

## Autophagy, citrullination and cancer

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Abstract

A cell needs to maintain a balance between biosynthesis and degradation of cellular components to maintain homeostasis. There are two pathways, the proteasome, which degrades short-lived proteins, and the autophagy/lysosomal pathway, which degrades long-lived proteins and organelles. Both of these pathways are also involved in antigen presentation or the effective delivery of peptides to MHC molecules for presentation to T cells. Autophagy (macroautophagy) is a key player in providing substantial sources of citrullinated peptides for loading onto MHC-II molecules to stimulate CD4<sup>+</sup> T cell responses. Stressful conditions in the tumor microenvironment induce autophagy in cancer cells as a mechanism to promote their survival. We therefore investigated if citrullinated peptides could stimulate CD4<sup>+</sup> T cell responses that would recognize these modifications produced during autophagy within tumor cells. Focusing on the intermediate filament protein VIM (vimentin), we generated citrullinated VIM peptides for immunization experiments in mice. Immunization with these peptides induced CD4<sup>+</sup> T cells in response to autophagic tumor targets. Remarkably, a single immunization with modified peptide, up to 14 days after tumor implant, resulted in long term survival in 60-90% of animals with no associated toxicity. These results show how CD4<sup>+</sup> cells can mediate potent antitumor responses against modified self-epitopes presented on tumor cells, and they illustrate for the first time how the citrullinated peptides produced during autophagy may offer especially attractive vaccine targets for cancer therapy.

Classically MHC-I peptides, presented to CD8<sup>+</sup> or killer T cells, are generated by proteasomal degradation of intracellular proteins. In contrast MHC-II bound peptides, presented to CD4<sup>+</sup> or helper T cells, classically originate from extracellular antigens, phagocytosed by antigen-presenting cells and degraded by lysosomal proteolysis. More recently it has been shown that dendritic cells can present extracellular antigens to MHC-I, and sequencing of peptides eluted from MHC-II reveals that 2-30% of peptides originate from intracellular sources. Autophagy is a key player in providing substantial sources of intracellular antigens either from self-proteins or intracellular pathogens for loading onto MHC-II molecules. The latter are not a problem, but presentation of peptides from the

former could lead to the induction of autoimmunity. Presentation of modified peptides may alleviate this problem.

Recently autophagy has been shown to play a role in the presentation of citrullinated peptides from hen egg white lysozyme to CD4<sup>+</sup> helper T cells. This model antigen was overexpressed within antigen-presenting cells resulting in strong presentation of the citrullinated epitope. Inhibition of autophagy by 3MA or *Atg5* siRNA silencing specifically inhibits the presentation of citrullinated peptide. Citrullination of arginine involves the conversion of the positively charged aldimine group (=NH) group of arginine to the neutrally charged ketone group (=O) of citrulline. Citrullination is mediated by peptidylarginine deiminases (PADIs), which are a family of calcium-dependent enzymes found in a variety of tissues. PADIs are present in the cell nucleus where they are involved in gene regulation but are also expressed within the cytoplasm and PADI activity has been detected within autophagosomes. PADIs can be activated by cell death, lipopolysaccharide/LPS, TNF and formyl-methionyl-leucyl-phenylalanine/fMLP but it is unclear how they are activated within autophagosomes. PADIs usually require millimolar concentrations of calcium for their activation and are involved in protein modification in dying cells. However, as they also have a role in gene regulation, it has been speculated that their requirement for calcium may be reduced in the presence of cellular binding proteins perhaps also explaining their role in autophagy.

We have shown that citrullinated VIM peptides stimulate strong antitumor immunity, but there is no toxicity, despite numerous cells expressing VIM and the fact that citrullinated proteins play a role in the etiology of autoimmune diseases. Indeed, anti-citrulline antibodies are used to diagnose rheumatoid arthritis. This may be the key, as the peptide vaccines do not stimulate antibody responses, whereas they are essential to the development of autoimmune disease. Damaged cells activate PADI enzymes, which convert arginines to citrullines, thereby, changing charge and leading to unfolding of proteins. This results in precipitation of proteins that are recognized by B cells and the induction of an IgM response. Processing of the citrullinated proteins within autophagosomes in B cells, results in their presentation on MHC-II. Similarly the citrullinated proteins can be degraded via autophagy within antigen presenting cells to present the citrullinated epitopes to helper CD4<sup>+</sup> cells. They then recognize the same epitope on the triggered B cells resulting in affinity maturation and subclass switching of the antibody response.

If autophagy is triggered during normal cellular homeostasis or to promote cell survival, the triggering of a CD4<sup>+</sup> helper response would be unhelpful. In contrast, under nutrient depletion, hypoxia or intracellular infections these cells may have a vital role to play. The key may be interferons that induce class II expression. Although immune cells constitutively express MHC class II, other cells, including epithelial cells, mesenchymal cells and endothelial cells, lack expression unless induced by interferons. Thus, autophagy induced in the presence of interferons would allow presentation of peptides on newly synthesized MHC-II molecules. Infections or cellular damage that induce an interferon response would stimulate a T cell response. If interferons also activate PADIs then these T cells would recognize citrullinated peptides.

Tumors upregulate autophagy in response to stresses such as nutrient deprivation, oxygen deprivation, redox stress and DNA damage. However, as tumors evolve they acquire many anti-inflammatory mechanisms including the inhibition of interferon release from antigen-presenting cells. Vaccination with citrullinated peptides leads to a rapid increase in the number of pro-

inflammatory CD4<sup>+</sup> T cells targeting the tumor resulting in interferon release, which ultimately flips the tumor environment from anti- to pro-inflammatory and results in tumor clearance.

Thus, in conclusion, citrullinated CD4<sup>+</sup> T cell epitopes are excellent targets for anti-tumor immunity. Activation of PADI, autophagy and induction of MHC-II expression are all essential for target recognition. Due to extreme cellular stress these can all be induced in tumor cells but not on normal cells, leading to a safe nontoxic vaccine.