Citrullination and PAD Enzyme Biology in Type 1 Diabetes – Regulators of Inflammation, Autoimmunity, and Pathology

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The generation of post-translational modifications (PTMs) in human proteins is a physiological process leading to structural and immunologic variety in proteins, with potentially altered biological functions. PTMs often arise through normal responses to cellular stress, including general oxidative changes in the tissue microenvironment and intracellular stress to the endoplasmic reticulum or immune-mediated inflammatory stresses. Many studies have now illustrated the presence of 'neoepitopes' consisting of PTM self-proteins that induce robust autoimmune responses. These pathways of inflammatory neoepitope generation are commonly observed in many autoimmune diseases including systemic lupus erythematosus, rheumatoid arthritis, multiple sclerosis, and type 1 diabetes (T1D), among others. This review will focus on one specific PTM to self-proteins known as citrullination. Citrullination is mediated by calcium-dependent peptidylarginine deiminase (PAD) enzymes, which catalyze deimination, the conversion of arginine into the non-classical amino acid citrulline. PADs and citrullinated peptides have been associated with different autoimmune diseases, notably with a prominent role in the diagnosis and pathology of rheumatoid arthritis. More recently, an important role for PADs and citrullinated self-proteins has emerged in T1D. In this review we will provide a comprehensive overview on the pathogenic role for PADs and citrullination in inflammation and autoimmunity, with specific focus on evidence for their role in T1D. The general role of PADs in epigenetic and transcriptional processes, as well as their crucial role in histone citrullination, neutrophil biology and neutrophil extracellular trap (NET) formation will be discussed. The latter is important in view of increasing evidence for a role of neutrophils and NETosis in the pathogenesis of T1D. Further, we will discuss the underlying processes leading to citrullination, the genetic susceptibility factors for increased recognition of citrullinated epitopes by T1D HLA-susceptibility types and

provide an overview of reported autoreactive responses against citrullinated epitopes, both of T cells and autoantibodies in T1D patients. Finally, we will discuss recent observations obtained in NOD mice, pointing to prevention of diabetes development through PAD inhibition, and the potential role of PAD inhibitors as novel therapeutic strategy in autoimmunity and in T1D in particular.

ORIGIN AND IMPLICATIONS OF POST-TRANSLATIONAL MODIFICATIONS IN AUTOIMMUNITY

At a simplistic level, the success of immunity relies on distinguishing 'self' from 'non-self', originating primarily by purging the autoreactive repertoire both in central lymphoid organs, the thymus and bone marrow, as well as by peripheral tolerance mechanisms. Classical mechanisms of immune tolerance rely on the processing and presentation of self-peptides by antigen presenting cells (APCs), resulting in deletion or anergy of the autoimmune repertoire. However, it is clear that many self-proteins, or post-translational modifications (PTMs) to self-proteins, are not expressed in primary lymphoid organs. For example, PTMs specific to peripheral tissues may not be expressed in the thymus and thus these PTMs never tolerize or delete the emerging thymocytes. While we know that proteins are fundamentally assembled from 20 amino acid structures, the addition of PTMs pushes that group above 140 structurally unique amino acids (1). These observations have altered the concepts and the breadth of immune tolerance to self-proteins, particularly in the perspective of autoimmune syndromes. Many factors influence the rate at which PTMs arise in self-proteins, including the amino acid sequence, flanking amino acid motifs, and variables such as the tissue microenvironments. PTMs also alter specific peptides that arise by antigen processing and, subsequently, the specificity of ongoing B- and T-lymphocyte immunity (2–4).

Some PTMs arise by enzymatic processes, as with citrullination reviewed herein, or with N-linked glycosylation or phosphorylation, critical to biological functions of many host proteins. In contrast, some modifications arise spontaneously, under physiologic pH and temperature, such as the formation of isoaspartyl modifications [reviewed by (5)]. Also, some modifications can be processed either enzymatically or spontaneously, such as deamidation (6). As illustrated herein, PTMs may trigger aberrant autoimmunity as well as alter the biological functions of self-proteins in selected tissues, including transcriptional and translational events. The text that follows will review the conditions that elicit citrulline PTMs, including their role in biological and immunological processes, with specific focus on their implications in type 1 diabetes (T1D).

Citrullination, also known as deimination, is a PTM in which an arginine residue is converted into a citrulline (**Figure 1**). This modification, which was first described in 1939 (7), leads to a loss of one positive charge and a reduction in mass of 0.984 Da per modified arginine. This type of modification can alter intra- and inter-molecular interactions of the protein (8), having an impact on its structure, function and its interaction with other proteins (9). Citrullination is catalyzed by peptidylarginine deiminase (PAD) enzymes in a Ca^{2+}-dependent manner (10), which is thought to be an irreversible process (11).

A role for citrullinated self-proteins has been associated with several autoimmune diseases, such as T1D, rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), multiple sclerosis (MS), psoriasis, Sjögren's syndrome (SS), antiphospholipid syndrome (ALS) and inflammatory bowel disease (IBD) (**Table 1**). Among these different autoimmune diseases, citrullination as an autoimmune biomarker in RA has been most extensively described. RA is a chronic autoimmune disease characterized by inflammation of the synovial joints. Proteomic analysis of the cellular and soluble components of RA synovium identified the full RA citrullinome, with more than 100 citrullinated proteins, amongst which vimentin, enolase, fibrinogen and fibronectin (46). Some of these were shown to induce autoantibodies and/or autoreactive T-cell responses in RA (11, 22, 29, 30). The presence of anti-citrullinated protein antibodies (ACPAs) in the serum of RA patients is one of the most specific diagnostic marker for the disease (47). ACPAs can be detected years before clinical symptoms appear (48). Apart from being a prognostic biomarker for disease development, some ACPAs have been described as useful in predicting the severity of joint destruction during the first five years after RA onset (49). ACPAs can also be detected in a small percentage of patients with SS (3 to 9.9%), and the presence of such

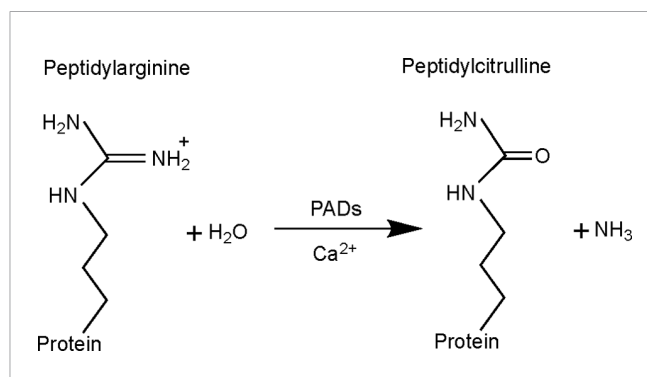**FIGURE 1** | Citrullination reaction catalyzed by peptidylarginine deiminase (PAD) enzymes. With the conversion of arginine into citrulline, the primary ketamine group of arginine (=NH) is replaced by a ketone group (=O), with production of ammonia as a side-product. This results in a mass difference of 0.984 Da and loss of one positive charge. Citrullination is catalyzed by PAD enzymes, requiring Ca2+ as cofactor.

TABLE 1 | Citrullination in autoimmune diseases.

Diseases	Target proteins	References
Type 1 diabetes (T1D)	GAD65	(12–14)
	IA-2	(13)
	GRP78	(15–17)
	IGRP	(13)
	IAPP	(17, 18)
Rheumatoid arthritis (RA)	fibrinogen	(19–21)
	vimentin	(22–24)
	histone H1, H2B, H3 and H4	(25–27)
	type 2 collagen	(28)
	α-enolase	(29)
	GRP78	(30)
Systemic lupus erythematosus (SLE)	LL37	(31)
	histone H1 and H3	(26, 32)
Multiple sclerosis (MS)	MBP	(33–36)
	GFAP	(36)
	neurogranin	(37)
	histone H3	(38)
Psoriasis	keratins	(39)
	filaggrin	(40)
Sjögren's syndrome (SS)	histone H1	(41)
	α-enolase	(42)
Antiphospholipid syndrome (ALS)	vimentin	(43)
Inflammatory bowel disease (IBD)	vimentin	(44, 45)

GAD65, glutamic acid decarboxylase 65; IA-2, islet antigen-2; GRP78, glucose-regulated protein 78; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; IAPP, islet amyloid polypeptide; MBP, myelin basic protein; GFAP, glial fibrillary acidic protein.

autoantibodies indicates a risk of developing RA (50). As listed in **Table 1**, emerging citrullinated target proteins are proposed as autoimmune disease specific biomarkers. Interestingly, the serological level of citrullinated vimentin in patients with ulcerative colitis (UC) was decreased compared to patients with another important IBD, Crohn's disease (CD), and non-IBD control subjects. Therefore, citrullinated vimentin has also been suggested as differentiating marker between CD and UC to improve the diagnostic accuracy in IBD (44).

PEPTIDYLARGININE DEIMINASES: TYPES AND MECHANISM OF ACTION

Peptidylarginine deiminases are a group of 5 enzymes encoded by genes localized on chromosome 1p36.1 in human and chromosome 4E1 in mice, located in a cluster of 350 kb and 240 kb, respectively (51). They were first described in 1977 as the enzymes mediating the conversion of arginine into the non-classical amino acid citrulline in proteins, from studies performed in mammalian hair follicles (52). The enzyme responsible for this reaction was for the first time partially purified by Fujisaki and Sugawara in 1981 and named peptidylarginine deiminase (PAD) (53). Since then, five different PAD isozymes have been described in mammals, designated as PAD1 to PAD4 and PAD6, which display 50-70% sequence identity (54). PADs replace the primary ketamine group of arginine (=NH) by a ketone group (=O) and yield ammonia as a side-product (**Figure 1**), leading to the loss of one positive charge of the target protein. This can lead to changes in the function and fate of the citrullinated target protein.

Although the PAD enzymes are widely expressed throughout the body, and have been implicated in a variety of physiological processes, each isozyme has specific tissue distribution, functions, and substrates under physiological conditions (8) (**Figure 2** and **Table 2**). As such, PAD1 is predominantly expressed in skin epidermis, uterus and hair follicles, targeting keratin and filaggrin (86). PAD2 is the ubiquitous member of the family, being expressed in multiple tissues such as brain, skeletal muscle, spleen, uterus, secretory glands and leukocytes (60, 65, 71). Importantly, PAD2 and PAD4 are the only PAD isozymes expressed in immune cells. Amongst other targets, PAD2 citrullinates myelin basic protein (MBP) in brain, vimentin in skeletal muscle and macrophages, actin in neutrophils and histones in various cell types (71). PAD3 is found in hair follicles and epidermis, and citrullinates filaggrin, trichohyalin, apoptosis-inducing factor (AIF) and vimentin (86). PAD4 is expressed in leukocytes, mainly granulocytes (like neutrophils and eosinophils), and monocytes and macrophages (63, 73). It is the only PAD isotype with a nuclear localization signal sequence, which is located at its N-terminus. PAD4 targets several nuclear proteins, such as histones, nucleophosmin and nuclear lamin C (71). With its high expression in neutrophils, PAD4 plays an important role in the generation of neutrophil extracellular traps (NETs) and thereby in the first line of defense against bacterial pathogenic invaders (see in more detail below). PAD6 is mainly expressed in eggs, embryo and ovary. It is the only PAD isozyme for which until today no protein substrates have been identified (86) and, in vitro, no catalytic activity can be measured (89). Also, no known association with autoimmunity or other diseases has been reported for PAD6.

Although all 5 PAD enzymes target arginine residues in proteins, they do have different substrate specificities. The basis
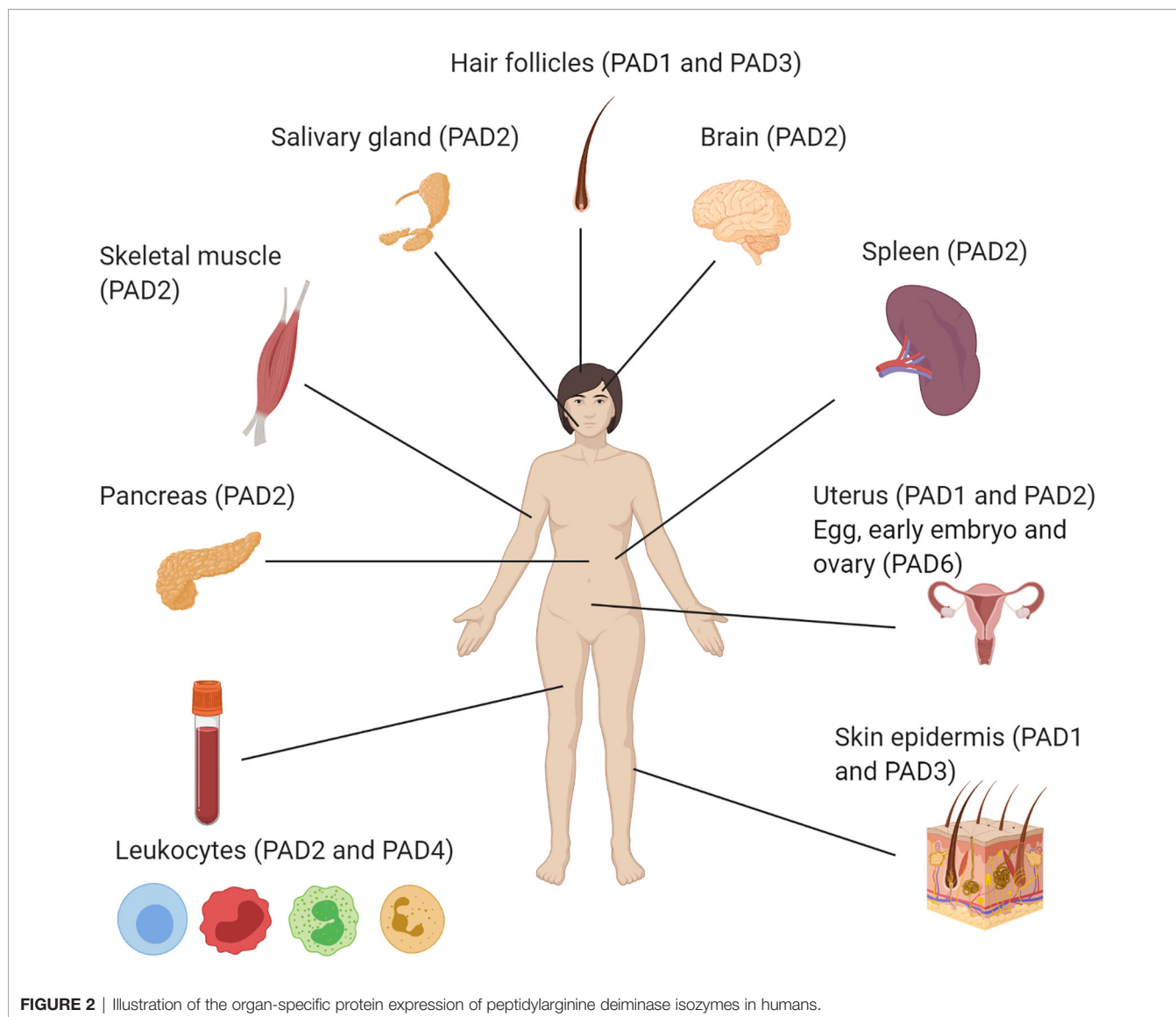
FIGURE 2 | Illustration of the organ-specific protein expression of peptidylarginine deiminase isozymes in humans.

TABLE 2 | PAD isozymes tissue distribution, target substrates, physiological functions and disease association.

Isozyme	Tissue distribution (Protein level)	Target substrates	Physiological functions	Disease association
PAD1	Skin epidermis, uterus (55) and hair follicles (56)	Keratin and filaggrin (57, 58)	Skin keratinization (59)	Psoriasis (39)
PAD2	Brain, skeletal muscle, spleen, spinal cord, uterus, secretory glands and pancreas (55, 60–62), leukocytes [macrophages (63), neutrophils (64) and T cells (65)]	Myelin basic protein (66), vimentin (63), actin (67), histones (68), fibrinogen and α- enolase (69)	Disassembly of vimentin filaments (70), CNS plasticity (9), epigenetic and transcriptional regulation (71), immune response (65, 72)	Rheumatoid arthritis, multiple sclerosis (73), Alzheimer disease (74) and prion diseases (75)
PAD3	Skin epidermis and hair follicles (55, 56)	Filaggrin, trichohyalin (56, 57), apoptosis-inducing factor (76)	Regulation of epidermal functions (57)	Unknown
PAD4	Leukocytes [mainly granulocytes, such as neutrophils and eosinophils (77, 78), monocytes, macrophages (63) and T cells (65)] and neurons (79)	Histones, nucleophosmin (80), nuclear lamin C (81), antithrombin (82), ING4 (83), NF-Kb (84), fibrinogen and α- enolase (69)	Epigenetic and transcriptional regulation (71), NET formation (85), immune response (65, 85)	Rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis (86) and cancers (87)
PAD6	Egg, early embryo and ovary (88)	No substrates identified; no activity *in vitro* (89)	Oocyte cytoskeletal formation and female fertility (90)	Unknown

ING4, inhibitor of growth 4; CNS, central nervous system; NET, neutrophil extracellular trap.

for this difference is not fully understood, but cannot solely be explained by their difference in tissue distribution or subcellular location (54). Additional influencing factors include the enzyme kinetics, the conformation of the secondary structure of the target protein and the flanking amino acid composition surrounding the arginine residues, as revealed from *in vitro* studies with recombinant PAD enzymes. In regard to the latter, it was shown for instance that a glutamic acid accompanying the arginine residue decreases the chance of being citrullinated, whereas a flanking aspartic acid residue increases the citrullination level (91). In this regard, PAD4 was found to have a higher substrate specificity as compared to PAD2 (92, 93).

The most important regulator of PAD activity is calcium, with micro to millimolar calcium concentrations needed for their full activation (53, 54, 94). However, under physiological conditions, with intracellular cytosolic calcium concentrations usually around 100-fold lower, a basal PAD activity can also be measured, in line with the occurrence of low levels of citrullination and their role during normal physiological processes. This raises questions as to how exactly calcium is mediating the activity of PADs. One thought is that calcium may alter the confirmation of PADs. Under low calcium conditions, PADs may be present in a conformation that selects only high efficiency substrates, thus allowing citrullinations to happen, but limiting aberrant citrullination events (11). In the presence of high calcium, all calcium binding sites of the PAD enzyme are occupied, promoting extensive rearrangement and dimerization of the enzyme, leading to its full activation, as shown for PAD4 (95, 96). Alternatively or additionally, other factors are known to regulate PAD enzymes (9), which could act as cofactors modulating their calcium sensitivity and specificity (11). Considering the importance of calcium in PAD activation, pathways that alter intra- or extracellular calcium levels, such as endoplasmic reticulum (ER) and inflammatory stress, are important in many of the autoimmune diseases in which citrullination is implicated (see in more detail below) and underscore the important link between citrullination and stress pathways. Indeed, changes in calcium fluxes may lead to activation of PAD activity in stressed or dying cells. Moreover, the externalization and activation of intracellular PAD enzymes into the extracellular space may occur in the surroundings of dying cells. This may explain the citrullination of extracellular proteins, as observed for instance during NET formation (NETosis) (see in detail below) or the citrullination of beta-cell proteins as observed in T1D (15). Similar observations in RA show that PADs are highly activated by Ca^{2+} ion deposition in the inflamed joints, particularly during apoptotic cell death (97, 98), leading to elevated levels of citrullinated proteins and peptides.

PROTEIN CITRULLINATION DETECTION

The first antibody-based methodology for citrullinated protein detection was described in 1992 by Senshu et al. (99).

The antibody does not recognize citrulline residues directly but instead binds a chemically modified form, diacetyl monoxime and antipyrine derivatized-citrulline, now available in commercialized kit form. The approach utilizes protein samples separated by SDS-PAGE, transferred to a microporous membrane. The membrane bound citrulline-containing proteins are modified in the presence of 2,3-butanedione monoxime and antipyrine in acid condition. In a similar concept known as the "Senshu" method, Moelants et al. developed a sandwich ELISA format to detect citrullinated proteins utilizing antibody recognizing 2,3-butanedione-modified citrulline (100). Another citrulline-specific labeling chemical probe, rhodamine-tagged phenylglyoxal derivative (Rh-PG), was developed for detection of citrullinated proteins in both purified protein sample and complex mixtures including serum (101, 102).

There are now commercially available anti-peptidyl citrulline antibodies for detection of citrullinated proteins including mouse monoclonal IgM antibody, clone F95, from Millipore (MABN328), and rabbit polyclonal antibody from Upstate (07-377) or Abcam (ab10092 and ab6464). Clone F95 antibody is extensively used in tissue staining, immunoblot and ELISA (25, 103, 104). The specificity and sensitivity of the above commercial anti-peptidyl citrulline antibodies for differentiating arginine citrullination and lysine carbamylation were recently discussed (105). Of note, carbamylation is a non-enzymatic PTM converting lysine to homocitrulline, which shares similar structural features with citrulline. For specific citrullinated target proteins, a variety of commercially available or in-house produced antibodies are available, such as those specific to citrullinated-histones, hypoxia-inducible factor 1-α, vimentin, fibrinogen, MBP and GRP78.

Upon citrullination, 0.984 Da mass increase from the parent peptide can be identified by mass spectrometry. As this is exactly the same increase in mass as for deamidated peptides, caution is needed when analyzing liquid chromatography-tandem mass spectrometry (LC-MS/MS) data, not to misinterpret a citrullinated peptide from a deamidated peptide on a closeby N- or Q residue. On top of that, C^{13} isotopes, with an increase in mass of 1 Da compared to C^{13} parent ions, can also be wrongfully identified as deamidated or citrullinated residues, even with the use of very accurate mass spectrometry instrumentation and strict settings. A recent study by Callebaut et al. (6) outlines critical parameters to detect deamidated residues by LC-MS/MS, through minimizing artificial *in vitro* occurring deamidations and manual inspection of spectra. This same method can also be applied for critical evaluation of citrullinated peptides from LC-MS/MS analysis. Another issue to be considered is that the loss of one positive charge of the arginine residue due to citrullination will affect protease cleavage efficiency for proteases which cleave after arginine, such as trypsin. This will result in mis-cleavages by trypsin, in case the arginine is citrullinated (106). Of particular interest is the dual search delta score method developed by Qian laboratory which integrates several critical parameters for identifying citrullinated and deamidated peptides in an automated way, thereby decreasing false discovery rates (FDR) (107). A different method used for facilitating LC-MS/MS-based

detection of citrullinated proteins is chemical derivatization of citrulline residues by 2,3-butanedione alone or combined with antipyrine, resulting in a mass increase of 50 or 238 Da, respectively (108, 109). Chemical derivatization of citrulline residues can also be used for enrichment of citrullinated proteins, through the use of biotin-conjugated phenylglyoxal (BPG), prior to LC-MS/MS analysis (101, 102). This method, however, requires large amounts of starting material. In addition, a BPG-based ELISA platform recently developed will validate the mass spectrometry proteomic data for citrullination detection (110).

INFLAMMATORY PATHWAYS INCREASE CITRULLINATION AND OTHER PTMS

Simply put, autoimmunity is initiated when cellular and soluble components of the immune system interact to trigger the recognition and robust response to self-proteins leading to tissue pathology. Additionally, a large number of heritable genetic risk traits have been defined by genome wide association studies (GWAS) in many autoimmune syndromes. However, autoimmune syndromes and T1D, in particular, are not entirely explained by a defined collection of heritable genetic traits. Indeed, poorly defined environmental influences and epigenetic factors, which may or may not be inherited, also influence the early onset and progression of T1D (111). Of importance, the amplification of PTMs in T1D autoimmunity is clearly linked to oxidative tissue environments. Reactive oxygen species (ROS), including superoxide anion ($O_2^{\cdot-}$), hydrogen radicals (OH.), and hydrogen peroxide (H_2O_2) are a product of a dynamic balance of endogenous anti-oxidant cellular compounds that control their tissue concentrations and biological effects. These anti-oxidants include superoxide dismutase (SOD), glutathione peroxidase, catalase, peroxiredoxins, as well as other small molecule anti-oxidants, including vitamins E and C. There are potentially a number of sources of specific ROS in tissue autoimmunity, including the infiltration of activated phagocytic cells (neutrophils, macrophages and dendritic cells) which have been demonstrated to be important in the progression and tissue pathology of T1D as well as many other autoimmune syndromes.

Oxidative stress can amplify the modification of certain proteins, or protein motifs, or, alternatively, alter metabolic pathways. As described in detail below, there are secondary effects of ROS on apoptosis, NETosis, and cellular metabolic pathways, affecting the progression of autoimmune responses and tissue pathology. ROS affected proteins may be changed in solubility, reduce the ability of proteins to be cleared, or increase immunogenicity. As detailed below, oxidation and subsequent citrullination can also provoke changes at both the level of DNA transcription and translation.

Citrullination of histone H3 by PAD4 in granulocytes, leading to formation of NETosis, is a process which is promoted by intracellular ROS (85, 112). Also, exogenous H_2O_2 was shown to induce citrullination and NETosis in mouse neutrophils (85).

Depending on the stimulus used, the level and time course of ROS production was shown to be important for subsequent H3 citrullination and NET formation (113). However, seemingly contradictory to this notion, *in vitro* studies showed a direct inhibitory effect of PAD2 and PAD4 activity in the presence of H_2O_2 concentrations above 40 μM. In addition, PMA-stimulated leukocytes could effectively citrullinate recombinant fibrinogen, although this process was markedly enhanced when ROS formation was inhibited by the NADPH oxidase inhibitor diphenyleneiodonium (DPI) (114). These findings suggest that although ROS is important intracellularly for mediating citrullination of histones, supraphysiological levels of ROS may inhibit citrullination extracellularly. Discrepancies in different studies on the exact role of ROS in PAD activation and citrullination may need further examination to resolve the impact of intra- vs. extracellular ROS concentrations.

Another important factor mediating PAD activity is the local redox balance, with a reducing environment needed for efficient PAD activation. As such, *in vitro* studies, making use of the non-physiological reducing agent dithiothreitol (DTT), have shown that PADs can be activated under reducing conditions. Also more physiological reducing agents, like thioredoxin (115) and reduced glutathione (GSH) (116) can activate PAD enzymes. Kinetic characterization of PAD4 using thioredoxin as reducing agent produced results equivalent to those obtained with DTT (115).

Clearly, ER stress is a key factor in the amplification of PTMs in T1D. The insulin producing beta-cell, i.e. the immune targeted cell in T1D, is particularly sensitive to ER stress, because of its highly developed ER needed to cope with the high demands for protein translation and folding (117–119), in response to acute changes in blood glucose levels. As such, beta-cells can increase the translation of preproinsulin up to 25-fold (120), reaching almost 1 million molecules of preproinsulin per minute (121), when blood glucose levels are high. In order to restore the equilibrium between the cellular demand for protein synthesis and the ER folding capacity, cells under ER stress activate the unfolded protein response (UPR) (122). The UPR is activated by three transmembrane protein sensors, activating transcription factor 6 (ATF6), protein kinase RNA-like ER kinase (PERK) and inositol-requiring kinase 1 (IRE1). Under physiological conditions, these three sensors are kept in an inactive state through binding to the chaperone glucose-regulated protein 78 (GRP78; also known as binding immunoglobulin protein (BiP) or heat shock 70kDa protein 5 (HSPA5)) (123). When misfolded proteins accumulate in the ER lumen, GRP78 releases from these sensors inducing their activation and downstream signaling (123) leading to ER stress attenuation. When this UPR fails to restore ER homeostasis, usually in conditions of intense or chronic stress, this adaptive UPR will change to a terminal UPR (124), activating pro-apoptotic signaling pathways that lead to cell death (122).

Next to ER stress induced by high demands of protein translation, typical for secretory cells such as beta-cells, environmental factors associated with T1D can trigger

additional ER stress in beta-cells (125), including coxsackie viral infection, dysglycemia, inflammation, ROS and exposure to chemicals such as streptozotocin and alloxan (125, 126). Apart from inducing beta-cell dysfunction and death, ER stress can also induce PTMs in beta-cells, a process that has been described for thapsigargin induced ER stress, showing increased activation of tissue transglutaminase (TGM2) and PAD, both Ca^{2+} dependent

enzymes (127, 128). Also, inflammatory cytokines, which act at least in part through activation of ER stress pathways, were shown to induce citrullination of beta-cell proteins (15, 16, 118, 129) (**Figure 3**). Interestingly, thapsigargin induced ER stress of human beta-cells leads to increased immunogenicity, as measured by IFNγ response of T-cell clones specific for deamidated peptides (127, 128).
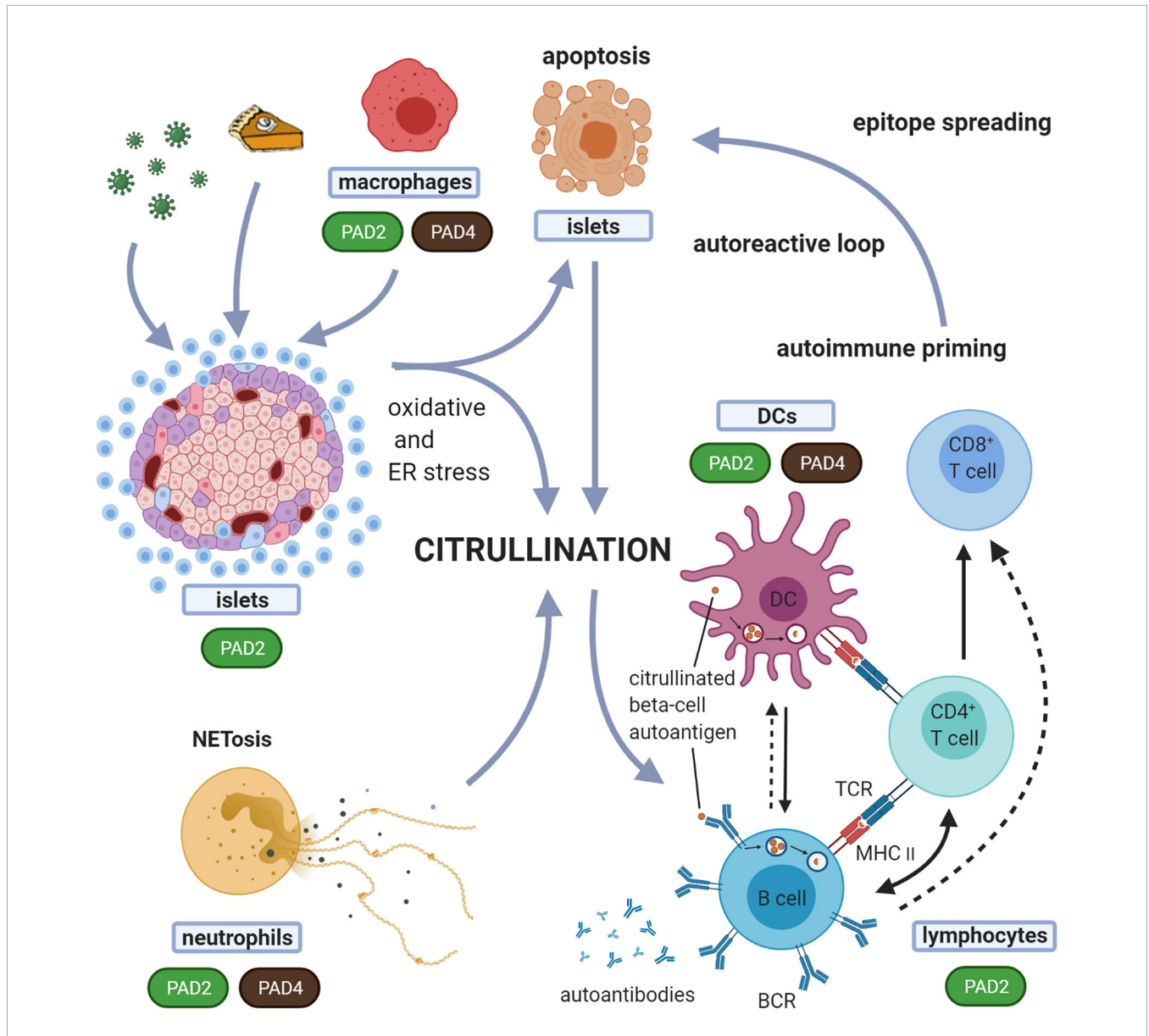

FIGURE 3 | Suggested model for the role of citrullination in the induction of autoreactive responses in T1D. In this proposed model, different forms of cell death, i.e. beta-cell apoptosis and neutrophil death (NETosis), are both implicated in the generation of citrullinated proteins. Any form of environmental stress, such as viruses, inflammatory cytokines or high metabolic demand, can induce oxidative and ER stress in the beta-cells. Oxidative stress induces NETosis. Both beta-cell apoptosis and NETosis have been shown to be involved in T1D initiation and propagation. With expression and activation of PADs during stress conditions, this model suggests that both beta-cells and neutrophils, can induce citrullination. These citrullinated peptides/proteins may be processed by the immune system in different immunogenic ways, forming a source of citrullinated autoantigens. Processing of these modified proteins/peptides by antigen presenting cells, and subsequent presentation to T cells, can in turn trigger several immune responses, including activation of B cells (producing islet autoantibodies) and islet antigen-specific (effector/memory) T cells that can directly kill beta-cells presenting citrullinated islet peptides. The expression of PAD enzymes in beta-cells and neutrophils, and their activation during processes of cellular stress, underscores the relevance of this model. Dashed arrows indicate the potential interactions between B cells and CD8+ T cells and between B cells and DCs. BCR, B cell receptor; TCR, T-cell receptor.

CITRULLINATED PROTEINS AND THEIR ROLE IN FUNCTIONAL PATHWAYS

Epigenetic Role and Biological Functions of Histone Citrullination

Histones are highly basic proteins, due to the abundance of lysine and arginine residues, assembling with DNA to form nucleosomes. Changes of the positive net charge of histone due to PTMs will affect its electrostatic interaction with chromatin and chromatin accessibility such as phosphorylation, acetylation and citrullination. Of note, citrullinated histones account for about 10% of all histones in granulocytes (80). Extensive studies demonstrate that PAD catalyzed histone citrullination regulates chromatin structure (condensation versus decondensation), transcriptional regulation, and a variety of biological pathways (**Table 3**).

Histone H1 function relies on basic amino acid residues to interact with chromatin and is responsible for the formation of higher-order chromatin structure. Linker histone H1 citrullinated at residue 54 (H1Cit54; mediated by PAD4) results in activation of several pluripotency genes, such as klf2, Tcl1, Tcfap2c and Kit, due to chromatin decondensation (130, 131). Linker H1Cit54 has also been found in breast cancer cells (138) and in activated neutrophils (41). Relevant to autoimmune syndromes, autoantibodies against citrullinated linker H1 were found in about 6% of sera from patients with SLE and SS (41).

The protein core of nucleosomes is composed by core histones, H2A, H2B, H3 and H4. H2A function is finely regulated by several PTMs such as acetylation, phosphorylation and methylation (139, 140). For example, protein arginine methyltransferase 5 (PRMT5) methylates H2A at residue arginine 3 (H2AR3) which serves as an epigenetic activator to promote prostate cancer growth (140). Of note, PAD4-catalyzed citrullinated histone H2ACit3, is identified in activated neutrophils (77). Compared to other histone proteins, citrullination on histone H3 has been extensively investigated. Different PAD isozymes catalyze H3 at different arginine residues and regulate various biological functions. Both PAD1

and PAD2 citrullinate histone H3 at 2, 8 and 17 residues, H3Cit2,8,17. Citrullination of histone tails at H3R2,8,17 and H4R3 was significantly reduced in 2- and 4-cell embryos after PAD1-morpholino knockout or treatment with a PAD1 specific inhibitor. Deficiency of PAD1 resulted in mouse embryo cells arrested at the 4-cell stage (133). In human mammary epithelium cells (MCF7 cells), PAD2-catalyzed H3R2,8,17 regulates gene expression of pleotropin (PTN) and melanoma associated antigen A12 (MAGEA12) (141). In addition, PAD4-mediated H3Cit8 diminishes the binding of heterochromatin protein 1α (HP1α) to methylated histone H3K9 and leads to the suppression of gene expression of human endogenous retroviruses (HERVs) and cytokines in MCF7 cells such as TNFα, IL-1A, IL-8, IL-16 and IL-23 (136). PAD2-catalyzed H3Cit26 results in chromatin decondensation and transcriptional activation of estrogen receptor α-regulated genes in breast cancer cells (68). Therefore, H3Cit26 is believed to be a potential prognostic biomarker for estrogen receptor positive (ER+) breast cancer (137). Similar to PAD1-catalyzed H3Cit2,8,17, PAD4-catalyzed H4Cit3 also facilitates early embryo genome transactivation (133). Moreover, PAD4-catalyzed H4Cit3 regulates the p53 pathway in apoptosis and in carcinogenesis (81).

Citrullination and Its Role in NETosis

NETosis is a cellular clearance mechanism distinct from apoptosis, which occurs when neutrophils encounter microorganisms and produce highly modified chromatin webs (142), immobilizing and killing the pathogens. The extruded DNA webs carry a number of bound bactericidal proteins (lactoferrin, elastase, proteinase 3, myeloperoxidase, cathepsin G, etc.) as well as histones and granule proteins. Thus, NETs serve as the first line defense mechanism of innate immunity to protect the host from bacteria, fungi, viruses and protozoa. Emerging evidence reveals that PTMs of histones in neutrophils regulate NETosis, and is associated with the development of autoimmune diseases such as RA, MS and SLE (**Table 3**). In leukocytes, both PAD2 and PAD4 are expressed (60), with PAD2 being mainly expressed in macrophages and PAD4 in

TABLE 3 | Biological functions of histone citrullination.

Histone	PAD isozyme	Physiological or pathological roles	References
Linker H1Cit54	PAD4	impairs binding to nucleosomal DNA	(130, 131)
		chromatin decondensation in pluripotent stem cells	
H2ACit3	PAD4	occurs in calcium ionophore A23187-stimulated neutrophils	(77, 132)
H3Cit2,8,17	PAD1	facilitates early embryo genome transactivation	(133)
H3Cit2,8,17	PAD2	regulates lactation associated genes during diestrus in mammary epithelial cells	(69)
H3Cit2,8,17	PAD4	neutrophil extracellular trap (NET) formation and NETosis	(134, 135)
H3Cit8	PAD4	transcriptional repression of cytokines genes and human endogenous retroviruses (HERVs) *via* heterochromatin protein 1α (HP1α)	(136)
H3Cit26	PAD2	chromatin decondensation and transcriptional activation of estrogen receptor (ER) α-regulated gene	(68)
		potential prognostic biomarker for ER positive (ER+) breast cancer	(137)
H4Cit3	PAD4	facilitates early embryo genome transactivation	(133)
		regulates p53 pathway in apoptosis and in carcinogenesis	(81)
		neutrophil extracellular trap (NET) formation and NETosis	(12–14, 134)

H, histone; Cit, citrullination; PAD, peptidylarginine deiminase.

monocytes, macrophages, eosinophils and neutrophils (63, 78). In neutrophils, citrullination is crucial for NET formation and release (85, 143). Activation of PAD4 leads to hypercitrullination of histones and consequently, decondensation and release of DNA structures coated with neutrophil granule proteins, the NETs (134, 143).

NETosis has been indicated as the major autoantigen source in SLE. NETs induce moderate levels of autoantibodies against H3Cit2, 17 and H3Cit26 in MRL/lpr mice, a spontaneous murine model of lupus (32). Moreover, autoantibodies against H1R53 were detected in patients with SLE (41). Recently, abundant citrullinated LL37 was identified in SLE target tissue (skin and kidney) and autoreactive T cells against both native and citrullinated LL37 were detected in patients with SLE, but not in RA (31). As mentioned earlier, NETosis is enhanced in RA circulating and synovial neutrophils and correlates with ACPA titers (144). H1Cit53 and H3Cit8,17,26 were found in RA and SLE neutrophil NETs (26). One study showed the presence of anti-citrullinated H2B antibodies in the anti-CCP2 positive sera from patients with RA (25). In addition, both H3Cit2,8,17 and citrullinated H4 from NETs were found as the targets of autoantibodies in patients with RA (27, 145).

The involvement of neutrophils and NETosis in T1D has been pointed out by several studies. In the non-obese diabetic (NOD) mouse, a spontaneous mouse model of autoimmune mediated beta-cell destruction and diabetes development, neutrophils and formation of NETs in the pancreas have been shown to be present during early stages of disease development and are required for diabetes development (146). In human T1D, one recent study illustrated a reduction in serum components of NETs [neutrophil elastase (NE) and proteinase 3 (PR3)], consistent with a reduced overall neutrophil count in early onset T1D (147). Conflicting studies report increases in these same NET components in T1D and a positive correlation of the circulating levels of these components with titers of autoantibodies against IA-2 and GAD65 (148). Neutrophil count is decreased in newly diagnosed T1D adult and pediatric patients (147, 149) as well as in pre-symptomatic autoantibody individuals (150). This reduction correlates with a decline in beta-cell function (151). Additionally, neutrophils infiltrate the pancreas before disease onset and during disease progression (151) and a significant fraction of these pancreas-infiltrating neutrophils forms NETs (54% and 50% in autoantibody-positive and T1D donors, respectively) (151). Relevant to citrullination, protein expression of PAD4 was elevated in neutrophils from patients with T1D and T2D (152). Given all these data indicating that neutrophils and NETosis are involved in diabetes development, the role of PADs and citrullination has also been implicated in T1D through their high expression in neutrophils and essential role in NET formation (**Figure 3**).

Of note, besides neutrophils, macrophages can release extracellular traps, a process called macrophage extracellular trap formation (METosis). The contribution of METosis in T1D has not been investigated yet, but METosis and PAD4 have been shown to contribute to self-antigen citrullination and ACPA production in autoimmune arthritis (153).

Citrullination in Transcriptional Regulation in Immune Cells

Besides NETosis, citrullination has other functions in the immune system. In neutrophils, citrullination of NF-kB p65 enhances its nuclear translocation and transcriptional activity, increasing Toll-like receptor (TLR)-induced expression of IL-1β and TNFα (84). Citrullination of the transcription factors GATA3 and RORγt by PAD2 determines the fate of differentiating Th cells. As such, citrullination of GATA3 on R330 weakens its DNA binding ability, thereby decreasing transcription of Th2 cytokines, attenuating differentiation of Th2 cells. On the other hand, citrullination of RORγt on R56,59,77, 90 strengthens its DNA binding ability, increasing the transcription of IL-17A/F thereby enhancing the differentiation of Th17 cells (72). Also, citrullination of RNA polymerase II by PAD2 favors an efficient transcription of genes related to cellular proliferation (154). Citrullination can also reduce the potency of chemokines, such as CXCL8, CXCL10, CXCL11 and CXCL12, when compared to their native form (155–157), thereby dampening inflammatory reactions. Additionally, PADs can citrullinate the cytokine TNFα (158), reducing its capacity to stimulate the production of inflammatory chemokines, and TNFα can induce the translocation of PAD4 from the cytosol to the nucleus (38).

Citrullinated Proteins and Their Role in Increasing Antigenicity in T1D

A primary function of the immune system is to differentiate between self and non-self-proteins. This is achieved by mechanisms that deplete the immune system of lymphocytes that react too strongly to self-antigens being present in the thymus and bone marrow, resulting in tolerance towards self-proteins. For achieving T-cell tolerance, the medullary thymic epithelial cells (mTECs) play an important role in this so-called negative selection (159). This mechanism works effectively for many self-antigens that are expressed in the thymus, through mTEC specific transcriptional regulator AIRE (autoimmune regulator), which drives expression of tissue-restricted genes such as islet specific genes, in the thymus (160). However, whether post-translationally modified self-proteins (**Tables 1** and **4**) are also expressed in the thymus has not been extensively investigated. If not, this could create a novel autoantigenic proteome for which immune tolerance has not been established in the thymus. This concept, previously described as 'autoantigenesis' is a term described to proteins that 'evolve' and acquire PTMs in a disease related target tissue, during the course of disease development, leading to B and/or autoreactive T-cell responses (4). Evidence for such antigenic modifications was shown in a mouse model of tissue-restricted self-antigen collagen type II, where the PTM reactive T cells escaped thymic selection (162). In regard to citrullination, it has been shown that PAD2 and PAD4 are expressed in murine mTECs, as measured by qPCR, thereby demonstrating that the prerequisites for negative selection of citrulline-specific T cells in the thymus are met in C57Bl6 mice (163). However, whether these enzymes are active and able to convert arginine into

TABLE 4 | Citrullination in T1D.

Target proteins	Affected immune responses	References
GAD65	target of autoreactive T cells (HLA-A2 and HLA-DRB1*04:01)	(12, 13, 17)
IAPP	target of autoreactive cells (HLA-DR and HLA-DQ8)	(17, 127, 161)
IA-2	potential target of autoreactive T cells (HLA-A2)	(13)
GRP78	target of autoreactive T cells (HLA-DRB1*04:01) recognized by autoantibody	(15–17)
IGRP	potential target of autoreactive T cells (HLA-A2)	(13)

GAD65, glutamic acid decarboxylase 65; IAPP, islet amyloid polypeptide; IA-2, islet antigen-2; GRP78, glucose-regulated protein 78; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein.

citrulline in proteins locally in the thymus, and whether this PAD expression and citrullination capacity is defective in autoimmune strains, such as the NOD mouse, needs further investigation.

In T1D, progressive loss of B and T-cell tolerance to beta-cell specific antigens leads to the destruction of insulin producing beta-cells. A growing number of studies suggest that immune recognition of non-conventional generated peptides/proteins (164), amongst which citrullinated proteins (12, 15, 16, 165) are an important component of that loss of tolerance (**Table 4**). As such, citrullinated proteins, generated through different stress pathways in beta-cells or neutrophils, as outlined above, could be a source of citrullinated antigens. The expression of PAD enzymes both in beta-cells and neutrophils, their activation during processes of cellular stress, and the role for both beta-cell apoptosis and NETosis in initiation and propagation of T1D, fit with such view (as schematically shown in **Figure 3**). The citrullinated peptides/proteins may be processed by the immune system in different immunogenic ways, forming a source of citrullinated autoantigens. Presentation of citrullinated proteins/peptides by antigen presenting cells, and subsequent presentation to T cells, can trigger several immune responses, including activation of B cells (producing islet autoantibodies) and islet antigen-specific (effector/memory) T cells that can directly kill beta-cells presenting citrullinated islet peptides.

Already in 1993 it was shown that the insulin B chain is prone to citrullination in the bacterial model *Porphyromonas gingivalis* (166), however, only during the last decade several publications described the link between citrullinated beta-cell proteins and autoreactive responses in T1D. In comparison with native peptides, citrullinated peptides present higher binding affinity to HLA-A2 (13) and HLA-DRB1*04:01 (12, 13, 167), diabetes-associated HLA class I and class II molecules, respectively. Of interest, there is a significant overlap in genetic susceptibility between T1D and RA, with HLA-DRB1*04:01 haplotype being a high-risk haplotype in both diseases. The antigenicity of citrullinated beta-cell antigens that bind to HLA-A2 has been demonstrated by *in vitro* activation of CD8^{+} T-cell clones, expanded from peripheral blood of HLA-A2^{+} T1D subjects, when cultured with citrullinated peptides derived from IA-2, GAD65 and IGRP (13). One citrullinated peptide of GAD65 with higher binding affinity to HLA-DRB1*04:01 was shown to be recognized by CD4^{+} T cells in the peripheral blood of T1D

patients making use of *ex vivo* tetramer assays. These CD4^{+} T cells exhibited an antigen-experienced phenotype and were less- or non-responsive to the native form of the epitope (12). Shortly thereafter, Rondas et al. revealed that the ER chaperone GRP78, a major ER chaperone and a key mediator of the UPR pathway, can be citrullinated in INS-1E beta-cells under inflammatory stress, as shown by 2D-Western blotting using a citrulline specific antibody (16). This confirmed earlier proteomic studies showing an increase in PTM for GRP78 in INS-1E after IFNγ plus IL1β exposure (129). Citrullinated GRP78 was shown to be immunogenic in diabetes-prone NOD mice with appearance of both autoreactive T cells and autoantibodies (16). Furthermore, it was shown that NOD mice have significantly higher levels of *Padi2* mRNA and PAD activity in islets, already at the age of 3 weeks, as compared to normoglycemic C57Bl6 mice (16, 165). Absence of IL1β and IFNγ expression at this young age, indicated no or minor immune infiltration in the islets, suggesting expression of PAD in the endocrine cells. Further proof for this was provided in a later study, in which CD45^{+} islet infiltrating immune cells from 10-week-old NOD mice were separated from beta and alpha cells by FACS sorting. Expression of *Padi2* mRNA was evident in all 3 fractions, with comparable levels in beta-cells and CD45^{+} immune cells (Sodré et al., 2021). Further *in vitro* studies showed that GRP78 was not only citrullinated upon inflammatory stress, but was also translocated from the ER to the plasma membrane and secreted, at which level the modification of GRP78 was even much more pronounced (16, 118). This opens the view that GRP78 may become citrullinated once exposed on the plasma membrane or even after its secretion into the extracellular space. First evidence for a role of citrullinated GRP78 in human T1D, came from the discovery of a CD4^{+} T-cell clone, isolated from an islet outgrowth of a deceased T1D patient, which recognized citrullinated GRP78 epitope 292-305 (citrullinated at position 297) (17). The same study also showed autoreactive CD4^{+} T cells against citrullinated IAPP. Direct evidence for citrullination of human GRP78 at arginine residue 510 in cytokine-exposed islets was shown by targeted LC-MS/MS. Using *ex vivo* tetramer and ELISA assays, the same study showed that a subpopulation of T1D patients presents higher frequencies of CD4^{+} T cells against a citrullinated GRP78 epitope and elevated titers of autoantibodies against citrullinated GRP78 compared to

healthy subjects (15). Moreover, T-cell responses and autoantibodies against citrullinated GRP78 were more frequent in long-standing T1D than in patients with new-onset T1D. Although the number of patients included in this study is too low to make hard conclusions, these last findings may indicate that immune responses against citrullinated GRP78 contribute to aggravation and/or acceleration of the disease rather than to the initiation of the disease development. Longitudinal studies on at-risk subjects are awaited to further evaluate the exact role of citrullinated antigens in disease staging.

PTMS CAN TRIGGER MORE EXTENSIVE AUTOIMMUNITY, SUCH AS EPITOPE SPREADING

Several investigators have identified significant differences between the T- and B-cell responses that develop against PTM self-proteins, or cryptic epitopes (5, 13, 128, 168–170). In general, T-cell responses to PTM determinants tend to be specific for the modified peptide only and more rarely cross-react with the unmodified form of the peptide. This concept was originally demonstrated in mice immunized with the isoaspartyl PTM form (isoAsp) of snRNP D lupus autoantigen protein, showing T cells only proliferate in response to the isoAsp PTM, but are unresponsive to the native (Asp) peptide (168). Alternatively, B-cell and autoantibody responses are often more promiscuous in their binding to both the PTM modified and native self-protein. This phenomenon may be due to the features of antibodies to bind flanking amino acid sequences in both modified and native protein forms. As one example, human SLE and lupus-prone MRL/lpr mice exhibit autoantibodies that bind both isoAsp and Asp forms of histone H2B (171). Moreover, it was demonstrated that autoimmune responses originating from the PTM self-protein diversify in an intra- and extra-molecular manner to other self-protein determinants. Therefore, breaking immune tolerance to a PTM self-protein promotes 'epitope spreading', a mechanism where autoimmunity diversifies to epitopes beyond the initial site(s) initiating the response (170). Similarly, there is both intra- and inter-molecular B- and T-cell epitope spreading in T1D, SLE, and MS (168, 172). Classical studies have described epitope spreading of autoantibody responses with the progression of autoimmune disease (173). As illustrated earlier, apoptotic and necrotic cells are rich sources of PTM-altered self-proteins in various microenvironments, notably conditions of oxidative stress (174) or altered pH (175).

In both of the collagen-induced arthritis (CIA) and experimental autoimmune encephalomyelitis (EAE) mouse models, citrullination of joint and brain proteins creates neoantigens that become additional targets in epitope spreading of autoimmune responses (172). Citrullination of aggrecan, vimentin, fibrinogen, and type II collagen, known target proteins in RA, initiates epitope spreading by promoting T-cell responses to both citrullinated peptides and the corresponding control peptides (176). In addition,

citrullination-induced conformational changes of HSP90 protein unmasks cryptic epitopes to bypass B-cell tolerance in RA (177). Epitope spreading may also apply for citrullinated epitopes in triggering T1D autoimmunity (**Figure 3**). This is shown for example for citrullinated GRP78, where circulating CD4$^+$ T cells and autoantibodies against both naïve and citrullinated GRP78 peptides are detectable in patients with T1D, suggesting that epitope spreading due to citrullination also occurs in T1D (15).

PTMS IN ANTIGEN PROCESSING AND PRESENTATION

It is clear that the specificity of ongoing immunity relies on the efficient and accurate antigen processing pathways in epitope generation (178). The presence (or absence) of a PTM of an amino acid residue has been shown to alter the recognition and cleavage by proteases. Additionally, the presence of PTM within an intracellularly processed peptide alters the binding to specific MHC motifs. For example, it has been demonstrated that the absence of N-glycosylation of the neuronal glutamate receptor subunit 3 in Rasmussen's encephalitis, a severe form of pediatric epilepsy, exposes a granzyme B cleavage site. This is just one example of how PTMs may create a novel autoantigen (neoepitope) (179). As another example, most proteases and peptidases *do not* recognize the peptide linkage connecting isoAsp residues to its neighboring amino acid (180). Finally, Moss and coworkers demonstrated that the deamidation of asparagine residues in tetanus toxin C fragment inhibits the processing by asparagine endopeptidase and results in decreased antigen processing (181). The role of PTMs in antigen processing was examined years ago in studies of model proteins in immunity (168, 182–184). An isoaspartylated form of cytochrome c protein is cleaved differently by cathepsin D compared to the (normal) aspartyl form of the same protein (2). Granzyme B cleavage of autoantigens may also generate new epitopes based on the presence or absence of PTMs in self-protein (185). Simply put, the presence of PTMs that affect proteolytic enzyme recognition generates a completely new repertoire of peptides during antigen presentation.

Specific subsets of APCs and even the compartments within the cells in which the antigen is processed may shape the type of PTM acquired and whether modified peptide is presented on MHC. Ireland and colleagues (186) demonstrated that B-cell autophagy was required for the generation and presentation of a citrullinated peptide (but not required for the non-modified peptide form). Mamula and others have demonstrated the unique APC functions of B cells in presenting antigen to T cells. In particular, it is clear that B cells both present antigens directly, as well as transfer antigens to other APC, such as macrophages and dendritic cells. Thus, different APCs may dictate the self PTM epitopes generated and eventually presented by the immune system (187–189).

It is not fully understood as to how a PTM will affect HLA binding. The 'fit' of the PTM peptide versus native peptide for an

HLA molecule can vary based on elements including charge and structural changes imposed by PTMs. The HLA binding of various PTM T1D autoantigens has been studied and reviewed by James et al. (12, 13). For deamidated and citrullinated residues specifically, the introduction of a negative charge enhances the binding affinity at specific positions in HLA-DR or HLA-DQ molecules. As such, HLA-DRB1*04:01 and DRB1*15:01 prefer citrulline at key positions of their binding motifs, whereas deamidation results in preferential loading onto HLA-DQ molecules (13). This was also shown for citrullinated peptides of vimentin, a RA autoantigen, which has greater affinity for HLA-DRB1*0401 than the unmodified peptide (190).

Among other autoimmune syndromes, specific PTMs of MBP result in either low, intermediate or a similar affinity for MHC compared with the corresponding wild-type peptide (191). Notably, acetylated MBP peptide (Ac 1-11) is required to incite pathogenic T cells in murine MS, though the unmodified peptide binds MHC with virtually identical kinetics. Similarly, isoaspartic acid residues in cytochrome c or snRNP D peptides (SLE autoantigens) bind MHC class II in a manner identical to the unmodified peptides (168). However, immune tolerance is maintained to the native peptides. The overall lesson is that PTM self-peptides alter processing and binding by MHC in distinct manner from unmodified (native) peptide.

TARGETING CITRULLINATION: TYPES OF PAD INHIBITORS AND THEIR POTENTIAL AS THERAPEUTIC AGENT IN PRECLINICAL MODELS OF AUTOIMMUNITY

Given the involvement of citrullination and PAD enzymes in several autoimmune diseases, it has been of great interest to generate compounds targeting PAD activity, i.e. PAD inhibitors. The development of such compounds was made possible with the discovery of the crystal structure of PAD enzymes (95, 192). Since then several pan-PAD inhibitors have been developed, with increasing potency, specificity and metabolic stability, of which the 4 most extensively studied irreversible inhibitors are shown in **Figure 4**. More recent efforts also include the development of specific PAD inhibitors, targeting for instance PAD2 or PAD4, as well as non-covalent reversible inhibitors. For a detailed overview of all developed PAD inhibitors we refer to a recent review specifically focusing on these developments (197).

Considering that PAD dysregulation is associated with several autoimmune diseases (86), numerous studies have evaluated the efficacy, safety and mechanism of action, mainly making use of irreversible pan-PAD inhibitors in animal models of autoimmunity, with promising results. As such, amelioration or even reversal of disease in case of intervention therapies, and delayed initiation or complete protection in case of preventive therapies, was shown in mouse models of RA, MS, SLE, UC, inflammatory bowel disease, and recently also in T1D [reviewed by (198, 199)]. In general, mechanistic insights from these

studies have taught us that targeting PAD making use of pan-PAD inhibitors effectively decreases protein citrullination levels in the inflamed target tissues, as measured mainly by LC-MS/MS, PAD activity assays or Western blotting using anti-citrulline Ab (200–203). Such decreased protein citrullination can evidently have an effect on the autoreactive responses against citrullinated autoantigens, both in terms of autoantibody and T-cell responses, as shown in some published studies (200, 201). Next to this, it has become clear that other mechanisms are involved in the observed protection, pointing towards more general effects on innate and adaptive immunity. Not surprisingly, based on the high levels of PAD4 in neutrophils and their important role in histone 3 citrullination and induction of NETosis, as outlined above, *in vivo* PAD inhibition was shown to reduce NET formation and associated NET-induced tissue damage (204, 205). Other ameliorating disease effects were associated with a decrease in circulating pro-inflammatory cytokine levels, such as IL-6, TNFα and IL-1β (206, 207), an increase in Treg populations (200) or a shift in T-lymphocyte populations from Th1/Th17 towards Th2 (201, 208). The latter is thought to be mediated through direct inhibition of citrullination of the transcription factors RORγT and GATA3 (see also above) (72).

Of interest in the field of T1D, a recent study from the Overbergh laboratory showed a complete protection against diabetes development in the NOD mouse, by daily subcutaneous injections of BB-Cl-amidine (1µg/g body weight) (200). BB-Cl-amidine is a pan-PAD inhibitor that, similar to its mother compound (Cl-amidine), irreversibly inactivates PAD enzymes through covalent modification of an important cysteine for the activity of the enzymes (209). Remarkably, diabetes protection was observed when starting treatment at 8 weeks of age, a time point at which insulitis is already ongoing, but hyperglycemia has not developed (200). This observation tempts us to conclude that citrullination may play a role in amplification of the disease rather than being an initial trigger in breaking immune tolerance. BB-Cl-amidine treatment was associated with decreased citrullination levels in the pancreas as demonstrated by LC-MS/MS and western blotting with anti-citrulline Ab, as well as decreased levels of circulating autoantibodies against citrullinated GRP78, a known citrullinated autoantigen in T1D (as outlined above, **Table 4**) (15, 16). These findings confirm the effective direct action of BB-Cl-amidine on inhibition of PAD activity. Furthermore, bone-marrow derived neutrophils isolated from BB-Cl-amidine treated mice showed less potential for spontaneous NET formation when compared to the control group. Disease protection was associated with preservation of pancreatic insulin levels, although only a marginal reduction in insulitis was observed, suggesting a less aggressive form of insulitis, in line with reduced CD4^{+} effector memory T cells and reduced IFNγ-producing T cells in the pancreas infiltrates. In the periphery, a shift from Th1 towards Th2 cytokine levels and increased frequency of regulatory T cells was observed.

Taken together, the promising results obtained with pan-PAD inhibition in the NOD mouse, as well as in other autoimmune mouse models, indicate that disease protection is
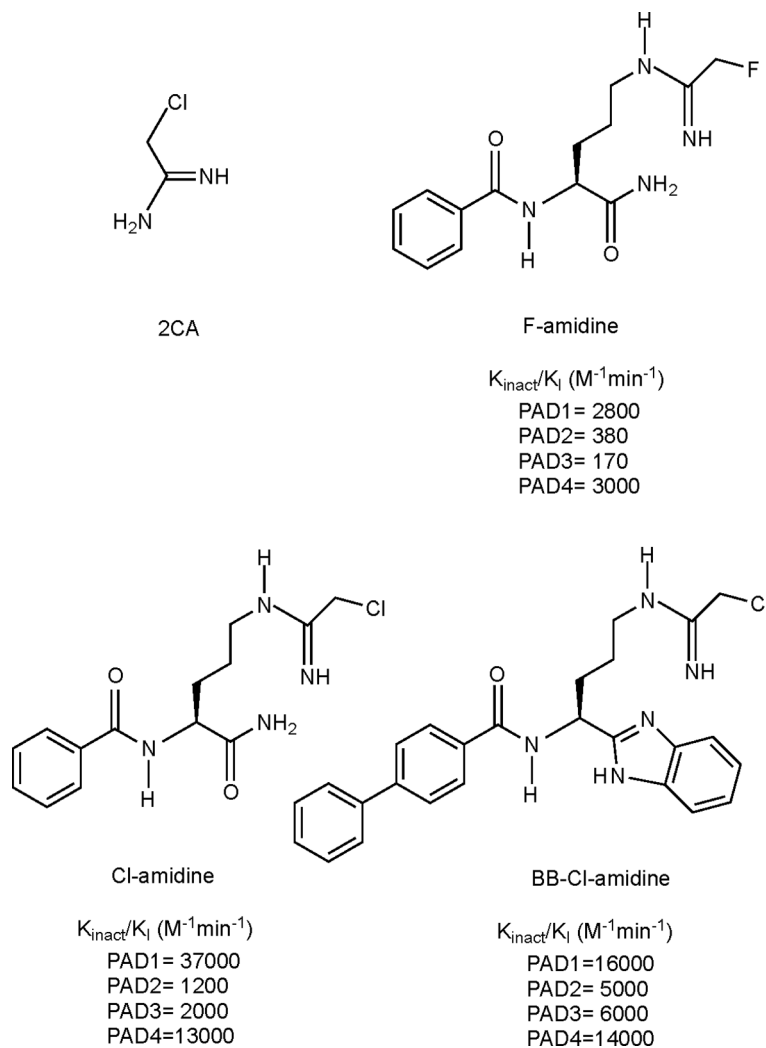
FIGURE 4 | Structures and k_{inact}/K_I values for some irreversible pan-PAD inhibitors. 2CA (193), F-amidine, Cl-amidine (194) and BB-Cl-amidine (195). k_{inact}/K_I values have been described as the best measure of potencies for irreversible inhibitors (196). k_{inact}: rate of enzyme inactivation; K_I: inhibition constant.

mediated by effective inhibition of citrullination in the target inflamed tissue, leading to decreased autoreactive responses. This further underscores the observation that citrullination is not a specific disease-related event, but rather an inflammation-dependent process occurring preferentially in autoimmune target tissues (210). Next to this, the extensive data in different preclinical autoimmune models indicate that pan-PAD inhibitors also have more general effects on immune cells, thereby dampening inflammation and reshaping the immune response towards tolerance. Whereas this may be an additional benefit for the use of PAD inhibitors, caution is also warranted. Since pan-PAD inhibitors act both intra- and extracellularly, and citrullination is important in many physiological processes, such as epigenetic and transcriptional regulation, as outlined above, the concern about possible adverse side effects cannot be neglected. Therefore, more studies are needed to evaluate more specifically potential adverse effects before translating to the human situation. Of interest in this regard is the development of isozyme-specific PAD inhibitors, which are hoped to still be protective in disease development, but allow physiological citrullination to occur. Great progress has been made in this path, with development of a high number of PAD specific inhibitors. For instance, a PAD1-selective inhibitor (211), D-Cl-amidine, and a specific PAD2 inhibitor (194), AFM30a, both based on the structure of Cl-amidine. PAD4-specific inhibitors include TDFA (212) and GSK199, with GSK199 being a reversible PAD4 inhibitor that targets the apo state of the enzyme, showing potent inhibition of PAD4 at low concentration of calcium (0.2mM) (143). GSK199 has been demonstrated to be effective in blocking murine arthritis (213), however, more studies are needed to evaluate the efficacy and safety of such specific inhibitors.

CONCLUDING REMARKS

In summary, we have attempted to illustrate and summarize various biochemical, immunologic, and transcriptional aspects of citrulline modifications. How citrullination alters these unique biologic processes in spontaneous autoimmune syndromes are beyond the prediction of simple genetics or other stochastic factors. Citrullination, as well as many other PTMs and cellular pathways, are affected by inflammatory cytokines and reactive oxygen species that inhabit the tissue microenvironments in T1D and other autoimmune diseases. More indirect pathways are also affected by citrulline modified proteins, including downstream transcriptional events. The emerging technologies of detecting citrulline modifications as well as other PTMs in proteomics and tissue analyses will undoubtable change the landscape of autoimmunity in the coming months and years. These analyses, notably the presence of serum anti-citrulline autoantibodies, have already contributed to the clinical diagnoses and assessment of the progression of disease, and tissue pathology. With the identification of specific biomarkers and an understanding of their origins, the field will now have potential therapeutic pathways as targets to modify these autoimmune diseases, including the reducing tissue inflammation and use of inhibitors of citrullination prior to destruction of the pancreatic islets.

AUTHOR CONTRIBUTIONS

M-LY, FS, MM, and LO conceived the concept and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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