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Non-mutational neoantigens in disease

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Abstract

The ability of mammals to mount adaptive immune responses culminating with the establishment of immunological memory is predicated on the ability of the mature T cell repertoire to recognize antigenic peptides presented by syngeneic MHC class I and II molecules. While it is widely believed that mature T cells are highly skewed towards the recognition of antigenic peptides originating from genetically diverse (for example, foreign or mutated) protein-coding regions, preclinical and clinical data rather demonstrate that novel antigenic determinants efficiently recognized by mature T cells can emerge from a variety of non-mutational mechanisms. In this Review, we describe various mechanisms that underlie the formation of *bona fide* non-mutational neoantigens, such as epitope mimicry, upregulation of cryptic epitopes, the usage of non-canonical initiation codons, alternative RNA splicing, defective ribosomal RNA processing, as well as both enzymatic and non-enzymatic post-translational protein modifications. Moreover, we discuss the implications of the immune recognition of non-mutational neoantigens for human disease.

Introduction

MHC proteins present a sampling of the cellular proteome at the cell surface for inspection by T cells as part of the immune surveillance of the body for infection or malignancy. Generally, MHC class I proteins sample peptides generated by the proteasomal degradation of cytosolic proteins, whereas MHC class II proteins sample peptides generated by the endosomal or lysosomal proteolysis of (1) cell surface proteins, (2) proteins taken up from the extracellular environment through receptor-mediated or fluid-phase endocytosis, and (3) proteins from cellular organelles engulfed by autophagosomes. Thus, the MHC system

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accesses all major protein degradation pathways and intracellular compartments as a means to inform T cells about the cellular state.[1]

Positive and negative thymic selection have evolved to ensure that the mature T cell repertoire of each individual specifically recognizes peptides that (1) are presented by autologous MHC class I and II molecules, and (2) are not generally presented by healthy tissues.[2] However, central tolerance as generated by thymic selection is leaky, implying that mature T cell repertoires contain many T cell clones that are specific for unmodified self antigens.[3] Importantly, a panel of mechanisms exist to prevent the activation of such autoreactive T cell clones, including (but not limited to) (1) the requirement of multiple signals beyond TCR activation for T cells to acquire effector and memory functions, and (2) the existence of antigen-specific immunosuppressive T cell populations such as $CD4^+CD25^+FOXP3^+$ regulatory T (T_{REG}) cells.[4]

Defects in these mechanisms of peripheral tolerance (for example, *FOXP3* mutations) have been associated with various autoimmune conditions, such as IPEX syndrome.[5] Moreover, modern MHC elution techniques coupled with the development of algorithms that can link peptide mass spectra with specific peptide structures have unveiled the existence of many antigenic epitopes that – because of structural modifications or overrepresentation – become able to be efficiently presented by MHC class I and II molecules, and hence elicit reactivity, but do not originate from DNA mutations.[3] Such ‘non-mutational neoantigens’ can originate from a variety of post-transcriptional and post-translational mechanisms, including epitope mimicry, the usage of non-canonical initiation codons, alternative RNA splicing, defective ribosomal RNA processing, as well as both enzymatic and non-enzymatic post-translational protein modifications, such as phosphorylation, citrullination, glycation and many others.[3] Importantly, preclinical and clinical data suggest that non-mutational neoantigens are involved in a number of disorders ranging from autoreactive conditions to cancer, with multiple host-related risk factors (Box 1).

In this Review, we summarize the molecular mechanisms leading to the generation of the autoreactive non-mutational MHC immunopeptidome and their emerging role in human disease. Conversely, we will not cover the details of physiological MHC class I and II antigen processing and presentation (reviewed elsewhere),[1] nor autoreactivity to non-peptide antigens presented by non-canonical MHC proteins such as CD1 family members and major histocompatibility complex, class I-related (MR1) (reviewed elsewhere).[6, 7]

Epitope mimicry

Autoreactivity against non-mutational neoantigens can emerge from so-called ‘epitope mimicry’, the ability of some microbial antigens to elicit cross-reactive T or B-cell responses against the host (Figure 1).[8] Mimicry can obviously result from a considerable overlap in the primary amino acid sequence between microbial and host epitopes, but also from structural similarities in the context of minimal sequence overlaps.[9] Autoreactive conditions associated with epitope mimicry encompass: (1) multiple sclerosis, in which myelin basic protein (MBP) is recognized upon Epstein Barr virus (EBV) infection;[10, 11] (2) rheumatic fever, which is unleashed upon infection by *Streptococcus spp.* and consequent cross-reactivity

against cardiac glycoproteins;[12] (3) Guillain-Barré syndrome, as driven by autoreactive responses against neuronal proteins elicited by EBV, cytomegalovirus (CMV) or type I herpes simplex virus (HSV-1) infection;[13] (4) type 1 diabetes, in which pancreatic β cell antigens are targeted upon infection with enteroviruses or lymphocytic choriomeningitis virus (LCMV);[14, 15, 16] (5) rheumatoid arthritis, which is promoted by cross-recognition of synovial tissue and cartilage antigens upon infection by type I human immunodeficiency virus (HIV-1), hepatitis B virus (HBV) or hepatitis C virus (HCV);[17, 18] as well as (6) systemic lupus erythematosus (SLE), which is facilitated by DNA- and Smith (Sm)-targeting antibodies emerging after EBV, CMV, HCV or parvovirus infection.[19, 20, 21] Similarly inflammatory cardiomyopathy seems to be promoted by cardiac myosin-specific T_H17 cells elicited in the intestine by a commensal *Bacteroides spp*. epitope.[22] Also, SLE, the Guillain-Barré syndrome and other autoimmune conditions such as pediatric inflammatory multisystemic syndrome or Kawasaki-like disease in children have also been linked to epitope mimicry upon SARS-CoV-2 infection.[23, 24, 25]

That said, the majority of patients with infectious diseases do not develop autoimmune disorders. Moreover, a comparative analysis of the human and pathogen proteome identified up to 90% overlap in 6–8 identical amino acids stretches within 9-mer epitopes.[26] Thus, factors beyond epitope mimicry probably contribute to the development of autoreactive disorders after infection. Aside from polymorphisms in HLA-coding genes and other genes involved in immune regulation (Box 1),[27] such factors might include the differential presentation of antigenic epitopes as a consequence of infection-related perturbations of cellular homeostasis (for example, endoplasmic reticulum stress),[28] and the pathogenic relocalization of normally symbiotic microbes to ectopic locations (e.g., from the intestine to the liver).[29]

Of note, various microbial epitopes have also been shown to resemble tumor-associated antigens (TAAs).[30] For example, similarities have been documented between *Mycoplasma penetrans* HF-2 epitopes and MAGE family member A6 (MAGEA6),[31] CMV peptides and MAGEA10,[32] HSV-1 components and melan-A (MLANA),[33] as well as HBV antigens and transmembrane protein 161A (TMEM161A).[34] Supporting the notion that epitope mimicry is common in neoplastic conditions, a comparison between public databases of TAA-derived epitopes (https://caped.icp.ucl.ac.be/peptide/list) and viral proteomes (Viruses tazid:10239) identified almost 100 shared sequences.[35] The functional significance of epitope mimicry for patients with cancer is exemplified by studies identifying (1) cross-reactivity between microbial antigens and TAAs, (2) a positive association between microbial antigens and response to immunotherapy, and (3) the existence of T cells recognizing cross-reactive antigens.[36, 37] Cross-reactivity between microbial antigens and TAAs has been detected in patients with bladder cancer treated with Bacillus Calmette-Guérin, an attenuated strain of *Mycobacterium bovis*,[38] or with neoadjuvant immune checkpoint inhibitors (ICIs),[36] as well as in individuals with renal and lung cancer receiving a programmed cell death 1 (PD-1) blocker, where $CD8^+$ T cells recognizing an epitope from the opportunistic pathogen *Enterococcus hirae* cross-react with naturally-processed TAAs.[37] Considerable efforts are currently being dedicated to combining bacterial or viral peptides with other immunostimulatory maneuvers to elicit tumor-targeting immune responses that may be actionable with ICIs.[30] Considering that a particular TCR can recognize more than a million

different peptides,[39] and that different TCRs can recognize the same peptide (with different affinity),[40] issues of TCR degeneracy versus specificity,[41, 42] will have to be explored in the context of epitope mimicry.

In summary, although mimicry between pathogen-derived and self epitopes may elicit pathogenic T cell responses, other factors are normally required for the development of autoreactivity/autoimmunity against these antigenic determinants. Understanding these factors will enable the development of therapeutic strategies to prevent or harness epitope mimicry in patients.

Cryptic epitopes, DRiPs, SLiPs and unconventional translation products

Cryptic peptides are epitopes that are physiologically processed and presented by antigen-presenting cells (APCs) inefficiently, at low copy number, or not at all, hence failing to elicit central tolerance or peripheral T cell responsiveness.[43] However, various perturbations of cellular homeostasis, including alterations in the MHC class I and II machinery, can affect the processing of native proteins, potentially resulting in an increase in the number of cryptic peptides presented by MHC molecules and hence in the initiation of an autoreactive T cell response (Figure 1).[43] Conditions that may generate or upregulate cryptic peptides include: (1) reduced competition by other peptides for binding to MHC molecules; (2) enhanced protein availability (due to increased synthesis or decreased degradation); (3) protein unfolding exposing cleavage sites for alternative proteases (as in the case of endoplasmic reticulum stress); (4) changes in the cytosolic or reticular microenvironment (for example, during inflammatory conditions), resulting in activation/deactivation of specific proteases, and (5) quantitative changes in the expression of peptide processing and editing components of the MHC class I and II machinery.[43, 44, 45]

In the context of autoimmunity, one of the best examples of crypticity is provided by proteolipid protein 1 (PLP1), one of the main components of the myelin sheet of neurons that is targeted during autoimmune encephalomyelitis. Specifically, PLP1 exists in multiple splice variants, and the one presented in the thymus (DM20) lacks a major epitope that instead is presented in the periphery, resulting in lack of central tolerance and consequent T cell activation at neuronal terminals, and pathogenic demyelination.[46] Different splice variants and cryptic peptides of MBP have also been associated with multiple sclerosis,[47, 48] similar to novel PLP1 reactivities emerging after Theiler virus infection.[49] Along similar lines, cartilage-derived cryptic epitopes seem to be preferentially presented in the acute, inflammatory phase of rheumatoid arthritis, likely as a consequence of extracellular matrix (ECM) protein unfolding and processing by enzymes released by immune cells.[50] Finally, systemic lupus erythematosus (SLE) has been shown to involve T-cell and B-cell autoreactivity against cryptic epitopes from components of the complement cascade.[51] Similar findings have been obtained in the context of autoreactive responses as elicited by viral infection. Specifically, the HIV-1 membrane glycoprotein gp120 has been shown to uncover cryptic CD4 peptides resulting in pathogenic CD4-specific autoantibodies.[52] Moreover, ribosome profiling and proteomic mapping identified cryptic SARS-CoV-2 antigens associated with severe post-infection autoreactivity.[53, 54, 55] These observations exemplify the broad pathogenic relevance of autoreactivity against cryptic epitopes.

Potentially autoreactive epitopes can also originate from defective ribosomal products (DRiPs), short-lived proteins (SLiPs),[56] non-canonical (including non-AUG defined and novel unannotated) open-reading frames (ORFs), protein frameshift (for example, owing to the lack of a specific amino acid),[57, 58, 59, 60] as well as from the translation of 5'- or 3'-untranslated regions (UTRs).[61, 62] All these scenarios have been shown to be relevant for the composition of the MHC immunopeptidome.

DRiPs and SLIPs can be translated as efficiently as canonical proteins and have been shown to generate peptides 5-fold more efficiently per translation event.[57] Indeed, it seems that the MHC class I and less so class II immunopeptidome contain a large proportion of DRiPs and SLIPs as compared to cryptic epitopes derived from canonical proteins.[56] Importantly though, defining whether an MHC-bound epitope is a DRiP or a SLiP cannot be achieved with standard MHC elution and mass spectrometry, as these peptides share the same amino acid sequence of the corresponding full protein, calling for the use of dynamic SILAC and pulse chase quantitative mass spectrometric techniques or ribosomal profiling coupled with MHC elution.[63] DRiPs/SLiPs can arise from the translation of non-canonical (including non-AUG defined) open reading frames (ORFs),[59, 60] as well as from the translation of 5'- or 3'- untranslated regions (UTRs),[61, 62] two scenarios that result in the generation of novel epitopes.

DRiPs/SLiPs were initially characterized in the context of viral infection, potentially offering a rapid mechanism for infected cells to enable MHC-restricted antiviral immunosurveillance.[62] Albeit the vast majority of defined viral peptides derive from standard ORFs of stable proteins, several viral DRiPs and SLiPs have been shown to derive from errors in protein translation, trafficking, folding or other mostly unknown mechanisms.[64, 65] For example, peptides translated from a non-AUG defined ORF have been shown to potently elicit alloreactive T cells in the context of eukaryotic translation initiation factor 2 subunit alpha (EIF2S1, best known as eIF2α) phosphorylation,[66] which is one of the first cellular reactions to viral infection,[67] as well as in the presence of pro-inflammatory cues including interferon gamma (IFNG).[68] Of note, DRiP/SLiP-generated viral epitopes generally coupled to specific cytotoxic T cell responses[69] have been documented in the context of infection by a number of viral pathogens, including influenza virus, coxsackie virus, HSV and numerous retroviruses.[69, 70, 71] Moreover, at least some viral pathogens (for example, EBV, SARS-CoV-2) promote the presentation of host DRiPs/SLiPs, ultimately resulting in autoreactivity.[72, 73]

Non-canonical translation leading to DRiPs/SLiP accumulation has also been linked to overt autoimmunity.[61] For example, Reiter's syndrome has been associated with autoreactive T cells specific for an interleukin-10 (IL10) epitope created by a non-canonical ORF.[74] Along similar lines, DRiPs/SLiPs generated from an alternative ORF in the insulin (INS)-coding mRNA have been shown to efficiently load on MHC class I and class II molecules, generating both humoral and cellular autoreactivity of pathogenic relevance in type 1 diabetes.[75] In line with this notion, ribosomal profiling of human pancreatic β cells exposed to inflammatory conditions identified numerous DRiPs/SLiPs generated from non-canonical ORFs that are not present in healthy pancreatic β cells.[76]

Most (but not all) non-mutated cancer-associated epitopes that have been associated with natural cytotoxic T cell responses, or successfully harnessed to elicit at least some degree of tumor-specific autoreactivity, represent *bona fide* cryptic epitopes, as their immunogenicity largely reflects differential expression levels in malignant versus normal tissues.[77] For example, melanoma-infiltrating lymphocytes seem to be highly enriched in T cells recognizing epitopes from differentiation and cancer-testis antigens,[78] but also contain T cells specific for epitopes from absent in melanoma 2 (AIM2) and alpha-1,6-mannosylglycoprotein 6-beta-N-acetylglucosaminyltransferase (MGAT5) generated by non-canonical ORFs emerging from incomplete splicing.[79] Importantly, such autoreactive T cells could be isolated from the peripheral blood of patients with melanoma, but not healthy volunteers or patients with neoplasms other than melanoma,[79] corroborating the crypticity of these epitopes. Similar results have been obtained with atypical teratoid/rhabdoid tumors, which were shown to present several MHC class I- and II-associated cryptic epitopes (shared with glioblastomas but not extracranial tumors) associated with $CD8^+$ and $CD4^+$ T cell reactivity,[80] as well as colorectal cancers.[81]

Supporting the presentation of cryptic epitopes by malignant cells, a number of clinical trials testing TAAs or epitopes thereof as therapeutic vaccines (delivered via various technologies) have documented at least some degree of tumor-targeting immune reactivity.[82, 83] These studies include trials testing: (1) erb-b2 receptor tyrosine kinase 2 (ERBB2, best known as HER2) epitopes in women with $HER2^+$ breast cancer,[84] (2) glypican 3 (GPC3) epitopes in patients with hepatocellular carcinoma,[85] (3) a multiepitope vaccine in individuals with melanoma,[86] and (4) a premelanosome protein (PMEL, best known as gp100) epitope in patients with melanoma.[87] Moreover, a cryptic NY-ESO-1 epitope (159–167) was shown to induce robust immunodominant reactivity over two other HLA-A2-binding NY-ESO-1 peptides that were associated with at least some clinical activity in HLA-$A2^+$ patients with melanoma expressing cancer/testis antigen 1B (CTAG1B, best known as NY-ESO-1).[88, 89] That said, it is now clear that therapeutic vaccination has limited clinical efficacy in patients with cancer, reflecting the potently immunosuppressive microenvironment that most neoplasms generate.[77] Interestingly, diagnostic applications have also been proposed for DRiP-based and SLiP-based preparations. Specifically, it has been suggested to use naturally processed DRiPs and SLiPs contained in tumor-derived autophagosomes (which originate from an intracellular mechanism for the lysosomal degradation of cytoplasmic material)[90] to interrogate tumor-targeting reactivity in cancer patients prior to immunotherapy.[91]

Taken together, these observations suggest that cryptic epitopes including peptides generates by DRiPs, SLiPs and non-canonical translation products are abundantly presented by cells experiencing perturbations of homeostasis. Although these epitope seem to contribute to pathogenic autoreactivity in the setting of infectious and autoimmune disorders, they might represent a therapeutically actionable mechanism for cancer therapy.

Protein modifications

Antigenic epitopes can also originate via multiple post-transcriptional mechanisms other than the usage of non-canonical ORFs, alternative splicing and the generation of non-conventional ribosomal products. These mechanisms reflect not only direct amino acid

substitution at ribosomes and so-called ‘peptide splicing’, i.e., the fusion of conventional proteolytic products, but also other enzymatic and non-enzymatic post-translational modifications (PTMs) (Figure 2).

Amino acid substitution and peptide splicing.

When a specific amino acid is scarce, the correct aminoacyl tRNA can be replaced by another, resulting in the generation of mutated proteins during translation, despite the usage of purely wild-type mRNAs.[92] This mechanism has been shown to promote T cell reactivity in melanoma cells depleted of tryptophan, generally resulting in phenylalanine substitutants.[92] Along similar lines, approximately 1% of methionine residues in the physiological cellular proteome are inserted via non-methionyl-tRNAs, a fraction that remarkably increases in the presence of viral or chemical cues.[93] Whether these findings can be extended to the depletion of other amino acids remains to be investigated. Moreover, the effect of amino acid substitution on autoimmune conditions has not been formally interrogated yet.

The term peptide splicing (or transpeptidation) refers to the generation of a polypeptide by fusion of shorter peptides from non-contiguous regions of the same (*cis*) or different (*trans*) proteins.[94] Originally described for MHC class I epitopes, spliced peptides have also been reported for MHC class II epitopes, a setting in which they are often described as ‘hybrid’ peptides, reflecting an apparent preference for *trans* over *cis* splicing.[94] While the cytoplasmic proteasome seems to be the major cellular site responsible for transpeptidation of MHC class I epitopes,[95] resulting from the high local concentrations of peptide products in the proteasome interior that compete with water for hydrolysis of acyl-enzyme intermediates,[96, 97] endosomal cathepsins and proteasomes have also been reported to efficiently catalyze this reaction.[94, 98] Interestingly, viral and bacterial proteins also have been reported to undergo *cis*-splicing to generate novel antigenic determinants associated with cellular immunity.[99, 100, 101] However, the reported abundance of spliced peptides in the MHC immunopeptidome ranges from as much as 25–30% (Refs. 102, 103) to as little as 1–3% (Refs. 56, 104, 105), with many epitopes originally attributed to splicing being alternatively explained as originating from novel unannotated ORFs.[104, 106] Thus, the contribution of spliced peptides to the MHC immunopeptidome is controversial.

Supporting the relevance of spliced peptides for human autoimmune disorders, T cells from patients with type 1 diabetes have been shown to recognize an MHC class II-associated epitope resulting from the *trans*-splicing of pro-insulin (aa 64–71) and islet amyloid polypeptide (IAPP, aa 74–80),[107] as well as MHC class I-restricted epitopes derived from the *cis*-splicing of IAPP[108] or secretogranin V (SCG5).[109] In all these settings, splicing is believed to occur in pancreatic β cell secretory granules, which contain very high concentrations of secretory hormones and processing enzymes. Data from non-obese diabetic (NOD) mice provide mechanistic support for the pathogenic relevance of these neoantigens.[109, 110, 111]

Immunogenic neoantigens generated by amino acid substitution and transpeptidation have also been described in the context of cancer.[112] For example, an asparagine-to-aspartate substitution in tyrosinase (TYR) has been shown to generate a neoepitope resulting in

the activation of melanoma-targeting T cell responses.[113] Moreover, the *cis*-splicing of fibroblast growth factor 5 (FGF5) reportedly elicits immunoreactive epitopes in human renal cell carcinoma cells,[96] as does the *cis*-splicing of gp100 and TYR in melanoma cells.[97, 114, 115] Several other spliced neoantigens associated with tumor-targeting immune responses have been reported.[112]

Post-translational modifications.

Hundreds of PTMs have been documented in mammals, greatly expanding the diversity of the cellular proteome.[116] These modifications encompass enzymatic alterations that are inbuilt in normal physiology and responses to stress, such as phosphorylation, ubiquitination, acetylation, and enzymatic oxidation, as well as non-enzymatic changes imposed by intracellular or extracellular conditions, such as glycation, carbonylation, and non-enzymatic oxidation.[116] At least theoretically, shifts in PTMs can generate new antigenic determinants either directly, by modifying a previously existing non-immunogenic epitope, or indirectly, by influencing virtually any step of antigen presentation from proteasome processing through MHC binding and exposure. In line with this notion, an expanding literature demonstrates that both enzymatic and non-enzymatic PTMs associated with perturbations of cellular homeostasis linked to various human disorders can generate autoreactive responses of pathological significance.[3]

Citrullination is a PTM of arginine that can occur upon non-enzymatic oxidation, which is common in inflamed and aging tissues,[117] or via the catalytic activity of protein arginine deiminase (PAD) family members.[116] Citrullinated peptides have been associated with the generation of novel antigenic determinants and pathogenic humoral or cellular autoreactivity to epitopes from extracellular matrix proteins in rheumatoid arthritis,[118, 119] glucokinase (GCK) in type 1 diabetes[120] and MBP in multiple sclerosis.[121] Whether these epitopes uniquely emerge from enzymatic or non-enzymatic citrullination, however, is unclear. Phosphorylation is a critical event in multiple signaling cascade, and phosphorylated epitopes from peripherin (PRPH) and small RNA binding exonuclease protection factor La (SSB) have been linked to the generation of pathogenic autoantibodies in type 1 diabetes[122] and SLE.[123, 124] Acetylation is a PTM with broad activity in cellular biology, notably in the regulation of gene expression.[125] Acetylation of the amino-terminal peptide of MBP is required for induction of autoimmunity in experimental allergic encephalomyelitis, a mouse model of multiple sclerosis.[126, 127] Antibodies against acetylated histones are common in patients with SLE, and their titer generally correlates with disease severity.[128]

Cellular stress as imposed by aging, inflammation and metabolic alterations such as hyperglycemia can result in an increased abundance of reactive species that promote non-enzymatic oxidation, carbonylation, and glycation.[129] Oxidized epitopes from multiple components of lipoproteins have been shown to promote B cell and T cell autoreactivity with pathological significance for atherosclerosis and other cardiovascular conditions.[130] Along similar lines, oxidative protein modifications as driven by malondialdehyde elicit autoantibodies that promote rheumatoid arthritis at least in part by stimulating osteoclast activation.[131] Juvenile idiopathic arthritis has been associated not only with an increase in carbonylated albumin and immunoglobulins in the circulation,[132] but also with

carbonylation-related alteration in the affinity of a transthyretin (TTR)-derived epitope for HLA-DR1, culminating in pathogenic autoreactivity.[133] More broadly, carbonylation has been shown to have pleiotropic effects on antigen presentation by dendritic cells (DCs), resulting in considerable qualitative and quantitative changes in their MHC class II immunopeptidome.[134] Glycation is particularly common in metabolic conditions associated with hyperglycemia such as type 1 and 2 diabetes.[134, 135] Aside from imposing broad qualitative and quantitative alterations to the DC MHC class II immunopeptidome,[134] glycation as driven by metabolic cues has been shown to favor the emergence of a neoantigenic determinant in protein disulfide isomerase family A member 3 (PDIA3) that elicits autoreactive antibodies promoting disease progression.[135] Finally, the presentation of iodinated, deaminated or nitrosylated thyroglobulin (TG) epitopes has been shown to trigger autoimmune thyroiditis.[136, 137, 138] These observations exemplify the broad pathogenic effect of enzymatic and non-enzymatic PTMs in human disorders with an autoreactive or autoimmune component.

On the other hand, PTM-derived neoepitopes might be beneficial for the development of therapeutic cancer vaccines, based on their increased abundance in malignant over non-malignant cells as a result of oncogene signaling.[139, 140] For example, phosphorylated epitopes from enolase 1 (ENO1), tumor protein p53 (TP53, best known as p53), insulin receptor substrate 2 (IRS2), and cell division cycle 25B (CDC25B), are overrepresented in pancreatic carcinoma (ENO1)[141] and multiple other tumors (p53, IRS2, CDC25B),[142, 143] whereas citrullinated epitopes from matrix metallopeptidase 21 (MMP21), ENO1 and vimentin (VIM), are overrepresented in melanoma[144, 145] and metastasizing carcinomas,[146] respectively. Supporting the actual immunogenicity of these PTMs, epitope-specific responses against citrullinated ENO1 or VIM peptides restricted to the MHC class II molecules HLA-DR4 or HLA-DP4 have been documented in 58% of patients with ovarian cancer.[146] Aside from confirming the immunogenicity of citrullinated ENO1 and mechanistically linking it to citrullination, mouse data demonstrate that this neoantigenic epitope can be employed successfully as a therapeutic vaccine against MHC class II-positive (but not MHC class II-negative) mouse tumors established in immunocompetent, syngeneic HLA-DR4 transgenic mice, an effect that is accompanied by $CD4^+$ T cell activation and acquisition of cytotoxic effector functions.[147]

Supporting the clinical relevance of these observations, an acetylated p53 epitope – but not its de-acetylated counterpart – has been shown to elicit HLA-DR-restricted $CD4^+$ T cell responses in peripheral lymphocytes from patients with cancer, but not healthy volunteers.[142] Moreover, various therapeutic vaccination approaches based on glycosylated mucin 1, cell surface associated (MUC1) have been associated with robust immunogenicity and at least some degree of efficacy in patients with a variety of tumors, although clinical efficacy remains marginal.[148, 149] Despite this and other limitations, the aforementioned observations suggest that a number of PTMs generate non-mutational neoantigenic determinants that – at least in principle – might be harnessed to drive anticancer immune responses.

Alternative processing.

Proteases (the genes for which account for approx. 3% of the human coding genome) are broadly classified into: (1) cysteine proteases (for example, papain, calpains, caspases, cathepsin B, C, H, F, L, K, S, O, W), (2) serine proteases (for example, chymotrypsin, trypsin, elastase), (3) threonine proteases (for example, proteasome-associated proteases and some acyltransferases), (4) aspartic proteases (for example, renin, cathepsin D and E), and (5) metalloproteases (for example, matrix metalloproteases [MMPs], collagenase, various aminopeptidases and carboxypeptidases).[150] In physiological conditions, MHC class I-restricted and class II-restricted peptides are mostly generated by (immuno)proteasomal and endolysosomal proteolysis, respectively.[151] MMPs, calpains and caspases are also known to contribute to the physiological MHC immunopeptidome, reflecting their important function in physiological cellular processes including matrix degradation during immune cell trafficking (MMPs) and programmed cell death (caspases and less-so calpains).[152, 153]

In the presence of perturbations of cellular or microenvironmental homeostasis (for example, inflammatory stimuli, metabolic cues, oxidative stress), the overall proteolytic activity of a cell can considerably change as a consequence of the activation or inactivation of various proteases, resulting in the formation of potentially antigenic (and hence potentially pathogenic) neoepitopes.[154] For example, robust effector T cell activation associated with abundant granzyme B (GZMB) release[155] has been shown to promote the GZMB-dependent generation of neoepitopes from lamin B (LMNB), poly(ADP-ribose) polymerase 1 (PARP1) and RNA, U1 small nuclear 1 (RNU1–1, also known as U1) in SLE,[156, 157, 158, 159] centromere protein C (CENPC) in scleroderma,[160] and SSB in Sjögren's syndrome,[161] most often resulting in pathogenic B cell and/or T cell autoreactivity.[158, 159, 161, 162] Along similar lines, MMPs released by immune cells responding to pro-inflammatory cytokines have been demonstrated to cleave ECM components to release neoantigenic determinants. As an example, matrix metallopeptidase 9 (MMP9) released in response to IL-1 and tumor necrosis factor (TNF) reportedly generates type II collagen-derived neoepitopes that contribute to rheumatoid arthritis[163, 164] Moreover, inhibition of endoplasmic reticulum aminopeptidase 1 (ERAP1) causes profound changes in the MHC class I immunopeptidome, resulting (at least in mice) in a substantial T cell response to a H2-Qa1-restricted neoepitope.[44, 165] In line with this notion, *ERAP1* polymorphisms have been associated with several autoimmune disorders including ankylosing spondylitis,[166] birdshot chorioretinopathy,[167] psoriasis[168, 169] and Beçhet disease.[170]

While alternative processing is likely to occur and generate non-mutational neoantigens also in malignant cells, the impact of this process on tumor-targeting immune responses is understudied. That said, neutrophil-derived proteases taken up by endosomes in lung cancer cells not only appear to improve antigen presentation by favoring the exposure of MHC class I molecules on the cell surface, but also seem to generate a number of neoantigenic determinants within these organelles.[171] Importantly, $CD8^+$ cytotoxic T cells recognizing some of these neoepitopes, including epitopes from minichromosome maintenance complex binding protein (MCMBP), ATP binding cassette subfamily A member 1 (ABCA1), and signal regulatory protein delta (SIRPD), were enriched in the tumor microenvironment of

patients with lung cancer as compared to the circulation,[171] suggesting at least some degree of specificity.

These observations exemplify the broad pathological effect of non-mutated neoepitopes generated by protein modifications.

Conclusion

Preclinical and clinical data suggest that non-mutational neoantigens are abundantly presented on both MHC class I and II molecules, and that humoral and cellular immune responses targeting these neoepitopes can have a major impact on disease. On the one hand, it is now clear that neoantigenic determinants generated by non-mutational sources elicit B cell and T cell responses that contribute to the etiology of various disorders with an autoimmune/autoreactive component, including (but not limited to) SLE, rheumatoid arthritis, autoimmune encephalitis, and diabetes (both type 1 and 2).[3] On the other hand, non-mutational neoantigens presented by malignant cells seem to elicit at least some degree of tumor-targeting immunity. While such a natural immune response is generally unable to arrest tumor progression because of multipronged immunosuppressive mechanisms established by developing neoplasms, non-mutational neoantigens offer a valid target for the development of tumor-specific immunotherapeutics including cancer vaccines and (at least in the case of surface-exposed neoepitopes) CAR T cells.[77]

Importantly, the accurate identification and quantification of non-mutational neoantigens cannot be achieved from DNA/RNA sequencing data, but relies strictly on peptide elution from MHC molecules coupled with mass spectrometry. This fact has at least two important implications. On the one hand, it highlights the crucial importance of the reference libraries employed for spectral matching. As an obvious example, a mammalian library is intrinsically incompatible with the identification of pathogen-derived epitopes. On the other hand, it underscores the complexity associated with the identification of non-mutational neoantigens in clinical samples. Indeed, in most cases, the number of cells obtained from tissue biopsies may not be sufficient to achieve a sufficient peptide elution yield for high-resolution immunopeptidome analysis by mass spectrometry.

Despite such limitations, we surmise that interrogating the non-mutational neo-immunopeptidomes in increased detail will not only provide additional mechanistic insights into disorders as diverse as autoimmunity and cancer, but also may suggest new therapeutic avenues against these and other human pathologies.

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Box 1.

Risk factors for autoreactivity driven by non-mutational neoantigens.

Numerous factors have been associated with an increased likelihood for the development of autoreactivity against non-mutational neoantigens. These factors include mutations in important genes underlying peripheral tolerance (for example, *FOXP3* mutations),[5] as well as polymorphisms in gene encoding cytokines (for example, *CCL21*, which is linked to rheumatoid arthritis), cytokine receptors (for example., *IL23R*, which is associated with Crohn's disease), death receptors and their ligands (for example, *FAS* and *FASLG*, which are linked to systemic lupus erythematosus [SLE]), Toll-like receptors (for example, *TLR3*, which is associated with type 1 diabetes) as well as intracellular pattern recognition receptors (for example, *NOD2*, which is also linked to Crohn's disease).[172, 173, 174] That said, HLA polymorphisms are by far the most common risk factors for autoimmune reactions against non-mutational neoantigens.[175] To name a few examples, this applies to ankylosing spondylitis (HLA-B27), psoriasis (HLA-C06), rheumatoid arthritis (HLA-DR4), Crohn's disease (HLA-C), SLE (HLA-DRB1, HLA-DQA1, HLA-DQB1), type 1 diabetes (HLA-DR4, HLA-DQ8, HLA-DR3/DQ2), multiple sclerosis (HLA-DR2), and celiac disease (HLA-DQ2, HLA-DQ8).[175] In each of these cases except for SLE and Crohn's disease, the disease-associated MHC allele has been shown to present an autoantigen recognized by self-reactive T cells, but the precise role of such autoreactivity in pathogenesis or disease progression is unclear. Other non-overlapping mechanisms to loosen central tolerance have been described, including increased thymocyte resistance to negative selection,[176] alternate peptide docking and binding register,[177, 178] low-affinity peptide binding,[179, 180, 181] reduced antigen density or HLA instability,[182] and abnormal regulation of HLA transcription.[183, 184, 185] Conversely, defects in cytokine and PRR signaling can impair peripheral tolerance by promoting disproportioned inflammatory responses in tissues.[186] Irrespective of the precise mechanism, all these risk factors predispose the host to autoreactive $CD4^+$ and/or $CD8^+$ T cell responses of pathological relevance.

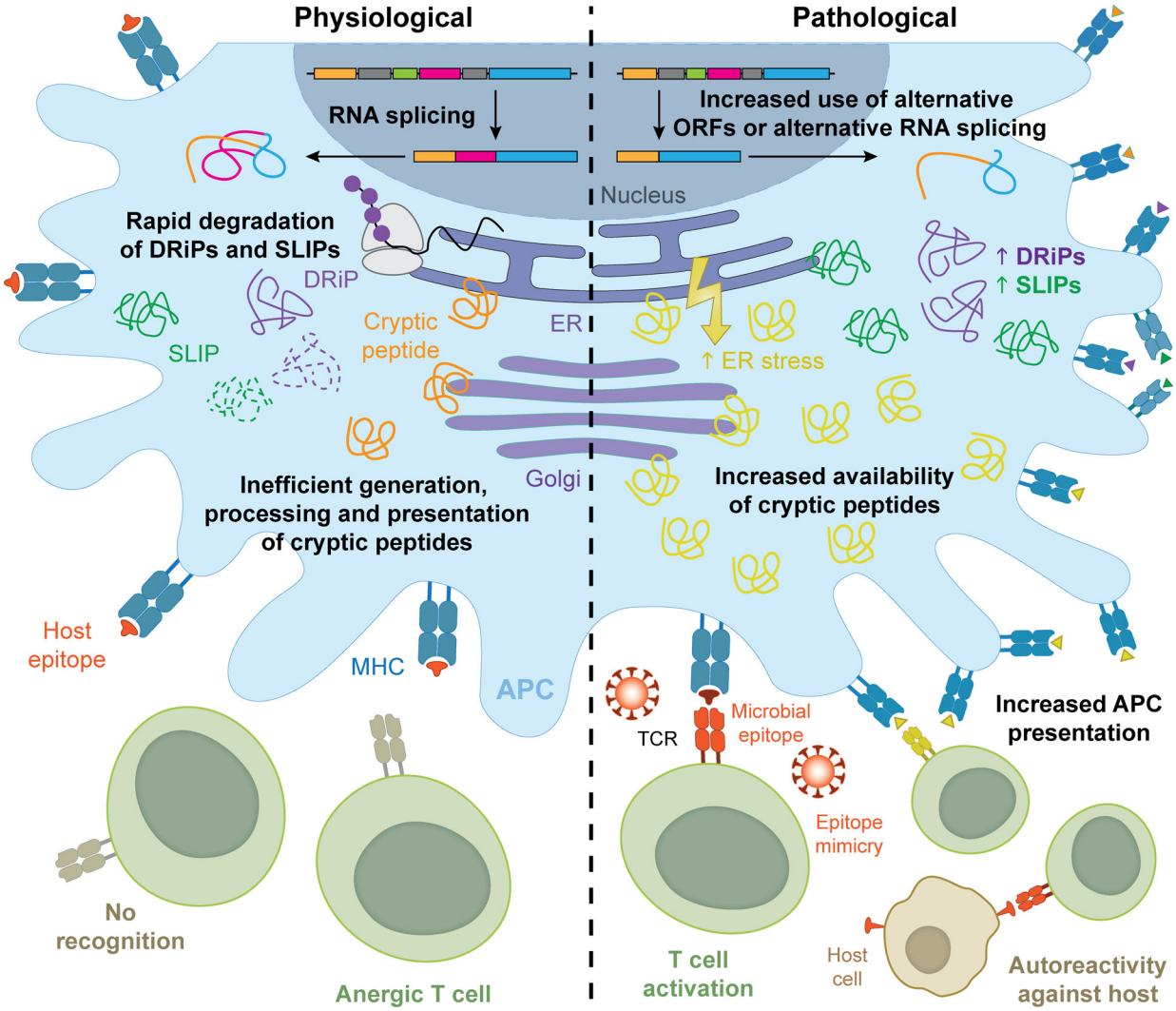

Figure 1. Mimicry and crypticity in non-mutational neo-antigenicity.

Autoreactivity against non-mutational neoantigens can emerge from a variety of mechanisms including epitope mimicry and crypticity. Such autoreactive responses are invariably driven by T cell clones escaping (leaky) thymic selection. On the one hand, pathogen-derived epitopes exhibiting considerable structural resemblance to self peptides can drive potent autoreactive responses, at least in individuals with one or more risk factors (**see** Box 1). On the other hand, purely self peptides (at least from a genetic standpoint), as well as defective ribosomal products (DRiPs) and short-lived proteins (SLiPs) that are normally not presented on MHC class I or II molecules, may elicit autoreactivity downstream of accrued antigen presentation as a consequence of: (1) usage of non-canonical open reading frames (ORFs), (2) alternative RNA splicing, (3) increased expression levels, (4) decreased competition for binding to MHC class I or II molecules, and/or (5) stress conditions that overall alter antigen processing and presentation. APC, antigen-presenting cell; ER, endoplasmic reticulum.

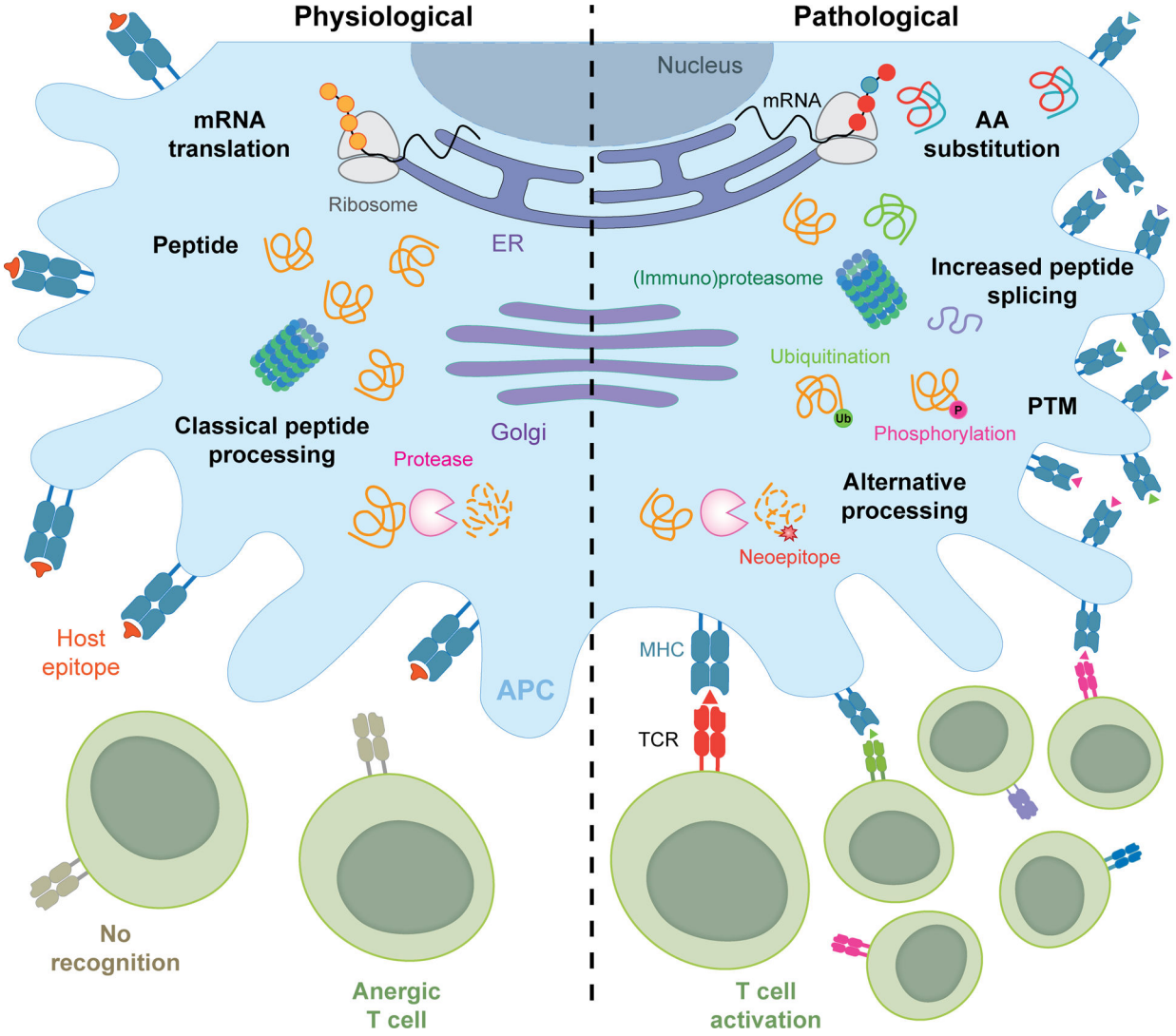

Figure 2. Protein modifications in non-mutational neo-antigenicity.

Loss of peripheral tolerance and consequent autoreactivity against non-mutated neoepitopes can emerge from a number of protein modifications associated with altered cellular homeostasis, encompassing: (1) the direct replacement of one or more amino acids during translation, (2) peptide splicing within the proteasome, endosomes/lysosomes, other protease-containing compartments, (3) a variety of enzymatic or non-enzymatic post-translational protein modifications (PTMs) such as citrullination, oxidation and glycation, and (4) altered protease activity and consequent generation of novel cleavage products. AA, amino acid; APC, antigen-presenting cell; ER, endoplasmic reticulum.