Chapter 2
Aspects of Peptidylarginine Deiminase Regulation that May Predispose to Autoreactivity Against Citrullinated Proteins

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2.1 Summary

Autoimmune diseases represent a long-standing puzzle. In an incompletely understood series of steps, the immune system loses immune tolerance to self and acquires the ability to recognize and respond to defined and characteristic autoantigens. The involvement of an infectious agent has been suspected to trigger this transition, but a specific etiologic stimulus has not been identified. Recent years have seen an evolution in the understanding of events that lead to autoimmunity. A central role has been assigned to posttranslational modifications of autoantigens during the initial, preclinical phase of autoimmune syndromes. In response to various infections or even under sterile inflammatory conditions, the innate immune system activates a characteristic set of enzymatic reactions, including the regulated conversion of certain arginine residues to citrulline residues. The conversion, carried out by the peptidylarginine deiminases (PADs), results in the conversion of arginine residues to citrulline residues in many notable autoantigens. In turn, an important category of autoantibodies, referred to as anti-citrullinated protein antibodies (ACPA), specifically recognizes the citrullinated form of these autoantigens. Thus, the concept is gaining acceptance that diverse infections (or sterile inflammation) result in the citrullination of self-proteins, which—given genetic predisposition or a conducive infectious microenvironment—break tolerance and trigger a self-perpetuating autoimmune process. This chapter highlights aspects of PAD regulation and the development of ACPA in order to propose a unifying principle for the induction of autoimmune disorders.

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2.2 Tolerance and Autoimmunity

Autoimmunity was anticipated as a possible outcome that could arise from the adaptive nature of the immune system (Ehrlich P 1800s). Because the immune system is capable of recognizing almost any foreign molecule, it appeared obvious to Paul Ehrlich that autoreactivity, or "horror autotoxicus," would also predictably appear. Indeed, several dozen human autoimmune disorders are currently recognized as distinct clinical entities, and new disorders continue to be assigned to the category of maladies that arise due to a malfunction of the immune system. Many of these disorders are quite common, such that, for example, rheumatoid arthritis (RA) affects nearly 1 in 100 persons. In aggregate, several percent of the human population develop a serious health condition because of the inappropriate anti-self-reactivity of the immune system.

Conversely, it may be even more astounding that more of us do not suffer from an autoimmune disease, given the enormous rates at which cells of the immune system proliferate and the large numbers of cells that comprise the innate and adaptive immune systems. Moreover, our immune systems are constantly stimulated by microbes that survive in our environment and form part of our extensive microbiota. In view of the continued microbial challenge, it is important to consider ways in which our immune systems distinguish foreign pathogens from self. The mechanism that safeguards against the formation of autoreactive B and T lymphocytes is called immune tolerance. During development and throughout their functional responses, lymphocytes are regulated by active mechanisms that suppress immune responses aimed at molecules that form part of ourselves. The encounter of autoreactive lymphocytes with self-molecules ensures the functional inactivation or correction of the inappropriate B and T cell antigen receptors. Multiple mechanisms are engaged in central tolerance, some of these lead to anergy, deletion, or receptor editing in autoreactive B cells (Radic and Zouali 1996), and others induce cell death of autoreactive T cells in the organs that support lymphopoiesis (Palmer 2003).

The sophistication and power of immune tolerance can best be appreciated by immunization methods in experimental animal species. Standard approaches can readily yield antibodies of high affinity and exquisite specificity for the antigen of choice, provided that the immunogen contains structural motifs (epitopes) that are distinct from the endogenous antigens. For example, a protein that is isolated from the hemolymph of a marine invertebrate, keyhole limpet hemocyanin, is a versatile carrier in numerous immunization experiments. However, if the immunogen of interest is highly related or identical to the animal's own molecular components, multiple immunizations may not elicit an immune response to the intended target. A notable example is provided by the collective experience of frequent but often futile efforts to raise antibodies to various members of the histone protein family (Rubin et al. 1990).

Histones are highly conserved, basic proteins that associate into octamers of two histone H3, two H4, and two each of H2A and H2B monomers (Moudrianakis and Arents 1993). Together these eight histones form the core of a structure that serves to organize approximately 147 base pairs of nuclear DNA into a single nucleosome, the basic unit of chromatin. Histones are among the most ubiquitous, abundant, and

highly conserved proteins in eukaryotes, given that histone primary amino acid sequences vary by less than 1% from humans to yeast. Thus, immunizations of mice or rabbits with histones elicit very little to no immune response due to the strong negative selection of lymphocytes against self-reactivity by immune tolerance. A similar resistance to immunization with DNA is observed, yet both DNA and histones are targets of anti-self-antibodies (Stollar 1971) in an autoimmune disease called systemic lupus erythematosus (SLE). Other conserved and abundant proteins are characteristic autoantigens in other autoimmune diseases. For example, myelin basic protein, a membrane-associated protein of neurons, is recognized by T cells in multiple sclerosis (Martin et al. 1992), insulin is a target of autoantibodies in diabetes (Michels and Nakayama 2010), and collagen or even the immunoglobulin molecule itself is attacked by the immune system in RA (Rowley et al. 2008).

But how does the immune system overcome ("break") immune tolerance and begin an autoimmune attack? Some early illustrative experiments with the multifunctional protein cytochrome c provided important clues. Immune tolerance prevents mice from responding to mouse cytochrome c. However, human cytochrome c, due to only six amino acid variations in sequence from the mouse protein, can induce a B cell response (Lin et al. 1991). Remarkably, following a subsequent immunization with a combination of mouse and human cytochrome c, the mouse B and T cells respond to the mouse cytochrome c protein. The conclusion from those experiments is that small molecular differences between autoantigens and immunogens can break tolerance and induce a self-sustaining autoimmune response, provided that B cells to the altered antigen arise which can present antigen to incompletely deleted T cells.

It was observed that small molecular differences between an immunogen and an autoantigen can also be introduced by various covalent modifications of autoantigens and that such differences can stimulate a response by the immune system. In pioneering experiments, Bill Weigle discovered that chemically modified albumin could break tolerance in rabbits tolerant of the unmodified albumin (Weigle 1962). More recent work from Mark Mamula's laboratory expanded this field of research and showed that isoaspartate racemization of self-antigens, including histones, increases their immunogenicity (Doyle et al. 2013). These experiments established the possibility that posttranslational modification (PTM) of autoantigens may contribute a possible mechanism to disrupt immune tolerance. In turn, studies in a number of laboratories identified autoantibodies that preferentially reacted against the modified form of a variety of autoantigens. This independent evidence solidified the idea that the covalent, enzymatic modifications of autoantigens play a central role in the induction of autoimmunity.

2.3 Discovery of Arginine Deimination

The first identification of citrulline residues in a protein was reported in 1958 by researchers from the Melbourne Wool Research Laboratories examining hair follicles in sheep (Rogers and Simmonds 1958). The discovery was notable for the fact

that citrulline is not incorporated into proteins during translation; thus, it must be introduced into proteins by posttranslational modifications. However, formal proof for this conclusion had to await the discovery of peptidylarginine deiminases (PADs), the enzymes that convert arginine residues in proteins to citrulline residues (Fujisaki and Sugawara 1981). Subsequent research identified additional members of this small protein family, which established the expression of five isoforms of PADs in mammals (Chavanas et al. 2004). PADs are a relatively recent evolutionary adaptation, which are present in vertebrates but absent from other eukaryotic subphyla (Vossenaar et al. 2003).

Research into the structure and function of PADs intensified following the discovery that autoantigens, including myelin basic protein, keratin, and histones, are substrates for PADs (Muller and Radic 2015). These hallmark autoantigens that have high diagnostic value for the detection and prognosis of a variety of difficult to treat autoimmune disorders previously had not been considered to be linked by a common pathway. The discovery of shared PTM between autoantigens in different autoimmune disorders immediately raised expectations that a common underlying mechanism may be at the root of this category of clinical entities. Most notably, a series of elegant studies traced the RA autoreactivity against cells from the oral mucosa to yet another citrullinated antigen. Schellekens and collaborators observed that autoantibodies in RA display a high degree of specificity and sensitivity for peptides derived from filaggrin but only if the arginine residue in these peptides is replaced by citrulline (Schellekens et al. 1998). The diagnostic utility of the serologic test for anti-citrullinated protein antibodies (ACPA) was demonstrated by the observation that ACPA can be detected months or even years ahead of the appearance of clinical manifestations of RA (Johansson et al. 2016). The inclusion of ACPA as classification criteria for RA further boosted basic and applied research into PADs (Liao et al. 2008).

The five PAD isozymes differ from each other in the cell types and subcellular locations where they are expressed and, in turn, by what substrates they modify. PAD1 is expressed in the epidermis and uterus (Terakawa et al. 1991), PAD3 is active in hair follicles (Kanno et al. 2000), and PAD6 is most abundant in the oocyte and during embryonic development (Yurttas et al. 2008). PAD2 and PAD4 are of greatest relevance in cells that comprise the immune system (Vossenaar et al. 2004). Notably, PAD2 is also expressed in the central nervous system and in muscles (van Beers et al. 2013). PAD4 is highly expressed in leukocytes, the white blood cells of the innate immune system, along with other immune cells (Anzilotti et al. 2010). PAD4 is the only PAD with a distinct nuclear localization sequence, and thus, it is the probable isozyme in charge of modifying nuclear proteins, including histones (Nakashima et al. 2002). Accordingly, activation of PAD4 makes important contributions to chromatin structure modifications that regulate gene expression (Cuthbert et al. 2004; Wang et al. 2004). However, PAD2 has also been implicated in the modification of histones (Zhang et al. 2012), whereas PAD4 may also be active in the cytoplasm and even on extracellular substrates (see below).

All PADs require calcium for enzymatic activity, and maximal activity may require extracellular calcium influx to supplement intracellular calcium stores. Calcium ions organize the overall PAD structure and shape the conformation of the active site, as determined by X-ray diffraction patterns of PAD4 obtained in the presence and absence of the divalent cations (Arita et al. 2004). PADs are relatively promiscuous enzymes because they accept substrates with different amino acid sequences, provided the target arginine is preceded by a residue with a small side chain at the R-2 position and the polypeptide chain flanking the arginine can fold into a tight beta turn (Arita et al. 2006). Catalysis, which consists of the hydrolysis of the guanidino group on arginine to yield the ureido group on citrulline (Fig. 2.1), leads to the release of ammonia, the loss of arginine's positive charge, and the nearly exact gain of 1 Da in mass by the citrulline-containing product (Rohrbach et al. 2012b). Although PAD4 can accept methylated arginine as a substrate, it prefers unmodified arginine by a factor of over 100:1 in an in vitro reaction (Kearney et al. 2005; Raijmakers et al. 2007). Therefore, biochemically, one consequence of arginine citrullination is to preclude any further arginine modifications (Thompson and Fast 2006).

As the evidence for autoantibodies to citrullinated antigens accumulated, it became clear that useful applications would emerge from the development of PAD inhibitors. Insights into the conformation of the PAD4 active site were starting points for the design of different versions of PAD4 inhibitors. Work spearheaded by the Thompson lab used various chemical approaches to screen and confirm the efficacy, specificity, bioavailability, and mode of action of various compounds (Bicker and Thompson 2013). One of the earliest inactivators that were effective at inhibiting the action of several PAD enzymes was *N*-alpha-benzoyl-*N*5-(2-fluoro-1-iminoethyl)-L-ornithine amide, or F-amidine, which became available about 10 years ago (Luo et al. 2006), and was soon followed by several other irreversible and selective inhibitors (Knuckley et al. 2010). For example, a natural compound with antibiotic properties, streptonigrin, was identified as a selective PAD4 inhibitor (Dreyton et al. 2014), whereas the inorganic dye used in histology, ruthenium red,

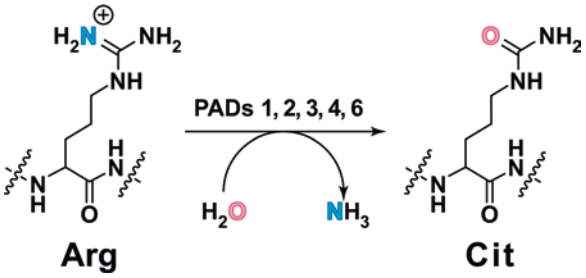

Fig. 2.1 Diagram of peptidylarginine deiminase (PAD)-mediated conversion of arginine to citrulline. One of five PADs converts arginine to citrulline by using oxygen from water and releasing nitrogen as ammonia

was a selective inhibitor of PAD2 (Lewallen et al. 2014). Ruthenium red binds to PAD2 at a site that normally accepts calcium, and thus, it prevents the activation of the enzyme. These inhibitors and inactivators are of immense value for determination of PAD activity and its consequences in vivo and in vitro.

Mounting evidence places PAD4 at the center of attention in events shaping the interactions between the innate and adaptive immunity. In particular, research indicates that PAD4 activation is intimately involved in the initial stimulation and subsequent tissue damage associated with autoimmune diseases. Nevertheless, additional open questions remain and deserve further attention. For example, although PAD4 can auto-citrullinate (Andrade et al. 2010; Mechin et al. 2010), the precise consequences of this modification are unclear, as the modified PAD shows little change in enzymatic activity on selected in vitro substrates (Slack et al. 2011). Clearly, one possibility in vivo is that auto-deimination may affect the binding with other interacting partners of the enzyme. More broadly, several unsolved questions of PAD4 regulation remain, and efforts at finding answers will drive much ongoing and future research. Some of the questions are highlighted in Box 2.1.

Box 2.1: Unsolved Questions in Cell Biology of PADs

1. Is PAD4 enzymatic activity controlled by other interacting proteins, many of which are yet to be identified?
2. Do PAD4 modifications regulate the access of calcium to its five binding sites?
3. What principles determine PAD4 recognition of other proteins and the cellular location of the enzyme?

2.4 Role of PAD4 in Innate Immune Responses

PAD4 supports fundamental functions in neutrophils, the most abundant type of granulocytes in blood. The PAD4 gene is expressed relatively late during neutrophil maturation but reaches high expression levels during a short window preceding the release of neutrophils from the bone marrow (Theilgaard-Monch et al. 2005). Even though PAD4 clearly can be transported into the nucleus and its most abundant cellular substrates are core histones, large quantities of PAD4 are also packaged into ficolin-1-rich neutrophil granules (Rorvig et al. 2013). The issue of subcellular localization can be addressed with the use of specific antibodies raised against PAD4. Figure 2.2 shows the results of probing human peripheral blood neutrophils with a monoclonal antibody to PAD4 (kind gift of Prof. Katsuhiko Nakashima). Using a preparation of unstimulated neutrophils, it can be seen that cells exhibit heterogeneous staining patterns, in which some cells have abundant nuclear PAD4, whereas others have a more cytoplasmic PAD4 distribution. In addition, particular cells exhibit a patchy nuclear distribution, perhaps indicating that PAD4 shows preference for certain chromosomal domains. The resolution of the confocal microscope does not allow the precise localization of PAD4 to cytoplasmic granules, but

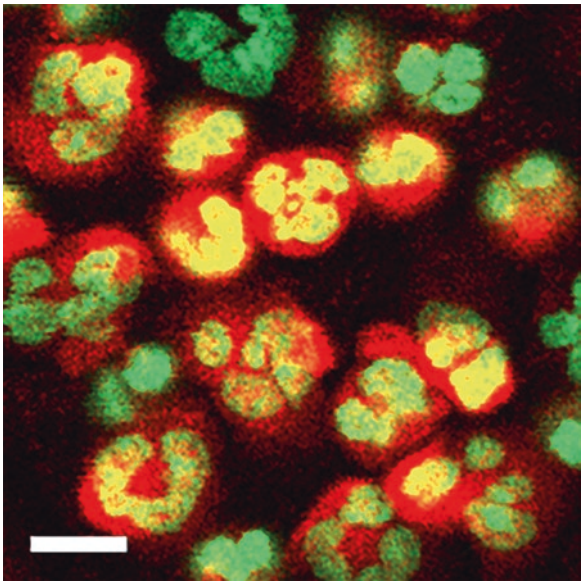

Fig. 2.2 Detection of PAD4 in purified human blood neutrophils. Human neutrophils were isolated from blood and purified using standard conditions. Fixed cells were permeabilized and incubated with a monoclonal antibody to PAD4 (kind gift from Dr. Nakashima, Japan). Antibody binding was visualized by secondary anti-mouse antibodies shown in *red*, and nuclear DNA was detected with *Sytox Green*. The overlap between the colors yields *yellow*. In this preparation, neutrophils were heterogeneous, and PAD4 was localized to both nuclei and cytoplasm. Bar is equivalent to 10 μm

that assignment is consistent with biochemical fractionation experiments carried out by Niels Borregaard's laboratory in Copenhagen.

The protective function of neutrophils in the immune system is to act as sentinels for infections or inflammation. As white blood cells, neutrophils circulate throughout the body, yet they are exquisitely sensitive to any local signs of inflammation. The presence of IL-8 (CXCL8) and expression of selectins on endothelial cells lining the blood vessels induce neutrophil attachment to the vessel wall (Riese et al. 2014) and their extravasation (migration) into tissues. Neutrophils express metalloproteases such as MMP-9 which transiently dissociate tight junctions that bind endothelial cells to each other, thus generating gaps that neutrophils exploit to squeeze through the endothelium and reach the inflamed tissues (McColl et al. 2008). Once in tissues, neutrophils convert into highly motile cells capable of chemotaxis toward increased concentrations of microbial patterns such as f-MLP and various cytokines, such as IL-1 or TNF. Neutrophils also migrate toward signals from damaged cells, such as ATP, ADP, and related molecular messengers that promote inflammation (McDonald et al. 2010). Neutrophils integrate these diverse signals by expression of multiple cell surface receptors that operate in a hierarchical system of dominant vs. supplementary signaling cascades (Heit et al. 2002; Mocsai et al. 2015).

The functional transition of a neutrophil from passive transport in the blood to an active, directional movement in tissues is mirrored in a notable increase in gene transcription. In part, the upregulation of gene expression reflects the activation of cell surface adhesion molecules, such as Mac1 integrins, which are important in the process of extravasation as well as in the subsequent migration in tissues (Kobayashi et al. 2002). The coordinated migration of neutrophils in tissues has been described as "swarming" which can be viewed in vivo following a focused tissue injury (Lammermann et al. 2013). Expression of Mac1 is required for the formation of tight clusters that comprise the center of swarms and assist in the repair of damaged extracellular matrix components. A variety of gene products is induced, most notably several pro-inflammatory cytokines and chemokines that serve to condition the tissue microenvironment and promote the migration of other cell types to the site of inflammation (Nathan 2006).

PAD4 expression is induced by several of the signals that promote inflammation, including TNF (Neeli et al. 2008) and IL-8 (Gupta et al. 2005). Moreover, PAD4 expression is stimulated by neutrophil adhesion to extracellular matrix components via Mac1 and is sustained by an active and functional cytoskeleton (Neeli et al. 2009). Most importantly, PAD4 expression is strongly induced by substances emanating from pathogens or by direct contact with microbes. Bacteria, fungi, and viruses that upregulate PAD4 activity include clinically important pathogens such as *Staphylococcus aureus* (Kolaczkowska et al. 2015) and *Shigella flexneri* (Li et al. 2010). It is therefore tempting to say that PAD4 activation begins with the stimulation of neutrophils in the blood, progresses through the stages of active migration toward a site of infection, and culminates during the precise moment that contact with an invasive pathogen is made. Upon encounter with microbes, the neutrophils may induce alternative responses. Neutrophils are active phagocytes capable of the internalization and destruction of a pathogen. Alternatively, neutrophils can release bactericidal products and reactive oxygen species (ROS). Although the precise role of PAD4 during phagocytosis is not known, it has been reported that various secreted cytokines and chemokines are modified by deimination (Proost et al. 2008; Moelants et al. 2013). It thus appears that PAD4 is intimately involved in essential functions of the innate immune system, for which it regulates and fine-tunes many complementary but independent processes.

2.5 PAD4 and NETs

An additional and unexpected innate defense mechanism was initially described in 2004 (Brinkmann et al. 2004). These authors observed that in response to various bacterial pathogens, neutrophil cell membranes rupture and release nuclear chromatin into the cellular surroundings. The released chromatin forms a matrix that was named a "neutrophil extracellular trap" (NET) because it has the ability to bind and immobilize the pathogens that it contacts. Subsequent studies have more carefully examined the steps in NET release (Fuchs et al. 2007). Morphologically, it was observed that the nuclear chromatin relaxes, and, as a result, the lobed nucleus

expands until it is roughly spherical. Thereupon, the nuclear envelope distends until gaps appear, the cytoplasmic granules break open, and granule contents associate with the chromatin that breaks out from the confines of the nucleus. Ultimately, the plasma membrane ruptures and NETs that consist of nuclear chromatin and associate with several granule constituents, such as myeloperoxidase and elastase (Papayannopoulos et al. 2010), are deployed. It is assumed that the granule contents such as proteases and antibacterial peptides, which decorate the NETs, contribute to their bactericidal potential. Interestingly, certain strains of bacteria express nucleases that allow them to escape from neutrophil traps (Buchanan et al. 2006). In vivo, these bacteria are more virulent than variants that are deficient in nuclease production.

Separate experiments have provided evidence for the release of NETs in response to certain viruses and fungal infections. Infections with poxviruses identified a protective function of NETs in the microvessels of the liver (Jenne et al. 2013). In that study, NETs were visualized in vivo and provided effective containment of viruses in liver sinusoids which protected the body from viral dissemination. Interestingly, neutrophils also release NETs in response to fungi such as *Aspergillus* (Gazendam et al. 2016) and *Candida* (Byrd et al. 2013). In fact, the relative size of a pathogen may determine, in part, whether the neutrophil will release NETs or undergo an alternative innate immune defense mechanism (Branzk et al. 2014). Cell wall glucans of *Candida* are also effective inducers of neutrophil swarming in vitro (Byrd et al. 2013). These and other in vivo studies determined that there is a type of NET release that does not result in the massive rupture of the cell but, in fact, leaves the neutrophil able to continue chemotaxis (Yipp et al. 2012). It was further determined that this alternative form of NETs may be composed of mitochondrial DNA (Yousefi et al. 2009). If so, the mitochondrial NETs may lack histones and perhaps other granule-derived bactericidal components of classical NETs. Clearly, important characteristics of the mitochondrial nucleoid NETs and their ability to damage or destroy pathogens remain to be established.

In the classical form of NET release, numerous independent and consistent studies demonstrated that PAD4 carries out an essential function. In pioneering studies, our lab showed that various inflammatory stimuli induce histone deimination and identified deiminated histones as integral components of NETs (Neeli et al. 2008). Subsequent studies confirmed these results (Wang et al. 2009) and established that PAD4 activity is essential for the regulated release of NETs, as neutrophils deficient in PAD4 fail to deploy extracellular chromatin (Li et al. 2010; Rohrbach et al. 2012a). These results are consistent with evidence that PAD4 inhibitors are effective at blocking NET release (Lewis et al. 2015). However, it is not clear how PAD4 contributes to NETosis. It is plausible, albeit unproven, that PAD4 contributes an essential function for NET deployment by modifying arginine residues in histones. The amino termini of core histones extend from the nucleosome core particle in unstructured fashion. Each of the four core histones has extended amino termini of about 18–25 residues in length that contain numerous positively charged arginine and lysine side chains (Radic and Muller 2013). Through these extensions, each of the eight histones in a nucleosome can contribute to bind adjacent nucleosomes into

condensed and relatively inert chromatin. A fifth histone, the linker histone H1, does not form part of the nucleosome core particle yet contributes in an important way to chromatin structure. Histone H1 binds DNA at the entry and exit points from the nucleosome and thus controls the angle of the linker DNA that connects adjacent nucleosomes. In that way, H1 is at a crucial position to regulate chromatin structure (Izzo and Schneider 2016). The histone tails, together with H1, regulate structural transitions in chromatin, which facilitate access of transcription factors, RNA polymerases, and other proteins to particular DNA sequences. Many histone PTMs serve to organize chromatin according to the functional needs of the cell.

By converting arginine residues to citrulline residues, PAD4 removes the positive charge from the amino termini of core histones (Fig. 2.1) and thus diminishes the attractive forces between histones and DNA. As a result, histone deimination loosens the structure of chromatin. Yanming Wang and colleagues used defined chromatin templates to show their structural relaxation upon treatment with PAD4 (Wang et al. 2009), and a similar transition may provide the force that expands the nucleus and ultimately ruptures the nuclear envelope to release NETs. In their sequence, H1 histones contain a large number of lysine residues but only a few arginine residues. Therefore, we asked whether one or more of these arginines are deiminated by PAD (Dwivedi et al. 2014). We prepared highly modified histone H1 and subjected it to tandem mass spectrometry. Peptide analysis determined that the most highly conserved arginine, R53, in the winged helix domain of H1, is a substrate of PAD4. In independent studies by the laboratory of Tony Kouzarides in Cambridge, deimination of R53 was reported to occur during transcriptional reprogramming that accompanies the development of cell lineages from pluripotent stem cells (Christophorou et al. 2014). The deimination of H1 by PAD4 thus was shown to play a crucial and conserved role in the developmental program of higher eukaryotes. Strikingly, the same H1 modification that facilitates global changes in gene expression also was co-opted toward a unique mechanism of innate immune response.

The myriad of stimuli that lead to NET release and the potential existence of different forms of NETs make it difficult to identify the signaling pathways that participate in the activation of PAD4. Signals from Gram-positive bacteria, including lipopolysaccharide (LPS) acting on the Toll-like receptor 4 (TLR4), may transmit signals via MyD88 and its associated catalytic subunits to IRAK1 (Huang et al. 2015). Through the activation of distinct IKK subunits, the pro-inflammatory axis of NFkappaB is engaged, leading via MEK1 to the further activation of ERK1 and ERK2 (Yu et al. 2015). Alternatively, it was reported that FcgammaRIIIb, acting through TAK1, leads to the activation of ERK1/ERK2 (Aleman et al. 2016). Additional feed-forward signals may involve activation of PLCgamma and the formation of its messenger IP3, followed by calcium release from endogenous ER stores (Numaga et al. 2010). Alternatively, a calcium-activated potassium channel may directly engage signals leading to NETosis (Douda et al. 2015). Calcium could act as an additional signal by activating PKC subunits, which have been shown to have a direct effect on NET release. Our own studies revealed an unexpected complexity of PKC involvement in NETosis. Experiments with an inhibitor

of classical PKC, chelerythrine, as well as a structurally related compound, sanguinarine, demonstrated that classical PKC enzymes may block activation of PAD4, yet an atypical PKC, most likely PKCzeta, exerts an activating role upstream of PAD4 (Neeli and Radic 2013). The opposing effects of two PKC isoforms argue for very precise regulation of PAD4 in neutrophils. Through as yet incompletely understood mechanisms, these enzymes contribute to the disruption of granule and nuclear membranes, chromatin relaxation, and, ultimately, NET release. Some of the remaining questions regarding the regulation of NET release are summarized in Box 2.2.

The activation of PAD4 can most easily be seen by monitoring histone deimination (citrullination), for which specific antibody reagents are commercially available. However, discovery of new substrates will require the availability of additional approaches. One method to detect the activation of PAD4 in cells is by isolating neutrophil proteins and exposing them to a compound that selectively reacts with the ureido group of citrulline. Under acidic conditions, phenylglyoxal reacts with citrullines to form a covalent bond that is stable during subsequent manipulations at neutral pH (Lewallen et al. 2015). By conjugating rhodamine to the phenylglyoxal probe (Rh-PG), citrulline-containing proteins become fluorescently labeled. We have used such an approach to explore the diversity of neutrophil proteins that are substrates for PAD4 (Fig. 2.3).

The results demonstrate that, in purified human neutrophils, certain proteins react with Rh-PG following incubation in calcium-containing buffer (lanes 1). However, the intensity of Rh-PG reactivity increases following neutrophil activation with ionophore (lanes 2). In particular, histones in ionophore-activated neutrophils become quite reactive, indicating deimination by PAD4. An increased intensity of reactivity is observed following ionophore stimulation in the presence of a classical PKC inhibitor (lanes 3). Purified calf thymus histones that were deiminated in vitro by recombinant PAD4 (kind gift from Paul Thompson) indicate the migration of histones (lane labeled H). The specificity of Rh-PG labeling is tested by comparison to the reactivity of antibodies reactive against modified citrulline (lane MC). There is extensive agreement between these two approaches (compare lanes 3 to MC). An alternative method could use a mouse monoclonal antibody, F95, which was raised against a deca-citrulline peptide and which reacts with citrulline-containing antigens (Nicholas and Whitaker 2002). Each of these should be added to the list of reagents that will play a crucial role in the further identification of PAD4 functions in NETosis.

Box 2.2: Unsolved Issues of PAD4 Activation in Classical NETosis

1. By using a defined NETosis stimulus, the precise series of contingent activation steps leading to PAD4 activity need to be established.
2. The precise relation between PAD4 activation and nuclear chromatin relaxation should be demonstrated.
3. The possible activation of PAD4 during the mitochondrial NET release and/or swarming should be examined.

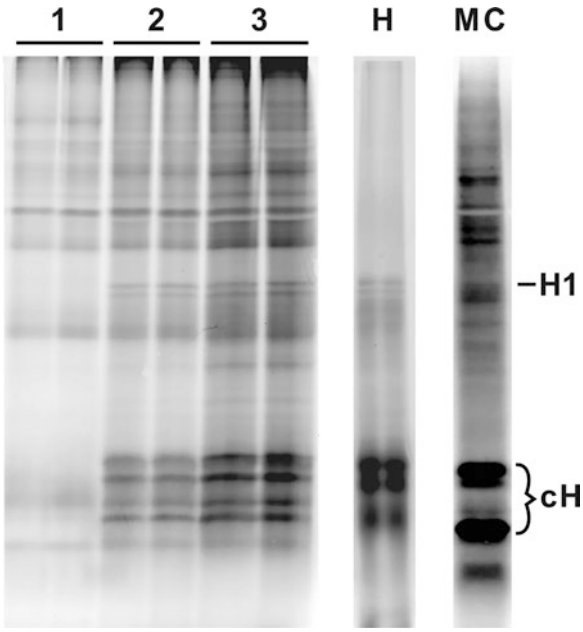

Fig. 2.3 Detection of deiminated proteins in neutrophils. Purified neutrophils were incubated with calcium buffer (lanes 1), calcium plus ionophore A23187 (lanes 2), or as in 2 with the addition of chelerythrine for 2 h (lanes 3). Cells were frozen and thawed, incubated with 20% TCA, sonicated, and reacted with Rh-PG as described by Bicker et al. Proteins were resolved on SDS-PAGE, and citrulline-containing bands were visualized on a Typhoon imager. For comparison a lane with purified deiminated histones is shown (H), and total neutrophil proteins were also reacted with a modified citrulline antibody, as recommended by supplier (Millipore). Approximate positions of H1 histone and core histones (cH) are indicated

2.6 NETs in Autoimmunity

Work in our laboratory, with essential contributions from collaborators, established the first indications that deiminated histones are important autoantigens in various autoimmune diseases that affect connective tissues. By using in vitro deiminated or unmodified histones for screening by ELISA or Western blots, Dwivedi et al. found that certain autoimmune IgG prefers to bind deiminated histones, often showing additional preference for specific core or linker histones (Dwivedi et al. 2012). This observation was confirmed with subsets of sera from SLE and Sjögren's syndrome patients (Dwivedi et al. 2014). Most notably, sera from patients with Felty's syndrome, a more severe variant of arthritis with neutropenia, splenomegaly, and anti-histone antibodies, showed a dramatic preference for deiminated histones

(Dwivedi et al. 2012). Thus, immunochemistry accounted for the intense preference of autoimmune sera for activated neutrophils and NETs over freshly isolated blood neutrophils that is observed in immunofluorescence microscopy. Histones are well-known autoantigens in SLE, and anti-histone antibodies also arise in Felty's, Sjögren's, and RA. Surprisingly, investigators had not previously evaluated relative binding to deiminated histones. Following the publication of our study, the lab of Paola Migliorini confirmed that a certain subgroup of RA patients express autoantibodies that preferentially, even exclusively, bind to the deiminated antigens (Pratesi et al. 2014).

The list of PAD substrates which react more vigorously with autoantibodies upon deimination continues to expand and currently includes over one dozen proteins (Muller and Radic 2015). Similar to peptides from deiminated filaggrin that show over 70% sensitivity and 96–98% specificity in the diagnosis of RA (Schellekens et al. 1998), autoantibodies from RA sera also bind to deiminated peptides from fibrin and from histone H4 with similar sensitivity and specificity (Pratesi et al. 2014). Other autoantigens that are recognized in citrullinated form include additional extracellular substrates, including collagen type II (Haag et al. 2014). One important MS autoantigen that forms part of the axon sheath and insulates neurons to facilitate their function over distance is myelin basic protein, which is modified by PAD2 (Bradford et al. 2014). Cytokines, in particular CXCL8 (Proost et al. 2008) and CXCL10 (Loos et al. 2008) that stimulate inflammation in vivo, are additional substrates of deiminases. A consequence of deimination is the decrease of the specific activity of the cytokines, suggesting that PAD activity also has the capacity to dampen an inflammatory response. Cytoplasmic substrates such as vimentin (Van Steendam et al. 2010) and f-actin-capping protein (Matsuo et al. 2006) are modified by PADs, and their deimination likely affects the proper function of the cytoskeleton. Notably, nuclear and cytoplasmic enzymes provide additional substrates for PAD, which include enolase (Wegner et al. 2010) and PAD4 itself (Andrade et al. 2010). These examples serve as evidence for the diversity of cellular functions that are affected by deiminases. Moreover, the initial hypothesis that citrullination provides new epitopes which break tolerance is strongly supported by the accumulated evidence.

The role of NETs in the pathogenesis of RA is an area of research that has yielded much information to date, although NETs are suspected as important contributors to disease manifestations in an increasing number of disorders, including SLE, vasculitis (Yoshida et al. 2013), colitis (Chen et al. 2008), multiple sclerosis (Bradford et al. 2014), Felty's syndrome, and gout (Schauer et al. 2014). In RA, neutrophils infiltrate into affected joints just before, or concurrent with, the first signs of pain, stiffening, and inflammation (Patel and Haynes 2001). The long-term consequences of RA are damage to the cartilage and bones of the affected joints, leading to permanent deformation and loss of function. The influx of neutrophils into the fluid-containing capsule surrounding the joints, which is called the synovium, therefore deserves close scrutiny. Early studies of joints from animal models of RA revealed that neutrophils represent the most numerous cellular infiltrate and that sections of

the inflamed joints demonstrate extracellular PAD (Damgaard et al. 2014) and citrulline-containing immunoreactive material (van Beers et al. 2013). These observations motivated efforts to more closely characterize RA synovial fluids and their cellular infiltrates.

Synovial fluids can be prepared from swollen joints of RA patients, a procedure that is clinically effective because it provides a temporary relief of pain. The fluid is rich in pro-inflammatory cytokines, including TNF and IL-17, and contains high numbers of activated neutrophils that migrate to this location. A comparison between synovial neutrophils and those from peripheral blood of the same individual, or synovial neutrophils of osteoarthritis patients, revealed that RA synovial neutrophils are much more prone to release NETs (Khandpur et al. 2013). This tendency held true whether additional NET-inducing cytokines were added to the cultures or not. As expected, RA neutrophils displayed increased PAD activity, and purified NETs contained citrullinated enolase and vimentin. Importantly, NETosis of RA neutrophils was enhanced by addition of synovial fluids or autologous patient serum, and antibodies present in RA sera alone had stimulatory activity. Release of NETs from patient neutrophils was dependent on deiminase activity and reactive oxygen species, as shown by the use of inhibitors of PAD4 or NADPH oxidase.

A separate study reported increased deimination in neutrophils exposed to cytolytic agents and high extracellular calcium. Incubation of neutrophils with perforin and granzymes, proteins expressed by cytolytic T cells, induced extensive deimination, resembling synovial cell extracts prepared from RA patients with active disease (Romero et al. 2013). Proteomic analysis of synovial extracts identified deiminated cytoskeletal proteins, such as actin, tubulin, myosin, vinculin, vimentin, talin, and coronin, secreted proteins, such as serpin B6, heat-shock protein 60, and myeloperoxidase, along with histones H2A and H4. Interestingly, the analysis also identified deiminated plasminogen, fibrinogen, and collagen type 1, suggesting that the experimental treatment also led to the release of active PAD to the extracellular space, consistent with cell rupture. This finding would explain the observed deimination of extracellular substrates, many of which are recognized as autoantigens in autoimmune diseases.

A set of recent studies explored neutrophil NETs in the pathogenesis of lupus. Both studies (Caielli et al. 2016; Lood et al. 2016) examined the response of neutrophils to anti-ribonucleoprotein autoantibodies and observed that the NETs released under these conditions consisted mainly of oxidized mitochondrial DNA. Perhaps SLE-specific stimuli determine the type of neutrophil response, and, in turn, the components of NETs affect the clinical manifestations of the autoimmune response. Previously, oxidized mitochondrial DNA was shown to be particularly effective at stimulating the Toll-like receptor pathways and inducing the activation of dendritic cells (Pazmandi et al. 2014). The recent neutrophil analyses clarified features of neutrophil mitophagy and possible deviations in this process that may lead to lupus (Muller and Radic 2016). Overall, the study of PAD4 contributions to autoimmunity portends many additional insights and raises new questions, some of which are listed in Box 2.3.

Box 2.3: Questions Concerning the Role of PAD4 in Autoimmunity

1. What is the comprehensive proteome of PAD4 substrates following specific stimuli?
2. Which autoantigens contain binding determinants that are focused on and depend on citrullines?
3. What is the contribution of environmental factors that predispose to autoimmunity (e.g., diet, obesity, injuries, or infections) toward the activation of PAD4?

2.7 Model of PAD4 Activation in the Pathogenesis of Autoimmune Disease

The release of NETs represents an immune mechanism that may act as a final barrier to disseminated disease. However, NET release carries with it a major risk, which is the release of extracellular proteases and reactive oxygen species that contribute to tissue damage. In addition, the enzymatic reactions that make essential contributions to NET release also lead to the release of modified autoantigens to the exterior of the cells. Thus, sequestration of nuclear autoantigens is disrupted, and chromatin and other nuclear autoantigens become accessible to cell surface receptors of antigen-presenting cells. Moreover, the release of NETs occurs in close apposition with immunostimulatory cytokines and pathogen-derived, adjuvant-like molecules that may enhance the development of autoreactivity. As argued by others, environmental triggers associated with choices in lifestyle (Makrygiannakis et al. 2008) and diet (Mohanan et al. 2013) also may promote activation of PADs along with the release of NETs. Given the currently available evidence, it appears likely that, over time, chronic stimulation of neutrophils by infections or inflammation provides conditions that enhance the production of deiminated autoantigens both inside and outside of tissue-infiltrating neutrophils. A good example of the progression of disease is provided by patients with Felty's syndrome. Usually, Felty's develops after several years of progressive RA (Dwivedi and Radic 2014). Patients are likely to receive medication that leads to immunosuppression, leaving the individuals more prone to infections. The recurrent infections likely lead to frequent neutrophil activation and aggravated NET production. Additional stimulation of the adaptive immune system by deiminated autoantigens gives rise to ACPA. In turn, these autoantibodies promote NET release. Secondary effects will likely include splenomegaly due to the clearance of spent neutrophils and additional stimulation of the adaptive immune response. Overall, these interactions mutually enhance each other, and the individually protective adaptations mutually synergize to drive autoimmunity.

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