Oxidative Modifications in

Tissue Pathology and Autoimmune Disease

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Abstract

Various autoimmune syndromes are characterized by abnormalities found at the level of tissues and cells, as well as by microenvironmental influences, such as reactive oxygen species (ROS), that alter intracellular metabolism and protein expression. Moreover, the convergence of genetic, epigenetic, and even environmental influences result in overt clinical expression of disease, as evidenced by the presence of specific B and T lymphocyte autoimmunity and tissue pathology. This review will describe how oxidative stress to cells and tissues may alter posttranslational protein modifications, both directly and indirectly, as well as potentially lead to aberrant gene expression. For example, it has been clearly observed in many systems how oxidative stress directly amplifies carbonyl protein modifications. However, ROS also lead to a number of non-enzymatic spontaneous modifications including deamidation and isoaspartate modification as well as enzyme-mediated citrullination of self proteins. ROS have various effects on DNA methylation, leading to influences in gene expression, chromosome inactivation, and the silencing of genetic elements. Finally, reactive oxygen species can alter a number of other cellular pathways, including the initiation of apoptosis and NETosis, triggering the release of modified intracellular autoantigens. We will discuss the importance of understanding various protein posttranslational modifications (PTMs), mechanisms that mediate the ability of 'modified self' to induce autoimmunity, and how these PTMs, or products of ROS, may be important biomarkers of tissue pathogenesis.

Introduction

At its most basic level, autoimmunity arises when components of the immune system, including cells and soluble factors, initiate the recognition and robust response to self proteins and tissues leading to immune-mediated pathology. In addition, various genome wide association studies (GWAS) now conducted in virtually all autoimmune syndromes have implicated a large number of heritable genetic traits that predispose individuals to autoimmunity. However, autoimmunity, and Type 1 diabetes (T1D) in particular, is not entirely explained by a defined collection of genes. For example, many non-heritable factors including poorly defined environmental influences and so-called 'epigenetic' factors, which may or may not be inherited, also shape the onset and development of disease. The collection of manuscripts included in the present issue of *Antioxidants and Redox Signaling* examines the features of T1D autoimmunity linked to oxidative tissue environments. Reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen radicals (OH^-), and hydrogen peroxide (H_2O_2) are a result of dynamic balance with natural anti-oxidant cellular products that control their concentrations and biological effects. These anti-oxidants include superoxide dismutase, catalase, glutathione peroxidase, peroxiredoxins, as well as other neutralizing small molecule substances, including vitamins E and C. While not elaborated within this review, the sources of ROS at sites of tissue autoimmunity are many, including the invasion of activated immune phagocytic cells (neutrophils, macrophages and dendritic cells) which are undeniably critical to the onset, progression, and tissue pathology of type 1 diabetes as well as many other autoimmune syndromes. Though ROS cellular stress induces many protein PTMs [reviewed in (131)], the present work will focus on those specific pathways relevant in inflammatory autoimmune syndromes.

'Oxidative stress' can trigger direct modification of certain proteins, or protein motifs, or, alternatively, as secondary modifications due to indirect metabolic pathways affected by ROS. These secondary effects include the role of ROS on apoptosis, NETosis, and intracellular metabolic pathways, all of which may affect the outcome of autoimmune responses and/or inflammatory tissue pathology. Affected proteins may be altered in solubility, in their ability to be digested or cleared, or altered in immunogenicity. As illustrated in this review, oxidation can provoke a number of cellular changes at both the DNA and protein level, the latter of which includes both spontaneous and enzyme-mediated modifications to self proteins that are relevant biomarkers of tissue pathology and autoimmunity in T1D. Herein, we will examine features of the onset and progression of T1D autoimmunity that are influenced by oxidative pathways, including various posttranslational protein modifications, as well as the role of oxidation at the level of DNA transcription and translation.

Posttranslational Modifications of Self Antigens in Autoimmunity. One principal function of the immune system is to differentiate between the self and non-self proteome. There are a variety of mechanisms in place to deplete the immune system of lymphocytes that react too strongly to self-antigens that are present in the thymus and bone marrow. While a variety of self-antigens are expressed in the thymus (28), the posttranslational modification (PTM) of self-antigens (Tables 1 and 2) can in effect create a novel self antigenic proteome for which immune tolerance has not been established either in the thymus or periphery. This concept, previously described as 'autoantigenesis' is a term described to proteins that 'evolve' and acquire PTMs over the course of disease and provoke B and T cell autoimmunity (36). Such is the case with a number of autoimmune diseases, including multiple sclerosis, rheumatoid arthritis, SLE, and type 1 diabetes [Table 1 and refs (35,36)]. The PTM autoantigens illustrated in Tables 1 and 2

represent many of the notable modified self proteins that trigger autoreactive B and T cell responses and, in many cases, are specific diagnostic biomarkers as well as a reflection of disease pathology. Other PTMs (Figure 1) can be directly affected by tissue ROS and/or inflammatory microenvironments (carbonylation, methylation, isoaspartylation, deamidation), or be influenced by more indirect downstream pathways affected by ROS (acetylation, glycosylation, phosphorylation, citrullination).

Citrullination. One recent and well-studied example of an immune response to a posttranslationally modified self-antigen is the autoantibody response that develops to citrullinated proteins, notably in rheumatoid arthritis (RA). Citrulline is the consequence of the deimination of arginine residues by peptidylarginine deiminase (PAD) (8). More importantly, in the case of rheumatoid arthritis, PADs (PAD1, PAD2, PAD3, PAD4) are highly activated by Ca^{2+} ion deposition in an inflamed joint during apoptotic cell death (86,155) and lead to the generation high levels of citrullinated proteins/peptides. Sera from patients with rheumatoid arthritis recognize citrullinated autoantigens, such as fillagrin, vimentin, collagen, and □-enolase. Anti-citrulline antibodies arise early in RA, correlate with disease severity (69,154), and are now routinely used for the diagnosis of RA (155). As noted below, citrulline modification has been directly linked to redox metabolism and ROS in various tissues. In particular, circulating citrulline, arising from arginine modification, is a byproduct in the synthesis of nitric oxide (47,94,166). As detailed herein (Figure 1, Tables 1 and 2) and in accompanying monographs of this issue, B and T cell responses directed at PTMs have become important diagnostic tools for many autoimmune diseases, including and emerging group of PTM biomarkers important in T1D (Table 2). A recent review from Nguyen and James (115) has carefully defined the biological implications of citrulline modifications and autoimmunity arising from pancreatic beta cell

proteins in the development of T1D. In particular, citrullinated GAD65 and GRP78 both elicit a vigorous B and T cell autoimmune response in human T1D and NOD murine disease, respectively (101,125). The latter studies were marked by a significant upregulation of PADI2 in the islets of NOD mice, supported by genetic risk in the *Idd25* locus of mouse chromosome 4. These citrulline modifications of T1D autoantigens were linked to cytokine and/or ROS stress effects on the endoplasmic reticulum, a recurrent theme in fostering many PTMs.

Carbonyl PTMs. Protein carbonylation is a major product of tissue proteins in response to oxidative stress. Increased carbonylation of self proteins arise due to either decreases in antioxidant defense pathways, increases in ROS production, or an inability to remove or repair oxidized self proteins, as previously reviewed by Nystrom (116). The classical antioxidant (ROS) defense mechanisms, superoxide dismutase, catalases, and peroxidases all protect against the induction of carbonyl modifications. A number of diseases have already been associated with aberrant carbonylation of self proteins, including Parkinson's and Alzheimer's disease and cancer. Carbonylation is a marker of cellular senescence and is increased in aging cells and tissues. Carbonylation is a metal catalyzed (free iron) oxidative modification of the side chains of proline, arginine threonine, and lysine (Figure 1). Carbonyl modifications are typically more difficult to induce relative to other oxidative modifications. This modification has many deleterious effects to various intracellular enzymatic mechanisms and mitochondria are particularly vulnerable to ROS induced carbonylation (116). There is evidence to suggest that carbonylation is one protection mechanism of cells for directing damaged proteins into proteolytic degregation pathways, as these modified proteins are conformationally unstable. Carbonylation is an irreversible modification, thus the biological functions of these modified proteins are unrepairable (37). All of these properties make carbonylated self proteins

recognized in an autoimmune response, particularly if they fail to find their way to normal cellular degredation pathways.

It has been observed that increases in ROS contributes to insulin resistance and metabolic dysfunctions in adipose tissue of both animal models and human T1D (41,128,135). While publications have profiled carbonylated plasma proteins as potential biomarkers in type 2 diabetes (T2D) (40,48) though little is known about carbonylation in the progression of T1D. Our recently submitted studies from M.L. Yang, et al. defined a group of pancreatic beta cell proteins with carbonyl PTMs, all bound by autoantibodies from human and NOD T1D antisera. Among this group were both novel and established biomarkers of T1D, including protein disulfide isomerase (PDI) isoforms, 14-3-3 protein isoforms, glucose-regulated protein 78 (GRP78), chymotrypsinogen B and malate dehydrogenase. Of interest, carbonylated prolyl-4-hydroxylase beta (P4Hb, also known as protein disulfide isomerase A1; PDIA1) was found to be an early autoantigen in both human and murine models of T1D. P4Hb is required for the appropriate folding of insulin from pancreatic beta cells. Our data suggest a novel role of modified P4Hb, both as an early target of autoimmunity as well as a pathway that provokes autoimmunity to insulin and/or proinsulin. In fact, autoimmunity to P4Hb always preceded autoimmunity to insulin in both NOD and human T1D, as defined by the pathway described in Figure 5. Carbonylation is amplified by either oxidative or cytokine stress to beta cells. Carbonylated P4Hb fails in its ability to accurately fold and process proinsulin to insulin. We hypothesize that misfolded proinsulin levels accumulate in the cell or serum and lead to linked autoimmune responses to insulin itself. Aberrant carbonylated P4Hb biological functions in the beta cell provide an explanation for recent observations of increased proinsulin to insulin ratios in the progression of T1D.

ROS and PTMs in NETosis. NETosis is a cellular clearance mechanism distinct from apoptosis, which occurs when neutrophils encounter microorganism and produce highly modified chromatin webs (26). 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. This pathway is marked by the release of mitochondrial DNA, a process dependent on cellular ROS. In particular, NETs are thought to be one source of immunogenic histone H2B. A study by Liu and colleagues (83) characterized the PTMs that occur within histones from NETs. These PTMs included citrullination of H3 and H4, as well as methylation and acetylation of H2B leading to autoantibody responses. It is interesting to note that apoptotic and NETotic cells display different PTMs, and perhaps it is the combination of both of these events and their PTMs that break immune tolerance to modified self proteins. The role of NETs in T1D have yet to be fully resolved; one recent study illustrating a reduction in serum components of NETs (neutrophil elastase and proteinase 3), consistent with a reduced overall neutrophil count in early onset T1D (121). Conflicting studies report increases in these same NET components in T1D (152).

A growing number of cytoplasmic proteins that undergo posttranslational modifications are the targets of autoantibodies in SLE (Table 1). Tissue pathology, inflammatory cytokines and ROS, creates an ideal milieu that favors the generation of PTMs. Processes whose purpose are to clear inflammatory material, apoptosis and neutrophil extracellular traps (NETosis), may drive the PTM of self-antigens, being viewed as 'foreign' to the immune system and are amplified by ROS. Apoptotic cells and NETosis have long been considered a source of autoantigens in SLE, and their clearance is impaired in lupus patients (52,53,72,139). As noted above, NETs are enriched in various PTM self proteins, including citrullination (65). In addition, specific lupus

autoantigens, such as nucleosomal DNA, Ro/SSA, La/SSB, and snRNPs, migrate to surface blebs of apoptoic cells (15), a reservoir of PTM self proteins. There are a number of self-antigens that also undergo PTM during apoptosis, including phosphorylation/dephosphorylation, transglutamination, ADP-ribosylation and proteolysis [reviewed in (141)]. Notably, SLE patient sera bind phosphorylated SR proteins (pre-messenger RNA splicing factors) but not non-phosphorylated peptides relating to the SR domain (114).

Isoaspartic acid, the result of the spontaneous isomerization of aspartic acid increases in the presence of ROS as well as cells that undergo necrosis and/or apoptosis (Figure 1 and details below) (19,22). Both lupus-prone MRL mice and human SLE patients have high titers of autoantibodies that react with isoAsp modified histone H2B (32). SLE patient sera also bind to the C-terminus of snRNP, which contain symmetric dimethyl arginines, again, another methylation pathway amplified by ROS (13).

We and others have identified significant differences between the T and B cell responses that develop against PTM self-proteins. For example, T cell responses to PTM determinants tend to be specific for the modified peptide only and are not cross-reactive with the native (unmodified) form of the protein. This concept is illustrated in mice immunized with the isoaspartyl-modified form (isoAsp) of snRNP D where T cells only proliferate in response to the isoAsp modified snRNP D peptide, but are unreactive to the Asp form of peptide (91). T cell autoimmunity to PTM self proteins will be more fully addressed by other authors in this issue of *Antioxidants and Redox Signaling*. In contrast, B cell and autoantibody responses tend to be more promiscuous in their binding to both the modified and native self-protein. This phenomenon may be due to the ability of antibodies to bind flanking amino acid sequences in both modified and native protein forms. For example, human SLE and lupus-prone MRL/lpr

mice that possess autoantibodies that bind both an isoAsp and Asp form of H2B p21-35 (32). It was demonstrated that responses are initiated with the PTM self protein/peptide, and thereafter spreads in an intra- and extra-molecular manner to other determinants. Thus, the introduction of a modification in a self-protein promotes 'epitope spreading', a mechanism by which the immune response diversifies to include epitopes beyond the site(s) that initiate the response [Figure 2 and (78)]. There is both intramolecular and intermolecular B and T cell epitope spreading in to self-antigens in T1D, SLE, and multiple sclerosis (66,91). Epitope spreading of autoantibody responses are associated with the development and progression of autoimmune disease (3). One likely mechanism is that posttranslationally modified self-antigens, viewed as foreign, triggers a limited autoimmune response, even prior to the onset of symptoms, followed by the progressive accumulation and successive rounds of additional targeted epitopes (Figure 2). As illustrated above, apoptotic and necrotic cells are certainly early sources of altered self proteins in various microenvironments, notably conditions of oxidative stress (44) or altered pH (45).

PTMs in antigen processing and presentation. One explanation as to why immune responses develop to PTM self proteins is from alterations or defects in the specific negative selection of immune cells. It is clear that efficient and accurate antigen processing plays a major role in epitope generation (93). The presence of a PTM of an amino acid residue critical for recognition and cleavage by certain proteases affect the antigenic peptides generated and/or the rate in which they are generated (Figure 3). Moreover, the presence or absence of PTM within an intracellular processed peptide affects the binding to MHC. It has been demonstrated that the lack of N-glycosylation of the neuronal glutamate receptor subunit 3 in Rasmussen's encephalitis, a severe from of pediatric epilepsy, exposes a granzyme B cleavage site, thus

creating a novel autoantigen (neoepitope) (43). Most proteases and peptidases *do not* recognize the □-peptide linkage connecting isoAsp residues to its neighboring amino acid on the carboxyl side (62). Moss and coworkers demonstrated that the spontaneous deamidation of asparagine residues in tetanus toxin C fragment inhibits the processing by asparagine endopeptidase and results in decreased antigen processing (110). Simply put, the lack of proteolytic enzyme recognition generates a completely new repertoire of peptides during antigen presentation (Figure 3). These observations were confirmed several years ago in studies of model proteins in immunity (87-90), now confirmed by more recent work with disease relevant PTM autoantigens. An isoaspartylated from of cytochrome c protein is cleaved differently by cathepsin D compared to the (normal) aspartyl form of the same protein (34). Granzyme B cleavage of autoantigens may also generate new epitopes based on the presence or absence of PTMs in self-protein (16).

Specific APCs in which the modified self-protein are processed, and even the acidified compartments within the cells in which the antigen is processed, may determine the type of PTM acquired and whether modified peptide is presented on MHC. Studies by Ireland and colleagues (60) demonstrated that autophagy was required in B cells for the generation and presentation of a citrullinated peptide, while it was not required for the non-modified form. Our laboratory and others have demonstrated the unique APC functions of B cells in presenting antigen to T cells. For example, B cells transfer antigens to other APC, such as macrophages and dendritic cells, emphasizing that different APC may dictate the self epitopes generated and eventually presented by the immune system (49,50,122). The implications of these observations are that different subsets of APCs may process and present different peptides from the same PTM autoantigen (23,24,110)

After peptides have been generated by antigen processing, it is not obvious as to how a PTM will affect MHC binding. The 'fit' of the modified peptide versus native peptide for a MHC molecule can vary. As one example, different PTM of myelin basic protein (MBP) result in either low, intermediate or a similar affinity for MHC compared with the corresponding wild-type peptide (25). Acetylated MBP peptide (Ac 1-11) triggers pathogenic T cells in murine multiple sclerosis, though the unmodified peptide binds MHC with identical kinetics. Similarly, isoaspartic acid residues in cytochrome c or snRNP D peptides bind MHC class II in a manner identical the unmodified peptides (91), yet immune tolerance is maintained to the native peptide. In contrast, citrullinated peptides of vimentin, a RA autoantigen, has greater affinity for HLA-DRB1*0401 than the unmodified peptide (55). The overall message to be learned is that PTM self peptides may or may not be processed and bound by MHC in a manner found with unmodified (native) peptide. Moreover, is cannot be predicted whether TCR and BCR will bind PTM self peptides or proteins and cross react with the corresponding unmodified antigen.

ROS alters the methylation of DNA and protein. A variety of intracellular methylation reactions regulate pathways critical in the normal function of immune responses. Transmethylation is accomplished by enzymes that catalyze the addition of a methyl group from *S*-adenosyl-methionine (SAM) to substrates of DNA, proteins, or lipids. The family of methyltransferases include DNA methyltransferase (DNMTs), protein arginine methyltransferase (PRMTs), protein lysine methyltransferase (PKMTs), and protein isoaspartate methyltransferase (PIMT). Reactive oxygen species alter all of these methylation pathways (10,109,160). The most well-studied transmethylation substrates in regulating epigenetics are DNA and histone proteins. However, proteomic techniques developed within the post-genomic era identify other protein methylations as critical regulators in a broad array of biological pathways including

transcriptional regulation, signaling pathways, and immune cell differentiation. In fact, lymphocytes rely more heavily on accurate methylation in cell activation compared to most other cell types (Table 3) (46). Protein methylation is required efficient for TCR responses, downstream signaling, and cytokine regulation (such as STAT1 in IFNα/β-induced transcription and Vav1 involved Th1/2 cytokine production; Table 3). T cell development relies on the accurate methylation of histone proteins and CpG dinucleotide sequences within promoter regions in regulating gene expression. Inhibition of protein arginine methyltransferase (PRMT) alters DNA binding ability of phosphorylated STAT1 (signal transducers and activators of transcription) after INF stimulation (113). Of interest, CD28 costimulation of T cells increases PRMT activity and Vav1 methylation in human and mouse T cells (9). The recurring theme of this review are the effects of ROS in these various, and sometimes indirect, biological pathways. Recent studies have demonstrated PRMT1, a major protein methyltransferase in humans, is downregulated by ROS in the microenvironment causing the release of asymmetric dimethylarginine (ADMA) into the serum, a biomarker of tissue pathology (109).

Transmethylation reactions are also highly associated with autoimmune disease. The failure to maintain the methylation status of CpG dinucleotide triggers T cell autoreactivity and results in the pathogenesis of SLE (134). As mentioned above, symmetric dimethylated ribonucleoproteins, SmD1 and SmD3, are autoantigens recognized by anti-Sm autoantibodies in lupus patients (13) are autoantigens. As another example, arginine-methylated myelin basic protein provokes an autoimmune response (autoantibodies and T cells) in multiple sclerosis (MS) (120).

In type 1 diabetes, it is attractive to hypothesize that DNA and/or protein methylation pathways are influenced by ROS as they are by inflammatory cytokines (IL-1b, IFNg, or IL-6) in

the pancreatic islet microenvironment (127). From the time that autoantibodies are first detected in individuals at genetic risk for T1D, the course of disease is highly variable (144). Many individuals do not progress to overt disease, and those that do may do so over different time periods ranging from a few months to decades. This variability has largely focused on the immune effector and regulatory cells that are involved in β cell killing and maintenance of tolerance (12,99). However, it is equally likely that β cells respond to the immune attack and environmental factors such as ROS in ways that may accelerate or retard disease progression (Figure 4). In addition to the modification of these proteins, there may also be modifications of the enzymes responsible for the epigenetic changes themselves. TET2 was reported to be modified by acetylation during oxidative stress and DNMT3a has been reported to be SUMOylated which modulates its repression of transcription (82,168). DNMT3a is known to control HDAC9 gene expression and in the setting of inflammation, TET2 physically associates with certain HDACs (27). TET2 is required to resolve inflammation due to IL6 (80). Identifying the effects of PTMs on epigenetic enzymes and PTMs in the absence and presence of epigenetic enzymes is not yet fully understood.

Herold, et al., have studied individuals who were at very high risk for diabetes by virtue of finding two or more positive autoantibodies and dysglycemia. Historically, approximately 75% of these subjects progress to overt diabetes within 5 yrs. These studies measured the relative levels of unmethylated insulin *INS* DNA (released from dying β cells) in the serum compared to the amount of methylated *INS* DNA (representing dying non-β cells). It was found that the frequency of increased levels of unmethylated *INS* DNA measurements (taken about every 6 months) was low in the at-risk subjects followed for up to 4 years, and not significantly different in progressors and non-progressors to diabetes. However, the levels and frequency of elevated

levels of unmethylated *INS* DNA was significantly higher in the very high risk subjects suggesting that there were high levels of β cell killing in the peridiagnosis period. These observations, and others from clinical studies of β cell function, have refined our understanding of the kinetics of progression, originally suggested by Eisenbarth in 1986.

In summary, our studies of β cells during progression of T1D in NOD mice and human β cells in vitro indicate that there is induction of DNMTs as well as PTMs that can remodel or affect survival of β cells. An underlying theme to these studies is that the processes of epigenetic protein modifications are interconnected, possibly both in response to inflammatory mediators or more directly in which one process modifies the others. An overview of this converging pathways is illustrated in Figure 4.

Protein arginine methyltransferase (PRMTs). In 1996, PRMT1 was the first PRMT enzyme member to be cloned and characterized (81). To date, there are at least 11 PRMT enzymes utilizing S-adenosyl methionine (SAM) as the intracellular methyl donor. Generally, the PRMTs will methylate in the preferred sequence motif, GAR domains (glycine-arginine-rich). PRMTs are divided into four classes depending on the transmethylation product as shown in Table 3. Type I, II and III PRMTs catalyze the methylation in the *terminal* guanidine nitrogen atoms of arginine. Type IV PRMT catalyzes the methylation in the *internal* guanidine nitrogen atoms of arginine. All PRMT members will catalyze mono-methylation in arginine residue as the intermediate or final product. However, Type I PRMTs will form asymmetric di-methylarginine and type II PRMTs will form symmetric di-methylarginine (7).

Arginine methylation has recently attracted the attention of immunologists since accumulating evidence demonstrates that PRMTs play a critical role in B and T cells immune responses (Table 3). PRMT1, the most abundantly expressed PRMT in all cells, is responsible

for over 85% of arginine methylation (7). In T lymphocytes, PRMT1 regulates cytokine production (IL-2 and IL-4), mediated by the modulation of the TCR signaling pathway (Vav1, STAT1/6 and Akt/PKB) (9,103,113,119) and by transcriptional activation/repression (NIP45 and **N**uclear **F**actor of **A**ctivated **T** cells; NFAT) (112). PRMT1 can bind directly to the intracytoplasmic domain of type 1 interferon receptor. In B lymphocytes, PRMT1 is believed as a key factor to regulate B cell differentiation mediated via PI3K kinase pathway. The acetylation of core histones is essential to maintain the "active" chromatin status. Nonetheless, PRMT1 modulates transcriptional activation via the typical histone modification. PRMT1 depleted cells fail to methylate histone H4R3 of the nucleosome and suppress H3 and H4 acetylation (57,79,150). Relevant to lupus autoimmunity, hypoacetylation of histone H3 and H4 is found T cells of lupus patients and is correlated with disease activity (56). PRMT2 is also highly involved in T cell functions, including Th17 differentiation and T helper cells (145). Loss of PRMT4 (CARM1) results in hypomethylation during thymocyte differentiation (2). PRMT5 is associated to IL-2 gene expression, histone methylation (H2A and H4) and B cell lymphoma. PRMT7 is abundantly expressed in thymus and dendritic cells and catalyzes histone H4 methylation (41).

At another level, T cells from either mouse or man appear to have intrinsic abnormalities, mediated by ROS and/or inflammatory cytokines, that contribute to their ability to drive autoimmunity. For example, aberrant TCR signaling is a key phenotype in lupus T cells reflected as increased phosphorylation of tyrosine residues in signaling molecules, increased Syk/ZAP-70, ERK, and PI3K kinase activity, increased calcium and lipid raft clustering and decreased PKC, PKA and MAPK kinase activity (21,111). As illustrated here, ROS may initiate a chain reaction of downstream effects of methylation and phosphorylation leading to aberrant T

cell biology.

In addition, the serum levels of IL-4, IL-17, and IFN□are significantly elevated while IL-2 is reduced in lupus patients (1,140). These cytokines and/or receptors are regulated by PRMTs via transcriptional factors or histone modifications (Table 3).

Repair of posttranslational modifications induced by ROS. Protein L-isoaspartyl (D-aspartyl) methyltransferase (PIMT) is the only known enzyme to repair isoaspartyl (isoAsp) modification, which is known to contribute the onset of autoimmune B and T cell responses and autoimmune pathology in murine SLE as described above (33,162). This repair pathway is essentially a response to ROS and inflammation in tissues, seeking to repair deleterious isoaspartyl PTMs to the aspartic acid isoform in self proteins. PIMT is a highly conserved repair enzyme, found in both prokaryotes and eukaryotes, thus emphasizing its importance in maintaining cellular health. PIMT is usually known as a soluble intracellular protein but some studies demonstrated that PIMT activity can be detected in extracellular compartment such as cerebrospinal fluid (14,107). The isoAsp-modified PTMs are notable among several autoantigens in SLE, including histone H2B and snRNPs (91,164). The latter authors have postulated that an inability to repair isoAsp PTMs, due to PIMT polymorphisms in SLE patients, may contribute to the antigenicity of self-proteins.

PIMT deficiency results in 2-6-fold higher levels of isoAsp residues systemically including thymus, spleen and lymph nodes (33,84). In the lupus-prone MRL model, isoAsp modified proteins are elevated over time in the brain and kidney (162). In particular, PIMT deficiency leads to T cell hyperproliferation and increased phosphorylation of selected members of the TCR cellular signaling pathways in a manner similar to T cell hyperproliferation observed in both human SLE and in the MRL mouse (33). Recently, we found that the content of isoAsp

modification in erythrocytes is correlated with PIMT activity (specific PIMT polymorphisms) in patients with SLE (unpublished data). Moreover, we have found that isoAsp modification does occur at several sites in ZAP70 (unpublished data) suggesting that PIMT repair defects and/or isoAsp-modified ZAP70 protein signaling mediates abnormal (hyperproliferative) T cell functions in SLE.

Regarding type 1 diabetes, PIMT is shown to be expressed in pancreatic beta cells and the transformed insulinoma cell line, INS-1. Induction of PIMT expression delays the appearance and reduces the severity of T1D in the BB rat model of T1D (148). The *PIMT1* gene maps to a region, 6q24-25, linked to the *IDDM5* site studied in genome analyses of human T1D (149). Four PIMT1 polymorphisms were found to be in linkage disequilibrium, with *PCMT1* promotor activity increased in response to cytokine stimulation. Collectively, the work supports an interaction between *PCMT1* and both *HLA* and *SUM04* in the genetic risk for T1D. As yet, however, specific PIMT polymorphisms already defined to exist in humans (29) (20) (30), have not yet been clearly associated with any autoimmune syndrome. Moreover, the expression of PIMT protects from Bax-induced cellular apoptosis, perhaps yet another mechanism that evolved to prevent the release of isoaspartyl-modified self proteins (58). The emerging picture is that ROS and/or cytokine induced inflammation of tissues triggers various PTM pathways, followed by protection mechanisms initiated by the cell to prevent both its destruction and to repair aberrant self proteins. Unfortunately, the inflammatory storm found in autoimmune disease is often too vigorous to be impeded by these protective intracellular mechanisms.

Our study indicated that lack of PIMT-based repair represents an 'accelerating' factor of T cell abnormalities along with the multigenic risk factors of SLE. We have recently observed significant increases in cellular isoaspartyl levels in the islets of NOD diabetogenic mice as well

as in human pancreatic islets treated with physiologic levels of H_2O_2 or inflammatory cytokines (Yang and Mamula, unpublished data).

Targeting DNA and protein methyltransferases as a novel immunotherapy in autoimmune disease. The immune response can be modulated by the global inhibition of methyltransferases as extensively investigated by a number of investigators (75). An example in the clinical spectrum of SLE is with drug-induced lupus induced by procainamide and hydralazine (134), two compounds that inhibit DNA methylation in T cells. Simply put, procainamide is a specific DNMT1 inhibitor suppressing DNA methylation directly while hydralazine indirectly suppresses DNA methylation mediated by specific inhibition of the ERK pathway and subsequently prevents the upregulation of DNMT1 and 3a in T cells and B cells (134). Beyond SLE, global DNA hypomethylation is also found in patients with rheumatoid arthritis (RA) (64). These studies define the central role of transmethylation in immune responses and T cell mediated autoimmune syndromes. Thus, targeting methyltransferases for epigenetic regulation of gene expression is considered as a potential therapeutic approach in autoimmune disease.

All transmethylation reactions depend on the availability of an intracellular methyl donor group. *S*-adenosylmethionine (SAM) serves as the single major intracellular methyl donor. Inhibition of transmethylation can be simply achieved by the disruption of byproducts of SAM metabolism, including the levels of s-adenosylhomocysteine and 5'-deoxy-5'-methylthioadenosine (MTA) (75). Several SAH hydrolase inhibitors are demonstrated to exhibit immunomodulatory effects due to the reduction of intracellular protein methylation (42,75,156-158). All of these compounds have been shown to inhibit T cell proliferation in murine models of SLE, MS and type 1 diabetes (130,133,138,147,157,158). Moreover, transmethylation

inhibition can suppress delayed type hypersensitivity ear swelling and peptidoglycan polysaccharide-induced arthritis (130) and can slow the pathology of collagen-induced arthritis (157). Recently, our laboratory has demonstrated that MTA, as a feedback inhibitor of the SAM pathway, markedly ameliorates pathology of murine SLE. Administration of MTA to MRL/lpr mice caused suppression of T cell functions and reduced splenomegaly, lymphadenopathy, autoantibody titers as well as IgG deposition and cellular infiltration in the kidney (163).

The development of specific protein methyltransferase inhibitors is an attractive approach in immunotherapy. For example, AMI-1 and its derivatives, non-nucleoside specific small molecule inhibitors of PRMTs, can suppress T cell proliferation and reduce Th1/Th2 cytokine expression including IL-4 and IFNγ (11,18). Recently, Mowen and coworkers successfully identified two novel specific PRMT1 inhibitors by high-throughput screening (31). Future studies will undoubtedly reveal new mechanisms of how protein methylation may alter the course of autoimmune syndromes.

DNA methylation in autoimmunity. Clearly, various autoimmune syndromes, including T1D, SLE and RA, are a result of immune recognition of self-antigens. The diagnostic hallmarks include the obvious clinical sequelae combined with specific markers in the diagnostic laboratory. However, novel PTMs described earlier in this review as well as other biological elements including specific nucleotide sequences, sugar phosphate backbone modifications, and even DNA secondary structure require the reassessment of diagnostic substrates and/or the development of new biomarkers.

Epigenetics denotes modifications that occur during the transcription and translation of genes which influence the level of gene expression but do not affect DNA sequence (85). The conventional process of DNA methylation, of histones acetylation/deacetylation, of nucleosome

remodeling and of small non-coding RNA transcripts modification constitutes epigenetic factors. As noted throughout, posttranslationally modified proteins are linked to alterations in DNA methylation. Lupus patients and unaffected human controls were evaluated for methyl-cytosine residues which are the products of DNA methylation (5). This study revealed that global DNA hypomethylation is more prevalent in SLE as compared to healthy individuals. Massive global hypomethylation of DNA has been associated with classical autoimmune diseases such as SLE and RA.

Rui et al reported epigenetic modifications of β cells during progression of diabetes in NOD mice, the murine model of T1D (127). They showed that there was methylation of exons in *Ins1* and the promoter and exons of the *Ins2* gene. There was a strong inverse relationship between methylation of Ins2Exon1 and Ins2 mRNA levels in β cells. There was induction of DNMT3a in particular during disease progression which was shown to account for the epigenetic changes by siRNA silencing. They attributed the induction of DNMT3a to inflammatory cytokines that were found in the islets during progression of disease because culture of islets with IL-1b, IL-6, and IFNγ, which were present during insulitis, induced DMNT3a and cause methylation of Ins2 DNA. DNMTs are critically involved in β cell differentiation and subsequent studies suggested that there may be cellular changes resulting from the inflammation that may result from epigenetic modifications (126). They described a subpopulation of β cells in the islets of NOD mice that developed during progression of disease in which there was loss of normal differentiation features of β cells and stem-like characteristics. The novel subpopulation showed increased frequency of methylation of CpG sites in the Ins genes compared to normal β cells (unpublished). These cells were resistant to immunologic killing suggesting that the mechanism

of epigenetic modification to inflammatory mediators may represent a cellular protective response.

It is notable that epigenetic changes related to global hypomethylation of DNA (hypoacetylation of histone H4 and trimethylation of H4) is also used as early detection of cancer, Alzheimer's disease, and a variety of autoimmune syndromes including acute disseminated encephalomyelitis, Addison's disease, ankylosing spondylitis, antiphospholipid antibody syndrome (APS), Grave's disease, MS, and mixed connective tissue diseases (54).

Concluding Remarks

In summary, we have attempted to illustrate and summarize several specific pathways beyond the genetics of disease that influence the onset and progression of autoimmunity. Many of the PTMs and antigen processing pathways described herein are spontaneous in nature and beyond the prediction of genetics. It is now obvious that many PTMs and cellular pathways are affected by reactive oxygen species. Indeed, it may appear that ROS is at the 'center of the universe' of triggering autoimmunity. ROS may affect PTM of self proteins in direct ways, such as with carbonyl modifications. Indirect pathways are affected by ROS, as with methylation of DNA or proteins and with amplifying enzyme mediated PTMs (citrullination) and by triggering PTM repair mechanisms (PIMT). While we have defined some specific PTMs in T1D, SLE and many other autoimmune syndromes, this dynamic and quickly changing field does not allow us to enumerate and detail all published PTM autoantigens. Moreover, we have not attempted to define the fine specificity of B and T cell responses or specific epitope modifications of many PTM autoantigens. For those details, we would refer the reader to other manuscripts within this volume of *Antioxidants and Redox Signaling* or to the original publications. Studies by

Piganelli, et al. (reviewed in this volume have carefully defined the role of inflammatory and oxidated stress in pancreatic tissue and the endoplasmic reticulum in the course of T1D autoimmunity (95,96). The emerging technologies in proteomics and tissue analyses will undoubtable change the landscape of this field in the coming months and years. These analyses have already 'modified' how the clinical community diagnoses and assesses 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.

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Declaration of Interest:

The authors report no conflicts of interest.

Abbreviations used in this paper:

AMI-1, protein arginine methyltransferases 7,7′-carbonylbis(azanediyl)bis(4-hydroxynaphthalene-2-sulfonic acid; CARM1, coactivator-associated arginine methyltransferase 1; DNMT, DNA methyltransferase; NFAT, Nuclear factor of activated T cells; NETs, neutrophil extracellular traps; PAD, peptidylarginine deiminase; PIMT, protein isoaspartate methyltransferase; PRMT, protein arginine methyltransferase; PTM, posttranslational modification; SAM, *S*-adenosylmethionine.

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Table 1. Posttranslational protein modifications associated with autoimmune disease.

Modification	*Disease*	*Antigen*	*References*
Phosphorylation	EAE/MS	aB-crystallin	(143)
	SLE	nucleophosmin	(74)
		snRNP	(108)
Glycosylation	CIA	Type II collagen	(38)
Citrullination	EAE/MS	MBP	(161)
(Deimination)		CapZα1	(98)
		Histone H4	(104)
		Fibrin	(97)
		Type I, II collagen	(137)
		α-enolase	(68)
	T1D	GRP78	(124)
		GAD65	(100)
Acetylation	EAE	MBP Ac1-11	(167)
	SLE	Histone H2B	(83)
	RA	Vimentin	(63)
Hydroxylation	CIA	Type II collagen	(6)
Methylation	SLE	Sm D1, D3	(13,17)
Deamidation	Celiac disease	Gliadin	(73)
	T1D	Preproinsulin/Proinsulin	(102,142)
IsoAsp formation	SLE	snRNP D	(91)
		Histone H2B	(32)
Oxidation	SLE	oxLDL	(51)
	T1D	Insulin	(92)
Carbamylation	RA	A1T1	(146)
		Vimentin	(117)
		GRP78	(165)
Carbonylation	T1D	P4Hb	(unpublished data)

EAE, experimental autoimmune encephalomyelitis; MS, multiple sclerosis; SLE, systemic lupus erythematosus; CIA, collagen-induced arthritis; RA, rheumatoid arthritis; MBP, myelin basic protein; LDL, low density lipoproteins; CapZα1, F-actin capping protein alpha-1 subunit; GRP78, glucose-regulated protein 78; A1T1; alpha 1 anti-trypsin; GAD65, glutamic acid decarboxylase 65; P4Hb, prolyl-4-hydroxylase.

Table 2. Posttranslational protein modifications in type 1 diabetes.

Target proteins	*Modification*	*References*
GAD65	citrullination	(4,101)
Preproinsulin/ Proinsulin	deamidation	(102,142)
Insulin	oxidation	(92)
IA-2	citrullination	(101)
	deamidation	(142)
GRP78	citrullination	(4,124)
ZnT8	citrullination	(101)
	deamidation	(142)
IAPP	citrullination	(4)
IGRP	citrullination	(101)
	deamidation	(142)
ICA69	deamidation	(142)
SERCA2a	carbonylation	(132)
P4Hb	carbonylation	(unpublished data)

GAD65, glutamic acid decarboxylase 65; IA-2, islet antigen-2; GRP78, glucose-regulated protein 78; ZnT8, zinc transporter 8, IAPP, islet amyloid polypeptide; IGRP, islet-specific glucose-6-phosphatase catalytic subunit-related protein; ICA69, islet cell autoantigen 69; SERCA2a, sarco/endoplasmic reticulum Ca^{2+}ATPase; P4Hb, prolyl-4-hydroxylase.

Table 3. PRMTs associated with immune responses.

	Methylation product	PRMTs member	Epigenetic regulation	Refs
Type I	methylation in the terminal guanidine nitrogen atoms monomethylation asymmetric dimethylation	1 (RMT1 in yeast) 2 (?) 3 (RMT3 in yeast) 4 (CARM1) 6 8	Th2 cytokine production (IL-2 and IL-4) (PRMT1) Signal transduction in T cells Vav1, STAT1/6, AKt/PKB, NIP45, and NFAT (PRMT1) and STAT3 (PRMT2) Interaction to Interferon receptor (PRMT1) P13K kinase pathway in B cell differentiation (PRMT1) T helper cells function (PRMT2) thymocyte differentiation (PRMT4) Th17 differentiation (PRMT2, 4 and 6) Histone methylation, H4R3 (PRMT1) and H3R2, R8, R17 and R26 (PRMT4)	(136) (70) (157) (76, 112, 113, 129) (153) (59) (61) (67) (71) (7, 159)
Type II	methylation in the terminal guanidine nitrogen atoms monomethylation symmetric dimethylation	2(?) 5 9(FBXO11) Hsl7	IL-2 gene expression (PRMT5) Histone methylation, H2A, H4 (PRMT5) B cell lymphoid cancer (PRMT5)	(123) (39, 118) (151)
Type III	methylation in the terminal guanidine nitrogen atoms monomethylation	7	Histone methylation, H4 (PRMT7) Thymus and dendritic cells (PRMT7)	(105) (77, 106)
Type IV	methylation in the internal guanidine nitrogen atoms monomethylation	RMT2 (in the yeast)		

Figure Legends

Figure 1. Structures of common posttranslational protein modifications in autoimmune disease.

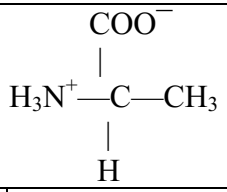
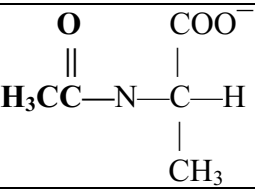
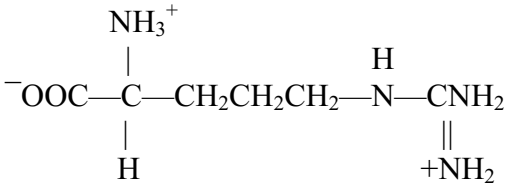
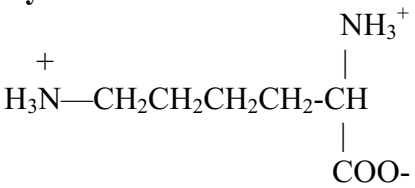
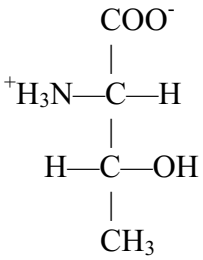
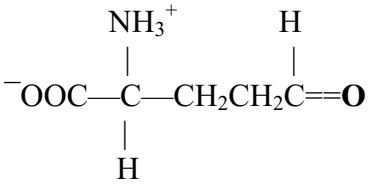
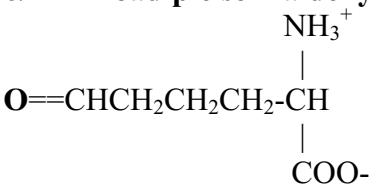
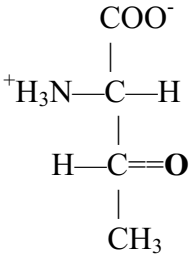
Figure 2. ROS and inflammation initiate cycles of autoimmunity and epitope spreading. Posttranslationally modified self proteins arise in tissues during cellular stress, including ROS, inflammatory cytokines and/or infection. PTMs are released into the milieu and phagocytized by antigen presenting cells (either macrophages, dendritic cells, or B cells). Neoantigenic PTM self peptides are then presented to autoreactive T and B cells that have escaped negative selection in the thymus and bone marrow. This occurs because the modified peptide is typically not presented during selection in non-inflammed secondary lymphoid organs. Subsequently, autoreactive T and B cells infiltrate host tissue where an autoimmune response develops, leading to a second round of PTM generation and/or altered DNA methylation.

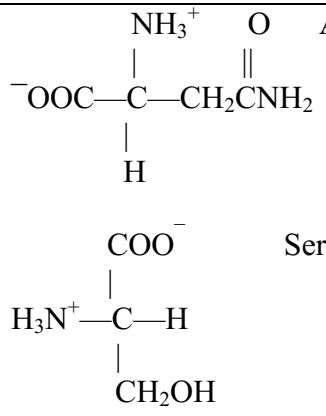
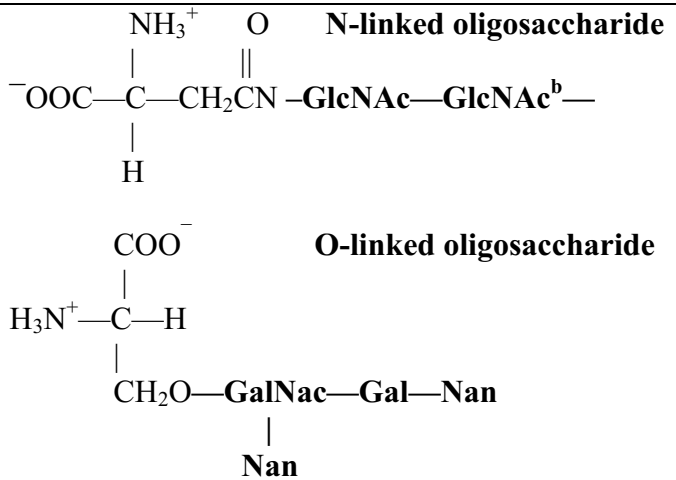
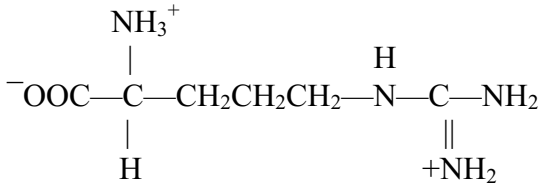
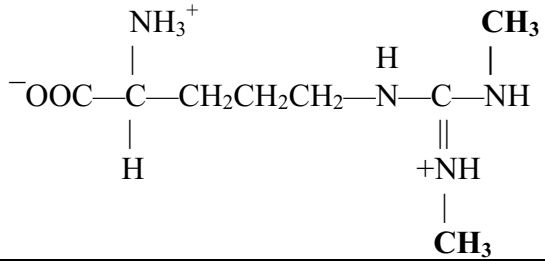
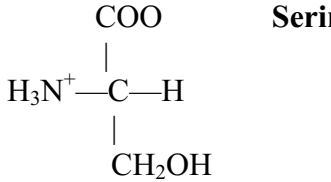
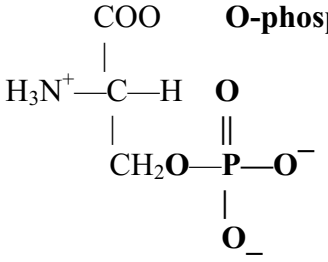
Figure 3. Antigen processing is altered by posttranslational modifications. (a) Native isoforms of self-antigens are cleaved by intracellular proteases (as represented by "X") into distinct peptides. Under most conditions, negative selection eliminates T and B cells that recognize these normal isoform peptides due to clonal deletion and anergy. (b) Posttranslationally modified sites are often not accurately recognized or cleaved by proteases, thereby creating novel self peptides to which immune tolerance does not exist. Novel peptide presentation by APCs primes T cells which, provide help to B cells in secreting autoantibodies.

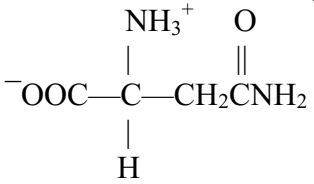
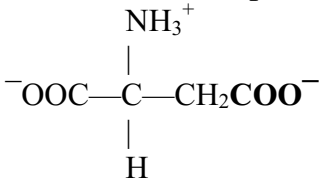
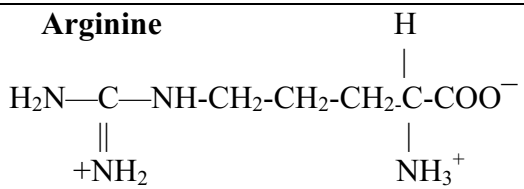
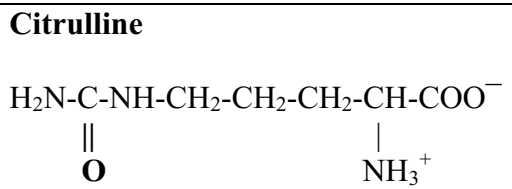
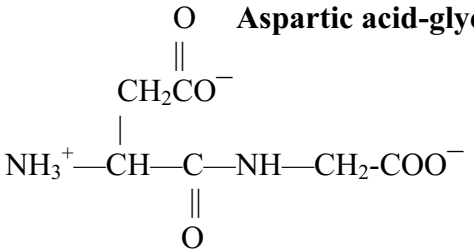
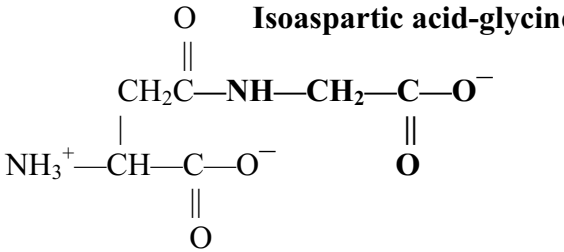
Figure 4. The generation of PTMs in pancreatic beta cells in T1D. Immune cell infiltration of pancreatic islets includes macrophages, CD4 and CD8 T cells, and NK cells. Direct attack to the beta cell from CD8 and NK cells occurs, while ROS is released from resident macrophages. Other inflammatory cytokines, including TNFa, IL-1b, IL6, and IFNg all contribute to PTMs generated inside of the beta cell. The response to ROS and cytokine stress include various PTMs such as carbonylation, oxidation, citrullination, and protein methylation. In addition, DNMTs and TETs altered by the presence of ROS cause various defects in DNA methylation and subsequent downstream translational regulation.

Figure 5. ROS and inflammation induce carbonyl modification of the chaperone protein, P4Hb. The oxidative and cytokine stress in the pancreatic islet microenvironment induces carbonyl modification of beta cell proteins (see text) and P4Hb. P4Hb is one chaperone protein responsible for the accurate folding and processing of proinsulin to insulin in the beta cell. Carbonyl modified P4Hb is a neoantigen that induces autoreactive B and T cells found in early onset human T1D and in the NOD mouse. In addition, carbonyl P4Hb fails to accurately process proinsulin leading to reduced insulin secretion.

Figure 1. Common posttranslational modifications[a].

Modification	Normal	Modified
Acylation	 **Alanine**	 **N-acetylalanine**
Carbonylation	**Arginine**  **Lysine**  **Threonine** 	**Glutamic semialdehyde**  **α-Aminoadipic semialdehyde**  **α-Amino-β-ketobutyric acid** 

Glycosylation		
Methylation		
Phosphorylation		

Deamidation	**Asparagine** 	**Aspartic acid** 
Deimidation/ Citrullination	**Arginine** 	**Citrulline** 
Isoaspartyl	**Aspartic acid-glycine** 	**Isoaspartic acid-glycine** 

[a] The majority of these modifications are mediated by specific enzymes that for the sake of clarity are omitted from this table.

[b] Abbreviations: GlcNac, N-acetyl-D-glucosamine; GalNac, N-acetyl-D-galactosamine; Gal, D-galactose; Nan, N-acetyl-neruaminic acid

Figure 2.

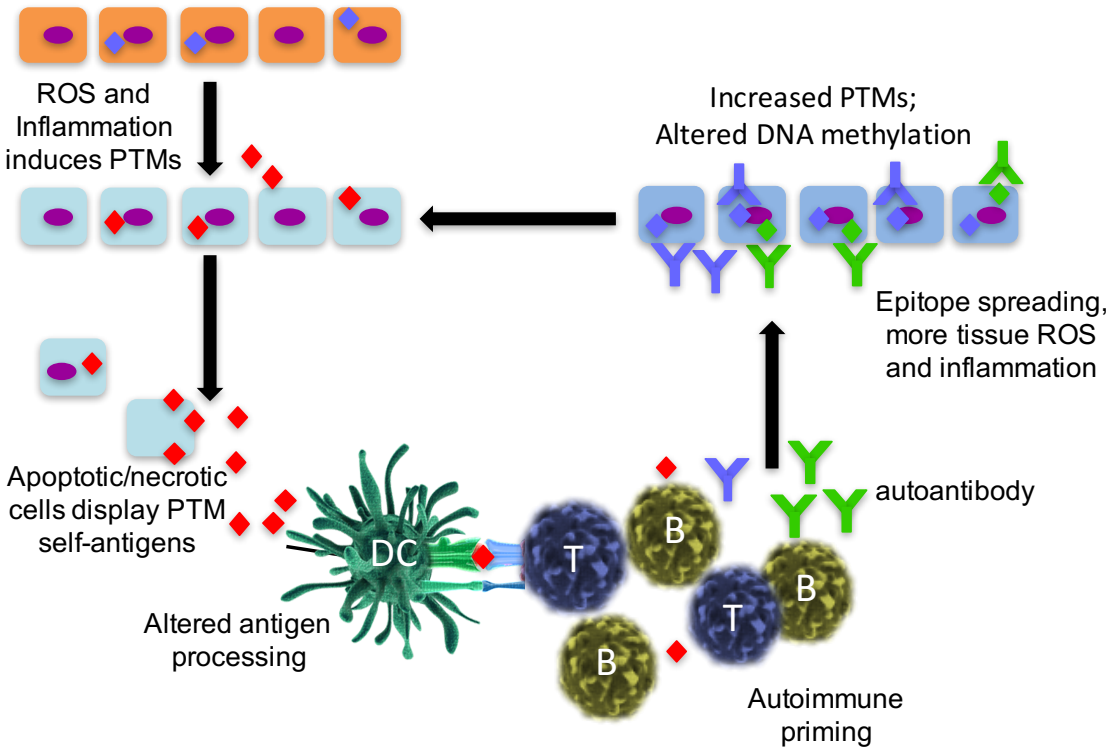

ROS and
Inflammation
induces PTMs
Increased PTMs;
Altered DNA methylation
Epitope spreading,
more tissue ROS
and inflammation
Apoptotic/necrotic
cells display PTM
self-antigens
DC
T
B
T
B
B
autoantibody
Altered antigen
processing
Autoimmune
priming

Figure 3.

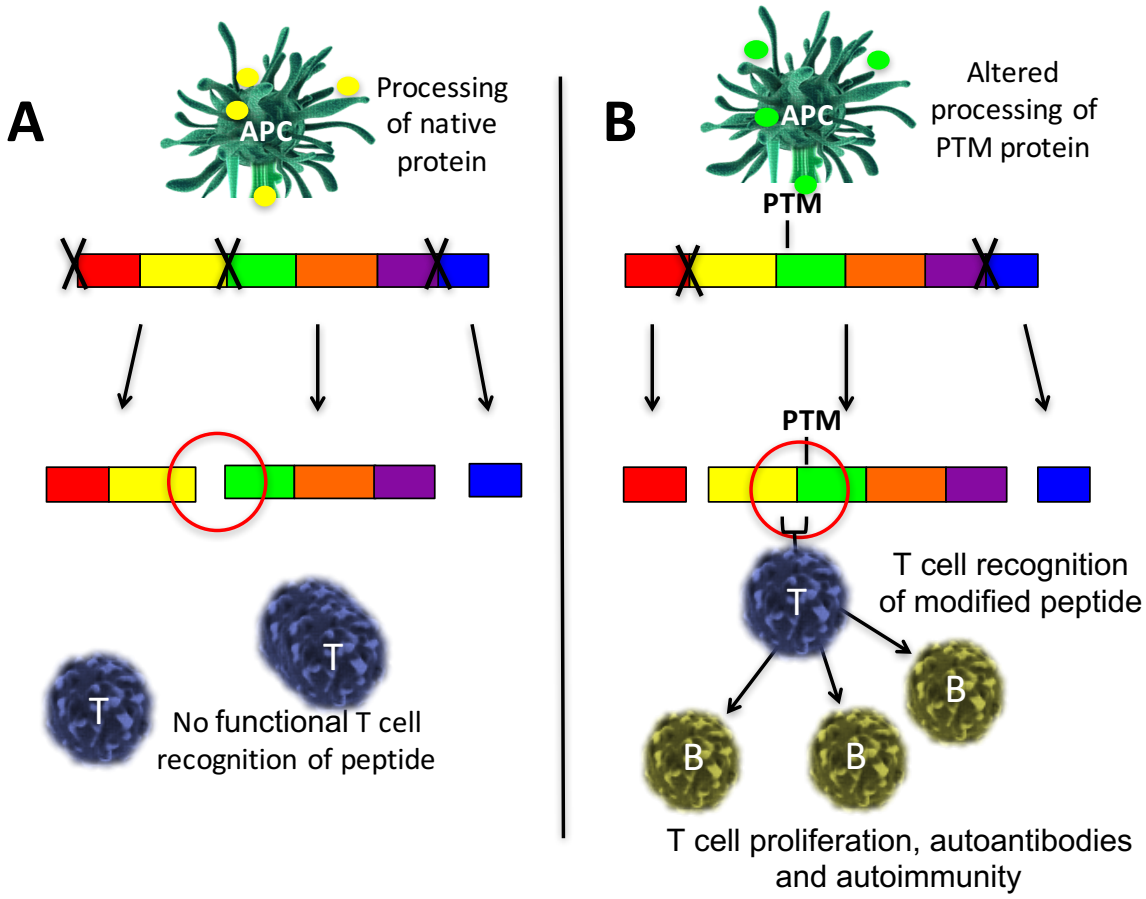


Figure 4.

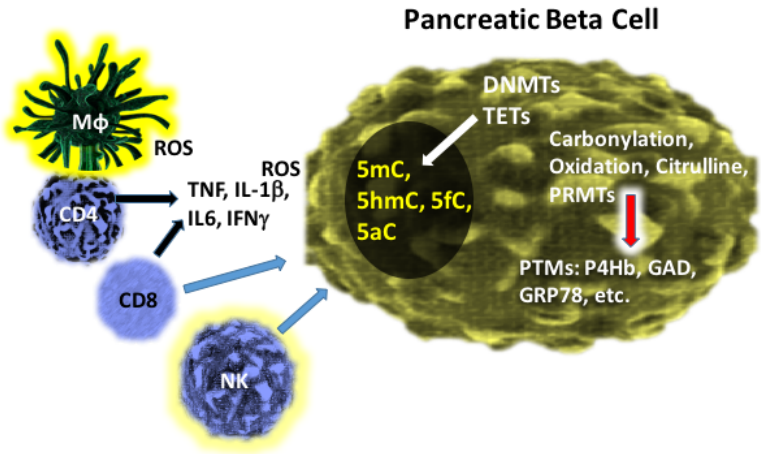
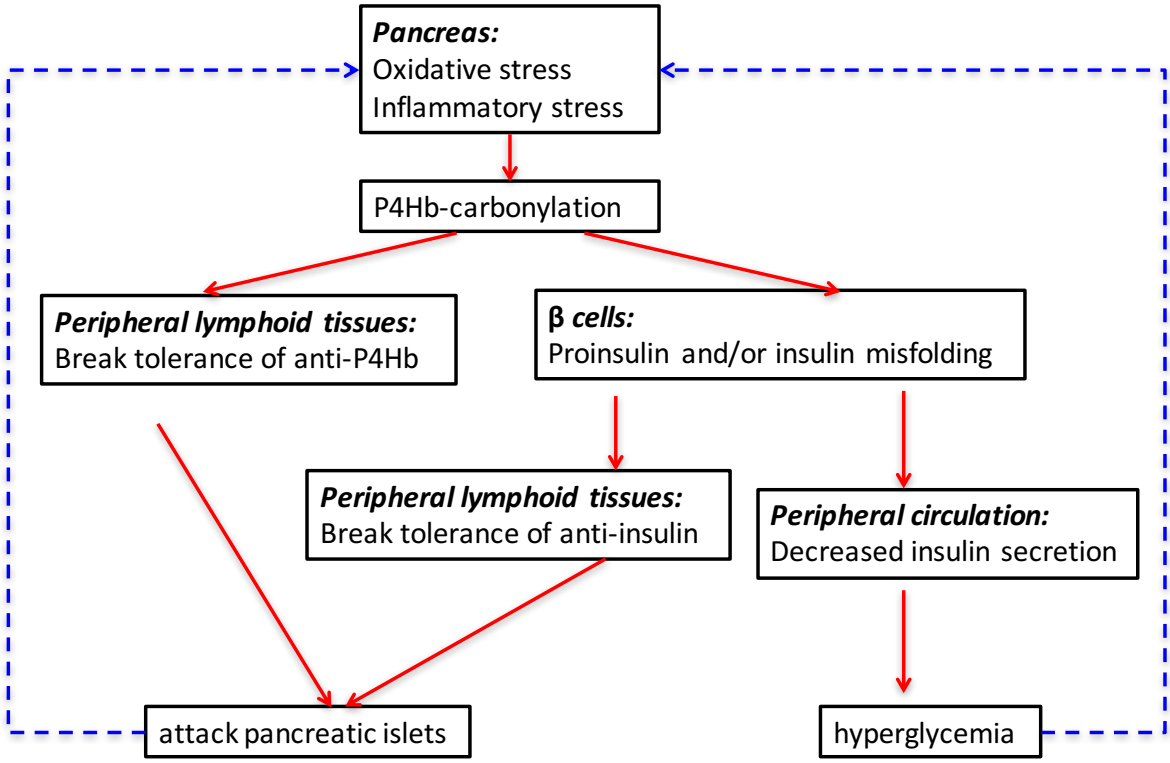


Figure 5.


Pancreas:
Oxidative stress
Inflammatory stress
P4Hb-carbonylation
Peripheral lymphoid tissues:
Break tolerance of anti-P4Hb
β cells:
Proinsulin and/or insulin misfolding
Peripheral lymphoid tissues:
Break tolerance of anti-insulin
Peripheral circulation:
Decreased insulin secretion
attack pancreatic islets
hyperglycemia