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Review

Post-translational modifications such as citrullination are excellent targets for cancer therapy

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ABSTRACT

Under conditions of cellular stress, proteins can be post-translationally modified causing them to be recognized by the immune system. One such stress-induced post-translational modification (siPTM) is citrullination, the conversion of arginine residues to citrulline by peptidylarginine deiminase (PAD) enzymes. PAD enzymes are activated by millimolar concentrations of calcium which can occur during apoptosis, leading to precipitation of proteins, their subsequent uptake by B cells and stimulation of antibody responses. Detection of anti-citrullinated protein antibodies (ACPAs) is a diagnostic of rheumatoid arthritis (RA), where immune complexes stimulate inflammation around the joints. More recently, autophagy has been shown to play a role in the presentation of citrullinated peptides on MHC class II molecules to $CD4^+$ helper T cells, suggesting that citrullination may be a way of alerting immune cells to cellular stress. Additionally, inflammation-induced IFNγ and concomitant MHC class II expression on target cells contributes to immune activation. Stressful conditions in the tumor microenvironment induce autophagy in cancer cells as a pro-survival mechanism. Cancer cells also over express PAD enzymes and in light of this the hypothesis that citrullinated peptides stimulate $CD4^+$ T cell responses that would recognize these siPTM's produced during autophagy has been investigated. The induction of potent citrullinated peptide-specific CD4 responses has been shown in both humans and HLA transgenic mouse models. Responses in mouse models resulted in potent anti-tumour responses against tumours expressing either constitutive or IFNγ-inducible MHC class II. The anti-tumour effect relied upon direct recognition of tumours by specific CD4 T cells suggesting that citrullinated peptides are attractive targets for cancer vaccines.

1. Citrullination, a PAD-dependent enzymatic process

Cellular stress conditions affect post-translational protein modifications such as citrullination - the Ca^{2+}-driven enzymatic conversion of arginine residues to citrulline. Citrulline is a modified, non-coded, amino acid, the generation of which relies on the action of peptidylarginine deiminases (PAD), a family of enzymes found in a wide range of tissues. The term "citrullination" or "deimination" refers to the modification of the primary ketimine group (=NH) to a ketone group (=O) of the arginine side chain, yielding ammonia as a side-product (Fig. 1).

Replacement of the positively charged arginine by the electrostatically neutral citrulline increases protein hydrophobicity with important consequences for protein structure and function. Humans possess five PAD isoenzymes, namely PAD1, 2, 3, 4 and 6, with 70–95 % sequence homology and only partially overlapping tissue distribution as well as substrate specificity. The tissue-specific distribution of the enzymes can be summarized as follows: PAD1 - epidermis and uterus; PAD2 - multiple organs including brain, female reproductive tract, skeletal muscle and haematopoietic cells; PAD3 - restricted to hair follicle and epithelium; PAD4 - hematopoietic cells, lung, oesophagus, breast and ovary carcinomas; PAD6 - oocytes and pre-implantation embryos [1]. Although the PAD enzymes are largely cytosolic enzymes, PAD4 has a nuclear localization sequence and can thus citrullinate nuclear targets such as histones. PAD2 on the other hand lacks this canonical sequence, but can nevertheless translocate into the nucleus at increased cellular Ca^{2+}- concentrations [2]. Aligned with the tissue distribution, each isoenzyme appears to target partially overlapping sets of cellular proteins, linked with distinct physiological functions: PAD1 and PAD3 regulating cellular architecture, whereas PAD2 and

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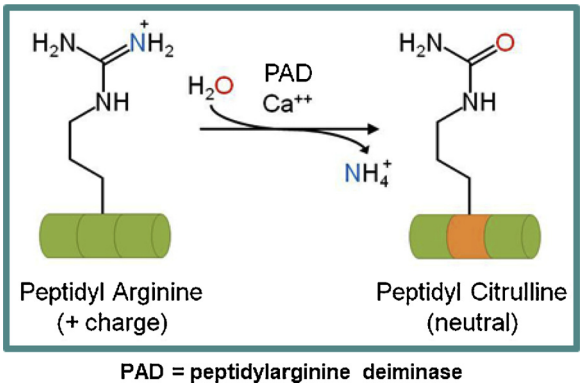

Fig. 1. Schematic of the citrullination or deamidation of arginine.

PAD4 are involved in gene regulation, apoptosis and NET-formation. Interestingly, not all solvent-accessible arginine residues are targets for citrullination by PAD, with evidence of preferred secondary structures, clustering and flanking residues. For example, PAD4 prefers small non-polar amino acids in positions −2 and +2, whereas proline residues flanking the arginine prevent citrullination [3,4]. Given the potentially potent and irreversible consequences on protein function, citrullination must be tightly regulated. *in vitro*, PAD enzymes require millimolar (10^{-3}–10^{-4}M) calcium concentrations for full activity, hence under physiological concentrations (10^{-8}–10^{-6}M) the enzymes are thought to be inactive or sub-optimally active, or may require other cofactors [5,6]. The high intracellular calcium concentration requires membrane barrier disruption or occurs within specialised subcellular compartments such as during apoptosis or autophagy, respectively. Additionally, reducing conditions have also been shown to be essential for PAD activity in order to maintain the reduction of the active site cysteine thiol, implying redox balance as a necessary requirement for activity [7].

2. Citrullination and autoimmunity (AI)

Citrullination plays a role in the pathogenesis of numerous AI conditions such as rheumatoid arthritis (RA), characterized by the production of antibodies to citrullinated proteins (so-called 'ACPA') in two-thirds of patients, multiple sclerosis (MS), systemic lupus erythematosus (SLE), autoimmune encephalitis and type I diabetes (T1D) [8–11]. The significance of the citrullinome to RA pathogenesis and ACPA is still not fully understood, but both features suggest the prevalence of $CD4^+$ autoreactive T cells targeting citrullinated epitopes [12,13]. Additionally, the association of $ACPA^+$ RA with HLA-DRB1 shared epitope (SE) alleles, such as HLA-DR*0401 (HLA-DR4), suggests that citrullinated peptides are preferentially bound by these HLA types [12,14,15]. ACPA targets include filaggrin, collagen, α-enolase, fibrinogen and vimentin and are used as specific markers to diagnose the disease [16–18]. More recently, a number of other citrullinated proteins including histone, nucleophosmin, B23, co-activator complex, antithrombin, aggregan, elongation factor 1α, adenylcyclase associated protein, glucose regulated protein, mitochondrial aldehyde dehydrogenase, cartilage intermediate layer protein (CLIP), aldolase, phosphoglycerate kinase 1, calreticulin, HSP60, HSP90, GRP78, far upstream element-binding proteins 1 and 2, asporin, cathepsin D, heparin binding protein, β-actin, F-actin, capping protein α-1 subunit, albumin, histamine receptor, and protein disulphide-isomerase ER60 precursor have also been identified as targets for antibodies in RA patients [16–18].

Hypercitrullination as a result of membranolytic (bacterial or host-instigated) or cell death processes give rise to increased intracellular calcium and PAD activation, with *de novo* citrullinated protein generation and/or extracellular release of PAD enzymes and their targets. These events can also lead to protein precipitation, enabling recognition by B cells resulting in antibody production, immune complexes and the inflammation associated with RA. Recently, the citrullination of NF-kB p65 by PAD4 led to enhanced NF-kB p65 nuclear localization and toll-like receptor (TLR)-induced interleukin 1 beta (IL-1β) and tumour necrosis factor alpha (TNFα) production, providing further evidence for a direct link between citrullination and inflammation [19]. Additionally, this study also demonstrated that an RA-prone PAD4 variant was more efficient in enhancing NF-kB activity. Neutrophils are the most likely, but not exclusive source of citrullination, as other immune cells including synovial fibroblasts and monocytes are also capable of citrullination and importantly, autophagy-induced generation of citrullinated peptides in human synoviocytes has been demonstrated [6,20–22]. More recently, both bone marrow-derived dendritic cells (DCs) and peritoneal macrophages have been shown to express PAD2 and PAD4 [23].

Equally relevant to the disease process is the relocation of citrullinated proteins such as actin and vimentin to the osteoclast plasma membrane inducing further bone loss through ACPA-mediated osteoclast activation [24–26]. Recognition of surface expressed citrullinated GRP78 on monocytes and macrophages by ACPAs is also thought play a role in the exacerbation of disease [27].

3. Citrullination in cancer pathogenesis

Citrullination as part of the inflammatory process is only just beginning to be explored, with mediators of cell stress or cellular damage, via TLRs, damage-associated molecular pattern (DAMP) receptors or heat shock proteins, enabling activation of PAD enzymes both within antigen-presenting cells (APCs) and within target cells, such as infected cells or tumour cells, leading to a breach of tolerance to modified self-epitopes and induction of immune responses.

Citrullinated proteins have been detected in cancer cells [28], by 2DE gel electrophoresis and mass spectrometry. It has been more difficult to determine citrullination of specific proteins in fixed human tumour tissues as the ACPAs are frequently cross reactive recognizing motifs in multiple citrullinated proteins [29]. However, citrullination correlates with increased expression levels of PAD enzymes so they can be used as a surrogate of citrullination. Indeed, PAD expression is higher in tumours compared to healthy tissue suggesting that citrullination does occur in cancer cells [30,31]. PAD2 and PAD4 are the most commonly expressed PAD enzymes and in one study, 1673 cancers of different origins revealed overexpression of PAD4 in carcinomas of the ovary, uterus, colon, bladder, breast, liver, lung, oesophagus, kidney and soft tissue tumours. Smaller studies also showed over-expression of PAD2 in prostate and small cell lung cancers [32,33].

The role citrullinated proteins play in tumour progression has recently been reviewed [34]. PADs can regulate gene transcription by demethylation of histones (or other proteins) and converting the proteins to their basal states [35]. Proteins that are directly citrullinated include p53, ING4, nucleophosmin, β-catenin and GSK-3β leading to altered cell signalling, cellular differentiation and epithelial to mesenchymal transition (EMT; [36,37].

Citrullination can occur as a result of autophagy, a dual stress response and housekeeping mechanism, involving the degradation and recycling of cellular content [38,39]. Due to their rapid growth, high levels of hypoxia and loss of contact inhibition most tumours express high levels of autophagy [40]. Macroautophagy in particular, being a pro-survival response and involving double membrane-sealed autophagosomes, provides the target proteins as well as the micro-environmental conditions for PAD-mediated citrullination. Importantly, autophagic pathways are also perfectly poised to deliver cellular cargo for antigen presentation [41,42]. Classically, MHC class I peptides, presented to $CD8^+$ or killer T cells, are usually generated by proteasomal degradation of intracellular proteins, whereas MHC class II bound peptides, presented to $CD4^+$ or helper T cells, originate from

extracellular antigens, phagocytosed by APCs and degraded by lysosomal proteolysis. More recently, significant cross-talk between both pathways has been demonstrated, with for instance DCs cross-presenting extracellular antigens onto MHC class I [42,43]. Additionally, sequencing of eluted peptides from MHC class II reveals that 2–30 % of peptides originate from intracellular sources. Autophagy is a key driver for the provision of intracellular antigens from self-proteins or intracellular pathogens for loading onto MHC class II molecules, with starvation-induced macroautophagy having a dramatic effect on the composition of MHC class II presented peptides [44]. Additionally, $CD4^+$ T cell selection in the thymus is mediated by thymic epithelial cells using macroautophagy to load intracellular antigen for MHC class II presentation. In the periphery the increased MHC class II presentation of self-peptides could lead to a breach in immune tolerance and induction of AI.

Recently, autophagy was shown to be a key cellular event in the presentation of citrullinated peptides from hen egg white lysozyme to $CD4^+$ helper T cells [38]. This model antigen was overexpressed within APCs, resulting in strong presentation of the citrullinated epitope. Inhibition of autophagy by 3-methyadenine (3MA) or *Atg5* siRNA silencing specifically inhibited the presentation of the citrullinated peptide. Crucially, PAD activity was detected within purified autophagosomes, coinciding with accumulation of calcium in autophagic vesicles [45].

Collectively, autophagy-induced citrullination may be a way of alerting immune cells to cellular stress including carcinogenesis. In the context of inflammation, exposure to IFNγ and subsequent MHC class II expression by target cells, will allow immune recognition, instigating the question whether citrullinated epitopes are good targets for anti-tumour immunity?

4. Preclinical studies using citrullinated epitopes for cancer immunotherapy

Citrullination in cancer constitute neo-epitope-like targets for tumor therapy. It was therefore investigated if vaccination with citrullinated peptides could stimulate $CD4^+$ T cell responses that would recognize these modifications produced during autophagy within tumor cells. Unlike bona-fide neo-epitopes, vaccination with citrullinated peptides would not be a form of personalised therapy, but could be used to treat a wide range of cancer types, notably being of value in tumours with low mutational burden.

4.1. Citrullinated vimentin

Initially, the work focused on the intermediate filament protein vimentin, as it is known to be citrullinated and is over-expressed by a wide variety of cancers [46–51], especially during EMT [52]. The transition of carcinoma cells from an epithelial to mesenchymal-like phenotype via EMT is recognized as an important step in the metastasis of solid tumours (Fig. 2).

We observed robust $CD4^+$ T cell responses in HLA-transgenic mice vaccinated with citrullinated peptides (Fig. 3), that generated potent anti-tumour immune response [53]. Remarkably, a single immunization with a citrullinated peptide up to 14 days after tumor implant, resulted in long term survival in 60–90 % of animals. Immunized mice demonstrating strong tumour rejection, displayed no evidence of toxicity suggesting that healthy cells do not present these citrullinated epitopes. Antibody responses to the citrullinated peptides or joint erosion were also absent, suggesting that T cells alone cannot induce RA.

These results demonstrated that CD4 T cells could mediate potent anti-tumor responses against modified self-epitopes presented on tumor cells, and illustrated for the first time how citrullinated peptides produced during autophagy may offer an attractive vaccine target for cancer therapy.

4.2. Citrullinated enolase

α-Enolase (ENO1) is a glycolytic enzyme that catalyzes the penultimate step in glycolysis [54]. Many tumors switch to generating their energy via glycolysis in a process termed the “Warburg effect”. ENO1 has been found to be overexpressed in a wide range of tumors [55–58]. Due to its ubiquitous expression and abundance in most cells, ENO1 is often degraded during autophagy; previous studies have shown that ENO1 can also be citrullinated [59,60]. Therefore, like vimentin, ENO1 represents a good target for anti-tumor immunity [61]. Immunization of mice with two citrullinated ENO1 peptides induced strong Th1 responses that recognized the post-translationally modified peptide, but not the wild type unmodified peptide. Citrullinated ENO1 peptides induced anti-tumor responses in C57Bl/6 mice implanted with melanoma tumors (B16F1, 50 % survival $p = 0.0026$) and in HLA-DR4 transgenic mice (B16-DR4, 50 % survival $p = 0.0048$). In addition, ENO1 peptides induced an anti-tumor response in HLA-DR4 transgenic mice implanted with pancreatic (Pan02-DR4 50 % survival $p = 0.0076$) or lung (LLC/2-DR4 40 % survival $p = 0.0142$) tumors expressing the matched HLA-DR4 allele. The unmodified epitope did not induce an anti-tumor response in any of these models.

4.3. Human T cell responses to citrullinated peptides

$CD4^+$ T cells specific to citrullinated peptides have been shown in autoimmune patients with RA, T1D and MS as well as during influenza viral infection [62]. A study comparing responses to citrullinated peptides in RA patients and healthy donors revealed that healthy donors also show evidence of $CD4^+$ T cells to citrullinated peptides although they differ in phenotype with RA patients showing more Th1 memory which is associated with disease severity [12]. In animal models, vaccination with citrullinated peptides has been shown to stimulate cytotoxic CD4 T cells responses that result in dramatic tumor regression/eradication and greatly improved survival times. To translate this animal work into clinical application, it would be necessary to examine in more depth the T cell repertoire to citrullinated peptides in healthy donors and cancer patients. Studies using a proliferation based assay (Fig. 4) show that CD4 T cells within peripheral blood mononuclear cells isolated from healthy individuals and cancer patients proliferate when cultured with citrullinated peptides [53,61,63].

Our recent (unpublished) findings show that proliferative responses in healthy individuals are either increased or unmasked following the depletion of CD25 positive cells, indicating a degree of regulatory control. Furthermore, cell surface and intracellular phenotyping by flow cytometry shows that the proliferating cells express the activation marker CD134, produce IFNγ and granzyme B and are largely comprised of effector memory and/or highly cytotoxic T_{EMRA} [64] populations (as described by expression of the lymphoid homing molecule CD197 and CD45RA). In some donors these citrullinated peptide-driven proliferative responses are lost upon depletion of “memory” ($CD45RO^+$) T cells but in others they are lost following depletion of naive T cells ($CD45RA^+$), these findings are in agreement with our animal studies where response to citrullinated peptides were seen at day 2 post immunization. These results imply a pre-existing component to some of these citrullinated peptide-specific responses. Sorting of citrullinated peptide stimulated $CD4^+/CFSE^{high}$ T cells (non-proliferating) and $CD4^+/CFSE^{low}$ T cells (proliferating) was performed and subsequent hi-throughput TCR repertoire sequencing of each population revealed a more clonal, less diverse repertoire in the proliferating population (Fig. 5).

This data supports the hypothesis that healthy individuals have a T cell repertoire specific to citrullinated peptides that in some donors appears to be pre-existing. We have shown previously [53] and in recent unpublished data that a similar repertoire exists in cancer patients and suggest that this repertoire could be boosted by immunization to assist in tumor eradication. This would be in line with studies in RA

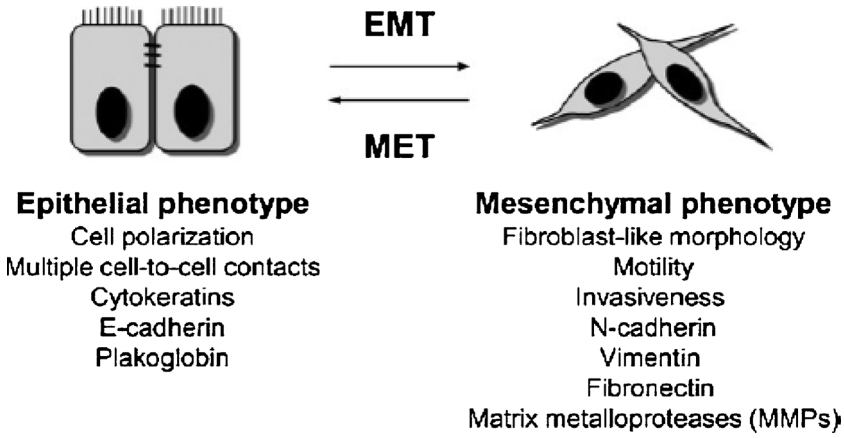

Fig. 2. Relevant phenotypic changes defining the EMT and its reverse process, the mesenchymal-to-epithelial transition (MET: [52]).

where oligoclonal expansion of the TCR repertoire was seen in response to citrullinated type II collagen peptides [65].

4.4. HLA restriction

In previous studies, we have shown that most healthy donors can generate a CD4 T cell response to one or more citrullinated vimentin and enolase peptides suggesting that this is a common occurrence [53,61] and not restricted to certain HLA alleles. As mentioned above, citrullinated peptides can be presented on MHC molecules via autophagy which is increased under stressful conditions. The presentation of citrullinated peptides on MHC molecules could be a plausible mechanism to alert the immune system to recognise and remove stressed cells. Therefore, the assumption is that citrullinated peptides must be generated and presented on a wide range of different HLA alleles. Analysis of the proliferation responses to citrullinated peptide stimulation appeared highly oligoclonal in TCR repertoire and profiles also varied between peptides, suggesting that these cells were selectively responding to each peptide; although the numbers tested were insufficient to infer any bias in Vβ usage. In addition, the predominant clones in the proliferating populations were of very low frequency in the non-proliferating subset. Evidence for the presentation of citrullinated peptides in the thymus has been reported [66] and the detection of responses in healthy donors indicates that the T cells responding to the citrullinated peptides are positively rather than negatively selected. The lack of negative selection could indicate an avidity range that is prohibitive for negative selection and/or insufficient level of presentation, as well as unusual modes of TCR binding to self-peptide/MHC in the thymus [67]. Examination of responses to citrullinated peptides in RA patients suggests an association of disease with HLA-DR*0401 and associated alleles namely SE alleles [12,14,15]. However, in our previous studies in cancer patients [53,61] and healthy donors we do not see this association of HLA restriction with response [63]. The best observed correlation with response was expression of HLA-DP4, with 91 % of the responding patients expressing this haplotype. There have been a few publications suggesting citrullinated peptides can also preferentially bind to HLA-DR9 and HLA-DQ2,7 and 8 [68,69]. None of the donors described in our previous work expressed HLA-DR9, HLA-DQ7 or HLA-DQ8, 54 % of the responding donors expressed HLA-DQ2 suggesting this HLA allele could have presented the citrullinated peptides in these donors. It has been previously thought that the conversion of arginine to citrulline enhances the binding of peptides to HLA-DR and DQ alleles [69,70]. Our previous work confirmed the ability of HLA-DP4 to present citrullinated peptides and showed that the citrullinated vimentin and enolase peptides bind more strongly to HLA-DP4 than the arginine containing peptides [63]. This implies the suitability of HLA-DP4 as a restriction allele for these citrullinated peptides. To confirm these observations, we also demonstrated that HLA-DP4 transgenic mice generated strong Th1 responses specific to the citrullinated vimentin and enolase peptides after vaccination. The presentation of peptides via HLA-DP molecules has been shown in the context of infectious disease, allergy and cancer [71–74]. The diversity of HLA-DP alleles is more restricted than DR or DQ alleles, with only 5 alleles frequently expressed in the worldwide

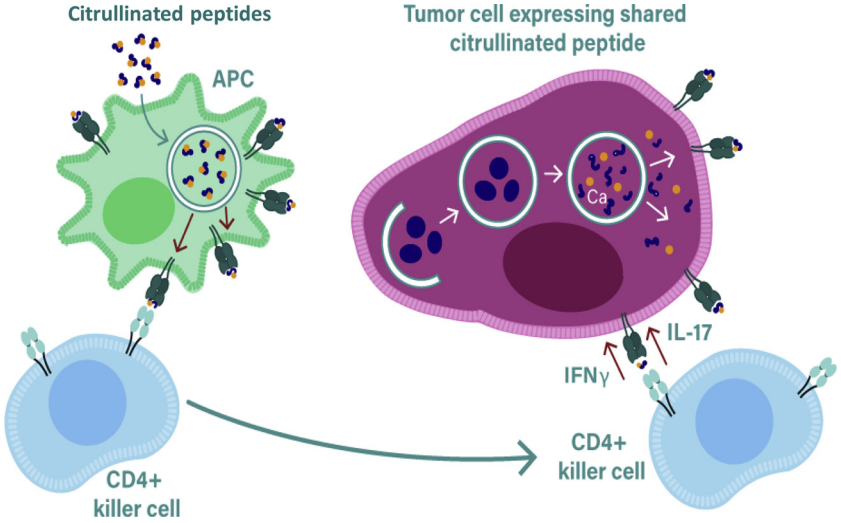

Fig. 3. Citrullinated peptides activate killer CD4 T cells which mediate anti-tumour immunity. Citrullinated peptides are administered with adjuvant to allow presentation on APCs. Primed killer CD4 T cells enter the tumour and are reactivated by APCs presenting citrullinated peptides from tumours. They release IFNγ which upregulates expression of MHC class II on the stressed tumors, undergoing autophagy and citrullination, thus allowing direct recognition and lysis by the killer CD4 T cells.

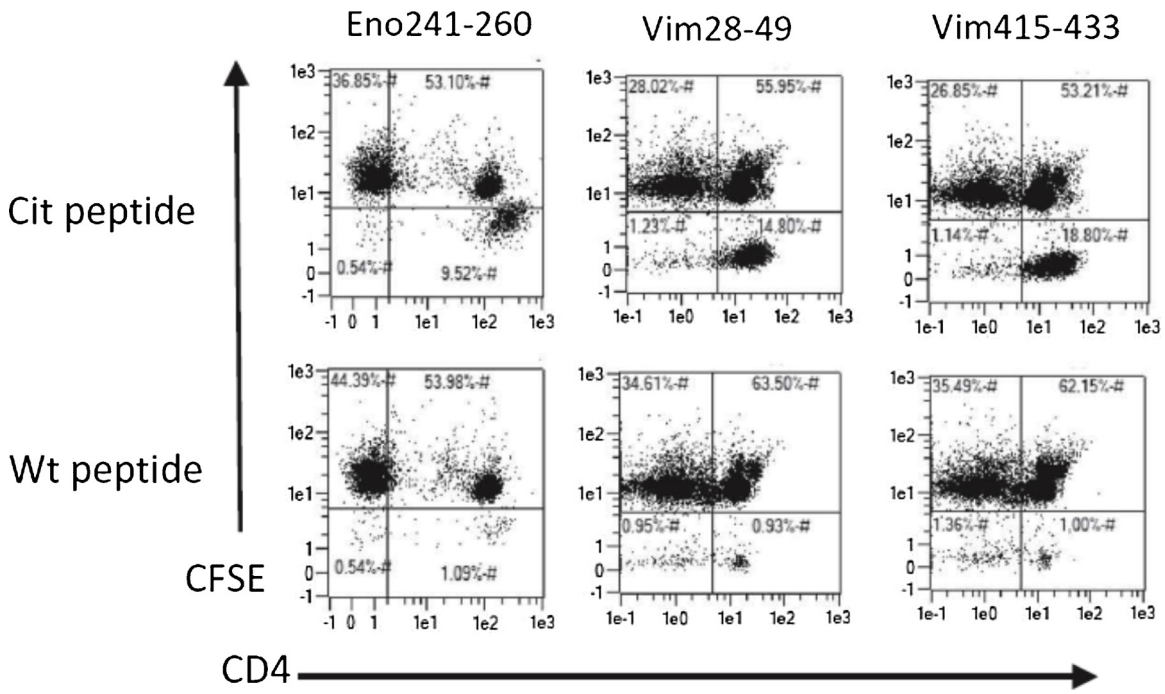

Fig. 4. CD4 T cell proliferation *ex vivo*. Example plots showing proliferation of $CD4^+$ T cells to citrullinated (cit) and native (wt) peptides [63].

population covering approximately 90 % of individuals [75]. A study by Sidney et al. [75] also demonstrates a shared HLA supertypic binding specificity between these common HLA-DP alleles and therefore it is plausible that the citrullinated peptides could show binding to a range of HLA-DP alleles. The potential to alert the immune system to cellular stress through presentation of citrullinated peptides via MHC class II would be beneficial as 'stressed cells' would require clearance by the immune system. HLA-DP alleles have the potential to play a role in the clearance of stressed cells and the reduced polymorphism among HLA-DP alleles perhaps suggests an evolutionary role in the presentation of citrullinated peptides in this universal process. Studies have shown that HLA-DP peptide-binding motifs differ from those of (ER-loaded) MHC molecules, so HLA-DP is not likely to compete with classical MHC class II-binding peptides [76]. HLA-DP alleles also do not bind CLIP fragments [77]. Suggestions are that HLA-DP is more accessible to peptides produced during autophagy and van Lith et al. have shown that it does not require invariant chain or HLA-DM to form stable dimers [78,79]. HLA-DP molecules are reported to have lower expression levels compared to HLA-DR and DQ molecules [80,81] which likely plays a role to avoid AI and promote self-tolerance. The co-expression on HLA-DP4 and an HLA SE allele may, however, push T cells over the threshold resulting in autoimmune disease.

In responding healthy donors and HLA-DP4 transgenic mice immunised with the citrullinated, vimentin and enolase peptides a Th1 CD4 T cell response is induced [63]. These CD4 T cells mediate an anti-tumour response in HLA-DP4 transgenic mice similar to that seen in our prior work described above [53,61]. Anti-tumor responses were similar in both HLA-DP4 and HLA-DR4 mice suggesting both alleles can present the citrullinated epitopes. As yet unpublished data from our group shows a single immunization with the combination of citrullinated vimentin and enolase peptides was sufficient to induce significant regression of established B16 melanoma within 4 days of vaccination (Fig. 6).

Anti-tumour responses were not restricted to B16 melanoma model, similar results were also obtained against a HLA-DP4 or HLA-DR4 positive Lewis lung carcinoma line, LLC/2, an HLA-DP4 positive ID8 ovarian line as well as HLA-DR4 positive Pan02 pancreatic line which all express vimentin and enolase. These studies suggest citrullinated vimentin and enolase peptides could be used to stimulate strong anti-tumour immune responses in either HLA-DR4 or HLA-DP4 individuals.

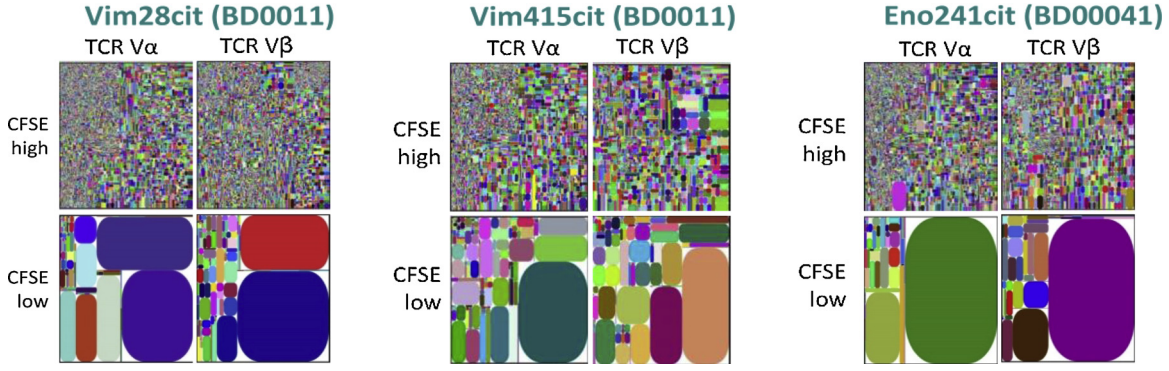

Fig. 5. TCRα and β diversity in $CD4^+$ $CFSE^{high/low}$ cells responding to citrullinated peptides. Tree maps depicting TCR α and β chain CDR3 clonotype usage in relation to repertoire size in $CD4^+$ $CFSE^{high/low}$ cells on incubation with citrullinated peptides vimentin 28–49 and 415–433 from donor BD0011 and enolase 241–260 from donor BD0041. Each rectangle in a tree map represents a unique CDR3 nucleotide sequence and the size of each rectangle denotes the relative frequency of an individual sequence. The colours for the individual CDR3 sequences in each tree map plot are chosen randomly and thus do not match between plots.

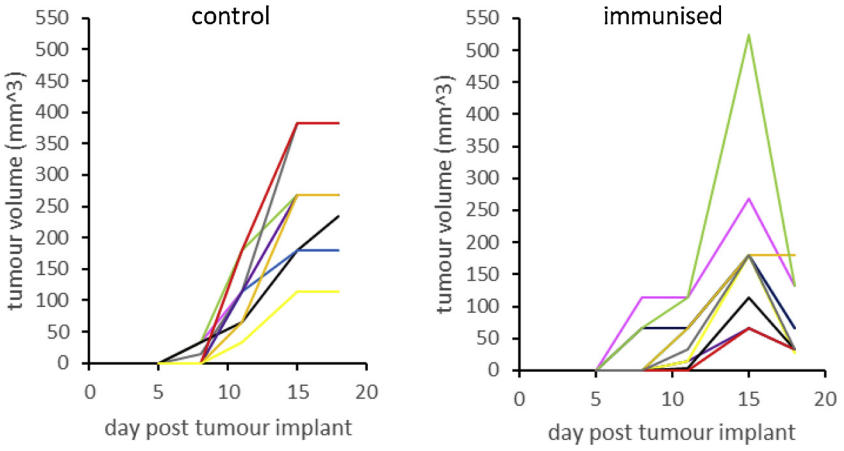

Fig. 6. Citrullinated peptide vaccination causes rapid tumour regression. HLA-DP4 transgenic mice challenged with B16 cells expressing DP4 under an IFNγ inducible promoter were immunised with combination of citrullinated Vim28–49, Vim415–433 and Eno241–260 peptides in CpG/MPLA at day 15 when tumours reached 5 – 10 mm diameter compared to unimmunised control. Tumour size was monitored. Data from a representative study is shown.

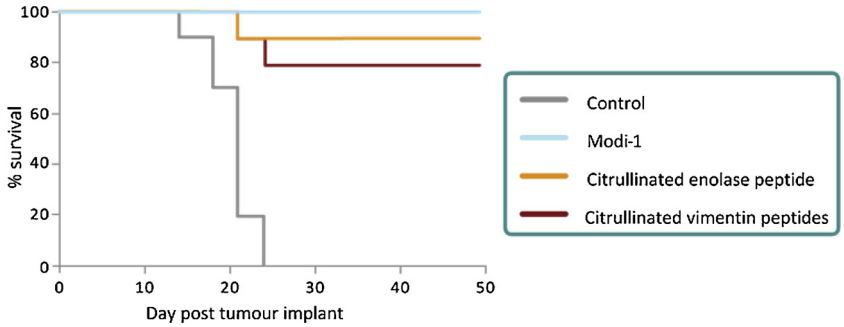

Fig. 7. Citrullinated vimentin and enolase peptides provide tumor therapy. HLA-DP4 mice were challenged with B16F1 melanoma cells expressing HLA-DP4 on day 1. On days 4, 11 and 18 mice were immunized with citrullinated peptides in 5 μg each of CpG ODN 1826 and MPLA. Tumor growth and survival was monitored.

To translate these studies into the clinic the two citrullinated vimentin peptides and the citrullinated enolase peptide (Modi-1 vaccine) will be used in combination to prevent tumour antigen escape. Combining epitopes can lead to dominant responses to at least one of the included epitopes with subdominant or absent responses to others [82–86], it is therefore important to carefully select combinations to generate the most potent anti-tumour immune response. We have shown in both HLA-DR4 and HLA-DP4 transgenic mice that combination of two citrullinated vimentin peptides and one enolase peptide (Modi-1) can generate specific CD4 T cell responses without any associated immunodominance, resulting in potent anti-tumour responses against both B16 melanoma and ID8 ovarian tumours expressing either constitutive or IFNγ inducible DR4 or DP4 (Fig. 7). In contrast, there was no anti-tumour response from wild type peptides or adjuvant alone [63].

In viral infections, immunodominant CD4 T cell responses often predominate skewing the immune response [87]. In our work with citrullinated peptides there is no apparent immunodominance to the citrullinated epitopes. When presented with a number of immunogenic proteins the immune system carefully selects for a few dominant epitopes which often appear as the most abundant peptides bound to the MHC class II molecules. The relative abundance of an epitope is due to several factors including appropriate protein cleavage, the resistance to HLA-DM and/or cathepsins in the system whilst HLA loading, as well as removal of nondominant epitopes, which are susceptible to action of both HLA-DM and cathepsins. Once selected, the dominant epitopes in complex with MHC molecules are recognized by their cognate T cells [88]. Regulatory T cells and indoleamine 2,3-dioxygenase are known to further regulate these responses [89]. Although the citrullinated epitopes in our studies have shown stronger binding to MHC class II than the native arginine containing epitopes, their binding affinity remains moderate to low [63]. Self-antigens known to stimulate AI have been shown to have low binding affinity to MHC class II and can be edited by HLA-DM. Although they have these properties, they are also resistant to proteolytic cleavage which permits rebinding in the absence of stronger foreign epitopes which are likely to be degraded [90–92]. The modification of arginine to citrulline is known to alter proteolytic cleavage patterns and can therefore generate neo-epitopes. Many tumours do not constitutively express MHC class II and therefore the expression of citrullinated self-epitopes on tumour cells is complex. These tumours need to be induced to express MHC class II during stress/inflammation and also may not express HLA-DM. The increased resistance to protease cleavage observed with modified self-antigens and the absence of HLA-DM editing may be favourable for the presentation of citrullinated peptides.

5. Role of CD4 T cells in tumor regression

The focus of anti-tumour immune responses has largely been focused on the generation of tumour-specific CD8 T responses, this is in part due to the lack of MHC class II expression by most solid tumours. These tumours therefore constitute better targets for CD8 T cells than CD4 T cells. However, therapies involving CD8 T cells have elicited only modest and short-lived responses in patients. It has been known for many years that CD4 helper T cells play a pivotal role in the induction of epitope-specific immune responses, whether antibody or CD8 T cell mediated. Importantly, memory CD8 T cell responses are impaired in the absence of CD4 T cell help [93,94]. This was initially believed to be due to their secretion of IL-2 [95] but more recently it is believed to also be due to modification/activation of DCs, which in turn activate CD8 T cells [96–99]. In most cases, this help is provided by foreign CD4 T cell epitopes originating from pathogens or incorporated in vaccines. However, tumour-specific CD4 T cell responses are also required at the tumour site to enhance inflammation, resulting in enhanced recruitment and retention of CD8 T cells, NK cells and other inflammatory mediators of anti-tumour immunity [100–103]. The involvement of

CD4 helper T cells in cancer immunity is further construed from studies using CD4 T cells or MHC class II deficient mice, where tumour progression ensues, indicating the importance of CD4 T cells in the eradication of tumours. Recent reports in the literature suggest the importance of tumour-reactive CD4 T cells, that play a direct role in tumour eradication [104–107]. We have demonstrated the induction of potent CD4 T cell responses to citrullinated antigens, with both direct and indirect effects on anti-tumour immunity. Our findings in the mouse models suggest that the anti-tumour response is mediated by CD4 T cells with no role for CD8 T cells, as the depletion of CD4 T cells abrogates the response whereas the depletion of CD8 T cells had no effect. Also the crucial role of direct CD4 T cell mediated killing for the anti-tumour responses was demonstrated by the lack of an anti-tumour response in the absence of tumour MHC class II expression. More recently, we have also shown that there is a correlation of tumour regression with increased CD4 T cell infiltrate and a concomitant reduction in myeloid-derived suppressor cells.

High-frequency and high-avidity $CD4^+$ T cell responses to tumour-specific neo-epitopes can be generated [108–110]. These neo-epitopes are patient-specific but can be successfully exploited as personalised vaccine or immunotherapy targets [111–113]. When the T cell repertoire is subject to self-tolerance, the induction of epitope-specific $CD4^+$ T cell responses is much more limited and resulting responses are of lower frequency and avidity. Citrullinated self-epitopes may be an exception as $CD4^+$ T cells to these epitopes are positively selected in the thymus and may be crucial in detecting stressed cells. The immunosuppressive tumour environment may inhibit these responses but vaccination may reinvigorate them.

6. Conclusion

Citrullination is a widely expressed, novel, siPTM that is a potent target for cancer vaccines.

- There is a repertoire of cytotoxic $CD4^+$ T cells in mice and humans that recognizes citrullinated epitopes
- Tumors over-express PAD enzymes
- Inflammation and stress induces high levels of autophagy and MHC class II presentation of citrullinated epitopes on tumors
- Citrullinated peptides induce potent anti-tumour immunity with no evidence of associated toxicity and will shortly enter the clinic.

Disclosure statement

This work was funded by Scancell Ltd, UK. VA Brentville, RL Metheringham and LG Durrant have ownership interest in patent WO2017013425 A1. LG Durrant is a director and CSO of Scancell Ltd has ownership interest (including patents) in Scancell Ltd. All authors are employees of Scancell Ltd.

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