchmark Microplate Reader

(BIO-RAD) and data were analyzed using Microplate Manager III software. The cytokine content of culture supernatants was measured in triplicate wells and expressed as mean ± the SEM (background cytokine production in unstimulated samples were subtracted from the cytokine production of antigen-stimulated samples).

2.2.6 Adoptive transfers. Splenic lymphocytes were isolated from donor spleens as described above and lymphocytes were purified using lympholyte-M (Cedarlane, Hornby, Ontario, Canada) following the manufacturer's instructions. Naïve recipient DR4 tg mice were injected intraperitoneally (i.p.) with 1×10^7 lymphocytes in 100 µl of PBS. The number of cells was chosen based on literature on adoptive transfer of arthritis in the spontaneous arthritis model in SKG mice and the proteoglycan aggrecan-induced arthritis model. (27, 28). In addition all recipient mice received an intraarticular (i.a.) injection of 5 µg of CithFib in 5 µl PBS into one knee. The contralateral control knee received 5 µl of PBS or 5 µg of hFib.

2.2.7 Clinical assessment of arthritis in recipient mice. All mice were observed for clinical signs of arthritis for 30 days after the final treatment with CTLA4-Ig or control hIgG1. Ankle swelling of recipient mice measured using calipers was determined three times per week.

2.2.8 Serum antibody detection. Serum antibodies were measured by ELISA. Wells of Immulon II microtitre plates (Nunc, Rochester, New York, USA) were coated overnight at 4°C with 100 µl of protein antigen (CithFib, hFib, CitmFib, mFib) at a concentration of 10 µg/ml in 0.1 M carbonate-bicarbonate buffer (0.15 M Na_2CO_3, 0.35 M $NaHCO_3$, 0.03 M NaN_3 pH 9.6) or carbonate coating buffer containing 0.1% BSA (Sigma) to monitor background reactivity. After two washes with wash buffer WB, the wells were blocked

with 200 µl of blocking solution (0.1% BSA in PBS) per well for 30 minutes at room temperature (RT). Excess blocking solution was removed by two washes with WB. The collected sera were diluted appropriately in diluent (0.1% BSA, 0.05% Tween 20 in PBS) and 100 µl were added to the wells. The plates were incubated for 2 hours at 37°C followed by four washes with WB. The secondary antibody (diluted 1:10000 in diluent) was biotin-conjugated goat anti-mouse IgG, Fc-specific (Sigma-Aldrich, Canada). This secondary antibody was pre-conjugated with streptavidin-horseradish peroxidase polymer - ultrasensitive (diluted 1:4000 in diluent) (SHRP) (Sigma-Aldrich, Canada) for 15 minutes. One hundred µl was added to each well and plates were incubated for 30 minutes at RT. After four washes to remove unbound secondary antibodies, the antibody binding was revealed by adding 100 µl/well of 3,3',5,5'-Tetramethylbenzidine (TMB) (Sigma-Aldrich, Canada) and incubating for 10 minutes at RT. The SHRP–TMB reaction was stopped after 10 minutes by adding 50 µl/well of 2M H_2SO_4 (Sigma). The plates were read at 450 nm using a Benchmark Microplate Reader (BIO-RAD Canada, Mississauga, Ontario, Canada) and data were analyzed using Microplate Manager III software. Known sera previously assayed to be antigen-reactive and antigen-non-reactive were included in each ELISA test to control for variability of the assay. The quantity of antibodies in serum samples was expressed as mean optical density (OD) at 450nm. The antigen specific IgG responses were calculated by subtracting background OD values from OD values for antigen-coated wells.

Anti-cyclic citrullinated peptide (CCP_2) antibodies were measured using an anti-CPP_2 ELISA kit (Euroimmune) following the manufacturer's instructions except that the

peroxidase-conjugated goat antibody was against mouse immunoglobulins (IgG, IgA, IgM) (Cappel, Ohio) as used by Kawane et al. (37).

2.2.9 Histological evaluation. Mice from each treatment group were sacrificed 30 days following the final administration of CTLA4-Ig or control hIgG1. Similarly, the recipients of adoptively transferred splenic lymphocytes were sacrificed 21 days following transfer. The hind paws were excised and fixed in 10% buffered formalin solution (VWR). Fixed tissues were de-gloved (skin removed) and then decalcified for 12 hours in RDO rapid decalcifier (Apex Engineering Products), followed by dehydration and paraffin embedding. Sagittal sections (3 μm) were stained with hematoxylin and eosin (H&E). H&E-stained sections were assessed for joint pathology in arthritic DR4 tg mice that were treated with either CTLA4-Ig or control hIgG1, or left untreated. Images of the prepared slides were captured using an Olympus Q color 3 camera attached to an Olympus BX61 microscope and collected using Image Pro-plus 5.0 software (MediaCybernetics, Bethesda, MD).

2.2.10 Statistical analysis. Differences between mean responses of treatment groups were determined by either the Student's *t*-Test or the Chi-squared Test (Microsoft Excel, Redmond, WA), with a value of $P < 0.05$ being considered significant.

2.3 RESULTS

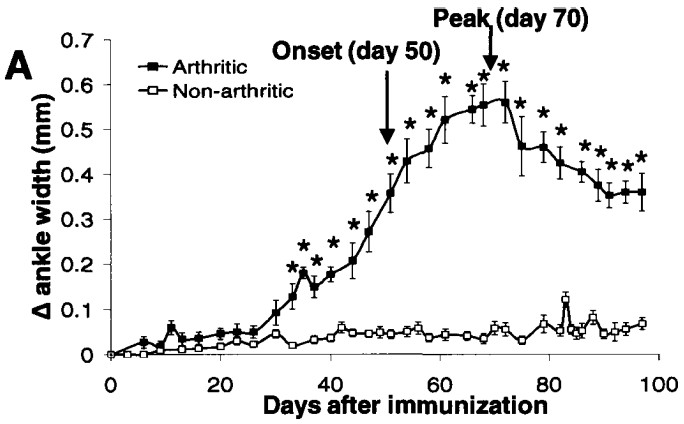
2.3.1 Effect of CTLA4-Ig treatment on the clinical and histopathologic progression of citrulline-induced arthritis. CithFib immunization of DR4 tg mice induced arthritis in 37% of mice, which was comparable to the frequency previously seen (24). The course

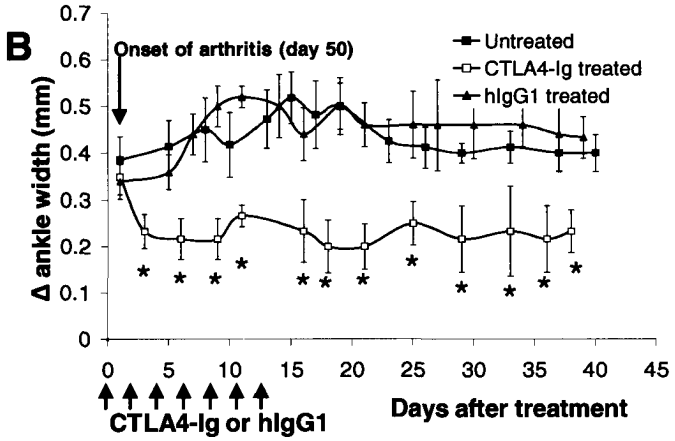
of arthritis in CithFib-immunized DR4 tg mice progressed in a predictable and consistent manner with a gradual increase in joint swelling that persisted (Figure 8A). The earliest point that the joints of arthritic and non-arthritic mice could confidently be distinguished by caliper measurements of joint swelling was day 50 (onset of arthritis). At this point arthritic mice displayed a progressive increase in joint swelling, which peaked around day 70 (Figure 8A).

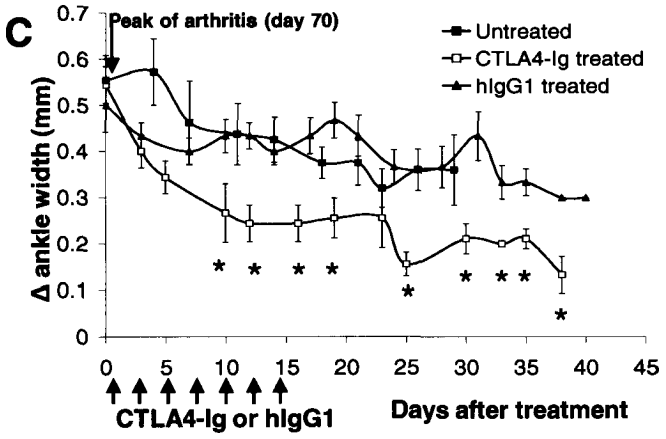
Arthritic DR4 tg mice at the onset (day 50) and peak (day 70) of disease were either treated with seven doses of CTLA4-Ig or with hIgG1 (100 μg/dose), or left untreated to determine whether CLTA4-Ig treatment had any effect on disease progression. Treatment with CTLA4-Ig at the onset of disease significantly reduced the clinical signs of arthritis (joint swelling) in arthritic mice compared to untreated or control hIgG1 treated mice (Figure 8B). Mice treated with CTLA4-Ig at the peak of established arthritis also demonstrated similar clinical improvement (Figure 8C). Separate animals received preventive treatment with seven doses of CTLA4-Ig or hIgG1 at both the time of primary immunization and at boosting with CithFib in order to assess if early suppression of T cell activation would decrease disease frequency. Preventive treatment blocked the development of citrulline-induced arthritis in all treated mice ($P < 0.03$) (Figure 8D). In contrast, hIgG1 treated and untreated mice developed arthritis at a frequency of 43% and 37%, respectively, as expected (24).

Following the monitoring period after arthritic mice received treatment, the ankle joints of mice were assessed for histopathological damage. The joints of mice treated with CTLA4-Ig at the onset of arthritis maintained a normal histological appearance (Figure 9B) compared to untreated and hIgG1 treated mice (Figure 9A and C). The

Figure 8. CTLA4-Ig treatment suppresses citrulline-induced arthritis in DR4 tg mice. **A,** Course of arthritis in non-treated mice. Joint swelling in arthritic (n=14) and non-arthritic (n=24) DR4 tg mice was determined by caliper measurements of the ankle width and was assessed 3 times a week. **B,** Course of arthritis when CTLA4-Ig is used to treat early disease. Arthritic mice were treated at the onset (day 50) of disease with 7 doses of 100 μg of CTLA4-Ig (n=6) or hIgG1 control (n=5) every other day, or were left untreated (n=14). **C,** Course of arthritis when CTLA4-Ig is used to treat established disease. Arthritic mice at the peak of disease (day 70) were also treated as described above in (B) with CTLA4-Ig (n=9), hIgG1 (n=3) or left untreated (n=14). **D,** Arthritis development when mice are left untreated (n=8) or treated with CTLA4-Ig (n=5), or hIgG1 (n=7) at the time of immunization (day 0) and boost (day 21). Bars show mean and SEM. * = $P <$ 0.05 arthritic vs. non-arthritic (A), hIgG1 or untreated controls vs. CTLA4-Ig treatment by Student's *t*-Test (B and C), or Chi-squared test (D).





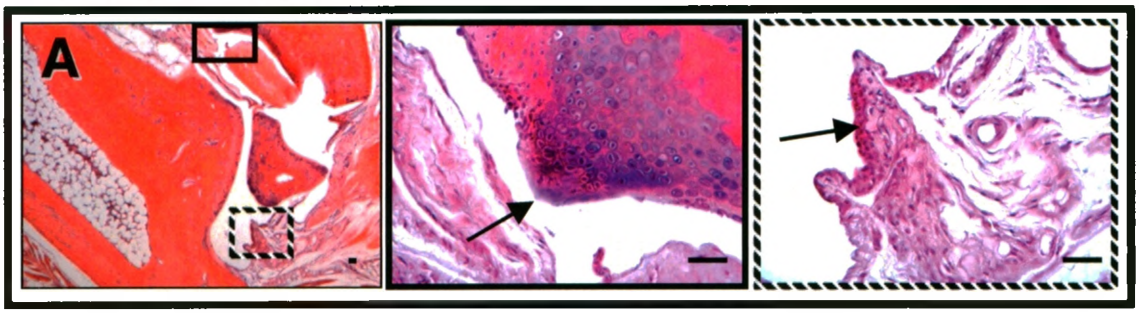


D

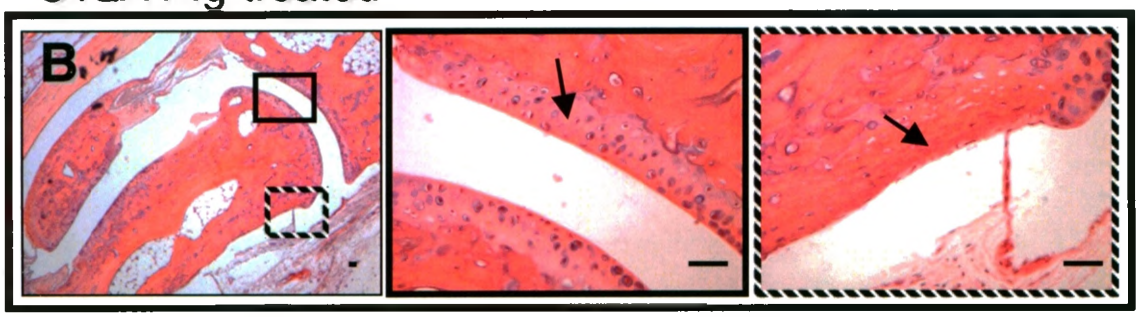
Preventive treatment regimen	Arthritis at Day 70	Chi-squared test
Untreated	3/8	*P*=0.906
CTLA4-Ig-treated	0/9	*P*<0.03
hIgG1-treated	3/7	*P*=0.684

Figure 9. CTLA4-Ig treatment at the onset of arthritis (day 50) ameliorates the pathologic joint destruction in citrulline-induced arthritic DR4 tg mice. Representative H&E stain of ankle joints of two mice from each group of arthritic mice treated with CTLA4-Ig, hIgG1 control, or left untreated (from Figure 8B). **A**, Untreated arthritic mice. Arrows in the magnified images indicate sites of reactive bone changes and synovial hyperplasia. **B**, hIgG1 control treated arthritic mice. Arrows in the magnified images show reactive bone changes and bone erosion. **C**, CTLA4-Ig treated arthritic mice. Magnified images show normal joint cartilage and bone appearance as indicated by the arrows. *Measurement bar=100μm.*

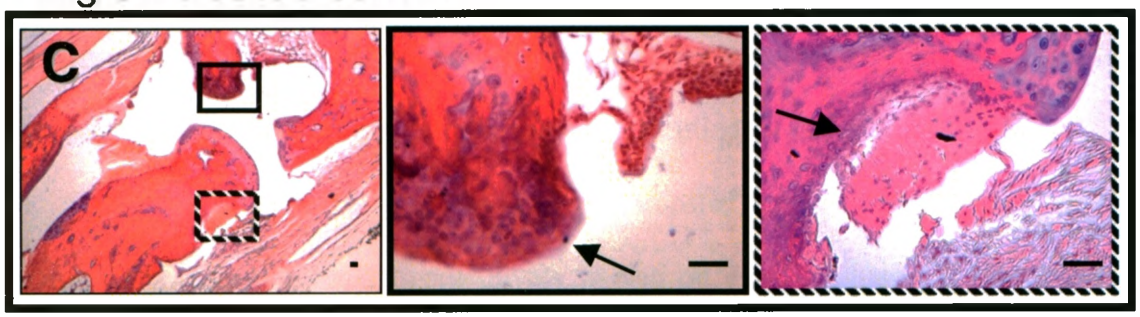
Untreated control



CTLA4-Ig-treated



hIgG1-treated control



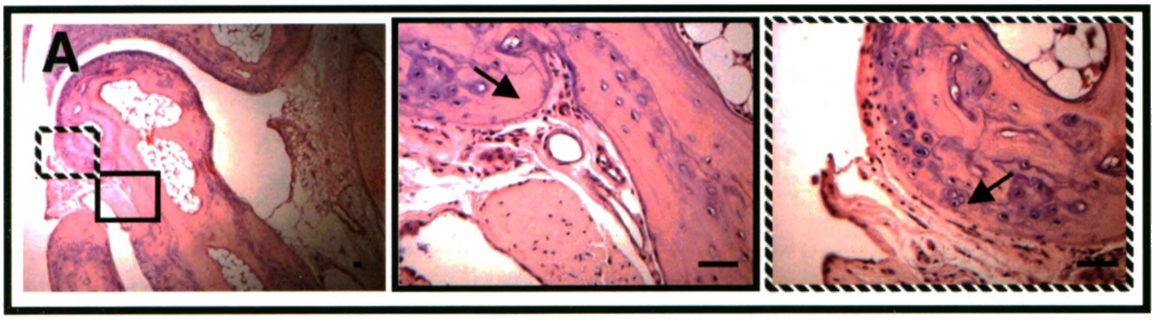
histological appearance of the joints from untreated and hIgG1 treated mice revealed reactive bone changes, synovial hyperplasia (Figure 9A), and bone erosions (Figure 9C) typical of what we have previously reported (24). CTLA4-Ig treatment at the peak of disease (day 70) similarly reduced joint damage compared to untreated and hIgG1 treated groups (Figure 10).

2.3.2 Effect of CTLA4-Ig treatment on the arthritogenicity of lymphocytes from DR4 tg mice. Splenic lymphocytes from arthritic mice at the onset of disease (day 50) or from non-arthritic mice at the same time point were transferred i.p. into naïve syngeneic DR4 tg mice. Recipient mice also received CithFib i.a. in one limb and, PBS or hFib control in the contralateral limb. The lymphocytes from arthritic mice induced joint swelling within two days following transfer in only the i.a. CithFib-injected limb of recipient mice (Figure 11A). In contrast, mice receiving splenic lymphocytes from non-arthritic mice showed no significant ankle swelling ($P < 0.05$) (Figure 11A). Furthermore, lymphocytes from arthritic donors, but not non-arthritic donors induced chronic arthritis, which resulted in reactive bone changes, synovial hyperplasia as well as ankylosis (Figure 12A and B). These experiments therefore demonstrated that the splenic lymphocytes from arthritic but not non-arthritic mice are capable of transferring arthritis to naïve syngeneic recipients also receiving an i.a. citrullinated target.

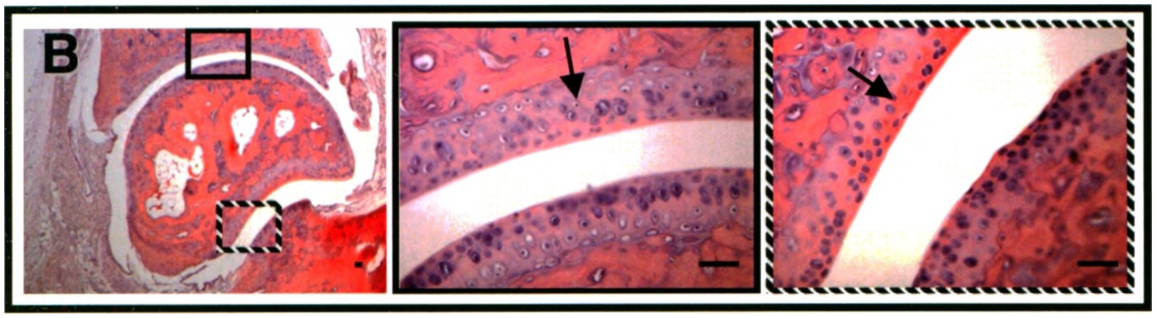
The effect of CTLA4-Ig on the ability to transfer arthritis was examined using splenic lymphocytes from mice preventively treated on day 0 (primary immunization) and day 21 (secondary boost), as well as from arthritic mice treated on either day 50 (disease onset) or day 70 (disease peak) or that were left untreated. Splenic lymphocytes from arthritic mice treated with CTLA4-Ig either at the onset or peak of disease as well as

Figure 10. CTLA4-Ig treatment at the peak (day 70) of established arthritis ameliorates the pathologic joint destruction in citrulline-induced arthritic DR4 tg mice. Representative H&E stain of ankle joints of two mice from each group of arthritic mice treated with CTLA4-Ig, hIgG1 control, or left untreated (from Figure 8C). **A,** Untreated arthritic mice. Arrows in the magnified images indicate sites of bone erosion and reactive bone changes. **B,** CTLA4-Ig treated arthritic mice. Magnified images show normal joint cartilage and bone appearance as indicated by the arrows. **C,** hIgG1 control treated arthritic mice. Arrows in the magnified images show synovial hyperplasia with reactive bone changes and bone erosion. Measurement bar=100μm.

Untreated control



CTLA4-Ig-treated



hIgG1-treated control

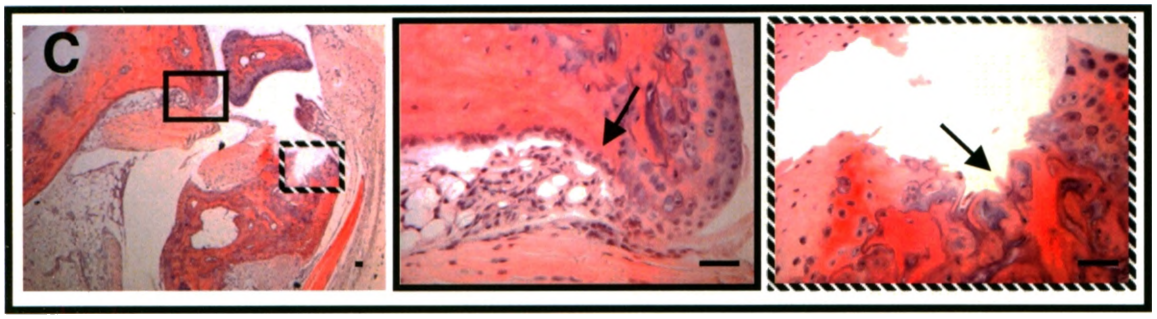
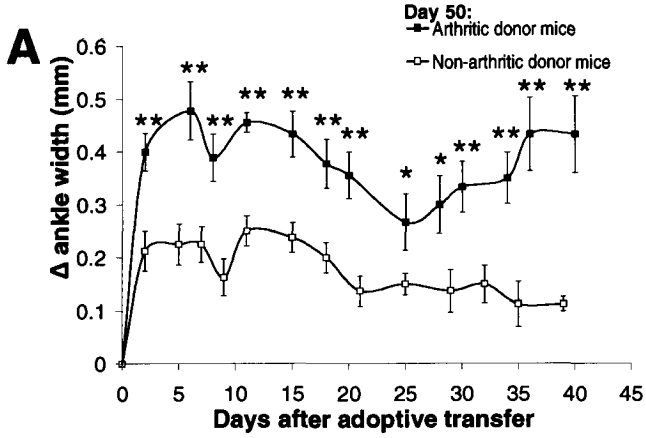
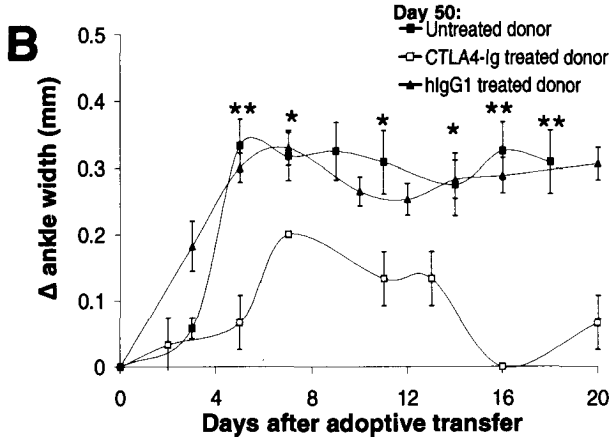
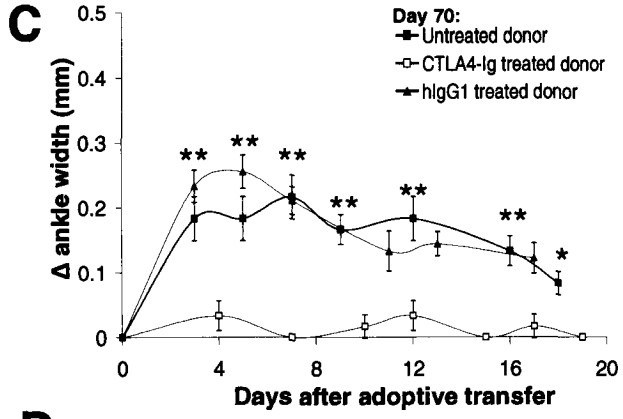


Figure 11. CTLA4-Ig blocks the adoptive transfer of arthritis from citrulline-induced arthritic DR4 tg mice to naïve syngeneic recipients. **A**, Transfer of splenic lymphocytes from arthritic mice (n=9) or non-arthritic mice (n=8) sacrificed at day 50. Arrows indicate ankylosis and synovial hyperplasia. **B**, Transfers of splenic lymphocytes from day 50 CTLA4-Ig treated (n=3), hIgG1 treated (n=12) mice or untreated (n=17) arthritic mice. **C**, Transfers of splenic lymphocytes from day 70 CTLA4-Ig treated (n=9), hIgG1 treated (n=6) mice or untreated (n=6) arthritic mice. **D**, Transfers of splenic lymphocytes from preventive (on day 0 and 21) CTLA4-Ig treated (n=10), hIgG1 treated (n=5) mice or untreated (n=3) mice. All recipients (in A, B, C and D) received ten million splenic lymphocytes with i.a. CithFib and their ankle swelling was monitored. Error bars show mean and SEM. * = $P < 0.05$, ** = $P < 0.01$ arthritic vs. non-arthritic and hIgG1 or untreated controls vs. CTLA4-Ig treated (by Student's *t*-Test).


A
Day 50:
Arthritic donor mice
Non-arthritic donor mice
Δ ankle width (mm)
Days after adoptive transfer


B
Day 50:
Untreated donor
CTLA4-Ig treated donor
hIgG1 treated donor
Δ ankle width (mm)
Days after adoptive transfer


C
Day 70:
Untreated donor
CTLA4-Ig treated donor
hIgG1 treated donor
Δ ankle width (mm)
Days after adoptive transfer

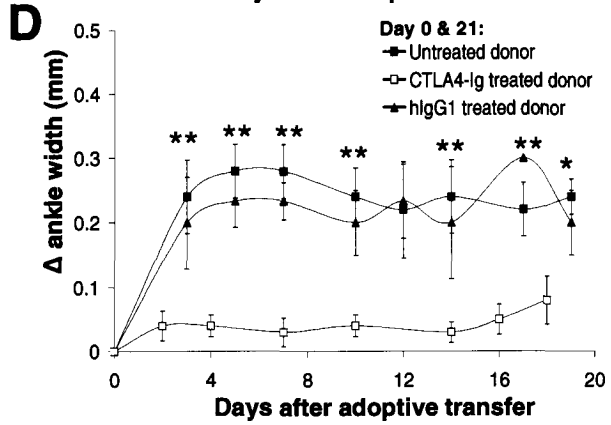
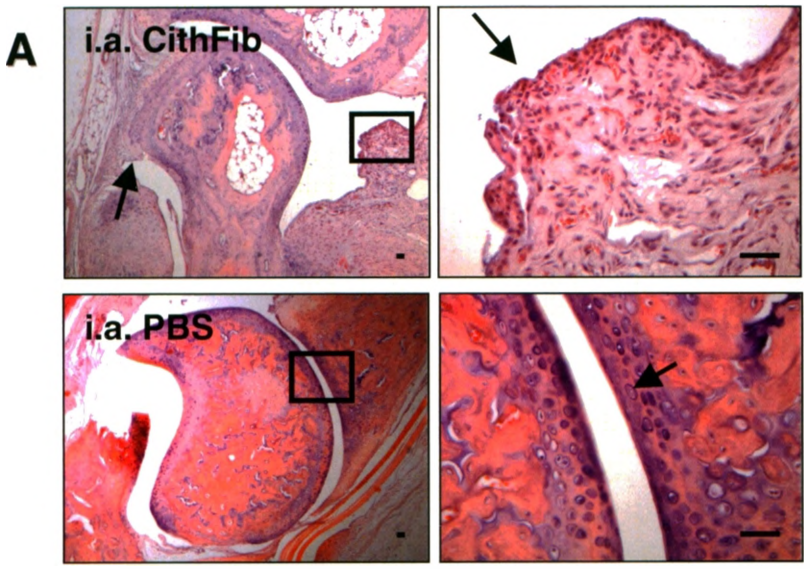
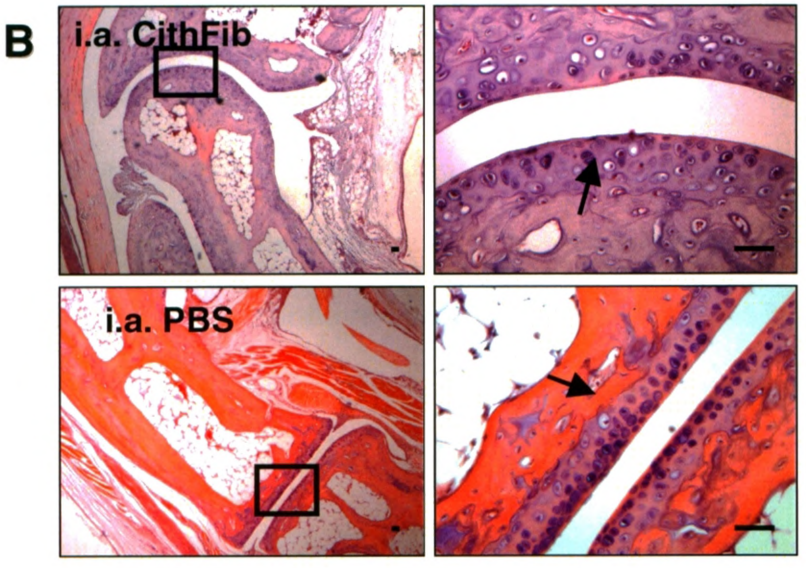

D
Day 0 & 21:
Untreated donor
CTLA4-Ig treated donor
hIgG1 treated donor
Δ ankle width (mm)
Days after adoptive transfer

Figure 12. Adoptive transfer of arthritis by citrulline-induced arthritic, but not non-arthritic DR4 tg mice to naïve syngeneic recipients. H&E stains were performed on the joints of recipient mice receiving lymphocytes from **(A)** arthritic and **(B)** non-arthritic mice at day 50. Recipient mice also received an intraarticular injection of citrullinated human fibrinogen and ankle swelling was monitored. Arrows indicate **(A)** ankylosis and synovial hyperplasia in the i.a. CithFib limb and normal bone and cartilage in the i.a. PBS limb, **(B)** normal joint bone and cartilage in both the i.a. CithFib and i.a. PBS limbs. Representative image of histology from two recipients of each group. Measurement bar=100μm.

Recipient of day 50 arthritic lymphocytes



Recipient of day 50 non-arthritic lymphocytes



from mice preventively treated with CTLA4-Ig, were unable to transfer arthritis to the recipient mice (Figure 11B, C and D) nor did they induce visible joint pathology (Figure 13B, 14B). In contrast, mice receiving lymphocytes from untreated or hIgG1 treated mice showed significant joint swelling (Figure 11B, C and D) and pathology (Figure 13A and C, 14A and C) and thus retained the ability to transfer arthritis.

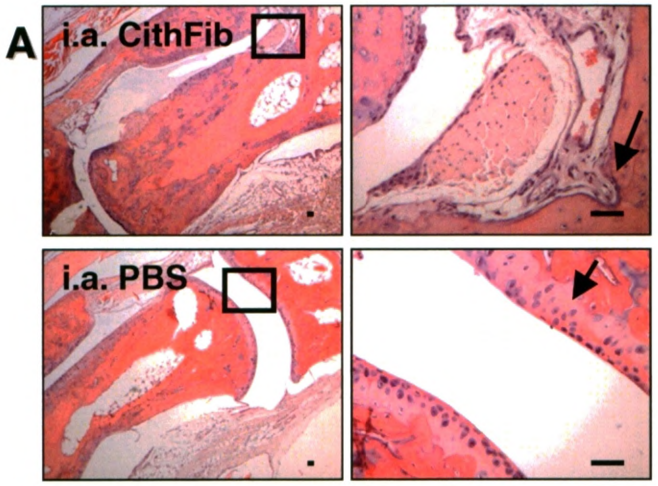
2.3.3 Effect of CTLA4-Ig treatment on the activation of T cells in DR4 tg mice.

We first examined the *in vitro* CTLA4-Ig effect on the proliferation of T cells from CithFib-immunized mice. Splenocytes obtained at day 50 from CithFib-immunized DR4 tg mice that had not received CTLA4-Ig were stimulated *in vitro* with CithFib and hFib in the presence or absence of CTLA4-Ig or control hIgG1 treatment. Both untreated and control treated splenocytes showed similar proliferative responses as predicted (24). However, when these stimulated cells were concurrently treated *in vitro* with CTLA4-Ig, the T cell proliferative response was significantly lower than that of untreated ($P < 0.0001$) and hIgG1 treated ($P < 0.0001$) groups (Figure 15A).

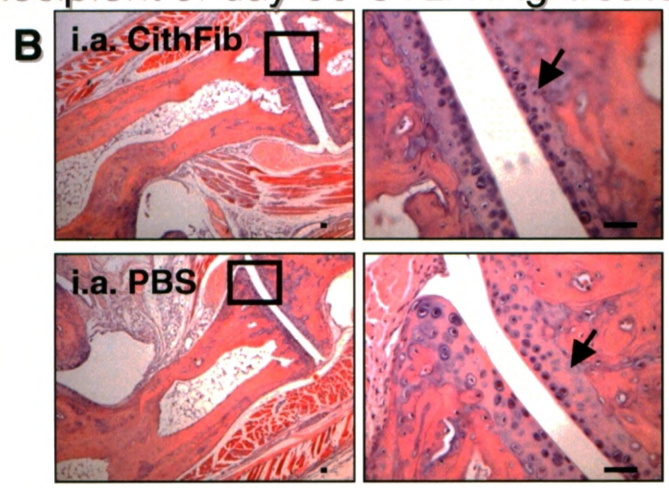
The effect of *in vivo* CTLA4-Ig treatment on *in vitro* T cell proliferation was examined next. Splenocytes were obtained from arthritic mice that were treated and untreated. CTLA4-Ig treatment at the onset of arthritis resulted in a significant reduction in T cell proliferation in response to the immunizing antigen compared to untreated ($P < 0.03$) and hIgG1 treated ($P < 0.04$) arthritic mice (Figure 15B). CTLA4-Ig treatment administered at the peak of disease also demonstrated a similar trend compared to untreated and hIgG1 treated mice (Figure 15C). CTLA4-Ig preventive treatment administered at both immunization and boost also resulted in suppression of T cell activation compared to untreated and hIgG1 treated mice (Figure 15D). Additionally,

Figure 13. Day 50 CTLA4-Ig treatment of Cit-induced arthritic DR4 tg mice blocks the pathologic joint destruction in adoptively transferred recipient mice. H&E stains were performed on the joints of recipient mice receiving lymphocytes from arthritic mice treated at the onset of disease (day 50) with **(B)** CTLA4-Ig, **(C)** hIgG1 or **(A)** left untreated. Recipient mice also received an intraarticular injection of citrullinated human fibrinogen and ankle swelling was monitored. Arrows indicate **(A)** bone erosion in the i.a. CithFib limb and normal bone and cartilage in the i.a. PBS limb, **(B)** normal joint appearance in both i.a. CithFib and PBS limbs, **(C)** synovial hyperplasia in CithFib limb and normal bone and cartilage in i.a. PBS limb. Representative image of histology from two recipients of each group. Measurement bar=100μm.

Recipient of day 50 untreated control



Recipient of day 50 CTLA4-Ig-treated



Recipient of day 50 hIgG1-treated control

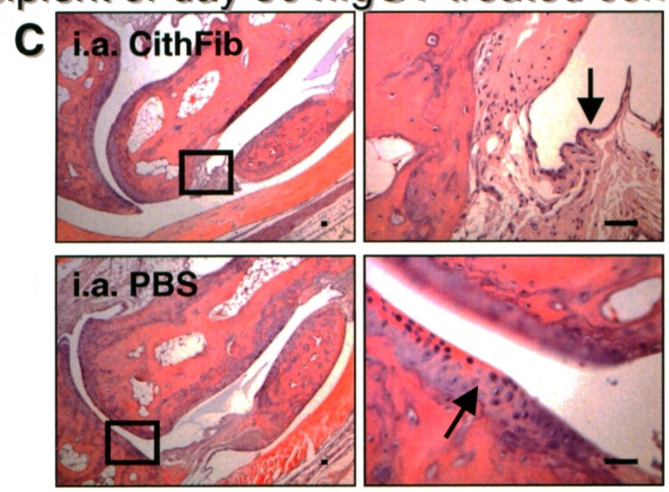
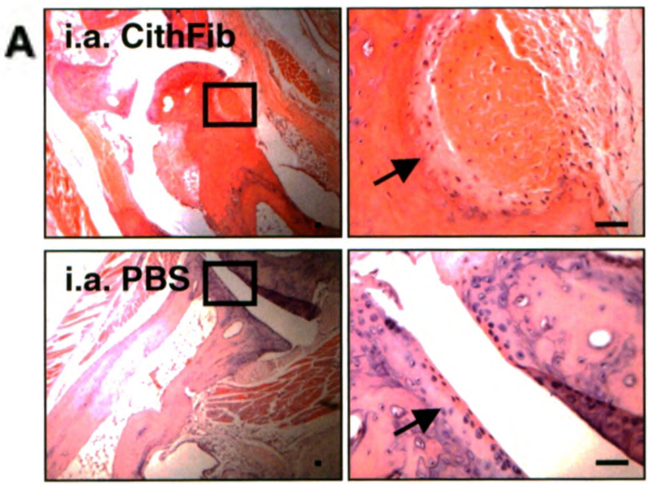
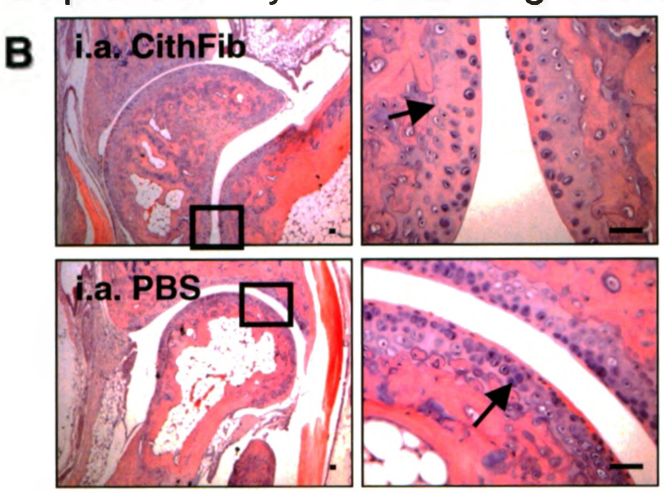


Figure 14. Day 70 CTLA4-Ig treatment of Cit-induced arthritic DR4 tg mice blocks the pathologic joint destruction in adoptively transferred recipient mice. H&E stains were performed on the joints of recipient mice receiving lymphocytes from arthritic mice treated at the peak of established disease (day 70) with **(B)** CTLA4-Ig, **(C)** hIgG1 or **(A)** left untreated. Recipient mice also received an intraarticular injection of citrullinated human fibrinogen and ankle swelling was monitored. Arrows indicate **(A)** bone erosion in the i.a. CithFib limb and normal bone and cartilage in the i.a. PBS limb, **(B)** normal joint appearance in both i.a. CithFib and PBS limbs, **(C)** synovial hyperplasia in CithFib limb and normal bone and cartilage in i.a. PBS limb. Representative image of histology from two recipients of each group. Measurement bar=100μm.

Recipient of day 70 untreated control



Recipient of day 70 CTLA4-Ig-treated



Recipient of day 70 hIgG1 treated-control

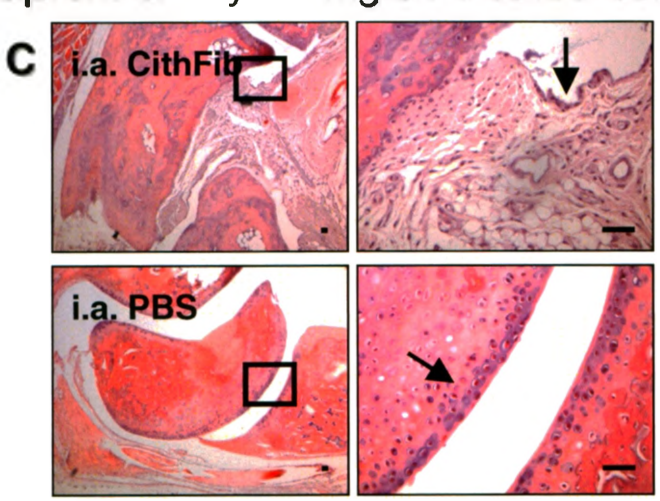
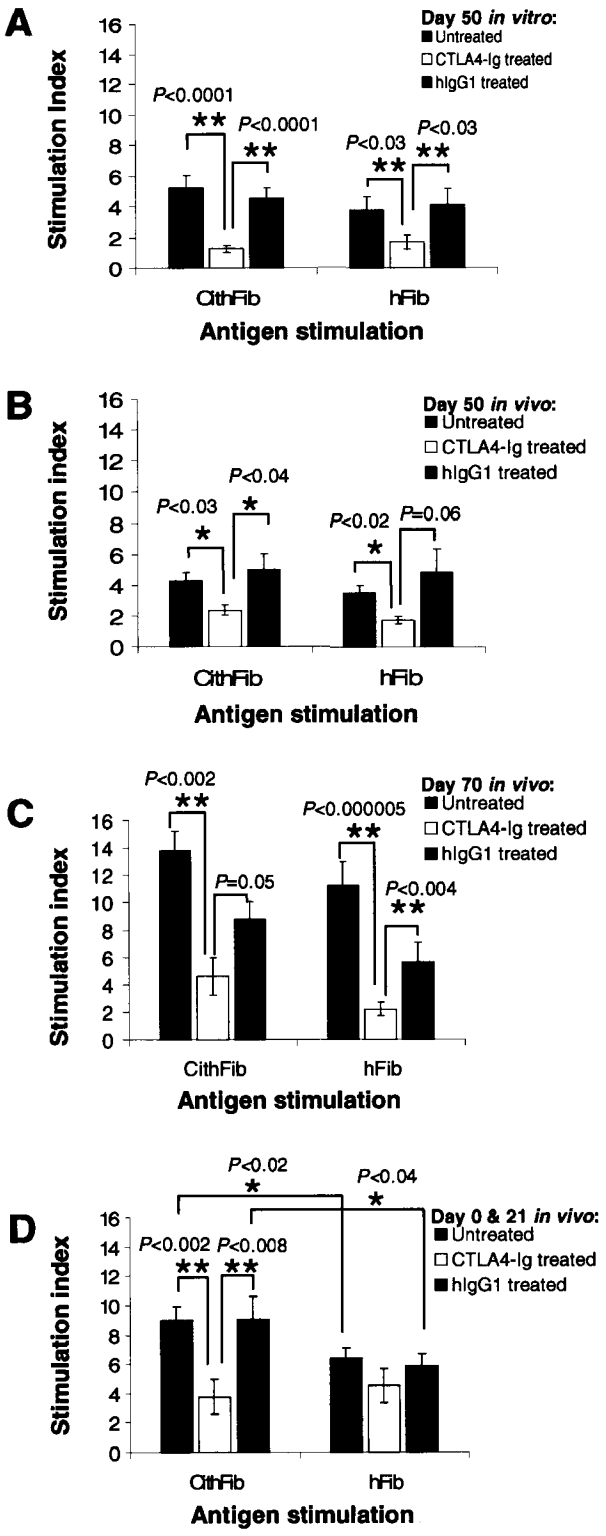


Figure 15. CTLA4-Ig treatment suppresses T cell activation. **A**, Proliferative response of splenocytes from CithFib-immunized (n=12) mice on day 50 post-CithFib immunization. The splenocytes were stimulated *in vitro* with either CithFib or hFib in the presence or absence of CTLA4-Ig or hIgG1 treatment. **B**, Proliferative responses to CithFib and hFib of splenocytes from arthritic mice treated *in vivo* with CTLA4-Ig (n=6), hIgG1 (n=5) or untreated (n=5) at the onset of disease (day 50). **C**, Proliferative response to CithFib and hFib of splenocytes from arthritic mice, treated *in vivo* with CTLA4-Ig (n=9), hIgG1 (n=3), or untreated (n=3), at the peak of disease (day 70). **D**, Proliferative response to CithFib and hFib of splenocytes from mice untreated (n=8) and preventively treated with CTLA4-Ig (n=5), or hIgG1 (n=7), at the time of CithFib immunization (day 0) and boost (day 21). The T cell proliferative responses were assessed by tritiated thymidine incorporation. Bars show mean and SEM. * = $P < 0.05$, ** = $P < 0.01$ hIgG1 or untreated controls vs. CTLA4-Ig *in vitro* treatment (by Student's *t*-Test).


A
Stimulation Index
Day 50 in vitro:
Untreated
CTLA4-Ig treated
hIgG1 treated
P<0.0001
P<0.0001
P<0.03
P<0.03
CithFib
hFib
Antigen stimulation
B
Stimulation index
Day 50 in vivo:
Untreated
CTLA4-Ig treated
hIgG1 treated
P<0.03
P<0.04
P<0.02
P=0.06
CithFib
hFib
Antigen stimulation
C
Stimulation index
Day 70 in vivo:
Untreated
CTLA4-Ig treated
hIgG1 treated
P<0.002
P=0.05
P<0.000005
P<0.004
CithFib
hFib
Antigen stimulation
D
Stimulation index
Day 0 & 21 in vivo:
Untreated
CTLA4-Ig treated
hIgG1 treated
P<0.02
P<0.04
P<0.002
P<0.008
CithFib
hFib
Antigen stimulation

CTLA4-Ig preventive treatment suppressed the citrulline-specific proliferative response seen in untreated and hIgG1 treated arthritic DR4 tg mice (Figure 15D).

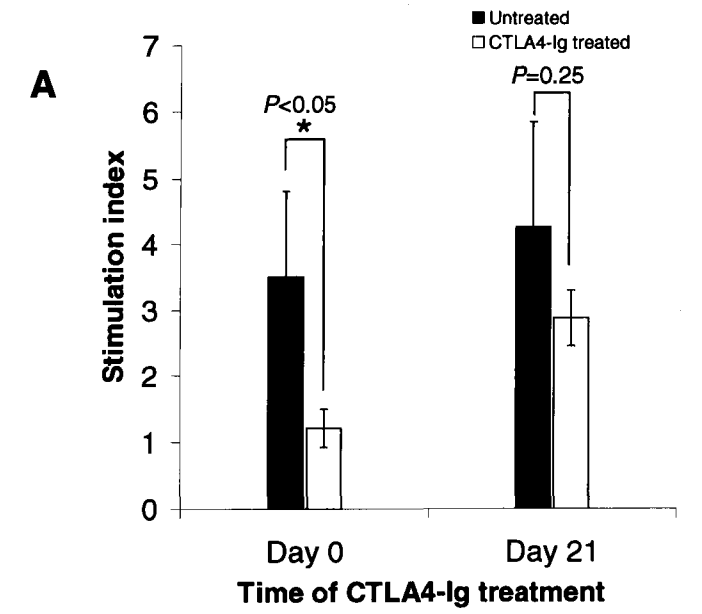
Thus, these experiments demonstrate that in DR4 tg mice, *in vitro* and *in vivo* CTLA4-Ig treatment inhibited the T cell immune response induced by CithFib immunization. In the preventive *in vivo* setting, CTLA4-Ig also inhibited the citrulline-specific T cell response in these mice.

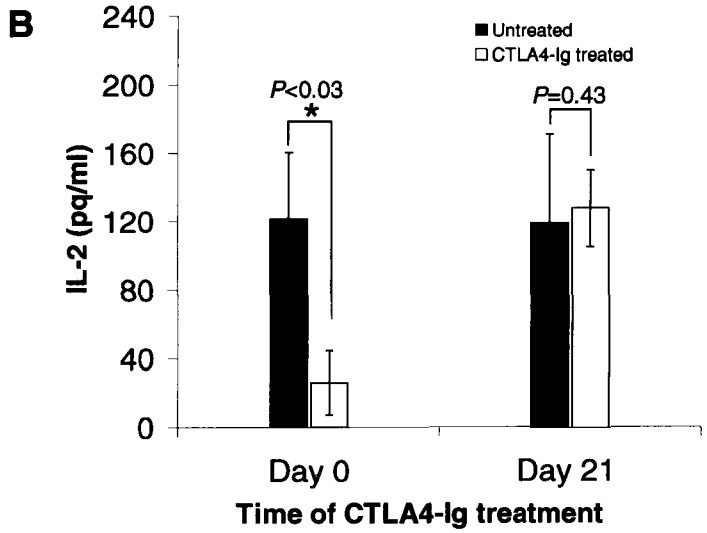
2.3.4 CTLA4-Ig treatment suppresses a primary but not a secondary T cell immune response to a foreign antigen in DR4 tg mice. Since CTLA4-Ig treatment was effective in preventing and blocking arthritis progression as well as suppressing T cell activation, it was of interest to investigate whether the target of *in vivo* suppression was the primary or secondary T cell immune response or both.

DR4 tg mice were treated with seven doses of CTLA4-Ig (100 μg/dose) either at the time of primary immunization only or at the time of secondary boost with a foreign antigen (hFib) only. One day following the final CTLA4-Ig treatment, splenocytes were obtained to assess the effect of treatment on the T cell response. Splenocytes from mice that received a primary immunization with hFib and were left untreated proliferated strongly in response to hFib (Figure 16A) and secreted IL-2 (Figure 16B). However, concomitant treatment with CTLA4-Ig at the time of primary immunization resulted in a significant suppression ($P < 0.05$) of both the T cell proliferative response and IL-2 secretion (Figure 16A and B). In contrast, CTLA4-Ig treatment at the time of secondary boost suppressed neither T cell proliferation nor IL-2 secretion.

Thus, these experiments indicate that CTLA4-Ig treatment predominantly suppressed primary activation of T cells, and had a negligible effect on already

Figure 16. CTLA4-Ig treatment suppresses a primary but not a secondary T cell immune response to a foreign antigen in DR4 tg mice. **A**, CTLA4-Ig *in vivo* treatment blocks a primary, but not secondary T cell proliferative response in hFib-immunized DR4 tg mice. **B,** CTLA4-Ig *in vivo* treatment blocks IL-2 secretion during re-stimulation following a primary, but not a secondary immunization with hFib in DR4 tg mice. Lymphocytes were obtained and stimulated *in vitro* with hFib. The T cell proliferative response was assessed by tritiated thymidine incorporation (**A**) and IL-2 secretion in the supernatants were assessed by ELISA (**B**). The sample size for all groups in (**A**) and (**B**) are n=4Bars show mean and SEM. * = $P < 0.05$ vs. CTLA4-Ig *in vivo* treatment (by Student's *t*-Test).


A
Untreated
CTLA4-Ig treated
P<0.05
*
P=0.25
Stimulation index
7
6
5
4
3
2
1
0
Day 0
Day 21
Time of CTLA4-Ig treatment


B
Untreated
CTLA4-Ig treated
P<0.03
*
P=0.43
IL-2 (pq/ml)
240
200
160
120
80
40
0
Day 0
Day 21
Time of CTLA4-Ig treatment

established primed T cell immune responses, consistent with the fact that primary responses are more costimulation-dependent than secondary responses.

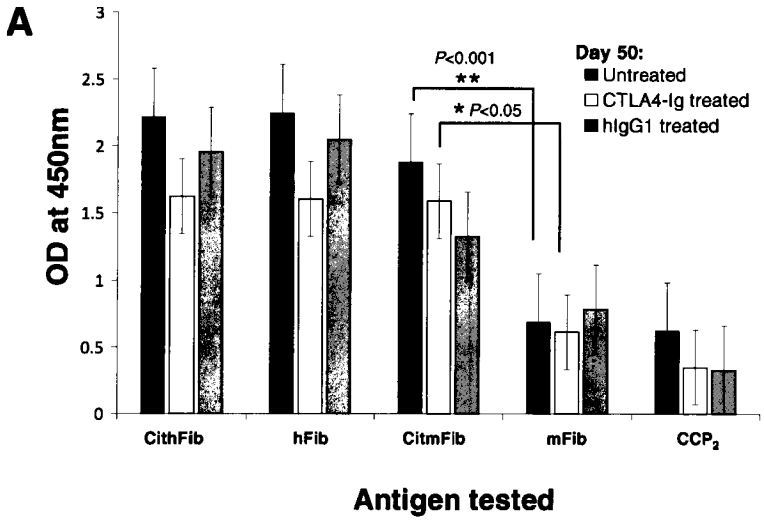
2.3.5 Effect of CTLA4-Ig treatment on B cell immune response in DR4 tg mice.

Serum samples were collected from CithFib-immunized DR4 tg mice preventively treated on day 0 and day 21 and also from arthritic DR4 tg mice treated at the onset and peak of disease with either CTLA4-Ig, control hIgG1, or from those left untreated. Serum antibody responses to CithFib, hFib, CitmFib, mFib, and CCP_2 were assessed to determine the effect of CTLA4-Ig treatment on antibody responses. A citrulline-specific response to CitmFib compared to mFib was observed as previously described (24). However, there were no significant changes in antibody levels to the tested antigens between the different groups regardless of whether CTLA4-Ig was administered at the onset or peak of disease, or co-administered at both the time of immunization and boosting (Figure 17A, B and C). This suggests that at the times of CTLA4-Ig administration, the ability of animals to effectively mount an antibody immune response remained intact.

2.4 DISCUSSION

The immune responses to citrullinated proteins/peptides are believed to be important in RA pathogenesis (4, 7-9, 11). Our citrulline-induced model of RA in DR4 tg mice provides a unique opportunity to investigate the mechanisms involved in this response. CTLA4-Ig was used to explore the role of T cells in mediating citrulline-induced arthritis since we previously found that immune responses to a panel of

Figure 17. Effect of CTLA4-Ig treatment on the antibody response in arthritic CithFib-immunized DR4 tg mice. **A**, On day 50 (onset of arthritis) arthritic mice were treated with CTLA4-Ig (n=6), hIgG1 control (n=5), or were left untreated (n=5). Following the treatment and monitoring period, serum antibody levels to CithFib (1in8000), hFib (1in8000), CitmFib (1in500). mFib (1in500), and CCP_2 (1in50) were determined at the indicated serum dilutions. **B**, Antibody levels to these antigens were also tested in the sera of mice treated at day 70 (peak of disease) with CTLA4-Ig (n=9) or hIgG1 control (n=3), or left untreated (n=3). **C**, Antibody levels to these antigens were also determined in the sera of mice treated both at the time of immunization (day 0) and boost (day 21) with CTLA4-Ig (n=5), hIgG1 control (n=7) or left untreated (n=8). No significant differences were detected in the level of antibody among the treatment groups. * = $P < 0.05$, ** = $P < 0.01$ CitmFib vs. mFib within a treatment group (by Student's *t*-Test).


A
OD at 450nm
3
2.5
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P<0.001
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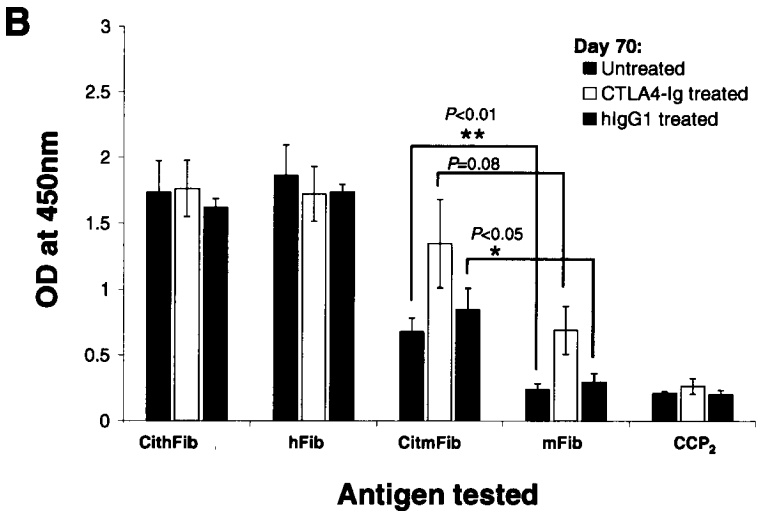
* P<0.05
Day 50:
Untreated
CTLA4-Ig treated
hIgG1 treated
CithFib
hFib
CitmFib
mFib
CCP2
Antigen tested


B
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3
2.5
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P<0.05
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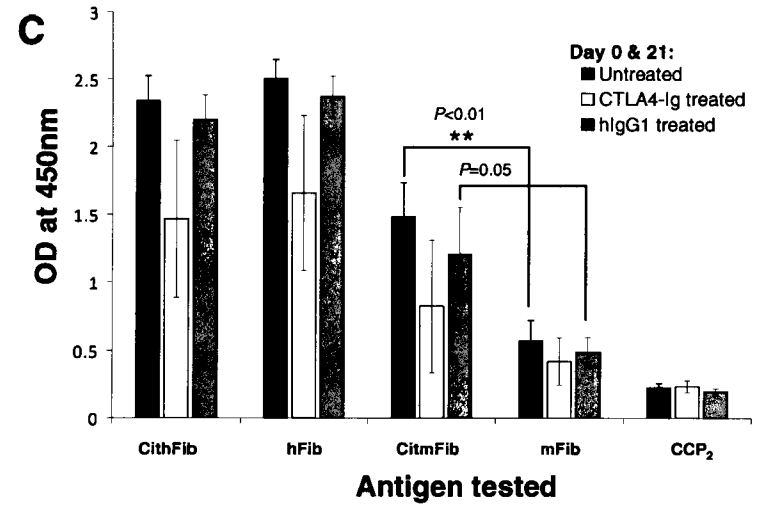
Day 70:
Untreated
CTLA4-Ig treated
hIgG1 treated
CithFib
hFib
CitmFib
mFib
CCP2
Antigen tested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P=0.05
Day 0 & 21:
Untreated
CTLA4-Ig treated
hIgG1 treated
CithFib
hFib
CitmFib
mFib
CCP2
Antigen tested

citrullinated proteins/peptides was similar in arthritic and non-arthritic DR4 tg mice (24). We found that CTLA4-Ig blocked the progression of citrulline-induced arthritis, significantly decreasing joint swelling and preventing joint damage. CTLA4-Ig treatment also suppressed citrulline-specific T cell activation *in vivo*. These results suggest that citrullinated peptide(s)-specific T cells are actively involved in the development and progression of citrulline-induced arthritis and that modulation of T cell costimulation with CTLA4-Ig may lead to the amelioration of arthritis.

An important observation was that CTLA4-Ig suppressed both early and established citrulline-induced arthritis as well as prevented disease when co-administered during immunization and boost. Furthermore, suppression of arthritis, when treated at the onset and peak of disease, was maintained for at least 30 days after the cessation of treatment. The observed benefit of CTLA4-Ig treatment for both early and established disease is similar to its effectiveness in the treatment of human RA (38) and to the treatment of EAE and CIA (31, 33). As well, the prolonged effect of CTLA4-Ig is consistent with the study by Finck et al. (32) where CTLA4-Ig treated, but not control lupus mice survived for 3 months post-treatment. These experiments provide evidence that CTLA4-Ig has an extended beneficial effect at multiple stages of disease and suggests a long-term modulatory effect on the citrulline-induced immune response.

The finding that CTLA4-Ig treatment prevented pathological joint damage is consistent with human RA trials where it has been demonstrated to dampen the development of erosions (38) and also with the findings of Webb et al. (33) and Knoerzer et al. (39) where it prevented similar damage in CIA models. The reduced joint damage

could have resulted from the suppression of T cell activation as activated T cells have been shown to induce osteoclastogenesis (40). Additionally, CTLA4-Ig might have inhibited RANKL- and tumor necrosis factor (TNF)α-induced osteoclastogenesis (41) leading to the reduced joint damage.

We also showed that in this citrulline-induced RA model, splenic lymphocytes from arthritic but not non-arthritic mice were able to transfer disease into naïve syngeneic recipients. This transfer of disease was shown to be citrulline-specific by induction of joint swelling in only the i.a. CithFib but not hFib-injected limb of recipient mice. These observations imply an arthritogenic anti-CithFib peptide response along with a functional difference between the immune cells of arthritic vs. non-arthritic mice. As well, CTLA4-Ig was found to prevent arthritis transfer by these immune splenic lymphocytes. CTLA4-Ig costimulation blockade suppressed T cell activation, which suggested that this suppression was responsible for inhibiting the arthritogenic properties of splenic lymphocytes from arthritic mice. Several mechanisms may explain these observations. Razmara et al. (42) showed that CTLA4-Ig can induce the conversion of naïve T cells into T regulatory (Treg) cells. We explored this possibility but found no difference in the percentage of Treg cells when comparing spleens from untreated vs. CTLA4-Ig treated mice, nor in arthritic vs. non-arthritic mice (Appendix 2). It has been suggested that a deficiency in number and function of invariant natural killer T (*i*NKT) cells may be involved in a number of autoimmune diseases including RA (43). However there was no difference in the frequency of splenic *i*NKT cells in arthritic vs. non-arthritic, or between untreated vs. CTLA4-Ig treated mice (Appendix 3). Despite the similar frequencies of

Treg and *i*NKT cells, we can not rule out the possibility of functional differences in these subsets between different groups.

While the T cell proliferative response in CTLA4-Ig treated mice was suppressed, a corresponding suppression of the antibody response induced by CithFib was not observed. Previous studies indicate that CTLA4-Ig can suppress T cell-dependent antibody production (29, 33). However this difference may be due to different treatment regimens. In previous studies, CTLA4-Ig was often used continuously for an extended period of time starting at the initiation of an immune response (29, 32, 33, 44). In such situations of continual costimulation blockade, the development of both T and B cell immune responses would be very limited. In contrast, with our citrulline-induced model, arthritis does not develop until day 50 post primary immunization, at which time a strong IgG response is established. There are several possible reasons for the lack of an observable change in serum IgG levels. First, it is possible that CTLA4-Ig did not have an effect on IgG levels due to the timing or treatment regimens used. Alternatively, it is possible that CTLA4-Ig did have a suppressive effect; however, at the time serum was collected, no difference was observed because IgG levels had fallen to a lower stable level (45). It could also be that the antibody levels remained similar in the serum but not in the joints of CTLA4-Ig treated vs. control and untreated mice. Such a situation would be similar to the increased autoantibody levels found in the synovial fluids vs. the sera of RA patients (46).

The ability of CTLA4-Ig to block primary and secondary T cell immune responses to a foreign antigen was tested to investigate how CTLA4-Ig was affecting the citrulline-induced immune response. Primary but not secondary T cell immune responses

were found to be suppressed by CTLA4-Ig, suggesting that the observed benefit of CTLA4-Ig treatment in arthritic DR4 tg mice resulted from suppression of primary T cell activation rather than of previously primed T cells. This is in line with the notions that CTLA4-Ig predominantly targets costimulation, as opposed to reverse signaling in antigen presenting cells (47), and that naïve T cells are more dependent on costimulation than primed memory T cells (48). These observations then imply that CTLA4-Ig suppression of ongoing T cell activation led to the observed reduction of the clinical and pathological signs of arthritis.

During inflammation, citrullination of proteins can occur when peptidyl arginine deiminase is released due to apoptosis (49, 50). In those with a genetic predisposition to develop an immune response to citrullinated antigens, similar to our DR4 tg mice carrying the SE, activation of citrulline-specific T cells could perpetuate a pathogenic process of inflammation and formation of new citrullinated targets. Blocking the activation of naïve T cells by new epitopes or neoantigens formed through processes such as citrullination, may have then contributed to the observed amelioration of disease. Finally, since CTLA4-Ig appeared to predominately affect primary immune responses, treatment with CTLA4-Ig would likely not compromise previously established and beneficial immune responses elicited for example by vaccinations against infectious agents.

In our preventive treatment experiments, it was found that the citrulline-specific T cell immune response was suppressed along with disease development. The observations that CTLA4-Ig predominantly targeted primary T cell activation and suppressed the citrulline-dependent transfer of disease by splenic lymphocytes suggest that CTLA4-Ig

suppression of an ongoing pathogenic citrulline-specific immune response may have led to the reduction of the clinical and pathological signs of arthritis.

Collectively, these studies provide evidence that ongoing CD28 costimulation-dependent T cell responses are directly involved in the development and progression of citrulline-induced arthritis. The finding that blocking costimulation with CTLA4-Ig suppressed only primary T cell responses supports the idea that continual *de novo* T cell activation, such as to citrullinated antigens, is important for maintaining arthritis in this model. Finally, these studies point to T cell immune responses directed at citrullinated target(s) as being pathogenic and to a distinct difference between immune lymphocytes from arthritic vs. non-arthritic mice in this arthritis model.

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CHAPTER 3

DISCUSSION AND CONCLUSIONS

3.1 RA and the immune response to citrulline

The finding that the serum autoantibodies to citrulline are specifically identified in RA patients (1) has generated considerable interest in whether the immune response to citrulline plays a pathogenic role in RA. Association of this response with MHC class II genes (e.g., HLA-DRβ1) expressing the SE (2), the strongest genetic risk factor for disease (2, 3), was an important finding. This suggested a potentially pathogenic mechanism, according to which SE expression may lead to the development of an anti-Cit immune response. The observation that ACA can predate and predict the severity of disease has provided further support for a pathogenic role for the immune response to Cit (1, 4-7). Finally, the finding that specific citrullinated forms of proteins including fibrinogen and vimentin are specifically targeted in RA patients (8, 9) as well as present in RA joints (10, 11) further implicates a pathogenic link between the immune response to Cit and RA. The autoantibodies to Cit are now routinely tested by commercial kits, and have become of crucial diagnostic importance for RA.

Although previous studies have led to the development of ELISA kits that reliably detect the highly RA-specific ACAs, thus a very important diagnostic tool, understanding the underlying mechanism of RA remains largely unknown. Furthermore, the role of both antibody and T cell responses to Cit in the pathogenesis of RA has yet to be definitively demonstrated. If it is found that the immune response to Cit is pathogenic, it would have important therapeutic implication for RA since strategies to suppress this specific response could be instrumental to controlling the disease process.

3.2 Citrulline, the SE and the two-hit hypothesis

Cit is generated through post-translational modification of arginine residues situated within peptides via deimination (12). Deimination is mediated by peptidyl arginine deiminase (PAD) which is an intracellular calcium-dependent enzyme (12, 13). Deimination results in the reduction of the positive charge from the side chain of arginine residues to polar and uncharged Cit residues (12, 13). The SE, which is a consensus amino acid motif (QK/RRAA), carries a positive charge in the P4 binding pocket due to the lysine or arginine residue (K/R) (14). As a result, it has been shown that MHC class II allomorophs containing the SE can preferentially bind to and present the Cit version of peptides over the corresponding arginine version (2). Given the association between ACAs and the expression of the RA SE, the most important genetic factor in this disease, this preferential presentation of Cit peptide antigens provides a mechanistic explanation for the observed association.

In a search to understand how these different findings regarding the immune response to Cit are related to arthritis development, a two-hit hypothesis for RA pathogenesis was proposed by us and others (15, 16).

In this model the first hit would be the development of an immune response to Cit. Though citrullination does occur during the natural process of apoptosis (17), it can also specifically occur during inflammation (18). Thus during the process of inflammation and/or apoptosis, Cit-specific T cells could become activated and provide help to B cells, which will in turn produce antibodies to Cit. Low-grade inflammation and the consequent citrullination occur commonly on a day-to-day basis; however only a

small proportion of the population develops ACAs (19). This suggests the importance of SE expression in restricting the development of an immune response to citrullinated proteins formed during inflammation or under other environmental influences. Smoking is an environmental factor that can lead to inflammation, and is interestingly associated with disease in RA patients with both the SE and ACAs, but not in those lacking one of these entities (20). In this case, smoking is thought to activate and prime cells for apoptosis, thereby leading to citrullination of antigens in the lung (20). In individuals with the SE, this could lead to an immune response to Cit and production of ACAs. This provides a compelling example of how genetic and environmental factors may interact to influence the risk for disease development. As the immune response progresses in individuals expressing the SE, activated T cells could provide help to B cells, leading to class switching and the characteristic IgG ACA response. However the development of a systemic immune response to Cit alone is perhaps not sufficient for the development of RA.

The second hit in this model may occur when Cit antigens become available in the joint, providing an ia target for the immune response. Deposition of Cit as a target for the immune response could arise from a number of different scenarios leading to inflammation and thus citrullination, including infection or trauma. Inflammation could lead to the ia recruitment of immune cells including granulocytes, monocytes, and macrophages, which are known to express PAD4 and PAD2, the enzymes necessary to catalyze the process of citrullination (21). Therefore, following the eventual turnover and apoptosis of these cells, citrullination of both intracellular and extracellular proteins such as fibrinogen and vimentin could occur. Circulating ACAs could recognize these Cit

antigens and target ACAs in the joint to form immune complexes. Immune complex formation could then lead to subsequent complement activation as well as increased APC activation through the binding of Fc receptors (22). This would in turn begin a vicious cycle in which inflammation could cause more Cit antigen to be created, providing more targets for ACAs, and thus leading to an inflammatory response which results in the accompanying pathology characteristic of RA.

Although ACAs have been characterized extensively, the role of the T cell response to Cit has received far less attention. This is likely in part due to the hypo-responsiveness of T cells from RA patients, which limits our ability to address this question (23).

3.3 T cell costimulation blockade in citrulline-induced arthritis

The idea of targeting costimulation has had a great appeal in the field of autoimmunity. When targeting costimulation, the immune response is not blocked, but rather can be modulated. Furthermore, by targeting one of the key signals required for optimal T cell activation, costimulation blockade can be used effectively without knowing which foreign or self antigen is involved in the immune response of interest.

The evidence supporting the importance of the immune response to Cit in RA has been strongly established and is growing continually. However as mentioned previously, the role of T cells in this immune response to Cit and its involvement in mediating RA has yet to be thoroughly investigated. Our group has developed a Cit-induced model of RA using DR4 tg mice expressing the human SE (24, 25). Thus the use of CTLA4-Ig in

our DR4 tg RA model should provide a unique system to investigate the role of costimulation and the T cell immune response to Cit in mediating arthritis.

A number of exciting observations were noted in our studies using T cell costimulation blockade in the DR4 tg Cit-induced arthritis model. We found that CTLA4-Ig blocked the progression of Cit-induced arthritis both in early and established disease. The administration of CTLA4-Ig also significantly decreased joint swelling compared to control mice and prevented the characteristic joint damage associated with the disease. Furthermore, CTLA4-Ig treatment suppressed T cell activation both *in vitro* and *in vivo* as well as blocked the adoptive transfer of disease by splenic lymphocytes into naïve recipient mice. Interestingly however, changes in IgG or IgG subclass antibody levels were not observed. Finally, in an effort to understand how CTLA4-Ig was having its effect on the immune response to Cit, we investigated the effect of treatment on an *in vivo* primary vs secondary T cell response to a foreign antigen. We found suppression of the primary T cell immune response and a negligible effect on the secondary T cell response.

The observed suppression by costimulation blockade affected only the primary T cell response to antigen. This suggests that the observed T cell suppression seen in these DR4 arthritic mice with CTLA4-Ig treatment was predominantly a result of suppression of primary T cell activation. Interestingly, *in vitro* re-stimulated Cit primed immune cells from immunized mice were suppressed when CTLA4-Ig was added directly into culture. This indicates that primed and/or memory T cells, though less costimulation dependent, could still be affected by CTLA4-Ig at an *in vitro* dose of 20 μg/ml. Since *in vivo* CTLA4-Ig treatment predominantly affected primary immune responses, this suggests that the effects of CTLA4-Ig may be due to suppression of a continual T cell immune

response to Cit. The suppression of T cell activation and, likewise, of arthritis implicates T cells as key mediators in the progression and maintenance of Cit-induced arthritis.

Though there is evidence suggesting that CTLA4-Ig can suppress T cell-dependent antibody production (26, 27), a change in the level of antibodies was not observed in our treatment groups. This may be due to the fact that in previous studies, CTLA4-Ig was often used continuously for an extended period of time starting at the initiation of an immune response (26-29). In such a situation of continual T cell costimulation blockade, the development of both T and B cell immune responses would be very limited. In contrast, with our Cit-induced model, arthritis does not develop until day 50 post primary immunization. As a result, a strong IgG response is established following the boost and serum levels by the time of sacrifice (~ 50 days post treatment) may have reached a stable level (30). Thus CTLA4-Ig-mediated costimulation blockade might not effectively result in a noticeable difference in antibody levels with the treatment regimen used.

There also exists the possibility that while total IgG antibody levels did not change, there might be suppression of a subclass of IgG antibody due to a shift from a Th1 to a Th2 type response (31, 32). Th1 responses are associated with IgG2c subclass antibodies in C57B/6 mice, whereas Th2 responses are associated with IgG1 antibody subclass (33). An analysis of serum antibodies from the different treatment groups revealed no significant change in IgG subclass antibody levels between our experimental groups (Appendix 4). The observation that IgG Ab levels as well as IgG subclasses to a variety of antigens did not correlate with disease severity suggests that serum Ab levels in this Cit-induced model do not appear to be indicative of disease development.

Our previous work with this Cit-induced DR4 tg model of RA have not revealed a difference in the immune response of arthritic vs non-arthritic mice. In this study however, I showed that splenic lymphocytes from arthritic but not non-arthritic mice were able to transfer arthritis when an ia Cit target was provided. This suggests a distinct cellular difference in arthritic vs non-arthritic splenic lymphocytes. Furthermore, CTLA4-Ig treatment was able to suppress this ability to transfer arthritis. This suppression was possibly due to the suppressed T cell proliferative response observed in treated arthritic mice. As suggested by CTLA4-Ig's predominant effectiveness on primary activation, this suppression was likely due to inhibition of a continual immune response. The decreased T cell proliferative response could reflect fewer T cells being activated, and thus fewer antigen-specific effector cells in circulation that could target Cit. It is then possible that the proportion of arthritogenic T cells in the transferred splenic lymphocytes from treated donors did not meet the threshold necessary to cause arthritis in the recipient mice. Since it has also been suggested that CTLA4-Ig treatment could induce Treg cell formation (34), it is also possible that the induction of Treg cells could play a role in the observed suppression of arthritis as well as suppression of arthritis transfer.

To try to determine a mechanism for the difference in arthritogenicity between lymphocytes from arthritic and non-arthritic mice, the number of Tregs and *i*NKT cells were also investigated. No noticeable difference in the frequency of either cell type in the spleen was found. However this does not exclude the possibility of a functional difference between cells from arthritic vs. non-arthritic mice.

The suppression of T cell activation by costimulation blockade may in large part account for the reduced joint swelling and destruction. Activated T cells have been shown

to induce osteoclastogenesis and thus suppressing T cell activation could result in reduced joint destruction (35). In addition, CTLA4-Ig has been shown to induce DCs to express IDO, which is also known to suppress T cell activation (36). Finally alternate mechanisms for CTLA4-Ig action have also been suggested. For example a reduction in bone changes has recently been implicated as a direct effect of CTLA4-Ig in blocking osteoclastogenesis independent of T cells (37). These studies indicate that T cell costimulation is crucial in the progression of Cit-induced arthritis.

3.4 Insight into how the T cell immune response to Cit is involved in RA

It is important to note that there is a key difference between mouse and human CD28 expression. All mouse T cells express CD28 (38), while in humans only approximately 80% of T cells express CD28 (all CD4+ cells and about 50% of CD8+ cells) (39). As a result, this may lead to a greater dependence of mouse T cells on costimulation, and thus potentially a stronger effect of costimulation blockade than would be seen in humans. Despite this, the efficacy of CTLA4-Ig in the treatment of human RA with its similar outcomes (40) would support the relevance of my findings here.

These studies support the idea of an ongoing immune response during disease pathogenesis in which T cells are involved in its maintenance. As suggested in the two-hit hypothesis, it is possible that chronic inflammation leads to citrullination and the formation of neoantigens. This subsequently leads to activation and recruitment of more unprimed Cit-specific immune cells as well as the perpetuation of the inflammatory response. In similar models of arthritis and autoimmunity such as CIA and EAE,

evidence for the spreading of immune reactivity to an increased number of citrullinated antigen targets during disease progression has be shown (41). The efficacy of CTLA4-Ig treatment *in vivo* as well as it predominant effect on primary activation *in vivo* suggests the involvement of T cells in this continual response to Cit. Suppression of T cell activation in CTLA4-Ig-treated mice responding to potential Cit neoantigens formed during inflammation would then result in fewer antigen-specific effector cells and thus a decreased number of lymphocytes capable of trafficking into the joint. The presence of fewer lymphocytes in the joint in turn could mean decreased levels of inflammatory cytokines as well as T cell-induced osteoclastogenesis, which would otherwise perpetuate the chronic inflammatory response.

The lack of an effect of CTLA4-Ig on antibody levels, but suppression of disease further implicates an arthritogenic role for T cells in this model independent of providing help to activate B cells. Despite the specificity and thus diagnostic and prognostic value of ACAs in human RA, their role in the pathogenicity of RA is still not entirely clear. ACA as well as RF levels do not appear to consistently fluctuate with disease activity or remission in humans (42-46), which is similar to our findings in the Cit-induced arthritis model. These observations suggest that serum levels of autoantibodies might not be ideal in assessing current disease activity. However recent work in our laboratory has directly implicated human ACAs in the pathogenicity of RA. Transfer of purified ACAs from human RA patients induced arthritis in FcγRIIB -/- mice, whereas equivalent amounts of IgG from normal patients did not (47). These findings may indicate that though our arthritis model has a number of relevant similarities to human disease such as expression

of the human SE as well as development of an immune response to Cit and arthritis, there are also important differences.

Finally, the finding that adoptive transfer of arthritis into naïve recipients was dependent on the provision of a Cit target further provides support for the second hit in the two-hit hypothesis. However, it is also interesting to note that arthritis was only transferred by lymphocytes from arthritic but not non-arthritic mice. These adoptive transfer experiments provide a powerful example of the differences in the immune response to Cit in arthritic vs. non-arthritic DR4 tg mice. Thus further investigation into this immune response might provide insight into individuals with ACAs, but no signs of disease.

3.5 Summary and conclusions

Much work has gone into studying how Cit and the SE are involved in the pathogenesis of RA. Although T cells have long been thought to play a role in the disease, their role in the highly RA-specific immune response to Cit has largely not been addressed. Our current studies using CTLA4-Ig costimulation blockade in our Cit-induced model of RA have extended the understanding of the efficacy of costimulation blockade as well as how T cells fit into and support different aspects of the current views on RA pathogenesis. The importance of the involvement of T cells in this immune response to Cit as a potential mediator of disease became especially apparent in the ability of only arthritic lymphocytes to transfer disease.

In summary, these studies imply that T cell responses to Cit have a direct role in the progression of Cit-induced arthritis from its development into its chronic stages. Furthermore, the use of the Cit-induced model provided insight into how costimulation blockade might be mediating its beneficial effects in human RA patients. Finally, our work points to a distinct difference in the arthritogenicity of splenic lymphocytes from arthritic vs. non-arthritic mice despite an identical genetic background and environment. Further understanding of how Cit-specific T cells, the highly diagnostic ACAs, and environmental factors interact and are involved in RA pathogenesis will provide a foundation for the development of new therapeutic treatments in the future.

3.6 Future directions

A number of questions have arisen from these studies, which should be addressed in the future. First, it is still unclear how costimulation blockade through CTLA4-Ig treatment is mediating its effects on the disease. Our results indicate that primary T cell activation was predominantly suppressed by this treatment as opposed to an already primed T cell response to Cit. This could have clear implications in understanding the effects of costimulation blockade on previous beneficial immune responses such as those elicited to a vaccine, as well as its effectiveness on pathological immune responses driven primarily by a few immunodominant epitopes. It would thus be important to verify this *in vivo* observation of CTLA4-Ig's effect on primary immune responses. This could be accomplished by using TCR transgenic mice, such as ovalbumin (OVA)-specific TCR transgenic mice (48). Mice could be immunized with OVA, thus priming all T cells

expressing this TCR transgene. Following immunization, CTLA4-Ig could be administered *in vivo* with the secondary booster immunization. Following treatment, splenocytes would be stimulated *in vitro* with OVA and stained with carboxyfluorescein succinimidyl ester (CFSE). CFSE is a fluorescent cell staining dye which is partitioned equally to daughter cells and thus could be used to determine the effect of treatment on the rate of proliferation in CTLA4-Ig treated compared to untreated or control treated mice (49).

Another aspect that warrants further investigation is the adoptive transfer experiments. It was interesting to see that CTLA4-Ig treatment suppressed the arthritogenic properties of lymphocytes from arthritic mice, while lymphocytes from control-treated and untreated groups were still able to transfer disease. It would be important to characterize these lymphocytes being transferred to enumerate different cell populations, such as Treg cells that have been suggested to be induced by CTLA4-Ig treatment via IDO expression in DCs (34, 36, 50). Furthermore, the difference in arthritogenicity between splenic lymphocytes from arthritic and non-arthritic mice calls for further study. Though there were no differences in the numbers of Treg and *i*NKT cells between arthritic and non arthritic mice, functional characterization of these cell types was not studied.

The next step following our murine studies would be to verify our findings in human RA patient lymphocytes. Determining how Cit-specific T cell responses of peripheral blood lymphocytes from RA patients are affected by CTLA4-Ig treatment could be studied. It would also be of interest to investigate whether current disease

activity of patients is related to T cell responses to different Cit antigens, which are often targeted in RA patients such as CithFib, citrullinated α enolase, or citrullinated vimentin.

These results could provide crucial insight into the mechanisms underlying the beneficial effects of CTLA4-Ig treatment as well as strategies in the use of costimulation blockade in general. Verification in the human system could aid in understanding the mechanism of human disease and hopefully lead to the development of novel RA therapies.

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APPENDICES

Western

01.01.09
***This is the 3rd Renewal of this protocol**
*A Full Protocol submission will be required in **2009**

Dear Dr. **Cairns**

Your Animal Use Protocol form entitled:

Induction and prevention of Rheumatoid Arthritis in DRB1*0401 transgenic mice

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **01.01.09 to 12.31.09**

The protocol number for this project remains as **2005-085**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

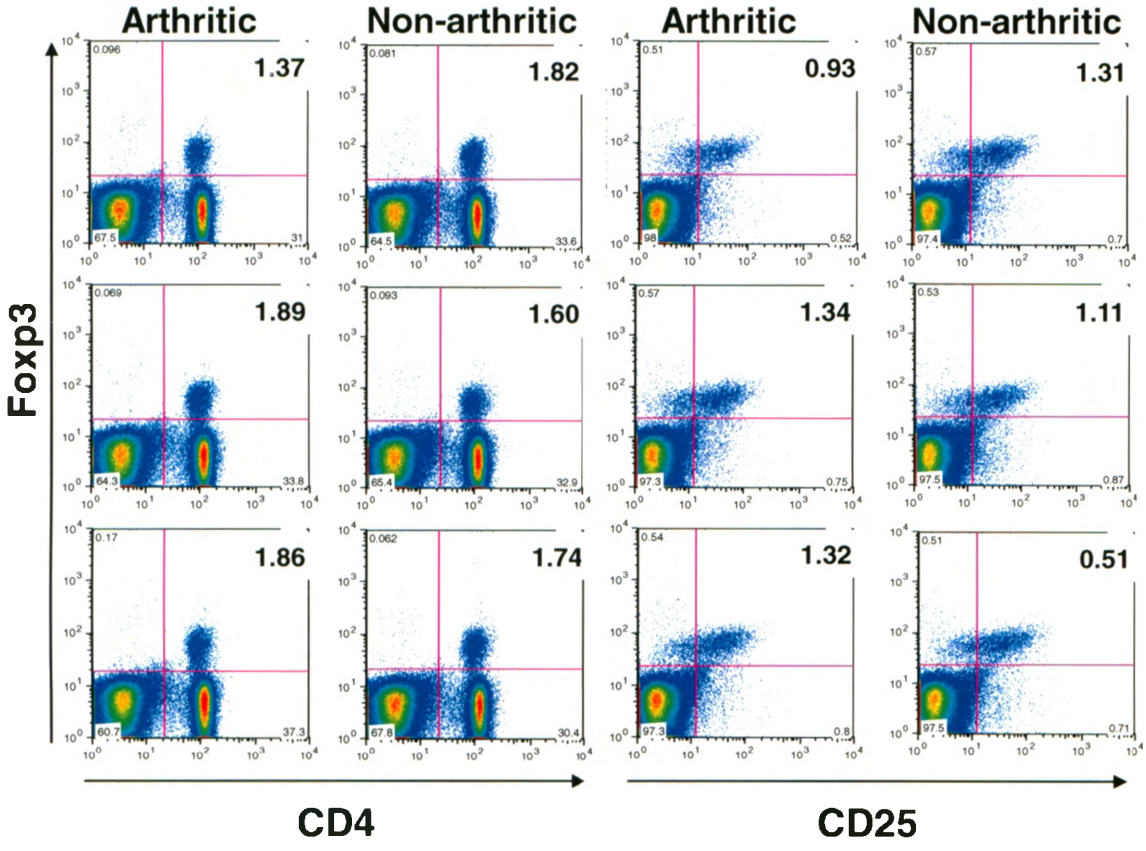
REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.

c.c. W Brintnell, W Lagerwerf

***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. 86770 • FL 519-661-2028 • www.uwo.ca / animal

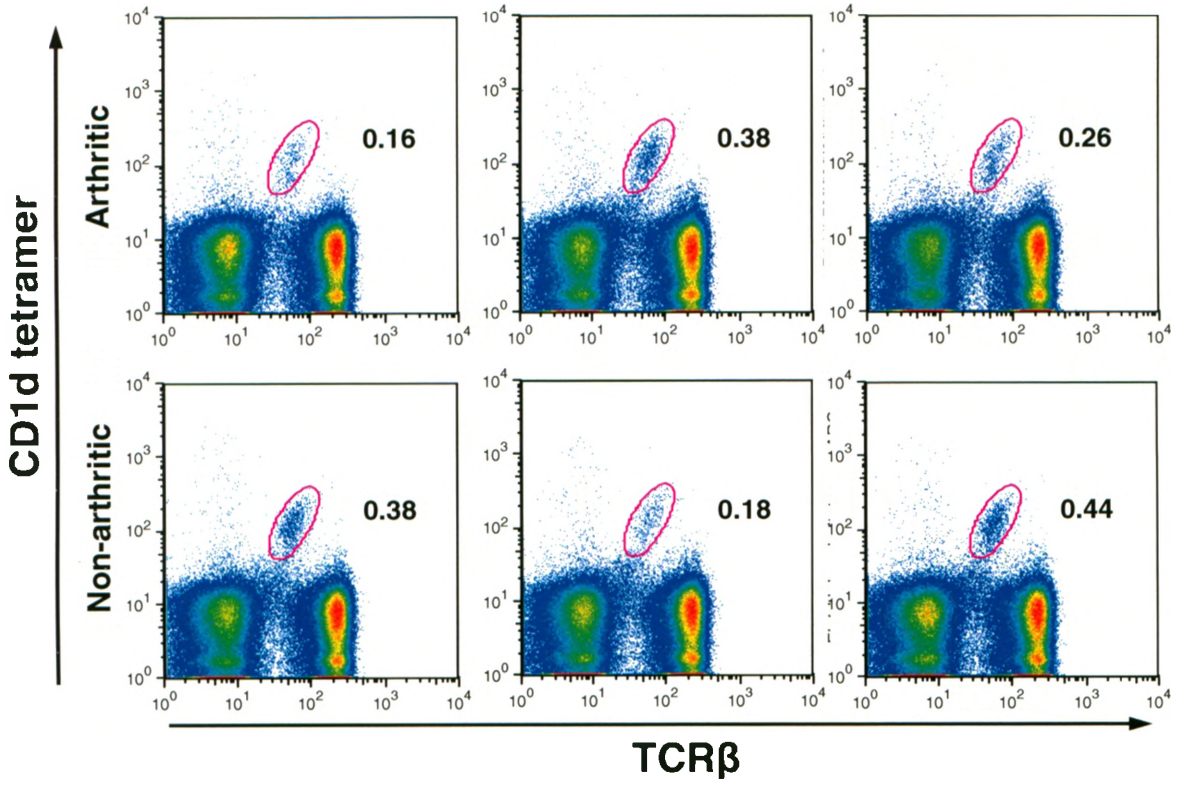
Appendix 1. Ethical approval for use of animal subjects

Appendix 2. T regulatory cell frequency in arthritic and non-arthritic CithFib-immunized DR4 tg mice. There is no difference in the number of T regulatory cells in arthritic vs non-arthritic DR4 tg mice at the onset of arthritis. Lymphocytes were obtained from arthritic (n=3) and non-arthritic (n=3) DR4 tg mice at the onset of disease (day 50). Cells were stained with anti-Foxp3-allophycocyanin(APC), anti-CD4-fluorescein isothiocyanate(FITC), and anti-CD25-Phycoerythrin(PE) (Mouse regulatory T cell Staining Kit, eBioscience) and analyzed by flow cytometry.



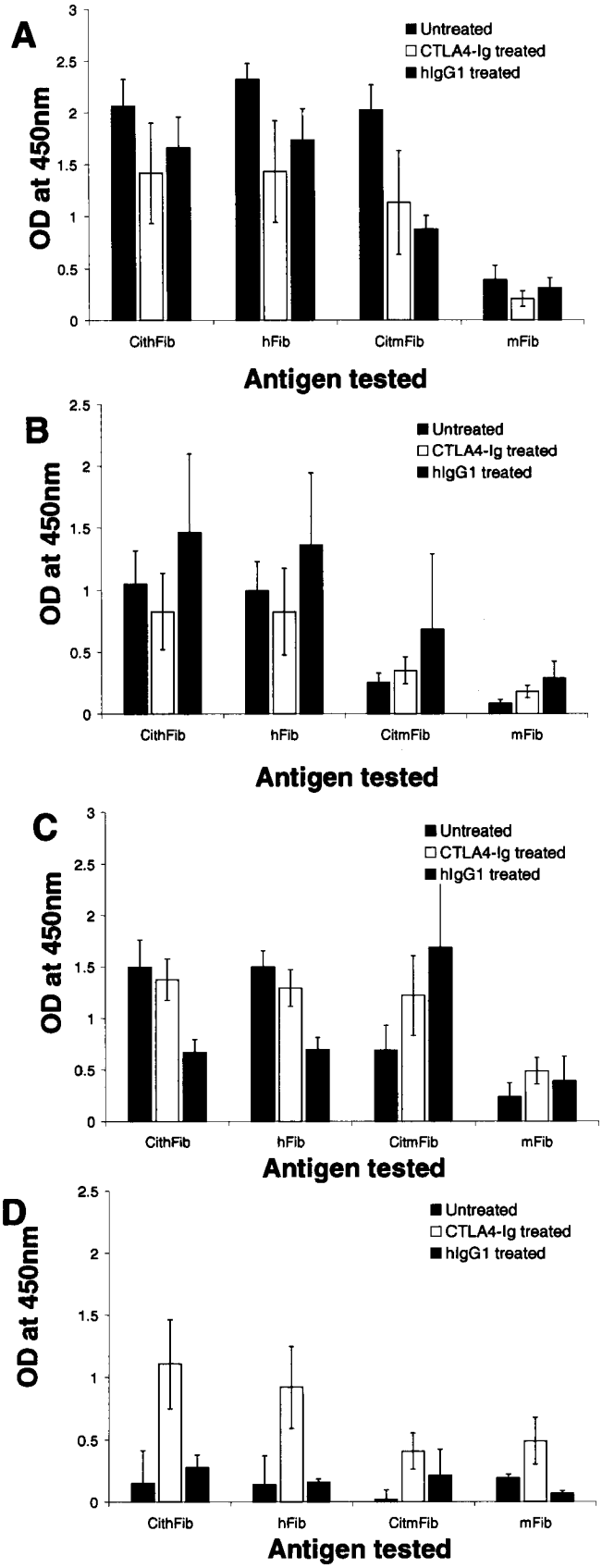
	Mean percent of stained cells (%)		
Disease status	**CD4-CD25**	**CD4-Foxp3**	**CD25-Foxp3**
Arthritic	1.307	1.707	1.197
Non-arthritic	1.357	1.720	1.230

Appendix 3. Invariant NKT cell frequency in arthritic and non-arthritic CithFib-immunized DR4 tg mice. There is no difference in the number of *i*NKT cells in arthritic vs non-arthritic DR4 tg mice at the onset of arthritis. Lymphocytes were obtained from arthritic (n=3) and non-arthritic (n=3) DR4 tg mice at the onset of disease (day 50). Cells were stained with anti-TCRβ-FITC (eBioscience) and mouse PBS-57-loaded CD1d tetramer-APC (National Institutes of Health Tetramer Core Facility, Bethesda, MD) and analyzed by flow cytometry.



	Mean percent of stained cells(%)
Disease status	**Tetramer-TCRβ**
Arthritic	0.267
Non-arthritic	0.333

Appendix 4. Effect of CTLA4-Ig treatment on antibody class in arthritic CithFib-immunized DR4 tg mice. **A-B,** On day 50 (onset of Cit-induced arthritis) arthritic mice were treated with CTLA4-Ig (n=6), hIgG1 control (n=5), or were left untreated (n=5). Following the treatment period, serum IgG1 **(A)** class antibody levels to CithFib (1in100000), hFib (1in100000), CitmFib (1in4000) and mFib (1in4000) were determined at the indicated serum dilutions. As well, IgG2c **(B)** antibody levels were determined to CithFib (1in5000), hFib (1in5000), CitmFib (1in200) and mFib (1in200) at the indicated serum dilutions. **C-D,** Similarly sera were also collected form mice treated at day 70 (peak of disease) with CTLA4-Ig (n=9), hIgG1 control (n=3) or were left untreated (n=3). IgG1 **(C)** and IgG2c **(D)** class antibody levels to these antigens were determined at the previously indicated serum dilutions. Testing was done using ELISA as described in the materials and methods, except that a secondary antibody against either IgG1 (Invitrogen, Camarillo, CA) or IgG2c (Beckman Coulter, Fullerton, CA) was used.


A
Untreated
CTLA4-Ig treated
hIgG1 treated
OD at 450nm
3
2.5
2
1.5
1
0.5
0
CithFib
hFib
CitmFib
mFib
Antigen tested
B
Untreated
CTLA4-Ig treated
hIgG1 treated
OD at 450nm
2.5
2
1.5
1
0.5
0
CithFib
hFib
CitmFib
mFib
Antigen tested
C
Untreated
CTLA4-Ig treated
hIgG1 treated
OD at 450nm
3
2.5
2
1.5
1
0.5
0
CithFib
hFib
CitmFib
mFib
Antigen tested
D
Untreated
CTLA4-Ig treated
hIgG1 treated
OD at 450nm
2.5
2
1.5
1
0.5
0
CithFib
hFib
CitmFib
mFib
Antigen tested