Toll-like Receptor Ligands Energize Peptide Vaccines through Multiple Paths

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Abstract

The potential of vaccines for cancer therapy or prevention has yet to be realized. Recently, we showed that using an immunologic adjuvant composed of a Toll-like receptor (TLR) ligand can increase the production of antitumor CTLs produced by a peptide vaccine in a mouse model of breast cancer. By increasing the cross talk between the innate and adaptive immune systems, TLR ligands can drive expansion and memory of CTLs that can destroy cancer cells. [Cancer Res 2007;67(17):7945–7]

The Yin and Yang of Peptide Vaccines

For more than a decade, synthetic peptides representing epitopes for antigen-specific CTL have been proposed as prime candidates for vaccine development (1). However, the ability of a peptide vaccine to induce a CTL response that translates into a clinical benefit (e.g., antitumor effect or antiviral protection) depends on a large number of factors, from the binding affinity of the peptide to an MHC molecule to how the peptide is presented to the naïve CTL precursors. *In vivo* administration of peptides representing CTL epitopes, in some cases, can activate T-cell responses, but in other circumstances, it can induce T-cell tolerance/anergy (2). The outcome of the CTL response to a peptide challenge (activation versus tolerance) is dictated, in great part, by the type of antigen-presenting cell (APC) and its activation status. Other factors such as the presence of $CD4^+$ T cells that either enhance (T helper cells) or suppress (T regulatory cells) immune responses are also important for the generation of an effective antitumor CTL response to a peptide or any other kind of vaccine. Thus, to generate a clinically effective antitumor CTL response, it becomes necessary, in addition to simply administering a peptide injection, to manipulate various components of the immune system.

Effects of Toll-like Receptor Ligands in CTL Responses

Molecules that stimulate members of the Toll-like receptor (TLR) family have been shown to activate the immune system in multiple ways (3). Synthetic oligodeoxynucleotides containing CpG motifs (hereafter referred to simply as CpG) stimulate TLR-9 on APC such as dendritic cells, enhancing their capacity to present antigen to naïve CTL. TLR-stimulated APC express higher amounts of MHC molecules, enhancing their ability to present peptide to the CTL ("signal 1"). In addition, TLR-activated APC also express high levels of costimulatory molecules (e.g., CD80/CD86, or "signal 2") and secrete cytokines (e.g., interleukin-12, type I IFN; "signal 3") that mediate CTL proliferation and maturation into effector cells (4, 5). We previously reported that repeated administration of CpG (nine daily injections of 100 μg/mouse) conferred protection to T cells against death-mediated lymphokine withdrawal or activation-induced cell death (AICD) through an increased expression of antiapoptotic mediators such as Bcl-xL and c-FLIP (6). Interestingly, the death protection effect of CpG on T lymphocytes was not antigen specific and affected all subsets (CD4, CD8, naïve, memory, and effector). In view of all of the pleiotropic effects of TLR ligands on the immune system, these compounds have been used as immune adjuvants to enhance CTL responses to various types of cancer vaccines. Earlier work from our group indicates that peptide vaccination with CpG induces potent CTL responses with significant effects against transplantable tumors (7).

Evaluation of Peptide Vaccines with TLR Ligands against Breast Tumors

Recently, our group evaluated the antitumor effect of peptide vaccination with CpG injections in a mouse model of breast cancer (8). Female BALB-neuT mice (BALB/c background) express the product of the activated rat *neu* oncogene, RNEU, on their mammary glands at puberty (~4 weeks); by 15 weeks of age, most animals have already developed *in situ* mammary carcinomas (9). A peptide epitope from the RNEU sequence corresponding to positions 66 to 74 (p66) was shown to induce potent CTL responses when administered together with five daily injections of CpG (5XCpG) in BALB/c. However, the CTL responses to the same vaccine were minimal in BALB-neuT mice, indicating the presence of immune tolerance (8). Notwithstanding, treatment of BALB-neuT mice with either anti-CD4 or anti-CD25 monoclonal antibodies (mAb) before vaccination resulted in CTL responses of a similar magnitude as those observed in BALB/c mice, indicating that $CD4^+/CD25^+$ T regulatory cells were involved in the p66 CTL tolerogenic state. Whereas p66/5XCpG vaccination against an established $RNEU^+$ breast tumor cell line (TUBO) in BALB-neuT mice significantly delayed tumor growth, pretreatment with anti-CD25 mAb abolished tumor cell growth, confirming that inhibition of T regulatory cells improves the effectiveness of the vaccine. On the other hand, treatment of BALB-neuT mice with anti-CD4 mAb, although effective in the reversal of the CTL unresponsive state, did not improve, but in fact reduced, the effectiveness of the p66/5XCpG vaccine. These results can be explained by the fact that anti-CD4 mAb treatment not only depletes T regulatory cells but also eliminates conventional T helper cells, which play a critical role in the long-term persistence of a CTL response (10). Studies by Janssen et al. (11) showed that "helpless" CTL (i.e., CTL primed in the absence of T help) fail to expand after re-encountering antigen.

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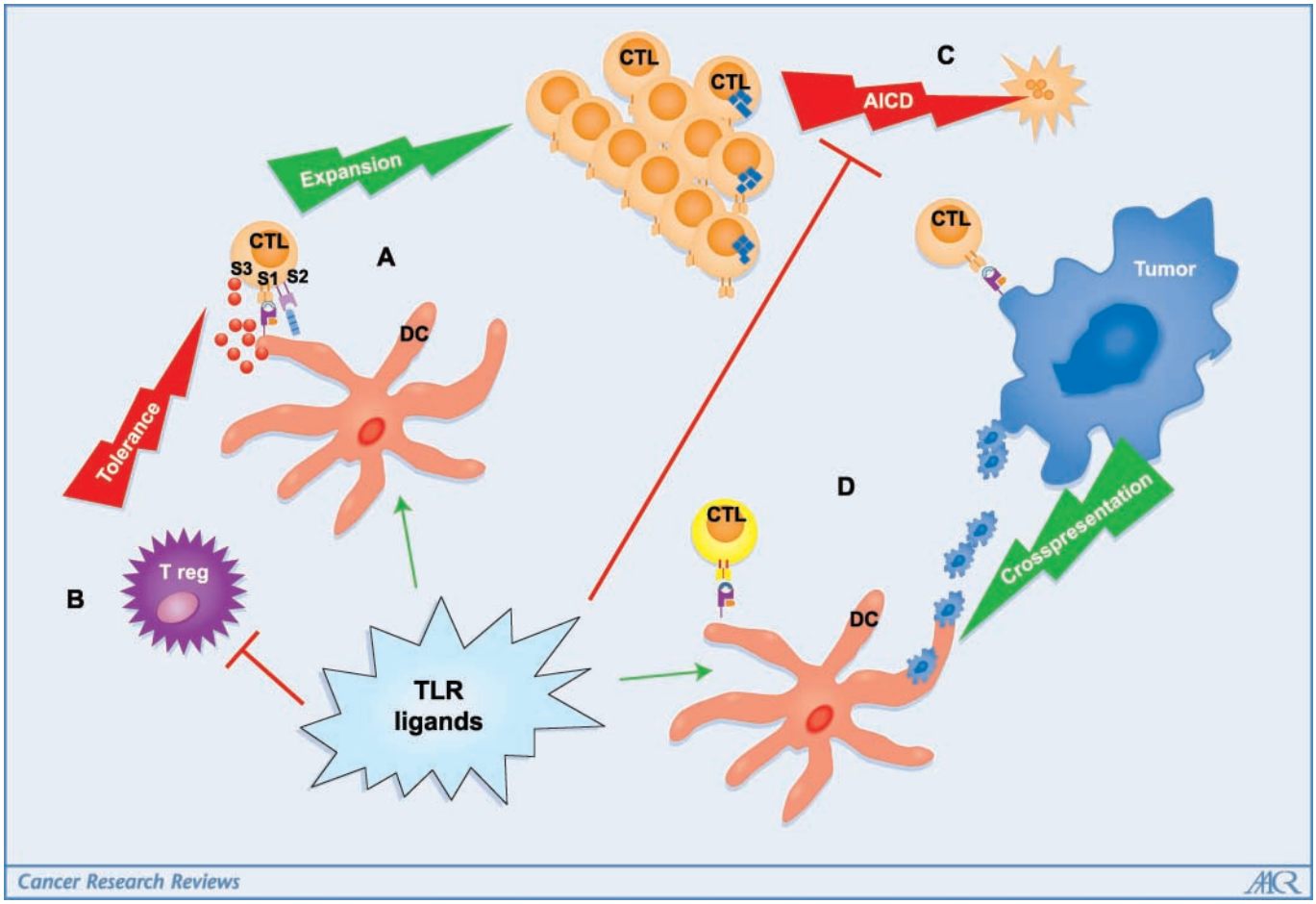

Figure 1. Pleiotropic effects of TLR ligands in potentiating CTL responses to peptide vaccines. Coadministration of TLR ligands with synthetic peptide may enhance CTL responses through numerous mechanisms: *A,* TLR ligands stimulate dendritic cells (*DC*) to increase expression of peptide/MHC complexes [signal 1 (*S1*)], CD80/CD86 [signal 2 (*S2*)], and cytokines [signal 3 (*S3*)], all of which enhance CTL activation, proliferation, and maturation. *B,* immunologic tolerance may be overcome by TLR ligands that inhibit T regulatory (*T reg*) cell function. *C,* TLR ligands such as CpG prevent AICD in CTL by increasing the expression of antiapoptotic mediators such as Bcl-xL and c-FLIP (*small blue diamonds*), allowing these cells to survive and migrate into the tumor site. *D,* TLR ligands activate dendritic cells at the tumor site and enhance tumor antigen cross-presentation, leading to epitope spreading and the generation of new CTL responses. *Red arrows,* inhibitory; *green arrows,* stimulatory.

The failure of helpless CTL to accumulate after antigen restimulation is due to AICD mediated by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL; ref. 12).

We observed that repeated p66/5XCpG vaccination (prime plus two boosts) in BALB-neuT mice was able to generate an effective antitumor CTL response without the need of anti-CD25 mAb treatment. Interestingly, this vaccination schedule was effective against established transplantable breast tumors even in circumstances where $CD4^+$ T cells had been depleted (8). Thus, the prime, two-boost vaccination protocol somehow helped overcome the suppressive effect of T regulatory cells and may have prevent the establishment of helpless CTL. With respect to the latter, we observed that the p66/5XCpG prime, two-boost vaccination protocol resulted in an expansion of antitumor CTL.[1] Thus, we believe that the prolonged administration of CpG (total of 15 injections of 100 μg) used in the in the prime, two-boost protocol could be responsible for overcoming both T regulatory suppressive function and AICD associated with T helplessness. In agreement with this assumption are the reports that persistent TLR stimulation can reverse T regulatory cell–mediated CTL tolerance (13, 14). In addition, in our model system, repeated CpG administration could also have prevented TRAIL-mediated AICD observed on helpless T cells through the up-regulation of c-FLIP. Thus, persistent TLR stimulation with some ligands such as CpG during peptide vaccination may enhance CTL responses through more than one way (Fig. 1). Prolonged TLR signals during an acute infection could be the main warning signal to the immune system not to shut down a response and to continue with the expansion of immune effector cells. On the other hand, persistence of TLR signals by endogenous ligands (heat shock proteins and unmethylated DNA) could be related to the presence of chronic autoimmune disorders (15).

Perhaps the most significant findings of our recent peptide vaccine publication (8) were the observations done in the setting of the spontaneous breast tumors that arise in BALB-neuT mice. First, we observed that a single peptide vaccination to young (9-week-old) BALB-neuT mice delayed significantly (by ~5 weeks) the appearance of autochthonous breast tumors as compared with

[1] Unpublished results.

unvaccinated controls (8). More striking were the results obtained in anti-CD25 mAb–treated mice, where spontaneous breast tumors were prevented up to 35 weeks of age. The p66/5XCpG prime, two-boost protocol (w/o prior mAb treatment) was also effective in delaying (~15 weeks) the appearance of measurable tumors, even when administered at 15 weeks of age, when carcinoma *in situ* is already present in these mice. Notably, the therapeutic effect of p66/5XCpG vaccination against spontaneous tumors was accompanied by the generation of CTL responses to additional RNEU CTL epitopes (8). We believe that this phenomenon known as epitope spreading is caused by the presentation of new CTL epitopes by CpG-activated APC that capture antigens released by tumor cells killed by the first wave of p66-specific CTL (Fig. 1).

Implications and Additional Challenges for Peptide Vaccine Development

A different picture is observed when peptide/5XCpG vaccines (prime, two-boost) are given to BALB-neuT mice that already have measurable tumors (~5-mm diameter at 17–19 weeks of age). In one half of the mice, the tumors cease to grow or slow down (disease stabilization) but tumors are not eliminated. In the other half of the mice, tumors continue to grow at a fast rate (disease progression). When CTL responses are assessed, it becomes evident that the mice that display disease stabilization have significant CTL responses, whereas the mice with progressive disease fail to show any CTL activity.[1] The suboptimal CTL response associated with lack of antitumor effects is probably due to immunosuppressive activities that are associated with the tumor burden. For example, CTL responses can be strongly inhibited by myeloid-derived suppressor cells (MDSC; ref. 16) that are generated by tumor-derived factors (e.g., vascular endothelial growth factor, granulocyte macrophage colony-stimulating factor, prostaglandin E2). In fact, large numbers of MDSC accumulate in blood, lymphoid organs, and tumors in BALB-neuT mice (17), supporting the possibility that inhibitory cells are responsible for the suboptimal CTL responses to peptide vaccination when there is measurable disease. Other powerful immunosuppressive strategies used by tumors to counteract CTL function include the expression of molecules such as B7-H1 and B7-H4 that can directly transmit inhibitory/proapoptotic signals to tumor-infiltrating CTL (18). In view of these additional challenges, it becomes clear that further therapeutic strategies will have to be developed to enhance the antitumor effects of peptide vaccines (or any other type of immunotherapy) in the advanced disease setting. For example, cyclooxygenase-2 inhibitors and gemcitabine could be used to counteract the effects of MDSC (19, 20) and anti–B7-H1 and anti–B7-H4 mAbs could be used to block the negative signals to the CTL.

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