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Unconventional post-translational modifications in immunological signaling

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The activity of a cell is governed by the signals it receives from the extracellular milieu, which are 'translated' into the appropriate biological output, such as activation, survival, proliferation, migration or differentiation. Signaling pathways are responsible for converting environmental cues into discrete intracellular events. The alteration of existing proteins by post-translational modification (PTM) is a key feature of signal-transduction pathways that allows the modulation of protein function. Research into PTMs has long been dominated by the investigation of protein phosphorylation; other PTMs, such as methylation of lysine and arginine residues, acetylation, and nitrosylation of thiol groups and tyrosine residues, have received comparatively little attention. This Review aims to present an overview of these PTMs, with an emphasis on their role in cells of the immune system.

Cells that coordinate immune responses have evolved to sense and respond to invasion by microorganisms as well as to recognize altered self, such as that present during viral infection or the cancerous transformation of cells. The immune response is generally subclassified into two arms: innate immunity and adaptive immunity. Recognition by the innate arm of the immune system results in the release of proinflammatory cytokines and, in most cases, the clearance of pathogens. The detection of pathogen-associated molecular patterns by members of the Toll-like receptor (TLR) family is critical for driving the innate immune response[1]. Induction of the expression of type 1 interferons (IFN-α and IFN-β) is a common outcome of TLR ligation[1]. Type 1 interferons are a family of cytokines with pleiotropic effects that serve mostly antiviral, antiproliferative and immunomodulatory roles. When an infection escapes the innate response, the second arm (the adaptive immune response) takes the helm. Unlike the innate arm, the adaptive arm, which is composed of B lymphocytes and T lymphocytes, recognizes specific components from infectious agents via antigen receptors. Cells of the adaptive immune system consolidate signals generated by the ligation of antigen receptors with the cytokine environment to orchestrate an appropriate immune response.

Several signaling pathways are commonly used by cellular receptors of the innate and adaptive immune responses. The NF-κB family of transcription factors is important for mediating receptor signaling in both the innate immune system and the adaptive immune system, as these factors are situated downstream of the family of receptors for tumor-necrosis factor (TNF), the TLRs and receptors for antigens. In resting cells, NF-κB is retained in the cytoplasm by the IκB inhibitor of NF-κB. After stimulation of the cells, IκB is phosphorylated, polyubiquitinated and degraded, which thereby releases NF-κB to enter the nucleus[2]. Ligation of many cytokine receptors results in activation of the Jak tyrosine kinases family and their substrates that are members of the STAT family of transcription factors; this is collectively known as the Jak-STAT pathway[3]. STAT proteins that contain a Src-homology 2 domain represent a family of latent cytoplasmic transcription factors that are activated by a large number of extracellular signals, such as growth factors and cytokines. Tyrosine-phosphorylation of STAT proteins in response to activation via receptors mediates their homo- or heterodimerization through reciprocal interactions of phosphorylated tyrosine residues with Src-homology 2 domains and their subsequent translocation to the nucleus, where they activate the transcription of immediate-early-response genes[3]. The calcium-dependent signal-transduction pathway of the transcription factor NFAT is a central regulator for gene expression in many types of cells of the immune system[4]. NFAT resides in the cytoplasm of unstimulated cells as a highly phosphorylated molecule. Mobilization of calcium results in activation of the phosphatase calcineurin, which dephosphorylates NFAT, unmasking its nuclear-localization sequence and thus allowing NFAT to enter the nucleus, where it interacts with many different binding partners to induce genes encoding immunoregulatory molecules[4].

As illustrated in the description of the Jak-STAT, NF-κB and NFAT pathways above, post-translational modifications (PTMs) serve an important role in regulating signal-transduction pathways. In this context, phosphorylation and ubiquitination are widely appreciated PTMs; however, it has become increasingly clear that numerous other covalent changes to existing proteins—some of which are irreversible—can have an equally important effect on protein activity. Among those modifications is the methylation of the amino groups on lysine or arginine residues, as well as acetylation or nitrosylation of proteins. In this Review, we summarize the most prevalent of those PTMs (**Fig. 1**) and their roles in the immune system.

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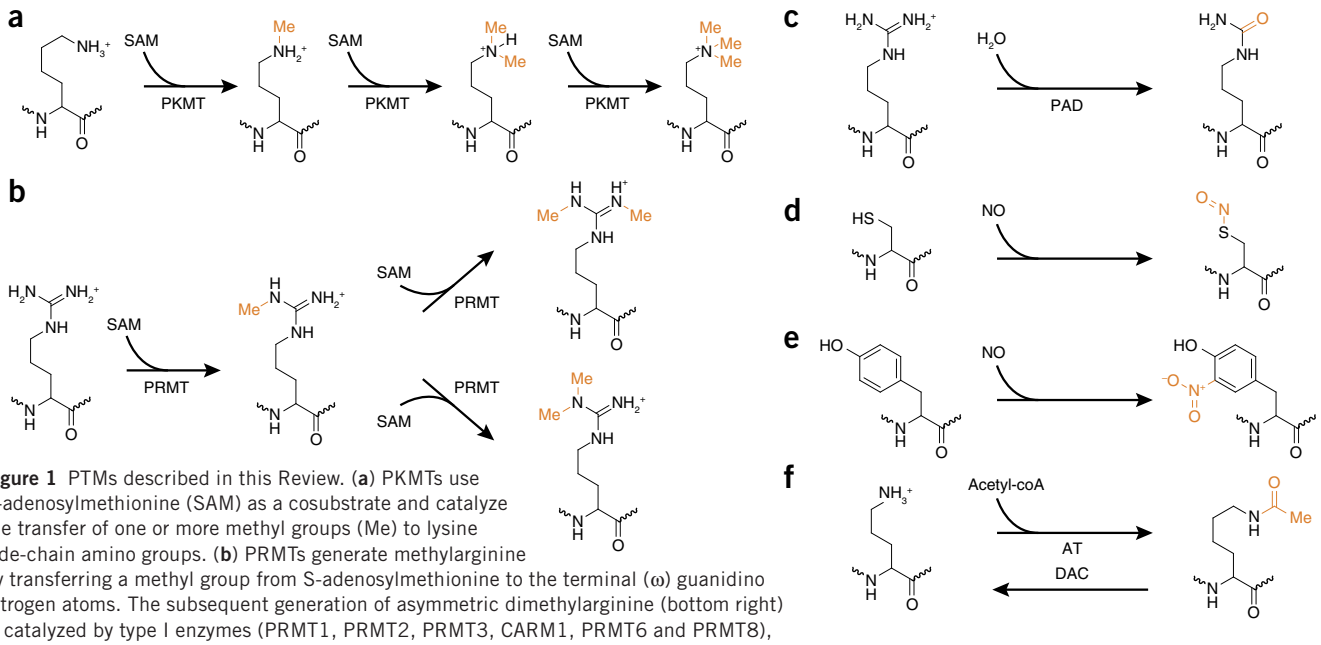

Figure 1 PTMs described in this Review. (**a**) PKMTs use S-adenosylmethionine (SAM) as a cosubstrate and catalyze the transfer of one or more methyl groups (Me) to lysine side-chain amino groups. (**b**) PRMTs generate methylarginine by transferring a methyl group from S-adenosylmethionine to the terminal (ω) guanidino nitrogen atoms. The subsequent generation of asymmetric dimethylarginine (bottom right) is catalyzed by type I enzymes (PRMT1, PRMT2, PRMT3, CARM1, PRMT6 and PRMT8), and the production of symmetric dimethylarginine (top right) is catalyzed by the type II enzyme PRMT5. PRMT7 functions as a type III enzyme, which generates only a monomethylarginine product (middle). (**c**) PAD enzymes convert arginine residues into citrulline residues by a hydrolysis reaction. (**d**) S-nitrosylation is a redox-mediated PTM that regulates protein function through the covalent reaction of NO-related species with a cysteine thiol group on the target protein. (**e**) The nitration of tyrosine residues consists of the addition of a nitro group to one of the two equivalent ortho carbons of the aromatic ring of tyrosine. (**f**) Acetyltransferases (AT) transfer the acetyl moiety is from acetyl–coenzyme A (Acetyl-coA) to the target lysine on the substrate; deacetylases (DAC) remove acetyl groups from acetylated lysine residues.

Methylation of lysine and arginine

Methyltransferases catalyze the transfer of a methyl group from the cofactor S-adenosylmethionine onto the side chains of lysine or arginine residues (**Fig. 1a**,**b**). Over 30 protein lysine methyltransferases (PKMTs), in nine related families, have been identified in humans. The enzymatic activity of PKMTs can result in the formation of monomethyl-, dimethyl- or trimethyl-lysine residues[5]. Except for the DOT1-like histone H3 methyltransferase, all PKMTs have a 131–amino acid catalytic SET domain. The methylation of lysine is a well-described PTM present on histones, where it serves a prominent function in regulating chromatin states and, thus, gene expression. The methylation of lysine residues on histones provides a motif recognized by epigenetic regulator proteins containing chromodomains, tudor domains and WD40-repeat domains[6]. Detailed discussion of the effect of such methylation on the immune system is beyond the scope of this Review[5]. Instead, we note that several excellent reviews on this subject have been published[7–10]. Our discussion here will focus on findings pertaining to the few described, non-histone targets of PKMTs that are most directly relevant to immunology.

There are nine members of the protein arginine methyltransferase (PRMT) family in humans. Three types of PRMTs have been subclassified on the basis of the symmetry of their reaction products. Type I PRMTs (PRMT1, PRMT2, PRMT3, PRMT4 (CARM1), PRMT6 and PRMT8) catalyze the asymmetrical methylation of arginine residues, and the type II PRMT PRMT5 catalyzes symmetric transfer of methyl groups to arginine residues[11] (**Fig. 1b**). Both type I and type II PRMTs catalyze monomethylation as a reaction intermediate. The type III PRMT PRMT7 catalyzes the monomethylation of arginine as its end product[12]. S-adenosylmethionine is the methyl donor for the reaction. Despite the similarity of its sequence to that of PRMT1, PRMT2 has not yet been demonstrated to have methyltransferase activity[11]. Like many PTMs, methylation of arginine regulates protein-protein interactions. For example, methylation of arginine in the proline-rich region of the RNA-binding protein Sam68 by PRMT1 prevents interactions with its SH3 domain–binding partners (Fyn, Lck and Itk) while permitting its interaction with WW domain–containing partners[13]. Since their discovery, PRMTs have been shown to regulate transcription, the subcellular localization of protein and RNA, RNA splicing, DNA-damage repair and signal transduction[11].

Methylation and immunological function

Several studies have highlighted the methylation of lysine in non-histone substrate proteins as an important regulator of immune system function (**Fig. 2**). The PKMT SETD6 monomethylates the NF-κB subunit p65 at Lys310. SETD6-mediated methylation of p65 leads to the attenuation of p65-induced transcription of genes encoding inflammatory molecules by creating a docking site for the histone methyltransferase GLP, an inhibitor of transcription[14]. Methylation of p65 at Lys37 by SET9 regulates the binding of p65 to DNA and facilitates the transcription of a subset of TNF-induced p65-regulated genes[15]. Modification of p65 at Lys218 and Lys221 by the PKMT NSD1 promotes p65-driven transcription, possibly by increasing the affinity of methylated p65 for DNA[16,17]. Additionally, the DNA-bound form of STAT3 is methylated at Lys140 by SET9 following stimulation with interleukin 6 (IL-6). Preventing such methylation through the introduction of a lysine-to-arginine substitution affects only a subset of STAT3-regulated genes[18]. Thus, methylation of lysine residues in p65 and STAT3 acts to 'fine-tune' their transcriptional activity.

PRMT1 can modify and regulate several critical immunomodulatory proteins. Coengagement of the costimulatory receptor CD28 and the T cell antigen receptor (TCR) enhances PRMT1 activity and the methylation of arginine residues in cellular proteins, including methylation of the guanine nucleotide–exchange factor Vav1; this results in augmented IL-2 expression[19]. Incubation with MDL 28842, an irreversible inhibitor of S-adenosylhomocysteine hydrolase, diminishes the methylation of Vav1, as well as IL-2 production[19]. Methylation of arginine residues in the NFAT cofactor NIP45 by PRMT1 in

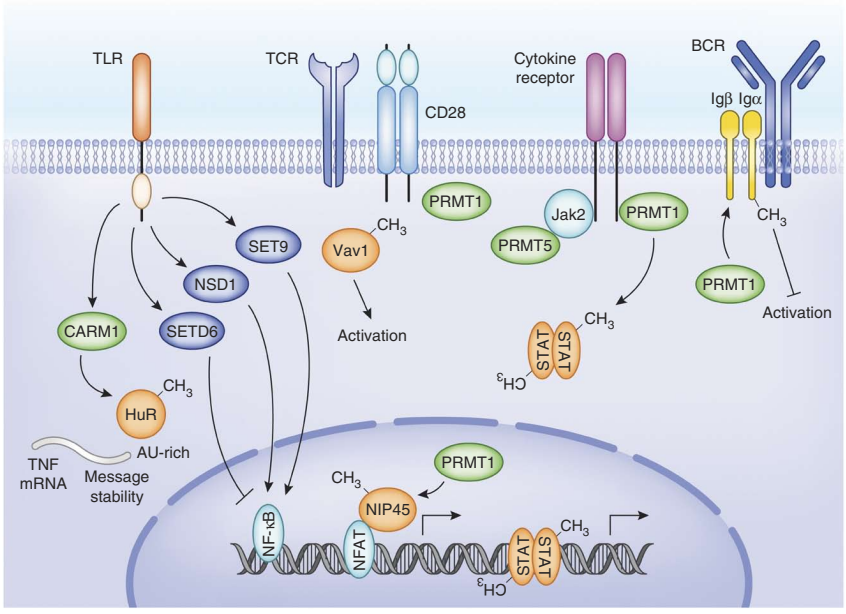

Figure 2 Protein methyltransferases regulate innate and adaptive immune responses. Engagement of the TLR leads to CARM1-mediated methylation (CH_3) of arginine residues in HuR, which correlates with stability of TNF mRNA. The PKMTs NSD1 and SET9 promote NF-κB-driven transcription, while SETD6 acts to impede NF-κB activity. In helper T cells, methylation of arginine residues in NIP45 by PRMT1 facilitates the interaction of NIP45 with NFAT and stimulates the expression of cytokine-encoding genes. The TCR signaling pathway controlled by CD28 has been shown to promote methylation of arginine residues in Vav1, which is associated with the activation of T cells. PRMT1 interacts with the cytoplasmic domain of the receptor for type I interferon, and depletion of PRMT1 alters IFN-α-induced growth arrest. Methylation of STAT1 by PRMT1 prevents the association of STAT1 with its inhibitor PIAS1; thus, PRMT activity serves to increase both cytokine expression and signaling. PRMT5 was first isolated through its ability to bind to Jak2. PRMT1 methylates the immunoglobulin α-chain (Igα) of the B cell antigen receptor (BCR) and thus negatively regulates PI(3)K pathways and promotes B cell differentiation.

helper T cells promotes its association with NFAT and thereby drives IL-4 expression[20,21]. A conserved arginine residue (Arg198) in the cytoplasmic tail of the immunoglobulin α-chain in the B cell antigen receptor is methylated by PRMT1, and that modification interferes with activation of the lipid kinase PI(3)K mediated by the B cell antigen receptor. Introduction of an immunoglobulin α-chain with substitution of lysine for arginine at position 198 into pre-B cells inhibits IL-7-facilitated B cell differentiation[22]. Collectively, these results demonstrate that PRMT1-induced methylation of arginine residues can regulate key signaling events downstream of antigen receptors.

Type I interferons bind to receptors consisting of the subunits IFNAR1 and IFNAR2 present on most cell types[23]. PRMT1 has been identified as an IFNAR1-binding protein in a yeast two-hybrid screen[24]. Abrogation of PRMT1 expression through the use of antisense constructs diminishes the antiviral and antiproliferative effects of IFN-α and IFN-β[24]. In addition, studies using genome-wide RNA-mediated interference to screen for additional regulators of the Jak-STAT pathway in *Drosophila* cells have revealed a potential regulatory function for the *Drosophila* CARM1 homolog Dart4 (ref. 25). Through the use of yeast two-hybrid technology, PRMT5 was originally cloned as a Jak2-binding protein, although a function for PRMT5 in Jak-STAT signaling has not been defined[26]. Methylation of STAT1 at Arg31 prevents its interaction with PIAS1, a negative regulator of the STAT1 dimer complex, and thereby promotes IFN-α- and IFN-β-induced STAT1-dependent gene expression[27]. Although there has been controversy about methylation of arginine residues in STAT1 (ref. 28), several studies have also reported amino-terminal methylation of arginine residues in STAT3 and STAT6 (refs. 29–31). Liver biopsies of patients infected with hepatitis C virus have revealed defective interferon signaling with less methylation of STAT1 and more STAT1-PIAS1 association[32]. Finally, *STAT1* mutations identified in patients with chronic mucocutaneous candidiasis, too, lead to diminished methylation of arginine residues in STAT1, enhanced association of STAT1 with PIAS1 and a diminished response to IFN-γ following restimulation. Thus, methylation of arginine residues in STAT1 has an important role in regulating the cellular response to stimulation with interferon.

The PRMT CARM1 (PRMT4) was originally described as a transcriptional coactivator that associates with the p160 family of coactivators in nuclear receptor–mediated transcription[33]. Transcriptional coactivation by CARM1 is mediated by the methylation of histones, a modification correlated with active transcription[34]. CARM1 directly associates with p65, and stimulation of *Carm1*$^{-/-}$ mouse embryonic fibroblasts with TNF or lipopolysaccharide results in impaired expression of a subset of p65 targets that encode proinflammatory molecules[35]. Recruitment of CARM1 facilitates p65-driven gene expression through the deposition of transcriptionally permissive histone-methylation marks[35]. CARM1 can also regulate the expression of genes encoding proinflammatory molecules, such as TNF, through post-transcriptional targeting of the RNA-binding Hu proteins. The Hu protein HuR binds to AU-rich elements in the TNF transcript and stabilizes this mRNA[36]. HuR is a substrate of CARM1, and the presence of the methylated form of HuR correlates with stability of HuR target mRNA[37]. Together these results demonstrate an important role for CARM1-mediated methylation of arginine residues in inflammation and suggest that CARM1 might be a target for the treatment of inflammatory diseases.

Analysis of mice lacking CARM1 has revealed a function for the methylation of arginine residues in regulating the development of cells of the immune system. CARM1-deficient mice have diminished lung airspace due to unrestrained proliferation of pulmonary epithelial cells and succumb to respiratory distress during the perinatal period[38]. Thymocytes purified from CARM1-deficient embryos are arrested between $CD4^-CD8^-$ double-negative stage 1 and double-negative stage 2, which is accompanied by a profound decrease in thymic cellularity[38,39]. Coculture of *Carm1*$^{-/-}$ fetal liver cells with OP9 stromal cells that express the Notch ligand DL1 has demonstrated that CARM1 is essential for the survival of thymocytes, which may explain the blockade at double-negative stage 1 in *Carm1*$^{-/-}$ thymocytes[39]. Notably, reconstitution of CARM1-sufficient hosts with *Carm1*$^{-/-}$ hematopoietic progenitor cells from fetal liver or

bone marrow has revealed that the developmental block in *Carm1*$^{-/-}$ thymocytes is cell autonomous[39]. The identification of CARM1 substrates in the hematopoietic lineage will be important for determining the molecular defect that underlies the T cell–developmental defect of *Carm1*$^{-/-}$ mice.

Targeting methylation for cancer treatment

Epigenetic regulation mediated by enzymes of the PKMT and PRMT families have an essential role in tumorigenesis, and several members of these families have been linked to malignancies derived from cells of the immune system. In some cases of multiple myeloma, chromosomal translocation results in the control of *NSD1* expression by strong enhancers of the gene encoding the immunoglobulin heavy-chain complex[40,41]. Tumorigenic, activity-enhancing mutations in the gene encoding the PKMT enzyme EZH2 can be found in non-Hodgkin lymphoma. The activity of EZH2 mutants leads to the generation and disposition of the repressive methylated H3K27 mark on genes encoding tumor suppressors. Selective targeting of EZH2 mutants offers a promising therapeutic strategy for such patients[42]. PRMT5 is overexpressed in some subtypes of non-Hodgkin B cell lymphomas. The epigenetic marks induced by PRMT5 are repressive and lead to the silencing of genes encoding tumor suppressors, which suggests that compounds that block PRMT5 activity may be useful for the treatment of lymphoma[43–45]. Conversely, a constitutively active, oncogenic version of Jak2, Jak2V617F, found in myeloproliferative neoplasms, phosphorylates PRMT5 and impairs its activity, which contributes to the oncogenic processes driven by Jak2V617F[46]. Thus, in the context of some oncogenic mutations, blockade of PRMT5 can drive neoplastic growth, which will need to be considered in the development of anti-PRMT5 therapies.

Mixed-lineage leukemia is a very aggressive and predominantly pediatric blood cancer characterized by fusion proteins produced by a chromosomal translocation between the gene encoding the PKMT MLL and genes encoding a variety of fusion partners, which results in the formation of an oncogenic transcriptional complex. That translocation destroys the enzymatic activity of MLL, and instead the fusion protein and its binding partners confer new epigenetic functions[47]. Many fusion partners of MLL recruit the PKMT DOT1L, which contributes to the activation of genes that drive leukemogenesis[47]. PRMT1 is an essential component of the MLL-EEN fusion-protein transcription complex, and direct fusion of MLL with PRMT1 enhances the self-renewal ability of hematopoietic cells. The transformative ability of the MLL-PRMT1 fusion requires the enzymatic activity of PRMT1 and is specific because fusion with CARM1 does not promote MLL-mediated transcriptional activity[48,49]. These data support the proposal that targeting PKMT and PRMT enzymes may be a successful strategy for treating malignancies of the immune system.

Citrullination of arginine

Peptidylcitrulline is generated through the hydrolysis of arginine residues by the calcium-dependent peptidylarginine deiminase (PAD) enzymes (**Fig. 1c**). The conversion of arginine to the amino acid citrulline (called 'citrullination' or 'deimination') results in only a small change in molecular mass (less than 1 dalton) and in the loss of a positive charge, which can have considerable consequences on protein structure and protein-protein interactions. Five PAD enzymes (PAD1–PAD5) have been identified in mammals, and they exhibit fairly high amino acid sequence homology (~41–55%)[50]. Members of the PAD family require high calcium concentrations for their activity[51]. Treatment with the calcium ionophore ionomycin induces endogenous PAD activity and subsequent protein citrullination[50]. In fact, structural analysis of the PAD4 has revealed five calcium-binding sites that are reasonably conserved among all PAD enzymes (except PAD6)[52,53]. Binding of calcium leads to a change in conformation that moves the key catalytic residue Cys645 to the enzyme's active site[53]. The expression pattern of members of the mammalian PAD family is fairly tissue restricted[50]. Of the PAD family, PAD2 and PAD4 are the only members detected in hematopoietic cells[54,55], and therefore this Review will focus on what is known about PAD2 and PAD4 in immunological function.

Of all the PAD enzymes, PAD2 has the most ubiquitous expression, with high expression documented in the central nervous system, monocytes, macrophages and mast cells[50,56]. Studies have provided insight into the function of PAD2 in various types of cells of the immune system. In macrophages, PAD2 has been shown to target IKKγ, a kinase upstream of NF-κB activation, and citrullination of IKKγ suppresses activation of NF-κB[57]. The NF-κB pathway is downstream of many proinflammatory pathways, which suggests that under some circumstances, PAD2 may be important for resolving inflammation[58].

Injured cells release high concentrations of ATP, which can act as a damage-associated molecular pattern and can provide an immunostimulatory signal through stimulation of the receptor for P2X7 (refs. 59,60). Stimulation of that receptor induces calcium flux and downstream signaling via the receptor, which leads to the production of several inflammatory molecules[61] (**Fig. 3a**). Stimulation of the receptor for P2X7 by ATP also induces PAD2-mediated citrullination of proteins[56]. P2X7-mediated activation of PAD2 regulates the expression of transcripts encoding the TNF receptor TNFR2, the metalloproteinase ADAMTS9 and the GTPase Rab6B in mast cells. Interestingly, PAD2 and its citrullinated substrate proteins are released from mast cells after activation with ATP[56]. The identity of the citrullinated proteins released and the function of extracellular PAD2 remain unknown.

PAD4 expression is restricted mainly to hematopoietic cells, especially granulocytes, and PAD4 has been linked to the regulation of inflammation[54,55]. After being recruited to the site of infection, neutrophils can kill invading pathogens by phagocytosis, by the release of preformed microbicidal granules and by the generation of reactive oxygen species (ROS)[62,63]. Neutrophils can also kill extracellular pathogens by 'weaponizing' their nuclear contents, releasing neutrophil extracellular traps (NETs)[64]. NET structures are composed of decondensed chromatin decorated with antimicrobial mediators such as defensins, neutrophil elastase, myeloperoxidase and citrullinated histones[64]. PAD4 is essential for the production of NETs and NET-associated citrullination of histones, and PAD4-deficient neutrophils are unable to form NETs[65,66]. PAD4-mediated citrullination of histones is a hallmark of NET formation and is thought to serve a mechanical role in NET formation whereby the conversion of positively charged arginine residues into neutral citrulline residues by PAD4 promotes chromatin decondensation[67] (**Fig. 3b**). PAD4-mediated NET formation is critical for the control of at least a subset of bacterial infections, as PAD4-deficient mice are more susceptible to infectious disease in a model of necrotizing fasciitis[66]. The formation of NETs by neutrophils, although critical for full activation of the innate immune response[68], has also been linked to the pathogenesis of inflammatory diseases, including rheumatoid arthritis (RA)[69] and lupus[70].

Citrullination of arginine in autoimmunity

Multiple sclerosis (MS) manifests as a chronic inflammatory demyelinating disease[71]. Citrullination of proteins in the brain and spinal cord

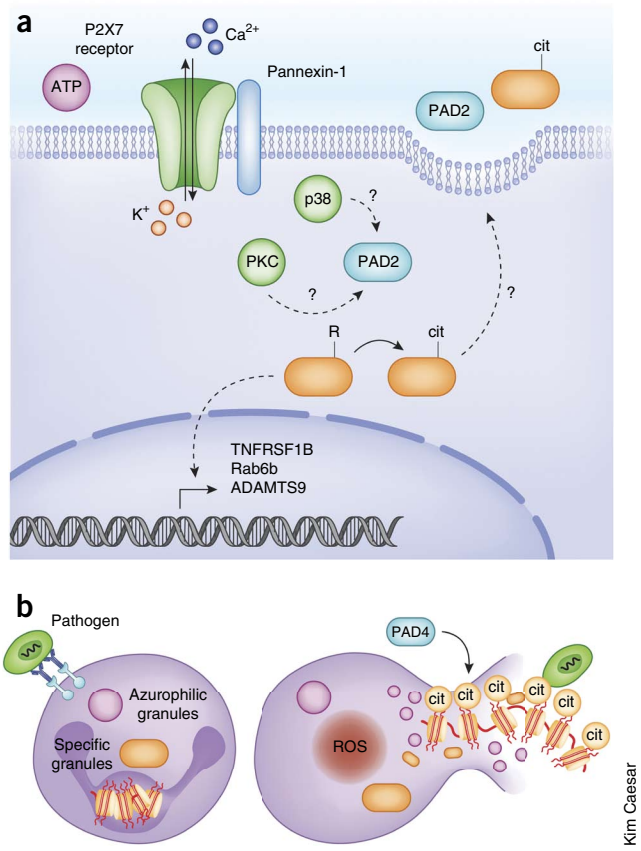

Figure 3 PAD-activation pathways in the immune system. (**a**) Activation of the receptor for P2X7 by the inflammatory 'danger signal' ATP induces PAD2 activity and robust citrullination (cit) of arginine (R) in proteins. P2X7-mediated activation of PAD2 is sensitive to inhibitors of p38 and protein kinase C (PKC), and PAD2 regulates the expression of transcripts encoding TNFR2, Rab6b and ADAMTS9 in mast cells. PAD2 and its citrullinated substrate proteins are released from mast cells after activation with ATP. (**b**) Pathways that activate the formation of NETs are known to require NADPH oxidase activity and the activation of PAD4 and subsequent citrullination of histones.

is a hallmark of MS[51] and of the mouse model of MS, experimental autoimmune encephalomyelitis (EAE)[72]. Both PAD2 and PAD4 are localized to the myelin sheath in patients with MS and in EAE[73]. The present model for the role of citrullination in MS is that citrullination of the protein components of myelin interferes with their association with lipids and is thought to directly contribute to the instability and degradation of myelin[51]. PAD activity is upregulated in mice with transgenic expression of extra copies of the myelin proteolipid protein DM20, and these mice develop spontaneous demyelination[74]. Expression of PAD2 has also been documented in the central nervous system, including microglia[75], astrocytes[75,76] and oligodendrocytes[77]. Indeed, mice with transgenic overexpression of PAD2 under control of the promoter of the gene encoding myelin basic protein exhibit increased citrullination of myelin basic protein and spontaneous demyelinating disease[78]. While detectable citrullination of proteins in the central nervous system of mice with EAE is entirely dependent on the presence of PAD2, PAD2-deficient mice are susceptible to EAE[79]. The role of PAD2 in EAE and in MS is probably complex, and other members of the PAD family, such as PAD4, might participate in disease pathogenesis.

RA is a chronic autoimmune inflammatory disease of the synovial joints. A major advance in the RA field was provided by the discovery that the main target of serum autoantibodies that recognize keratin and perinuclear structures is actually citrullinated fibrin[80]. Autoantibodies directed against citrullinated epitopes, called 'anti–citrullinated peptide antibodies' (ACPAs), exhibit high specificity and sensitivity as diagnostic markers of RA. ACPAs can appear before disease onset and correlate with the most erosive form of RA[69]. Indeed, there is a strong association between ACPAs and the so-called 'shared epitope' HLA-DRB1 major histocompatibility complex (MHC) class II molecules that are linked to susceptibility to RA[69]. The conversion of arginine to citrulline increases the binding affinity of peptide-MHC for one of the HLA-DRB1 molecules[69], and transgenic DR4-IE mice carrying one of the *HLA-DRB1* alleles develop an autoimmune arthritis response to citrullinated fibrinogen but not to uncitrullinated fibrinogen[81]. ACPAs develop in the mouse model of collagen-induced arthritis, and immunization with citrullinated collagen or administration of ACPAs can enhance the disease course of arthritis in mouse models[82,83]. The development of autoantibodies to citrullinated epitopes in RA suggests that aberrant PAD activity contributes to this disease.

Plasma and synovial biopsy specimens from patients with RA contain high concentrations of citrullinated proteins[69]. PAD2 and PAD4 are the only PAD enzymes detected in hematopoietic cells and in the RA synovium[54,55]. However, so far only polymorphisms in *PADI4* (which encodes PAD4) have been specifically linked to RA in studies of patients from Asian populations and in a large European cohort[69,84,85]. PAD enzymes can presumably act on extracellular proteins, since collagen and fibrinogen are both substrates of PAD enzymes[86,87], but the mechanism by which the PAD molecules gain access to the extracellular space is unknown. The association of PAD4 with NET structures and the release of PAD2 following activation of the receptor for P2X7 in mast cells indicate possible mechanisms by which PAD2 and PAD4 may be liberated from the cell to generate citrullinated antigens and exacerbate inflammation[88,89]. However, the cascade of events that lead to the activation of PAD enzymes during autoimmune-mediated inflammation is unknown at present. Thus, there is strong evidence supporting a pathogenic role for both PAD2 and PAD4 in RA, but the exact mechanism(s) by which PAD2 and PAD4 contribute to the pathophysiology of RA is (are) unclear.

S-nitrosylation and tyrosine nitration

A common feature of inflammatory diseases is increased stress due to overproduction of the highly reactive free radicals ROS and reactive nitrogen species. Nitric oxide (NO) is one such molecule that can not only function as a signaling mediator under physiological conditions but also cause a cellular stress response when overproduced in an inflammatory environment. PTM of proteins due to an increase in the abundance of reactive nitrogen species encompasses the nitrosylation of cysteine residues, as well as nitrosylation of the aromatic ring in tyrosines (**Fig. 1d**,**e**). The generation of NO from L-arginine is facilitated by the NO synthase enzymes, which include endothelial, neuronal and inducible NO synthase[90]; the expression of inducible NO synthase is substantially upregulated during inflammatory processes. Because of the short half-life of reactive nitrogen species, nitrosylation is compartmentalized as a short-distance signaling mechanism.

The S-nitrosylation of proteins is a PTM whereby the NO group is covalently bound to the cysteine SH group to produce the S-nitroso derivative (**Fig. 1d**); that can be reversed via denitrosylating enzymes[91]. S-nitrosylation can lead to both activation of enzyme function and its inactivation[33,92,93], and structural features that accommodate S-nitrosylation have been identified[94]. Several studies have linked

S-nitrosylation to apoptotic events. For example, apoptosis is affected by the nitrosylation of XIAP, the most prevalent member of the IAP family of antiapoptotic proteins. S-nitrosylation of XIAP inhibits its E3 ligase and antiapoptotic activity[95]. S-nitrosylated caspases can transfer their NO group to XIAP in a process called 'trans-nitrosylation'[96]. Apoptosis is also induced through S-nitrosylation of the kinase Erk, which prevents the phosphorylation needed for its activation[43]. Yet another example of the initiation of apoptotic responses via S-nitrosylation is found in the nitrosylation of glyceraldehyde phosphate dehydrogenase, which results in augmented binding and stabilization of the E3 ligase SIAH[97]. However, S-nitrosylation seems to affect immune responses beyond the modulation of apoptosis. *O*-linked *N*-acetylglucosaminyltransferase exists in an S-nitrosylated form in resting cells and is subject to denitrosylation and concomitant activation in response to lipopolysaccharide[98]. Although only a few other S-nitrosylated proteins have been reported, a large-scale proteomics study has revealed that S-nitrosylation is a rather common PTM[99].

Similar to S-nitrosylation, the nitration of tyrosine residues (**Fig. 1e**) also alters the activity of various enzymes or transcription factors. At present, nitration of the aromatic ring in tyrosine seems to be an irreversible modification, and denitrating enzyme activity has yet to be identified. For example, nitration of tyrosine inhibits the activity of the superoxide dismutase MnSOD[100]; intriguingly, that modification can be triggered by the immunosuppressive drug cyclosporin A[101]. Furthermore, nitration of tyrosine residues in STAT1 prevents its phosphorylation in response to IFN-γ and thus attenuates the activation of macrophages[102]. An important aspect of modulation of the immune response via the nitration of tyrosine residues lies in the increased immunogenicity of such modified polypeptides. In mice with transgenic expression of pigeon cytochrome *c*, a prominent immune response can be elicited by immunization with cytochrome *c* peptides that have tyrosine nitration but not by unmodified peptides[103]. Confirmatory data have been obtained by the immunization of mice with transgenic expression of hen-egg lysozyme[104], which indicates that this PTM of tyrosine is sufficient to break central tolerance and might promote the development of autoimmunity. Indeed, as with citrulline, antibodies to 3-nitrotyrosine have been found in human blood. In addition to modification of antigens, nitration of tyrosine also occurs in the TCR–coreceptor CD8 complex; peroxynitrate produced by myeloid cell–derived suppressor cells causes the nitration of TCR-CD8, which makes such T cells unable to bind their cognate peptide-MHC complex. As a result, the nitration of tyrosine residues in the TCR-CD8 complex promotes evasion of the immune system by tumors due to increased tolerance by $CD8^+$ T cells[105].

Acetylation of lysine

The acetylation of lysine residues (**Fig. 1f**) represents a PTM executed by acetyltransferases that affects a large number of histone and non-histone proteins; it is reversible through the activity of deacetylases. The alteration of chromatin structure via the acetylation of histone and its effect on gene transcription is well recognized, and excellent reviews on this topic have been published[8,106,107]. However, an increasing number of non-histone substrates have been reported as targets of acetylation, which affects the stability of mRNA and protein, their subcellular localization and their interaction with other proteins or DNA. Several such targets of acetylation are known to have decisive roles in the development and function of the immune system.

After recognizing pathogen-associated molecular patterns, TLRs initiate downstream signaling events that lead to the production of many different types of immunomodulatory molecules, including type I interferons[1]. TLR signaling is affected by acetylation of the dual-specificity mitogen-activated protein kinase phosphatase MKP-1. Acetylation of MKP-1 promotes the interaction of this with its 'preferred' substrate, the mitogen-activated protein kinase p38, and thus limits TLR responses to prevent excessive inflammation[108]. Similarly, deacetylase activity is also required for TLR-mediated transcription of interferon-stimulated genes via activation of the transcription factor IRF3 in response to viral infection[109]. Finally, HMG-A1, a member of the HMG ('high-mobility group') family of chromosomal proteins, is subject to acetylation at Lys65 and Lys71 by the acetyltransferases CBP and PCAF, respectively. While the first modification results in decreased IFN-β expression, the latter positively affects transcription of the gene encoding IFN-β[110]. Thus, acetylation of lysine residues is important for 'fine-tuning' TLR-induced signaling processes and the resulting innate immune response.

Transcription factors of the NF-κB family have crucial roles in both the development and the function of the immune system, and p65, among others, is required for the transcriptional induction of genes encoding proinflammatory mediators, especially downstream of TLRs[2]. Activation of NF-κB is regulated not only by the phosphorylation and ubiquitination of its inhibitor IκB but also through direct targeting for modification, including modification by the CBP-p300 coactivator family[111]. The acetylation of lysine in NF-κB has been reported to lead to increased or dampened transcriptional activity, depending upon the lysine residue targeted[2].

Members of the STAT family are commonly activated by ligation of cytokine receptors, and, not surprisingly, the STAT transcription factors have important functions in both innate immune responses and adaptive immune responses[3]. STAT1 is indispensible for the biological functions of IFN-α, IFN-β and IFN-γ, and acetylation of STAT1 interferes with the expression of interferon-stimulated genes[109,112]. Furthermore, acetylation of STAT1 enables its binding to p65 and its attenuation of the expression of NF-κB-controlled genes[113]. In contrast to the apparently inhibitory effect of acetylation on STAT1 function, STAT3-mediated responses are promoted by its acetylation. Thus, acetylated STAT3 undergoes more dimer formation and, consequently, displays enhanced DNA binding and transactivation of cytokine-responsive target genes[114,115].

Transcription factors of the GATA and Foxo ('forkhead box') families are also regulated by acetylation. Members of the GATA family have vital roles in hematopoiesis[116], and Foxo transcription factors have been shown to serve critical roles in the development of B cells and T cells[117–119]. GATA-2, which is crucial for the survival and population expansion of hematopoietic stem cells, is acetylated by p300 and PCAF, which leads to increased DNA binding and transcriptional response[120]. Acetylation of GATA-3 by p300 promotes its transactivation potential. As GATA-3 is essential for T cell development, the loss of acetylation of GATA-3 negatively affects T cell homeostasis and homing of T cells to secondary lymphoid organs[121]. Acetylation of Foxo transcription factors in response to cytokines or growth factors via CBP-p300 or PCAF lessens their ability to bind DNA and consequently attenuates their transactivation ability[122].

Closing remarks

The catalog of post-translational alterations explored in this Review and their effect on the immune system is far from complete. Sulfation, ribosylation of ADP, hydroxylation, isomerization of proline and carboxylation are but a few examples of additional modifications that contribute to the regulation of protein function. In the past, research into such unconventional PTMs has been (and to a large extent is still) hampered by the lack of adequate (immunological) reagents

that specifically recognize the modified proteins rather than their naive counterparts. Consequently, investigation into such modifications is dependent mainly on the use of mass spectrometry, which, although it is more reliable, is also not impervious to errors. High-throughput proteomics has been used to identify large sets of proteins with methylation of arginine residues in T cells; such studies will be important for the identification of previously unknown roles for unconventional PTMs in regulating immunity[123]. Bioinformatics analysis of such data sets not only will potentially reveal consensus sequences surrounding the modified amino acid (information that is now largely unavailable) but will also probably shed light on the regulation of the enzymes responsible for the unconventional PTMs. As more and more attention is directed toward such unconventional covalent protein modifications, it would not be surprising to find that they rival phosphorylation and ubiquitination in their importance as regulatory adaptations used by cells to respond to changes in their environment, and that they have important implications for the development and function of the immune system.

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