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Presentation Of Citrullinated Peptides Derived From Unmodified Protein By Antigen Presenting Cells.

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PRESENTATION OF CITRULLINATED PEPTIDES DERIVED FROM UNMODIFIED PROTEIN BY ANTIGEN PRESENTING CELLS.

by

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ABSTRACT OF THE DISSERTATION

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Autoimmune responses to citrullinated proteins have been associated with rheumatoid arthritis; however, little is known of the mechanism by which tolerance to citrullinated proteins is established or the biology of citrullination by cells of the immune system.

We find that immunization of B10.BR mice with HEL in CFA gives rise to T cells that specifically recognize citrullinated epitopes. We generated T cell hybridomas by fusing these T cells. T cell hybridomas that recognize citrullinated peptides from hen egg white lysozyme (HEL) were used to detect presentation of citrullinated peptides. Using this technique, we show that antigen presenting cells (APC) present citrullinated peptides after processing whole unmodified protein.

As expected, APC isolated from the draining lymph node of mice immunized with HEL in complete Freund's adjuvant elicit a response from the citrullinated peptide specific T cell hybridomas. Examination of APC from HEL transgenic mice revealed that splenic dendritic cells, macrophages and thymic dendritic cells presented citrullinated peptides constitutively. Conversely we did

not detect a response from B cells pulsed with HEL or from HEL transgenic mice or from a B lymphoma line.

The reported localization of the enzymes that convert peptidylarginine to peptidylcitrulline is either the cytosol or nucleus and not in antigen processing and loading compartments. The role of autophagy, or the mechanism by which cytosolic proteins and senescent organelles are taken up in to vesicles for degradation in lysosomes, in citrullination of antigen was examined.

Treatment of dendritic cells and macrophages with 3-Methyladenine (3MA), a class III PI3 kinase inhibitor known to inhibit autophagy, blocked presentation of citrullinated peptides but presentation of unmodified peptides was not affected.

B cell receptor engagement has been shown to induce autophagy in primary B cells. We examined presentation of HEL by B cells from anti-HEL transgenic mice, in which all B cell receptors bind HEL, and found that, in contrast to B cells from B10.BR mice, they presented citrullinated peptides. B cells from mHEL mice present citrullinated peptides after BCR engagement with anti-IgM or anti-IgG antibodies. Induced presentation of citrullinated peptides was blocked by 3MA.

In addition, we find that C3.F6.mHEL B lymphoma cells present citrullinated peptides after serum starvation but blocked by 3MA treatment. C3.F6.mHEL cells that stably expressed shRNA targeting Atg5, a protein essential for autophagy, did not present citrullinated peptides after serum starvation. Higher levels of citrulline in naturally processed peptides from these

cells were detected biochemically after culture in serum starved conditions. Our findings demonstrate a role for autophagy in citrullination of antigen during processing by antigen presenting cells.

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ABBREVIATIONS

3MA	3-Methyladenine
ACPA	anti-citrullinated protein antibodies
APC	antigen presenting cell
BCR	B cell receptor
BMDC	bone marrow derived dendritic cell
CCP	cyclic citrullinated peptide
CFA	complete Freund's adjuvant
DAMO	diacetyl monoxime
DC	dendritic cell
EAE	experimental allergic encephalomyelitis
FCS	fetal calf serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
HEL	hen egg white lysozyme
HPRT	hypoxanthine-guanine phosphoribosyl transferase
LC3	light chain 3
MBP	myelin basic protein
MHC	major histocompatibility complex
MS	multiple sclerosis
PAD	peptidylarginine deiminase
PEC	peritoneal exudate cells
PTM	post translational modification
RA	rheumatoid arthritis
RF	rheumatoid factor
SE	shared epitope
TCR	T cell receptor

CHAPTER 1
INTRODUCTION

Citrullination and Peptidylarginine deiminase

The process by which the immune system establishes tolerance to self-proteins bearing post-translational modifications is poorly understood. One post-translational modification, citrullination, has been implicated in the pathophysiology of two autoimmune diseases, rheumatoid arthritis and multiple sclerosis [1-6]. This body of work examines the ability of antigen presenting cells to impart this modification on protein antigen while processing for presentation on MHC class II. In this section, we will review what is known about citrullination in normal biology as well as the evidence for a role for citrullination in autoimmunity.

Citrullination

Citrulline is not a natural amino acid encoded in DNA and there is no tRNA specific to this amino acid. It was first described in 1914 when it was isolated from watermelon by Koga and Odake in Japan and in 1932 found by Krebs and Henseleit to be an intermediary of the urea cycle. Citrulline is not incorporated into proteins as they are synthesized. Citrulline in proteins was first described in 1930 by another Japanese group who described its presence in tryptic digests of caseinogen (Wada 1930). The presence of citrulline in proteins is due to the

enzymatic deimination of arginine residues in a process called citrullination. Citrullination is the hydrolytic deimination of arginine residues to yield ammonia and peptidylcitrulline. The change from arginine to citrulline leads to a net loss of one atomic mass unit and a loss of positive charge. Citrulline lacks the strongly basic character of arginine and is a neutral amino acid similar to glutamine. The loss of positive charge leads to altered inter and intra-molecular interactions and the functional consequences of this change have many important roles in normal biology [7, 8]. The detection of citrulline in biological samples has been largely facilitated by chemical derivatization of the ureido functional group of citrulline [9] that leads to the formation of a colored product.

Peptidylarginine deiminase

A family of proteins called peptidylarginine deiminases catalyzes this reaction [10-16]. There are five PAD isoform that have been identified (EC 3.5.3.15). The genes are clustered on chromosome 1p35-36 and conserved with synteny on mouse chromosome 4E1. The substrate specificity of these enzymes seems to be limited and the activity of the enzymes is regulated by the requirement for high calcium levels as well as by expression. The tissue expression of these proteins has been characterized. PAD2 is ubiquitously expressed. PAD1 and 3 are found in the skin and hair follicles as well as in the uterus. PAD4 is expressed by cells of the immune system. PAD6 is found in eggs, ovaries and early embryos. [17-24] These enzymes require high levels of

calcium (10^{-5} M) for biological function due to 3 conserved calcium binding sites that when bound induce a conformational change that reveal the active site and allow substrate binding [25]. The mechanism by which this enzyme catalyzes this reaction has been proposed by two independent groups. Arita proposed that the cysteine attacks the guanidino group and acts as a tetrahedral adduct while ammonia is released. A water molecule then acts as a nucleophile and attacks the adduct, releasing the regenerated cysteine and forming a ureido group [25]. In agreement with this report, Paul Thompson's laboratory introduced a series of mutations to provide evidence for a reverse protonation reaction in which the active site cysteine exists as a thiolate that is stabilized by forming an ion pair with a nearby histidine [26].

PAD Isoforms and roles for citrullination in biological processes.

PAD1, 2, &3- Hair and Skin

Perhaps the most well studied processes, as well as one of the earliest described roles for this post translational modification, are those of the cornification of skin and the hardening of the hair follicle.

The presence of citrulline in hair was described in 1962 by Rogers et al [27], who later went on to report that it was a Calcium dependent enzymatic activity that lead to the presence of citrulline in these proteins [28, 29]. The major citrullinated protein in the inner root sheath was found to be trichohyalin. Deimination of trychohyalin by PADs 1and 3 increases its solubility, releasing it

from the trychohyalin granules and allowing it to be linked to type1 keratins by transglutminases [8, 21].

In the epidermis, PAD enzymes 1, 2, and 3 are expressed and are involved in terminal cornification as well as maintenance of healthy skin through its role in the production of natural moisturizing factor. Deimination of the basic Keratin K1 and thus the loss of positive charge occurs during the process of differentiation from keratinocyte to corneocyte [30] and it is speculated that this alters the interaction with the acidic keratin K10, which is also deiminated, and facilitates relaxation of the corneocyte fibrous matrix [31]. In addition to Keratin in the skin, filaggrin is also deiminated. Filaggrin is synthesized as a profilaggrin, a large molecule that upon differentiation is deiminated and cleaved into individual filaggrin units which can then act to bundle keratin filaments into an organized matrix (Pearton 2002). Citrullination of filaggrin leads to increased susceptibility to proteolysis and allows it to be cleaved into amino acids that make up natural moisturizing factor [32] [33] [16].

Apoptosis

Citrullination may play an important role in apoptosis. Intermediate filaments are more stable than the dynamic microfilaments and the regulation of their polymerization has yet to be worked out. The arginine residues in vimentin, an intermediate filament expressed in various cell types, are required for polymerization. In vitro citrullination of vimentin filaments causes disassembly

[34, 35]. During apoptosis, where cytosolic calcium levels are very high, PAD enzymes deiminate vimentin and may be involved in apoptotic morphological changes [35].

In addition to regulating intermediate filament disassembly during cell death, citrullination may have a role in the induction of apoptosis. Over-expression of PAD4 in cell lines induced apoptosis through increased P53 and mitochondrial membrane depolarization[36]. Deimination of histones H2A, H3, H4 and nucleophosmin/B23 may alter chromatin organization and DNA susceptibility to fragmentation. This may lead to the induction of apoptosis through DNA damage signals as well as play a role in DNA fragmentation during apoptosis [22, 37, 38]. In cells undergoing calcium ionophore induced apoptosis, 70kDa nuclear protein associated with the nuclear lamina was found to be deiminated [39]. Citrullination of this protein reduced its isoelectric point and reduced its affinity for the inner nuclear membrane, which contributes to the degeneration of the nuclear lamina during apoptosis.

Myelin Basic Protein

PAD2 and PAD4 are expressed in the central nervous system [4, 40-44]. Myelin basic protein (MBP) is expressed by oligodendroglial cells and is a key component of the myelin sheath. It associates with lipids through ionic interactions and acts to help organize the lipids into compact multi-layered structures. There are several isoforms of MBP that undergo various

posttranslational modifications including citrullination [45]. These modifications regulate the strength of the interactions between the positively charged MBP and the negatively charged lipids [4, 41]. Posttranslational modifications of the different isoforms of MBP produce different charge isomers and this influenced the fluidity of the myelin sheath [46-48]. The ratio of deiminated MBP to total MBP is important for CNS function [49] and it changes through development of the central nervous system. In humans for the first two years nearly all of the MBP is deiminated [50-52] and the ratio of citrullinated MBP to MBP gradually goes down until the age of four where it remains at about 18%. This change corresponds to decreased plasticity of the brain as it develops. In addition, the areas of the CNS associated with more rudimentary function have been shown to have higher levels of citrullinated MBP [53].

Epigenetic regulation of gene expression

PAD4 is the only PAD isoform that can localize to the nucleus and does so by virtue of a monopartite nuclear localization signal [22]. Posttranslational modification of histones is a mechnism by which gene expression is regulated. The most well known histone modifications are methylation, acteylation and deacteylation [54-56]. PAD4 is also involved in chromatin remodeling. This enzyme has been shown to deiminate nuclear proteins including histones H2A, H3 H4 and nucleophosmin/B23 and has been shown to regulate gene expression

by this mechanism. In addition, hypercitrullinated histones have been detected in decondensed chromatin [57, 58].

It had previously been thought that histone methylation was an irreversible process as no histone demethylating proteins had been identified [59]. However PAD4 has been shown to be able to reverse histone methylation by converting arginine to citrulline and releasing methylamine [57].

PAD4 expression in the uterus and pituitary gland was shown to be dependent on expression of 17β-estradiol [60]. Interestingly, PAD4 acts as a transcriptional corepressor of estrogen responsive genes. Activation of estrogen responsive genes is partially due to the methyltransferase activity of CARM1 and PRMT1, which methylate histones and enhance gene expression [61, 62]. Wang et al. showed that these histones (specifically at the pS2 gene) were methylated in MCF-7 cells treated with estrogen and a concomitant increase in expression was observed. However after 40-60 minutes the amount of PAD4 at these sites doubled and citrullination at these sites was observed and coincided with transcriptional silencing [57]. There is specificity to PAD4 transcriptional silencing as no PAD4 was observed at control genes. It is not clear what mediates this specificity. One recent paper from the Fuks group has found that PAD4 physically associates with histone deacetylase1 and that these two proteins collaborate to regulate the cyclical expression of estrogen responsive genes [63].

Citrullination and Disease

Rheumatoid Arthritis

Autoantibodies to self proteins have long been used as a diagnostic marker for rheumatoid arthritis. One of the most compelling findings linking responses to post-translationally modified self-proteins to autoimmunity is the antibody response to citrullinated proteins in RA. Antibodies to self-antigens, previously known as anti-perinuclear factor [64] [65] [66], anti-keratin [67] [68] [69] antibodies , and anti-Sa antigen [70] have been found in patients with RA and were later shown to recognize citrullinated proteins [71, 72], specifically fillagrin, fibrinogen [73, 74] and vimentin [75]. A cyclic peptide derived from filaggrin has been used as an antigen in an ELISA used in clinical practice to diagnose RA with high specificity [76]. These anti-cyclic citrullinated peptide (anti-CCP) antibodies can be found early in disease and levels can be correlated with disease severity [77] [78] [79, 80] [77-79, 81] [7, 82]. Their concentration is higher in the arthritic synovium, which possibly reflects a local ongoing antigen driven immune response [83] [84, 85]. These results are highly suggestive that a response to a citrullinated antigen could be playing a role in the disease process.

A report by the Holers group demonstrated that in the collagen induced arthritis model there are anti CCP antibodies that develop with the same kinetics as antibodies to Type II collagen and appear before joint inflammation. In addition, they were able to partially tolerize mice with a peptide derived from human fillagrin containing one citrulline substitution, while the unmodified peptide

had no effect. They identified monoclonal antibodies that bound citrullinated fibrinogen as well as other citrullinated proteins. When they transferred these antibodies into mice that had been given a submaximal dose of a cocktail of monoclonal antibodies against type II collagen (Arthrogen) they found that the mice developed a more severe disease. Transfer of the antibodies was also able to overcome the tolerizing effect of the citrulline containing peptide. They showed that there was an increase in citrullination of proteins in the synovium of mice with CIA. They observed an increase in citrullinated protein in the lymph nodes of mice with CIA. They concluded that presentation of citrullinated antigen in the lymph node was driving the generation of the antibodies though they did not evaluate how this might occur. They speculated that PAD enzymes might be released by infiltrating neutrophils or macrophages, leading to citrullination of antigen [86].

There is a strong association between the presence of the shared epitope (SE) and development of anti-CCP antibodies [87, 88]. Hill et al. showed that peptides derived from vimentin with citrulline substitutions at residues that lie in the P4 pocket of MHC containing the shared epitope had a much higher affinity than the same peptide with arginine at that position. Further, they found that immunization with citrullinated peptides in CFA elicited bulk T cell responses while immunization with unmodified peptide did not, which suggested that the mice were not tolerant to citrullinated vimentin [89].

In more recent work, Hill et al. demonstrated that immunization of DR4 transgenic mice with citrullinated fibrinogen, but not unmodified fibrinogen

induced an arthritis-like disease. Control mice (C57Bl/6) were not susceptible, suggesting that the HLA transgene played a key role in the response to citrullinated proteins. There was a notable difference in the pathology of these mice in that there was no infiltration of polymorphonuclear cells. They examined T cell specificity based on peptides of fibrinogen that were predicted to bind to HLA DR4 and found T cells that were reactive to citrullinated peptides [90]. This study represents an attempt to directly show that there is a lack of tolerance to neoepitopes generated by citrullination of antigen. However, a major caveat to this conclusion comes from studies that demonstrate that derivatization of protein antigen can break tolerance [91, 92]; much in the same way that Type B responses to self antigen are achieved [93, 94]. It may be that the process of citrullination of fibrinogen denatures the protein [8] rendering it available for presentation in a less stable conformation and priming responses of T cells that have escaped negative selection. Nevertheless, this report is intriguing and the results merit further study. The notion that citrullination of self-antigen represents a mechanism by which tolerance can be breached is attractive because it provides a simple explanation for a potential cause of RA. However, as this report demonstrates, this idea is a gross oversimplification of a very complex process that likely has many contributing factors. A basic understanding of the biology of citrullination within the cells of the immune system is necessary to fully begin to understand what role if any this may play in the autoimmune process.

Multiple Sclerosis

Multiple sclerosis (MS) is a debilitating chronic progressive disease that is characterized by the destruction of the myelin sheath. This leads to a deficiency in saltatory conduction that contributes to the neurological symptoms experiences by patients with MS. As the disease progresses there is eventual axonal destruction and this leads to paralysis and ultimately death [95, 96].

The underlying cause of MS is unknown; however the disease is marked by lymphocytic infiltration accompanied by macrophages microglia and astrocytes suggesting an autoimmune component [97]. In addition there is some evidence to suggest that primary neurodegeneration is followed by an autoimmune response [98-101] Citrullination has been implicated in both of these aspects of the etiology of MS.

MBP citrullination influences the strength of the interactions with the membrane and fluidity of the myelin sheath (See above). It has been reported that there is an increase in the amount of citrullination in MS lesions and that levels of citrullinated MBP and glial fibrillary acidic protein (GFAP- an intermediate filament) can be correlated to the severity of the disease [44] [50] [102]. MBP that is heavily citrullinated cannot organize the compact multi-layered lipid structures that are a key component of the myelin sheath [103] due to the alteration of the electrostatic interactions between the lipid bilayer and the charged MBP. This leads to a decrease in the stability of the multi-layered lipid structure of the myelin sheath [4, 41]. In addition, MBP that is citrullinated has a

looser and more disordered conformation and is therefore more susceptible to degradation by myelin associated proteases, specifically Cathepsin D. [104] [8] [48] [41]. These observations have also been seen in the murine autoimmune model of MS, experimental allergic encephalomyelitis (EAE) [105] [106] as well as in DM20 transgenic mice, a model for degenerative demyelination [107].

Both PAD2 and PAD4 have been found to be expressed in the CNS. PAD2 is expressed in oligodendroglial cells, astrocytes, and microglial cells. PAD4 can also be detected in the brain and was recently shown to be detected in both the cytoplasm and the nucleus of oligodendroglial cells. PAD2 [42] and PAD4 [108] expression levels are higher in diseased brain, both in human MS and murine EAE. Interestingly, PAD2 knockout mice are still susceptible to EAE [109, 110]. PAD4 is active in these mice and can be localized to the target cells and citrullination of MBP still occurs although at fewer sites in the protein [42].

The immunological aspects of MS indicate that an autoimmune component may be involved. The similarities between EAE, a T cell mediated disease and MS provide some clues to the autoimmune process that may be occurring in MS. The pathology of the MS plaque includes a robust inflammatory infiltrate that includes T lymphocytes [101, 111]. MBP has been identified as a candidate autoantigen in MS [112, 113]and T cells that recognize MBP have been isolated from peripheral blood of patients with MS [114-116]. The degradation of citrullinated MBP by Cathepsin D releases an immunogenic peptide [43, 117].

As in the case with RA, it has been speculated that citrullination increases immunogenicity of self-proteins and therefore has some role in the pathogenesis of MS. The evidence for this comes from the isolation of T cell lines from patients with MS and normal controls that were shown to recognize citrullinated MBP [118]. More recent work from the same group has elaborated on this finding [119]. They evaluated 5 subject pairs of one healthy control and one MS patient. Peripheral blood lymphocytes were cultured with MBP-C8 (citrullinated MBP) and recall responses to either MBP-C1 (unmodified MBP) or MBP-C8 were assessed. They found that in each pair the MS patients had a higher stimulation index, a higher number of reactive T cells, or both. These findings indicate that T cell responses to citrullinated MBP are elevated in patients with MS but still present in healthy controls. As in the case with RA, the foundational understanding of the biological roles for citrullination in the immune system must be established before questions of tolerance can properly be addressed.

Autophagy and Antigen Presentation

Autophagy is a ubiquitous catabolic process by which cytosolic components are shuttled in to vesicular compartments for degradation in lysosomes. This process has been conserved through evolution and is involved in the homeostatic degradation of cytosolic proteins and turnover of senescent organelles [120-128]. The extra cellular signals that lead to the initiation of autophagy include growth factor removal, amino acid starvation, infection, DNA

damage, and ER-stress among many others. This is in addition to the constitutive autophagy that cells undergo to maintain homeostasis. The intra cellular cascade that initiates and mediates autophagy is very complex and includes a growing number of proteins most of which have been named Atg proteins.

The autophagosomal membrane has been suggested to derive from a poorly characterized organelle called the phagophore [129-132]. The formation of the of the phagophore is dependent on a class III phosphoinositide 3-kinase, Vps34, that forms a part of a large complex made of several proteins including Atg6 (also called Beclin 1), Atg14 and Atg15. This complex is required for the generation of the nascent autophagosome [133-135]. Vps34 is the target of chemical inhibition of autophagy by treatment with 3-Methyladenine and wortmannin [136].

There are two ubiquitin-like conjugation systems that converge on the formation of a fully formed autophagic vesicle, the Atg12/5 and the Atg8/PE systems. The process of extending the phagophore into a vesicle, or elongation, starts with Atg12 conjugation to Atg5 by Atg7 (E1-like). The Atg12/5 complex remains only until the autophagosome is formed and then dissociates. This complex associates with Atg16L [137, 138]. Microtubule–associated protein 1 light chain 3 (LC3- also known as Atg8) is the major player in the second conjugation system. The 23 amino acids at the C-terminus of LC3 are cleaved by the cysteine protease Atg4 leaving an exposed glycine residue and forming LC3I. This is then modified by a series of interactions with Atg3 and Atg7 to form LC3II 16 (kDa) and ultimately conjugated to phosphatidylethanolamine [139]. LC3

remains associated with the autophagosome and is the only known marker specific for the autophagic vesicle.

The observation that peptides from cytosolic sources were presented on MHC class II is not a new one. In 1993 Brooks et al. reported that APC expressing cytosolic HEL could elicit responses from 3A9 [140]. Brazil in 1997 showed that C5 could be presented to T cell if excessive degradation was prevented by treatment with low doses of the lysosomotropic agent ammonium chloride. Furthermore they showed that inhibition of autophagy with 3MA blocked this presentation [141]. Studies evaluating naturally processed peptides from MHC II molecules have revealed that peptides derived from cytosolic proteins are presented at significant levels [142] [143] [144] [145] [146]. This was directly addressed in a study by Dengjel et al. that evaluated the repertoire of naturally processed peptides isolated from cells that had been treated to induce autophagy. In addition to showing a marked increase in the amount of peptides derived from cytosolic sources, they also reported an effect of starvation on peptides from the same source proteins and found a decrease in the activity of various cathepsins in the starved cells indicating that starvation may have some effect on MHC II processing [147]. Nimmerjahn et al., using EBV transformed B cells expressin cytosolic and renal cell carcinomas that expressed cytosolic neomycin phosphotransferase, demonstrated presentation to T cells through autophagy as inhibition with 3MA blocked presentation. They excluded cytosolic processing by using inhibitors of Class I processing and directly showed the protein in endosomes and lysosomes by western blots of subcellular fractions

[148]. Paludan et al. showed that presentation of a viral nuclear antigen was dependent on autophagy, indicating that nuclear components can access autophagic compartments [149].

It is clear based on these studies that autophagy contributes to antigen loading compartments. Work by Dorothy Schmid evaluated the prevalence of this in various APC and found that there was continuous input from autophagic compartments into MCH II processing vesicles in dendritic cells, macrophages, B cells, and Class II positive eptithelial cells. Interestingly they were able to strikingly enhance presentation of a protein by targeting it to the autophagosome via fusion to LC3.

In mice that express a green fluorescent protein LC3 fusion it was found that thymic epithelial cells have a relatively high level of constitutive autophagy. Thymi taken from embryonic Atg5 knock out or control mice were transplanted in to the kidneys of wild type mice. Using a series of T cell receptor transgenic mice they found differences in positive selection for CD4 T cells but not in CD8 T cells. These thymi were transplanted in to athymic nude mice and it was found that recipients of Atg5 knock out thymi developed a severe multi-organ autoimmunity. This disease was transferable by adoptive transfer of T cells [150].

This report shows that autophagic contribution to the MHCII peptide repertoire in the thymus is involved in the T cell selection and plays a significant role in the deletion of autoreactive T cells.

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CHAPTER 2

This chapter is taken from our first report. It is titled: Cutting Edge: Unique T Cells That Recognize Citrullinated Peptides Are a Feature of Protein Immunization by Jamie Ireland, Jeremy Herzog and Emil R. Unanue and was published in the Journal of Immunology in volume 177 on pages 1421-1425 in 2006. It is reprinted with the permission of the Journal of Immunology.

It is the first description in the literature of T cells clones that exclusively recognize citrullinated epitopes and the ability of APC to citrullinated antigen during processing for presentation on MHC II molecules. This project was initiated to evaluate presentation of citrullinated peptides. Our first aim was to develop a system by which we could detect presentation of citrullinated peptides. Since citrullination leads to such a small change in molecular weight (1 atomic mass unit), mass spectrometry was not a practical choice for a first general evaluation of antigen presenting cells. We chose to use HEL as a model antigen because processing and presentation of HEL on I-A^k has been well studied and we have access to several potentially useful reagents. The major findings of this paper are:

1- Immunization of B10.BR mice with HEL in CFA gives rise to a T cell response that includes T cells that specifically recognize citrullinated peptides. This suggests that APC that are priming T cells are presenting some level of citrullinated peptides.

2- Two panels of hybridomas were examines in this study to two citrullinated variants of immunodominant epitopes of HEL on I-A^k; 48-62cit61 and 114-129cit125. In Figures 1 and 2 of this paper we demonstrate that the reactivity of these hybridomas is specific to citrullinated peptides and there is no cross reactivity to unmodified peptides. We further show that the T cell contacts are still required for reactivity.

3- Citrullination of 48-62 leads to little change in binding. However the change of 114-129 to 114-129cit125 leads to an increase in binding affinity by removing the Arginine at P7 which is a hindering residue. A similar increase in binding affinity has also been reported in some peptides binding to RA susceptible HLA alleles that contain the shared epitope. The results or our binding assays are summarized in Table 1.

4- Peritoneal macrophages and bone marrow derived dendritic cells present citrullinated peptides after processing whole unmodified HEL. Figure 3 shows 3 hybridomas that recognize 48-62cit 61 respond to presentation of HEL. There some small enhancement in the presence of LPS, however further studies have shown that this is not specific to presentation of citrullinated peptide and is also seen with presentation of the unmodified peptide.

Together these findings demonstrate that citrullinated peptides are presented after processing and suggest APC may play role in citrullination of antigen *in vivo.* Furthermore, we establish the utility of our hybridomas as a reagent to study presentation of citrullinated peptides.

Abstract

Abs against citrullinated proteins are present in patients with rheumatoid arthritis. In this study, we describe a unique cohort of T cells that selectively responded to citrullinated variants of two epitopes of hen egg-white lysozyme, a major and a minor one, bound to the MHC molecule, I-A^k. In addition, we show that when given an intact, unmodified lysozyme protein, dendritic cells and peritoneal macrophages presented citrullinated peptides and stimulated modification-specific T cells. Thus, presentation of citrullinated-peptide-MHC complex is a feature of immune responses to protein Ags.

Introduction

Antibodies to a variety of citrullinated proteins are found in a very high number of patients with rheumatoid arthritis (RA)[3] and can be used as a highly specific clinical index of the disease. Citrullination of arginines is a posttranslational change resulting from the action of peptidylarginine deiminases (PAD), a family of enzymes found in different cells including neutrophils, monocytes, and macrophages (1, 2, 3, 4, 5, 6). The immunological consequences of deimination of arginines are therefore of great interest, particularly with respect to their possible pathogenetic significance in RA. The issue of whether citrullinated proteins may be autoantigenic has been raised, not only in RA but also in cases of inflammatory disease of the nervous system where citrullination occurs in myelin basic protein (7, 8). Citrullinated proteins have been found to be antigenic (9, 10).

Posttranslational changes have been reported in MHC-bound peptides (11, 12). We recently called attention to nitration of tyrosine residues (13) and oxidation of tryptophans on peptides from the protein, hen egg-white lysozyme (HEL), bound to the class II histocompatibility molecule I-A^k (14). These changes took place when APCs were activated during immunological reactions including infections. T cells highly specific to the changes were generated in vivo, and some escaped negative selection in HEL transgenic mice.

With the findings of citrullinated proteins in RA, we searched for such a modification in MHC-bound peptides using approaches akin to those mentioned above. Again, using HEL as a model Ag, we show in this study that T cells specifically reactive to citrullinated epitopes were among the responding repertoire to immunization with an unmodified HEL protein. We also found that APC presented modified peptides when provided an intact, unmodified HEL ex vivo. Finding T cells specific to citrullinated peptides adds another dimension to the reports of Abs in RA.

Materials and Methods

Cell culture

CD4 T cell hybridomas were made by fusing cells obtained from popliteal lymph nodes 7 days postimmunization with 10 nmol of HEL in CFA. First, lymphocytes were cultured for 1 wk in the presence of 1 μM peptide. The cells were then stimulated with fresh peptide and irradiated splenocytes as APC. Three days

later, the cells were fused to BW5147α-β-thymoma cells line, as described previously (15).

C3.F6 B lymphoma cells were used as APC for examining the response to the various peptides. Peritoneal macrophages were obtained by i.p. injection with 100 µg of Con A. After 4 days, peritoneal exudate cells (PEC) were harvested. Dendritic cells (DC) were derived from bone marrow cultured for 6 days in GM-CSF containing medium as described previously (16). Hybridoma activation was measured by IL-2 secretion as assayed by CTLL proliferation. ELISPOT assays were conducted according to the BD Biosciences protocol.

For limiting dilution cloning, mice were immunized with 10 nmol of HEL in CFA in the hind footpads. Seven days later, popliteal lymph nodes were harvested. Lymphocytes were plated in round-bottom plates with 5 x 10^5 irradiated (2000 rad) splenocytes with 50 U/ml rIL-2 and 5 µM 48–62cit61 peptide. Clonal growth-positive wells were maintained in these culture conditions.

Mice and reagents

B10.BR mice, usually 6- to 12-wk-old, of both sexes were obtained from The Jackson Laboratory and maintained in facilities at Washington University. B10.BR-Tg(KLK4mHEL)6Ccg mice expressing membrane HEL under the class II Eα promoter were generated and maintained by our laboratory (15). HEL was obtained from Sigma Chemical and purified to eliminate contaminant proteins and

LPS. Peptides were synthesized using Fmoc techniques. LPS (Sigma-Aldrich) was used at a final concentration of 1 μg/ml.

RT-PCR

RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer's instructions. The RNA was treated with DNA-free (Ambion), then reverse-transcribed using Superscript First-Strand Synthesis (Invitrogen Life Technologies), and amplified using the following primer sequences: PAD2, TACAGATTCCCGTACACGTTGCGT and AACTGGCCAGAGAATTGAGGACCA with an expected amplicon of 245 bp; and PAD4, CCAAGAAAGCCAAGTGCAAGCTGA and TTCCCGATGAGAATTCTGCCCAGT with an expected amplicon of 316 bp.

Relative binding affinity

Peptide-binding affinity for I-A^k was measured as described previously (17), using baculovirus-purified I-A^k molecules. Results indicate the concentration of unlabeled peptide that inhibited by half the binding of an ^{125}I-labeled peptide standard.

Results

Immunization with HEL gives rise to T cells that are specific to citrullinated epitopes

Mice were immunized with HEL in CFA, and 1 wk later the draining lymph node cells were isolated and screened for CD4 T cells to citrullinated HEL epitopes. After a 7-day culture in the presence of citrulline (either HEL 48–62cit61 or HEL 114–129cit^{125}) (Table I), followed by a 3-day stimulation with irradiated splenocytes, the cells were fused to create T cell hybridomas. In a different approach, the lymph node cells of the mice immunized with HEL were placed in an ELISPOT assay against the modified peptides, or were cloned by limiting dilution.

Peptide 48–62 represents the major chemically dominant segment of HEL selected from its processing (18). This family of peptides centers on the nine-residue core from 52 to 60. Arginine, underlined, is the tenth residue on this peptide: DYGILQINSRW. The crystal structure of the 48–62 peptide bound to I-A^k showed that the P10 arginine and the P11 tryptophan were solvent exposed (19). As expected, changing the arginine 61 to citrulline did not change the binding of the 48–62 peptide to purified I-A^k molecule (Table I).

Several hybridomas were found that responded to 48–62 with a citrullinated P10, and not to the wild-type arginine residue. Three representative T cells are shown in Fig. 1*A*, labeled Pepe, Granny, and Marvin, and each responded with varying degrees of sensitivity to 48–62 but only with a citrulline in

place of the arginine at P10. These hybridomas did not secrete IL-2 in response to stimulation with wild-type peptide. All of the citrulline-specific hybridomas showed an absolute dependence on the presence of the tryptophan at P11, that is, responded to 48–62cit61 but not 48–61cit61. This finding is not surprising as the screening process selected for a population of T cells whose receptors recognized C-terminal residues. About half of the T cells that responded to the 48–62 segment of HEL also required the presence of Trp^{62} (20, 21).

It is known that most of the T cells that interact with the 52–60 core segment interact with three TCR contact residues, Tyr^{53}, Leu^{56}, and Asn^{59} (19, 21, 22). Such was the case for the T cells that recognized cit61 (Fig. 1*B*). None recognized the alanine substitutions for Tyr53 or Asn59. Additionally, the response was severely hindered when leucine at the P5 position, a centrally positioned TCR contact, was replaced by an alanine. Whether the TCR directly interacted with the cit^{61} and Trp^{62}, or the latter imparted a conformational change of the three TCR contact residues of the core segments is not known.

To further define the specificity of the hybridomas reactive with 48–62cit61, we assessed the effects of several amino acid substitutions at the Arg61 residue (Table II). Replacement of the Arg61 with alanine or glycine resulted in a markedly diminished response. The substitution of glutamine, an amino acid with structural similarities to citrulline, induced a similar or smaller response among the various T cell hybridomas. (Fig. 1*A* and Table II). In contrast, substitution at P10 with lysine completely abrogated the response in data not shown. T cell

hybridomas that recognize unmodified 48–62 were tested for reactivity to the modified peptides: we found no difference in the responses to stimulation with the various substitutions.

ELISPOT analysis of spleen cells from mice immunized with HEL indicated that frequency of T cells reactive with 48–62cit61 is ~1:36,000. The ELISPOT, however, did not distinguish those T cells that exclusively reacted with the citrullinated peptides. The frequency of T cells responding to 48–62 was ~ 1:25,000. We therefore conducted cloning of primary T cells from HEL-immunized mice by limiting dilution. Each clone was tested against 48–62 and 48–62cit61. Analysis of 23 primary T cell clones showed that 8, or 35%, reacted only to 48–62cit61, whereas the other 15 responded to both 48–62cit61 and 48–62.

Peptide 114–129 binds weakly to I-A^k molecules, although it induces a relatively strong T cell response (23). The binding core in 114–129 is from residues 119–127: DVQAWIRGC. Arginine is at P7 in the peptide and is known to be a negative or hindering residue. Peptide 114–129cit125 bound with an IC_{50} of 1 μM, whereas the unmodified peptide bound at 3.8 μM (Table I). These findings are in agreement with a recent report on the binding of a citrullinated peptide to HLA DRB1*0401 (24).

Immunization with HEL and selection for T cells that recognized a citrulline for the Arg125 resulted in T cells that specifically recognized the modification. Two representative T cells specific for the 114–129 peptide with citrulline at

residue 125, C68 and Everardo, are shown in Fig. 2. Unlike the hybridomas that recognize 48–62cit[61], these did not react with peptides in which the arginine at 125 was replaced with glutamine. Similarly, hybridomas that recognized the 114–129 epitope did not recognize the citrullinated peptide (Table II). Two ELISPOT assays were done. The number of 114–129-reactive T cells after HEL immunizations were 1:24,539 and 1:36,000. The numbers of 114–129cit125-reactive T cells were much less: 1:70,000 and 1:142,857. Collectively, these data indicated that citrullinated peptides were presented in vivo, from HEL processing, and elicited specific T cell responses.

Primary APC present modified peptides when given whole HEL

We tested the abilities of different APC to present the modified peptides when cultured with HEL. To this end, we examined bone marrow-derived DCs (BMDC), adherent PECs, or the C3.F6 B lymphoma line (Fig. 1*A*), as APC. C3.F6 pulsed with whole HEL did not stimulate the hybridomas that were specific to 48–62 cit61. The hybridomas specific for 48–62cit61 responded to whole HEL when presented by BMDC (Fig. 3*A*) or PEC (Fig. 3*B*). The response was increased after addition of LPS to the cultures: with Marvin the response was only detected after LPS stimulation. In addition, DC ($CD11b^{-}$, $CD11c^{+}$) and macrophages ($CD11b^{+}$, $CD11c^{-}$) isolated from B10.BR spleens pulsed with HEL presented 48–62cit61.

In data not shown, less sensitive hybridomas responded weakly or not at all, suggesting that the levels of 48–62cit61 may be limiting. In support of this idea, the 114–129cit125-specific hybridomas, C68 and Everardo, did not respond to HEL presented by BMDC or PEC. Both of these hybridomas are much less sensitive. The levels of the 114–129 epitope of HEL presented on I-A^k are low relative to the levels presented by the 48–62 family. Lastly, in data not shown we found that BMDC and PEC from lysozyme transgenic mice stimulated the 48–62cit61 hybridomas. The B lymphoma line C3.F6 did not present to either set of T hybridomas (Figs. 1 and 2). The conversion of arginine to citrulline requires the activity of PAD enzymes, which are cytosolic or nuclear and are active only at high calcium concentrations, ≈10^{-5} M (4). There have been several reports indicating that monocytes express PAD enzymes (25, 26, 27, 28), and in this study we indicate that both BMDC and peritoneal macrophages expressed PAD2 and PAD4 by RT-PCR. C3.F6 expressed PAD2 but not PAD4 (Fig. 3*C*).

Discussion

The surprising observation made in this study is the presence of T cells to citrullinated peptides upon immunization with the highly immunogenic HEL protein. HEL was given in CFA, which induces a strong inflammatory response. In a previous study, we found that, in the draining lymph node, ≈1% of macrophages and DC contained the HEL following similar conditions of immunization as used here (29). Actually, by ELISPOT, the number of T cells reactive with the citrullinated peptides was relatively high. We conclude that

strong immunization measures can drive the process in APC that leads to citrullination of protein segments that are processed into peptide-MHC complexes. Now that we recognize that such T cells exist, we are actively searching for them in different specific tissue autoimmunities and inflammatory conditions as well as outlining the mechanisms of citrullination of Ag during presentation by APC.

The steps involved in citrullination of Ag require analysis, taking into consideration the properties of the PAD enzymes. The mechanism by which presentation of the modified epitopes occurs is puzzling considering the high level of calcium required and the localization of the enzymes in the cell. A high calcium level could take place during apoptosis, although we have not discerned an obvious apoptotic response in our cultures. The lack of presentation by C3.F6 suggests that PAD4 may be the critical enzyme, but this conclusion needs to be substantiated in experiments where this enzyme is removed from DC or macrophages. Such experiments are in progress.

Having identified specific T cells to citrullinated proteins as part of the response to immunization now begs the question of their immunopathologic role. What is their meaning in the context of the findings in RA? Because citrullination of proteins in the joint is such a striking feature, will T cells to the modified epitopes be enriched there? If such T cells develop in inflamed joints against protein components, will they be pathogenic? The issue of whether these responses are involved in disease pathogenesis or a byproduct of a more general

immunological reaction must be clarified before steps to target deimination as a treatment are taken.

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Footnotes

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[3] Abbreviations used in this paper: RA, rheumatoid arthritis; PAD, peptidylarginine deiminase; HEL, hen egg-white lysozyme; PEC, peritoneal exudate cell; DC, dendritic cell; BMDC, bone marrow-derived DC.

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Table 1. Peptide binding results

Peptide	Sequence	IC50 (μM)
48-62	DGST *D YG I L QI N S* RW	0.07
48-62 cit61	DGST *D YG I L QI N S* citW	0.06
114-129	RCKGT *D VQ A W IR G C* RL	3.8
114-129 cit125	RCKGT *D VQ A W Icit G C* RL	1

Figure 1.

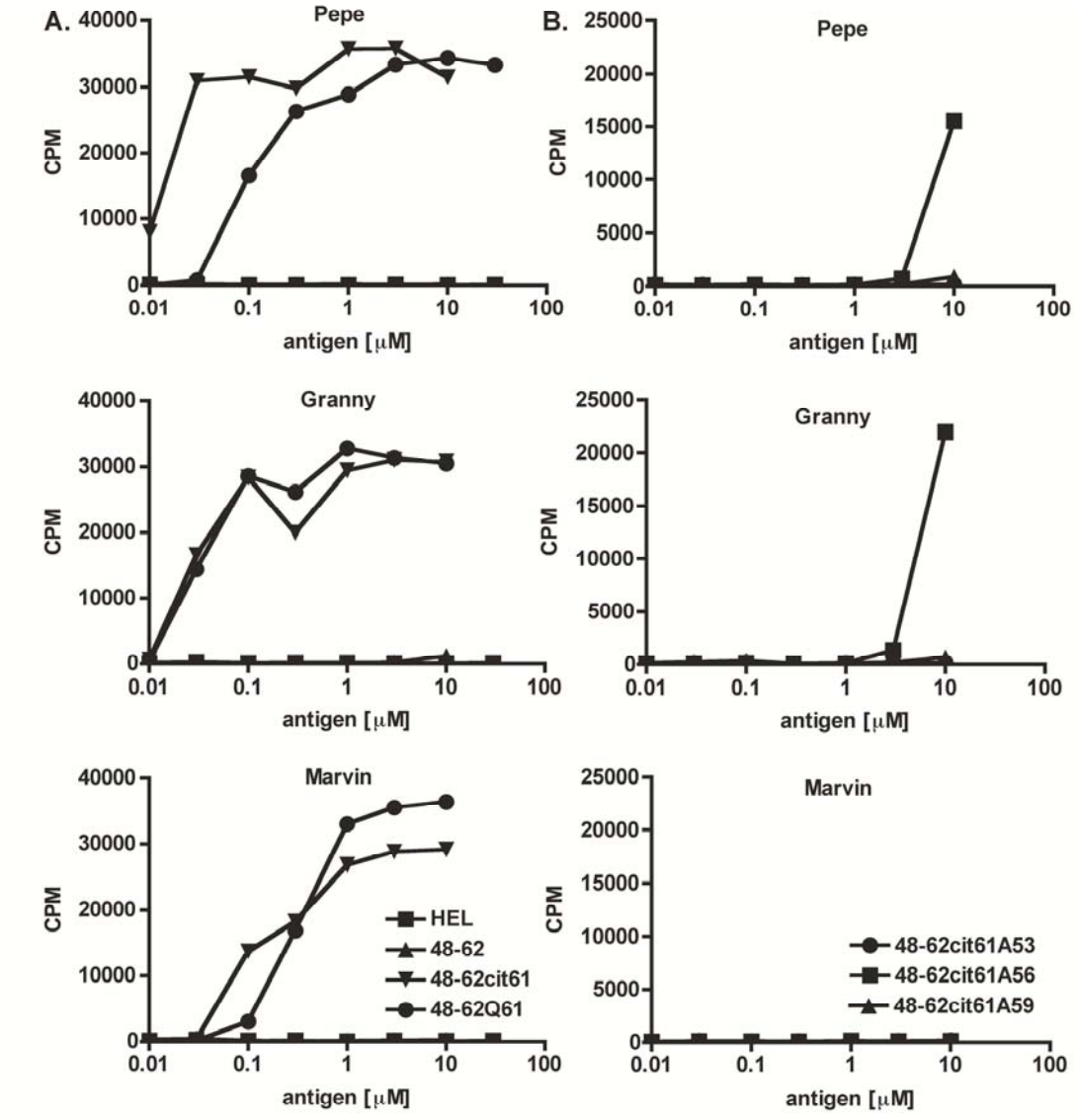
A.
Pepe
CPM
antigen [μM]
B.
Pepe
CPM
antigen [μM]
Granny
CPM
antigen [μM]
Granny
CPM
antigen [μM]
Marvin
CPM
antigen [μM]
HEL
48-62
48-62cit61
48-62Q61
Marvin
CPM
antigen [μM]
48-62cit61A53
48-62cit61A56
48-62cit61A59

Figure 1.

T cell hybridomas are reactive to the 48–62cit61 peptide. *A*, Hybridomas were cocultured with C3.F6 as APC and tested with 1.5 log dilutions of 48–62 unmodified or with citrulline or glutamine replacements at P10 or whole HEL. *B*, The response of the hybridomas to 48–62 cit61 with alanine substitutions at TCR contact positions.

Figure 2.

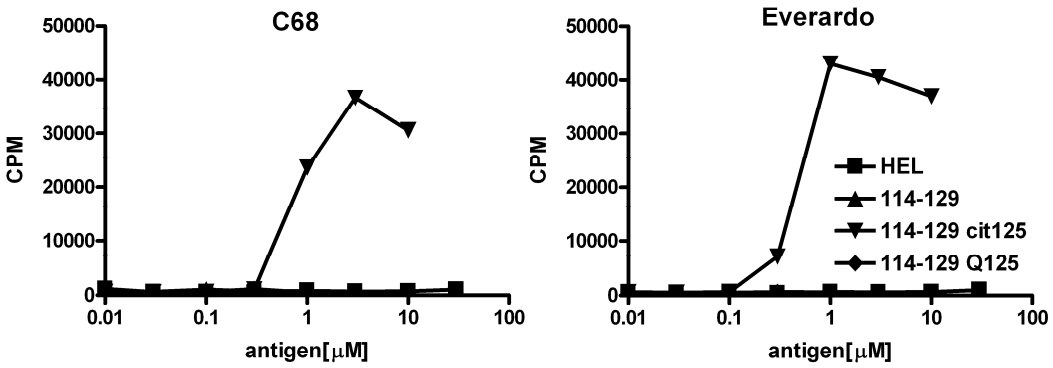
C68
Everardo
CPM
50000
40000
30000
20000
10000
0
0.01
0.1
1
10
100
antigen[μM]
HEL
114-129
114-129 cit125
114-129 Q125

Figure 2.

C68 and Everardo are reactive to citrullinated 114–129. Hybridomas were cocultured with C3.F6 as APC. HEL or peptide was added to the cultures in 1.5 log dilutions, and IL-2 secretion was assayed by proliferation of CTLL cells.

Figure 3.

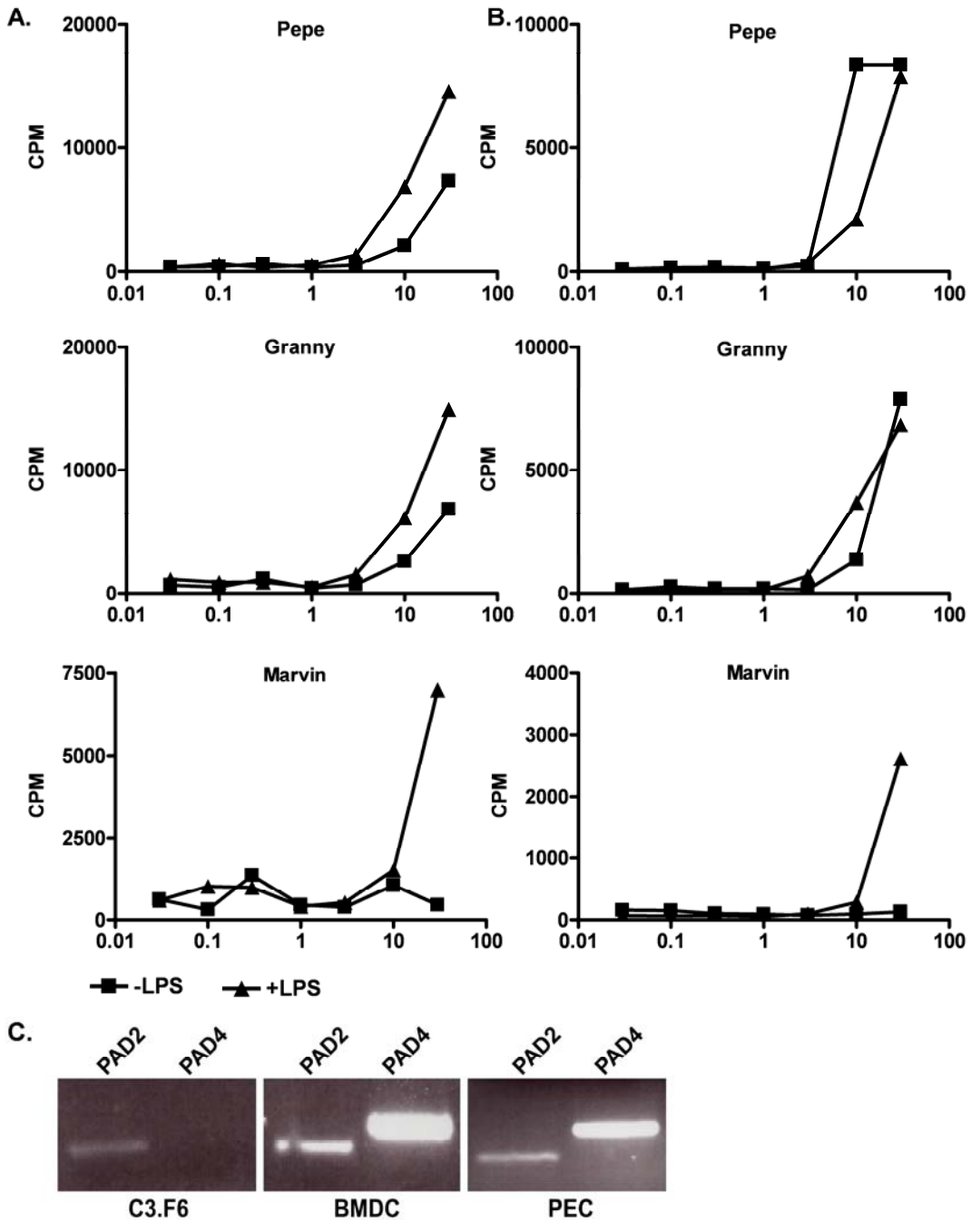
A.
B.
Pepe
Granny
Marvin
CPM
20000
10000
0
5000
7500
2500
4000
3000
2000
1000
0.01
0.1
1
10
100
-LPS
+LPS
C.
PAD2
PAD4
C3.F6
BMDC
PEC

Figure 3.

APC present modified peptides from HEL. BMDC (*A*) or PEC (*B*) were cultured overnight with dilutions of HEL. The cells were washed and cocultured with the hybridomas, and IL-2 secretion was assayed. *C*, Expression of PAD 2 and PAD 4 in APC used in this study. Total RNA was isolated from cells, reverse-transcribed, and amplified using internal primers to semiquantitatively detect mRNA levels.

CHAPTER 3

Presentation of citrullinated peptides by class II histocompatibility molecules is associated with autophagy.

This chapter consists of our second report as we prepare to submit for publication. In this chapter, we demonstrate a role for autophagy in citrullination of antigen by APC. To avoid redundancy, we omit the introduction as the material has been covered in chapter one of this thesis.

Presentation of citrullinated peptides by class II histocompatibility molecules is associated with autophagy.

Presentation of citrullinated peptides by class II histocompatibility molecules is associated with autophagy.

Abstract

Autoimmune responses to citrullinated proteins have been associated with rheumatoid arthritis; however, little is known of the mechanism by which tolerance to citrullinated proteins is established or the biology of citrullination by cells of the immune system. Using T cell hybridomas that recognize citrullinated peptides from hen egg-white lysozyme (HEL) we have shown antigen presenting cells (APC) present citrullinated peptides after processing whole unmodified protein. APC isolated from the draining lymph node of mice immunized with HEL in complete Freund's adjuvant presented citrullinated peptides. Examination of

APC from HEL transgenic mice revealed that splenic dendritic cells, macrophages and thymic dendritic cells presented citrullinated peptides constitutively. Treatment of APC with 3-Methyladenine (3MA) blocked presentation of citrullinated peptides but presentation of unmodified peptides was not affected. Presentation of citrullinated peptides was not detected on splenic B cells or B lymphoma cells under normal culture conditions. B cell receptor engagement has been shown to induce autophagy in primary B cells. We examined presentation of HEL by B cells from anti-HEL transgenic mice, in which all B cell receptors bind HEL, and found that, in contrast to B cells from B10.BR mice, they presented citrullinated peptides. B cells from mHEL mice presented citrullinated peptides after BCR engagement with anti-IgM or anti-IgG antibodies. Induced presentation of citrullinated peptides was blocked by 3MA. We find that C3.F6.mHEL B lymphoma cells presented citrullinated peptides after serum starvation and this was also blocked by 3MA treatment. Higher levels of citrulline in naturally processed peptides from these cells were detected biochemically after culture in serum starved conditions. Furthermore, C3.F6.mHEL cells that stably express shRNA targeting Atg5, a protein essential for autophagy, did not present citrullinated peptides after serum starvation. Our findings demonstrate a role for autophagy in citrullination of antigen during processing by antigen presenting cells and a link between antibodies to immunoglobulin and presentation of citrullinated peptides.

Results

Primary APC present citrullinated peptides *in vivo*.

We generated a panel of hybridomas that exclusively recognize citrullinated peptides by fusing T cells isolated from the draining lymph nodes of mice immunized with whole unmodified HEL in CFA. The responses of these cells provide a useful reagent with which to detect the presentation of citrullinated peptides. In the series of experiments described here we compare the response of Granny, which exclusively recognizes the HEL peptide, 48-62cit61 on I-A^k [1], to 3A9 which recognizes the 48-62 peptide (DGSTDYGILQINSRW).

In order to determine if presentation of citrullinated peptides by class II-MHC molecules occurs *in vivo*, we examined APC from mHEL mice, which express HEL linked to the transmembrane region of L^d under the I-E promoter and therefore in every MHC class II expressing cell. Presentation of HEL 48-62 by APC in these mice is strong, found to be 3,400–20,000 pMHC complexes per cell [2]. We isolated $CD11c^+$ cells from the thymus and spleen of these mice. In addition to eliciting a robust response to the dominant HEL peptide 48-62 (using as indicator cell the CD4 T cell hybridoma 3A9) (Fig. 1b&d), the APC also presented 48-62cit61 (using Granny as the indicator T cell) (Fig. 1a&c). In contrast, splenic $CD19^+$ cells did not elicit a response from the 48-62cit61

reactive hybridoma though the response of 3A9 was strong (Fig. 1e&f). This last finding is in concordance with our previously published results demonstrating that a B cell lymphoma did not present citrullinated peptides after processing HEL [1].

Since T cells that recognize citrullinated peptides were first isolated from lymph nodes of mice after immunization with HEL in adjuvants, we verified the presentation of citrullinated peptides by APC in the draining lymph nodes. B10.BR mice were immunized with 10nmol HEL in adjuvant (Complete Freund's) and their APC from draining lymph nodes were isolated 24 hours later and then examined for their presentation to the indicator T cells. These APC, in addition to presenting HEL 48-62 (Fig. 1 g&h) presented HEL 48-62cit61.

Serum starvation induces presentation of citrullinated peptides by B lymphoma cells.

C3.F6.mHEL is a B lymphoma line that expresses HEL with a trans-membrane linker and constitutively presents high levels of HEL peptides on I-A^k molecules [3]. Such a line did not present the citrullinated HEL peptides and was used here to examine the reasons for such a negative response and the conditions that may induce it. Attempts to induce citrullination by activating the C3.F6.mHEL line with endotoxin, tumor necrosis factor or interferon-gamma failed. However there was a striking response by Granny after the cells were serum starved by reducing the amount of fetal calf serum (FCS) from 5% to 1%

or without FCS for a limited period of time (Fig. 2a). We did not observe a significant increase in dead cells by trypan blue exclusion in the cultures that had been serum starved. A similar positive response was found after irradiation of the cells by ultraviolet light.

This response led us to question whether the B cell line was undergoing autophagy as a result of the stress signals. The serum starved cells were treated with 3-methyladenine (3MA), a class III PI3 kinase inhibitor during the period of serum starvation. 3MA was previously shown to inhibit autophagy [4, 5], although effects on other components of intracellular protein processing and or catabolism cannot be ruled out. The response by Granny to the serum starved cell line was completely blocked by treatment with 3MA (Fig. 2a). In contrast to the striking effect by 3MA on the presentation of the citrullinated peptide, the response to the unmodified 48-62 peptide by 3A9 was not affected at all (Fig. 2c). There was no significant difference in presentation of synthetic peptide indicating that the levels of MHC molecules were not altered by serum removal or 3MA treatment (Fig. 2b).

We confirmed that the culture of C3.F6.mHEL without FCS induced an autophagy response by finding an increase in the levels of LC3 II which was reduced by 3MA treatment (Fig. 2d). Thus the 3MA effect was highly selective for presentation to the cell line that recognized the citrullinated derivative of HEL. The specific effect of 3MA only on presentation of the citrullinated epitope and

not on the unmodified one strongly pointed to autophagy as participating in the presentation of citrullinated peptides in these APC.

Presentation of citrullined peptide by primary APC is inhibited by 3-Methyladenine.

In culture, PEC and BMDC pulsed with HEL presented the 48-62Cit 61 epitope. We next addressed the possibility that the presentation of citrullinated peptides by PEC and DC would also be a target of 3MA, perhaps dependent on constitutive autophagy. Several reports indicated that APC constitutively underwent autophagy and that this process contributed to the repertoire of peptides presented on MHC class II molecules [6-12]. Both PEC and BMDC were pulsed with 3MA and HEL overnight, then assessed for their presentation of peptides to the two T cell hybridomas. The addition of 3MA profoundly inhibited the presentation of citrullinated peptides to Granny (Fig. 3 a&c). The inhibition was dependent on the dose of 3MA (Fig. 3 b&d). In contrast, presentation of HEL peptides to 3A9 was not effected at all over a range of concentration. Additionally, presentation of synthetic peptide was not changed by 3MA treatment. Thus the 3MA inhibition of HEL presentation by PEC and BMDC, also was highly selective only affecting that of the 48-62cit61 epitope. 3MA was not having a non-specific inhibition of HEL processing.

Knockdown of expression of Atg 5 inhibits presentation after serum starvation of B lymphoma cells

Although 3MA is generally accepted to inhibit the autophagy pathway, there have been reports that it may have some pleiotropic effects. In neuronal cells 3MA was shown to inhibit apoptosis and mitochondrial permeability transition [13, 14]. In colon cancer cells there was a minor increase in the mitochondrial protein, oligomycin sensitivity conferral protein, only in amino acid deprived cells [15]. In isolated hepatocytes 3MA was shown to induce glycogen breakdown through an increase in cyclic AMP and decreased the ability of the cells to accumulate chloroquine [16]. In addition it has been observed that in some situations, 3MA may actually induce autophagy [17]. Our data show that treatment of APC with 3MA had no effect on presentation of 48-62 to 3A9, even at low antigen doses; however, we wanted to completely rule out the possibility that the effect we observed with 3MA could be due to an effect independent of autophagy. Thus, we assessed whether an alternative method of inhibition of autophagy would recapitulate our findings. To this end we designed shRNA constructs to target expression of Atg5, a protein essential for autophagy, and cloned them into a lentiviral delivery system in which infected cells express green fluorescent protein (GFP) and can be purified by flow cytometry. C3.F6.mHEL cells were infected and the GFP expressing cells were sorted and examined. As a negative control, cells were infected with virus containing shRNA that targets Luciferase expression. The shRNA system effectively reduced Atg5 expression

by western blot (Fig. 4e). rtPCR indicated a 72% decrease in Atg5 message relative to Luciferase knockdown controls. The cells exhibited reduced levels of LC3 II conversion.

We cultured these cells in media with various amounts of FCS for 3 hours before changing to DMEM with 5% FCS and adding T cell hybridomas. In the control cells in which Luciferase expression was targeted, there was strong presentation of citrullinated peptides only upon serum starvation. The same cells cultured in 5% FCS did not present (Fig. 4a). Inhibition of expression of Atg5 affected presentation of citrullinated peptides in serum-starved cells (Fig. 4b). In contrast, neither serum starvation nor expression of Atg5 had any bearing on presentation of the unmodified epitope, HEL 48-62 (Fig.4 c&d). This result is consistent with our data demonstrating 3MA inhibition of presentation of citrullinated peptides and supports the conclusion that autophagy played a role in citrullination of antigen by APC.

Primary B cells present citrullinated peptides after BCR engagement.

It has been shown that B cell receptor (BCR) engagement induces autophagy in primary B cells [18, 19]. We purified B cells from anti-HEL B cell transgenic mice and cultured them overnight with HEL +/- 3MA. We found that, in contrast to B cells from B10.BR mice, they do elicit a response from Granny after processing whole unmodified HEL (Fig. 5a). 3MA did not inhibit presentation of HEL 48-62cit61 when it was removed from the culture before the addition of T

hybridomas (Fig 5b); however, we observed inhibition of presentation of citrullinated peptides after processing HEL in these B cells when they are fixed immediately after 3MA removal (Fig 5c). The effect of 3MA treatment is rapidly reversible [4] and we speculate that the signals that drive autophagy induced by antigen binding are still in effect when the cells are washed for the addition of T cells. The response of 3A9 to presentation of HEL by transgenic B cells is strongly enhanced when compared to B10.BR as expected as BCR mediated uptake is known to lead to highly efficient presentation of antigen [20].

To determine if BCR mediated uptake for presentation is required for trafficking of antigen to a particular compartment for citrullination or if BCR engagement induced autophagy is sufficient for presentation of citrullinated peptides we examined B cells from mHEL mice. Treatment of these B cells with anti-IgM induced presentation of HEL 48-62cit61 (Fig. 5d). We observed identical results with $F(ab')_2$ fragments. These results are not due to endotoxin contamination in the antibody as LPS treatment of these cells does not induce presentation of citrullinated peptides. Interestingly, treatment of B cells with anti-IgG also induced presentation of citrullinated peptides. Induced presentation of citrullinated peptides by both anti-IgM and anti-IgG was inhibited by treatment with 3MA, indicating that autophagy is required. In support of this conclusion, we find that LC3 conversion is induced by treatment with anti-IgM and anti-IgG and is inhibited by 3MA treatment (Fig. 5f). To examine the intracellular events that were occurring after BCR engagement, we examined B cells from GFP-LC3

mice. As expected, treatment with anti-IgM lead to capping and internalization of surface IgM. After treatment with anti-IgM, LC3 positive vesicles were observed which colocalized with MHC II vesicles and internalized IgM (Fig. 5g), thereby demonstrating an association of autophagic vesicles with BCR mediated uptake and processing for presentation on MHC II.

PAD2 and PAD4 levels are not affected by serum starvation or treatment with 3MA.

PAD activity has several levels of regulation; transcriptional, post-transcriptional [21], post-translational [22], and though a requirement for Ca^{++} [23]. In our own experiments, we found that over-expression of PAD4 in B lymphoma cells did not lead to presentation of citrullinated peptides however; we raised the question of whether treatment with 3MA, serum starvation, or decrease in Atg5 expression was modulating presentation of citrullinated peptides by influencing PAD expression levels. Figure 7 shows expression of PAD2 and PAD4 mRNA relative to untreated cells. There was no difference in PAD expression levels as determined by rtPCR. The data shown are normalized to beta actin levels and are similar to results we found when we normalized to Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 18s rRNA, and Hypoxanthine-guanine phosphoribosyl transferase (HPRT). We conclude that the

effect of serum starvation and 3MA treatment on autophagy was not influencing PAD expression in these cells.

Biochemical evaluation of naturally processed peptides demonstrates the positive effect of serum starvation.

A biochemical method that has been used to detect citrulline in biological samples targets its unique ureido group and can be used to quantify citrulline by a colorimetric assay [24, 25]. This method is based on the reaction of diacetyl monoxime (DAMO) with urea under acidic conditions and a second reaction with antipyrine. The product of this reaction has been recently identified to be 3-butyl-4-[(2,3-dihydro-1,5-dimethyl-3-oxo-2phenyl-1*H*-pyrazol-4-yl)methyl]-5-1*H*-imidazol-2(3*H*)-one [26]. We cultured C3.F6.mHEL in DMEM supplemented with 5% or 1% serum and, after lysis, isolated I-A^k peptide complexes. The peptides were separated from the Class II molecules and chemically modified to label citrullinated peptides. Table 1 summarizes the results of these experiments. Though there is some variability between experiments, the amount of citrulline detected in peptides from cells incubated in 1% FCS was higher than that from cells cultured in 5% serum. These data support our findings with citrullinated peptide reactive T cell hybridomas and our conclusion that serum starvation leads to an increase in presentation of citrullinated peptides.

Discussion

Autophagy is a ubiquitous catabolic process by which cytosolic components are shuttled in to vesicular compartments for degradation in lysosomes. This process has been conserved through evolution and is involved in the homeostatic degradation of cytosolic proteins and turnover of senescent organelles [27-35].

Studies evaluating naturally processed peptides from MHC II molecules have revealed that peptides derived from cytosolic proteins are presented at significant levels [6, 7, 36-40]. There have been several recent reports describing the contribution of the autophagic pathway to MHC II peptides [8, 10-12].

We found that APC, including thymic DC, from HEL transgenic mice present citrullinated peptides *in vivo* which was inhibited by 3MA. Inhibition of autophagy through 3MA has been shown to be relatively specific with only a few reports citing other effects [13-16]. We show here that 3MA has no effect on presentation of 48-62 to 3A9, even at low antigen doses. 3MA does not inhibit processing of HEL in general and the effect is specific to citrullination of antigen.

Primary B cells from B10.BR mice did not present HEL 48-62cit61 after processing whole HEL under normal conditions. The poor presentation by B cells and B lymphoma cells could be attributed to an absence or poor expression of

the PAD enzymes which convert arginine to citrulline. PAD2 and PAD4 are expressed in APC. Based on a study examining expression of PAD in leucocytes [21] and our own published results [1], PAD4 is expressed at lower levels in B cells and C3.F6.mHEL. We over expressed PAD4 in C3.F6 and found no difference in presentation of citrullinated peptides. In addition, we observed that treatment with 3MA, serum starvation, and inhibition of Atg 5 expression had no effect on PAD2 and PAD4 expression levels. These data indicate that the limiting factor in citrullination of antigen by APC is not PAD expression levels alone.

Primary B cells presented citrullinated peptides derived from HEL taken up via the BCR. In addition, we found that engagement of the BCR with antibody lead to presentation of HEL 48-62cit61 in cells that express membrane linked HEL. This data raises the question of how rheumatoid factor may factor in to the generation of autoimmune responses to citrullinated self-proteins. It is possible that induced presentation of citrullinated self-proteins, especially in the context of MHC II molecules that effectively bind these peptides, as has been shown with shared epitope containing alleles, could be one of the precipitating events that lead to the initiation of an autoimmune process.

The exact mechanism by which autophagy contributes to citrullination of antigen is unknown. We speculate that it acts by allowing PAD enzymes to gain access to antigen loading compartments. In addition, autophagic degradation of senescent organelles may act as a source of Ca^{++} to allow PAD activity.

PAD2 has been reported in the literature to be localized to the cytosol and PAD4 in the cytosol or nucleus [41-43]. A report by Mastronardi et al. showing translocation of PAD4 from the cytosol to a vesicular compartment and then to the nucleus of oligodendroglial cells after treatment with tumor necrosis factor prompted us to determine if we could detect PAD4 in $LC3^{+}$ vesicles by confocal microscopy [44]. As expected, we found that PAD4 primarily localized to the nucleus. We observed some cytosolic staining but no obvious staining in $LC3^{+}$ or Class II $^{+}$ vesicles. Since PAD proteins are enzymes, small amounts can potentially produce significant observable activity. In our panel of hybridomas that we generated we found that only the most sensitive hybridomas to 48-62cit61 responded to presentation of whole HEL by APC [1]. We would not expect to see high levels of PAD enzymes in antigen loading compartments based on the levels of citrullination we observe by APC.

What is the reason behind citrullination of antigen by APC? There are several possible teleological explanations for this. It is known that citrullination alters intramolecular interactions and can lead to increased accessibility to proteolysis [45, 46]. This may be a mechanism to assist in protein degradation within lysosomes. In addition, the change from arginine to citrulline has been shown in some cases to alter peptide MHC affinity and the conformation of the peptide in the binding groove [47-49] and may have a role in expanding the repertoire of peptides that can be presented on a particular MHC haplotype.

Finally, constitutive, low level presentation of citrullinated self-peptides may play a role in the establishment of tolerance. Citrullination plays a role in several normal biological processes and citrullinated proteins are present in healthy individuals.

Though it is likely a gross oversimplification, the notion that citrullination of self-antigen represents a mechanism by which tolerance can be breached is attractive because it provides a clear explanation for a potential cause of RA. The idea that adaptive responses are occurring to proteins that have been citrullinated and then made available to APC for processing and presentation has been the basis of much of the research on the role of citrullinated epitopes in disease. We present here evidence that suggests that APC could be a source of citrullinated antigen and that the local environment may potentially contribute to enhanced presentation of citrullinated epitopes. This is an additional factor that one should consider when thinking about citrulline and autoimmunity.

In summary, using T cell hybridomas that exclusively recognize citrullinated peptides we have shown that induction of autophagy can induce presentation of citrullinated peptides while inhibition of autophagy, both chemically using 3MA and by inhibiting Atg5 expression blocks presentation of citrullinated peptides. B cells that have taken up antigen via the BCR present citrullinated peptides. Interestingly, BCR engagement by anti-immunoglobulin antibody induces presentation of citrullinated peptides by B cells. Biochemical

analysis of naturally processed peptides from cells grown in serum starved conditions confirms that there is an increase in citrulline levels. We conclude that autophagy is associated with citrullination of antigen by APC.

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Figure 1

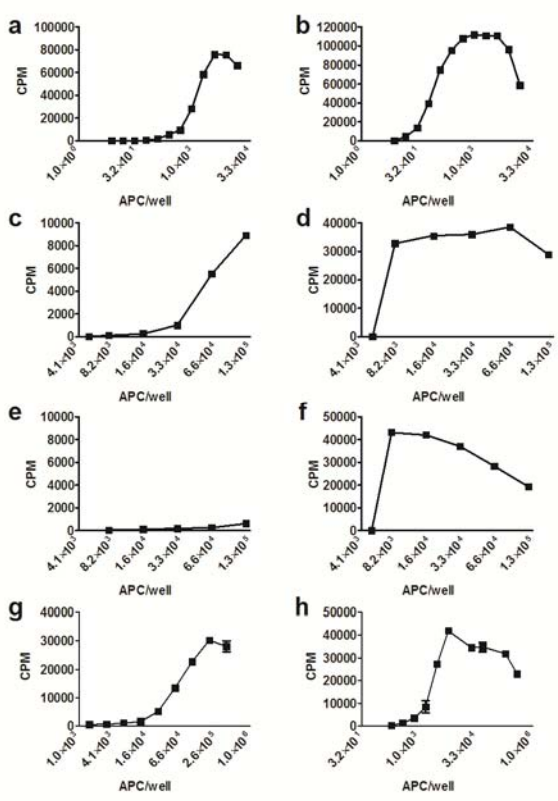

Figure 1 Antigen presenting cells present citrullinated peptides *in vivo*.

$CD11c^+$ cells isolated from thymi (a,b) or spleen (c,d) and $CD19^+$ cells isolated from spleens (e,f) of mHEL mice using magnetic bead purification. B10.Br mice were immunized in the hind footpad with 10 nmol HEL in CFA. After 24 hours, the popliteal lymph nodes were collected. $CD11c^+$ and $CD11b^+$ cells were purified by magnetic bead purification and used as APC (g,h). Cells were cultured with either Granny (a,c,e,g) or 3A9 (b,e,f,h) overnight. Cell were co-cultured overnight and supernatants were collected. IL-2 was measured by CTLL proliferation assay.

Figure 2

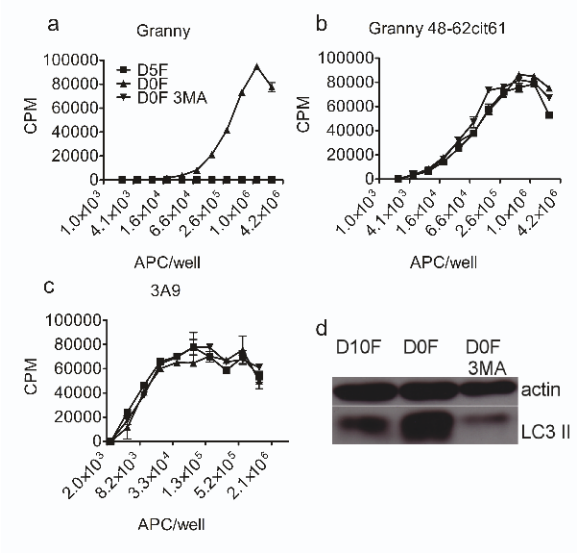
a
Granny
D5F
D0F
D0F 3MA
CPM
APC/well
b
Granny 48-62cit61
CPM
APC/well
c
3A9
CPM
APC/well
d
D10F
D0F
D0F 3MA
actin
LC3 II

Figure 2. C3.F6.mHEL present citrullinated peptides after serum starvation.

C3.F6.mHEL were cultured in DMEM supplemented with 5% fetal calf serum or no serum overnight. The culture medium was then changed to DMEM with 10 % fetal calf serum and T cell hybridomas were added. Synthetic peptide [3μM] was added as a positive control (b). After overnight incubation the culture supernatants were collected and IL-2 levels were measured by CTLL proliferation assay. D-C3.F6.mHEL were cultured in DMEM supplemented with either 10 % serum or no serum and treated with chloroquine for the last hour of culture. Levels of LC3 II and actin were measured by western blot.

Figure 3

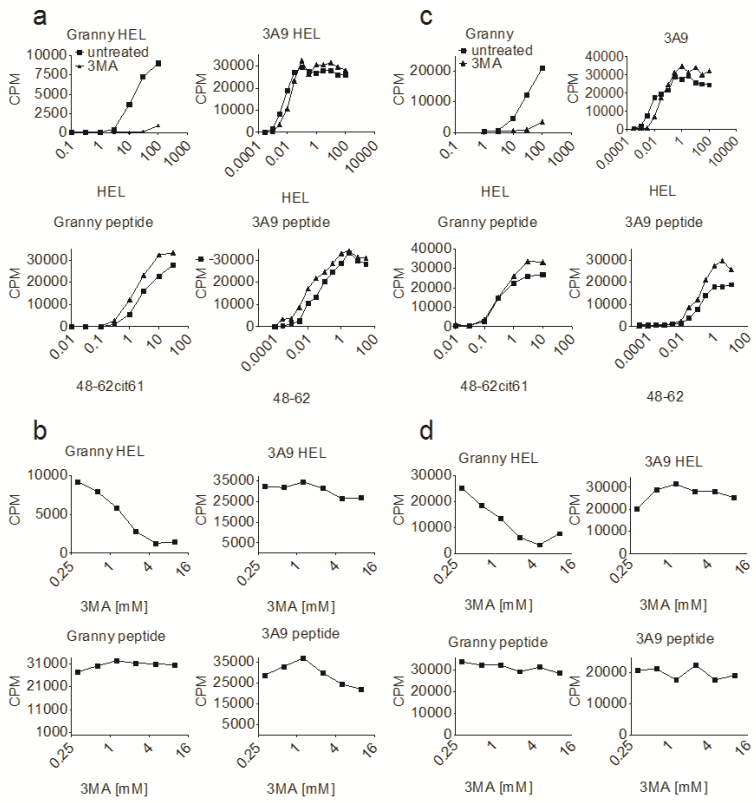
a
Granny HEL
untreated
3MA
CPM
HEL
3A9 HEL
HEL
Granny peptide
48-62cit61
3A9 peptide
48-62
c
Granny
3A9
b
d
3MA [mM]

Figure 3. Treatment with 3-Methyladenine inhibits presentation of citrullinated peptides.

BMDC (A and B) or PEC (C and D) were cultured overnight with 3-Methyladenine [10mM] and dilutions of HEL or peptide in 96 well plates(A and C) or HEL or peptide and dilutions of 3-Methyladenine (B and D). They were washed and cultured overnight with T cell hybridomas that recognize HEL 48-62 presented by I-A^k. After incubation overnight, the culture supernatant was collected and IL-2 levels were measured by CTLL proliferation assay.

Figure 4

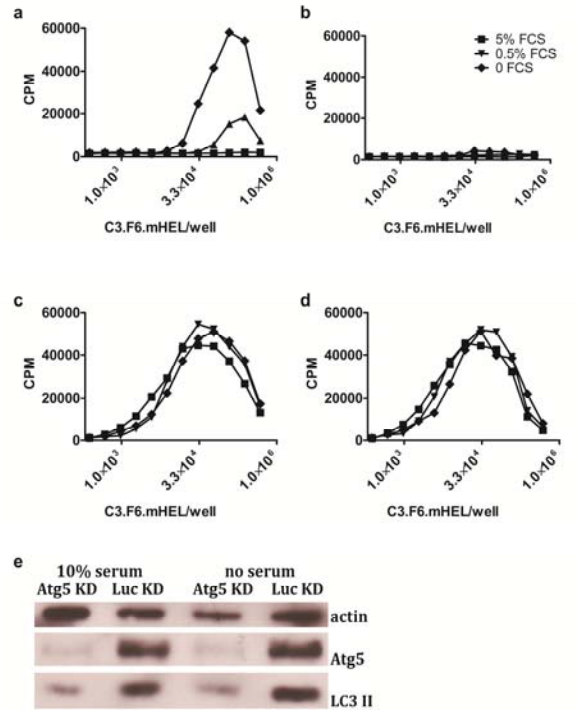
a
b
c
d
e
CPM
60000
40000
20000
0
1.0×10³
3.3×10⁴
1.0×10⁶
C3.F6.mHEL/well
5% FCS
0.5% FCS
0 FCS
10% serum
no serum
Atg5 KD
Luc KD
actin
Atg5
LC3 II

Figure 4. Targeted knockdown of Atg5 expression has an inhibitory effect on citrullination of antigen.

C3.F6.mHEL cells expressing either shRNA targeting Lucifierase (Luc) expression (a and c) or Atg5 expression (b and d) were cultured in DMEM supplemented with 5% FCS, 1% FCS, or no FCS for 2 hours. The culture medium was replaced with DMEM supplemented with 5% FCS and T cell hybridomas were added and IL-2 in the culture supernatant was measured by CTLL proliferation assay. e- Atg5 and LC3 II levels in Luciferase knockdown control cells and Atg5 knockdown cells were assessed by western blot.

Figure 5

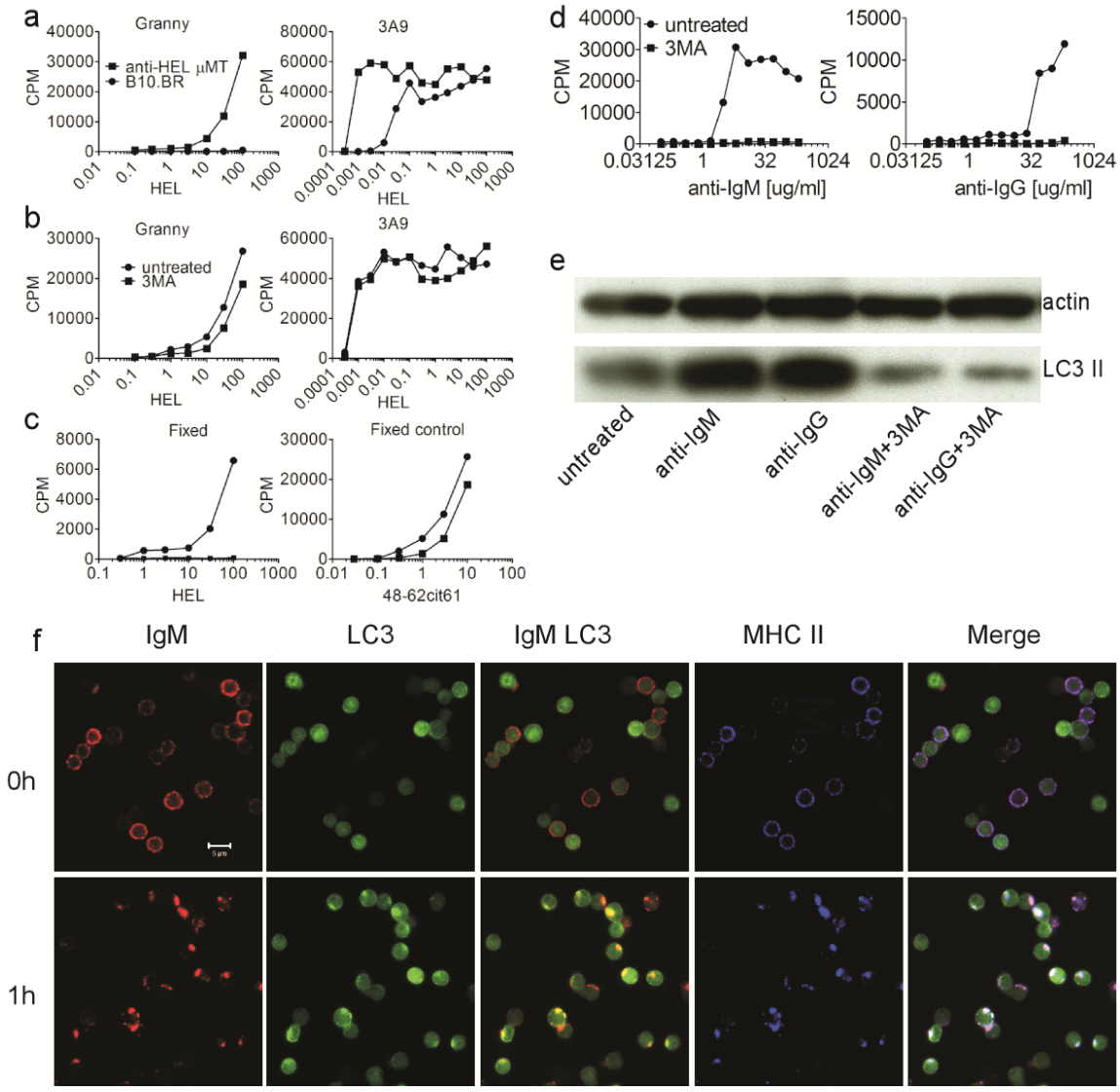
a
Granny
3A9
anti-HEL μMT
B10.BR
CPM
HEL
b
Granny
3A9
untreated
3MA
c
Fixed
Fixed control
48-62cit61
d
untreated
3MA
anti-IgM [ug/ml]
anti-IgG [ug/ml]
e
actin
LC3 II
untreated
anti-IgM
anti-IgG
anti-IgM+3MA
anti-IgG+3MA
f
IgM
LC3
IgM LC3
MHC II
Merge
0h
1h

Figure 5. B cells present citrullinated peptides after BCR mediated uptake or treatment with anti-IgM or anti-IgG.

(a)Splenic CD19+ cells from B10.BR or anti-HEL mice were cultured with HEL and either Granny or 3A9. (b) B cells from anti-HEL mice were cultured with HEL +/- 3MA overnight then washed before Granny was added to the culture. (c) B cells treated as in b but fixed before addition of Granny. (d) Splenic $CD19^{+}$ cells from mHEL mice were cultured with dilutions of anti-IgM or anti-IgG and Granny. IL-2 production was measured by CTLL assay. (e) LC3 conversion was assessed by Western blot in B cells treated with anti-IgM or anti-IgG +/- 3MA. (f) Splenic B cells from GFP.LC3 mice were labeled with anti-IgM on ice and fixed at 0h and 1h after transfer to 37°.

Figure 6

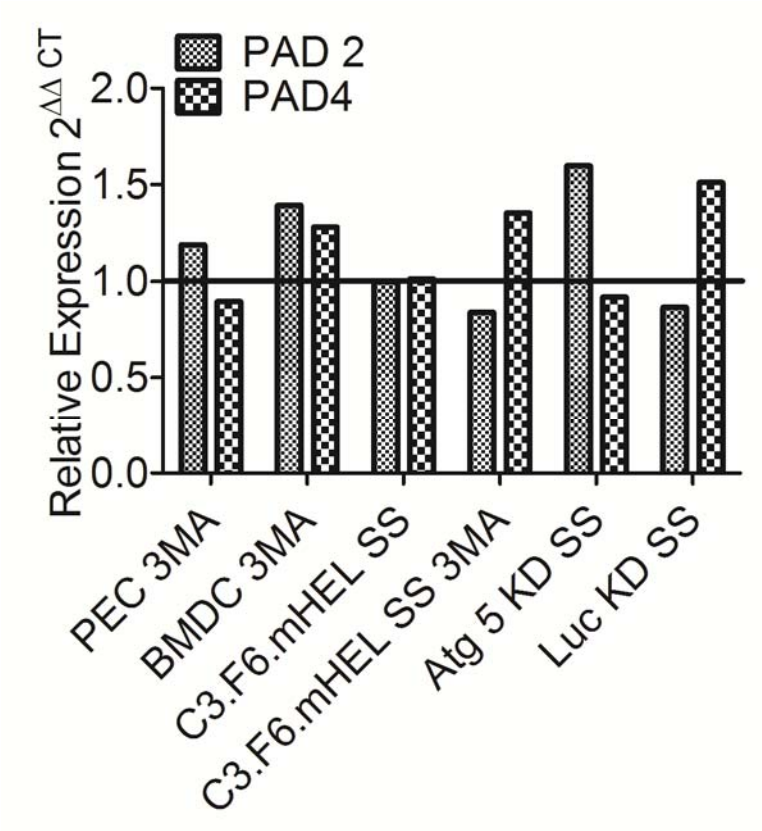
PAD 2
PAD4
Relative Expression $2^{\Delta\Delta CT}$
2.0
1.5
1.0
0.5
0.0
PEC 3MA
BMDC 3MA
C3.F6.mHEL SS
C3.F6.mHEL SS 3MA
Atg 5 KD SS
Luc KD SS

Figure 6. Relative Expression of PAD2 and PAD 4 after treatment.
PAD2 and PAD4 expression was measured in cells after treatment with 3MA, serum starvation (SS) or serum starvation and 3MA treatment. Delta delta CT values are relative to untreated cells and normalized to actin.

Experiment	Culture	nmol cit/10e9 cells	Fold increase
1	1% serum	5.05	2.46
	5% serum	2.06	
2	1% serum	2.62	8.73
	5% serum	0.30	
3	1% serum	18.19	13.50
	5% serum	1.35	
4	1% serum	2.37	2.25
	5% serum	1.05	

Table 1. Quantitation of citrulline in naturally processed peptides.

Chapter 4

Concluding Remarks and Future Directions

Summary

The aim of this project was to evaluate citrullination by APC. The system we developed to detect presentation of citrullinated peptides is a specific and sensitive response by T cell hybridomas. We demonstrate here that APC have an intrinsic ability to citrullinate antigen both constitutively, as in the case of dendritic cells and macrophages, and in response to certain conditions, as in the case of B cells.

Our interest in citrullination in the context of antigen processing and presentation stemmed from studies that suggest that an adaptive response to citrullinated self-epitopes may be involved in autoimmunity. A major obstacle in studying citrulline in naturally processed peptides is that of detection. This project began with the question- Can we detect an adaptive immune response to citrullinated epitopes in our model? A biological assay is relatively reliable and practical when compared to alternative methods by which to detect presentation of citrullinated peptides.

The 3 main findings of this project are the following-

1- T cells that recognize citrullinated peptides are among those responding after protein immunization. We fused T cells taken from several lymph nodes of immunized mice and generated hybridomas that exclusively respond to presentation of citrullinated peptides.

The specificity of these hybridomas to the citrullinated peptides allowed us to detect presentation of citrullinated peptides.

2- APC citrullinated antigen while processing for presentation. The T cells we fused to generate our panel of hybridomas had been primed *in vivo* after protein immunization- the citrullinated peptide had been presented *in vivo*. This conclusion was borne out when we examined APC isolated from the draining lymph node of immunized mice.

   Dendritic cells and macrophages present citrullinated peptides constitutively, while B cells do not present citrullinated peptides under normal conditions.

3- Autophagy plays a role in citrullination of antigen by APC. 3MA inhibits presentation of citrullinated peptides. Serum starvation induces presentation of citrullinated peptides. Atg5 knockdown inhibits serum starvation induced presentation of citrullinated peptides. Induction of autophagy in primary B cells through BCR engagement allows presentation of citrullinated peptides.

This body of work has brought to light the role of APC in citrullination and raises several questions for future studies to address. The system that we have developed has proven to be a useful method by which citrullination by APC can be detected.

T cells that can exclusively recognize citrullinated peptides are among those responding to protein immunization.

Citrullination leads to a minor change in molecular weight- only 1 amu. However, the loss of positive charge leads to significantly altered interactions with adjacent proteins. In the context of antigen presentation, this change has the potential to alter peptide binding affinity or T cell reactivity. In addition to our findings, several studies have addressed this effect in various systems.

In examining subtypes of the human HLA B allele, B*2705 and B*2709, which present a peptide from protein vasoactive intestinal peptide type 1 receptor in drastically different conformations, crystal structure revealed that the citrullinated peptide was similarly presented in different conformations by B*2705 and B*2709. However, the conformation of the citrullinated peptide was completely different from the unmodified peptide. They further determined that cytotoxic T lymphocytes that respond to presentation of the unmodified peptide did not recognize the citrullinated variant [1]. The crystallographic data directly demonstrates how citrullination can impact the conformation of the peptide in the binding groove.

Several studies have examined T cell responses to citrullinated epitopes. In examining T cell responses in rats, de Haan et al. showed that T cells could discriminate between unmodified peptides and citrullinated peptides [2]. In contrast to the previous study where the impact of citrulline was in the binding conformation, in this case it was the TCR contacts that were changed.

While there is substantial evidence that citrullination can have a profound effect on antigen presentation, our data demonstrating that T cells that

exclusively respond to citrullinated protein are primed *in vivo* after protein immunization raised the question of how the epitope is modified.

APC citrullinated antigen while processing for presentation.

The role of APC in citrullination of antigen has been largely ignored and this body of work constitutes the first direct evidence that antigen presenting cells can citrullinated antigen while processing for presentation. Our data demonstrate that APC present citrullinated peptides after processing whole unmodified HEL.

The fundamental issue in presentation of epitopes that carry post translational modification is that of tolerance. The mechanism by which tolerance to self proteins that have been post translationally modified is unknown. Since citrullination is part of several normal biological processes it is clear that there must be some level of tolerance to citrullinated self-proteins. We show that thymic DC from mHEL mice present citrullinated peptides. While we have not addressed tolerance in this study, it is logical to conclude that thymic DC may play a role in establishing tolerance to some citrullinated proteins. In addition, constitutive, low level presentation of citrullinated peptides may have a role in peripheral tolerance.

One of the strongest genetic association linked with RA is a class of MHC alleles that contain the so called "shared epitope", a 5 amino acid sequence, Q/R, K/R, R, A, and A at positions 70–74 of the β-chain in the third hypervariable region that forms part of the P4 pocket [3].

It is this presence of these positively charged amino acids that accounts for the enhanced binding of peptides that carry a citrulline residue at the P4 position, while a positively charged arginine residue hinders binding. An example of this was demonstrated by Hill et al. who, using transgenic mice that expressed HLA DRB1*0402, showed that a citrullinated peptide from vimentin bound with much higher affinity. They further showed that T cell responses could be detected in mice immunized with citrullinated self peptides, however, the focus of this paper was on binding and they did not carefully characterize the T cell specificity [4]. In a subsequent report, this group was able to show that immunization of these transgenic mice with citrullinated fibrinogen induced an arthritis-like disease [5]. In humans, found that the SE alleles predisposed patients to develop antibody responses to citrullinated vimentin but not fibrinogen [6, 7]. There are several examples of the association with SE alleles and the formation of anti-citrullinated protein antibody (ACPA) responses [8-16]. However some of the most intriguing and relevant work comes from the Toes laboratory which has shown that ACPA positive RA is exclusively associated with SE positive HLA DRB1 alleles and ACPA negative RA with HLA DRB1*03, a "protective" allele to be exclusively associated with ACPA negative RA [17].

Collectively these data suggest that presentation of citrullinated peptides is playing a significant role in the development of ACPA responses that are associated with RA. One of the outstanding questions is how these proteins are citrullinated as there is no physiological role for citrullination in the function of most of the target proteins. Our findings introduce the additional factor of APC in

citrullination of antigen especially when we consider the induced presentation of citrullinated peptides by B cells as discussed below.

Autophagy is required for citrullination of antigen.

The evidence given here for the requirement for autophagy in citrullination of antigen by APC raises several intriguing points related to the role of autoimmune responses to citrullinated self antigen. In particular, it is interesting to consider the possibility that influence of the local milieu may have some bearing on the level of presentation of citrullinated peptides.

In an EAE model, immunization with a citrullinated MOG peptide gave rise to a T cell response that included T cells that either cross reacted to unmodified peptide or exclusively responded to the citrullinated peptide. The mice did not develop EAE after transfer of T cells that only responded to the citrullinated peptide and induction of tolerance to the citrullinated epitope had no effect on disease. However, once disease was established, transfer of the citrullinated peptide reactive clones did exacerbate disease, indicating that the citrullinated peptide was presented in an inflammatory environment [18]. In a related study in rats, it was found that citrullinated MBP could induce disease similar to unmodified protein. Interestingly, in rats that had resolved disease after immunization with unmodified protein, secondary immunization with citrullinated protein induced a increase in disease severity when compared to rats that had been re-immunized with unmodified protein [19]. Finally, in a clinical study, it was

found that T cells could recognize citrullinated myelin basic protein and that there are a higher number of citrullinated peptide reactive T cells in patients with multiple sclerosis [20].

These results raise the question of how the disease process contributes to presentation of citrullinated peptides. One possibility is that the stressed environment induces an increase in autophagy which leads to presentation of citrullinated peptides.

B cell receptor (BCR) engagement has been shown to induce autophagy [21, 22] as one of the many striking cellular changes that occur after antigen binding by B cells. Our findings that demonstrate that antigen that has been captured and taken up via the B cell receptor is processed through a pathway that allows citrullination. Furthermore, we present data that shows the BCR engagement with antibody leads to presentation of citrullinated peptides derived from endogenously expressed HEL in mHEL B cells, all of which is inhibited with 3MA treatment. We demonstrated that the BCR localizes to an MHC and LC3 positive compartment. This result has some significant implications.

Beside ACPA, another serological marker for RA and other rheumatic diseases is rheumatoid factor (RF). It was first discovered in the 1940's with the observation that there were antibodies present in the sera of patients with RA that reacts with autologous IgG [23] [24] [25]. These antibodies are typically pentameric IgM and react with the fc domain of IgG, however RF has also been shown to bind to other isotypes of immunoglobulin, including IgM [26]. Our results that show that BCR engagement by antibody induces presentation of

citrullinated peptides. The combination of the presence of RF and SE containing HLA alleles could constitute conditions that lead to enhanced peripheral presentation of citrullinated self-peptides that may potentially trigger a breach of tolerance and lead to the initiation or enhancement of pathological adaptive responses. BCR induced presentation of citrullinated peptides may be the missing piece linking RF to RA.

Finally, smoking has been associated with RA [27, 28] [29, 30]. Bronchoalveolar lavages from smokers were shown to contain higher levels of PAD2 expression and citrulline [31], which could stem from autophagy induced presentation of citrullinated peptides. The additional association of smoking as a risk factor for ACPA positive RA in patients with SE containing alleles is further evidence that supports the connection between antigen presentation and ACPA development [32].

Future directions

The next steps in this project focus on the cellular biology of citrullination and the role of responses to citrullinated proteins in autoimmunity. A detailed understanding of the cellular mechanisms of citrullination by antigen presenting cells and the methods by which tolerance to citrullinated proteins can be breached may help to develop our understanding of diseases in which responses to citrullinated self-antigen could be involved.

We speculate that autophagy allows entry of PAD enzymes into antigen loading compartments. This will be directly evaluated using a combination of immunofluorescence and biochemical and enzymatic analysis of subcellular fractions.

Which enzymes are involved?- PAD2 and PAD4 knockout mice have been generated. They can be crossed on to a B10.BR background and examined for citrullination by APC.

Data links single nucleotide polymorphisms in PAD4 to RA susceptibility in certain populations, which makes the idea that this is the key enzyme provocative. However it is possible that both enzymes are involved as there is some data in the literature that demonstrates that PAD4 can compensate for the loss of PAD2 [33]. Further, we will evaluate the entry of the relevant enzyme(s) in to an autophagic compartment by subcellular fractionation and microscopy.

Does citrullination of antigen play a role in autoimmunity?- We have several reagents available to us in the form of HEL transgenic mice. In addition to the HEL model, our group is evaluating the role of citrullination of natural antigen in an EAE model.

The role of RF in citrullination by APC will be evaluated using an RF transgenic mouse [34]. We show that DC in the thymus present citrullinated peptides in mHEL mice. We would like to extend these findings to determine if these APC are involved in presenting citrullinated peptides derived from blood borne antigen.

While there remain several unanswered questions, the role of APC in citrullination of antigen adds a new dimension to the problem of citrullination of antigen. We hope that in the years to come some of the issues that have been raised here will be resolved.

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