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"Flagellated" cancer cells propel anti-tumor immunity

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The use of innate immune receptor agonists in cancer therapies has suffered from many drawbacks. Our recent observations suggest that some of these hurdles can be overcome by introducing flagellin into tumor cells to promote tumor antigen presentation by dendritic cells (DCs) and simultaneously trigger two types of pattern recognition receptors (PRRs).

Pathogens possess a variety of molecular structures [collectively termed pathogen-associated molecular pattern (PAMPs)] that are perceived by the immune system through PPRs, which signal the presence of an infection. Thus, various PAMPs—in particular those for Toll-like receptors (TLRs)—have been included in cancer vaccine compositions with the goal of mobilizing immunity against tumor cells in a manner similar to that against a life threatening infection.[1] The rationale was based on the knowledge that PRR engagement induces co-stimulatory molecule expression and secretion of pro-inflammatory cytokines critical for effective immunity.[2] While developing these strategies, it was neglected that most PAMPs are in fact physically or spatially associated with microbial antigens, and therefore, end up in the same phagocytic compartment of antigen-presenting cells (APCs) where antigens are loaded onto major histocompatibility complex molecules (MHC). A few years ago, Blander and Medzhitov uncovered a phagosome autonomous mechanism of MHC Class II (MHC-II) presentation in DCs whereby TLR signaling via PAMPs present within phagosomes induces the selective loading of antigens within those phagosomes allowing for the selection of microbial antigens for presentation to T cells within a co-stimulatory and inflammatory context.[3] This type of "associative recognition"[2] of antigen with PAMPs thus became an important biological process that could potentially be exploited in designing tumor vaccines. Hence we reasoned that introducing a PAMP within tumor cells would supply the pattern characteristic of pathogens, allowing APCs to mark tumor-associated antigens (TAAs) for successful priming of tumor-specific T cells. We forced expression of flagellin, a bacterial protein that triggers TLR5, into various tumor cell lines to ensure its co-delivery into phagosomes that also contain tumor antigens.[4] This strategy efficiently induced tumor cell clearance by innate immune cells, prevented tumor growth and activated tumor-specific $CD8^+$ and $CD4^+$ T-cell responses in mice (**Fig. 1**). When used as an anti-tumor treatment or cancer vaccine, irradiated flagellin-expressing tumor cells efficiently impaired parental tumor growth. In sharp contrast, co-administration of recombinant flagellin with tumor cells did not reproduce these effects underscoring the importance of co-delivery of TLR ligand and antigen to the same antigen processing compartments (**Fig. 1**). This can be achieved either via direct linkage of the fagellin to a TAA of choice, or more simply via the expression of flagellin within tumor cells such that tumor antigens are physically present with flagellin within the same phagosomal space.[4] In fact, our studies demonstrated that expression of flagellin-antigen fusion proteins within tumor cells was not necessary for protection against tumor.[4]

One of the mechanisms by which TLR signaling controls antigen presentation by MHC-II is through inducing proteolytic degradation of the invariant chain (Ii or CD74), which protects the MHC-II binding groove from premature peptide binding.[5] This could explain how $CD4^+$ T cells are primed by flagellin-expressing tumor cells. However, tumor-specific $CD8^+$ T cell cross-priming was also enhanced upon injection of flagellin-expressing cells, but not when flagellin was co-injected suggesting that physical association of TLR ligand and antigens also induces cross-presentation. How TLRs regulate this process is still imperfectly understood.[5] A recent study uncovered a role for CD74 in MHC-I trafficking from endoplasmic reticulum to endolysosome in cross-presentation.[6] This raises the possibility that a mechanism similar to the one described for MHC-II mediated $CD4^+$ T cell priming may also ensure TLR regulation of $CD8^+$ T cell cross-priming.

Another advantage of choosing flagellin as a PAMP to introduce into tumor cells is that it is sensed by NAIP5 (Neuronal apoptosis inhibitor protein 5) when localized in the cytosol of myeloid cells. In turn, NAIP5 recruits NLRC4

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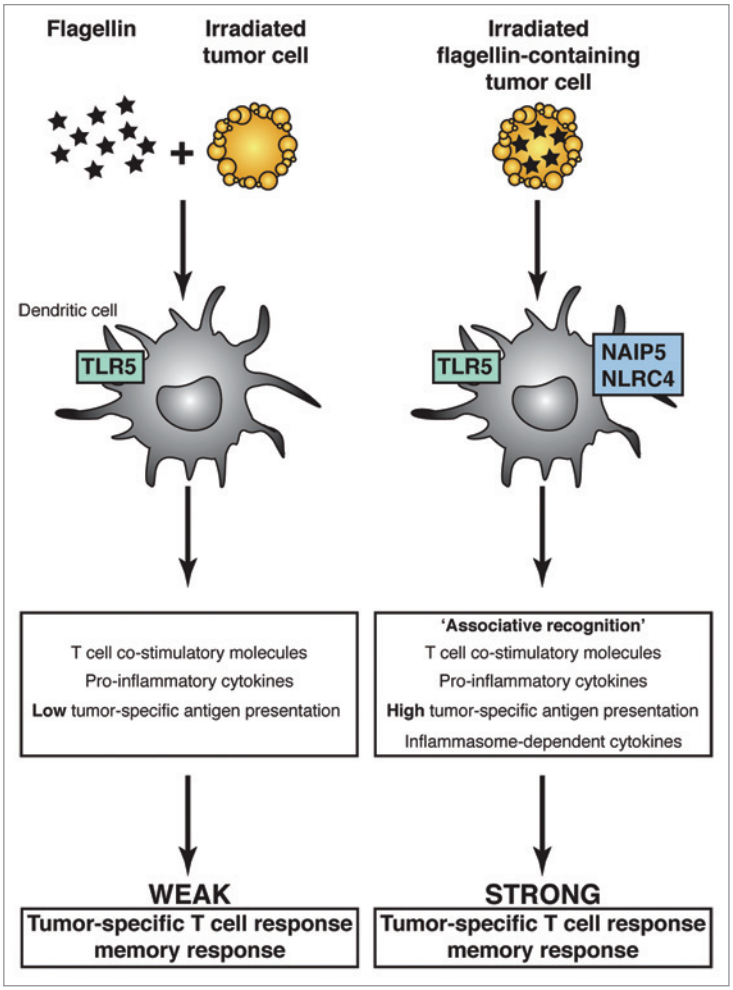

Figure 1. Schematic showing the potential clinical application of introducing flagellin within tumor cells for the purpose of inducing anti-tumor immunity. Left part: the administration of recombinant flagellin with irradiated tumor cells can induce DC maturation, expression of T cell co-stimulatory molecules and pro-inflammatory cytokine secretion. However, this is not effective at inducing a potent tumor-specific immune response. Right part: engineering the expression of flagellin within tumor cells followed by their irradiation and administration would ensure dual triggering of the Toll-like receptor 5 (TLR5) and the Neuronal apoptosis inhibitory protein 5 (NAIP5)/Nod-like receptor, CARD domain containing protein-4 (NLRC4) pathways in DCs that phagocytose the tumor cells. This would result in associative recognition of tumor-specific antigens with the TLR5 ligand leading to optimal antigen presentation to tumor-specific T cells and within an inflammatory environment that favorably supports robust anti-tumor immunity.

(NLR family, CARD domain-containing protein 4) to assemble a multiprotein complex, called the inflammasome, which controls caspase-1-dependent proteolytic maturation of the inflammatory cytokines interleukin (IL)-1β and IL-18.[7] However, transcription of the pro-forms of these cytokines is a limiting event that is strongly induced by TLR signaling. Therefore, flagellin offers the dual advantage of stimulating TLR5-dependent synthesis of these cytokine pro-forms and triggering their maturation by activating the NAIP5/NLRC4 inflammasome. We thus investigated whether NLR signaling was required for anti-tumor immunity generated in response to flagellin-expressing tumor cells.[4] Mutation of key residues for NAIP5/NLRC4 activation within the C-terminal domain of flagellin restored the ability of flagellin-expressing cells to form tumors in vivo. More surprisingly, it also abrogated the antitumor T cell responses and impaired vaccine capacity of irradiated flagellin-expressing tumor cells. Recent studies have uncovered a role for various NLRs, including NLRC4, in preventing colitis-associated tumor,[8,9] but how NLRs control T cell priming is still elusive. Work from the groups of L. Zitvogel and G. Kroemer has demonstrated importance of the NLRP3 inflammasome in priming tumor-specific $CD8^+$ T cells via controlling IL-1β secretion in response to chemotherapy induced tumor cell death.[10] In light of these findings, it would be important to address whether inflammasome-dependent cytokines are also required for adaptive antitumor immune responses generated by flagellin-expressing cells.

We thus propose two approaches to improve the clinical use of TLR agonists in cancer vaccines. First, promoting associative recognition of tumor antigens with a TLR ligand can easily be achieved once a TAA has been identified or by introduction of flagellin in tumor cells following tumor resection to enhance presentation of several TAAs. Second, TLR ligands may be combined with NLR ligands to evoke inflammasome-dependent immune responses. However, careful assessment of which combinations of TLR and NLR ligands provide the highest application potential in the clinic will be a critical step toward the design of new adjuvant-based cancer immunotherapies.

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