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Transcriptional regulation of peptidylarginine deiminase type IV: implications for rheumatoid arthritis

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology and Immunology

TRANSCRIPTIONAL REGULATION OF PEPTIDYLARGININE DEIMINASE TYPE IV: IMPLICATIONS FOR RHEUMATOID ARTHRITIS

by

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Abstract

High titers of anti-citrullinated protein antibodies have been detected in sera of rheumatoid arthritis (RA) patients, implicating citrullinating enzymes in the pathogenesis of RA. Peptidylarginine deiminase type IV (PAD4) is a member of the PAD family of enzymes that catalyze the post- translational modification of arginine to citrulline and has been linked with RA. However, little is known about its transcriptional regulation. Therefore, our aim was to determine how transcription of PAD4 is activated in the myeloid lineage. Using bioinformatics, a potential nuclear factor kappa B (NF-kB) binding site was identified on the PAD4 promoter. Luciferase assays were used to test promoter activity in human and murine myeloid cells. Interestingly, mutation of the NF-κB binding site significantly lowered promoter activity in WEHI-3B cells, but significantly increased it in both HL-60 and THP-1 cell lines. In addition, PAD4 mRNA was significantly lowered in response to TNF-α treatment in HL-60 cells, but increased in WEHI-3B cells. Finally, chromatin immunoprecipitation (ChIP) using anti-p50 and anti-p65 antibodies revealed that there was a significant increase in p50 enrichment at the PAD4 promoter, but not p65 in cells treated with TNF-α. Our results suggest that NF-κB may play an important role in the transcriptional regulation of PAD4 in human and murine immune systems.

Keywords

PAD4, NF-κB, Rheumatoid Arthritis, Autoimmunity, Transcription, Citrullination

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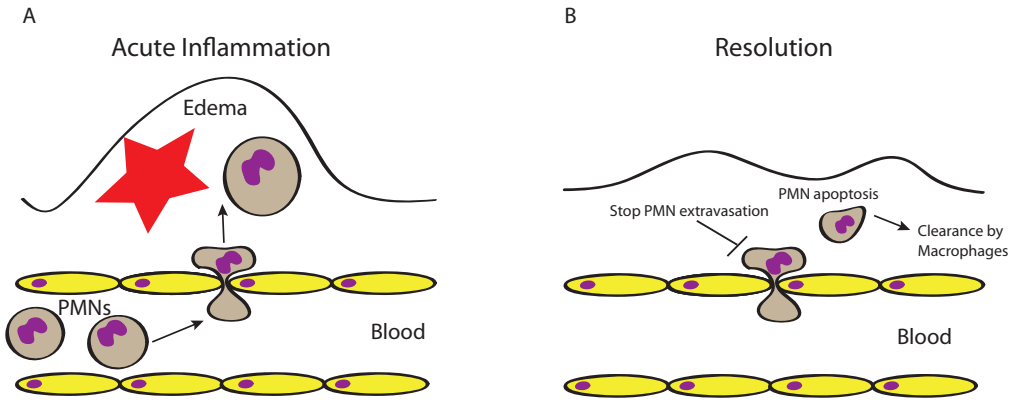
Chapter 1: Introduction

1.1 Inflammation

The human body is protected from pathogens and other harmful substances by an arsenal of effector cells and molecules that together make up the immune system. In general, the immune system carries out four chief tasks in order to protect the individual against disease: recognition, effector functions, immune regulation, and immunological memory (1). For the most part, the physical and chemical barriers erected by the body against pathogens are sufficient to prevent infection. These include antimicrobial proteins secreted at mucosal surfaces, as well as innate defenses such as the complement system (1, 2). In the event that these barriers are overcome, other components of the innate immune system come into action to firstly recognize the foreign agent via detection of pathogen associated molecular patterns (PAMPs) and secondly to eliminate the pathogen, which occurs via the inflammatory response (3). Inflammation is the process by which proteins and cells from the blood are recruited into infected tissues in order to facilitate the killing of pathogen. More specifically, inflammation is traditionally described by the latin words *calor, dolor, rubor,* and *tumor*, meaning heat, pain, redness, and swelling. Each of these features reflects an effect of cytokines or other inflammatory mediators on local blood vessels. Heat, redness, and swelling result from the dilation and increased permeability of blood vessels during inflammation, leading to increased local blood flow and leakage of fluid and blood proteins into tissues, whereas pain is accounted for by the migration of cells into the tissue and their local actions (Fig. 1.1) (3). The main cell types seen in the initial stages of inflammation are macrophages and neutrophils, with

Figure 1.1 The acute and resolution phases of inflammation. (A) Illustration of the acute phase of inflammation. Polymorphonuclear cells (PMNs) are recruited (among other cell types) to the site of infection and extravasate from blood vessels into affected tissue (resulting in edema) where they mediate inflammation. (B) Resolution phase. PMN infiltration ceases and those PMNs still present in the tissue undergo apoptosis. Adapted from *Isobe Y et al.* (4).

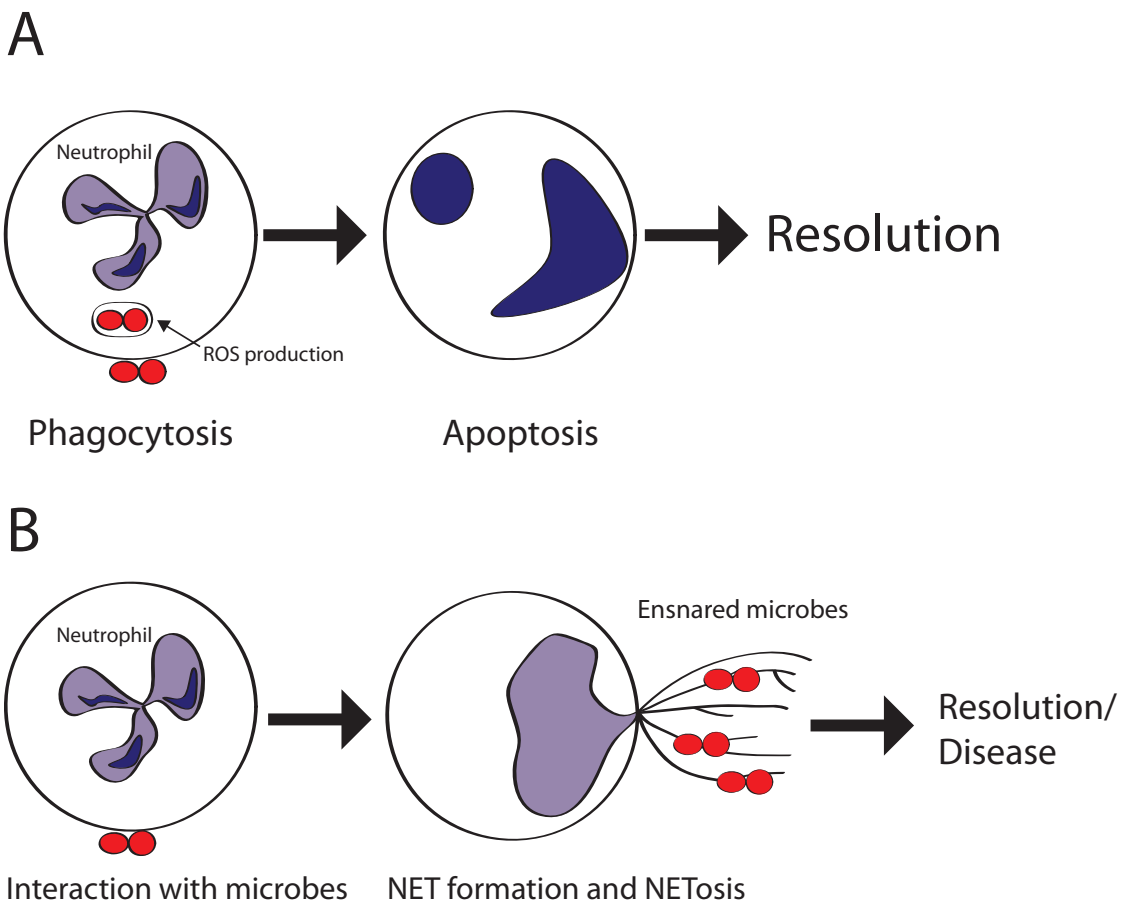


neutrophils forming the larger component of the two. Both cell types are the principal cells that engulf and destroy the invading microorganisms. Once the pathogen is eliminated, the resolution phase of inflammation follows in which various mechanisms are enabled to terminate inflammation and prevent damage to healthy tissue surrounding the point of infection. Some mechanisms include apoptosis of inflammatory cells (5), production and release of interleukin 10 (IL-10) (6), as well as the production of anti-inflammatory lipoxins (7). If the resolution of inflammation is not achieved correctly, this may result in chronic inflammation and continued damage to otherwise healthy tissues of the body.

1.2 Neutrophils in inflammation

Neutrophils are important components of innate immunity vital to the maintenance of homeostasis of the organism. They are short-lived polymorphonuclear granulocytes (PMNs) that form the primary defense against microbial infections. During acute inflammation, neutrophils circulating within the bloodstream are rapidly recruited to the site of infection (Fig. 1.1) in response to chemotactic factors released by pathogens or host cells. After attachment to the endothelium, neutrophils migrate from blood vessels and move in line with the chemotactic gradient toward the site of infection. At the site of inflammation, activated immune cells acquire the ability to kill pathogens. To carry out the killing of bacteria, fungi, and protozoa**,** neutrophils use a number of strategies such as phagocytosis and the recently discovered formation of neutrophil extracellular traps (NETs). During phagocytosis, internalized pathogens are translocated to phagosomes where the antimicrobial factors derived from granules and reactive oxygen species (ROS) create a killing environment for pathogens. However, while the cytotoxic factors produced by neutrophils are effective in combating pathogens, they can also be extremely damaging to surrounding host tissue if they are not neutralized effectively. The engulfment of pathogens by neutrophils is followed by apoptosis, which is a form of programmed cell death that ultimately promotes the resolution of inflammation (Fig. 1.2A). Neutrophils may also combat pathogens by the formation of NETs. During NET formation and action (a process termed NETosis), neutrophils extrude networks of decondensed chromatin decorated with citrullinated histones and granular antimicrobial proteins such as proteinase 3 (PR3), myeloperoxidase (MPO), and α-defensins, among others (8).

Figure 1.2 Overview of phagocytosis and NETosis. (A) Phagocytosis involves the recognition and internalization of pathogens by neutrophils. Following degradation in phagosome, neutrophils undergo apoptosis, which promotes resolution of inflammation. (B) Upon recognition of some pathogens neutrophils are triggered to release NETs that ensnare and kill the pathogen. However, this is accompanied with lysis of the neutrophil and release of cyototoxic molecules that may or may not be cleared by the host. Adapted from *Lu, T et al.* (9).



NETs act as a mesh that traps microorganisms and facilitates their interaction with neutrophil-derived effector molecules, limiting the spread of rapidly disseminating pathogens (10). NET formation can be induced by phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide (LPS), and bacteria (10). In particular, NETs have been shown to trap and kill pathogenic bacteria such as *Shigella flexneri* (10) and *Streptococcus pyogenes* (11). Moreover, NETs can induce the production of antimicrobial cytokines such as interferon-α (IFN-α) (12-14), a relevant cytokine in the control of viral, bacterial, and protozoal infections (15).

Since NETosis fundamentally involves the extracellular exposure of intracellular components (Fig. 1.2B), the discovery of NETs has sparked renewed interest in potential links between neutrophils and autoimmune disease. For example, the neutrophils of systemic lupus erythematosus (SLE) patients have been demonstrated to be more likely to form NETs (16), a finding that correlates with increased levels of circulating DNA in the plasma of SLE patients as well as the presence of antibodies towards other proteins released in NETs (17). Indeed, 74% of NET proteins have been reported to autoantigens in several systemic autoimmune diseases, notable among which are SLE, rheumatoid arthritis, and vasculitis (18). Thus, while NET formation has been revealed to be a unique effector mechanism of neutrophils against pathogens, it raises new questions and opens new doors into the study of the origin and pathogenesis of systemic autoimmune diseases.

1.3 Autoimmune disease

Although the main function of the immune system is to protect the human host from pathogenic agents, autoimmune diseases result when immune responses are directed at self antigens in the absence of any pathogen (19). These immune responses resemble normal immune responses in that they are specifically directed towards antigens, in this case self antigens or autoantigens, and give rise to autoreactive effector cells and to antibodies called autoantibodies against self antigens. Autoimmune diseases can broadly be categorized as organ specific (ex. diabetes and multiple sclerosis) in which cases the immune system targets autoantigens specific to particular organs, or systemic (ex. rheumatoid arthritis) in which cases the inflammation ensues in multiple tissues because the autoantigens causing the response are found in several (if not all) tissues of the body. In turn, a hallmark feature of systemic autoimmune diseases is the circulation of autoantibodies that recognize intracellular antigens thought to be expressed by all cells, yet are associated with specific disease phenotypes and outcomes. Since fundamentally autoimmune diseases occur because of a failure of the immune system to distinguish self from non-self correctly, several factors – both genetic and environmental – are implicated in their development. Genetic factors include genes important in the recognition of antigen such as those coding for immunoglobulins, T-cell receptors, as well as the major histocompatibility complex (MHC) (1). In particular, various MHC II allotypes have been associated with specific diseases; for instance, HLA DR2 is associated mostly with multiple sclerosis (20) whereas HLA DR4 shows high association with rheumatoid arthritis (21).

1.4 Rheumatoid arthritis

Rheumatoid Arthritis (RA) is a chronic, systemic autoimmune disease characterized by inflammation and progressive destruction of synovial joints (22). The most common form of inflammatory arthritis, RA affects nearly 1% of the world's population (23). Patients suffering from RA will present with a variety of symptoms ranging from mild pain and swelling in peripheral joints to widespread inflammation and joint destruction caused by a "runaway" immune response involving the development of autoantibodies and resulting in significant morbidity and mortality (23, 24).

Although RA etiology remains unclear, several factors – both genetic and environmental – have been associated with disease incidence, and it is thought that genetic predisposition to immune system dysregulation, coupled with an infectious event or the development of autoantibodies, may be at the center of the autoimmune responses (24). Genetic predisposition to RA is supported by twin studies in which higher concordance rates of disease were observed among monozygotic twins than dizygotic twins, as well as by sibling studies that show a 2-17% increase in the risk of RA contraction among siblings than the general population (25). The gene considered to be the major determinant of RA susceptibility (accounting for up to 30% of disease susceptibility) is human leukocyte antigen (HLA), *HLA-DRB1* (25, 26). One of the most polymorphic genes in the human genome, *HLA-DRB1* encodes the β-chain of the MHC class II protein. Several subtypes of the gene (termed shared epitopes) have been associated with RA in different populations around the world (25). However, while *HLA-DRB1* was thought to be the only RA susceptibility gene for nearly three decades, the last ten years

have seen the discovery of nearly 30 new RA susceptibility genes (22). Other genetic risk factors that have since been identified include the genes coding peptidylarginine deiminase type IV (*PAD4*), protein tyrosine phosphatase non-receptor type 22 (*PTPN22*), signal transducer and activator of transcription (*STAT4*) as well as several others (22).

Several environmental factors have been proposed as "triggers" for genetically susceptible individuals to develop RA. Among the most well known of these is smoking. Beginning in the 1980s, studies emerged demonstrating a higher incidence of RA in smokers (27). Furthermore, a positive correlation was found between smoking and seropositivity for rheumatoid factor (RF), the previous gold standard for rheumatoid arthritis (28, 29). More recently, it was demonstrated that smoking enhances the risk of RA in patients expressing anti-citrullinated protein antibodies (ACPAs) – the newly established gold standard for detecting and diagnosing RA (see below; (21)). Indeed, smoking was shown to enhance the risk of RA only in those individuals expressing ACPAs, and had no effect on individuals not expressing ACPAs (21) – an effect that has since been demonstrated in European as well as South American populations (30-32). Although the exact mechanism of how cigarette smoking contributes to RA, inhaled particulate matter (such as that from cigarette smoking) has been shown to trigger translocation of the transcription factor NF-κB (33) and may thus initiate pro-inflammatory cytokine expression contributing to RA.

Another environmental factor linked to the pathogenesis of RA is exposure to the bacterium *Porphyromonas gingivalis* and its associated infection periodontitis. Periodontitis has been demonstrated to increase the risk of RA in non-smokers and is also associated with ACPA production (34). Intriguingly, the bacterium is proposed to be able

to do this because it expresses a peptidylarginine deiminase (PAD) gene, and it has been demonstrated that its ability to express a fully functioning PAD protein – which produces citrullinated proteins – is responsible for its facilitation of ACPA driven RA development (35). *P. gingivalis* is the only bacterium known to express PAD, and its own enzyme is able to citrullinate both host and bacterial peptides (34). Indeed, ACPAs to the protein CEP-1 have been shown to bind the corresponding region of *P. gingivalis* enolase, indicating that microbial mimicry may play a role in the etiopathology of RA (34).

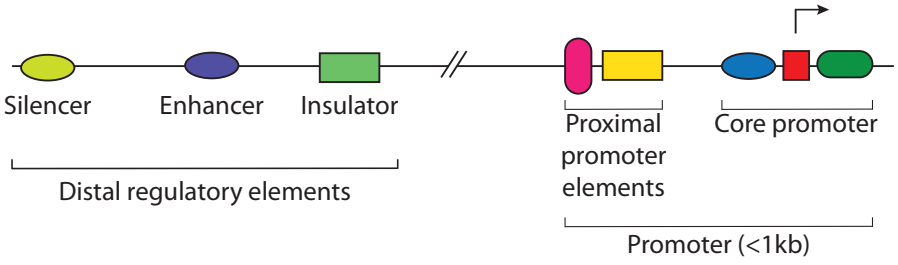
1.5 Transcriptional regulation

The proteins a cell produces are a result of each protein's mRNA levels, the frequency at which the mRNA is translated, as well as the stability of the protein itself. The start point, however, is the first process of the Central Dogma: the transcription of DNA into RNA – the first step towards gene expression. Although there are several steps at which the process of gene expression can be regulated to give rise to differential gene expression (and the resultant differences in cell properties and functions), it is the first step – transcription – which is the most important mechanism in for determining whether or not most genes are expressed and how much of the encoded mRNAs are produced (36, 37). The proteomic character of a cell is thus determined by which genes are transcribed and their rate of transcription in the given cell type. Therefore, it is the differential transcription of different genes that largely determines the actions and properties of cells.

Regulation of gene expression via transcriptional regulation is of paramount importance because it affects the execution of precise biological processes such as development, proliferation, inflammation, apoptosis, aging, and differentiation. Transcriptional

regulation of genes is achieved through the collective action of various *cis*-regulatory elements that are located proximally to genes. These elements include core promoters and promoter-proximal elements – located close to the transcription start site (TSS) – as well as other elements that are located distantly from the TSS including enhancers, silencers, insulators, and tethering elements (Fig. 1.3; (36, 38)). Gene promoters are particularly important since they are immediately adjacent to TSSs and are the sites that position the transcription initiation complex (PIC) consisting of RNA polymerase and other proteins (38). In eukaryotes, RNA polymerase II (RNAPII) is responsible for transcribing all protein coding genes – that is, it is responsible for the production of mRNA (38). Since this responsibility includes such a hugely diverse array of genes, RNAPII is itself controlled in many different ways, with one of these being differing classes of RNAPII promoters (38).

Figure 1.3 A typical gene regulatory region. The promoter spans an area typically less than 1 kb pairs and is composed of a core promoter and proximal promoter elements. Distal (upstream) regulatory elements can be located up to 1 Mb pairs from the promoter and may include enhancers, silencers, and insulators. Adapted from *Maston et al.* (37).



Another important aspect of the process of transcriptional regulation is the integration of several protein signals that culminates in the recruitment of RNAPII and the initiation of transcription. Chief among these protein signals is the binding of sequence-specific transcription factors (TFs) at their respective transcription factor binding sites (TFBS) either within the proximal promoter region or else at enhancers, which functions to recruit and stabilize the PIC and drive transcription forward (37, 38). Indeed, the sequence variability of TFBS forms another layer of transcriptional regulation in themselves. For instance, many transcription factors form heterodimers and/or homodimers, with the precise combination of subunits a key factor in the TF's binding specificity and regulatory output (37, 39). Furthermore, variations in the sequence may result in stronger or weaker interactions with TF's, and may also direct a preference for particular dimerization partners over others (37, 39). One example of this is the NF-κB family of TFs.

1.5 The NF-κB family

The NF-κB family is a key player in controlling both innate and adaptive immune responses and NF-κB activity is required for lymphocyte survival and activation, as well as for mounting normal immune responses (40, 41). NF-κB proteins are constitutively present in the cytoplasm in association with proteins that are known as inhibitors of NF-κB (IκBs). After activation by one of a variety of signals, the IκB proteins become phosphorylated, ubiquitinylated and degraded by the proteasome. Freed from their association with IκB, NF-κB proteins are able to translocate to the nucleus and bind their respective DNA binding sites to activate or repress the transcription of a vast array of

inflammatory genes, including cytokines, chemokines and a variety of antimicrobial peptides (42, 43). The constitutive activation of NF-κB pathways is often associated with inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis (MS) and asthma.

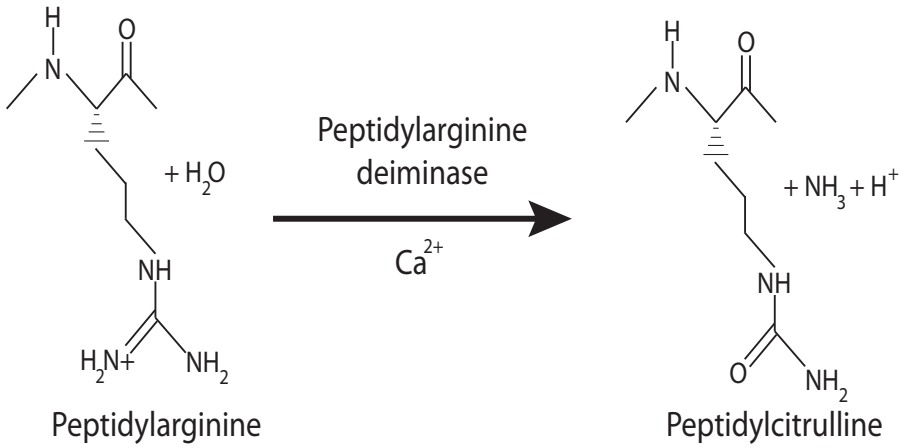
Together, the NF-κB family of proteins consists of five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and cRel; each member may form a homodimer or a heterodimer with any other member, and these dimers in turn have differential abilities to regulate gene transcription (44). These proteins have a structurally conserved amino-terminal 300-amino-acid region, which contains the dimerization, nuclear-localization and DNA-binding domains (Fig.1.3). The c-REL, RELB and p65 proteins also have a carboxy-terminal non-homologous transactivation domain, which strongly activates transcription from NF-κB-binding sites in target genes (41, 43). The main activated form of NF-κB is a heterodimer of the p65 subunit associated with either a p50 or p52 subunit. The p50 and p52 proteins are generated by proteolytic cleavage of precursor p105 and p100 proteins, respectively. Converse to the main dimers containing p65, p50 and p52 homodimers lack the transactivation domain, but still bind to NF-κB consensus sites in DNA and have been documented as repressing the transcription of some genes linked with inflammation (44-46). This function has been ascribed to the resolution phase of inflammation, in which it is important to suppress potentially toxic inflammatory factors (such as those released by neutrophils and eosinophils) in order to minimize damage to surrounding tissues (45). The precursor protein to the p50 subunit, p105, is especially interesting because it serves as both a NF-κB subunit precursor and an IκB protein (43).

With respect to the transcriptional functionality of NF-κB, The activation and nuclear translocation of classical NF-kB dimers (mostly p50-p65) is associated with increased transcription of genes encoding chemokines, cytokines, adhesion molecules [intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial – leukocyte adhesion molecule 1 (ELAM)], enzymes that produce secondary inflammatory mediators and inhibitors of apoptosis (42, 44). These molecules are important components of the innate immune response to invading microorganisms and are required for migration of inflammatory and phagocytic cells such as macrophages and nuetrophils to tissues where NF-kB has been activated in response to infection or injury.

1.6 The peptidylarginine deiminase family

Peptidylarginine deiminase (PAD) enzymes catalyze the conversion of arginine residues to citrulline residues in proteins (Fig. 1.4). Citrulline is a nonstandard amino acid, as it is not incorporated into proteins during translation and published reports that citrulline residues occur in proteins date as far back as 1939 (47). To date, five family members of PAD have been identified in humans. These are: PAD1, PAD2, PAD3, PAD4 and PAD6 (PAD6 does not appear to be active; (48, 49)). All these enzymes rely strongly on the presence of calcium ions for activity (Fig. 1.4) and are unable to convert free L-arginine into L-citrulline (a process catalyzed by nitric oxide synthase).

Figure 1.4 Citrullination of peptidylarginine by PAD. Schematic representation of the citrullination (deimination) reaction catalyzed by the PAD enzyme resulting in the net loss of positive charge. Adapted from *Vossenaar et al. (49).*



Although not much is known about the specificity of PAD towards protein substrates, studies with peptides indicate that certain amino acids flanking the arginine residue influence its susceptibility to citrullination by PAD. For instance, Arg flanked by two Pro residues is not citrullinated at all (50). The most noticeable difference between the isotypes is their tissue-specific expression. For example, PAD1 is expressed in epidermis and uterus, and has been shown to play a role in keratinocyte differentiation (49). During terminal differentiation of keratinocytes, keratins (K1 and K10) and the keratin-associated protein filaggrin are citrullinated and it is believed that the flexibility of the keratin cytoskeleton is reduced upon citrullination, stimulating the cornification of the epidermis (49, 51). Similar to PAD1, PAD3 is found mainly associated with the epidermis and in particular is localized in the inner root sheath cells of hair follicles (49). PAD2 is the most widely expressed of the family and while it can mainly be found in skeletal muscle, it is also expressed in brain (in particular the hypothalamus), spleen, and secretory glands as well as in macrophages (49).

1.7 Biological function of PAD4

PAD4 is the only member of the family that is located within the nucleus as it uniquely carries a nuclear localization sequence (NLS). Although PAD4 can be found broadly in white blood cells, it is mainly expressed in neutrophils where it has been shown to be essential to chromatin remodeling processes during neutrophil extracellular trap (NET) formation (49, 52, 53). More broadly however, PAD4 has gained increasing attention because of the unique role it plays in regulating immune function, gene transcription, as well as in maintaining the pluripotency of stem cells.

Within the inflammatory process, PAD4 plays an important role regulating the function of neutrophils. In response to a stimulus (e.g. lipopolysaccharide or LPS), a subset of neutrophils will undergo NET formation via the genome-wide citrullination of histones H1 and H3 (54, 55). Histone citrullination in cells undergoing NETosis leads to large-scale chromatin decondensation, which initiates the expulsion of DNA from the cell to form net-like structures that can "trap" invading bacteria. However, while NETosis is a defense mechanism against invading organisms, its pro-inflammatory nature often links it to a variety of inflammatory disorders. For instance, in chronic autoimmune diseases, this process is aberrantly upregulated and likely plays an important role in the etiology of RA, systemic lupus erythematosus, ulcerative colitis, atherosclerosis, and even cancer (56-58). In cancer, recent data indicate that aberrant NET formation promotes vascular inflammation, leading to thrombosis (58).

Histone citrullination by PAD4 has also been demonstrated to regulate gene transcription. In particular, citrullination of histones H3 and H4 has been demonstrated to be associated with decreased expression of genes under the control of the estrogen and thyroid receptors (53, 59, 60). Moreover, it was shown that treatment of U2OS cells, an osteosarcoma cell line, with the pan-PAD inhibitor Cl-amidine leads to decreased PAD4 activity that was associated with the increased expression of p53 as well as several p53-dependent genes, including p21, PUMA, and OKL38 as well as induction of apoptosis (61, 62).

More recently, an intriguing role for PAD4 as a mediator of pluripotency in stem cells has been under investigation. Whereas it was previously thought that PAD4 was only expressed in mature neutrophils and other myeloid cells, it has now been demonstrated

that PAD4 is expressed in mouse embryonic stem cells (ES), induced pluripotent stem cells (iPS), as well as the LSK (Lineage$^-$, Sca-1$^+$, c-kit$^+$) hematopoietic stem cells (63, 64). Christophorou et al. (63) showed that increased levels of citrullinated H3 are correlative with higher levels of the pluripotency genes *Klf2*, *Tcl1*, *Tcfap2c*, and *Kit*. To further demonstrate that PAD4 activity is required to generate pluripotent stem cells, the authors treated mouse ES cells with the PAD inhibitor Cl-amidine and showed that decreased histone H3 citrullination was correlated with decreased expression of *Nanog*, *Tcl1*, and *Klf5*. Further verifying the importance of PAD4 in maintaining pluripotency, Cl-amidine treatment also increased the expression of several differentiation genes, including Epha1, Prickle1, and Wnt8a. These data are in agreement with findings published by Nakashima et al. (64) which showed that PAD4 regulates multipotency by controlling expression of c-kit. In total, these data suggest an exciting new avenue in the study of PAD4 and its function outside the immune response.

1.9 PAD4, citrullination, and rheumatoid arthritis

PAD4 has for some time been investigated for a role in RA pathogenesis (65). *PAD4* is located on chromosome 1p36, and has been shown to be essential for NET formation (66). However, a serological examination of RA patients quickly reveals why PAD4 is being widely targeted as a factor in disease pathogenesis. As discussed above, a family of autoantibodies directed against proteins containing citrulline – termed anti-citrullinated protein antibodies (ACPAs) – can be found in high titers and with high specificity in the synovia of RA patients (67, 68). Some of these include anti-filaggrin autoantibody (AFA), anti-keratin antibody (AKA), anti-pernicular factor (APF), and anti-cyclic

citrullinated peptide antibody (anti-CCP) (68, 69). Indeed, diagnostic tests for ACPA's show >97% specificity and sensitivity of at least 82% (19, 67), and have even been shown to be predictive of disease onset and severity (70). These characteristics of ACPAs have led to their wide use in diagnostic laboratories in testing for RA (19).

The association between citrullinated proteins and onset of RA has stimulated great interest in the PAD enzymes, particularly PAD4. Studies in recent years have established that PAD4 is present in high levels in RA synovia and is itself a target of autoantibodies (69, 71). Furthermore, a meta-analysis of RA patients in Japan, North America, and Europe has established a positive correlation between polymorphisms in the *PAD4* gene and RA incidence (72). Intriguingly, a recently published pan-PAD inhibitor, Cl-amidine, was used to treat mice with collagen-induced arthritis, inducing a ~50% reduction in disease activity (73).

1.10 Hypothesis

While much work has focused on PAD4 and its role in RA pathogenesis, little has been done to find out how PAD4 is transcriptionally regulated. The purpose of this study was therefore to determine how transcription of the *PAD4* gene is regulated in the human myeloid lineage. Since it has been demonstrated to be a central mediator of the immune innate and adaptive immune responses, and since PAD4 has been shown to be essential to the functionality of neutrophils in the context of acute inflammation, it was hypothesized that NF-κB is an activator of *PAD4* transcription during inflammation in RA pathogenesis.

Chapter 2: Materials and Methods

2.1 Cell culture

The human HL-60 cell line (American Type Culture Collection [ATCC], Manassas, VA) was cultured in media consisting of Iscove's Modified Dulbecco's Medium (IMDM) with 4.5g/L glucose, HEPES buffer, and L-glutamine (Lonza, Shawinigan, QC) supplemented with 10% charcoal stripped fetal bovine serum (Wisent, St. Bruno, QC), penicillin (100 U/mL)/ streptomycin (100 μg/mL)/ L-glutamine (.292 mg/mL) stock combination (Mediatech, Manassas, VA), and $5x10^{-5}$ M β-2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO). The murine WEHI-3B and human THP1 cell lines (ATCC) were cultured in media consisting of RPMI-1640 medium with L-glutamine (Lonza) supplemented with 10% fetal bovine serum (Wisent), penicillin (100 U/mL)/ streptomycin (100 μg/mL)/ L-glutamine (.292 mg/mL) combination (Mediatech), and $5x10^{-5}$ M β-2-mercaptoethanol (Sigma-Aldrich).

2.2 LPS stimulation

Cultured human HL-60 cells were harvested and plated at 500,000 cells/well and incubated at 37°C for 24 h. The cells were then stimulated with LPS (Invitrogen) at a concentration of 1 μg/mL then incubated for 48h. Cultured murine WEHI-3B cells were harvested and plated at 500,000 cells/well in a 6-well plate and incubated at 37°C for 24 h. The cells were then stimulated with LPS (Invitrogen) at a concentration of 400 ng/mL

and incubated for 72h. Total RNA was isolated and real time RT-qPCR was used to measure levels of *PAD4* mRNA.

Table 2.1: PCR and RT-qPCR primer sequences

Primer	Name	Sequence
1	Murine PADI4 Real-Time PCR 5' Primer	5'-TCTTTGTGGGTCACGTGGATGAGT-3'
2	Murine PADI4 Real-Time PCR 3' Primer	5'-AGCTCCTGGAACAGCTGATAGCAA-3'
3	Murine GAPDH Real-Time PCR 5' Primer	5'-GAACATCATCCCTGCATCCA-3'
4	Murine GAPDH Real-Time PCR 3' Primer	5'-CCAGTGAGCTTCCCGTTCA-3'
5	TNF-α Real-Time PCR 5' Primer	5'-ATGAGCGAAAGCATGATCCGC-3'
6	TNF-α Real-Time PCR 3' Primer	5'-GTCTGGGCCATAGAACTGATGAGA-3'
7	Murine PADI4 Conserved Region 5' Primer	5'-CTTTGATGTGGAGCCAAAGGAGACCC-3'
8	Murine PADI4 Conserved Region 3' Primer	5'-GCTTATCTCTCTAGCAGATCTCTTGC-3'
9	PADI4 Promoter 5' Primer	5'-TAAGTGTGCTTGGGCAAGATGTGC-3'
10	PADI4 Promoter 3' Primer with *Hind*III	5'-GAAGCTTCCTTGCTCGCTCGGTCAGC-3'
11	Murine PADI4 NFκB Mutation 5' Primer	5'-GGAACCAGCCCAGCCGCTTCCTGCTGCC-3'
12	Murine PADI4 NFκB Mutation 3' Primer	5'-GGCAGCAGGAAGCGGCTGGGCTGGTTCC-3'
13	Human PAD4 Promoter 5'	5'-ACTGTGGGCATGAGGACCAGGACC-3'
14	Human PAD4 Promoter 3'	5'-AAAGCTTCGTCGGGCTAGCTCGTCCC-3'
15	Human PAD4 NFκB Mutation 5'	5'-GATATAAAGGAACCAGCCCAGCCGCTTCCTACAGCCAGAGGGAC-3'
16	Human PAD4 NFκB Mutation 3'	5'-GTCCCTCTGGCTGTAGGAAGCGGCTGGGCTGGTTCCTTTATATC-3'
17	Human PAD4 Real-Time PCR 5'	5'-AGGAGGTGTACGCGTGCAGTATTT-3'
18	Human PAD4 Real-Time PCR 3'	5'-ATCCTGCATCCACTGGTCATCCAT-3'
19	Human GAPDH Real-Time PCR 5'	5'-CATGTTCGTCATGGGTGTGAACCA-3'
20	Human GAPDH Real-Time PCR 3'	5'-AGTGATGGCATGGACTGTGGTCAT-3'

2.3 TNF-α stimulation

Cultured cells were passaged into flasks containing 10 mL of pre-warmed medium two days before the treatment. The cells were passaged again into two flasks prior to treatment to a final concentration of 1 x 10^6 cells/mL. TNF-α (eBioscience, San Diego, CA) was then added for a final concentration of 1.7 x 10^{-4} μg/mL to one flask and both flasks were incubated for 1 h at 37°C. Total RNA was isolated and real time qPCR was used to measure the levels of *PAD4* mRNA.

2.4 Retinoic acid stimulation

Cultured cells were passaged into flasks containing 10 mL of pre-warmed medium two days before the treatment. The cells were passaged again into two flasks prior to treatment to a final concentration of 3 x 10^6 cells/mL. The cells were then stimulated with retinoic acid (Sigma-Aldrich) dissolved in 95% ethanol. 10 μL of 3 μg/μL retinoic acid were added to one flask and 10 μL of 95% ethanol were added to the second. Both flasks were incubated at 37°C for 72 h. Total RNA was isolated and real time Q-PCR was used to measure the levels of *PAD4* mRNA

2.5 Bioinformatic analysis

The sequence of the upstream region of the human *PAD4* gene was obtained from the Ensembl database. Potential transcription factor binding sites were determined using the position-weight-matrices present in MatInspector (Genomatix, Munich, Germany).

2.6 RNA analysis

Total RNA was isolated from cells using the RNA-Bee Isolation Kit (Tel-Test, Friendswood, TX). The iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) was used to synthesize cDNA from 1μg of the extracted RNA. Real time PCR was performed using the iQ SYBR Green Supermix Kit (Bio-Rad) and analyses were done using a Rotor-Gene 6000 (Corbett Life Science, San Fransisco, CA). Murine *PAD4* transcript levels were measured and normalized in real time PCR analyses to murine *GAPDH*. Human *PAD4* transcript levels were measured and normalized in q-PCR analyses to human *GAPDH*. Gene expression analysis was done using REST 2009 Software via the comparative threshold cycle method (Technical University of Munich, Munich, Germany).

2.7 Plasmid construction

In total, two sets of three reporter plasmids were constructed: pGL3+NF-κB FWD, pGL3-Basic+NF-κB REV, and pGL3+NF-κB MUT. The first two constructs contained the predicted *PAD4* promoter in the forward and reverse directions, respectively. To obtain these first two constructs, the conserved region of the *PAD4* promoter was PCR amplified from C57BL/6 mouse DNA or from human HL-60 DNA. The PCR product was cloned using the StrataClone PCR Cloning Kit and cloned (Agilent Technologies, La Jolla, CA). Clones containing the target insert in the forward and reverse orientations were isolated to obtain the pGL3+NF-κB FWD and pGL3+NF-κB REV constructs, respectively. To obtain the pGL3+NF-κB MUT construct, the pGL3+NF-κB FWD construct was mutated using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies). Before use, all three plasmid constructs were transformed into DH5α cells

and transfection-quality DNA was made using the QIAGEN Plasmid MAXI Kit (QIAGEN).

2.8 Transient transfection analysis

For each transfection, 5 x 10^6 cells were transfected with 10 μg of luciferase reporter plasmid and 0.5 μg of pRL-TK (Promega, Madison, WI). After a 10 minute incubation, the cells were electroporated at 220 V and 950 μF using a GenePulser II with Capacitance Extender Plus (Bio-Rad). The cells were incubated for 24 h at 37°C following another 10 minute incubation at room temperature. Luciferase assays were performed using the Dual-Luciferase Reporter Assay System (Promega). The production of light was measured with a Lumat LB 9507 luminometer (Berthold Technologies, Oak Ridge, TN).

2.9 Chromatin ImmunoPrecipitation (ChIP)

HL-60 cells treated (or not) with TNF-α at 1.7 x 10^{-4} μg/mL were treated with 1% formaldehyde for 10 min at room temperature. Cross-linking was terminated with 125 mM glycine. The cells were lysed in lysis buffer (50 mM Tris-HCl [pH 8.1], 10 mM EDTA, 1% SDS) containing Halt™ protease inhibitor mixture (Thermo Scientific). Chromatin solutions were sonicated to yield DNA fragments in the range 300–700 bp using a Bioruptor 300 waterbath sonicator (Diagenode, Sparta, NJ). Sonicated chromatin was incubated with rabbit polyclonal anti-human p50 or anti-human p65 antibodies (Abcam, Cambridge, MA) conjugated to protein G DynaBeads (Invitrogen, Burlington, Ontario, Canada) overnight at 4°C. As a control, sonicated chromatin was incubated with rabbit polyclonal IgG (Abcam) conjugated to protein G DynaBeads. Magnetic bead-

bound complexes were enriched using a Dynal magnetic particle concentrator (Invitrogen). Bound beads were washed once with low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 150 mM NaCl), once with high-salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 500 mM NaCl), once with LiCl buffer (0.25 M LiCl, 1% Nonidet P-40, 1% Na-deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.0]), and twice with Tris-EDTA buffer at pH 8. Immunocomplexes were eluted with elution buffer (1% SDS, 0.1 M $NaHCO_3$). Cross-links were reversed in a final volume of 300 µl, containing 200 mM NaCl, overnight at 65°C. DNA was purified using a Wizard SV Gel and PCR purification Kit (Promega). Enrichment was measured using qPCR of DNA immunoprecipitated with anti-FLAG or mouse IgG, using primers indicated in Table 2.1. Fold enrichment was calculated using the comparative threshold cycle method.

2.10 Statistical Analysis

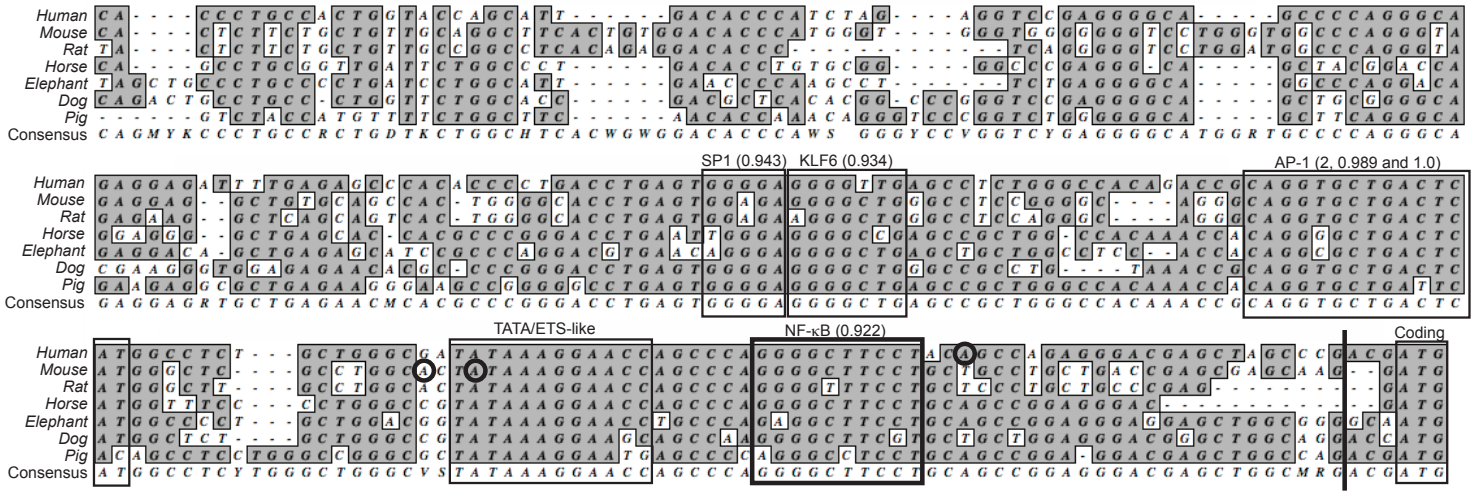
All data are reported as mean ± SD of the mean. Statistical significance of luciferase assay and ChIP results was determined using students *t* test. Statistical analysis of RT-qPCR results was done using REST 2009 software (Technical University of Munich).

Chapter 3: Results

3.1 Identification of the PAD4 promoter

Previous work in our laboratory was done to determine transcription start sites of the murine *PAD4* gene (74). Analyses done using RNA obtained from murine WEHI-3B cells revealed possible transcription starts sites at -52 and -55 relative to the translation start site (74). Furthermore, previous work done in our lab showed evidence that the transcription start sites identified in the *PAD4* gene were downstream of an active promoter (74). To test this region in human cells, the putative *PAD4* promoter was analyzed for conserved regions by comparing the DNA sequences of the *PAD4* locus from humans and six other species (Fig. 3.1). Regions that were highly conserved between species suggested that those regions represent functionally important regulatory elements (75, 76). A ClustalW alignment revealed several regions within 200 bp of the translation site that shared a high degree of sequence identity (Fig. 3.1), suggesting that this region may function as the *PAD4* promoter. Using the weight matrices available in MatInspector (Genomatix) which identify potential transcription factor binding sites based on known DNA binding patterns as well as biological function, four potential transcription factor binding sites in the *PAD4* promoter were located: Kruppel like transcription factor, vertebrate TATA binding protein, NF-κB and ETS1 (Fig. 3.1).

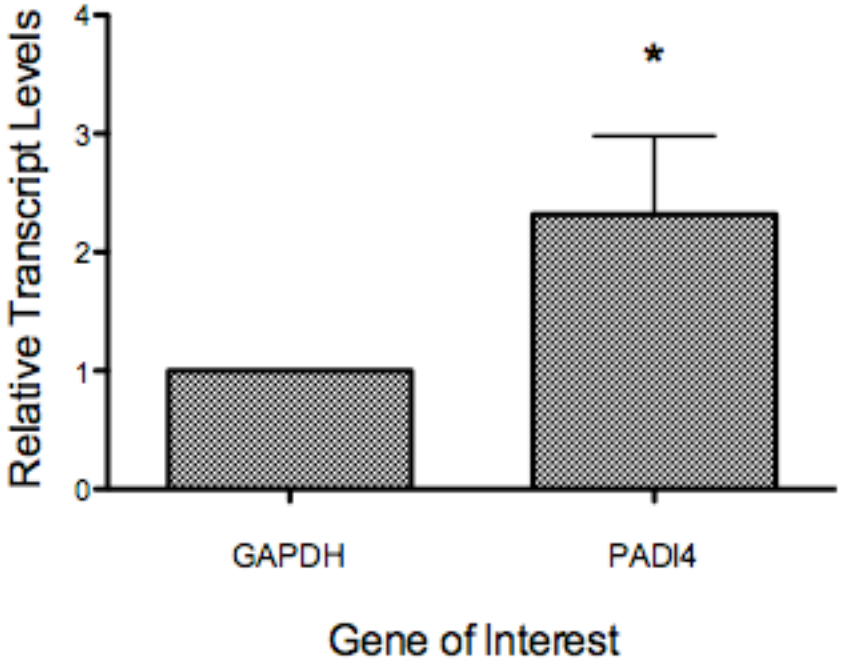
Figure 3.1 Potential transcription factor binding sites identified within the conserved region of the *PAD4* promoter. A region approximately 400 bp upstream of the *PAD4* ATG start codon contains two major conserved sequences and 150 conserved nucleotides. A ClustalW alignment of the *PAD4* promoter sequences from *H. sapiens* (human), *M. musculus* (mouse), *R. norvegicus* (rat), *E. ferus* (horse), *L. africana* (elephant), *C. familiaris* (dog), and *S. scrofa* (pig) is shown with the conserved sequences boxed. Bioinformatic analysis predicted binding sites for the transcription factors NF-κB, TATA-ETS-like, AP-1, KLF-6 as well as SP1.



3.2 PAD4 is inducible in human HL-60 cells

Before performing experiments based on *PAD4* expression, it was necessary to identify a human cell line that could express *PAD4*. It was previously reported that HL-60 cells can be induced with retinoic acid to express PAD4 (77), and we elected to repeat this. To that end, we stimulated cultured HL-60 cells with 3 μg/μL retinoic acid then extracted total RNA. Afterwards, cDNA was synthesized and real time qPCR was performed to analyze differences in *PAD4* expression between retinoic acid-treated cells and control cells. This experiment was repeated three times and there was an average 2.3 fold increase of *PAD4* transcripts in HL-60 cells treated with retinoic acid relative to untreated cells (Fig. 3.2). These results demonstrate that HL-60 express *PAD4* that is further inducible with retinoic acid.

Figure 3.2 *PAD4* is inducible in HL-60 cells treated with retinoic acid. HL-60 cells in two culture flasks were treated with retinoic acid or an equal volume of 95% ethanol. Shown are the mean transcript levels ± S.D. from three independent triplicate experiments. There was a 2.3-fold increase in PAD4 transcript levels in treated cells. *Asterisk* indicates significant difference ($p < 0.05$) as measured by a paired one-tailed students t-test.



3.3 The PAD4 promoter contains a potential NF-κB binding site

NF-κB is a multifunctional transcription factor that participates in the regulation of the immune response and inflammation that has also been implicated in the development of autoimmune diseases. Since our bioinformatics analysis predicted the presence of a NF-κB binding site in the *PAD4* promoter, we elected to test whether NF-κB could be important in the regulation of *PAD4* – itself an important mediator of innate immunity (40, 42, 52, 78).

NF-κB family members have been shown to interact with a DNA core motif containing the consensus RGGRNNHHYYB (79-81). To test the activity of the predicted NF-κB site in the *PAD4* promoter dual luciferase assays were carried out with various constructs. In brief, three reporter vectors were constructed (See 2.7-2.8). The first construct encoded the predicted NF-κB site in the forward orientation (FWD) directly upstream of the firefly *luciferace* gene, the second contained the predicted NF-κB site in the reverse orientation (REV) directly upstream of the firefly *luciferace* gene, and for the third construct site directed mutagenesis was used to convert the second and third guanosine residues into cytosines – a change expected to prevent NF- κB binding (Fig. 3.3B). Constructs containing the *PAD4* wildtype or mutated human NF-κB site were transfected into human and murine cells. The human *PAD4* promoter with a mutated NF-κB site displayed significantly lower biological activity ($P < 0.05$) than the wild-type when transfected into murine WEHI-3B cells (Fig. 3.4). Interestingly however, reporter vectors containing the human *PAD4* promoter with mutated NF-κB binding site transfected into

human HL-60 and THP-1 cells showed significantly greater activity than the wild-type (Fig. 3.5A and B; $P < 0.05$). To test whether mutating the murine *PAD4* promoter would have the same effect, constructs containing the murine *PAD4* promoter were transfected into human HL-60 cells (Fig. 3.6). Mutation of the potential NF-κB binding site in the murine promoter did not cause a significant change in luciferase activity relative to wild-type (Fig. 3.6).

To further test the importance of the NF-κB binding site in the the human promoter relative to other upstream TFBS, we elected to conduct a deletion analysis in which promoter vectors containing varying lengths of the *PAD4* promoter were transfected into human HL-60 cells. HL-60 cells transfected with constructs containing only the NF-κB binding site as well as the transcription start site (TSS) showed a modest but significant increase in luciferase activity relative to cells transfected with constructs containing only the TSS (Fig. 3.7). These results suggest that the NF-κB site in the human *PAD4* promoter is functional and may act as an activating or a repressive element in murine and human cells, respectively.

Figure 3.3 Overview of the pGL3-basic luciferase reporter plasmids. (A) The *PAD4* promoter region inserted into the vector contains the potential NF-κB binding site. Three constructs were made to test the site. One containing the *PAD4* promoter region in the forward orientation, another in the reverse, and the third with the *PAD4* promoter region in the forward orientation with a mutation in the putative NF-κB binding site. (B) Alignment depicting the mutation of the NF-κB binding site, with the second and third guanosines mutated to cytosines.

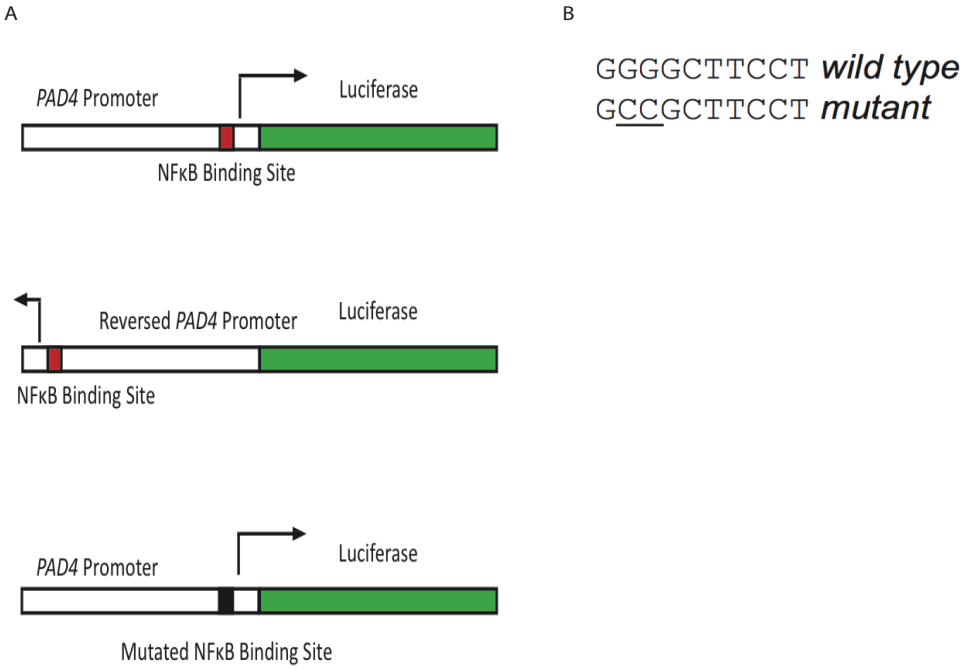


Figure 3.4 A predicted NF-κB site regulates *PAD4* transcription in murine WEHI-3B cells. Three constructs were made to test the site; one containing the murine *PAD4* promoter region in the forward orientation (FWD), another in the reverse (REV), and the third with the *PAD4* promoter region in the forward orientation with a mutation in the putative NF-κB binding site (MUT). The positive control (+) was a construct containing the viral SV40 promoter upstream of the *luciferase* gene. The negative control (-) was a promoter-less construct. Mutation of the human NF-κB results in decreased human promoter activity in murine WEHI-3B cells. Results shown are from three biological experiments performed in technical duplicate. Paired, one-tailed student's *t* test was used to compare luciferase activity from the FWD construct to that of the MUT construct.

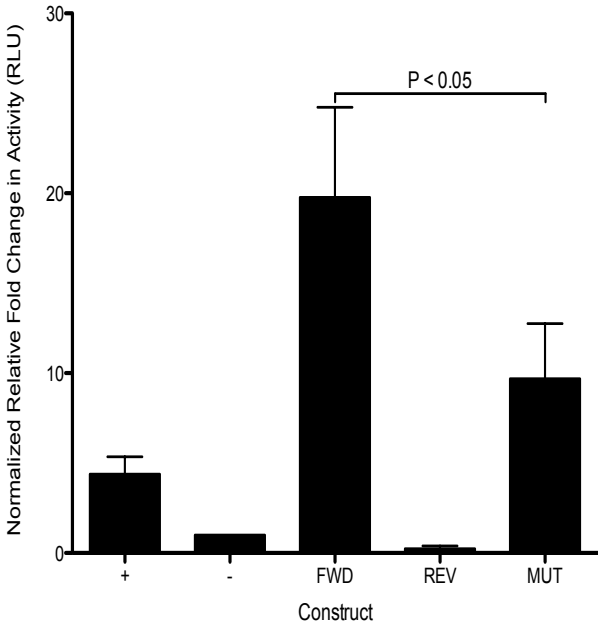
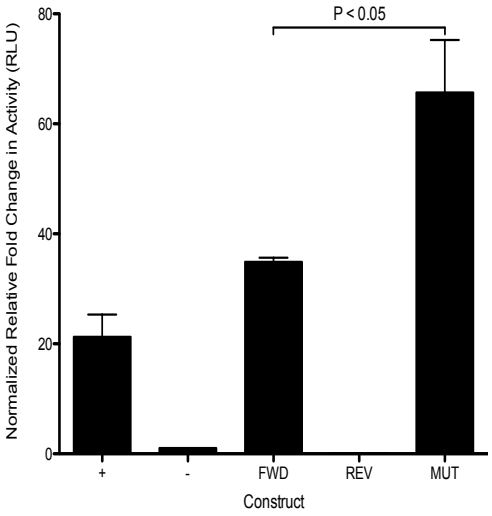


Figure 3.5 A predicted NF-κB site regulates *PAD4* transcription in human myeloid cells. Three constructs were made to test the site; one containing the human *PAD4* promoter region in the forward orientation (FWD), another in the reverse (REV), and the third with the *PAD4* promoter region in the forward orientation with a mutation in the putative NF-κB binding site (MUT). The positive control (+) was a construct containing the viral SV40 promoter upstream of the *luciferase* gene. The negative control (-) was a promoter-less construct (A) Mutation of the NF-κB binding site in the human *PAD4* promoter significantly increases promoter activity in human HL-60 cells. Shown is the relative fold difference in biological activity of each reporter normalized to the pGL3-basic promoter-less plasmid. Results are an average of three biological experiments performed in duplicate. (B) Mutation of the NF-κB binding site significantly increased human *PAD4* promoter activity in human THP-1 cells. Shown are the results of three biological replicates performed in duplicate. Paired, one-tailed student's *t* test was used to compare luciferase activity from the FWD construct to that of the MUT construct.

A


P < 0.05
Normalized Relative Fold Change in Activity (RLU)
80
60
40
20
0
+
-
FWD
REV
MUT
Construct

B

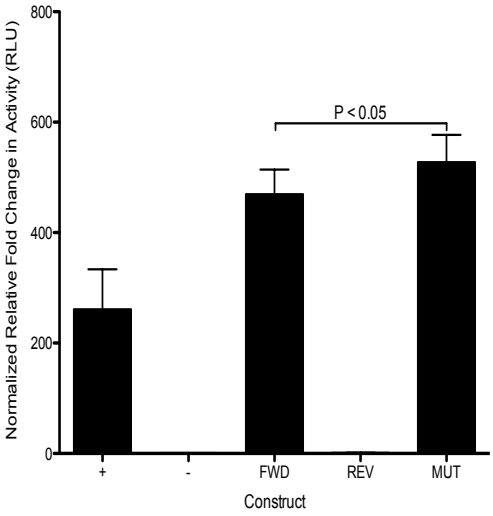

P < 0.05
Normalized Relative Fold Change in Activity (RLU)
800
600
400
200
0
+
-
FWD
REV
MUT
Construct

Figure 3.6 A predicted NF-κB site in the murine *PAD4* promoter does not affect transcription in human cells. Three constructs were made to test the site; one containing the murine *PAD4* promoter region in the forward orientation (FWD), another in the reverse (REV), and the third with the *PAD4* promoter region in the forward orientation with a mutation in the putative NF-κB binding site (MUT). The positive control (+) was a construct containing the viral SV40 promoter upstream of the *luciferase* gene. The negative control (-) was a promoter-less construct Mutation of the murine NF-κB binding site has no effect on promoter activity in human HL-60 cells. Shown is the relative fold difference in biological activity of each reporter normalized to the pGL3-basic promoter-less plasmid. A paired, one-tailed student's *t* test was used to compare luciferase activity from the FWD construct to that of the MUT construct and there was not a significant difference between the two. Results are an average of three biological replicates performed in duplicate.

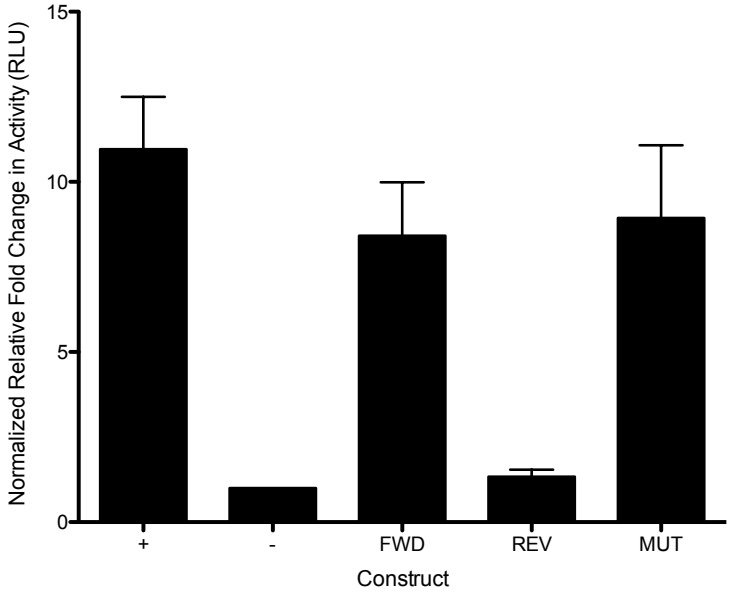
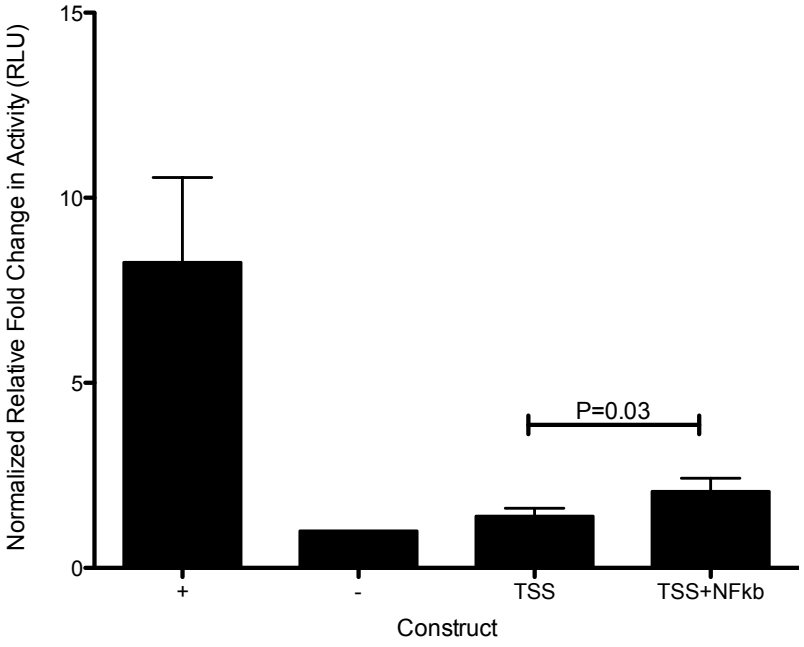


Figure 3.7 The predicted NF-κB site in the human *PAD4* promoter increases luciferase activity. Inclusion of the NF-κB binding site into the luciferase construct containing the PAD4 TSS (TSS+NF-κB) causes a significant increase in luciferase activity. Shown is the relative fold difference in biological activity of each reporter normalized to the pGL3-basic promoter-less plasmid. Results are an average of three biological replicates performed in duplicate. A paired, one-tailed student's *t* test was used to compare luciferase activity from the TSS construct to that of the TSS+NFkB construct.



3.4 Inflammatory stimuli regulate PAD4 expression

Since the luciferase results suggested the presence of a functional NF-κB binding site in the human and murine *PAD4* promoters (Fig. 3.4-3.7), we elected to test the effect of NF-κB activation on endogenous *PAD4* transcript levels. TNF-α or LPS – known activators of the NF-κB pathway – were used to stimulate human HL-60 and WEHI-3B cells (82, 83). Since both TNF-α and IL8 are markers of NF-κB activation, they were used as positive controls in these experiments (84). WEHI-3B cells treated with TNF-α displayed a 5.9-fold increase in *TNF-α* transcript levels and a 3-fold increase in *PAD4* transcript levels (Fig. 3.8). Human HL-60 cells treated with LPS did not show a significant change in *PAD4* or *IL8* transcript levels (Fig. 3.9A). Gene expression analysis was done using REST 2009 Software via the comparative threshold cycle method (Technical University of Munich,). This result suggests that human HL-60 cells grown in culture are not responsive to LPS treatment. Finally, HL-60 cells treated with TNF-α showed a significant 2.7-fold increase in *IL8* transcript levels — along with a significant 1.3 fold decrease in *PAD4* transcript levels (Fig. 3.9B; $P < 0.05$). These results were consistent with our previous findings and suggest that NF-κB may function as a transcriptional repressor of *PAD4* in humans while acting as an activator in murine cells.

Figure 3.8 Induction of NF-κB upregulates *PAD4* in mouse WEHI-3B cells. Fold induction of *GAPDH, PAD4* and *TNF-α* transcript levels in murine WEHI-3B cells treated with 1.7 x 10^{-4} μg/mL TNF-α. *TNF-α* transcript levels increased 5.88 fold while *PAD4* transcript levels increased 3.2 fold in treated cells. Gene expression analysis was done using REST 2009 Software via the comparative threshold cycle method (Technical University of Munich, Munich, Germany).

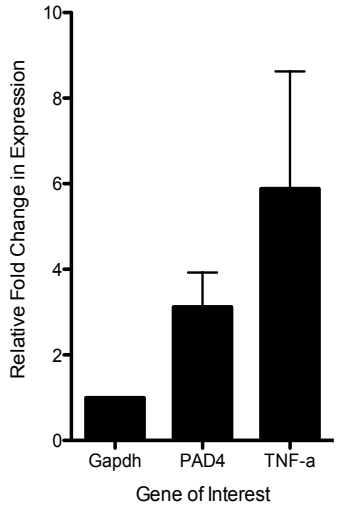
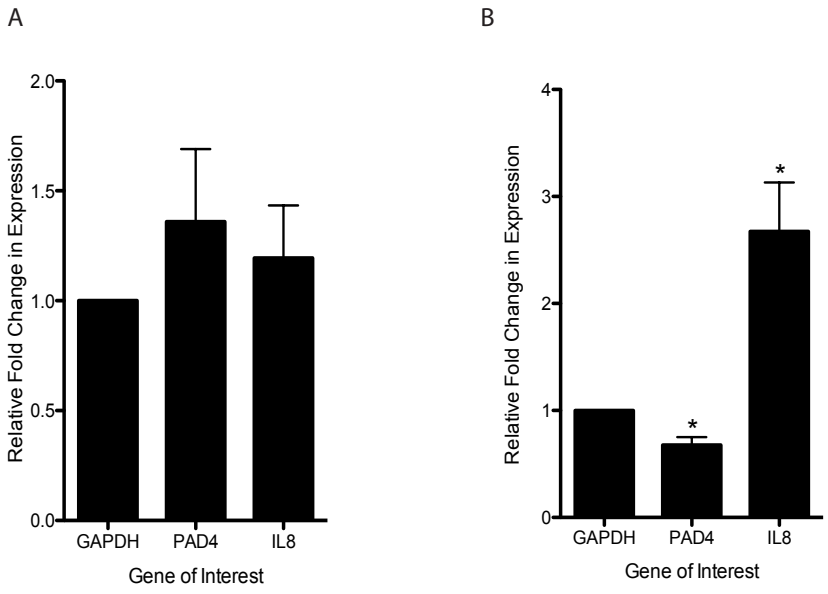


Figure 3.9 Induction of NF-κB downregulates *PAD4* in human myeloid cells (A) Fold induction of *GAPDH*, *PAD4* and *TNF-α* transcript levels in human HL-60 cells treated with LPS. Cells were treated with 400 ng/ml LPS and incubated for 72 hours. There was not a significant change in *PAD4* or *IL8* transcript levels. (B) Fold induction of *GAPDH, PAD4* and *TNF-α* transcript levels in human HL-60 cells treated or not with TNF-α. Shown are the mean transcript levels ± S.D. from three experiments performed in triplicate. *IL8* transcript levels increased 2.67 fold whereas *PAD4* transcript levels were reduced 0.71 fold in treated cells. * $p<0.05$



3.5 P50 binds to the PAD4 promoter

Finally, we investigated the potential role of NF-κB family members p50 and p65 in binding directly to the *PAD4* promoter. P50-p65 heterodimers form the "classical" NF-κB transcriptional activator responsible for most canonical NF-κB-mediated expression of inflammatory response genes (45, 85, 86). Conversely, p50 homodimers have been demonstrated to repress several different inflammatory genes controlled by NF-κB in a manner consistent with the resolution phase of inflammation (45, 46). Both p65-p50 heterodimers and p50 homodimers can be activated via the canonical pathway involving activation of the TNF receptor (TNFR) by TNF- α (44, 45). Therefore, we hypothesized that inducing the NF-κB pathway via TNFR stimulation would lead to production of the active forms of both p65-p50 heterodimers (the activating heterodimer) as well as repressive p50 homodimers. To determine if these NF-κB subunits bind the *PAD4* promoter, we performed chromatin immunoprecipitation (ChIP) analysis. Chromatin prepared from HL-60 cells treated or not with TNF-α was immunoprecipitated with either rabbit polyclonal anti-human p50 or anti-human p65 antibodies and rabbit polyclonal IgG to control for non-specific binding. qPCR was used to determine the relative amount of immunoprecipitated DNA from the *PAD4* promoter region, the *IL8* promoter region with which p50 and p65 is known to interact with as a positive control, and the negative control *C4ORF11*, a gene expressed at low levels and with no known binding to NF-κB (Fig. 3.11; (84, 87)). ChIP analysis confirmed that p65 was highly enriched at the *IL8* promoter, but not significantly enriched at the *PAD4* promoter upon TNF-α stimulation (Fig. 3.10A; B). However, p50 enrichment at the *PAD4* promoter was significantly greater in treated cells than untreated (Fig. 3.11A, B; p=0.017). Taken together, these

data are consistent with the possibility that p50 homodimers of NF-κB directly interact with the human *PAD4* promoter and act to functionally repress *PAD4* during inflammation.

Figure 3.10 The p65 subunit of NF-κB does not interact directly with the human *PAD4* promoter upon TNF-α stimulation. Human HL-60 cells were treated or not with TNF-α for one hour before chromatin immunoprecipitation (ChIP) analysis to examine p65 binding to the NF-κB site in the *PAD4* promoter. Real time quantitative PCR (RT-qPCR) was used to quantify fragments of DNA immunoprecipitated with rabbit polyclonal anti-human p65 and rabbit polyclonal IgG (control). (A) Representative figure from three anti-p65 ChIP experiments. Results are expressed as levels of DNA immunoprecipitated with anti-p65 IgG i.e. as a percentage of input DNA. Levels of p65 enrichment at the *IL8* promoter (positive control) were higher in treated cells than untreated. There was no change in levels of p65 enrichment at the *C4ORF11* promoter in treated and untreated cells. + and - refer to TNF-α treatment. (B) Results of all p65 (n=3) ChIP experiments. Data is expressed as fold enrichment of DNA immunoprecipitated with anti-p65 IgG normalized to DNA immunoprecipitated with IgG. There was not a siginificant change in p65 enrichment at the PAD4 promoter in treated cells relative to untreated. Significance was measured using student's *t* test.

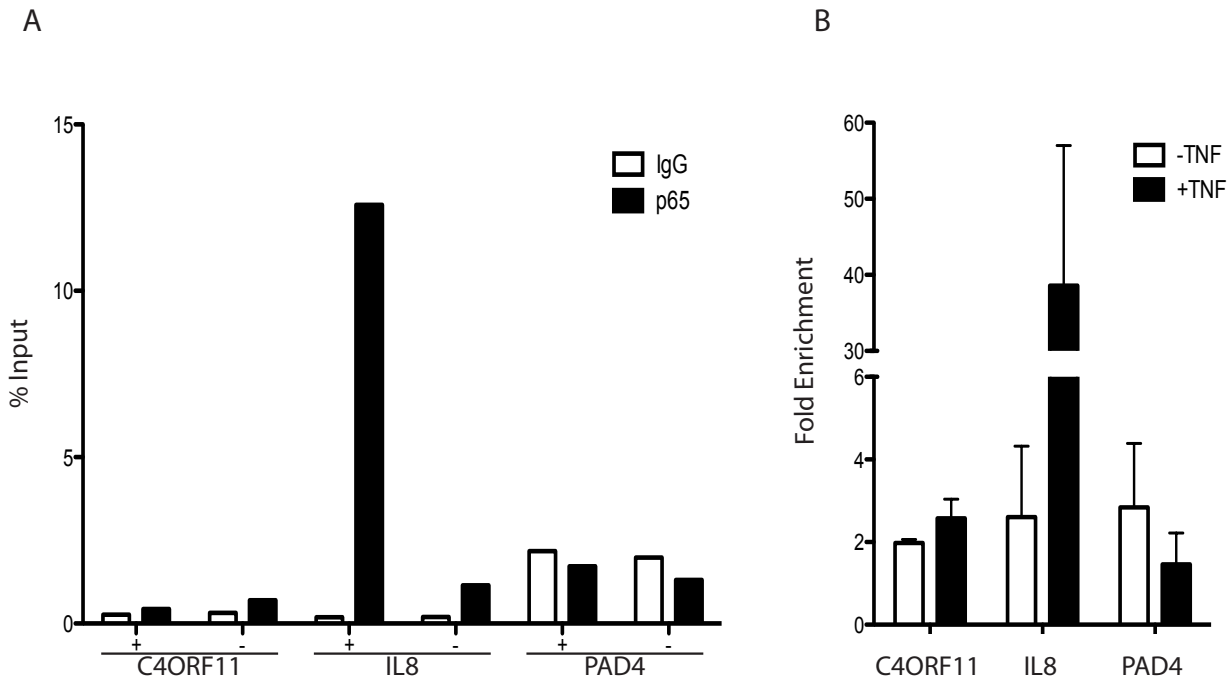
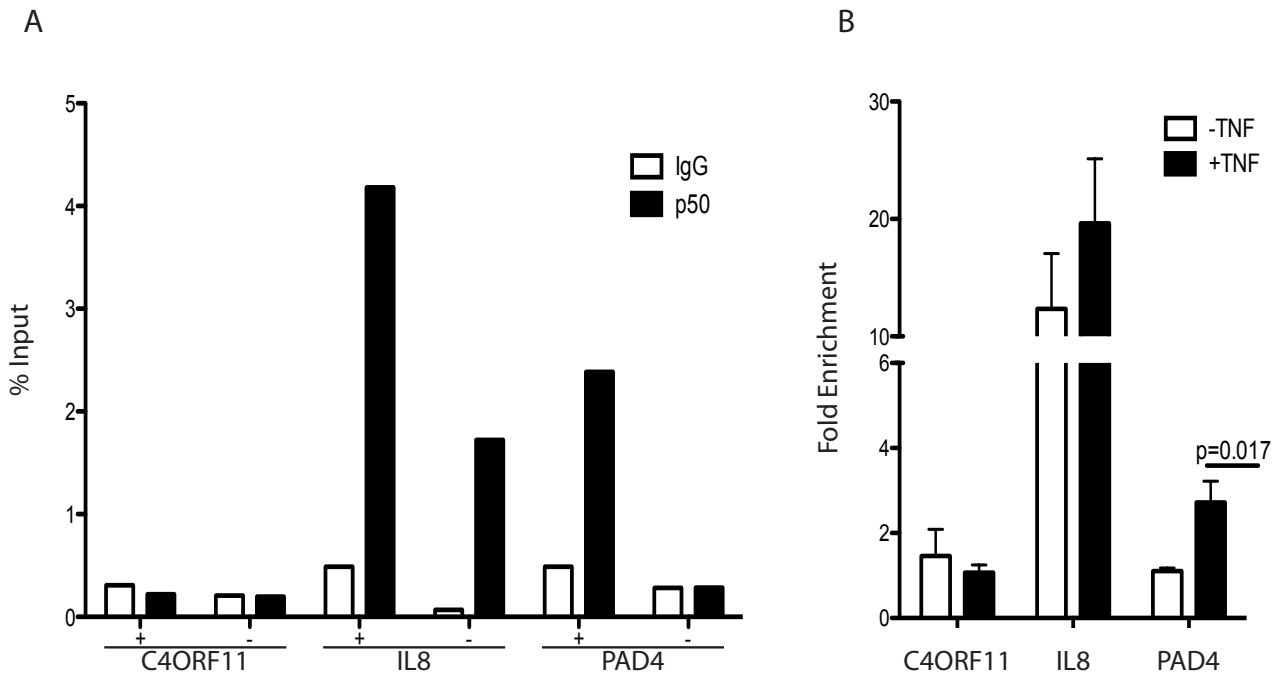

A
B
15
10
5
0
% Input
IgG
p65
+
-
C4ORF11
IL8
PAD4
60
50
40
30
6
4
2
0
Fold Enrichment
-TNF
+TNF
C4ORF11
IL8
PAD4

Figure 3.11 The p50 subunit of NF-κB interacts directly with the human *PAD4* promoter upon TNF-α stimulation. Human HL-60 cells were treated or not with TNF-α for one hour before chromatin immunoprecipitation (ChIP) analysis to examine p50 binding to the NF-κB site in the *PAD4* promoter. Real time quantitative PCR (RT-qPCR) was used to quantify fragments of DNA immunoprecipitated with rabbit polyclonal anti-human p50 and rabbit polyclonal IgG (control). (A) Representative figure from seven anti-p50 ChIP experiments. Results are expressed as levels of DNA immunoprecipitated with anti-p50 IgG i.e. as a percentage of input DNA. Levels of p50 enrichment at the *IL8* promoter (pos. control) were higher in treated cells than untreated. There was no change in levels of p50 enrichment at the *C4ORF11* promoter in treated and untreated cells. + and - refer to TNF-α treatment. (B) Results of all p50 (n=7) ChIP experiments. Data is expressed as fold enrichment of DNA immunoprecipitated with anti-p50 IgG normalized to DNA immunoprecipitated with IgG. There was a siginificant increase in p50 enrichment at the PAD4 promoter in treated cells relative to untreated. Significance was measured using student's *t* test.


A
% Input
5
4
3
2
1
0
IgG
p50
+
-
+
-
+
-
C4ORF11
IL8
PAD4
B
Fold Enrichment
30
20
10
6
4
2
0
-TNF
+TNF
p=0.017
C4ORF11
IL8
PAD4

Chapter 4: Discussion

4.1 Overview

Rheumatoid arthritis is an autoimmune disease whose etiology remains elusive because of the myriad environmental and genetic factors that have potential roles in its pathogenesis (22, 24). One of the potential genetic factors being considered for a role in RA is the gene encoding PAD4, an enzyme that catalyzes the post-translational modification of citrullination. PAD4 is particularly interesting because it is found in the rheumatoid synovial membrane, synovial fluid cells (for example, neutrophils), and extracellular synovial fluid (19, 88). Meta-analyses of Japanese, European and North American populations have established a correlation between polymorphisms in the *PAD4* gene and RA incidence (72). A family of autoantibodies directed against proteins containing citrulline – termed anti-citrullinated protein antibodies (ACPAs) – can be found in high titers and with high specificity in the synovia of RA patients (67, 68). Finally, PAD4 has itself been found to be a target of antibodies in RA (71). The goal of this study was to determine how transcription of PAD4 gene is directed in the human myeloid lineage, and whether NFκB is an activator of PAD4 transcription. Over the course of my thesis, I characterized the human *PAD4* promoter and demonstrated its activity. In turn, I analyzed both the human and murine *PAD4* promoters for possible transcription factor binding sites, located a possible NF-κB site, and determined that the site was active. I hypothesized that this NF-κB binding site could play a role in regulating *PAD4* transcription. I tested this idea by using inflammatory stimuli to activate the NF-κB pathway in cultured cells, showing that inflammatory stimuli caused changes

in *PAD4* transcription. Finally, I demonstrated that the NF-κB subunit p50 directly interacts with the *PAD4* promoter independently of p65, suggesting that p50 homodimers act to suppress *PAD4* transcription in the context of human inflammation.

4.2 NF-κB may be important for transcriptional regulation of PAD4

NF-κB is a particularly interesting candidate vis-a-vis rheumatoid arthritis because of the essential role it plays in human immunity. Indeed, several inflammatory diseases are characterized partly by activation of NF-κB including asthma, inflammatory bowel syndrome, atherosclerosis, and rheumatoid arthritis (89). Together, the NF-κB family of proteins consists of five members: NF-κB1 (p50), NF-κB2 (p52), RelA (p65), RelB and cRel; each member may form a homodimer or a heterodimer with any other member, and these dimers in turn have differential abilities to regulate gene transcription (44). In unstimulated cells, NF-κB is sequestered in the cytoplasm by IκB proteins. In turn, the IκB protein family consists of three functional groups: the typical IκB proteins IκBα, IκBβ and IκBε, all three of which are present in the cytoplasm of unstimulated cells and are degraded as a consequence of stimulation; the precursor proteins p100 and p105, which can be processed to form the NF-κB family members p52 and p50, respectively; and finally the atypical IκB proteins including IκBζ, BCL-3 (B-cell lymphoma 3) and IκBN, all of which are not normally expressed in the cytoplasm, but are expressed in response to activation and act to modulate transcription thereafter.

In response to activation, IκBα undergoes ubiquitin-mediated proteasomal degradation that results in the release of the bound NF-κB dimers. The cytoplasmic NF-κB (most

often in the form of p65-p50 heterodimers) then translocates to the nucleus and drives gene expression. NF-κB dimers are involved in both the activation and repression of a host of different genes involved in the immune response. The regulatory mechanisms regulating the activating or repressive function of NF-κB are complex and often involve substantial crosstalk between different pathways in context and cell-specific ways (90, 91). For instance, p50 and p52 homodimers have for some time been known to repress the transcription of some genes linked with inflammation (44, 45). However, this repression is often augmented by the presence of BCL-3, an atypical IκB family member, which is present in the nucleus and can either stabilize the repressive function of p50 homodimers already bound to DNA or else it may bind the homodimer and confer transcriptional activation ability (92, 93). Furthermore, the inflammatory context in which the cell is present has been advanced as another factor in the nature of NF-κB function. For example, the repressive function of p50 and p52 homodimers has been ascribed to the resolution phase of inflammation, in which it is important to suppress potentially toxic inflammatory factors (such as those released by neutrophils and eosinophils) in order to minimize damage to surrounding tissues (45). Thus the activation or repressive function of NF-κB dimers can be ascribed not only to the particular combination of subunits, but also to the stage of inflammation the cell is in.

Another factor in the function NF-κB is the physical sequence to which the transcription factor binds. While it was previously hypothesized that all NF-κB dimers bind a single consensus sequence, more recent data has emerged demonstrating that different dimers have slightly different sequence preferences (79, 94, 95). In particular, the GGAA motif has been shown to be most highly associated with p65 binding whereas p50 and p52

homodimers have less affinity for that motif and a higher preference for the motif GGGRY (R= purine, Y= pyrimidine) (79) – the motif present in the NF-κB site in the *PAD4* promoter (Fig. 3.1). This observation is consistent with the results of our experiments that suggest that p50 preferentially binds the *PAD4* promoter compared with p65 and that it may do so in the form of the repressive p50 homodimer (Fig. 3.9-3.11). The importance of the DNA sequence to which NF-κB is made even more clear by results in murine cells which indicate that, converse to the situation in the human cell lines, NF-κB may activate *PAD4* transcription in murine WEHI-3B cells (Figs. 3.4 and 3.8). This discrepancy may be explained by an insertion in the murine promoter that is not present in the human promoter approximately 300 bp upstream of the TSS (Fig. 3.1). In the murine promoter, this insertion is part of a larger sequence that includes the nucleotides GGGGGGTCCTG which falls within the consensus binding site for NF-κB (79-81, 96). Since this insertion is not present in the human promoter, no such binding site exists for the human *PAD4* promoter. Thus it may be NF-κB plays a differential role in regulating the transcription of *PAD4* in murine and human myeloid cells.

The results of our study are consistent with the previously established role of PAD4 in the inflammatory response. We demonstrated that NF-κB (in the form of p50) binds the *PAD4* promoter in human neutrophils, and correlates with repression of gene transcription thus providing a potential link between inflammation and the regulation of the *PAD4* gene.

4.3 The potential role of PAD4 during chronic inflammation in rheumatoid arthritis

The PAD4 enzyme has emerged as a potentially key participant in the pathogenesis of rheumatoid arthritis because of its importance to neutrophil function in innate immunity as well as its putative role in other autoimmune diseases. The *PAD4* gene is located on chromosome 1p36, and genome wide associate studies as well as a subsequent meta-analysis of these studies have demonstrated an association between polymorphisms in the *PAD4* gene and RA (72, 97-99). Interestingly, a genome wide screen of multiplex RA families found that genetic regions contributing to RA risk overlapped with those contributing to other autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus – a finding reflective of PAD4's implicated role in those diseases because of its role in inflammation (52, 100).

The PAD4 enzyme is located in the nuclei of neutrophils and has been shown to mediate citrullination of histones, a process that induces chromatin decondensation and, ultimately, NET formation (53, 101). NETosis is an effector mechanism of neutrophils unique from phagocytosis or apoptosis and involves the ejection of nuclear DNA decorated with a variety of modified nuclear and granular proteins that in turn ensnare extracellular bacteria and induce their death (10). NETs act as a mesh that traps microorganisms and facilitates their interaction with neutrophil-derived effector molecules, limiting the spread of pathogens (10). A number of studies have implicated NETs in the etiology of autoimmune conditions such as preeclampsia, Felty syndrome, SLE, multiple sclerosis, as well as RA (12, 13, 56, 102-105). In the context of RA, it has

been hypothesized that because of their ejection of intracellular (and citrullinated) contents to the extracellular space, NETs contribute to the generation of anti-citrullinated protein antibodies (ACPA), and may themselves also be targets of autoantibodies (56, 106). Furthermore, neutrophils isolated from RA patients have been shown to display a heightened propensity towards spontaneous and LPS-induced NETosis, which was in part mediated by TNF and IL-17 and could be inhibited by blocking NADPH oxidase or PAD4 (56, 106). Indeed, chemical inhibition of PAD4 significantly reduces histone citrullination and NET formation, and neutrophils of mice lacking PAD4 are unable to decondense chromatin and produce NETs leading to increased susceptibility to bacterial infections (66, 101, 107). Intriguingly, inhibition of NF-κB has recently been documented to reduce NET formation, compatible with a role for NF-κB in the regulation of *PAD4* expression (108).

4.4 Future directions

Future directions of this work include further elucidation of the role NF-κB has in regulating PAD4 expression over the course of inflammation. Moreover, the identification of other regulatory sites within the PAD4 gene, such as other transcription factor binding sites, is an important objective. Finally, although *in vitro* models of cell manipulation can be useful, it would be advantageous to test the connection between inflammation and PAD4 expression *in vivo* using mice. More specifically, although our results indicate a differing role for NF-κB in the regulation of *PAD4* in mouse and human cells, it would be useful to investigate the role of PAD4 in inflammation and NETosis in a mouse model of rheumatoid arthritis.

With respect to the role of NF-κB, one experiment that may be done to further clarify whether NF-κB regulates *PAD4* expression is to activate HL-60 cells with TNF-α (as done previously) and treat immediately with the NF-κB inhibitor JSH-23 (109). Additionally, to test the relative importance of the NF-κB binding site in the *PAD4* promoter, it is important to construct luciferase reporter plasmids containing the identified potential binding sites for AP-1 and SP1 and compare to data for NF-κB. Finally, to test the ability of PAD4 to citrullinate targets in the extracellular environment, an experiment that can be done is to induce primary human neutrophils to undergo NETosis using PMA and measuring PAD4 activity in the extracellular environment.

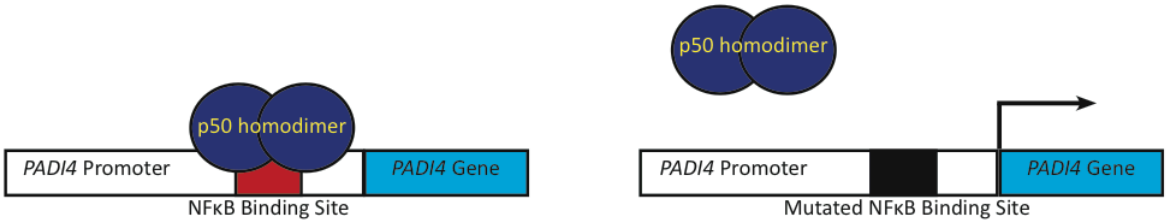
Our long-term goal is to understand the role played by PAD4 in rheumatoid arthritis in order to establish novel therapies and treatments for RA. While current treatments of RA aim to minimize the pain and inflammation caused by the disease, we have yet to develop measures that are adequate to the underlying auto-immunological cause. To that end, PAD4 has so far proven to be a plausible target for treatment; PAD inhibitors such as Cl-amidine have been used to treat mice with collagen-induced arthritis with notable success and continue to be studied as possible therapeutic agents (73).

4.5 Summary and conclusions

We speculate that repression of *PAD4* by NF-κB might be useful in the context of the resolution phase of inflammation, in which the activity of NETs must be checked in order to prevent chronic inflammation. Moreover, the negative regulation of *PAD4* might serve to limit NET formation during the acute phase of inflammation, thus reducing "collateral damage" caused by excessive NET formation as well as limiting the release of

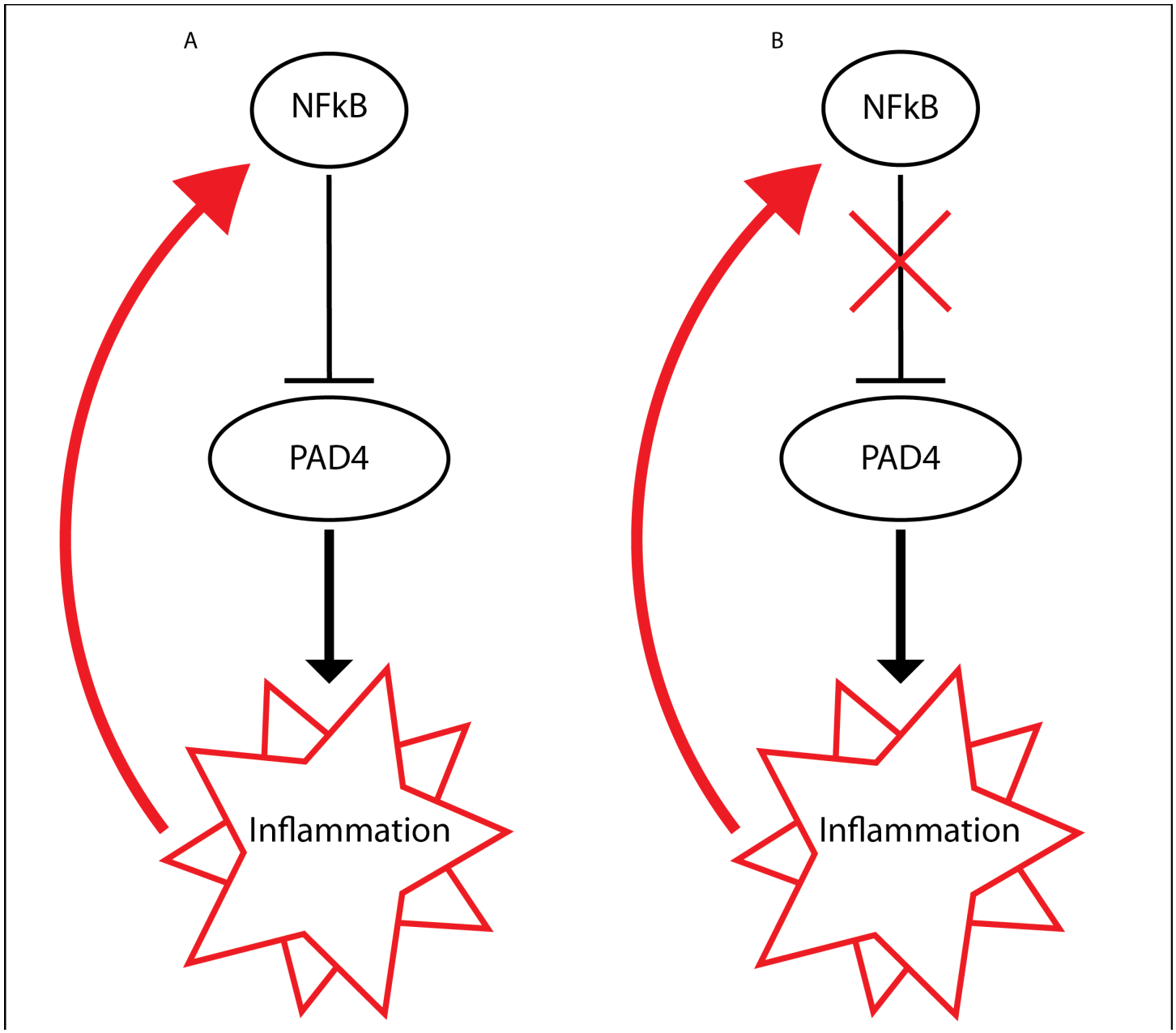
autoantigens including citrullinated proteins into the extracellular space. However, in patients with RA, it is conceivable that mutations in the *PAD4* promoter results in lowered p50 binding and continued, unattenuated expression of PAD4 (Fig. 4.1). This is turn may contribute to hypercitrullination of histones in the nuclei of neutrophils, leading to excessive NET formation and finally resulting in a "runaway" immune response involving accumulation of citrullinated products in the synovium and the breaking of immune tolerance.

Figure 4.1 A possible mechanism for dysregulation of PAD4 expression. In unaffected persons, p50 homodimers may bind the PAD4 promoter during the resolution of inflammation, suppressing the formation of NETs. Persons susceptible to RA may have a mutation that prevents p50 binding and results in overexpression of PAD4.



If, as our results suggest, NFκB functions as a repressor of *PAD4* expression in human immune cells, it is possible that in individuals unaffected by RA, an infection would begin in the inflammatory phase with the general activation of neutrophils and the release of NETs regulated by PAD4 (66). As inflammation moves into the resolution phase, NF-κB dimers involved in immunosuppression predominate, and this would involve formation of p50 homodimers. Since the PAD4 promoter contains the NF-κB motif that is most preferred by p50 subunits (79), it might be preferentially bound by p50 homodimers and its expression repressed, resulting in less NET formation and inhibition of neutrophil function in maintaining inflammation (Fi.g 4.1). In patients with RA, it is possible that a lack of p50 binding to the PAD4 promoter causes continued, unattenuated expression of PAD4, resulting in a "runaway" immune response involving accumulation of PAD4 in the synovium and breaking of immune tolerance (Fig.4.2).

Figure 4.2 Interplay between inflammation, NF-kB, and PAD4 expression (A) During the resolution phase of inflammation we hypothesize that NF-κB represses *PAD4* expression to reduce NET formation and thus "dial down" the inflammatory response, itself an activator of NF-κB. (B) Without the repression of PAD4, an uncontrolled positive loop would occur, leading to chronic inflammation.



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Curriculum Vitae

Name Ali Abbas

Education

Ongoing	M.Sc.	Western University, London, Canada Microbiology and Immunology, with Specialization in Developmental Biology
2012	B.Sc.	Western University, London, Ontario, Canada Honours Biology

Honours and Awards

2008 Valedictorian – Saunders Secondary School

2008 Western Scholarship of Excellence ($2000) – Western University

2009 Laurene Paterson Estate Scholarship ($2000) – Western University

2011 Microbiology and Immunology Summer Undergraduate Research Fellowship ($6500) – Western University
Infection and Immunology Research Forum Best Undergraduate Poster Presentation ($50)

2012 Schulich Department of Medicine Research Day Best Poster Presentation ($500)
Infection and Immunology Research Forum Best Poster Presentation ($100)

2013 Ontario Graduate Scholarship ($15, 000)

Research Publications

1. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP. Negative regulation of the peptidylarginine deiminase type IV promoter by NF-κB in human myeloid cells. *Gene*. 2014 Jan 1; 533(1): 123-31

2. Stephen K.H. Li., Ali K. Abbas, Gaëlle M.N. Groux, and Rodney P. DeKoter. TLR-Mediated Splenic B cell Proliferation Requires Transcriptional Activation of *Nfkb1* by PU.1 and Spi-B. Submitted to *Molecular Cell Biology*.

Abstracts and Presentations

1. Rheumatology Research Day. London, Ont. October 2011. Abstract and Oral Presentation. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

2. Infection and Immunology Research Forum. Western University. November 2011. Abstract and poster presentation. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

3. Ottawa Undergraduate Research Poster Competition. Abstract and poster presentation. Ottawa, Ont. January 2012. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

4. Ontario-Quebec Undergraduate Immunology Conference. Abstract and oral presentation. Toronto, Ont. May 2012. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

5. Schulich Department of Medicine Research Day. Abstract and poster presentation. London, Ont. June 2012. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

6. Canadian Society for Immunology Annual Meeting. Abstract and poster presentation. Whistler, BC. April 2013. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

7. Western University Developmental Biology Research Day. Abstract and poster presentation. London, Ont. June 2013. "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

8. American College of Rheumatology Annual Meeting. Abstract and poster presentation. San Diego, Ca. October 2013. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

9. Infection and Immunology Research Forum. University of Western Ontario. November 2013. Abstract and poster presentation. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

10. Western University Developmental Biology Research Day. Abstract and poster presentation. London, Ont. June 2014. Abbas, A.K, Le, K., Pimmett, B., Bell, D.A., Cairns, E. and DeKoter, RP: "Transcriptional Regulation of Peptidylarginine Deiminase Type IV (PAD4) in Rheumatoid Arthritis"

Teaching Experience

2013 Teaching assistant – Advanced Clinical Immunology 4300A, Microbiology and Immunology, Western University

Relevant Technical Experience

Chromatin Immuno-Precipitation (ChIP)
Transient transfection and luciferase assay
Reverse Transcriptase (RT) PCR
Real Time PCR
In vitro cell culture
DNA isolation

Voluntary Services

2010 Habitat for Humanity home builder

2010 Western Arab Student Association Executive – Vice President

2012- Muslim Community Centre Sunday School Teacher

2012- West London Soccer League Soccer Coach (Southend Soccer Club)