Citrullination and Immune Dysregulation in Rheumatoid Arthritis

By

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ABSTRACT

Rheumatoid arthritis is a chronic autoimmune arthritis characterized by severe joint inflammation. Inflammation during disease is complex, with activated cells including macrophages and synovial fibroblasts secreting pro-inflammatory stimuli, promoting immune cell influx. Stimuli can also induce neutrophil extracellular trap (NET) formation, which releases numerous proteins to the extracellular environment. Some NET proteins are autoantibody targets, including citrullinated proteins. Citrullination is the post translational deimination of arginine to citrulline, and citrullinated proteins are bound by anti-citrullinated protein antibodies (ACPAs), autoantibodies associated with rheumatoid arthritis. ACPA binding generates immune complexes capable of activating many cells, including macrophages, perpetuating inflammation. It is critical to understand this cycle to characterize pathways for potential targets for therapeutic intervention. Here, we focus on NETs and antibodies in normal and pathologic inflammation in rheumatoid arthritis. We investigate pathways regulating NET formation and identify stimuli that induce citrullinated and uncitrullinated NETs. We also determine the requirement of citrullinating enzymes peptidylarginine deiminase 2 (PAD2) and PAD4 in NETosis. Our findings demonstrate that NETs may not be a major source of citrullinated protein in rheumatoid arthritis and suggest other cell types should be investigated. We also evaluate antibodies in rheumatoid arthritis and address ACPA correlations with vaccine immunity. By examining response to the Tdap vaccine, we report that rheumatoid arthritis patients have significantly lower titers to the pertussis, but not tetanus, vaccine components. We provide evidence for a citrulline-biased immune response in rheumatoid arthritis in which patients have higher binding to citrullinated versus native pertussis. Lastly, we examine the sera of rheumatoid arthritis patients to identify novel autoantibodies in seronegative disease,

a subgroup difficult to diagnose due to the absence of classic serological markers. We determined that rheumatoid arthritis patients produce significantly more antibodies against native complement protein C8 than controls. Further, anti-C8 IgG antibodies are present in seronegative and seropositive rheumatoid arthritis, but not in patients with lupus or ankylosing spondylitis. We propose that anti-C8 IgG could be examined as a biomarker for seronegative disease. Together, this work analyzes two aspects of inflammation, namely NETosis and antibodies, to understand the complexity of disease in rheumatoid arthritis.

TABLE OF CONTENTS

CHAPTER ONE

Introduction

A. Rheumatoid Arthritis

Rheumatoid arthritis is a chronic autoimmune arthritis with a lifetime risk of ~3% (1) characterized by synovial inflammation and joint erosion. There is no cure for rheumatoid arthritis and extra-articular systemic disease involving the heart and lung is common, leading to pain, physical disability, and early mortality despite treatment (2-4). Current therapies are often partially effective or broadly suppress the immune system, and sustained remission is uncommon (5). Thus, rheumatoid arthritis is a major health concern with considerable personal and socioeconomic burdens (6). Improving diagnostics and designing novel interventions are paramount for reducing the impact and severity of disease.

Genome-wide association studies have identified numerous loci that contribute to increased risk of rheumatoid arthritis. Many loci include genes which regulate immune system functions including co-stimulatory pathways, innate immune cell activation, antigen presentation, and cytokine signaling. Genetic heritability (7) and epigenetics increase likelihood of disease, and environmental influences such as smoking (8) and periodontitis (9) are established risk factors. Microbes also contribute to disease, possibly through interactions from the gut or lung microbiome and infection with pathogens like *Porphyomonas gingivalis* (reviewed in (10)) .

The development of rheumatoid arthritis involves abnormal function of several cell types. In healthy tissue, the joint lining contains synovial fibroblasts and macrophages. Synovial

fibroblasts secrete factors that aid in lubrication or modification of extracellular matrix (reviewed in (11, 12)), and also secrete proangiogenic factors and chemoattractants that promote immune cell migration during tissue repair and wound healing (11, 13). In rheumatoid arthritis, synovial fibroblasts are hyperactivated and adopt an aggressive phenotype characterized by increased proliferation that results in synovial hyperplasia and subsequent thickening of the synovial lining (12). Hyperactivated synovial fibroblasts also increase secretion of pro-inflammatory factors like vascular endothelial growth factor (VEGF), IL-8, and IL-15 (13-15). The synovial inflammatory milieu recruits and activates circulating immune cells, including neutrophils and macrophages, which then further perpetuate inflammation by activating other cells, initiating the complement cascade, and displaying antigen that is recognized by autoantibodies (15). Therefore, to understand immune dysregulation in rheumatoid arthritis it is necessary to closely investigate multiple distinct components of inflammation (Figure 1).

B. Citrullination

Citrullination is the post-translational deimination of arginine residues to citrullines. In healthy tissue, citrullination has numerous roles and can contribute to embryogenesis (16), stem cell regulation (17), and transcription factor binding (18). Since arginine is positively charged and citrulline is neutral, citrullination can influence protein binding and conformation. Histone citrullination can disrupt associations to negatively charged DNA, contributing to chromatin decondensation and influencing availability of transcription

factor binding sites. During inflammation, citrullinated protein is increased (19) and citrullination has been linked to many diseases including rheumatoid arthritis, Alzheimer's disease, and multiple sclerosis (20-22). Unique to rheumatoid arthritis, citrullinated proteins are targeted by anti-citrullinated protein antibodies (ACPAs), present in ~75% of patients (21), which perpetuate inflammation.

Citrullination is catalyzed by the peptidylarginine deiminase (PAD) family of enzymes. There are five isozymes in the PAD family, PADs 1-4 and 6. PADs have about 50% sequence homology and are dependent upon calcium for enzymatic activity but differ in subcellular localization and tissue distribution. PAD1 is expressed in the skin and the uterus; PAD2 is the most widely distributed, with expression in immune cells, skeletal muscle, the brain, and other tissues; PAD3 is expressed in hair follicles; PAD4 is expressed in immune cells; PAD6 is regulated in embryos and oocytes (reviewed in (23, 24)). Interestingly, all PAD isozymes are cytosolic, but PAD4 can also be nuclear and is the only isozyme with a defined nuclear localization sequence (25). However, despite the inability to directly localize to the nucleus, PAD2 can deiminate histones (26) indicating a potentially undescribed mechanism for PAD2 nuclear transport. PAD enzymes can citrullinate a variety of proteins, and over 100 unique citrullinated proteins have been described in the arthritic joint alone including vimentin, fibrinogen, and fibronectin (27, 28).

Of the PAD isozymes, PAD2 and PAD4 are of particular interest due to expression in hematopoietic cells and the rheumatoid joint (29, 30). Single nucleotide polymorphisms

in PAD2 (31) and PAD4 are associated with increased risk of rheumatoid arthritis (32) and synovial PAD2 expression correlates with disease severity (33). Moreover, PAD2 and PAD4 independently contribute to murine inflammatory arthritis (34, 35). PAD4 influences autoantibody production, and PAD2 is required for a significant portion of citrullinated protein in the rheumatoid joint (34-36). Given that ACPAs are pathogenic in rheumatoid arthritis (37, 38), identifying sources of citrullinated protein could facilitate the development of future therapies for rheumatoid arthritis.

C. Neutrophil Extracellular Traps

Neutrophils are the most abundant innate immune cell and are prevalent in the rheumatoid joint. Neutrophil extracellular traps (NETs) are complex webs of protein and decondensed chromatin expelled from neutrophils during the programmed cell death process of NETosis (39). The classic NETosis pathway is initiated in response to stimulation of neutrophil receptors including toll-like receptor (TLR) 2, TLR4, FcγR, or Dectin 2 and subsequent activation of NADPH oxidase. NADPH generates reactive oxygen species (ROS) which then activate myeloperoxidase (MPO). MPO activates neutrophil elastase (NE), causing NE to translocate from azurophilic granules into the nucleus. Within the nucleus, NE and MPO disrupt associations between histones and chromatin causing the chromatin to unravel and swell within the nuclear membrane (40). Concurrently, nuclear membrane break-down allows decondensed chromatin to mix with cytosol and neutrophil granule proteins. The cell membrane then disintegrates and

chromatin, now covered in cellular proteins, is released from the cell (Figure 2; reviewed in (41)). Some NETosis pathways do not require NADPH, NE, or MPO activity and engage other proteins all together. For example, immune complexes can activate NETosis independent of NADPH oxidase and instead trigger mitochondrial ROS generation (42). Ionomycin is a calcium ionophore that induces influx of calcium into the cell, which activates PAD enzymes to citrullinate histone, leading to chromatin decondensation (43).

NET protein cargo largely depends on the neutrophil stimulant but NET structures can contain a variety of nuclear, cytosolic, granular, and cytoskeletal proteins including histones, catalase, MPO, and actin (37). Different strains of *Pseudomonas aeruginosa* alone can induce NETs with 33 common proteins and 50 distinct proteins (44). There are functional roles for NETs in pathogen clearance, and mice with impaired NETosis have higher likelihood of bacterial infection (45). NETs can also help to resolve inflammation in certain contexts of sterile inflammation, such as gout, due to the degradation of cytokines (46). Some classifications of NETs have been proposed using the presence of citrullinated protein as a key indicator of the effector NET pathway. One study suggests that a classic pathway of NETosis is uncitrullinated and pathologic to microbes; another pathway termed leukotoxic hypercitrullination (LTH) generates many citrullinated proteins and is utilized by microbes use to evade NET toxicity (9, 47). There are numerous different types of NETs, and individual pathways of formation could form NET structures with various physiological roles (reviewed in (41, 47)).

Although NETs have beneficial roles, NETs can also be pathologic (reviewed in (48)). NETs consist of a multitude of cellular components, many of which have been identified as targets of autoantibodies in the context of autoimmunity. For example, NETs can be targeted by anti-DNA autoantibodies generated by patients with systemic lupus erythematosus (49), or by anti-MPO autoantibodies generated by patients with antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (50, 51). Circulating NETs are increased in rheumatoid arthritis and neutrophils from rheumatoid arthritis patients more readily undergo NETosis (37, 52). In rheumatoid arthritis, NETs can be pathologic by displaying citrullinated proteins bound by ACPAs (39, 45, 52-56), suggesting that NETs may be a significant source of citrullinated protein in disease.

The conversion of positively charged arginine to neutral citrulline during histone citrullination can disrupt associations with negatively charged DNA (43, 57) and promote NETosis. PAD4 is required for histone citrullination and thus predicted to be significant in rheumatoid arthritis (43, 57). However, despite the absence of citrullinated NETs in PAD4$^{-/-}$ mice, overall protein citrullination in the joint is not reduced (34). Instead, PAD2 is required for joint citrullination, but is dispensable for the production of NETs activated by TNFα and LPS (34, 58). Neutrophils can also release PADs during NETosis or necrosis (59). However, PADs are inactivated in oxidative environments and are rendered inactive shortly after release from the cell (60). Because NETs are thought to be significant in rheumatoid arthritis, it is important to better understand diverse NET stimuli and the requirement of PAD2 and PAD4 in NETosis.

D. Antibodies

Rheumatoid arthritis is largely characterized by the presence of autoantibodies (reviewed in (61)). Among the many different autoantibodies generated during inflammation, rheumatoid factor (RF) and ACPAs are the most prominently investigated. RF recognizes the Fc portion of human IgG, while ACPAs recognize peptidyl citrulline. Further, RF and anti-cyclic citrullinated peptide (CCP) antibodies underlie rheumatoid arthritis diagnostics that contribute heavily to diagnostic criteria for disease (62). Classification criteria for rheumatoid arthritis is based on a scoring system involving multiple facets of disease (62). To be considered for a diagnosis of rheumatoid arthritis, a patient must have swelling of at least one joint that cannot be explained by a different diagnosis. Then, the patient must get a certain number of points based on symptoms. Joints that are swollen or tender are counted and patients receive more points for a higher number of involved joints and if the joints are small. Duration of joint inflammation is accounted for as well, and symptoms must be present for more than six weeks to gain a point. An additional point is given for serologic markers of inflammation including C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). Serological presence of RF and anti-CCP antibodies is also evaluated, and patients receive points for having positivity for each marker.

ACPAs are about 96% specific for rheumatoid arthritis and can be detected in the serum of patients up to ten years before diagnosis (63, 64). ACPA levels begin to rise shortly prior to the onset of disease and the repertoire of recognizable citrullinated epitopes also

expands (61, 63, 65). ACPAs promote inflammation in a variety of ways. NETosis can be stimulated by ACPAs, and NETosis correlates with ACPA abundance (37). Immune complexes formed by ACPAs and citrullinated protein are recognized by macrophage Fc receptors, eliciting secretion of the pro-inflammatory mediator TNFα (66). ACPAs and immune complexes can also initiate the complement cascade (67), generating chemotactic factors and the formation of membrane attack complexes onto nucleated cells causing subsequent secretion of pro-inflammatory factors (68, 69). Membrane attack complexes can also induce leukotoxic hypercitrullination (LTH) a process that appears to be a variant of NETosis, increasing the extracellular display of citrullinated protein (70).

Separate from autoimmune inflammation, the role of antibodies against citrullinated protein in microbial immunity is undefined. Rheumatoid arthritis patients are at an increased risk for infection (71). However, it is not known whether antibodies against citrullinated proteins could influence immunization and immunity. For example, if pathogens become citrullinated then perhaps recognition by ACPAs could aid in microbial clearance. Conversely, citrullination of vaccine components during immunization could decrease immunity to native pathogens. Since NETosis can be stimulated by vaccine adjuvant (72) and neutrophils in rheumatoid arthritis are more likely to undergo NETosis (37), during which PAD enzymes are released (59), patients could generate more antibodies against citrullinated vaccine components in addition to pre-existing circulating ACPAs. Therefore, it is important to investigate evidence for a citrulline biased response to vaccination in rheumatoid arthritis.

Although much work focuses on antibodies against citrullinated protein in rheumatoid arthritis, it is important to note that many patients do not test positive for these antibodies. About 25% of rheumatoid arthritis patients are seronegative for both serological diagnostic markers, RF and anti-CCP (73-75). Since CCP-RF- patients with a similar disease presentation as CCP+RF+ patients have fewer points and thus are less likely to meet classification criteria for rheumatoid arthritis, they often have delays in diagnosis and treatment (73). Seronegative rheumatoid arthritis patients would need high scoring in other categories such as number of swollen and tender joints and presence of inflammatory markers (including CRP and ESR) (62).

It is unknown whether seronegative disease is part of a spectrum of disease in rheumatoid arthritis or is another disease entirely that does not rely upon autoantibodies (reviewed in (76)). A recent study found that average autoantibody levels against thousands of different peptides are very low in seronegative patients (77), but it is unknown if high levels of autoantibodies exist against targets not evaluated in that study or if autoantibodies are present at very low, often undetectable, levels in seronegative disease in general. Interestingly, some seronegative patients will convert to seropositive overtime, suggesting that the diseases exist on the same spectrum. Further, some risk alleles are shared between seropositive and seronegative disease (76). Alternatively, seronegative disease may occur in an antibody independent manner that manifests similar to seropositive rheumatoid arthritis (reviewed in (78)). Pathology could be perpetuated by innate immune cells and by excess cytokines like TNFα (79), which activate inflammatory pathways including hyperactivity of macrophages and synovial fibroblasts that increase

secretion of additional pro-inflammatory cytokines. Dysregulation of many cell types can occur due to increased secretion of cytokines such as IL-1, Il-17, or IL-23. Further, dysregulated negative selection can increase circulation of autoreactive T cells (80). In mice, transfer of autoreactive CD4+ T cells to a naïve mouse can initiate inflammatory arthritis, indicating a dispensable role for IgG in disease initiation.

In recent years, significant work has focused on autoantibodies against post-translationally modified proteins in rheumatoid arthritis, including ACPAs (21, 81-83). However, rheumatoid arthritis patients also generate antibodies against a multitude of native peptides, including peptides from complement proteins (77). Autoantibodies against native proteins are understudied and could serve as the foundation for a better understanding of seronegative rheumatoid arthritis as well as the development of diagnostic tests in seronegative disease. Further work must investigate potential pathophysiologic roles for anti-complement antibodies in the cycle of inflammation and evaluate the potential for anti-complement antibodies in rheumatoid arthritis diagnostics.

E. Conclusions and Objectives

Rheumatoid arthritis is a vicious cycle of inflammation (depicted in Figure 1). Through genetic and environmental factors, activation of various cells and reactivity against self-antigen occurs in the synovium. Activated synovial fibroblasts and macrophages release pro-inflammatory mediators including vascular endothelial growth factor (VEGF), IL-8,

and TNFα, resulting in increased angiogenesis and recruitment of immune cells to the joint (13). In response to pro-inflammatory factors, innate immune cells become activated and neutrophils produce extracellular traps which display a variety of proteins, some of which are citrullinated. Simultaneously, the adaptive immune system is activated causing B cells to initiate secretion of autoantibodies which recognize self-antigen in the joint. Immune complexes can then initiate the complement cascade which terminates in the formation of membrane attack complexes in synovial fibroblasts, inducing production of more pro-inflammatory cytokines. Immune complexes are also bound by Fc receptors on macrophages and, in response, macrophages secrete TNFα to initiate the cycle again.

In order to develop interventions for rheumatoid arthritis, it is critical to investigate the inflammation cycle to identify pathways which could serve as potential therapeutic targets. Here we address two parts of the cycle, NETosis and antibodies in rheumatoid arthritis. In chapter 2, we investigate the production of NETs in response to a variety of stimuli to define activators which lead to citrullinated and uncitrullinated NETs. We also define the requirement for PAD2 and PAD4 in the production and citrullination of NETs. In chapter 3, we aim to better understand the influence of rheumatoid arthritis on immunization in part through investigating a citrulline biased immune response to the pertussis component of the Tdap vaccine. In chapter 4, we identify novel anti-complement autoantibodies in seronegative rheumatoid arthritis in an effort to improve disease diagnostics.

F. Figures

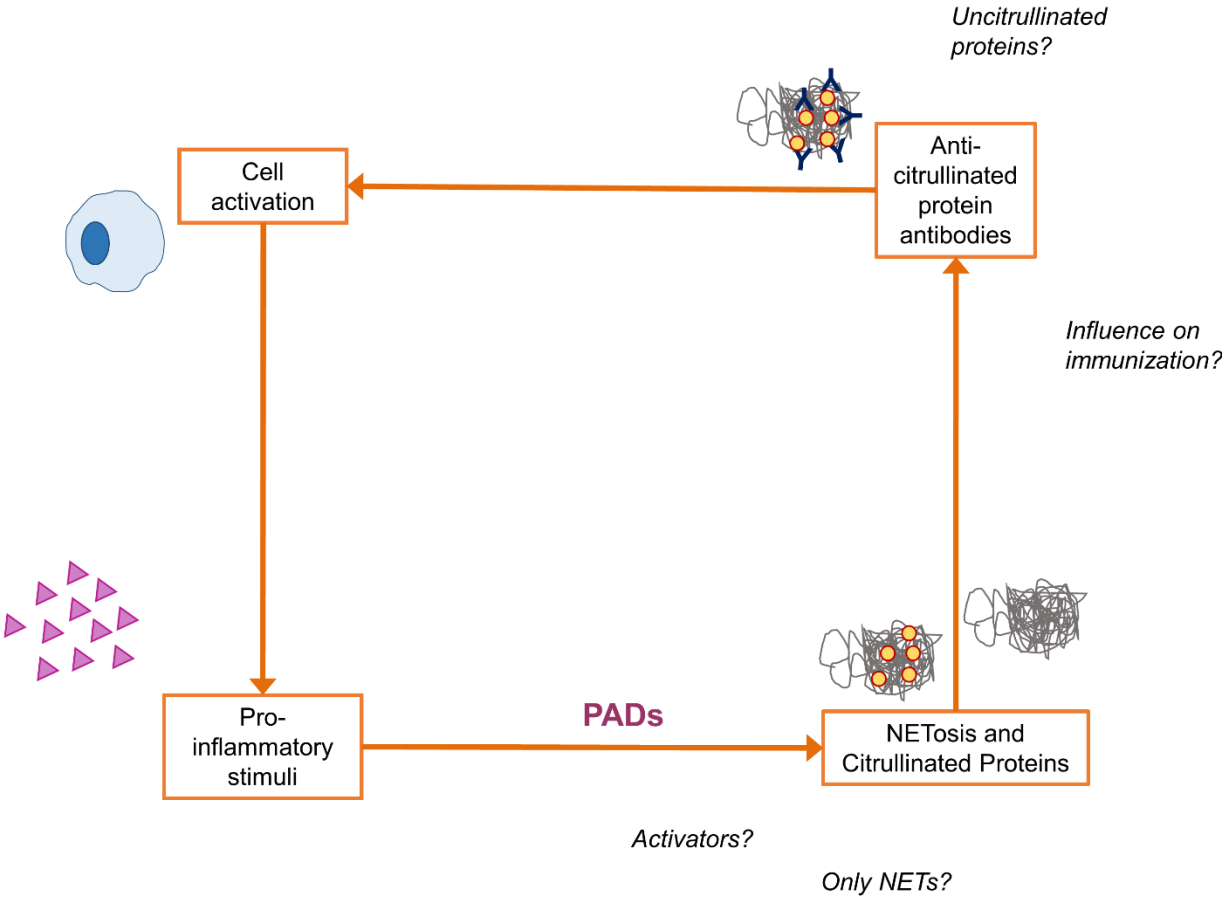

Figure 1. The cycle of inflammation in rheumatoid arthritis. Inflammation during rheumatoid arthritis is complex. Activated cells such as synovial fibroblasts and macrophages secrete pro-inflammatory factors that can activate NETosis. Citrullinated proteins, which can be found on NETs, are bound by ACPAs. Immune complexes formed by ACPAs then stimulate cells to secrete more pro-inflammatory factors, initiating the cycle again. However, there are multiple questions that remain such as: Which stimuli activate the production of NETs, both citrullinated and uncitrullinated? Are PAD2 and PAD4 required for NET production? Do macrophages produce citrullinated extracellular

traps? How does immune dysregulation in rheumatoid arthritis intersect with vaccination and is there a citrulline biased immune response to vaccination in rheumatoid arthritis? What native proteins are targeted by autoantibodies? Are autoantibodies against native protein present seronegative disease? This thesis will investigate these questions. (PAD: peptidylarginine deiminase; NET: neutrophil extracellular trap).

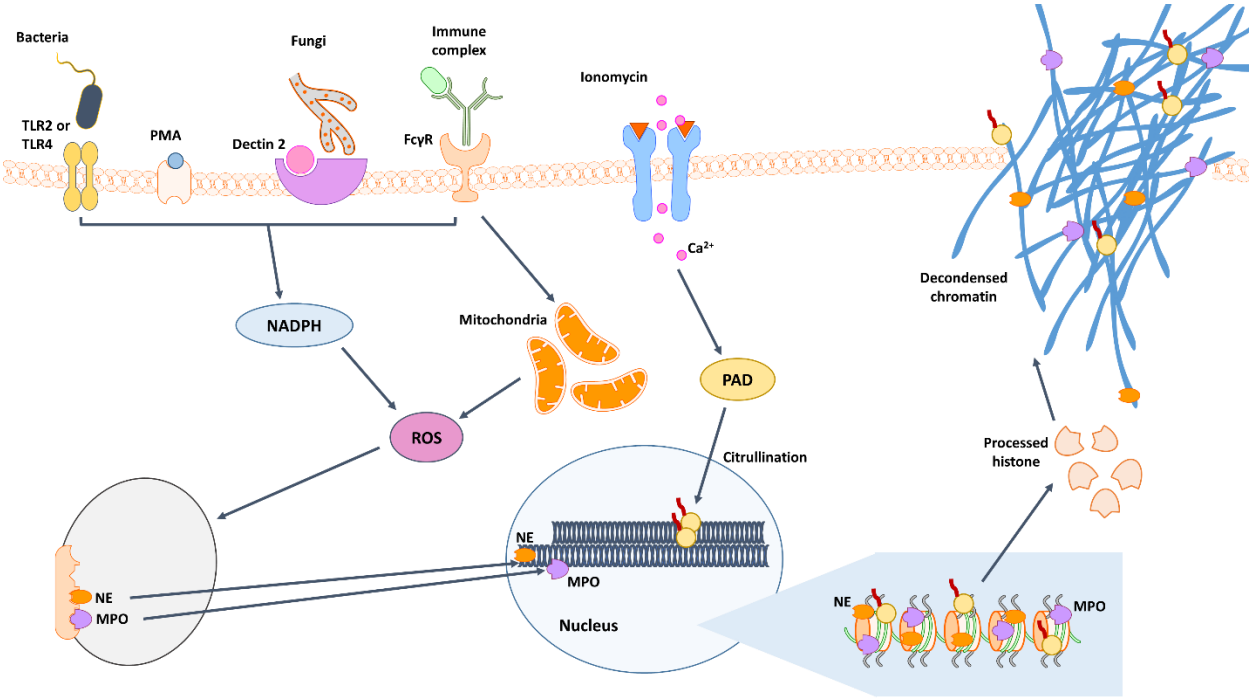

Figure 2. Common stimuli and pathways of NETosis. During NETosis, stimuli can activate NADPH which generates ROS. The ROS then activate MPO, which activates NE. MPO and NE then translocate to the nucleus and process histones, disrupting chromatin packaging and resulting in nuclear decondensation. Immune complexes can initiate NETosis independent of NADPH by stimulating generation of mitochondrial ROS. Ionomycin can induce an influx of calcium ions which activate PAD enzymes that

citrullinate histones, leading to chromatin decondensation. (TLR: toll-like receptor; PMA: phorbol myristate acetate; ROS: reactive oxygen species; MPO: myeloperoxidase; NE: neutrophil elastase; PAD: peptidyl arginine deiminase.)

CHAPTER TWO

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Insight into Neutrophil Extracellular Traps through Systematic Evaluation of Citrullination and Peptidylarginine Deiminases

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CLH contributed to the manuscript by performing about 25% of the human neutrophil experiments in Figure 1; performing all mouse experiments in Figures 2-4 and Supplementary Figures 2-5; developing methodology for quantifying NETs; analyzing and assembling all data for the manuscript; making all of the figures, writing the original manuscript, reviewing and editing the final manuscript.

A. Abstract

In rheumatoid arthritis, an autoimmune inflammatory arthritis, citrullinated proteins are targeted by autoantibodies and thus thought to drive disease. Neutrophil extracellular traps (NETs) are a source of citrullinated proteins and are increased in rheumatoid arthritis and therefore also implicated in disease pathogenesis. However, not all NETs are citrullinated. One theory aiming to clarify the intersection of citrullination, NETs, and rheumatoid arthritis suggests that specific stimuli induce different types of NETs defined by citrullination status. However, most studies do not evaluate uncitrullinated NETs, only citrullinated or total NETs. Further, the requirement for peptidylarginine deiminase (PAD) 2 and 4, two important citrullinating enzymes in neutrophils and rheumatoid arthritis, in the formation of different NETs has not been clearly defined. To determine if specific stimulants induce citrullinated or uncitrullinated NETs and if those structures require PAD2 or PAD4, human and murine neutrophils, including from PAD4$^{-/-}$ and PAD2$^{-/-}$ mice, were stimulated *in vitro* and NETs imaged and quantified. In humans, phorbol myristate acetate (PMA), ionomycin, monosodium urate (MSU), and *Candida albicans* induced NETs with MSU and *C. albicans* inducing primarily citrullinated, PMA primarily uncitrullinated, and ionomycin a mix of NETs. Only ionomycin and *C. albicans* were strong inducers of NETs in mice with ionomycin-induced NETs mostly citrullinated and *C. albicans*-induced NETs a mix of citrullinated and uncitrullinated. Interestingly, no stimulus induced exclusively citrullinated or uncitrullinated NETs. Further, PAD4 was required for citrullinated NETs only, whereas PAD2 was not required for either NET in mice. Therefore, specific stimuli induce varying proportions of both citrullinated and

uncitrullinated NETs with different requirements for PAD4. These findings highlight the complexity of NET formation and the need to further define the mechanisms by which different NETs form and their implications for autoimmune disease.

B. Introduction

Neutrophil extracellular traps (NETs) are complex webs of chromatin and proteins extruded from neutrophils during the programmed cell death process of NETosis (39). NETs can be antimicrobial (39, 45, 53, 54) and aid in the resolution of inflammation (46). However, NETs also appear to be pathologic in multiple autoimmune diseases including rheumatoid arthritis (37, 52), systemic lupus erythematosus (84, 85), antiphospholipid antibody syndrome (86, 87), and small vessel vasculitis (51). In rheumatoid arthritis, the pathology is thought to hinge on the presence of citrullinated proteins on NETs. Citrullination is the posttranslational deimination of arginine residues to citrullines, catalyzed by the peptidylarginine deiminases (PADs). Most patients with rheumatoid arthritis generate autoantibodies that bind citrullinated proteins (21). Since NETs are increased in rheumatoid arthritis (37, 52) and contain citrullinated proteins targeted by anti-citrullinated protein antibodies (37, 55, 56), NETs are hypothesized to be a significant source of citrullinated proteins in rheumatoid arthritis thus driving inflammation.

However, different stimuli can produce NETs with different composition and cargo (37, 88, 89) as well as potentially different types of NETs with different roles for citrullination

(47). For example, leukotoxic hypercitrullination (LTH) generates NETs characterized by hypercitrullination and can be induced by the membrane attack complex (70) or pore forming bacterial proteins (9). In contrast, phorbol myristate acetate (PMA) stimulates NETosis without citrullination (88, 89). Based on the literature, a categorization of NETs has been hypothesized with NETosis induced by several stimuli including PMA, fungi, and monosodium urate (MSU) without citrullination and LTH induced by pore-forming molecules with citrullination (47). Such a categorization is helpful for understanding different types of NETs, their mechanisms of formation, their functions, and their potentially different roles in autoimmune disease. For example, if LTH induced by the membrane attack complex leads to hypercitrullination and NETosis induced by *Candida albicans* does not involve citrullination, then membrane attack complex induced LTH might drive rheumatoid arthritis and *C. albicans* induced NETosis might not. However, there is variation among reports regarding which stimuli induce NETs. For example, some studies show that ionomycin and *C. albicans* induce extensive NETs and others report that these stimuli induce few to no NETs (54, 88, 90-94). Further, most studies evaluate either total or citrullinated NETs, so much less is known about uncitrullinated NETs. Given the gaps in the literature and the importance of understanding different types of NETs in autoimmune disease, it would be of benefit to determine which stimuli induce citrullinated and uncitrullinated NETs.

There are also questions regarding the roles of PAD2 and PAD4 in the formation of different types of NETs. These two PADs are found in neutrophils (95) and the rheumatoid joint (96) and each independently contributes to murine rheumatoid arthritis (34, 35).

Further, specific inhibitors of each of these PAD enzymes are being developed with consideration for treatment in rheumatoid arthritis (97, 98). Many NET studies have focused on PAD4, which citrullinates histones enhancing chromatin decondensation during NETosis (43, 57). Further, PAD4 was shown to be required for the production of NETs induced by various stimuli (34, 45, 99-103). However, PMA inhibits PAD4 while inducing NET formation (88) and PAD4 is not required for NETs formed in response to *Klebsiella pneumoniae* (104) or *C. albicans* (94), suggesting that PAD4 may not be required for the formation of all NETs. Much less is known about the role of PAD2 in NETosis. PAD2 is present on NETs (59), but is not required for the formation of NETs in response to TNFα and LPS (34). No other studies have investigated a requirement for PAD2 in NET formation, a problematic gap in knowledge since PAD2 appears to be required for the bulk of citrullination in a murine model of rheumatoid arthritis (34).

In this report, we systematically quantify murine and human NETs formed in response to ionomycin, PMA, MSU, and *C. albicans* and determine if they are citrullinated or uncitrullinated. We also evaluate if PAD2 or PAD4 is required for the NETs induced by these stimuli.

C. Results

Ionomycin (a calcium ionophore and pore-forming molecule), MSU crystals (which activate leukocytes via toll-like receptors and the inflammasome driving gout), PMA

(which activates protein kinase C and thus NF-κB), and *C. albicans* are diverse and common stimulants of NETosis with innumerable connections to autoimmune disease. To determine if these stimulants induce citrullinated and/or uncitrullinated NETs, human neutrophils were isolated from peripheral blood and incubated with no treatment or each stimulant for 4 hours followed by fixation, staining to detect DNA and citrullinated proteins, imaging, and quantification. As expected and as confirmation of a lack of stimulation upon purification, untreated neutrophils generated almost no NETs (Figure 1A, B). Ionomycin, MSU, PMA, and *C. albicans* all induced more NETs than untreated neutrophils (Figure 1A, B). As shown in Figure 1E, MSU and *C. albicans* induced primarily citrullinated NETs whereas PMA induced mostly uncitrullinated NETs. However, PMA also induced some citrullinated NETs, more than unstimulated neutrophils (Figure 1C). *C. albicans* induced more citrullinated NETs as well as more uncitrullinated NETs than untreated neutrophils (Figure 1C, D). The ionomycin-induced NETs were a mix of citrullinated and uncitrullinated (Figure 1E). Given the low levels of citrullination at 4 hours after PMA or ionomycin treatment, we also quantified NETs 8 and 20 hours after PMA or ionomycin treatment. Similar numbers of citrullinated NETs were seen at those time points as compared to the 4 hour time point (data not shown). Finally, because F95 may cross-react with homocitrulline, we repeated our experiments quantifying citrullinated NETs using an antibody against citrullinated histone H4. The numbers of citrullinated NETs and uncitrullinated NETs were similar using this antibody (Figure 1C, D versus Supplementary Figure 2B, C), although in general slightly fewer citrullinated and slightly more uncitrullinated NETs were detected for each condition leading to some differences in the

ratio of citrullinated versus uncitrullinated NETs (Figure 1E versus Supplementary Figure 2D).

Since mice are commonly used as an experimental model for autoimmune diseases involving NETs, we wanted to determine if findings would be similar in mice. We purified neutrophils from murine bone marrow and induced and quantified NETs as above. Unlike in human neutrophils, ionomycin was a very strong inducer of murine NETs and these structures were primarily citrullinated (Figures 2B, E). Also unlike in humans, MSU induced NETs variably in mice and murine MSU-induced NETs were a mix of citrullinated and uncitrullinated structures (Figures 2B, E). With murine neutrophils, PMA did not induce more NETs than untreated neutrophils (Figure 2B), which is also different than human neutrophils. Even when we stimulated with tenfold higher concentrations of PMA, similar results were seen (data not shown). However, like human neutrophils, the few NETs that were induced by PMA were uncitrullinated (Figure 2E). Also, PMA, and no other stimulant, led to the formation of ring-shaped structures of variable size in about 30% of murine neutrophils. Human neutrophils did not commonly make these structures as visualized by immunofluorescence, but similar structures could be seen by electron microscopy (Supplementary Figure 1) and perhaps could be called “doNETs” given their donut-like shape. As in human neutrophils, *C. albicans* was a strong inducer of NETs (Figure 2B) and *C. albicans* induced a mixture of citrullinated and uncitrullinated NETs (Figure 2E). Of note, both human and murine *C. albicans*-induced NETs were smaller in size compared to NETs induced by other stimuli. Finally, like human neutrophils, similar proportions of citrullinated and uncitrullinated NETs were seen with activation for 8 and

20 hours as compared to 4 hours (data not shown). Results for F95 agreed with results for anti-citrullinated histone H4 with very small differences seen only for PMA likely due to the low number of NETs with this condition (Supplementary Figure 3).

We then determined if PAD4 is required for the formation of NETs in response to the selected stimuli. Identical experiments as above were performed using bone marrow derived neutrophils from $PAD4^{+/+}$ and $PAD4^{-/-}$ mice. As shown in Figures 3A-D, $PAD4^{-/-}$ neutrophils generated almost no citrullinated NETs in response to any stimulus, with similar numbers of uncitrullinated NETs to $PAD4^{+/+}$ neutrophils for all stimuli. The absence of citrullinated NETs led to a loss of total NETs in response to ionomycin, which primarily induces citrullinated NETs in mice. We then used identical methods and $PAD2^{-/-}$ and $PAD2^{+/+}$ mice to determine if PAD2 is required for NETosis. As shown in Figure 4A-D, $PAD2^{-/-}$ mice showed no difference in the number of either citrullinated or uncitrullinated NETs induced by any stimulus. Repeating both the PAD4 and the PAD2 experiments using anti-citrullinated histone H4 showed the same findings: a loss of citrullinated NETs in the absence of PAD4 and no loss of NETs in the absence of PAD2 (Supplementary Figures 4 and 5).

D. Discussion

In this study, we quantified the formation of citrullinated and uncitrullinated NETs in response to ionomycin, PMA, MSU, and *C. albicans*. One conclusion from our studies is

that human peripheral blood and murine bone marrow derived neutrophils respond differently to stimuli. For example, ionomycin induced 66% of neutrophils to form NETs in mice and 28% in humans while PMA induced 9% of neutrophils to form NETs in mice and 77% in humans (Figures 1 and 2). Additionally, MSU was a strong inducer of NETs in human neutrophils and a variable inducer in murine neutrophils. Although some differences may be due to the location from which the neutrophils were purified (i.e. peripheral blood versus bone marrow) and thus maturation level, these findings suggest that NET production varies with the source of neutrophils, which may contribute to conflicting reports about the ability of different stimuli to induce NETs (90). Other studies have identified a species-specific difference related to myeloperoxidase (105).

Regarding citrullination status, mice and humans often diverged again. For example, ionomycin-induced murine NETs were primarily citrullinated, whereas ionomycin-induced human NETs were a mix of citrullinated and uncitrullinated. *C. albicans* was a strong inducer of NETs in both mice and humans as previously shown (53, 92, 94, 106, 107) with primarily citrullinated NETs formed in humans and citrullinated and uncitrullinated in mice. Similarly, MSU induced mostly citrullinated NETs in humans and a mix in mice. In addition to highlighting the differences between mice and humans, our findings and the findings of others (94), do not support the theory that *C. albicans* or MSU inhibits citrullination (47). In contrast and as expected (88, 89), for both humans and mice, PMA induced primarily uncitrullinated NETs, although some citrullinated NETs formed, particularly in humans. In neutrophils, PMA rapidly induces reactive oxygen species (89), which is required for PMA-induced NETs (103) and can inhibit PADs (108), perhaps

explaining the relative lack of citrullination in addition to a reported role for PMA-induced protein kinase C alpha in PAD4 inhibition [16]. Of note, no stimulant in this study induced exclusively citrullinated or uncitrullinated NETs, a novel observation. It is possible that some citrullinated proteins were not detected by the F95 antibody and the immunofluorescence methodology although F95 recognizes a variety of citrullinated proteins. We observed similar results using an anti-citrullinated histone H4 antibody (Supplementary Figures 2-5) although there were some differences, primarily in humans, potentially related to F95 detecting homocitrulline or the reactivity of anti-citrullinated histone H4 against only a single citrullinated protein.

Nonetheless, by quantifying both citrullinated and uncitrullinated NETs, we demonstrated that specific stimuli induce varying proportions of both citrullinated and uncitrullinated NETs in mice and humans, providing new insights into NETs. Although the citrullinated and uncitrullinated NETs could be categorized as resulting from LTH and NETosis, we did not observe that specific stimuli strictly induced either LTH with citrullination or NETosis without citrullination. Thus, the combination of stimulus and citrullination presence/absence may not be ideal for defining different NETs. Moreover, the generation of both citrullinated and uncitrullinated NETs in response to a single stimulus suggests that individual neutrophils may employ different pathways to generate NETs, sometimes involving citrullination and sometimes not. It will be important to further characterize the different mechanisms by which NETs with different characteristics form, since these differences may have important implications for autoimmune disease, especially rheumatoid arthritis with its citrulline-targeting autoantibodies.

Additionally, since we evaluated the requirement for PAD4 in both citrullinated and uncitrullinated NETs, whereas other groups evaluated either total or citrullinated NETs, we were able to demonstrate for apparently the first time that PAD4 is required for the production of citrullinated, but not uncitrullinated, NETs. These findings help to explain some of the discrepancies in the literature. Multiple studies have shown a requirement for PAD4 in NETosis (34, 45, 99-103). However, many of these studies quantified citrullinated NETs. More recently, PAD4 was shown to be dispensable for *Klebsiella*-(104) and *C. albicans*-induced NETs (94), in both cases with NETs detected primarily by DNA staining. Thus, some of the discrepancies among PAD4 studies may relate to whether only citrullinated NETs or total NETs were quantified. Indeed, PAD4 is required for histone citrullination induced by *Klebsiella* and *C. albicans* (94, 104). Other discrepancies related to the role for PAD4 in NETosis may be due to methodology. For example, in a study that concludes that PAD4 is not required for ionomycin-induced NETs (94), the NETs were quantified by increased Sytox fluorescence, not visualized NETs. Since ionomycin can form pores, perhaps those pores allowed Sytox entry and DNA staining without NET formation. Our observation that PAD4 is required for the production of only citrullinated NETs also suggests that the formation of different NETs can have different requirements. Thus, it is important to assess both citrullinated and uncitrullinated NETs.

Finally, we evaluated PAD2 in NETosis. Previously, we demonstrated that PAD2 is not required for NETs induced by LPS and TNFα (34). Here we found that PAD2 is not

required for the production or citrullination of murine NETs induced by ionomycin, MSU, PMA, or *C. albicans,* suggesting that PAD2 is not required for NETosis in general. Thus, although PAD2 is present in NETs (59), it is not required for their formation. This finding has interesting implications for rheumatoid arthritis. NETs have been hypothesized to be a significant source of citrullinated protein in rheumatoid arthritis (37, 52). We, and others, have shown that PAD4 is required for the formation of citrullinated NETs (34, 45, 109). However, in PAD4-deficient mice with inflammatory arthritis, total citrullination is not reduced in the serum, lung, or joint (34-36). In contrast, PAD2 is required for a significant amount of citrullination in the joints of mice with inflammatory arthritis (34), but is not required to form citrullinated NETs. Although there are challenges related to the quantification of citrullination, taken together, these studies suggest that although NETs display citrullinated proteins targeted by ACPAs (37), NETs may not be the main source of citrullinated proteins in rheumatoid arthritis. This theory is supported by the observations that PAD2 levels in synovial fluid correlate with total PAD activity and disease activity in rheumatoid arthritis (110), $PAD2^{-/-}$ mice have less central nervous system citrullination in experimental autoimmune encephalomyelitis (111), and PAD2 has less restrictive substrate specificity than PAD4 (112).

E. Conclusion

This study demonstrates that various stimuli induce a mix of citrullinated and uncitrullinated NETs in mice and humans. Further, PAD4 is required for citrullinated NETs

and PAD2 is not required for citrullinated or uncitrullinated NETs. Future studies are needed to further define different NETs, their mechanisms of formation, and their roles in the pathophysiology of autoimmune disease.

F. Materials and Methods

Human Subjects

This study was carried out in accordance with the recommendations of the Association for the Accreditation of Human Research Protection Program. The protocol was approved by the Institutional Review Board at the University of Wisconsin-Madison. All subjects gave written informed consent in accordance with the Declaration of Helsinki. Human subjects 18 years or older were recruited and provided a blood sample.

Animals

Age- and sex-matched wild-type, PAD2$^{-/-}$ (111), and PAD4$^{-/-}$ (45) mice back-crossed to a DBA/1J background (Jackson Laboratories, Bar Harbor, USA) were used. Animals were housed in a pathogen free facility. This study was carried out in accordance with the principles of the Basel Declaration and recommendations of the ARRIVE guidelines, The National Centre for the Replacement, Refinement and Reduction of Animals in Research. The protocol was approved by the University of Wisconsin Animal Care and Use Committee.

Purification and Stimulation of Human Neutrophils

Human blood was collected into EDTA tubes and neutrophils were purified using the EasySep Direct Neutrophil Isolation Kit (StemCell Technologies, Vancouver, Canada) according to the manufacturer's protocol. Neutrophil purity was at least 95% by flow cytometry. Neutrophils were plated onto acid washed, poly-L-lysine (Sigma Diagnostics, Livonia, USA) coated 12 mm glass coverslips at a concentration of 50,000 cells per coverslip in media containing RPMI 1640 (Thermo Fisher Scientific, Waltham, USA) with 2% fetal bovine serum (Atlanta Biologicals, Flowery Branch, USA) and 1% penicillin-streptomycin solution (Corning, Tewksbury, USA). Neutrophils were treated with the following and incubated for 4 hours at 37°C, 5% CO_2: 4 µM ionomycin (MilliporeSigma, Darnstadt, Germany), 560 µg/mL MSU crystals (InvivoGen, San Diego, USA), 25 nM PMA (Fisher BioReagents, Waltham, USA), or $1x10^6$ *Candida albicans* strain SC5314 (113).

Purification and Stimulation of Murine Neutrophils

Mouse femurs and tibias were flushed with the media described above and neutrophils were purified with the EasySep Mouse Neutrophil Enrichment Kit (StemCell Technologies) according to the manufacturer's protocol. Neutrophil purity was at least 91% by flow cytometry. Neutrophils were plated onto acid washed, poly-L-lysine coated 12 mm glass coverslips at a concentration of 70,000 cells per coverslip in the media described above. Neutrophils were incubated for 4 hours at 37°C, 5% CO_2 with the following stimuli: 5 µM ionomycin, 1200 µg/mL MSU crystals, 25 nM PMA, or $1x10^6$ *C. albicans* strain SC5314.

Candida albicans

C. albicans was prepared as previously described (34). Briefly, *C. albicans* was stored in 15% glycerol stock at -80°C with yeast extract-peptone-dextrose (YPD) medium supplemented with uridine (1% yeast extract, 2% peptone, 2% dextrose medium, 0.08% uridine) prior to experiments. Single *C. albicans* colonies were grown overnight in YPD with uridine at 30°C and orbital shaking at 200 RPM. Planktonic cells were used by diluting cultures 20-fold and incubating and shaking for an additional 2 hours. *C. albicans* was centrifuged and washed twice with the final concentration adjusted to $4x10^7$ cells/mL in phosphate buffered saline (PBS) before use.

Immunofluorescence

After stimulation, neutrophils were processed as previously (34) for immunofluorescence. Cells were incubated for 30 minutes at 4°C with 4% paraformaldehyde, 1% NP-40, and 0.5% Triton X-100 in PBS and then washed with PBS. Coverslips were then blocked overnight with 2.5% bovine serum albumin (BSA), 5% goat serum, and 0.5% Tween-20 in PBS followed by staining for 1 hour with anti-citrulline IgM (F95, MilliporeSigma) diluted 1:200 in blocking solution, washing with PBS, then incubating for 1 hour with anti-mouse-IgM-TRITC (SouthernBiotech, Birmingham, USA) diluted 1:200 and 4’6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, USA) diluted 1:1000 in blocking solution, and washing with PBS. Coverslips were mounted on glass microscope slides with Aquamount (Thermo Fisher Scientific). All staining was performed at room temperature. A Leica Fluorescence Microscope with Image Pro-Plus v.6.3 (Media Cybernetics,

Rockville, USA) was used to image five predetermined fields on the coverslip at 400x. For Supplementary Figures, processing, staining and imaging were identical as above, but F95 was replaced by anti-histone H4, citrulline 3 (MilliporeSigma) and anti-mouse-IgM-TRITC was exchanged for anti-rabbit IgG-TRITC (Jackson Laboratories).

Quantification of NETs

Neutrophils and NETs present in the five predetermined fields were counted by eye in a blinded manner. NETs were defined as neutrophils with significant enlargement of the DNA area beyond the size of a condensed nucleus (evident in unstimulated samples) with spread morphology and diffuse DNA structure (114). Citrullinated NETs also stained positively with F95.

Statistics

A t test was used to compare the percentage of NETs between untreated neutrophils and each stimulant as well as between wild-type and PAD-deficient neutrophils. A p value <0.05 was considered significant.

G. Figures

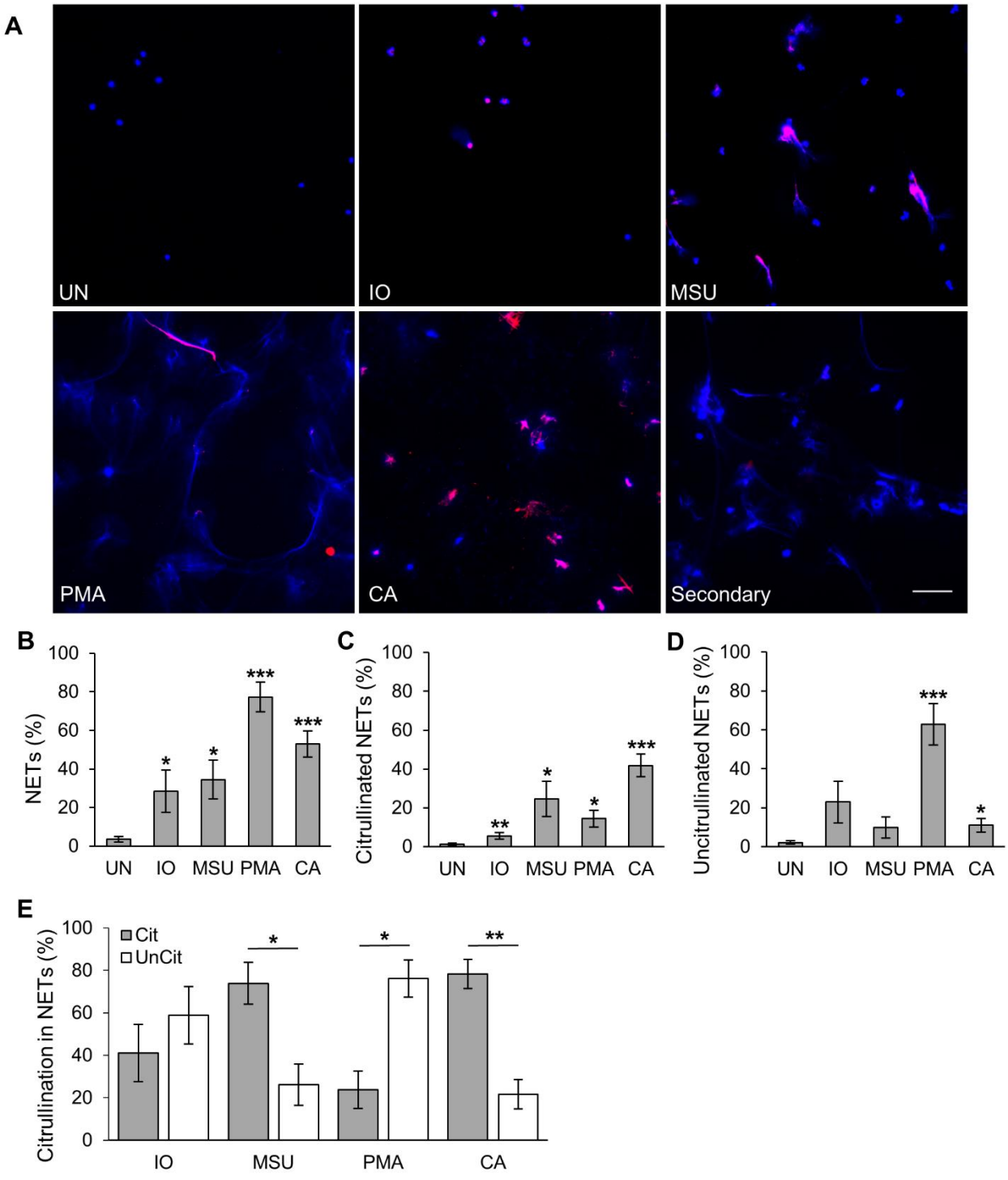

Figure 1. Induction of NETs in human neutrophils. Human neutrophils were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, or *C. albicans* (CA), fixed,

and stained with DAPI (blue) and anti-citrulline antibody (pink). Image labeled “Secondary” was created by stimulating neutrophils with *C. albicans* and staining without the F95 primary antibody and only the anti-mouse IgM TRITC secondary antibody as a negative control. (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (B), citrullinated NETs (C), and uncitrullinated NETs (D) for each condition with percent NETs for each stimulant compared to untreated. (E) The percent of citrullinated versus uncitrullinated NETs was compared for each stimulus with average and SEM graphed. For all panels: n=9, *p<0.05, **p<0.01, ***p<0.001.

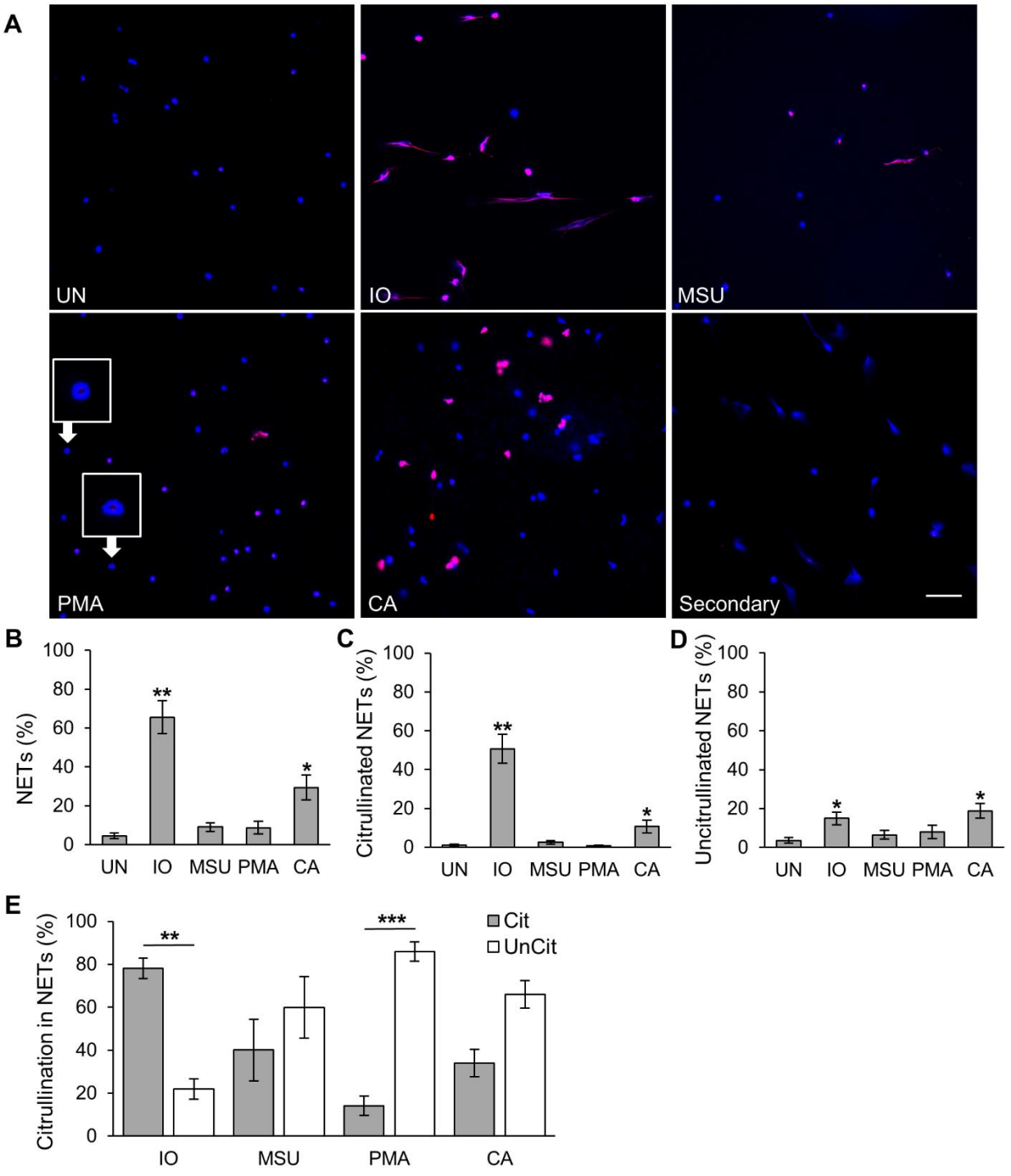

Figure 2. Induction of NETs in murine neutrophils. Murine neutrophils were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). Image labeled

"Secondary" was created by stimulating neutrophils with ionomycin and staining without the F95 primary antibody and only the anti-mouse IgM TRITC secondary antibody as a negative control. (A) Representative images at 400x, scale bar = 50μM. Enlarged insets demonstrate donut-like structures (doNETs). The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (B), citrullinated NETs (C), and uncitrullinated NETs (D) for each condition with percent NETs for each stimulant compared to untreated. (E) The percent of citrullinated versus uncitrullinated NETs was compared for each stimulus with average and SEM graphed. For all panels: n=6, *p<0.05, **p<0.01, ***p<0.001.

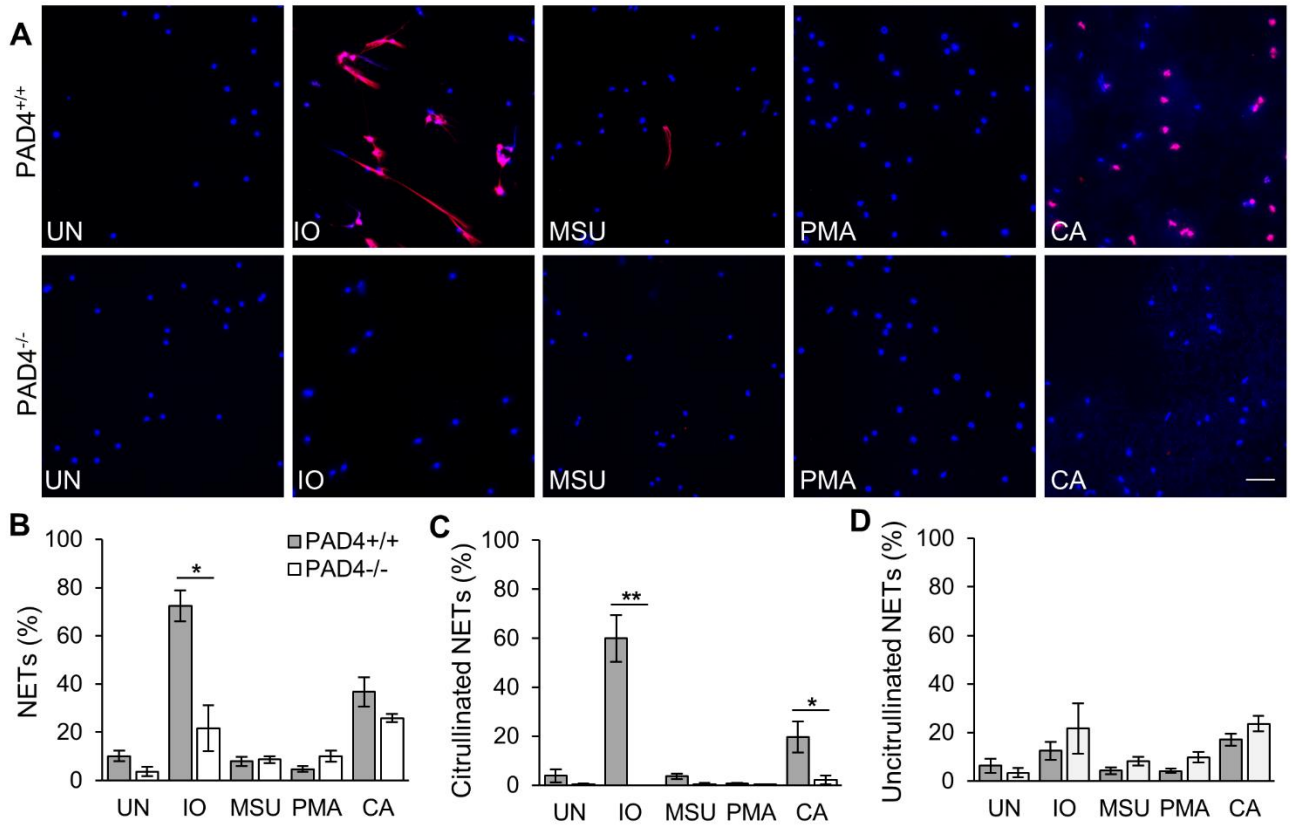

Figure 3. PAD4 is required for the formation of citrullinated NETs in murine neutrophils. Bone marrow neutrophils from PAD4$^{+/+}$ and PAD4$^{-/-}$ mice were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (B), citrullinated NETs (C), and uncitrullinated NETs (D) for each condition with percent NETs for each stimulant compared between PAD4$^{+/+}$ and PAD4$^{-/-}$ mice. For all panels: n=4, *p<0.05, **p<0.01.

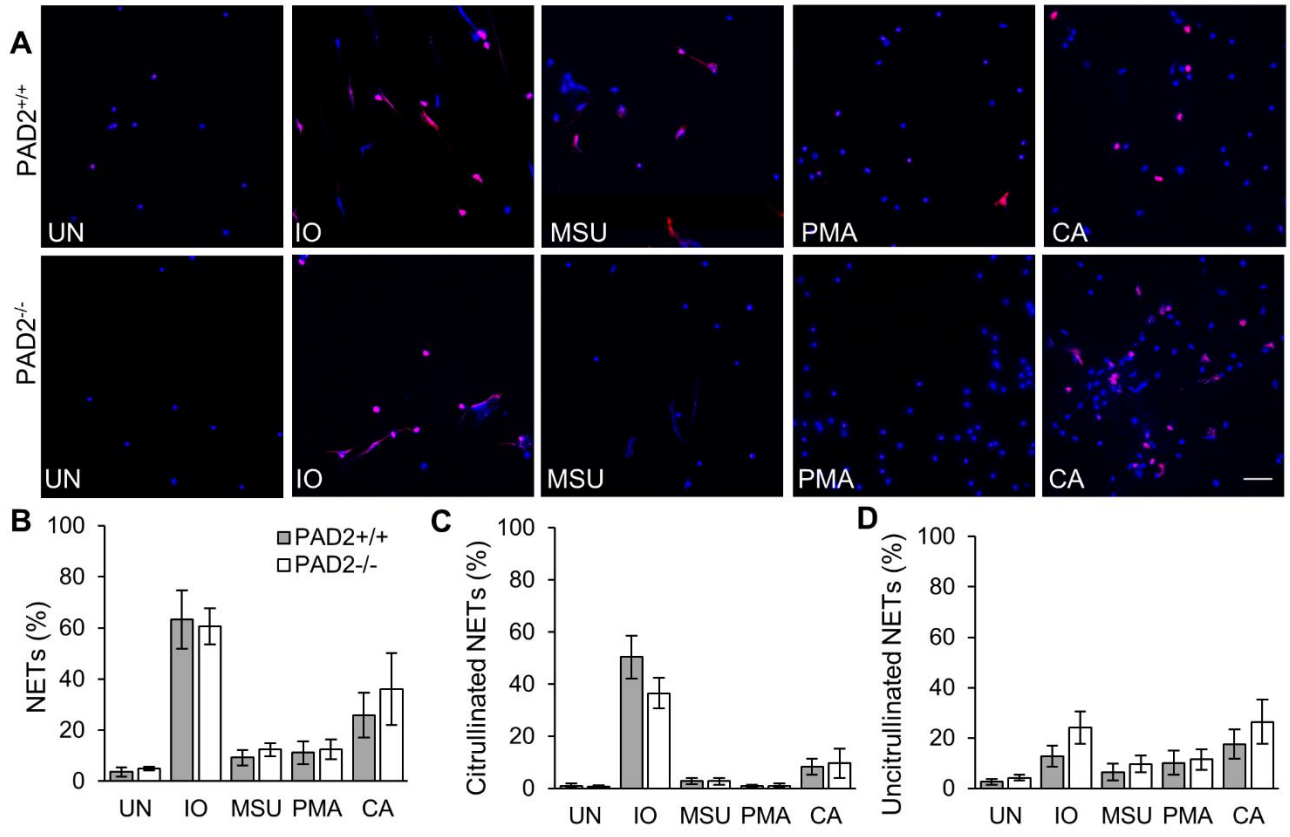

Figure 4. PAD2 is not required for the formation of NETs in murine neutrophils. Bone marrow neutrophils from PAD2$^{+/+}$ and PAD2$^{-/-}$ mice were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and *C. albicans* (CA), fixed, and stained with DAPI (blue) and anti-citrulline antibody (pink). (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed total NETs (B), citrullinated NETs (C), and uncitrullinated NETs (D) for each condition with percent NETs for each stimulant compared between PAD2$^{+/+}$ and PAD2$^{-/-}$ mice. For all panels: n=4, no comparisons were significant.

CHAPTER THREE

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Reduced IgG titers against pertussis in rheumatoid arthritis: evidence for a citrulline-biased immune response and medication effects

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Author Contributions:

CLH contributed to the manuscript by performing about 50% of the experiments in Figures 1 and 2 including subject selection; developing and analyzing regression modeling in Tables 1-4 and Supplementary Tables 1-2 under the supervision of a biostatistician; performing all experiments in Figure 3; analyzing and assembling all data for the manuscript; making all of the figures; writing the original manuscript and reviewing and editing the final manuscript.

A. Abstract

Background: The antibody response to pertussis vaccination in rheumatoid arthritis is unknown, a concerning omission given the relatively low efficacy of the pertussis vaccine, a rise in pertussis infections, and a general increased susceptibility to infection in rheumatoid arthritis. Additionally, the contributions from an intrinsically dysregulated immune system in rheumatoid arthritis and immune-suppressing medications to the response to pertussis vaccination is poorly defined. This study examines antibody titers against pertussis in vaccinated rheumatoid arthritis patients and controls as well as evaluates potential contributions from demographic factors, immune suppressing medications, and reactivity against citrullinated pertussis.

Methods: Serum IgG titers against native and citrullinated pertussis and tetanus were quantified by enzyme-linked immunosorbent assay in rheumatoid arthritis subjects and controls who were vaccinated within 10 years. Titers were compared by t-test and percent immunity by Fisher's exact test. Multivariable logistic regression was used to identify clinical factors that correlate with native pertussis titers.

Results: Compared to controls, rheumatoid arthritis subjects had lower titers against pertussis, but not tetanus, and reduced immunity to pertussis. These results were even more prominent at 5-10 years post-vaccination, when rheumatoid arthritis patients had 50% lower titers than controls and 2.5x more rheumatoid arthritis subjects were not considered immune to pertussis. Multiple logistic regression demonstrated that female sex and methotrexate use, but not TNF inhibiting medications, correlated with reduced immunity to pertussis. Finally, rheumatoid arthritis patients had higher IgG titers against

citrullinated pertussis than native pertussis.

Conclusions: Pertussis titers are lower in vaccinated rheumatoid arthritis patients with evidence for contributions from female sex, a citrulline-biased immune response, and methotrexate use.

B. Introduction

Patients with rheumatoid arthritis, a chronic progressive autoimmune disease with a lifetime risk of about 3% (115), are at increased risk for infection (71), but data are mixed regarding response to vaccination. Following influenza or pneumococcus immunization, which are both recommended for rheumatoid arthritis patients given overall efficacy (116, 117), rheumatoid arthritis patients have a normal response to some vaccine strains and serotypes and an impaired response to others (118-121), which may be improved by the use of adjuvant (122). Also, patients with rheumatoid arthritis have similar antibody levels against tetanus compared to controls, but differences in antibody affinity and subclass (123). Given the variability seen in the response of rheumatoid arthritis patients to different vaccines, it is necessary to separately assess the response to each vaccine. However, no studies have addressed the antibody response to the pertussis vaccine in rheumatoid arthritis.

Bordetella pertussis is a bacterial species that causes “whooping cough,” a severe respiratory infection characterized by violent and uncontrollable coughing associated with

high rates of rib fractures and syncope in adults and apnea, pneumonia, and death in babies. An estimated 16 million cases of pertussis were reported globally in 2008 and incidence in the United States has been rising since 2002 (124, 125). For adults in the United States, vaccination against pertussis is typically part of the Tdap (tetanus, diphtheria, and pertussis) vaccine, which is recommended to be administered every 10 years (126) since protection against pertussis from vaccination wanes after 4-12 years (127). Patients with inflammatory bowel disease were recently shown to have reduced titers against pertussis (128). Given the rise in the incidence of pertussis, the increased risk of infection in rheumatoid arthritis, and the reduced response of rheumatoid arthritis patients to some vaccines, it is important to determine if rheumatoid arthritis patients make a normal antibody response to pertussis vaccination.

The mechanism behind the altered response to some vaccines in rheumatoid arthritis is unclear. One possible mechanism is an inherently dysregulated immune system. People with rheumatoid arthritis generate autoantibodies against many different citrullinated proteins with overlapping specificity (56, 129-131) and strong reactivity against citrulline itself (132), starting years prior to the diagnosis of rheumatoid arthritis (63). This aberrant immune response extends to non-self antigens, since in rheumatoid arthritis, antibodies bind a citrullinated Epstein-Barr virus peptide more than the native peptide (133). However, this citrulline-bias has never been evaluated in the context of vaccine response. Additionally, patients with rheumatoid arthritis take immune suppressing medications that could reduce the response to vaccination (119, 134-136). Indeed, a brief discontinuation of methotrexate in rheumatoid arthritis patients can improve the response to vaccination

against influenza (137). The contributions of these mechanisms are unknown for the antibody response to pertussis vaccination in rheumatoid arthritis.

Here, we evaluate if antibody titers against pertussis in vaccinated rheumatoid arthritis patients are different than controls. We also examine demographics, immune suppressing medications, and reactivity against citrullinated pertussis to identify potential factors involved with the antibody response against pertussis in rheumatoid arthritis.

C. Results

Ninety-eight rheumatoid arthritis patients and seventy-seven controls who received the Tdap vaccine within 10 years of blood collection were selected from the UW Rheumatology Biorepository. Controls and rheumatoid arthritis patients were similar with regards to age, sex, race/ethnicity, smoking status, BMI, time since vaccination, and age at vaccination (S1 Table). Time since vaccination demonstrated a trend towards being slightly shorter for rheumatoid arthritis subjects as compared to controls, by an average of 9.6 months ($p=0.06$). Also, consistent with the one point given for a rheumatoid arthritis diagnosis in the scoring system, the Charlson comorbidity score was higher in rheumatoid arthritis subjects.

Sera from control and rheumatoid arthritis subjects were subjected to ELISA to detect IgG against pertussis and tetanus. As shown in Fig 1A (left panel), rheumatoid arthritis

subjects had lower pertussis IgG titers compared to controls. Moreover, more than twice as many rheumatoid arthritis patients were considered not immune to pertussis than controls (22% versus 10%, p=0.03, Fig 1A, right panel). As expected (123), no significant difference in tetanus IgG titers was observed between rheumatoid arthritis subjects and controls (Fig 1B).

Since pertussis immunity wanes after 4-12 years (127), we divided subjects by Tdap vaccination less than 5 years prior to serum collection or 5-10 years prior to serum collection and compared differences in pertussis IgG titers for rheumatoid arthritis versus control subjects. Rheumatoid arthritis subjects had only a trend towards reduced titers and reduced rates of immunity compared to controls <5 years after vaccination (Fig 1C). However, 5-10 years post-vaccination, rheumatoid arthritis subjects had about 50% lower titers compared to controls and 2.5 times more rheumatoid arthritis subjects were not immune (Fig 1D). Taken together, these data suggest that patients with rheumatoid arthritis have lower titers against pertussis and reduced rates of immunity against pertussis, particularly 5-10 years after vaccination.

To determine if clinical variables, such as use of immune suppressing medications, correlated with median pertussis titers, univariate and multiple logistic regression analyses were performed. When modeled for controls and rheumatoid arthritis subjects, a diagnosis of rheumatoid arthritis, female sex, and time since vaccination predicted lower than median pertussis titers in both univariate and multivariable analyses (Table 1). Obesity predicted higher than median titers in a univariate model, but this finding was not

significant in the multivariable model (Table 1). When rheumatoid arthritis subjects were evaluated separately, female sex was again predictive of lower than median pertussis titers (Table 2). Also, methotrexate use at the time of serum collection predicted lower than median pertussis titers in the univariate, but not the multivariable model (Table 2). In contrast, leflunomide use predicted higher than median pertussis titers in the univariate analysis only (Table 2). Time since vaccination again predicted lower than median pertussis titers and, interestingly, being diagnosed with rheumatoid arthritis at the time of vaccination (as opposed to not yet being diagnosed with rheumatoid arthritis at the time of vaccination) predicted greater than median pertussis titers in the univariate analysis with similar trends for both in the multivariable analysis.

We then performed univariate and multiple logistic regression analyses to identify clinical variables that correlate with immunity to pertussis according to cut-offs provided by the ELISA kit manufacturer. When modeled for controls and rheumatoid arthritis subjects combined, a diagnosis of rheumatoid arthritis and female sex predicted lower immunity to pertussis (Table 3), similar to the analysis for median pertussis titers (Table 1). Longer time since vaccination showed a trend towards predicting reduced immunity (Table 3), whereas time since vaccination clearly predicted lower than median titers (Table 1) perhaps due to titers falling over time, but not necessarily below the level required for immunity in this analysis. When modeled for rheumatoid arthritis subjects alone, female sex again predicted lower immunity (Table 4). Additionally, in multivariable analysis, methotrexate use at the time of serum collection predicted significantly lower immunity to pertussis (Table 4), similar to our univariate findings for median pertussis titers (Table 2).

Of note, medications prescribed at the time of vaccination were also analyzed in place of medications prescribed at the time of serum collection for subjects diagnosed with rheumatoid arthritis at the time of vaccination, but no medication significantly correlated with either greater than median pertussis titer or pertussis immunity when prescribed at the time of vaccination (S2 and S3 Tables).

We next determined if there was evidence of an inherently dysregulated immune system affecting the antibody response to pertussis vaccination in rheumatoid arthritis. We were intrigued by the univariate finding that a diagnosis of rheumatoid arthritis, as opposed to not yet being diagnosed, at the time of Tdap vaccination predicted greater than median pertussis titers (Table 2). The subjects without a diagnosis of rheumatoid arthritis at vaccination would be diagnosed 0.1 to 6.6 years later. Given known delays in diagnosis (138) and the common presence of anti-citrullinated protein antibodies (ACPAs) a decade prior to diagnosis (63), the subjects without a rheumatoid arthritis diagnosis very likely had undiagnosed rheumatoid arthritis or preclinical rheumatoid arthritis at the time of vaccination. However, none of these subjects were receiving immune suppression, providing an opportunity to evaluate a role for immune dysregulation in vaccine response. Thus, we compared pertussis titers in subjects with versus without a diagnosis of rheumatoid arthritis at the time of vaccination and found significantly lower titers in subjects not yet diagnosed with rheumatoid arthritis (Fig 2A). The undiagnosed subjects had a longer time since vaccination than subjects diagnosed with rheumatoid arthritis at the time of vaccination (average 1.8 years). Since a longer time since vaccination is also associated with lower pertussis titers (Fig 1 and Table 2), we divided subjects by

diagnosis or no diagnosis of rheumatoid arthritis at the time of vaccination and, for each group, plotted pertussis titer versus time since vaccination. As shown in Fig 2B, there is a trend towards reduced titers over time in both groups as well as lower titers in undiagnosed subjects suggesting that both time since vaccination as well as untreated, pre-diagnosed rheumatoid arthritis correlate with lower pertussis titers.

Given these findings suggesting that immune dysregulation might contribute to lower pertussis titers in rheumatoid arthritis, we then hypothesized that rheumatoid arthritis patients would have antibodies that bind citrullinated pertussis, potentially reducing the normal immune response against native pertussis. To test this hypothesis, we treated the pertussis- and tetanus-coated ELISA plates with PAD2 and PAD4 and quantified citrullination by ELISA. As shown in Fig 3A and 3B, pertussis was efficiently citrullinated, but tetanus toxoid was not, likely due to the detoxification of tetanus toxin with formaldehyde to generate tetanus toxoid, a process which modifies arginines (139, 140). We then demonstrated a lack of detectable binding of rheumatoid arthritis sera to the amount of PAD enzyme used to citrullinate (Fig 3C) and repeated our ELISAs to detect antibodies against citrullinated and native pertussis. Rheumatoid arthritis sera, and not control sera, had higher antibody binding to citrullinated compared to native pertussis (Fig 3D and 3E). Although we were unable to efficiently citrullinate tetanus toxoid *in vitro* and thus suspect that tetanus toxoid is also not citrullinated *in vivo*, we did see a trend towards increased citrullination and, thus, we determined if there was increased binding to the PAD-treated tetanus toxoid. As shown in Fig 3F and 3G, no increased binding was seen for rheumatoid arthritis or control sera against potentially citrullinated tetanus. Together,

these data suggest that pertussis can be citrullinated and antibodies in rheumatoid arthritis bind citrullinated pertussis more than native pertussis, whereas tetanus toxoid is resistant to citrullination and thus is not likely to be a target of ACPAs.

D. Discussion

In this report, we have demonstrated that pertussis IgG titers are significantly lower in rheumatoid arthritis subjects compared to controls. Moreover, the percent of rheumatoid arthritis subjects considered immune to pertussis is two-fold lower than controls. Both of these findings were even more prominent in subjects who received the pertussis vaccine 5-10 years prior to serum collection. These results could suggest that hundreds of thousands of rheumatoid arthritis patients in the United States alone may be susceptible to pertussis infection, despite receiving the Tdap vaccine according to national guidelines. Further, our findings, combined with the extremely low vaccination rates against pertussis in rheumatoid arthritis patients in Germany (141), could suggest that as the numbers of pertussis infections rise in general, pertussis could become a significant problem for rheumatoid arthritis patients.

Limitations of our study include its retrospective design and that it does not examine infection rates in rheumatoid arthritis. Additionally, while our results reflect immune status according to the cut-offs of a commercial assay, no laboratory correlate of definitive pertussis protection is known. It will be important for future studies to examine rates of

pertussis infection in patients with rheumatoid arthritis, particularly since we detected antibodies that react with citrullinated pertussis, which may or may not be protective. Moreover, prospective studies could determine if more frequent or higher dose vaccination would improve the antibody response to pertussis in rheumatoid arthritis.

We also provide evidence for potential mechanisms for these reduced titers, which are important for understanding how rheumatoid arthritis patients respond to immunization. One possible mechanism for reduced pertussis IgG titers is the use of specific immune suppressing medications. In multivariable analysis, methotrexate use at the time of serum collection significantly correlated with lower pertussis immunity in rheumatoid arthritis (Table 4) with a similar finding of predicting less than median pertussis titers in univariate analysis (Table 2). These findings are similar to results seen for several studies evaluating methotrexate use and pneumococcal and influenza vaccines (119, 134, 135), although another study did not identify a correlation between methotrexate use and reduced vaccine response (121). Additionally, a temporary pause in methotrexate usage at the time of influenza vaccination was shown to improve the response (137). In contrast, we did not observe a correlation between methotrexate use at the time of vaccination and reduced response to pertussis (Tables S2 and S3) potentially due to sample size, the retrospective nature of our study, or differences between the antibody response to pertussis versus influenza vaccines. Although the mechanism is not fully understood, methotrexate reduces T cell activity (142, 143). Since acellular pertussis vaccines likely require T cells to establish humoral memory (144, 145), the depletion of T cells by methotrexate may contribute to the lower pertussis IgG titers in subjects taking this

medication.

Interestingly, despite the high level of immune suppression attributed to TNF inhibiting medications, we did not see a correlation between TNF inhibitor use and reduced pertussis immunity or lower pertussis titers. This finding contrasts with a report that pertussis titers are lower in patients with inflammatory bowel disease using TNF inhibitors as compared to those using thiopurine (128). For influenza and pneumonia vaccines, some studies report a correlation between TNF inhibiting medications and reduced titers, some studies report no reduction in titers related to TNF inhibitor use, and some studies suggest that reduced titers could be due to co-administration of additional immunomodulating agents with the TNF inhibiting medication (121, 135, 136, 146-151), as is the case in many of our subjects. The reason for this variability has not been thoroughly investigated. One possibility is that there may be differences among TNF inhibitors. For example, infliximab (148) and golimumab (136) are associated with reduced response to influenza and pneumococcal vaccines, whereas in separate studies, adalimumab, certolizumab, and etanercept use correlate with a normal antibody response to these vaccines (149-151). Consistent with this theory, in our study, 34 of our 35 TNF inhibitor users were prescribed etanercept or adalimumab whereas in the inflammatory bowel disease study (128), 40% of the TNF inhibitor users were prescribed infliximab (F Caldera and M Hayney, University of Wisconsin-Madison, personal communication).

We also demonstrated that female sex significantly correlated with lower pertussis immunity and lower than median pertussis titers. This finding agrees with a study that

found a correlation between reduced seropositivity for pertussis toxin and female sex in Hungary (152). Sex differences in vaccine response occur for a variety of antigens although no clear pattern has been identified (153, 154). Females have a greater response to influenza, hepatitis A and B, smallpox, and Brucella whereas males show greater response to pneumococcal polysaccharide and yellow fever vaccines (153, 155-161). Both sexes respond similarly to the measles, mumps, and rubella (MMR) vaccine (162). It remains unknown if sex differences in vaccine response are due to hormones, epigenetics, environment, microbiome, or cultural influences (154). Future studies are needed to better characterize the role of sex in vaccine response and to optimize vaccine schedules based on gender and risk profiles.

Finally, our data suggest that an intrinsically dysregulated immune system may contribute to reduced pertussis titers in vaccinated rheumatoid arthritis subjects. We show that subjects vaccinated shortly prior to a diagnosis of rheumatoid arthritis (a disease phase associated with ACPAs as well as increased cytokines and chemokines (63, 163), but not immune suppressing medications) have lower pertussis titers than subjects diagnosed with rheumatoid arthritis at the time of vaccination. Moreover, we demonstrate that rheumatoid arthritis patients, but not controls, have greater binding to citrullinated than native pertussis, providing the first evidence that citrulline reactivity in rheumatoid arthritis extends to vaccine response. How antibodies against citrullinated pertussis develop and potentially contribute to reduced antibodies to native pertussis is not known. Vaccine adjuvants can attract neutrophils and induce neutrophil extracellular trap (NET) formation at the injection site (72). Citrullinating PAD enzymes are released during NETosis and

are present on NETs (59). Thus, pertussis at the injection site could become citrullinated by PADs released from NETs. Alternatively, antigen processing can generate citrullinated peptides (164). Rheumatoid arthritis patients may then preferentially generate antibodies against citrullinated as opposed to native pertussis antigens. Alternatively, given the cross-reactive repertoire of ACPAs in rheumatoid arthritis (56, 129-132), pre-existing ACPAs could cross-react with pertussis citrullinated by NETs leading to clearance and reduced antigen availability for the generation of an immune response. Further work is needed to fully reveal this and other potential mechanisms that may alter the immune response to pertussis in rheumatoid arthritis.

Finally, in contrast to our pertussis results, we found that rheumatoid arthritis patients had normal titers against tetanus, as previously reported (123). It is possible that immune suppressing medications do not sufficiently impair the immune system to reduce the antibody response to the highly effective tetanus vaccine, but do reduce the response to the less efficacious pertussis vaccine. Additionally, there may be inherent differences in the immune response against pertussis as compared to tetanus in rheumatoid arthritis. In support of this theory, we found increased IgG binding to citrullinated pertussis, whereas tetanus toxoid could not be efficiently citrullinated *in vitro* and thus is unlikely to be citrullinated *in vivo,* making the development of anti-citrullinated tetanus antibodies or cross-reactivity of ACPAs with tetanus extremely unlikely. Future studies are needed to examine differences in the immune response to vaccination against these different pathogens in rheumatoid arthritis.

E. Conclusion

We have shown that vaccinated rheumatoid arthritis patients have lower titers against pertussis than controls with a potential role for female sex, methotrexate, and a citrulline-biased immune response. Our findings have potential clinical importance since they are the first to identify lower pertussis titers in rheumatoid arthritis, suggesting that these patients could be more susceptible to pertussis infection and might benefit from more frequent vaccination. Additionally, we provide the first evidence that a citrulline-biased immune system may complicate the response to immunization in rheumatoid arthritis, providing a novel mechanism for abnormal vaccine response.

F. Materials and Methods

Human Subjects

Research was carried out and subjects gave written informed consent in compliance with the Declaration of Helsinki and as approved by the Institutional Review Board at the University of Wisconsin-Madison (#2015-0156). All clinical data and biologic samples were obtained from the University of Wisconsin (UW) Rheumatology Biorepository first described in (165). The biorepository contains clinical data (obtained from the electronic medical record and subject self-report) and serum from subjects at least 18 years old receiving primary care and rheumatology care (for rheumatoid arthritis subjects) in an

academic health system. Potential rheumatoid arthritis subjects were initially identified as individuals with two or more outpatient visits with rheumatoid arthritis associated ICD codes (ICD-9 codes 714.0-714.33, 714.9 or any ICD-10 code starting with M05, M06, or M08) within 24 months (166) or one visit and a positive anti-CCP (cyclic citrullinated peptide) antibody test. Rheumatoid arthritis diagnosis was confirmed based on manual review of the three most recent rheumatologist progress notes in the electronic medical record. Subjects were selected for this study if they had received a Tdap vaccine within 10 years of the blood collection for the biorepository. Rheumatoid arthritis subjects also had positive anti-CCP and rheumatoid factor tests with values twice the upper limit of normal. Since rituximab eliminates B cells preventing an antibody response, subjects using rituximab were excluded. Controls were matched by age and gender and excluded if they had any of the following diagnoses as determined by verbal screen or manual record review: systemic lupus erythematosus, Sjögren's Syndrome, scleroderma, multiple sclerosis, type I diabetes, psoriasis or psoriatic arthritis, ankylosing spondylitis, reactive arthritis, ulcerative colitis, Crohn's disease, cancer of the blood cells including leukemia or lymphoma.

For all subjects, the following variables were included as abstracted from the medical record for the time of serum collection unless otherwise noted: rheumatoid arthritis diagnosis, age, sex, smoking status and history, body mass index (BMI), Charlson comorbidity score (167), prescription of non-steroidal anti-inflammatory drugs (NSAIDs), and time since Tdap vaccination. For rheumatoid arthritis subjects, we also included age of rheumatoid arthritis diagnosis (self-reported since some subjects were diagnosed prior

to inclusion in our electronic medical record), which was used to determine if a subject had rheumatoid arthritis at the time of vaccination (for subjects self-reporting rheumatoid arthritis diagnosis within six months of vaccination date, diagnosis date by the subject's rheumatologist in the electronic medical record was used), and prescription of the following medications at the time of serum collection and vaccination as abstracted from the medical record: abatacept, hydroxychloroquine, leflunomide, methotrexate, sulfasalazine, and tumor necrosis factor (TNF) inhibitor (includes adalimumab, etanercept, and infliximab). No subjects were prescribed certolizumab or golimumab. Tofacitinib and tocilizumab were not included in the analysis since 4 and 0 subjects were prescribed this medication, respectively.

Serum Preparation

For the biorepository, blood was collected from subjects into serum separator tubes (Greiner Bio-One, Monroe, USA) and centrifuged at 1300x g for 10 minutes. Serum was transferred to a fresh tube and centrifuged at 2000x g for 5 minutes. The supernatant was then aliquoted and stored at -80°C.

Enzyme Linked Immunosorbent Assay (ELISA)

Pertussis and tetanus IgG titers were measured using *Bordetella pertussis* and Tetanus toxoid IgG ELISA kits according to the manufacturer's instructions (Immuno-Biological Laboratories, Inc., Minneapolis, USA). Per manufacturer's instruction, immunity to pertussis was defined as a pertussis IgG titer higher than 20 U/mL. For citrullination ELISAs, the precoated wells of pertussis and tetanus toxoid IgG ELISA kits were

incubated with citrullination buffer (100mM Tris-HCl pH7.5, 1mM DTT, and 5mM $CaCl_2$) alone or with buffer and 0.01µg/mL peptidylarginine deiminase (PAD) 4 and 0.01µg/mL PAD2 overnight at 37°C similar to previously (168). Wells were washed three times before proceeding with the ELISA per manufacturer's instruction. As a negative control, non-precoated 96 well plates (EIA/RIA Plate High Binding, Costar, Corning, USA) were exposed to buffer alone or buffer with PAD enzymes as above and used in ELISA to detect IgG in sera that binds to PAD enzymes. To assess citrullination efficiency, the pertussis and tetanus precoated wells exposed to buffer alone or buffer with PAD enzymes were washed three times before proceeding with the ELISA with these modifications: mouse anti-citrulline IgM (clone F95, EMD Millipore, Darmstadt, Germany) diluted 1:200 as primary antibody and anti-mouse IgM-HRP (SouthernBiotech, Birmingham, USA) diluted 1:5000 as secondary antibody.

Statistical Analysis

Mean pertussis titers were compared between rheumatoid arthritis subjects and controls using a t-test and antibody levels against native versus citrullinated antigen by paired t-test. Comparison for proportional immunity between groups was measured with a Fisher's exact test. To determine which clinical factors correlated with pertussis titer, univariate and multiple logistic regression analyses were performed. The multiple logistic regression models compared patients to median pertussis values across the entire cohort as well as proportions determined to be clinically immune to pertussis for odds ratio (OR) comparisons. For all statistical tests, $p<0.05$ was considered significant. Statistical analysis was performed using Stata version 14 (StataCorp LP, College Station, USA) and

Prism (GraphPad Software, San Diego, USA).

G. Figures and Tables

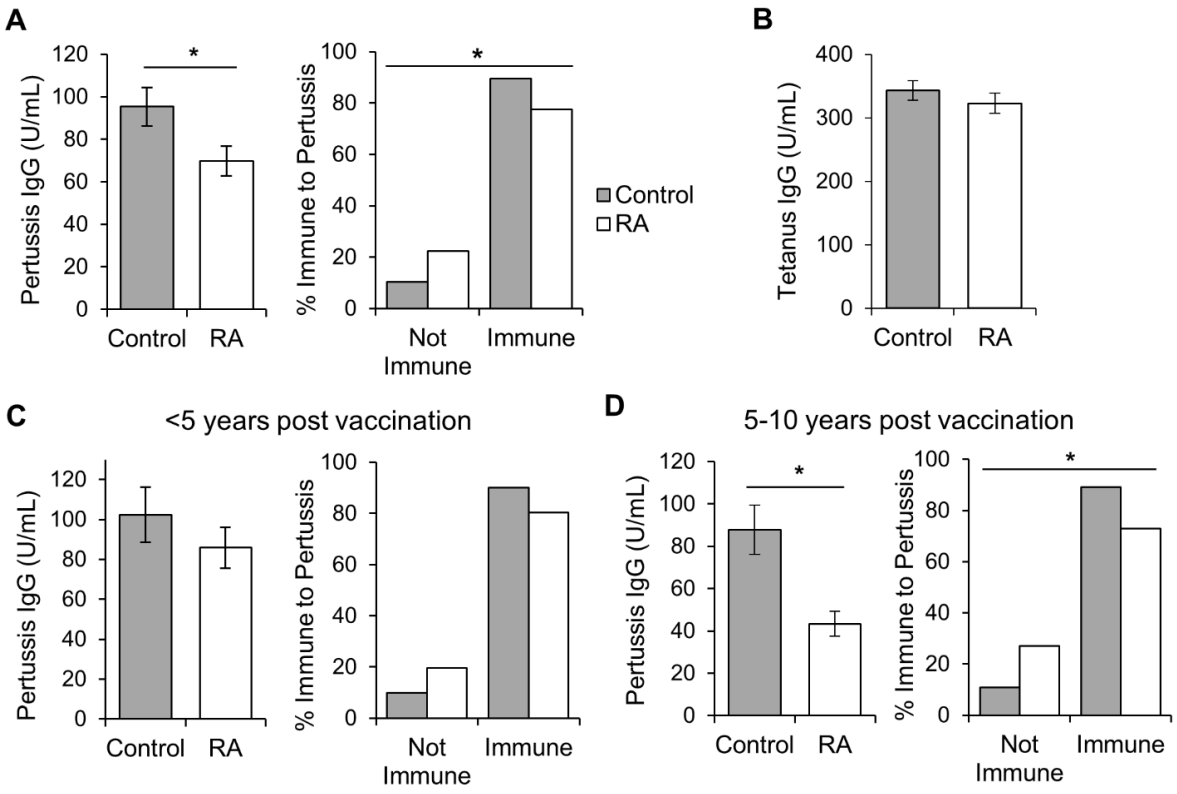

Fig 1. Pertussis titers are lower in rheumatoid arthritis subjects than controls especially 5-10 years post-vaccination. Sera from rheumatoid arthritis (RA) patients and controls were subjected to ELISA to detect IgG titers against pertussis (A) with averages and SEM graphed (left) as well as the percent of each group considered immune to pertussis (right) (controls n=77, RA n=98). (B) Sera were also subjected to ELISA to detect IgG titers against tetanus with average and SEM graphed (controls n=77, RA n=98). Subjects were divided into Tdap vaccination <5 years (C) or 5-10 years (D)

prior to serum collection with average pertussis IgG titers and SEM graphed (left) as well as the percent of subjects considered immune to pertussis (right). For subjects <5 years post-vaccination: control n=40, RA n=61. For subjects 5-10 years post-vaccination: control n=37, RA n=37. In all panels, *p<0.05, **p<0.01.

Table 1. Predictors of greater than median pertussis titer in rheumatoid arthritis and control subjects (n=98 rheumatoid arthritis and 77 controls)

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
Rheumatoid Arthritis	**0.48**	**(0.26, 0.89)**	**0.02**	**0.29**	**(0.13, 0.63)**	**0.002**
Age	1.00	(0.97, 1.02)	0.73	0.97	(0.93, 1.01)	0.12
Sex: Female	**0.38**	**(0.20, 0.75)**	**0.005**	**0.34**	**(0.16, 0.71)**	**0.005**
Smoking Status (Never)	Ref.			Ref.		
Current	1.53	(0.53, 4.41)	0.43	1.57	(0.49, 5.05)	0.45
Former	1.48	(0.77, 2.87)	0.24	1.34	(0.62, 2.91)	0.45
BMI (Normal)	Ref.			Ref.		
Overweight	1.49	(0.65, 3.44)	0.35	1.34	(0.53, 3.36)	0.53
Obese	**2.31**	**(1.09, 4.93)**	**0.03**	1.94	(0.85, 4.46)	0.12
Charlson Comorbidity Score	1.04	(0.90, 1.20)	0.57	1.19	(0.92, 1.54)	0.19
NSAIDs (n=77)	0.89	(0.49, 1.61)	0.70	0.92	(0.46, 1.85)	0.81
Time since Vaccination	**0.84**	**(0.75, 0.95)**	**0.007**	**0.83**	**(0.72, 0.95)**	**0.006**

Table 2. Predictors of greater than median pertussis titer in rheumatoid arthritis (RA) (n=98)

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
Age	1.01	(0.97, 1.04)	0.68	0.99	(0.93, 1.06)	0.85
Sex: Female	**0.28**	**(0.12, 0.67)**	**0.004**	**0.26**	**(0.09, 0.75)**	**0.01**
Smoking Status (Never)	Ref.			Ref.		
Current	1.71	(0.44, 6.62)	0.43	1.84	(0.36, 9.25)	0.46
Former	1.61	(0.66, 3.90)	0.29	2.02	(0.55, 7.45)	0.29
BMI (Normal)	Ref.			Ref.		
Overweight	2.35	(0.75, 7.36)	0.14	3.20	(0.82, 12.50)	0.10
Obese	2.48	(0.87, 7.08)	0.09	2.38	(0.66, 8.58)	0.19
Charlson Comorbidity Score	1.09	(0.90, 1.31)	0.40	0.85	(0.56, 1.30)	0.46
NSAIDs (n=54)	1.27	(0.57, 2.86)	0.56	1.09	(0.38,3.14)	0.87
Time since Vaccination	**0.80**	**(0.67, 0.96)**	**0.01**	0.81	(0.65, 1.02)	0.08
RA at Vaccination (n=70)	**3.67**	**(1.33, 10.14)**	**0.01**	3.51	(0.90, 13.72)	0.07

Abatacept (n=7)	0.53	(0.10, 2.89)	0.47	0.34	(0.05, 2.44)	0.29
Hydroxychloroquine (n=23)	0.86	(0.33, 2.24)	0.76	0.84	(0.21, 3.34)	0.80
Leflunomide (n=20)	**3.32**	**(1.19, 9.28)**	**0.02**	3.50	(0.78, 15.61)	0.10
Methotrexate (n=53)	**0.41**	**(0.18, 0.94)**	**0.04**	0.71	(0.21, 2.43)	0.59
Sulfasalazine (n=7)	1.95	(0.41, 9.21)	0.40	4.54	(0.41, 50.25)	0.22
TNF inhibitor (n=35) [a]	1.53	(0.67, 3.54)	0.32	1.16	(0.34, 3.95)	0.82

[a] For TNF inhibitor users: 17 were prescribed methotrexate, 4 leflunomide, 5 hydroxychloroquine, and 1 sulfasalazine with some subjects taking more than one of these medications.

Table 3. Predictors of pertussis immunity in rheumatoid arthritis and control subjects (n=98 rheumatoid arthritis and 77 controls)

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
Rheumatoid Arthritis	**0.40**	**(0.17, 0.96)**	**0.04**	**0.30**	**(0.11, 0.81)**	**0.02**
Age	0.99	(0.96, 1.03)	0.74	0.99	(0.94, 1.03)	0.55
Sex: Female	**0.30**	**(0.10, 0.91)**	**0.03**	**0.27**	**(0.08, 0.85)**	**0.03**
Smoking Status (Never)	Ref.			Ref.		

Current	3.75	(0.47, 30.02)	0.21	3.93	(0.47, 33.19)	0.21
Former	1.44	(0.59, 3.50)	0.42	1.32	(0.49, 3.51)	0.59
BMI (Normal)	Ref.			Ref.		
Overweight	1.03	(0.38, 2.84)	0.95	0.88	(0.30, 2.59)	0.82
Obese	1.73	(0.66, 4.57)	0.27	1.39	(0.50, 3.87)	0.53
Charlson Comorbidity Score	0.97	(0.81, 1.16)	0.74	1.04	(0.76, 1.42)	0.81
NSAIDs (n=77)	1.03	(0.47, 2.28)	0.94	1.02	(0.43, 2.43)	0.97
Time since Vaccination	0.91	(0.78, 1.07)	0.26	0.90	(0.76, 1.06)	0.21

Table 4. Predictors of pertussis immunity in rheumatoid arthritis (RA) (n=98)

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
Age	0.99	(0.95, 1.03)	0.69	0.99	(0.93, 1.06)	0.81
Sex: Female	0.34	(0.11, 1.11)	0.07	**0.23**	**(0.06, 0.91)**	**0.04**
Smoking Status (Never)	Ref.			Ref.		
Current	2.93	(0.34, 25.21)	0.33	3.91	(0.36, 2.71)	0.26
Former	1.12	(0.40, 3.14)	0.84	1.09	(0.31, 3.92)	0.89
BMI (Normal)	Ref.			Ref.		

Overweight	1.35	(0.39, 4.72)	0.64	1.55	(0.33, 7.29)	0.58
Obese	1.43	(0.46, 4.46)	0.54	1.48	(0.38, 5.69)	0.57
Charlson Comorbidity Score	1.00	(0.80, 1.26)	0.97	0.88	(0.61, 1.27)	0.49
NSAIDs (n=54)	1.03	(0.40, 2.67)	0.95	0.79	(0.26, 2.37)	0.67
Time since Vaccination	0.89	(0.73, 1.08)	0.24	0.85	(0.66, 1.09)	0.21
RA at Vaccination (n=70)	1.22	(0.44, 3.42)	0.70	1.02	(0.25, 4.13)	0.97
Abatacept (n=7)	0.35	(0.07, 1.71)	0.20	0.24	(0.03, 1.87)	0.17
Hydroxychloroquine (n=23)	1.06	(0.34, 3.26)	0.93	0.81	(0.20, 3.22)	0.76
Leflunomide (n=20)	0.84	(0.27, 2.63)	0.76	0.21	(0.03, 1.29)	0.09
Methotrexate (n=53)	0.47	(0.17, 1.27)	0.14	**0.15**	**(0.03, 0.82)**	**0.03**
Sulfasalazine (n=7) [a]	-	-	-	-	-	-
TNF inhibitor (n=35) [b]	1.25	(0.45, 3.44)	0.67	0.46	(0.11, 1.98)	0.30

[a] All subjects using sulfasalazine were immune to pertussis limiting calculations.

[b] For TNF inhibitor users: 17 were prescribed methotrexate, 4 leflunomide, 5 hydroxychloroquine, and 1 sulfasalazine with some subjects taking more than one of these medications.

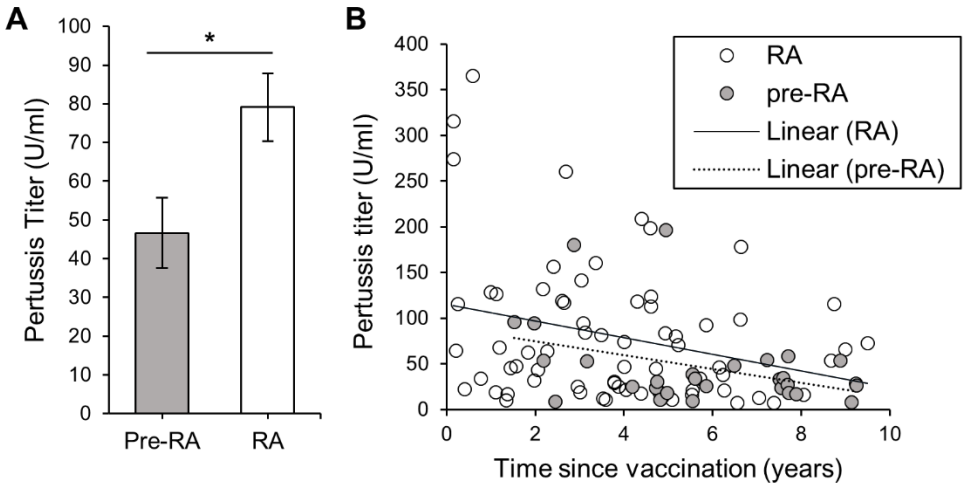

Fig 2. Lower pertussis titers in subjects not yet diagnosed with rheumatoid arthritis as compared to diagnosed with rheumatoid arthritis at the time of Tdap vaccination. A. Pertussis IgG titers were compared between subjects not yet diagnosed with rheumatoid arthritis (pre-RA) versus diagnosed with rheumatoid arthritis (RA) at the time of vaccination by t-test with averages and SEM graphed. B. Pertussis IgG titers and time since vaccination were graphed for pre-RA and RA groups with linear trendlines calculated. For all panels n=28 pre-RA, n=70 RA, *$p<0.05$.

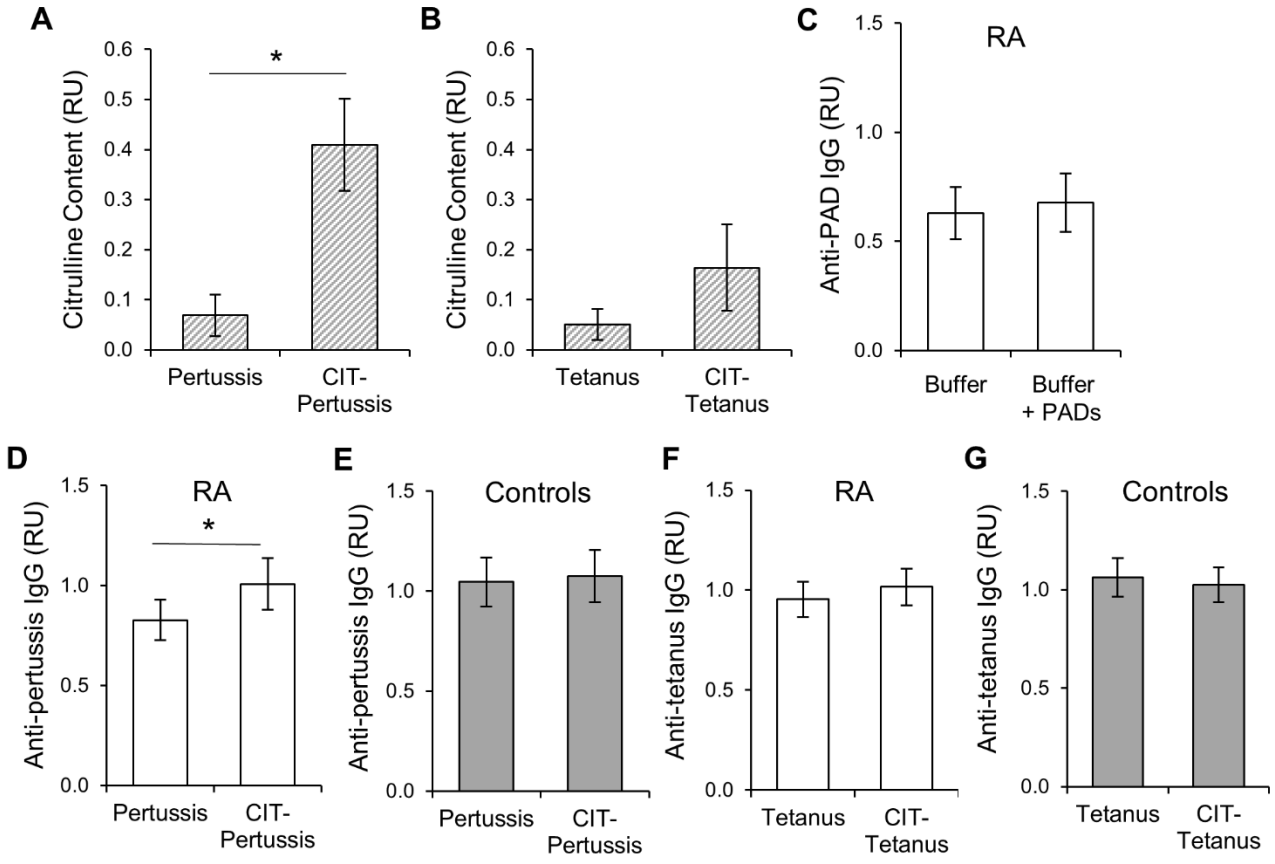

Fig 3. Rheumatoid arthritis patients have higher IgG binding to citrullinated than native pertussis. Following citrullination of pertussis-coated (A) and tetanus toxoid-coated (B) wells, the extent of citrullination was quantified using an anti-citrulline antibody with the relative units (RU) compared for untreated versus citrullinated (CIT) wells. Averages with SEM are graphed (n=5, *p<0.05). Sera from rheumatoid arthritis (RA) patients (C, D, F) and controls (E, G) were subjected to ELISA to detect IgG binding against PAD enzymes (C), native and citrullinated (CIT) pertussis (D, E), or native and potentially citrullinated tetanus (F, G) with averages and SEM graphed. For panels C-G, control n=30, RA n=31, *p<0.05.

CHAPTER FOUR

Anti-C8 IgG antibodies are present in seronegative rheumatoid arthritis

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Author Contributions:

CLH contributed to the manuscript by: developing and analyzing regression modeling in Tables 1 and 2 under the supervision of a biostatistician; performing validation of regression modeling in Figure 3; analyzing and assembling all data; making all of the figures, writing the original chapter, and reviewing and editing the final chapter.

A. Abstract

Pathologic autoantibodies contribute to joint inflammation in rheumatoid arthritis. Detection of rheumatoid factor (RF), antibodies against IgG, and anti-citrullinated protein antibodies (ACPAs), underlie the diagnostic RF and anti-cyclic citrullinated peptide (CCP) tests in rheumatoid arthritis. However, ~25% of patients are seronegative and do not test positive for either marker. Even in the absence of RF and CCP, seronegative rheumatoid arthritis patients can have high disease activity and the lack of serological markers leads to delays in diagnosis and treatment. Thus, it is critical to find new diagnostic markers that can detect seronegative rheumatoid arthritis. Using a high-density peptide array, we identified 15 native peptides from complement proteins that were bound twice as greatly by IgG in subjects with rheumatoid arthritis (including seronegative and seropositive subjects) compared to controls. Complement proteins and membrane attack complexes, the terminal complement structure, are increased in the rheumatoid joint. Here, we determine if subjects with rheumatoid arthritis, particularly seronegative disease, generate autoantibodies against complement as well as identify clinical correlates of increased anti-complement antibodies. We found that rheumatoid arthritis patients have higher anti-C8 IgG antibody levels in both CCP+RF+ and CCP-RF- disease than control subjects. However, anti-C8 antibody levels were similar between controls and patients with lupus or ankylosing spondylitis. Using univariate and multivariable analyses, we found that a diagnosis of rheumatoid arthritis and current smoking both correlate with higher anti-C8 IgG levels. Together, our data suggest that anti-C8 antibodies may be a

specific marker for rheumatoid arthritis that could improve detection of seronegative disease.

B. Introduction

Rheumatoid arthritis is an autoimmune disease in which pathologic autoantibodies contribute to joint inflammation (reviewed in (169)). Two autoantibody types, antibodies against IgG, called rheumatoid factor (RF), and anti-citrullinated protein antibodies (ACPAs), underlie the diagnostic RF and anti-cyclic citrullinated peptide (CCP) tests in rheumatoid arthritis (62). However, ~25% of patients are considered seronegative since they do not test positive for either RF or anti-CCP (73-75). Seronegative rheumatoid arthritis patients can have high disease activity despite the absence of classic serological markers (73, 74, 170). Moreover, the inability of diagnostic tests to identify these patients leads to delays in diagnosis and treatment of their disease (75). Thus, it is important to find new serologic markers that can detect these seronegative rheumatoid arthritis patients. Much attention in recent years has focused on autoantibodies against post-translationally modified proteins in rheumatoid arthritis (21, 81-83), particularly citrullinated proteins. However, antibodies in rheumatoid arthritis can also target native, unmodified antigens (77, 171-173). Thus, native peptides and proteins may be an understudied potential source of antigens for the development of better diagnostic tests.

Recently, using a high-density peptide array, we identified 49 native peptides bound twice as greatly by IgG in subjects with rheumatoid arthritis (including seronegative and seropositive subjects) as compared to controls (77). Interestingly, 15 of these 49 native peptides were derived from the complement proteins C4 (peptides common to both C4a and C4b), C5, C7, and C8. The complement cascade is part of the innate immune response that aids in the clearance of foreign pathogens and dead cells. Early classic complement pathway initiation occurs when the Fc portion of an antibody immune complex is bound by C1q. C1q activates C1r and C1s, which then cleaves C2 and C4 into C2a/C2b and C4a/C4b, respectively. C4b and C2a bind together to form C3 convertase which cleaves C3 into C3a and C3b. The C4bC2a complex is then bound by C3b to form C5 convertase which cleaves C5 into C5a and C5b. C5b then initiates assembly of C5b, C6, C7, C8, and C9 to form the membrane attack complex (MAC), the terminal result of complement activation (reviewed in (174)). The MAC integrates into cell membranes, forming a pore, and leading to cell lysis. Complement proteins and MACs are increased in the rheumatoid joint (175, 176). Further, pore forming proteins can induce hypercitrullination and have been hypothesized to be a source of the citrullinated proteins (70) that are increased in rheumatoid arthritis (177) and targeted by ACPAs. Antibodies against complement proteins C1q and C3b have been detected in lupus, but none have been reported in rheumatoid arthritis (178, 179).

Here, we evaluate if subjects with rheumatoid arthritis, particularly seronegative rheumatoid arthritis, generate autoantibodies against C4b, C5, C7, and/or C8 proteins and identify clinical correlates.

C. Results

As noted above, 15 native peptides derived from complement proteins C4, C5, C7, and C8 were identified as bound by IgG in rheumatoid arthritis (77). To more closely evaluate IgG binding to peptides across the entire complement proteins, we used the original peptide array data (77) and plotted the average fold increased binding of IgG from the CCP+RF+, CCP+RF-, CCP-RF+, and CCP-RF- rheumatoid arthritis sera over control sera against all peptides in each of those proteins. As shown in Figure 1, several peptides from each protein were bound more than control for each of the rheumatoid arthritis serogroups, including the seronegative CCP-RF- group.

Peptide binding is not always identical to protein binding in part due to hidden epitopes and structural epitopes in proteins. However, protein binding may be more relevant biologically. Therefore, we next evaluated IgG binding against C4b, C5, C7, and C8 proteins by ELISA. Unfortunately, despite multiple approaches to optimize the C5 ELISA, including heat inactivation of the subjects' sera, this ELISA was not reproducible and thus ultimately not included in this study. As displayed in Figure 2, there was no significant difference in IgG binding levels against C4b, C7, or C8 between the rheumatoid arthritis groups and controls. However, there was a trend towards an increase in anti-C8 IgG in CCP-RF- versus control. Suspecting that we did not reach the threshold for statistical significance due to an underpowered study, we increased the number of subjects and

focused on CCP+RF+ and CCP-RF- rheumatoid arthritis. The controls and rheumatoid arthritis subjects in the new larger cohort were similar apart from a higher Charlson Comorbidity Score (167) in rheumatoid arthritis patients, consistent with one point in scoring for a rheumatoid arthritis diagnosis (Table 1). As shown in Figure 3A, both CCP+RF+ and CCP-RF- rheumatoid arthritis patients had significantly higher anti-C8 IgG levels compared to controls with no detectable difference in anti-C8 IgG levels between CCP+RF+ and CCP-RF- subjects. These data suggest that antibodies against C8 could be a common feature of rheumatoid arthritis including both seronegative and seropositive disease.

Since seronegative rheumatoid arthritis patients have anti-C8 antibodies, these autoantibodies could enhance the sensitivity of diagnostics for rheumatoid arthritis. However, the presence of anti-C8 antibodies in other diseases like systemic lupus erythematosus, an autoimmune disease known to have antibodies against complement proteins C1q and C3b (178, 179) or ankylosing spondylitis, a seronegative inflammatory arthritis with different clinical characteristics than rheumatoid arthritis, would lower specificity and reduce utility. Therefore, we performed anti-C8 ELISA on sera from lupus and ankylosing spondylitis patients. There was no increase in anti-C8 IgG levels in patients with lupus or ankylosing spondylitis (Figure 3B-C), suggesting that anti-C8 antibodies could help differentiate between these autoimmune diseases.

We also hypothesized that anti-C8 antibodies might correlate with clinical and demographic features in our subjects. Thus, we performed univariate and multivariable regression analyses for rheumatoid arthritis patients and controls to identify clinical factors that correlated with greater than median anti-C8 binding. When modeled for rheumatoid arthritis subjects and controls combined, a diagnosis of rheumatoid arthritis predicted greater than median anti-C8 antibodies in the univariate and multivariable analyses. Being a current smoker was also predictive of greater than median anti-C8 antibody levels in both the univariate and multivariable analyses, while being a former smoker was predictive in the univariate analysis only (Table 2). There was no correlation between age, sex, and body mass index with greater than median anti-C8 IgG titer. We then modified the analysis to include only rheumatoid arthritis patients and variables relevant for disease. Again, current smoking was predictive of greater than median anti-C8 antibody levels in both the univariate and multivariable analyses, while being a former smoker was predictive in the univariate alone. Interestingly, glucocorticoid use showed a trend toward correlation with greater than median anti-C8 levels, whereas the use of TNF inhibiting medications or DMARDs did not correlate with anti-C8 IgG (Table 3).

To further evaluate the findings of the regression analyses, which categorized patients using a binary cutoff, we compared continuous values of anti-C8 binding for variables of interest. Consistent with the regression analyses, we found that rheumatoid arthritis patients (seronegative and seropositive combined) had higher anti-C8 antibody levels than controls (Figure 4A). Further, current smokers had higher anti-C8 antibody levels than never-smokers as well as a trend toward higher levels than former smokers. There

was no detectable difference in anti-C8 binding between patients who formerly smoked and patients with no smoking history (Figure 4B). Lastly, we found a trend for higher anti-C8 antibody levels in patients using glucocorticoids (p=0.08; Figure 4C).

D. Discussion

Rheumatoid arthritis patients generate autoantibodies to numerous antigens, including post-translationally modified and native proteins. Previously, using a high-density peptide array, we found two fold higher binding to native peptides from several complement proteins in rheumatoid arthritis patients compared to controls (77). Here, we found that IgG levels against C8 protein were higher in both CCP+RF+ and CCP-RF- rheumatoid arthritis compared to controls, but not in lupus erythematosus or ankylosing spondylitis (Figure 3). Thus, anti-C8 antibodies may be a unique marker of rheumatoid arthritis including seronegative disease. Future studies are needed to assess the utility of anti-C8 antibodies as an improved test for the diagnosis of rheumatoid arthritis.

The presence of anti-C8 antibodies may provide clues to rheumatoid arthritis specific pathology. The complement cascade is important in clearing microbes and dead cells and loss of complement pathways can be pathologic. For example, C8 deficient patients have a higher susceptibility to meningitis and pneumonia (180, 181). Since C8 is a member of the MAC, antibodies against C8 could potentially activate or inhibit MAC formation or activity with a variety of potential implications. MAC formation on the surface of synovial

fibroblasts has non-lethal effects including increased secretion of prostaglandin and collagenase (68, 69). MACs can also induce leukotoxic hypercitrullination (70) a process that appears to be a variant of NETosis (the formation of neutrophil extracellular traps). LTH is characterized by hypercitrullination, which may provide antigens bound by ACPAs as part of the development or perpetuation of rheumatoid inflammation. Given the potential pathways that anti-C8 antibodies could disrupt, it will be important for future studies to determine any functional effects of anti-C8 antibodies.

An additional unexplained question is why antibodies develop against C8. It is possible that the proximity of C8 with damage associated molecular patterns provides both an antigen and a danger signal leading to the potent activation of T and B cells and ultimately the development of anti-C8 antibodies. Another possibility is NETs. NET formation is increased in rheumatoid arthritis (37) and NETs display a number of proteins bound by autoantibodies including complement and citrullinated proteins. Although the role of NETs in the development of ACPAs is still undetermined, antibodies against C8 could form similarly to the formation of ACPAs.

We also investigated clinical variables that correlate with greater than median anti-C8 antibody levels using univariate and multivariable regression analyses. In control and rheumatoid arthritis cohorts, a diagnosis of rheumatoid arthritis predicted greater than median anti-C8 binding. Patients who were current smokers also had higher anti-C8 IgG levels, a trend not seen in former smokers. Rheumatoid arthritis patients who smoke produce more autoantibodies to multiple proteins (182), indicating a role for smoking in

the breaking of immune tolerance. Cigarette smoke generates reactive oxygen species that can modify proteins and increase the autoantibody binding to resulting epitopes (183). Although statistically insignificant, we also found a strong trend for glucocorticoid use and greater than median anti-C8 binding. Glucocorticoids are anti-inflammatory drugs widely used to treat rheumatoid arthritis, but may not influence the generation of autoantibodies (184). It is possible that the patients receiving glucocorticoids had higher disease activity and therefore anti-C8 antibody levels might correlate with higher disease activity. Of note, smoking also correlates with disease activity with cessation improving outcomes (185). Future studies are needed to address a potential link between anti-C8 antibodies and rheumatoid arthritis disease activity.

In conclusion, we have shown increased anti-C8 IgG antibody levels in rheumatoid arthritis patients compared to controls, including the seronegative rheumatoid arthritis subset. This finding was distinct from other autoimmune and inflammatory diseases and correlated with smoking. Future work is needed to further evaluate the role of anti-C8 antibodies in rheumatoid arthritis pathophysiology as well as its potential in improved diagnostics for rheumatoid arthritis.

E. Materials and Methods

Human Subjects

Research was performed in accordance with the Declaration of Helsinki and as approved by the Institutional Review Board at the University of Wisconsin-Madison (#2015-0156). All subjects gave written informed consent before participation in the study. All clinical data and biologic samples were obtained from the University of Wisconsin Rheumatology Biorepository as described in (77, 165, 186, 187). Briefly, all subjects are ≥18 years old and medically homed at UWHealth. Rheumatoid arthritis subjects were identified by having 2+ outpatient visits containing a rheumatoid arthritis International Classification of Disease (ICD) code within 24 months or one visit and a positive anti-CCP test (166). Diagnosis of rheumatoid arthritis was confirmed by manual review of rheumatology clinic notes. Only subjects with CCP+ and/or RF+ test results >2x the upper limit of normal were selected as CCP+RF+. CCP-RF- subjects had test results in the negative/normal range. Controls were excluded if they had known autoimmune or inflammatory diseases or hematologic malignancy. Patients with lupus or ankylosing spondylitis were initially identified for the study by their primary rheumatologist with diagnosis confirmed by manual review of the medical records (188).

The following variables were included as abstracted from the medical record or self-reported at the time of serum collection for all subjects: age at blood collection, sex, race/ethnicity, body mass index, Charlson Comorbidity Score (167), smoking status and history, and diagnosis of rheumatoid arthritis. The following variables were abstracted for rheumatoid arthritis patients only: CCP and RF values, presence of erosive disease (as determined manual review of all imaging), and prescription of the following medications at the time of serum collection: glucocorticoids (either orally within five days or

intravenously within the month prior to serum collection), TNF inhibitors (includes adalimumab, etanercept, certolizumab, and infliximab), or disease modifying anti-rheumatic drugs (DMARDs: hydroxychloroquine, leflunomide, methotrexate, or sulfasalazine). No subjects were prescribed golimumab.

Complement Protein Enzyme Linked Immunosorbent Assay (ELISA)

Individual wells of non-precoated 96 well plates (Costar 3590 RIA/EIA, Corning, USA) were coated overnight with 5µg/mL complement C7 or C8 protein (Sigma-Aldrich, St. Louis, USA) in PBS, or incubated with PBS alone, overnight at 4°C. Wells were then washed three times with 200µL 0.1% Tween20 in PBS (PBST) and blocked with 100µL 5% non-fat dry milk in PBST for 4 hours at room temperature. Wells were washed again with PBST and incubated for 2 hours at room temperature with 100µL of 1:50 serum samples diluted with blocking buffer. Wells were washed again with PBST and incubated for 1 hour and 45 minutes at room temperature with 100µL of 1:10,000 horseradish peroxidase-conjugated goat-anti-human IgG secondary antibody (Southern Biotech, Birmingham, USA) diluted in blocking buffer. Wells were washed again with PBST and incubated for 25 minutes at room temperature with 100µL TMB-Slow ELISA reagent (Pierce, Rockford, USA) until the reaction was stopped by addition of 100µL of 1.8M H_2SO_4. Absorbance at 450 and 540 nm for each well was read using a Synergy 2 (BioTek, Winooski, USA) plate reader controlled by Gen5.0 software (BioTek). Each serum sample was assayed in duplicate, in both coated and uncoated wells with results from uncoated wells subtracted from coated wells for each sample.

The C4b ELISA was performed as the C7 and C8 ELISA but with the following modifications: 10µg/mL C4b (Sigma-Aldrich, St. Louis, USA) in PBS to coat the plate, 1:100 serum dilution, 1:5,000 secondary antibody dilution with 2 hour incubation, and 30 minutes of color development.

Statistical Analysis

The following were measured using a Kruskal-Wallis test with a Dunn's multiple comparison: mean anti-C4b, C7, or C8 IgG levels between control, CCP+RF+, CCP-RF+, CCP+RF-, or CCP-RF- patients; anti-C8 IgG levels between controls, CCP+RF+, and CCP-RF- patients, and anti-C8 IgG levels between never, former, and current smokers. A Mann-Whitney test was used to compare anti-C8 IgG levels for controls versus rheumatoid arthritis, systemic lupus erythematosus, or ankylosing spondylitis patients and to compare anti-C8 IgG levels in rheumatoid arthritis patients using glucocorticoids. Univariate and multivariable logistic regression analyses were performed to identify clinical factors that correlated with greater than median anti-C8 antibody levels. For all statistical tests, $p<0.05$ was considered significant. Analysis was performed using Stata version 14 (StataCorp LP, College Station, USA) and Prism (GraphPad Software, San Diego, USA).

F. Figures and Tables

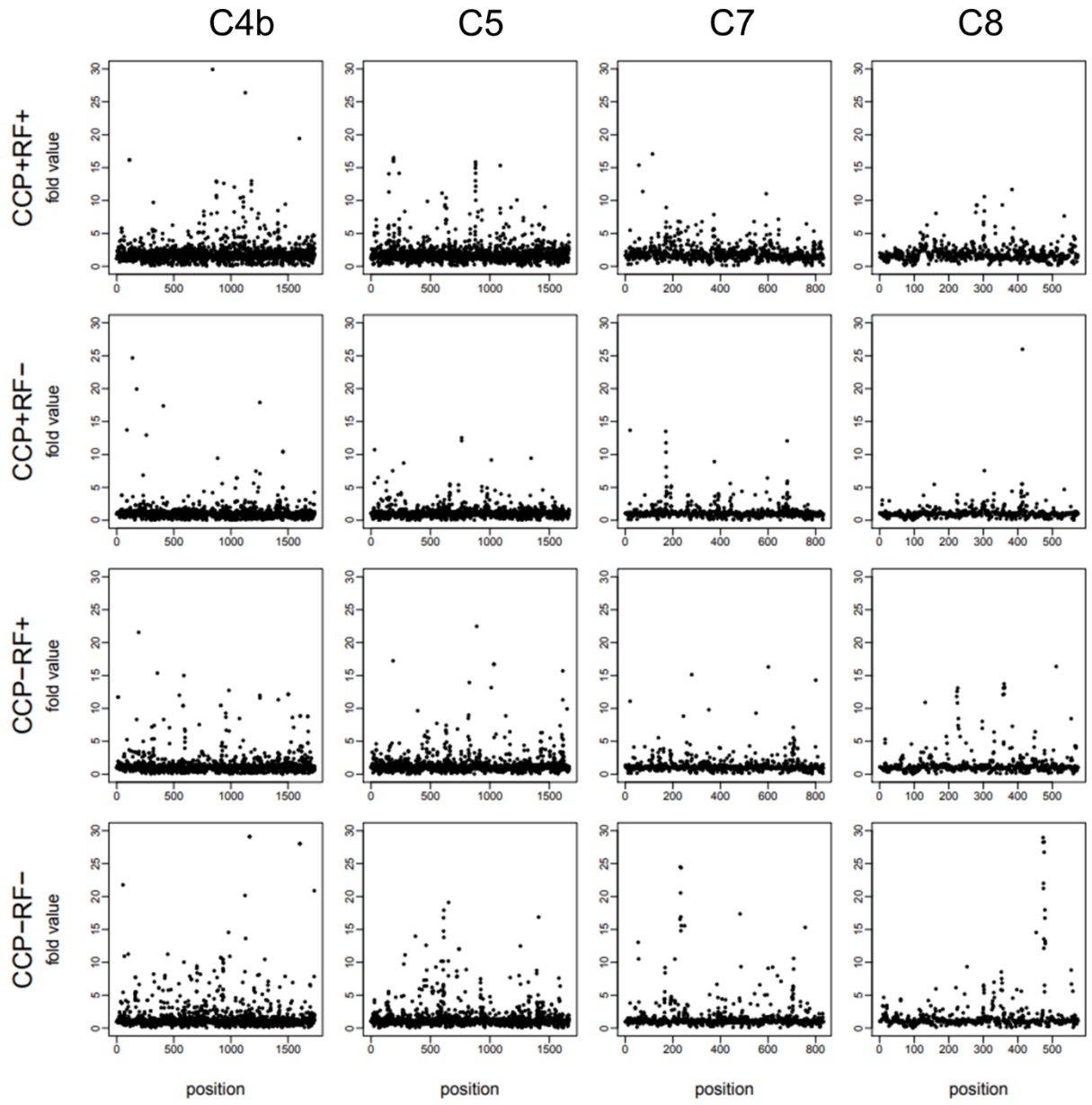

Figure 1. IgG from rheumatoid arthritis patient sera has increased binding to peptides from native complement proteins. Binding of IgG to peptides derived from four complement proteins for rheumatoid arthritis patients divided by controls is graphed for each peptide according to its position in the protein for CCP+RF+, CCP+RF-, CCP-RF+, and CCP-RF- subjects (n=12 subjects per group).

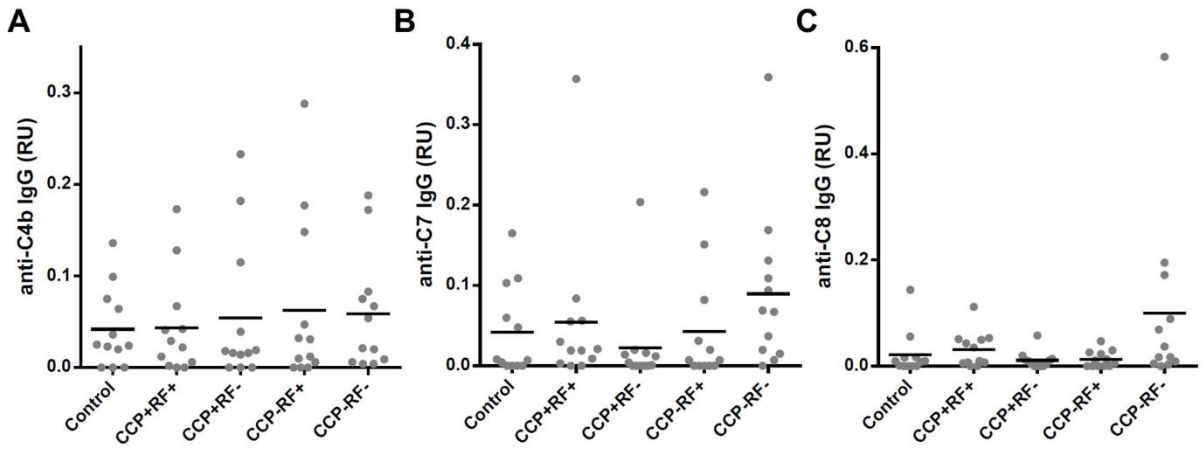

Figure 2. Anti-complement antibody binding is similar between controls and rheumatoid arthritis subgroups for C4b, C7, and C8. Sera from control, CCP+RF+, CCP+RF-, CCP-RF+, or CCP-RF- patients were analyzed by ELISA to detect IgG levels against C4b (A), C7 (B), or C8 (C) protein with individual absorbance values in relative units (RU), averages and SEM graphed. For all groups, n=12. No comparisons show a significant difference.

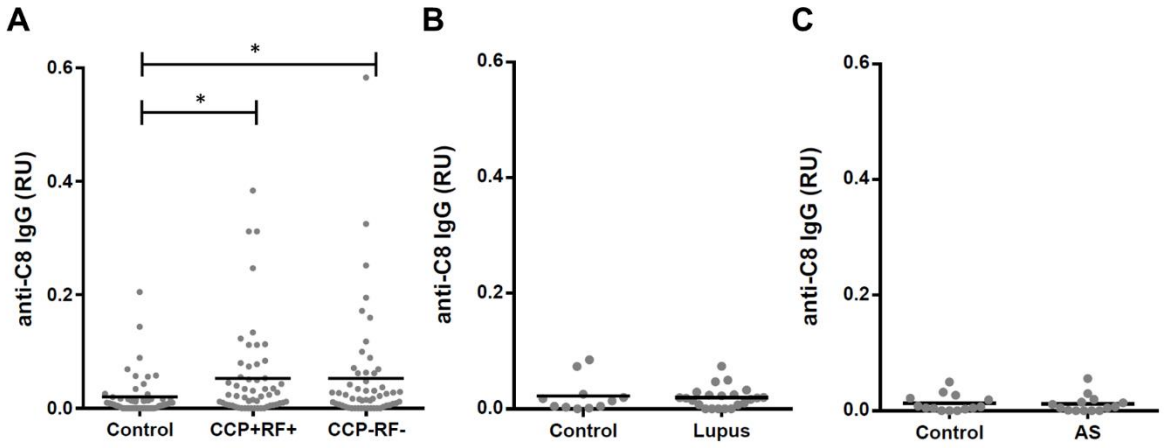

Figure 3. CCP+RF+ and CCP-RF- rheumatoid arthritis subjects have higher anti-C8 antibody levels than controls. Sera from control and CCP+RF+ or CCP-RF- rheumatoid arthritis (A), lupus (B), or ankylosing spondylitis (C) patients were analyzed by ELISA to detect IgG levels against C8. For A, n=55; for B, n=22; for C, n=14; for all, *p<0.05.

Table 1. Characteristics of rheumatoid arthritis (RA) and control subjects

	All Patients n=165, n (%)		
	Controls	RA	p value
	55 (33.3)	110 (66.7)	
Age, mean years ± SE	59.2 (1.6)	59.9 (1.1)	0.71
Sex, female (%)	46 (83.6)	93 (84.6)	1.00
Race/Ethnicity, number (%)			
White	50 (90.9)	98 (89.1)	0.76

Black	2 (3.6)	2 (1.8)	
Hispanic	1 (1.8)	6 (5.5)	
Native American	2 (3.6)	3 (2.7)	
Asian	0 (0.0)	1 (0.9)	
Pacific Islander	0 (0.0)	0 (0.0)	
Body Mass Index, mean ± SE	30.8 (1.0)	30.5 (0.8)	0.82
Charlson Comorbidity, mean ± SE	**2.1 (0.2)**	**3.4 (0.2)**	**<0.00**
Smoking Status, number (%)			
Never Smoked	34 (61.8)	56 (50.9)	0.44
Former Smoker	16 (29.1)	42 (38.2)	
Current Smoker	5 (9.1)	12 (10.9)	

Table 2. Predictors of greater than median anti-C8 antibody levels in rheumatoid arthritis (RA) and control subjects (n=165; control n=55, RA n=110).

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
RA	**2.26**	**(1.16, 4.40)**	**0.02**	**2.17**	**(1.09, 4.31)**	**0.03**
Age	1.02	(0.99, 1.04)	0.25	1.01	(0.98, 1.04)	0.56
Sex (Female)	0.68	(0.29, 1.59)	0.38	0.67	(0.27, 1.64)	0.38
Body Mass Index	1.00	(0.96, 1.04)	0.91	1.00	(0.96, 1.05)	0.91
Smoking (Never)						

Former	**2.13**	**(1.09, 4.16)**	**0.03**	1.91	(0.94, 3.86)	0.07
Current	**3.60**	**(1.17, 11.09)**	**0.03**	**3.54**	**(1.10, 11.42)**	**0.03**

Table 3. Predictors of greater than median anti-C8 antibody level in rheumatoid arthritis (RA) subjects (n =110).

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
CCP+RF+ status	1.00	(0.47, 2.12)	1.00	0.87	(0.37, 2.07)	0.76
Age	1.01	(0.98, 1.05)	0.38	1.00	(0.96, 1.04)	0.92
Sex (Female)	1.18	(0.42, 3.32)	0.76	1.13	(0.36, 3.62)	0.84
Body Mass Index	1.00	(0.95, 1.04)	0.84	0.99	(0.93, 1.04)	0.64
Smoking (Never)						
Former	**2.40**	**(1.05, 5.47)**	**0.04**	2.39	(0.97, 5.90)	0.06
Current	**14.67**	**(1.77, 121.51)**	**0.01**	**17.08**	**(1.94, 150.56)**	**0.01**
Erosive RA	0.73	(0.34, 1.55)	0.41	0.64	(0.27, 1.50)	0.30
Glucocorticoid	2.03	(0.89, 4.64)	0.09	2.29	(0.86, 6.12)	0.10
TNF Inhibitor (Current)	1.34	(0.59, 3.01)	0.48	1.88	(0.70, 5.06)	0.21
DMARD (Current)	0.71	(0.26, 1.97)	0.51	0.83	(0.26, 2.60)	0.75

TNF=tumor necrosis factor alpha, DMARD=disease modifying anti-rheumatic drug

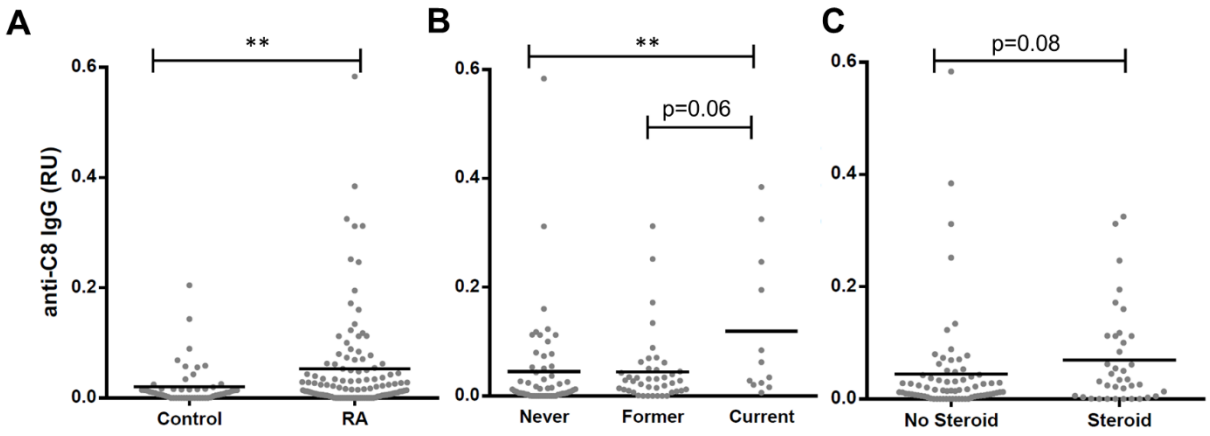

Figure 4. Rheumatoid arthritis patients and smokers have higher anti-C8 antibody levels. A. Sera from control or rheumatoid arthritis patients were analyzed by ELISA to compare IgG antibody levels against C8 (control n=55, rheumatoid arthritis n=110). Anti-C8 levels of rheumatoid arthritis patients were also plotted to compare never, former, and current smokers (B; never smokers, n=56, former smokers n=42, current smokers n=12), and glucocorticoid users versus non-users (C; no glucocorticoid use n=73, glucocorticoid use n=37). For all panels: *p<0.05, **p<0.01.

CHAPTER FIVE

Future Directions and Conclusions

In conclusion, the work detailed here investigates two major related components in the cycle of inflammation in rheumatoid arthritis, ETosis and antibodies (Figure 1). Our investigations have provided insight into how proteins, both citrullinated and uncitrullinated, are released into the extracellular environment and made available for detection by different autoantibodies.

First, we described specific stimuli that induce NETosis with or without citrullinated proteins. Our results indicate that ionomycin, MSU, PMA, and *C. albicans*, unique and diverse NET stimulants, induce a mixture of citrullinated and uncitrullinated NETs in both humans and mice. Further, we highlight that neutrophils from different species and maturity status may not undergo comparable NETosis, suggesting that studies should be clear in defining neutrophil origin when describing NET stimuli. Since the requirement of PAD2 and PAD4 in NETosis had not been clearly defined, we compared NETosis in wild type, $PAD2^{-/-}$, or $PAD4^{-/-}$ mice. Our studies agreed with previous reports that PAD4 is required for the production of citrullinated NETs. Interestingly, we also report that PAD4

is dispensable for the production of uncitrullinated NETs. Additionally, we found that PAD2 was dispensable for the production of citrullinated and uncitrullinated NETs.

These findings have important implications for the study of NETosis. Since each stimulant induced a mix of citrullinated and uncitrullinated NETs in both mice and humans, our results suggest that citrullination status and stimulant alone may not be ideal for defining NETs (Chapter 2, (58)). Previous classifications of NET effector pathways defined by the presence and absence of citrullinated protein should be reevaluated (47). Further, human and mouse neutrophils differ in their ability to form NETs to unique stimuli. Therefore, the comparison of NETs from different species may contribute to the conflicting reports on NET stimuli in the current literature.

Additionally, our study was the first to describe that PAD4 is required for citrullinated, but not uncitrullinated, NETs. Previous literature contained discrepancies regarding the requirement of PAD4 in NETosis. While multiple studies reported that PAD4 is required for NETosis (34, 45, 99-103), many investigations only examined citrullinated NETs. Conversely, other studies reported that PAD4 was dispensable for NETosis but examined total NETs via DNA staining (94, 104). By evaluating uncitrullinated NETs in our study, we provide a clearer picture into the requirement of PAD4 in citrullinated NETosis only. This work also highlights that different types of NETs can have requirement for different proteins and emphasizes a need to examine uncitrullinated NETs in future studies.

Finally, we also evaluated PAD2 in NETosis. PAD2 is required for the generation of a majority of citrullinated protein in murine models of inflammatory arthritis (34). Therefore, we determined if NETs could be a significant source of citrullinated protein by evaluating the requirement of PAD2 in NET citrullination. Since we found that PAD2 was dispensable for the production of citrullinated and uncitrullinated NETs, our study concludes that neutrophils may not be a major source of citrullination.

We then hypothesized that macrophages could be a major source of citrullination and that MET stimulants and cell signaling mechanisms should be explored in future studies, some of which have been initiated. From preliminary data (Appendix 1), we have identified ionomycin, monosodium urate crystals (MSU), and platelet activating factor (PAF) as potent activators of METosis, a process that generates citrullinated structures. Further, PAD2 appears to be required for the production of METs stimulated with MSU and PAF, which warrants further investigation. Interestingly, MSU and PAF are stimulants which engage inflammasome formation and subsequent execution of pyroptosis (189, 190). Thus, future studies should consider if METosis is an independent cell death process or if METs are a byproduct of pyroptosis. Future studies should also determine if other pyroptosis stimulants, such as ATP and nigericin, are inducers of METosis. The requirement of PAD4 in METosis also should be investigated.

We also investigated antibody production in rheumatoid arthritis to evaluate evidence for a citrulline-biased immune response. Our study found that rheumatoid arthritis patients have significantly lower titers to the pertussis component of the Tdap vaccine, but not the tetanus component, a finding that was particularly pronounced 5-10 years post vaccination. Using regression analysis, we identified clinical factors that correlate with lower pertussis titers and immunity including a diagnosis of rheumatoid arthritis, female sex, and methotrexate use. Interestingly, patients not yet diagnosed with rheumatoid arthritis had significantly lower pertussis titers than patients already diagnosed with the disease at the time of vaccination. Coexistence of immune suppressive medications and underlying immune dysregulation in rheumatoid arthritis patients has made determining the contributions of these factors to the increased rate of infections and reduced response to vaccination a challenge. This finding suggests that intrinsic immune dysregulation might contribute to lower pertussis titers since these subjects, who likely all had pre-clinical disease with its inherent immune dysregulation, had not yet been treated with immunosuppressive medications.

Finally, we discovered that rheumatoid arthritis patients have higher binding to citrullinated pertussis compared to native pertussis, providing evidence for a citrulline biased immune response in rheumatoid arthritis (Chapter 3, (186)). How and if rheumatoid arthritis patients generate antibodies specifically against citrullinated pertussis is unknown. It is possible that PADs secreted during NETosis at the site of vaccination citrullinate pertussis (59). Then, rheumatoid arthritis patients who have the

high risk HLA alleles associated with preferential presentation of citrullinated antigens (191) and thus the generation of ACPAs, preferentially generate antibodies against citrullinated pertussis. It is not known if such antibodies would be more or less effective in an immune response. It is also possible that pre-existing multispecific ACPAs in rheumatoid arthritis cross react with citrullinated pertussis, clearing pertussis from the site of vaccination before an immune response is mounted. Mechanisms involved in the intersection of ACPAs and vaccination should be further investigated in order determine how citrullination influences immunity and rates of infection. Other vaccines shown to have lower response in rheumatoid arthritis patients also could be evaluated to determine if a citrulline biased immune response is evident in other vaccinations.

We shifted from antibodies to autoantibodies in rheumatoid arthritis by discovering anti-C8 antibodies in seronegative and seropositive disease. Identification of novel antibodies is critical for detecting unique features of rheumatoid arthritis, especially in seronegative disease. For example, the presence of anti-C8 antibodies in seronegative disease suggests that “seronegative” rheumatoid arthritis may not truly be seronegative and rather may just have fewer autoantibodies, including potentially additional unknown autoantibodies. It will be important for future studies to more systematically evaluate autoantibodies in seronegative rheumatoid arthritis perhaps using novel technologies that would allow the evaluation of the entire human proteome. Such studies are needed to truly understand the spectrum of rheumatoid arthritis as well as the intersection of seronegative and seropositive disease. Importantly, we detected higher levels of anti-C8

IgG in both seropositive and seronegative rheumatoid arthritis over controls, indicating that these autoantibodies could be a feature that unifies these subsets of disease. Further, anti-C8 IgG was not detected in lupus or ankylosing spondylitis suggesting that anti-C8 IgG may be unique to rheumatoid arthritis highlighting a potential new pathophysiologic element. The presence of anti-C8 antibodies in seronegative rheumatoid arthritis as well as the absence in other rheumatologic diseases suggests that future studies could evaluate the clinical utility of anti-C8 antibodies to provide additional sensitivity to testing for rheumatoid arthritis with possible specificity given the absence in other rheumatologic diseases.

The physiologic role of anti-C8 antibodies is completely unknown and should be further explored. C8 is a member of the MAC and anti-C8 antibodies could augment or inhibit formation of the MAC. This could have significant implications for inflammation in the rheumatoid joint. MAC formation can activate the complement cascade and NETosis. Current investigations are underway to evaluate the influence of anti-C8 IgG in MAC formation.

This work highlights various aspects of immune dysregulation including the innate immune response, citrullination, and antibodies in rheumatoid arthritis. Future research must continue to focus on these areas in order to understand the immune system, break a link in the cycle of autoimmune inflammation, and better treat rheumatoid arthritis.

A. Figure

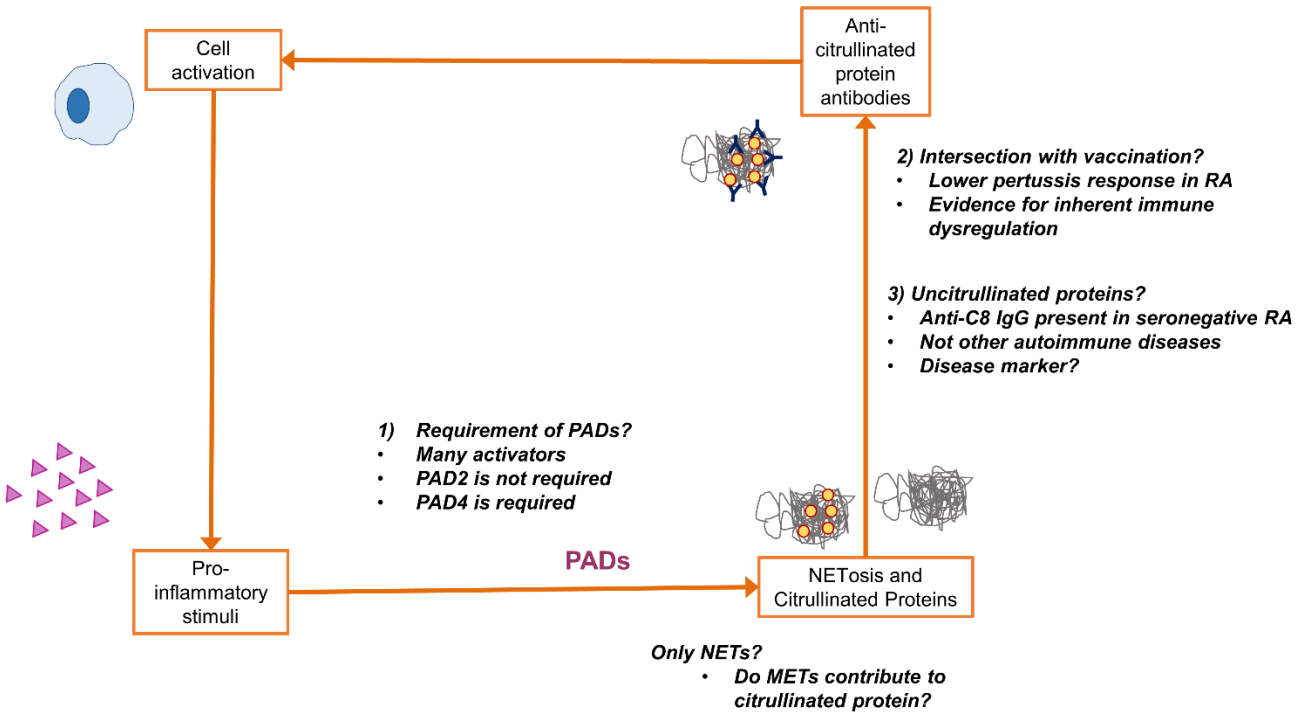

Figure 1. Examination of the cycle of inflammation in rheumatoid arthritis. Inflammation during rheumatoid arthritis is complex, as described in Chapter 1. In this thesis we investigated this cycle, providing new insights and also raising more questions. We found (1) Diverse stimuli activate both citrullinated and uncitrullinated NETs. PAD2 is dispensable for the production of NETs, both citrullinated and uncitrullinated. PAD4 is required for the production of citrullinated NETs only. These finding lead to new questions about the source of citrullinated proteins in rheumatoid arthritis such as METs. (2) Rheumatoid arthritis patients have lower response to pertussis, but not tetanus,

vaccination. Inherent immune dysregulation likely contributes to lower pertussis titers, and evidence was provided for a citrulline biased immune response to vaccination in rheumatoid arthritis. (3) Levels of anti-C8 IgG are higher in rheumatoid arthritis patients than controls or patients with lupus and ankylosing spondylitis. Anti-C8 IgG was present in both seropositive and seronegative subjects, suggesting it should be explored as a potential marker for disease. (PAD: peptidylarginine deiminase; NET: neutrophil extracellular trap; MET: macrophage extracellular trap).

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APPENDIX ONE

Macrophage Extracellular Traps

A. Macrophage Extracellular Traps

It is important to identify the source of citrullinated protein in rheumatoid arthritis, since involved molecules and cell types could be targeted by new therapies. PAD2 is required for the majority of citrullinated protein in mice (34), but not the formation of citrullinated NETs (58), suggesting that NETs may not be a major source of citrullinated protein in rheumatoid arthritis. Neutrophils are not the only cells that generate extracellular traps. Monocytes (192, 193) and macrophages (194-204) produce extracellular traps, although far less is known about these structures. Macrophages are involved in inflammation through immune surveillance and tissue remodeling, but are also linked to autoimmunity. Macrophages are abundant in the rheumatoid synovium (205) and prevalence correlates with disease activity (206). Inhibition of macrophage activity reduces severity of murine collagen induced arthritis (207). For patients, inhibition of TNFα, a major macrophage activating protein, is a common rheumatoid arthritis treatment that decreases disease severity in many patients.

In many ways, macrophage extracellular traps (METs) are similar to NETs (reviewed in: (208, 209)). In response to microbes, PMA, or acute kidney injury, macrophages produce METs which can be degraded by DNase, indicating extracellular DNA expulsion (196-

202, 204). MET structures capture bacteria and fungi (194, 196, 200-204, 210), but the antimicrobial activity of METs may be less potent than NETs (198, 201). Many different types of microbes can induce METosis (194, 196, 198-204), but aside from acute kidney injury and statin use (197, 203), non-microbial stimuli have not been investigated. This omission is concerning given the ability of neutrophils to produce NETs in response to many non-microbial stimuli (58). METs have also been associated with disease (195, 197, 200).

Different MET stimuli may induce signaling pathways with varying dependence on reactive oxygen species (ROS) activation (198, 199, 201, 204), although MET stimuli and signaling pathways are not fully described. METs contain similar proteins as NETs such as myeloperoxidase, histones, and lactoferrin (198, 200, 201, 211), but MET composition beyond a few proteins is unknown. Although some MET stimuli have been described, there are discrepancies. Aside from comparing METs across different species, studies have varying methods for purifying, activating, and quantifying METosis which may influence results. Some studies draw conclusions by comparing monocyte extracellular traps with METs, which is problematic for studying citrullination since monocytes and macrophages have different expression of PAD enzymes (30, 210) and differing abilities to produce extracellular traps to some stimuli (193, 196, 199). It is necessary to better understand MET formation and citrullination by systematically comparing stimuli using consistent experimental procedures. Identifying MET stimuli, signaling pathways, and protein cargo is critical to clarifying the role of METs in disease.

PAD2 and PAD4 are expressed and active in macrophages (30, 195, 210) and may be involved in METosis. After acute kidney injury, PAD4$^{-/-}$ mice have lower levels of circulating macrophage-dependent DNA, although this is not fully PAD4-dependent and the requirement of PAD2 in MET formation has not been defined (197). Some stimuli can induce citrullinated METs (195, 200), but this has been limited to a small number of reports and requires further investigation. Since macrophages express PAD, have citrullinated protein, produce extracellular traps, and are abundant in the rheumatoid joint, they may a major source of citrullinated protein in rheumatoid arthritis. Future studies should define stimuli and pathways leading to METs and the production of citrullinated protein in macrophages and determine if PAD2 and PAD4 are required for the production of METs and extracellular citrullination. These studies will contribute to a better understanding of rheumatoid arthritis through identifying novel roles of macrophages in citrullination.

B. Preliminary Data

A systematic approach to evaluating METs identifies several stimulants that induce METs. To elicit macrophages, 1mL of 4% thioglycolate was injected interperitoneally into BL6 mice. Four days later, mice were euthanized and cells retrieved with a peritoneal lavage and then allowed to adhere to non-tissue culture treated plastic for 20 hours. Flow cytometry before and after adhesion was performed to determine macrophage purity. On average, macrophage purity was 79% and neutrophils were only 0.1% of the cells. Macrophages were washed and plated onto uncoated coverslips for 1 hour before

stimulation with $5x10^6$ cells of heat killed *C. albicans* (HKCA), 20 μM ionomycin, 30 μg/mL lipopolysaccharide, 1 mg/mL monosodium urate (MSU) crystals, 10 μg/mL platelet activating factor (PAF), 50 nM phorbol myristate acetate (PMA), 400 ng/mL tumor necrosis factor alpha (TNFα), or no treatment. Stimuli were selected based on the ability to activate extracellular trap formation in macrophages, monocytes, or neutrophils (34, 58, 192, 193, 198) and dosages and activation time were determined by dose response curves. Moreover, these stimuli activate cells via different pathways. *C. albicans* binds surface TLR 2/4 and dectin-1 to initiate MyD88 and p38 MAPK signaling and results in activation of NF-κB with subsequent secretion of proinflammatory cytokines (212, 213). Ionomycin is a calcium ionophore which increases intracellular calcium concentration. LPS binds to CD14 and TLR4 to activate NF-κB through ERK1/2 and c-Jun kinase (214). MSU crystals bind CD16 and initiate MEK1/2 and ERK1/2 signaling (215). PAF binds to PAF receptor and increases intracellular calcium concentration and also activates ERK1/2. (216). PMA is a protein kinase C activator that results in activation of NF-κB. TNFα binds to TNFα receptor 1/2 to initiate NF-κB signaling through MEK 1/2 (217). *C. albicans,* MSU, and PAF can also activate the NLRP3 inflammasome, resulting in secretion of IL-1β (189, 190, 218). Cells were then incubated at 37°C for 20 hours before fixation, permeabilization, and staining with DAPI (blue) and an anti-citrulline antibody (anti-histone 4 citrulline 3, pink), and imaging at five predetermined locations as previously (58). DNase treated coverslips were incubated with 20 U/mL DNase for 20 minutes at 37°C. Percent METosis was quantified by imaging five preset fields and counting the number of METs divided by total cells. METs were identified as structures with significant enlargement, spread morphology, and extensive DNA diffusion compared

to untreated controls (114). METs were confirmed by the loss of identified structures after treatment with DNase, indicating that the structures were extracellular. Citrullinated METs had the same properties and stained positively with the anti-citrullinated histone antibody. Percent METosis was compared between untreated cells and each treatment by t-test with $p<0.05$ considered significant.

As shown in Figure 1A and B, ionomycin, MSU, and PAF activated extensively decondensed structures that were lost after DNase treatment, and were therefore classified as strong MET inducers. Interestingly, HKCA induced a significant amount of structures with decondensed DNA that were still present after DNase treatment. Thus, HKCA may activate macrophages to decondense their chromatin, but not activate METs. TNFα also induced a significant amount of decondensed structures that were lost after DNase treatment. Although this finding was statistically significant, TNFα induced 6.4% METosis compared to 3.8% seen in untreated controls. Since this increase was minimal, TNFα was not considered a strong inducer of METs and excluded from future analyses. We also found that MSU strongly induced citrullinated METs (Figure 1C). Ionomycin and PAF may also induce citrullinated METs, although our preliminary studies revealed only a trend. Interestingly, the MET stimuli identified in Figure 1B are activators of the NLRP3 inflammasome suggesting that future studies should examine the role of inflammasome assembly in MET formation. Also, since MSU induced citrullinated METs and can activate thioredoxin, which can activate PADs (219), the role of thioredoxin in citrullinated METosis should also be investigated.

A novel role for PAD2 in MET formation. Since ionomycin, MSU, and PAF induced citrullinated METs, we repeated the above experiments in PAD2$^{+/+}$ and PAD2$^{-/-}$ mice on the DBA1/J background to determine if PAD2 would be required for METs and citrullinated METs and thus be a potential source of PAD2-dependent gross protein citrullination (Figure 2A). In our preliminary studies, PAD2 is required for maximum METosis stimulated by MSU with the same trend in PAF (PAF p=0.08). There is also a trend towards PAD2 being required for citrullinated METs stimulated by MSU and PAF although these preliminary experiments are underpowered (MSU p=0.14, Figure 2C). Surprisingly, PAD2 is required for maximum production of uncitrullinated METs stimulated by MSU (Figure 2D). These data suggest that PAD2 may be required for citrullinated and uncitrullinated MET formation to certain stimuli and thus could be a source of citrullinated antigen in rheumatoid arthritis.

C. Figures

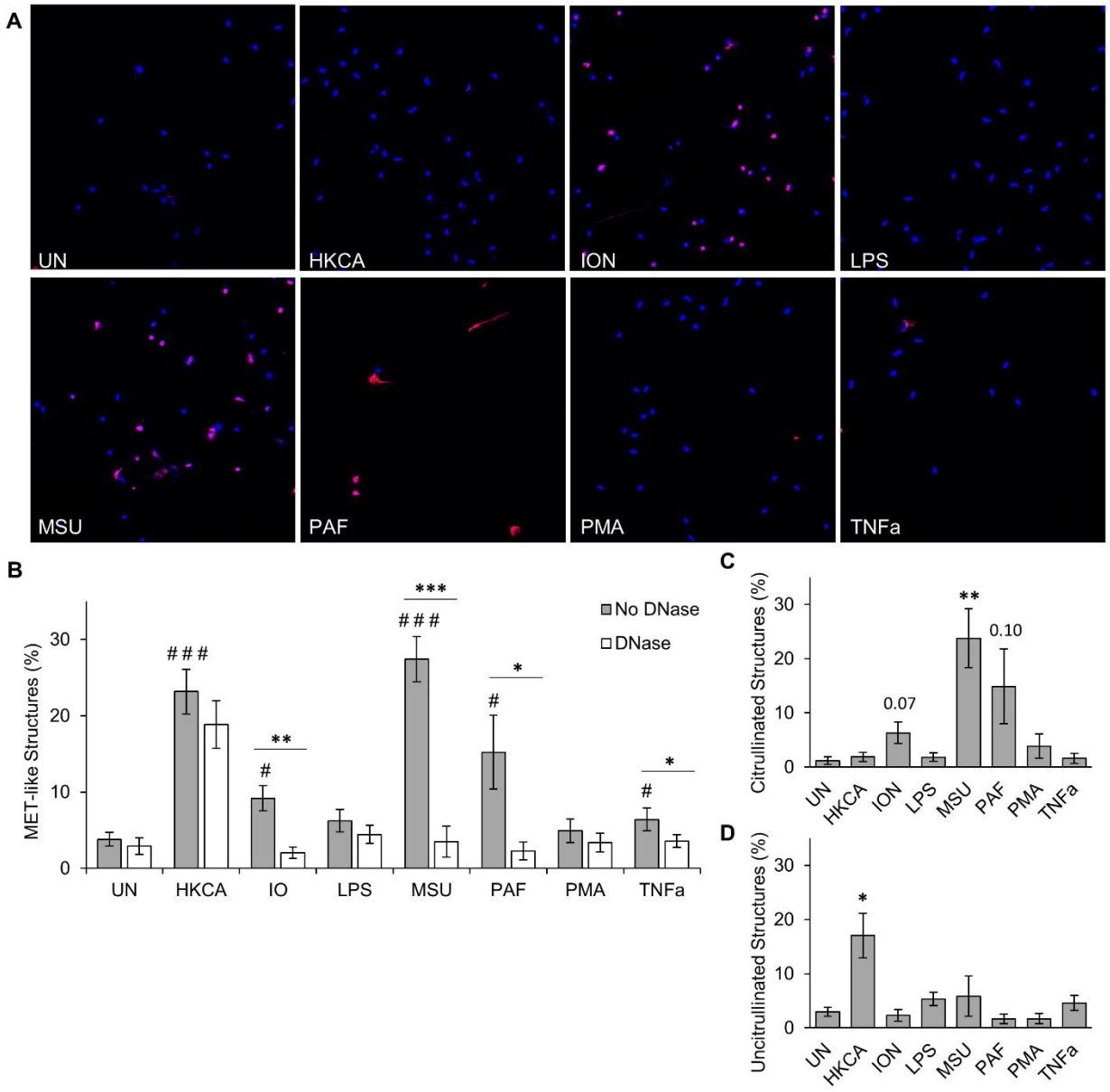


Figure 1. Induction of METs. Macrophages were untreated (UN) or treated with heat killed C. albicans (HKCA), ionomycin (ION), lipopolysaccharide (LPS), monosodium urate crystals (MSU), platelet activating factor (PAF), phorbol myristate acetate (PMA), or tumor necrosis factor alpha (TNFa), fixed, and stained with DAPI (blue) and anti-citrullinated histone antibody (pink). (A) Representative images at 400x. The number of macrophages

and METs were quantified. Graphs depict the average and SEM for percent of macrophages that formed total METs with and without DNase treatment (B), citrullinated METs in the absence of DNase (C), or uncitrullinated METs in the absence of DNase (D). For (A): n=10; #p<0.05, ###p<0.001 for each stimulant compared to the untreated control; asterisks (*) compare METs for each stimulant with and without DNase. For (B) and (C): n=7. For all images: *p<0.05, **p<0.01, ***p<0.001.

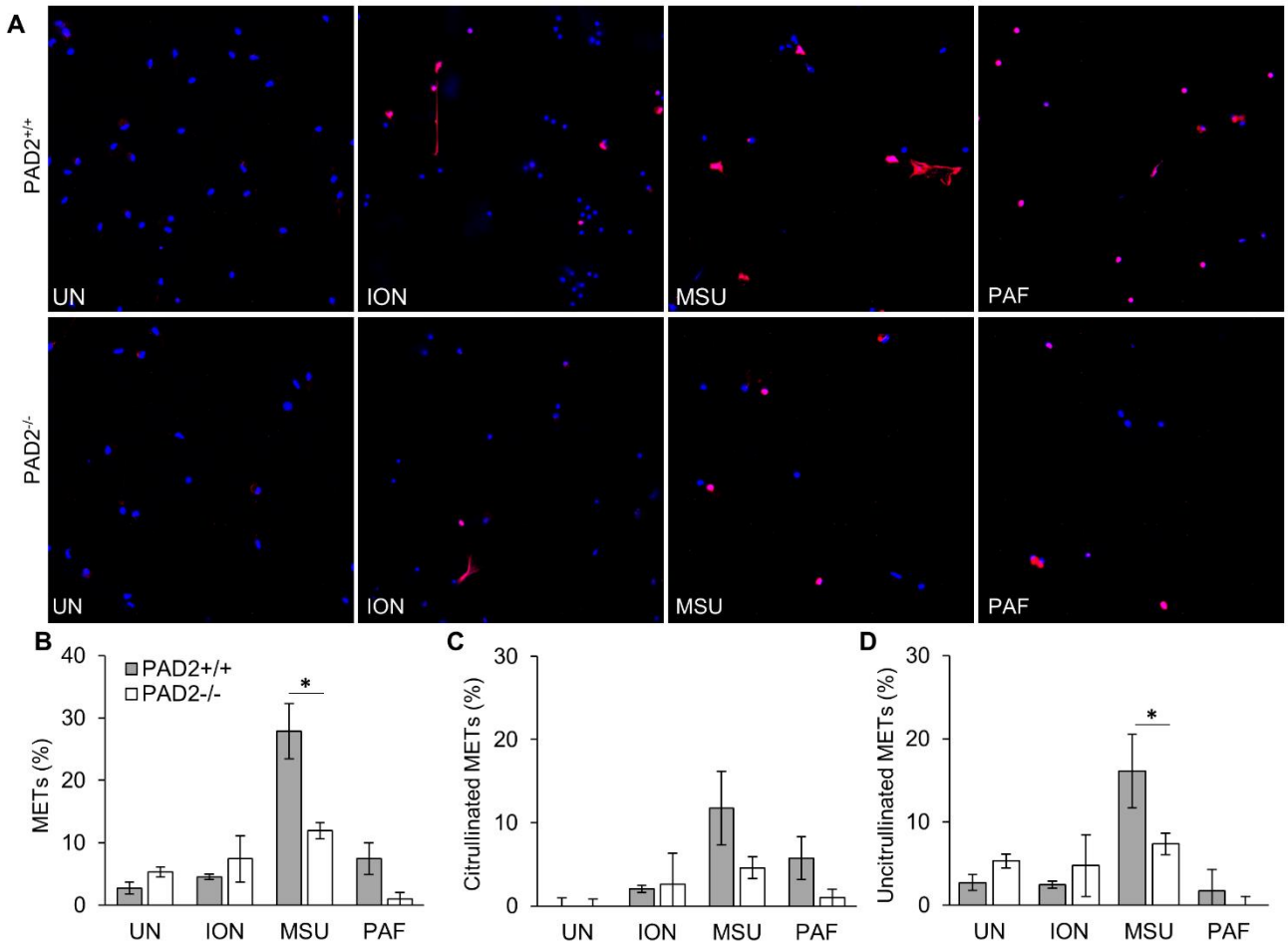
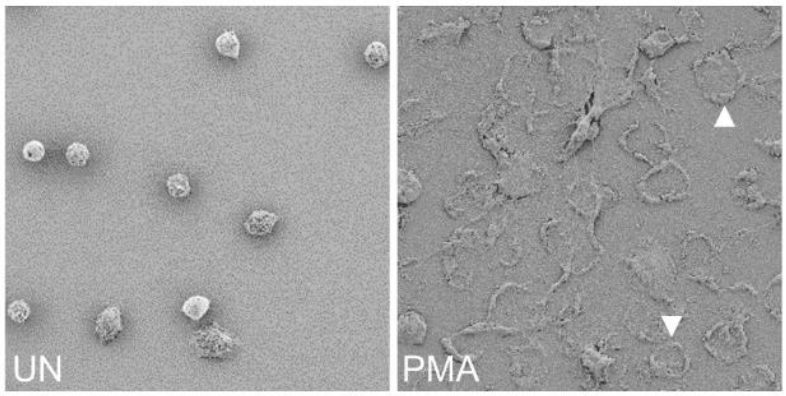


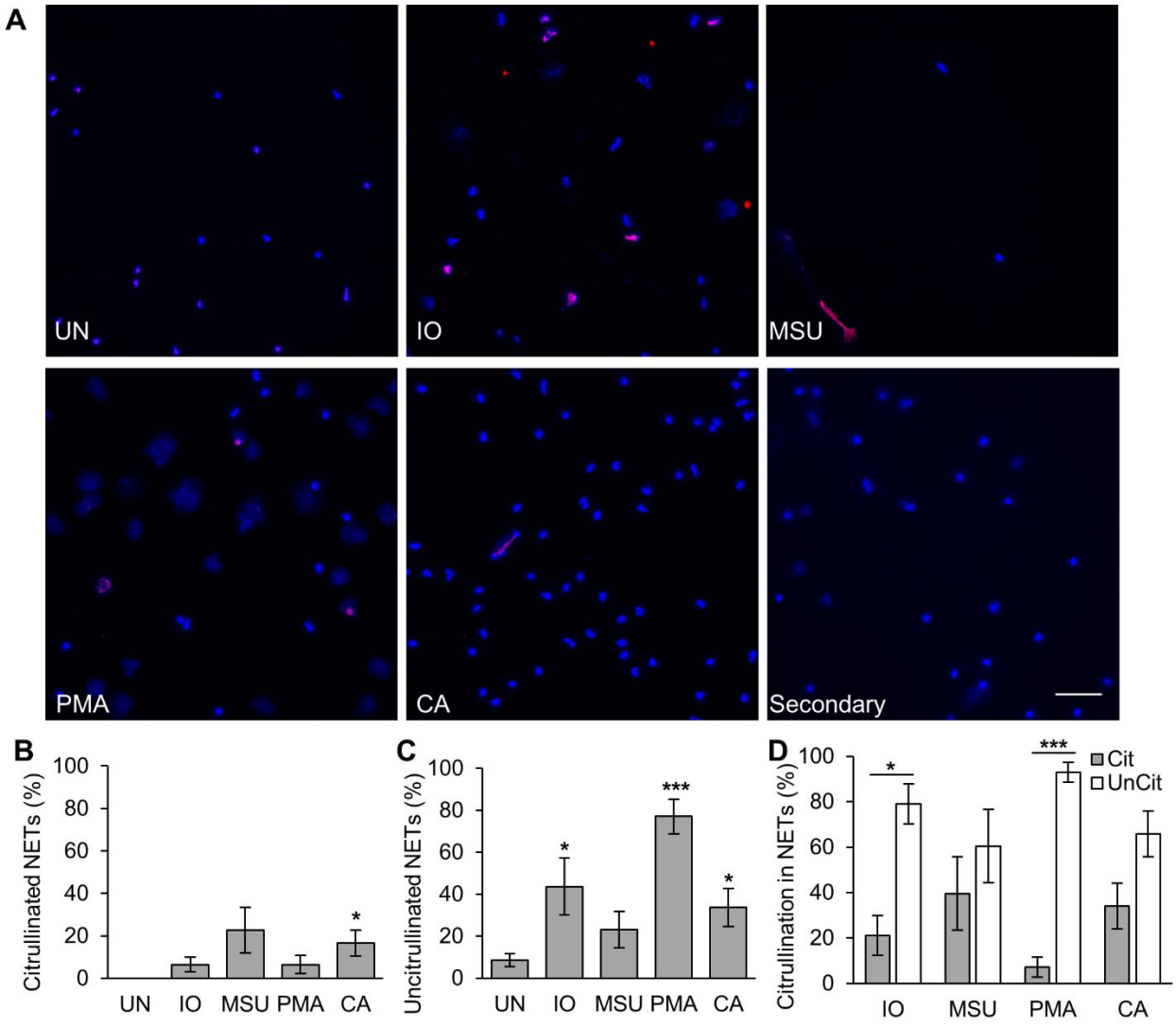
Figure 2. PAD2 is required for normal MET formation in response to some stimuli. Macrophages from PAD2+/+ and PAD2-/- mice were untreated (UN) or treated with ionomycin (ION), monosodium urate crystals (MSU), or platelet activating factor (PAF), fixed, and stained with DAPI (blue) and anti-citrullinated histone antibody (pink). (A) Representative images at 400x. The number of macrophages and METs were quantified. Graphs depict the average and SEM for percent macrophages that formed total METs (B), citrullinated METs (C), or uncitrullinated METs (D) with percent METs for each stimulant compared for PAD2+/+ versus PAD2-/- mice. For all images: n=3, *$p<0.05$.

APPENDIX TWO

Chapter 2 Supplementary Figures

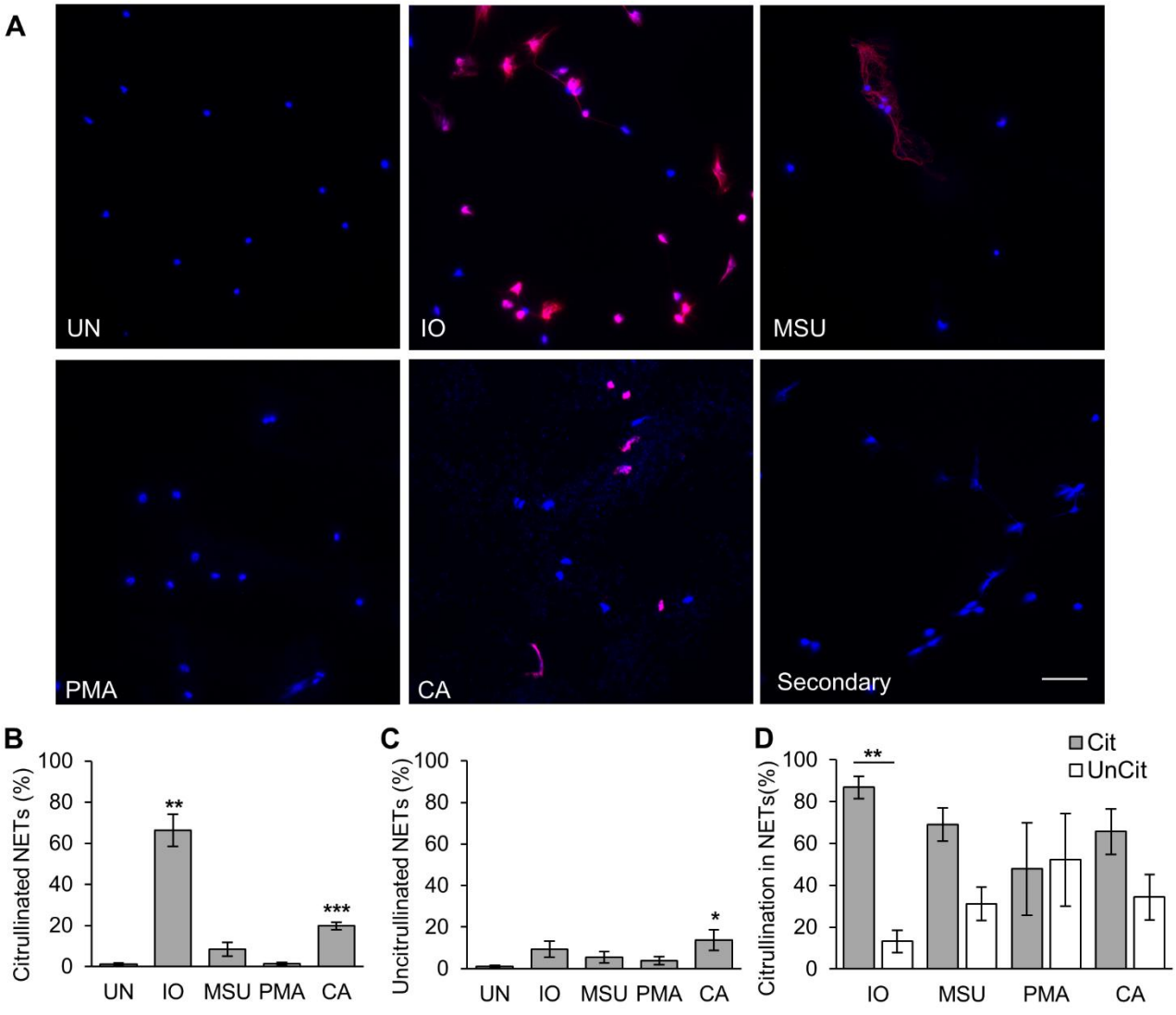


Supplementary Figure 1. Scanning electron micrographs of doNETs formed by PMA-stimulated human neutrophils. Treated neutrophils were washed with PBS, fixed overnight (4% formaldehyde and 1% glutaraldehyde in PBS), washed with PBS, treated with 1% osmium tetroxide, washed again, dehydrated via ethanol washes and critical point drying, coated with platinum, and imaged in a scanning electron microscope (LEO 1530-2 FESEM) at 3kV with SmartSEM software (Zeiss, Oberkochen, Germany). Representative images at 2,000x of untreated (UN) or PMA treated neutrophils are shown.

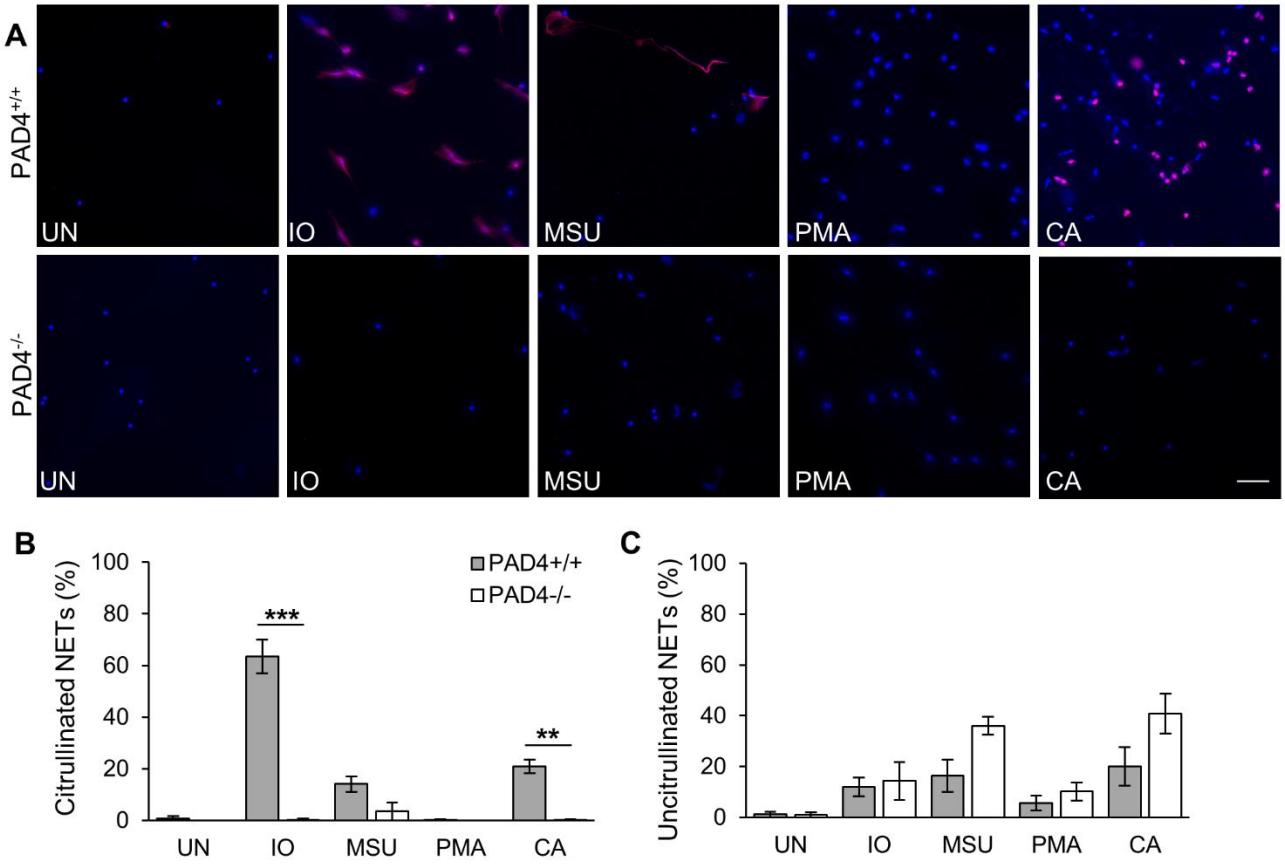


Supplementary Figure 2: Citrullinated NETs in human neutrophils detected by anti-citrullinated histone H4. Human neutrophils were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, or C. albicans (CA), fixed, and stained with DAPI (blue) and anti-histone 4 citrulline 3 antibody (pink). Image labeled “Secondary” was created by stimulating neutrophils with C. albicans and staining without the histone 4 citrulline 3 primary antibody and only the anti-rabbit IgG TRITC secondary antibody as a negative control. (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils

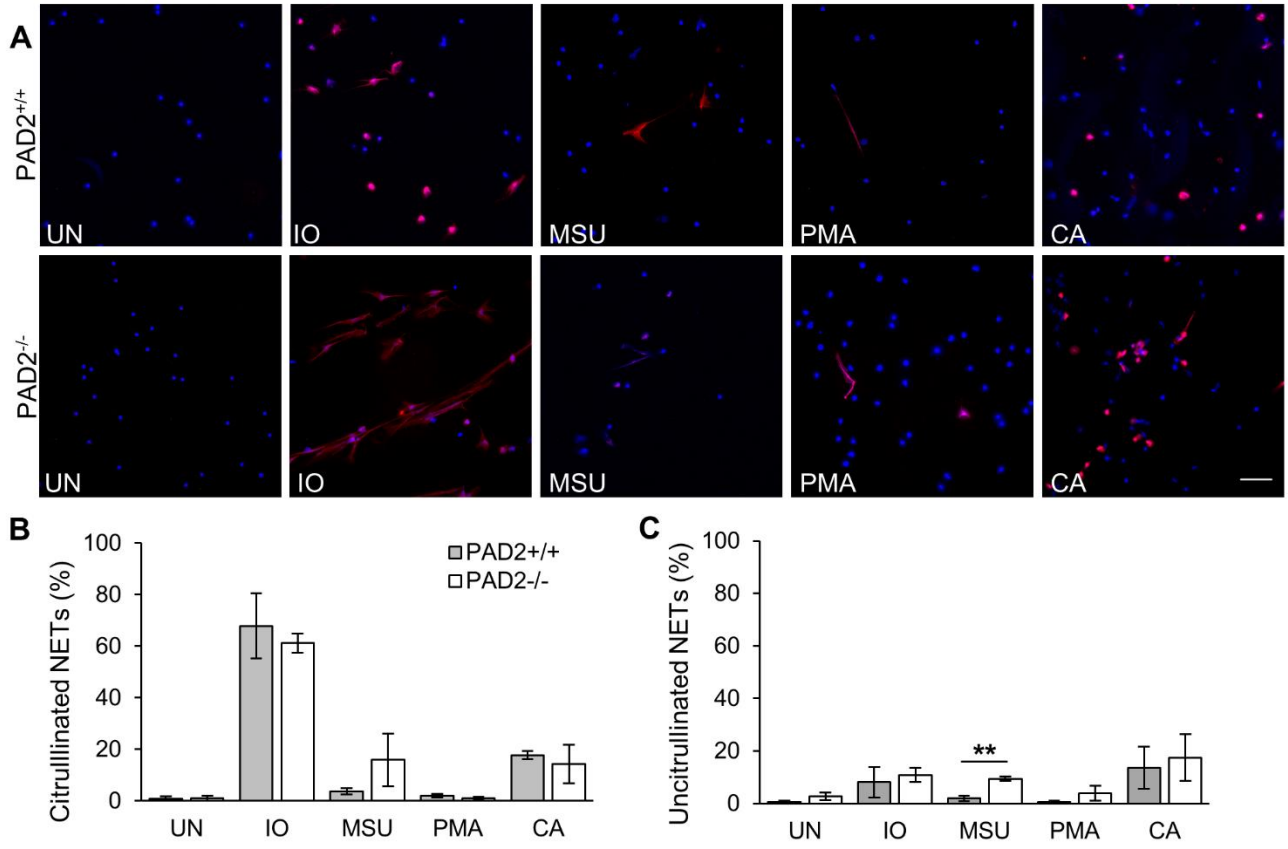
and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed citrullinated NETs (B) and uncitrullinated NETs (C) for each condition with percent NETs for each stimulant compared to untreated. (D) The percent of citrullinated versus uncitrullinated NETs was compared for each stimulus with average and SEM graphed. For all panels, n=6, *p<0.001.



Supplementary Figure 3: Citrullinated NETs in murine neutrophils detected by anti-citrullinated histone H4. Mouse neutrophils were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and C. albicans (CA), fixed, and stained with DAPI (blue) and anti-histone 4 citrulline 3 antibody (pink). Image labeled “Secondary” was created by stimulating neutrophils with ionomycin and staining without the histone 4 citrulline 3 primary antibody and only the anti-rabbit IgG TRITC secondary antibody as a negative control. (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed citrullinated NETs (B) and uncitrullinated NETs (C) for each condition with percent NETs for each stimulant compared to untreated. (D) The percent of citrullinated versus uncitrullinated NETs was compared for each stimulus with average and SEM graphed. For all panels, n=5, *$p<0.001$.



Supplementary Figure 4: PAD4 is required for the formation of citrullinated NETs in murine neutrophils as detected by anti-citrullinated histone H4. Bone marrow neutrophils from PAD4+/+ and PAD4-/- mice were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and C. albicans (CA), fixed, and stained with DAPI (blue) and anti-histone 4 citrulline 3 antibody (pink). (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed citrullinated NETs (B) and uncitrullinated NETs (C) for each condition with percent NETs for each stimulant compared between PAD4+/+ and PAD4-/- mice. For all panels, n=3, **p<0.001.



Supplementary Figure 5: PAD2 is not required for the formation of citrullinated NETs in murine neutrophils as detected by anti-citrullinated histone H4. Bone marrow neutrophils from PAD2+/+ and PAD2-/- mice were left untreated (UN) or were treated with ionomycin (IO), MSU, PMA, and C. albicans (CA), fixed, and stained with DAPI (blue) and anti-histone 4 citrulline 3 antibody (pink). (A) Representative images at 400x, scale bar = 50μM. The number of neutrophils and NETs were quantified. Graphs depict the average and SEM for percent of neutrophils that formed citrullinated NETs (B) and uncitrullinated NETs (C) for each condition with percent NETs for each stimulant compared between PAD2+/+ and PAD2-/- mice. For all panels, n=3, **p<0.01.

APPENDIX THREE

Supplementary Tables for Chapter Three

Supplementary Table 1. Characteristics of Rheumatoid Arthritis and Control Subjects

	Rheumatoid Arthritis (n=98)	Control (n=77)	p
Age, mean years (range)	60 (32-85)	59 (29-87)	0.44
Sex, female (%)	64 (65.3)	58 (75.3)	0.15
Race/Ethnicity, number (%)			
White	78 (79.6)	66 (85.7)	0.08
Black	3 (3.1)	3 (3.9)	
Hispanic	8 (8.2)	0 (0.0)	
Native American	8 (8.2)	5 (6.5)	
Asian	1 (1.0)	3 (3.9)	
Pacific Islander	0 (0.0)	0 (0.0)	
Smoking Status, number (%)			
Current smoker	10 (10.2)	6 (7.8)	0.80
Former smoker	31 (31.6)	23 (29.9)	
Never smoked	57 (58.2)	48 (62.3)	

Body Mass Index, number (%)			
Normal	26 (26.5)	17 (22.1)	0.71
Overweight	28 (28.6)	21 (27.3)	
Obese	44 (44.9)	39 (50.7)	
Charlson Comorbidity Score, mean (SE)	**3.5 (0.2)**	**2.1 (0.2)**	**<0.0001**
Time since Vaccination, mean years (SE)	4.4 (0.3)	5.2 (0.3)	0.06
Age at Vaccination, mean years (SE)	55.6 (1.2)	53.5 (1.5)	0.25

Supplementary Table 2. Predictors of greater than median pertussis titer in subjects diagnosed with rheumatoid arthritis at the time of vaccination including medications taken at the time of vaccination (n=70)

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
Age	0.98	(0.94, 1.02)	0.36	0.94	(0.86, 1.04)	0.23
Sex: Female	**0.31**	**(0.11, 0.88)**	**0.03**	**0.22**	**(0.06, 0.90)**	**0.03**
Smoking Status (Never)	Ref.			Ref.		
Current	1.54	(0.31, 7.79)	0.60	1.73	(0.24, 12.66)	0.59
Former	1.39	(0.49, 3.93)	0.54	2.42	(0.52, 11.34)	0.26
BMI (Normal)	Ref.			Ref.		
Overweight	2.33	(0.62, 8.82)	0.21	1.94	(0.38, 9.92)	0.43

Obese	**3.89**	**(1.18, 12.84)**	**0.03**	5.24	(1.00, 27.40)	0.05
Charlson Comorbidity Score	1.05	(0.84, 1.31)	0.66	1.14	(0.59, 2.18)	0.70
NSAIDs (n=37)	1.41	(0.55, 3.62)	0.47	0.67	(0.18, 2.55)	0.56
Time since Vaccination	0.88	(0.72, 1.08)	0.21	0.86	(0.64, 1.16)	0.34
Abatacept (n=4)*	-	-	-	-	-	-
Hydroxychloroquine (n=14)	1.43	(0.44, 4.67)	0.55	0.57	(0.08, 4.09)	0.57
Leflunomide (n=12)	2.30	(0.62, 8.48)	0.21	1.40	(0.21, 9.49)	0.73
Methotrexate (n=26)	0.61	(0.23, 1.62)	0.32	0.68	(0.20, 2.30)	0.54
Sulfasalazine (n=5)	4.39	(0.46, 41.04)	0.20	6.69	(0.31, 146.29)	0.23
TNF inhibitor (n=28)**	1.27	(0.49, 3.31)	0.63	0.68	(0.16, 2.84)	0.60

*All subjects taking abatacept had lower than median pertussis titers. **For TNF inhibitor users: 11 were prescribed methotrexate, 3 leflunomide, 4 hydroxychloroquine, and 1 sulfasalazine with some subjects taking more than one of these medications.

Supplementary Table 3. Predictors of pertussis immunity in subjects diagnosed with rheumatoid arthritis at the time of vaccination including medications taken at the time of vaccination (n=70)

	Univariate			Multivariable		
	OR	95% CI	p	OR	95% CI	p
Age	0.97	(0.92, 1.03)	0.35	1.01	(0.92, 1.12)	0.83
Sex: Female	0.59	(0.17, 2.09)	0.41	0.36	(0.06, 2.03)	0.25
Smoking Status (Never)	Ref.			Ref.		
Current*	-	-	-	-	-	-
Former	1.10	(0.32, 3.74)	0.88	1.79	(0.29, 11.01)	0.53
BMI (Normal)	Ref.			Ref.		
Overweight	1.50	(0.35, 6.50)	0.59	0.56	(0.08, 3.96)	0.56
Obese	2.31	(0.60, 8.94)	0.22	7.39	(0.94, 58.26)	0.06
Charlson Comorbidity Score	0.95	(0.74, 1.22)	0.67	0.82	(0.44, 1.56)	0.55
NSAIDs (n=37)	0.98	(0.31, 3.06)	0.97	0.46	(0.10, 2.06)	0.31
Time since Vaccination	0.89	(0.70, 1.12)	0.31	0.72	(0.50, 1.04)	0.08
Abatacept (n=4)	0.25	(0.03, 1.91)	0.18	0.03	(0.001, 1.01)	0.05
Hydroxychloroquine (n=14)	1.00	(0.24, 4.17)	1.00	0.15	(0.02, 1.22)	0.08
Leflunomide (n=12)	0.78	(0.18, 3.35)	0.74	0.10	(0.01, 1.23)	0.07
Methotrexate (n=26)	1.24	(0.37, 4.12)	0.73	1.31	(0.27, 6.30)	0.73
Sulfasalazine (n=5)**	-	-	-	-	-	-

TNF inhibitor (n=28)***	1.44	(0.43, 4.77)	0.55	0.20	(0.03, 1.67)	0.14

*All current smokers were immune to pertussis. **All subjects taking sulfasalazine were immune to pertussis. ***For TNF inhibitor users: 11 were prescribed methotrexate, 3 leflunomide, 4 hydroxychloroquine, and 1 sulfasalazine with some subjects taking more than one of these medications.