Regulatory T Cells and Toll-Like Receptors in Cancer Therapy

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Abstract

T regulatory (Treg) cells that suppress immune responses may limit the efficiency of cancer immunotherapy. Recent findings indicate that Toll-like receptors (TLR) directly regulate the suppressive activity of Treg cells. Linking TLR signaling to the functional control of Treg cells may offer new opportunities to improve the outcome of cancer immunotherapy by coadministration of certain TLR ligands. (Cancer Res 2006; 66(10): 4987-90)

T Regulatory Cells and Cancer

Naturally occurring CD4+CD25+ T regulatory (Treg) cells have been extensively studied in mice and humans. This subset of T cells represents a small fraction (5-6%) of the overall CD4+ T cell population, expresses a high level of GITR and Foxp3 molecules, and mediates immune suppression through a cell-cell contact mechanism (1). Depletion of this subset of Treg cells in normal hosts results in various types of autoimmune diseases because the host immune system is unchecked and goes on to attack the body’s own tissues (1, 2). However, tumor cells take advantage of Treg cells to protect themselves by suppressing immune responses elicited by vaccines. This may, at least in part, explain why current clinical trials with cancer peptides or dendritic cells pulsed with antigenic peptides can induce only weak and transient immune responses and fail to produce therapeutic benefit.

Treg cells are recruited and accumulated at tumor sites in animal models (3, 4). The removal of CD4+CD25+ T cells by an anti-CD25 antibody enhanced antitumor responses (5). The existence of CD4+ T cell–mediated immune suppression has also been shown in different types of human cancers (6–8). We recently showed the presence of tumor-specific CD4+ Treg cells in tumor-infiltrating lymphocytes (TIL) derived from fresh tumor samples (9, 10). More importantly, these Treg cells suppressed the proliferation of naive CD4+ T cells and inhibited interleukin-2 (II-2) secretion by CD4+ effector cells upon activation by tumor-specific ligands.

Antigen Specificity and Its Potential Role in Modulating Treg Cells

The generation and maintenance of Treg cells have long been speculated to require the presence of target antigen or tissues (11, 12), but the identity of natural ligands of Treg cells remains largely unknown (2, 13). This is may be due to the fact that bulk CD4+CD25+ T cell populations display very diverse specificities for autoantigens (tissue-specific self-antigens) and non–self-antigens (tumor antigens and pathogens), making it more difficult to obtain a T-cell population with recognition of a particular physiologic ligand. It is particularly true when one deals with the tissue specific CD4+ Treg cells in autoimmune diseases, in that they often have compromised function (14). In contrast, tumor-infiltrating CD4+ T cells provide an enriched source for establishing tumor-specific CD4+ Treg cells because they are frequently accumulated at tumor sites. Using this strategy, we have generated a larger number of tumor-reactive and perhaps self-specific T-cell clones with characteristics of naturally occurring Treg cell properties from bulk tumor-reactive T-cell lines. This has led to successful identification of several ligands for tumor-reactive Treg cells (9). Both LAGE1 (a homologue of NY-ESO-1) and ARTC1 (antigen recognized by Treg cell) are dominantly expressed in cancer and normal testis but not in other normal tissues tested, therefore providing the specificity for CD4+ Treg cells. Interestingly, Dna J-like 2, an autoantigen identified by recombinant expression cloning (SEREX), tends to elicit CD4+ CD25+ Treg cells for immune suppression in animals (15). Overall, these studies provide compelling evidence that antigen-specific CD4+ Treg cells are present at tumor sites and mediate antigen-specific, local immune suppression.

Although NY-ESO-1 is a LAGE1 homologue, it is one of the most immunogenic tumor antigens identified to date. It is not clear why some antigens preferentially stimulate CD4+ Treg cells, whereas others activate CD4+ Th cells. Intrinsic genetic factors, such as FoxP3, and extrinsic factors, such as antigens and cytokine environment, play critical roles in generation, development, and maintenance of Treg cells in the thymus and peripherals (16, 17). The potential role of antigens in induction and expansion of Treg cells has been clearly shown in vitro and transgenic mouse models. Using an in vitro system, we recently found that CD4+ T-cell clones with the same antigen specificity but with different T-cell functions can be generated (18). It seems that the same antigenic peptide can stimulate both CD4+ helper and regulatory T cells depending on peptide affinity, expression level, and cytokine milieu. Similar results were observed in HA and Ova transgenic (Tg) models (19, 20). In both cases, ~40% to 50% of the antigen-specific CD4+ T cells became CD4+CD25+ Treg cells; the remaining half developed CD4+CD25− effector cells (19, 21, 22). Therefore, CD4+ Treg and effector T cells with the same antigen specificity exist in T-cell receptor (TCR) Tg models. Furthermore, it seems that the levels of antigen expression dictates whether CD4+ T cells are deleted or become antigen-specific CD4+ Treg or Th cells. Recent studies showed that stimulation of T cells with a low antigen dose in the absence of costimulatory molecules favors the generation of Treg cells or conversion of CD4+CD25− effector cells into CD4+CD25+ Treg cells (23, 24). Therefore, the success of peptide-based immunotherapy against cancer and other diseases may likely depend upon our understanding of the nature of antigens/peptides that preferentially activate Treg or helper T cells, thus providing a new opportunity to modulating the balance between Treg and effector T cells.
Toll-Like Receptors and Cancer Vaccines

Toll-like receptors (TLR), which recognize a limited but highly conserved set of molecular structures, so-called pathogen-associated molecular patterns, have recently emerged as a critical component of the innate immune system for detecting microbial infection and activation of dendritic cell maturation programs to induce adaptive immune responses (25, 26). For example, lipopolysaccharide (LPS), which is unique to Gram-negative bacteria, activate TLR4, whereas peptidoglycan found in Gram-positive bacteria activate TLR2 signaling pathway. TLR3 recognizes double-stranded RNA produced during viral infection, whereas TLR9 recognizes an unmethylated CpG DNA motif of prokaryotic genomes and DNA viruses (26). TLR7 and TLR8 recognize single-stranded viral RNAs or guanosine-related analogues (loxoribine and imidazoquinoline; refs. 27, 28). TLR expression has been detected in many types of cells, including different subsets of dendritic cells, T cells, neutrophils, eosinophils, mast cell, monocytes, and epithelial cells (25). It is generally accepted that TLR2, TLR5, TLR7, TLR8, and TLR9 use MyD88 as their sole receptor-proximal adaptor to transduce signals, whereas TLR3 relies on a TRIF- and IFN- regulated factor 3–mediated pathway for the production of IFN-β in response to pathogen recognition (26). TLR4 uses both MyD88-dependent and MyD88-independent pathways (26). Thus, MyD88 is an essential component of the signaling activities from most TLRs to the downstream pathways.

Stimulation of TLR signaling pathways activates dendritic cells, which is a crucial step for the induction of adaptive immunity, and induces the production of proinflammatory cytokines, such as type-I IFN and IL-12, which may bias to Th1 response favoring cytotoxic T-cell development. Although it was reported that TLR signaling on dendritic cells by CpG or LPS renders effector cells refractory to Treg cell–mediated suppression (29), recent studies showed that stimulation of dendritic cells with TLR ligands significantly enhances the proliferation of naive and effector T cells, making it harder for Treg cells to inhibit them (30, 31). TLR-matured dendritic cells are also capable of stimulating the proliferation of Treg cells. Thus, it seems that the manipulation of TLR signaling pathways in murine dendritic cells can stimulate both naive CD4+CD25− T cells and CD4+CD25+ Treg cells to proliferate, thus producing potent adjuvant effect on vaccines. These findings may offer new strategies to develop more effective vaccines by manipulating TLR signaling pathway.

**TLRs and Reversal of Treg Cell Function**

A key question in cancer immunotherapy is how to eliminate or reverse the suppressive function of Treg cells. One approach...
is to eliminate CD25+ Treg cells by a specific antibody or Ontak (a IL-2-toxin fusion protein). However, because the CD25 marker is not specific for Treg cells, and because all activated T cells are positive for CD25 marker, this approach may not efficiently eliminate Treg cells or deplete both Treg cells and activated effector cells (32, 33). Although it has been shown that TLR signaling activation on dendritic cells can render naive T cells refractory to suppression mediated by Treg cells in mice, little or no evidence suggests the connection between TLR signaling and the suppressive function of Treg cells. To test this possibility, we recently showed that CpG-A (G*GGGGACGATCGTCG*G*G*G, where the asterisk denotes a phosphorothioate linkage) and Poly-goligonucleotides (G*GGGG*G*G*G*G*G*G) directly reverse the suppressive function of Treg cells in the absence of dendritic cells (34). Further experiments indicated that short Poly-G oligonucleotides, but not the CpG motif in CpG-A, were responsible for the observed reversal effect. More importantly, the ability of effective Poly-G oligonucleotides to reverse the suppressive function of Treg cells can be shown in both antigen-specific and naturally occurring CD4+CD25+ Treg cells. The suppressive activity of the purified naturally occurring CD4+CD25+ Treg cells could be reversed by Poly-G oligonucleotides but not by Poly-T10 (T*TTTT*T*T*T*T*T, where the asterisk denotes a phosphorothioate linkage). Using naive CD4+ T cells or T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), we found that the CFSE-labeled naive CD4+ T cells could proliferate in response to OKT3 antibody stimulation, whereas the CFSE-labeled Treg cells could not, regardless of the presence or absence of Poly-g2 oligonucleotides (34). The proliferation of CFSE-labeled naive CD4+ T cells was completely suppressed by naturally occurring CD4+CD25+ Treg cells as well as by Treg 102 cells, but this effect could be reversed by treatment with Poly-G2 oligonucleotides (AAG*GA; ref. 34). It seems that TLR ligands can act on Treg cells and dendritic cells to modulate immune responses (Fig. IA). Poly-G oligonucleotides can directly regulate human Treg cells in the absence of dendritic cells but have no effect on murine Treg cells. It is likely that other TLR ligands may stimulate murine dendritic cells to secrete IL-6 and unknown cytokines that promote the proliferation of naive and effector T cells and render them refractory to suppression mediated by Treg cells (Fig. IA).

To identify the receptor for Poly-G oligonucleotides, we used RNA interference to knock down TLR8, MyD88, and IRAK4 in Treg cells, which abolished the ability of Treg cells to respond to Poly-G oligonucleotides (34). Consistent with this result, reverse transcription-PCR revealed that TLR8 was the only receptor that was consistently expressed by naturally occurring as well as antigen-specific Treg cells. We further showed that natural ligands for human TLR8, ssRNA40 and ssRNA33 derived from HIV viral sequences (27), completely reversed the suppressive function of Treg cells, whereas ligands for other TLRs failed to do so. These results suggest that binding and activation of TLR8 by either guanosine-containing DNA or RNA oligonucleotides is the key step in the reversal of Treg suppressive function. Furthermore, although TCR stimulation by antigenic peptides or anti-CD3 antibody is required for suppressive function of Treg cells, activation of TLR8-MyD88 pathway by Poly-G oligonucleotides or ssRNA40 generate a negative signal to block suppressive activity of Treg cells (Fig. 1B).

To determine whether Poly-G oligonucleotides can permanently reverse the suppressive function of Treg cells, we found that after treatment of Treg cells with Poly-G oligonucleotides, Treg cells remained nonsuppressive for a few days and then became suppressive, suggesting that reversal of Treg cell function by Poly-G oligonucleotides is temporal. Further studies are needed to investigate regulatory mechanisms of Treg cells by Poly-G oligonucleotides.

To test whether Poly-G oligonucleotides can trigger murine TLR8 for functional reversal of Treg cells, we isolated and purified murine CD4+CD25+ Treg cells and assessed for their ability to respond to TLR ligands. Murine Treg cells were capable of suppressing the proliferation of naive CD4+ T cells, but Poly-G oligonucleotides could not reverse the suppressive activity of murine Treg cells1, which is consistent with previous findings that TLR8 is not functional in mice (35). Further studies are required to show the potential function of murine TLR8. For this reason, we developed a human tumor model by s.c. injecting human 586mel tumor cells into Rag1−/− (T and B cell deficient) mice for testing effects of reversal of human Treg cells by Poly-G on antitumor immunity in vivo. We selected human CD8+ TIL586 T cells that were isolated from a melanoma patient and had the ability to recognize and kill autologous tumor cell line 586mel as effector T cells (36), whereas Treg102 cells served antigen-specific Treg cells as previously described (9). Although 586mel tumor cells could grow progressively, their growth was inhibited when autologous tumor-specific CD8+ TIL586 cells were i.v. injected 3 days later. However, coinjection of tumor-bearing mice with CD8+ TIL586 and Treg102 cells led to more aggressive tumor growth than 586mel cells alone, suggesting that the Treg102 cells exerted inhibitory effects on antitumor immunity. By contrast, adoptive transfer of TIL586 cells and Poly-G10-treated Treg cells restored, and in some cases enhanced, antitumor immunity. These studies showed that treatment of Treg cells with Poly-G oligonucleotides could reverse immune suppression normally mediated by Treg cells, thus providing a rationale to further evaluate its in vivo function in cancer patients in future studies.

Significant Questions for Future Studies

Despite the important roles of CD4+ Treg cells in controlling immune responses to self-antigen and non–self-antigens, their natural ligands, suppressive mechanisms, and regulation remain poorly understood. Our studies show that the suppressive function of Treg cells can be reversed through manipulation of TLR signaling, thus linking this pathway to the control of Treg cell function. There are many key questions that remain to be answered. It is not clear how TLR8 signaling controls the suppressive function of Treg cells. Is TLR8 signaling unique in regulating Treg cell function? What is the identity of molecules responsible for immune suppression? Further studies are also needed to evaluate the clinical potential of TLR ligands to reverse Treg cell function and improve the efficacy of cancer vaccines.

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1 K.S. Voo and R.-F. Wang, unpublished data.
References


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In the Review article on regulatory T cells and Toll-like receptors in cancer therapy in the May 15, 2006 issue of Cancer Research (1), the author’s first address “The 2nd Hospital, Zhejiang University of Medical School, Hangzhou, China”, as well as “China NSF grant 30025039” in the Acknowledgments section, were not intended for publication and should not have been included in the article.


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