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Recognition of a New ARTC1 Peptide Ligand Uniquely Expressed in Tumor Cells by Antigen-Specific CD4⁺ Regulatory T Cells¹

Helen Y. Wang,* Guangyong Peng,* Zhong Guo,* Ethan M. Shevach,† and Rong-Fu Wang²*

CD4⁺ regulatory T (Treg) cells play an important role in the maintenance of immunological self-tolerance by suppressing immune responses against autoimmune diseases and cancer. Yet very little is known about the natural antigenic ligands that preferentially activate CD4⁺ Treg cells. Here we report the establishment of tumor-specific CD4⁺ Treg cell clones from tumor-infiltrating lymphocytes (TILs) of cancer patients, and the identification of an Ag recognized by Treg cells (ARTC1) gene encoding a peptide ligand recognized by tumor-specific TIL.164 CD4⁺ Treg cells. The mutations in a gene encoding an ARTC1 in 164mel tumor cells resulted in the translation of a gene product containing the peptide ligand recognized by CD4⁺ Treg cells. ARTC1 peptide-activated CD4⁺ Treg cells suppress the physiological function (proliferation and IL-2 secretion) of melanoma-reactive T cells. Furthermore, 164mel tumor cells, but not tumor lysates pulsed on B cells, were capable of activating TIL.164 CD4⁺ Treg cells. These results suggest that tumor cells may uniquely present an array of peptide ligands that preferentially recruit and activate CD4⁺ Treg cells in sites where tumor-specific self-peptide is expressed, leading to the induction of local and tumor-specific immune suppression. The Journal of Immunology, 2005, 174: 2661–2670.

Molecular identification of tumor Ags has provided new opportunities for the development of effective vaccines against cancer (1). Clinical trials using cancer peptides or peptide-pulsed dendritic cells (DCs)³ have produced some evidence of Ag-specific immunological responses in most patients as well as clinical responses in a small percentage of the treated patients, but overall, the immune responses in most patients have been weak and transient (1–3). Among many factors that may contribute to this failure, the lack of CD4⁺ Th (effector) cell response is thought to be critical, because such T cells are required for the priming and maintenance of CD8⁺ T cells, thus enhancing the immune response against cancer (4, 5). Recent studies support the notion that CD4⁺ T cells enhance the expansion of memory CD8⁺ T cells (6–8).

However, a growing body of evidence indicates that a small subset of CD4⁺ T cells, called regulatory T (Treg) cells, induces self-tolerance by suppressing host immune responses (9–11), thereby contributing to the prevention of organ-specific autoimmune diseases (10, 12) and the inhibition of antitumor immunity (13, 14). Thus, both CD4⁺ Treg and Th cells play important but opposite roles in regulating host immune responses against cancer and other diseases. Using animal autoimmune disease models, it has long been demonstrated that generation and maintenance of CD4⁺ Treg cells require the presence of target Ags or tissues (15–18), but the functional properties and Ag specificities of these Treg cells remain largely unknown in these animal models (9, 19, 20).

To elicit strong and long-lasting immune responses against cancer Ags in cancer patients, we believe that it is fundamentally important to understand how antitumor immunity is suppressed at tumor sites. We examined this issue by showing the presence of LAGE1-specific CD4⁺ Treg cells in a tumor-infiltrating lymphocyte (TIL) line generated from fresh tumor samples of a cancer patient (21). Several recent studies show that CD4⁺ Treg cells are overrepresented in tumor sites as well as metastatic melanoma lymph nodes, and inhibit the function of infiltrating T cells (22, 23), but the Ag specificity is unknown. The fact that tumor-specific CD4⁺ Treg cells are present at tumor sites suggest that these tumor-specific Treg cells may explain, at least in part, why tumor-specific immune responses elicited by peptide or peptide-pulsed DC vaccines in cancer patients are weak and transient. Although MHC class II-restricted cancer peptides are capable of stimulating CD4⁺ T cell response, it is completely unknown whether such peptides elicit Ag-specific CD4⁺ effector T cell or Treg cell responses, which may result in two opposite consequences. Thus, to understand the molecular mechanisms of immune suppression at tumor sites, it is critical to identify these peptide ligands differentially recognized by CD4⁺ Treg or effector cells. Besides LAGE1, it is not clear whether other peptide ligands also preferentially stimulate CD4⁺ T cell responses in different cancer patients. In this study, we established a panel of tumor-specific CD4⁺ Treg cell clones, and identified a tumor-specific peptide ligand derived from a new mutated Ag. Activation of CD4⁺ Treg cells by tumor cells appears to be critical in suppressing the proliferation of naive CD4⁺ T cells and IL-2 secretion of Ag-specific CD4⁺ T cells. These findings raise the possibility that cancer cells may function...
as APCs to induce immune tolerance through preferential activation of CD4⁺ Treg cells at tumor sites, and therefore negatively regulate antitumor immune responses.

**Materials and Methods**

**Cell lines and T cell cloning**

CD4⁺ TIL (TIL164, TIL1363 and TIL1558) were cultured from a fresh tumor sample. All TILs and T cell clones were grown in RPMI 1640 medium containing 10% human AB serum and rIL-2 (300 IU/ml). Melanoma cell lines and EBV-transformed B cell lines were maintained in RPMI 1640 with 10% FCS. T cell clones were generated from TILs by limiting dilution methods (at 0.3 cell/well) as previously described (24).

**FACS analysis**

The expression of GITR was determined after staining T cells with an anti-GITR Ab (R&D Systems) followed by a secondary goat anti-mouse mAb conjugated to FITC. T cells were maintained in the culture medium containing a low IL-2 (30 IU/ml) for at least 2 wk before FACS analysis. To determine the expression of CD4 and CD25, T cells were stained with the respective Abs (BD Biosciences and eBiosciences) conjugated to either PE or FITC. After washing, cells were analyzed by FACS.

**Ab blocking and cytokine release assays**

To determine whether T cell recognition could be blocked by specific Abs, we measured T cell activity in the absence or presence of various Abs, as previously described (25). These Abs, including L243 (anti-HLA-DR; HB55), IVA12 (anti-HLA-DR, DP, DQ; HB145), IVD12 (anti-HLA-DQ, DR; HB144), and W6/32 (HLA-A, B, C; HB95), were purified from American Type Culture Collection hybridoma supernatants. A total of 2 × 10⁵ irradiated tumor cells in 80 μl of T cell assay medium (RPMI 1640/10% human serum/120 IU of IL-2) were incubated with 20 μl of an Ab (200 μg/ml) for 30 min. A total of 2 × 10⁵ T cells in 100 μl of T cell assay medium were then added, and the mixture was incubated overnight. GM-CSF, IL-2, IL-4, IL-10, IFN-γ, and TGF-β release from T cells was measured in culture supernatants by ELISA kits (Pierce). For suppression assay for IL-2 secretion, all Treg and effector cells were cultured in the RPMI 1640 medium containing 30 IU/ml IL-2, 10% heat-inactivated human serum. CD4⁺ TIL64 Treg cells were cocultured (1:1 ratio) with TIL1363-C10 CD4 Th cells, respectively, in the presence of 293ECIDR1 pulsed with ARTC-PEP1 or a control ARTC-PEP2 peptide. IL-2 secretion in the culture supernatants was determined by ELISA after 18 h of incubation of the mixture of T cells (Treg and TIL1363-C10) with 1363mel cells. CD4⁻CS T cells established from normal donor PBMCs were used as a control.

**FIGURE 1.** Phenotypic and functional characterization of a tumor-reactive CD4⁺CD25⁺ T cell line. A, FACS analysis of a tumor-reactive CD4⁺ T cell line from a cancer patient. B, Functional analysis of tumor-reactive CD4⁺ TILs for their ability to suppress the proliferative response of purified naive CD4⁺ T cells to anti-CD3 Ab stimulation. C, Generation and characterization of Ag-specific CD4⁺ Treg clones. TIL164 T cell clones were tested for their ability to recognize various target cells. All T cell clones specifically responded to autologous tumor cells (164mel), but did not recognize other cell lines tested. D, Cytokine profiles of TIL164 T cell clones. T cell clones were incubated with their corresponding tumor cell lines. Supernatants from overnight cultures were harvested for measuring the secretion of various cytokines. TIL1363-C10 and TIL1558-C3 clones were included as controls for cytokine profiles of CD4⁺ effector cells. Representative data from two TIL164 Treg clones and three repeated experiments are shown. TIL164-C1 and -C4 T cell clones secreted GM-CSF, IFN-γ, and IL-10, but did not produce detectable levels of IL-2, IL-4, or TGF-β by ELISA. TIL1363 and TIL1558 T cell clones secreted GM-CSF, IFN-γ, and IL-2, but not IL-4, IL-10, or TGF-β. If cytokine release from T cells based on optical readings at 450 nm were higher than that of the highest level of standards (1000 pg/ml), the bars are indicated.
cDNA library construction and screening

Total RNA was extracted from 164mel cells using TRIzol reagent (Invitrogen Life Technologies). Poly(A) RNA was purified from total RNA by the polyATract system (Promega) and converted to cDNA using a cDNA construction kit (Invitrogen Life Technologies) with an oligo-dT primer. The cDNA inserts were then ligated to a pTSX vector containing an II fragment (aa 1–80) (25), and cDNA libraries were electroporated into DH10B cells. Plasmid DNA for cDNA library pools was prepared from bacteria, each consisting of ~100 cDNA clones. DNA transfection and GM-CSF assays were performed as previously described (25).

Real-time quantitative PCR analysis

Total RNA was extracted from 1 × 10⁷ T cells using TRIzol reagent (Invitrogen Life Technologies). A SuperScript II RT kit (Invitrogen Life Technologies) was used in reverse transcription, in which 20 μl of the reverse transcription mixture, containing 2 μg of total RNA, was incubated...
at 42°C for 1 h. Foxp3 mRNA levels were quantified by real-time PCR using ABI/PRISM7000 sequence detection system (PE Applied Biosystems). The PCR was performed using primers, an internal fluorescent TaqMan probe specific to Foxp3 or hypoxanthine phosphoribosyltransferase (HPRT), all purchased from PE Applied Biosystems. Foxp3 mRNA levels in each sample were normalized with the relative quantity of HPRT. All samples were run in triplicate.

Peptide synthesis and T cell epitopes

The peptides were synthesized by a solid-phase method using a peptide synthesizer (model AMS 422; Gilson). Some peptides were purified by HPLC and had >98% purity. The mass of some peptides was confirmed by mass spectrometry analysis. Peptides reactive with CD4+ T cells were identified and characterized as previously described (25).

Proliferation assays

CD4+CD25- T cells (2 × 10^5) purified from human PBMCs by Ab-coated beads (Dynal) were cultured for 60 h in U-bottom 96-well plates containing 5 × 10^4 CD3-depleted APCs, 0.5 μg/ml anti-CD3 mAb, and different numbers of CD4+ regulatory or effector T cells. The proliferation of responder T cells was determined by the incorporation of [3H]thymidine for the last 16 h of culture, as previously described (26). Cells were harvested and the radioactivity counted in a scintillation counter. All experiments were performed in triplicate. Transwell experiments were performed in 24-well plates with a pore size of 0.4 μm (Corning Costar). A total of 2 × 10^5 of the freshly purified naive CD4+ T cells were cultured in the outer wells of a 24-well plate in medium containing 0.5 μg/ml anti-CD3 Ab and 2 × 10^5 APCs. Equal numbers of Treg cells or nonregulatory CD4-C5 cells were added into the inner wells in the same medium containing 0.5 μg/ml anti-CD3 Ab and 2 × 10^5 APCs. After 56 h of culture, the cells in the outer and inner wells were harvested separately and transferred to 96-well plates. [3H]Thymidine was added, and the cells were cultured for an additional 16 h before harvest for counting of the radioactivity with a liquid scintillation counter.

Results

Suppressive activity of tumor-specific CD4+ bulk T cell lines

To test whether CD4+ Treg cells with distinct Ag specificity exist in different cancer patients, bulk T cell lines (TILs) as well as autologous tumor cell lines were generated from fresh melanoma samples surgically removed from patients. CD4+ T cells were purified by anti-CD4 Ab-coated beads and were then used to screen for their reactivity against autologous tumor cell lines. After screening 15 CD4+ TIL lines, CD4+ TIL164 T cell line was found to recognize the autologous 164mel cells and selected for further analysis in this study (data not shown). To identify CD4+ Treg T cells from the purified CD4+ TIL164 cells, we first tested the expression of CD25 and GITR molecules and functional properties of this cell line. FACS analysis revealed that 36% of CD4+CD25+ T cells were present in the CD4+ bulk TIL164 populations, while naive CD4+ T cells purified from human PBMCs by Ab-coated beads (Dynal) were cultured for 60 h in U-bottom 96-well plates containing 5 × 10^4 CD3-depleted APCs, 0.5 μg/ml anti-CD3 mAb, and different numbers of CD4+ regulatory or effector T cells. The proliferation of responder T cells was determined by the incorporation of [3H]thymidine for the last 16 h of culture, as previously described (26). Cells were harvested and the radioactivity counted in a scintillation counter. All experiments were performed in triplicate. Transwell experiments were performed in 24-well plates with a pore size of 0.4 μm (Corning Costar). A total of 2 × 10^5 of the freshly purified naive CD4+ T cells were cultured in the outer wells of a 24-well plate in medium containing 0.5 μg/ml anti-CD3 Ab and 2 × 10^5 APCs. Equal numbers of Treg cells or nonregulatory CD4-C5 cells were added into the inner wells in the same medium containing 0.5 μg/ml anti-CD3 Ab and 2 × 10^5 APCs. After 56 h of culture, the cells in the outer and inner wells were harvested separately and transferred to 96-well plates. [3H]Thymidine was added, and the cells were cultured for an additional 16 h before harvest for counting of the radioactivity with a liquid scintillation counter.
fresh PBMCs had only 5.6% CD4 \(^{+}\) CD25 \(^{+}\) T cells (Fig. 1A). We next determined whether CD4 \(^{+}\) TIL164 had suppressive activity on proliferation of the purified naive CD4 \(^{+}\) T cells from fresh human PBMC of healthy donors in the response to an anti-CD3 Ab. As shown in Fig. 1B, TIL164 T cell line clearly inhibited the proliferative activity of naive T cells, while control CD4 \(^{+}\)CD25 \(^{+}\) T cells enhanced such proliferative activity. These results suggest that tumor-specific TIL164 line contained CD4 \(^{+}\)CD25 \(^{+}\) Treg cells with suppressive activity.

**Establishment and characterization of Ag-specific CD4 \(^{+}\) Treg cell clones**

To characterize the Ag-specific CD4 \(^{+}\) Treg cells from CD4 \(^{+}\) TIL164, we generated CD4 \(^{+}\) T cell clones from the TIL164 line by a limiting dilution method, as described earlier (24). The clones were screened for tumor reactivity based on their secretion of GM-CSF or IFN-\(\gamma\). Of 25 tumor-reactive CD4 \(^{+}\) T cell clones identified from TIL164, 10 were expanded further. As shown in Fig. 1C, all TIL164 T cell clones recognized the autologous 164mel tumor cells, but did not respond to other targets, including MHC class II-matched 293EC IIDR1, tumor, and EBV-transformed B cell lines.

**FIGURE 1.** DNA sequence and the predicted open-reading frames of ARTC1. Although three mutations underlined at positions 236 (T \(\rightarrow\) C), 917 (C \(\rightarrow\) T) and 971 (A \(\rightarrow\) G) were identified based on comparison between ARTC1 in 164mel and the published sequences in databases. However, the mutations at positions 236 and 917 were also identified in 164fibroblasts, suggesting that these two nucleotide changes may result from DNA sequence polymorphism. Mutation at position 971 represents a true mutation, and create a new translational start codon. The peptide ligand recognized by TIL164 Treg cells is underlined. The GenBank accession number for human ARTC1 is AY527413.
Isolation of a cDNA encoding an antigenic ligand of TIL164 Treg cells

To identify the genes encoding the ligand(s) recognized by specific CD4+ TIL164-C1 and -C4 T cells, we exploited a genetic targeting expression system that had been used in previous studies to identify several MHC class II-restricted tumor Ags (25). T cell reactivity against 164mel tumor cells was specifically blocked by a mAb against HLA-DR, but not by anti-HLA-DQ, HLA-DP, or MHC class I Abs (Fig. 3A). HLA typing showed the expression of HLA-DR1 and HLA-DR11 molecules on 164mel cells. Using an hi-fusion cDNA library constructed from mRNA isolated from 164mel cells, we screened 2 x 10^5 cDNA clones in 293ECI-DR1 cells with CD4+ TIL164-C1 T cells, and identified a positive pool that stimulated the T cells to release an increased amount of IFN-γ (data not shown). The positive-pool DNA was then transformed into E. coli, and individual colonies were picked for the preparation of plasmid DNA. After re-screening of 400 individual plasmid DNAs, we identified five single cDNA clones that could stimulate cytokine release from T cells. Fig. 3B shows that cDNA clones 10-1 and -2 were capable of stimulating IFN-γ release from T cells, while cDNA clone 1 (control) failed to do so. This gene was designated the Ag recognized by Treg cells 1 (ARTC1). To test whether other TIL164 Treg cell clones recognized the same Ag, we transfected 293ECI-DR1 cells with the same positive cDNA clones and tested for their ability to stimulate T cells. As shown in Fig. 3C, 9 of the 10 TIL164 Treg cell clones recognized 293ECI-DR1 cells transfected with the 10-1 cDNA clone, but did not respond to the LAGE1-expressing 293ECI-DR1 cells, suggesting that the ARTC1 Ag is an immunodominant T cell Ag. Although TIL164-C5 T cells recognized 164mel tumor cells, they did not respond to any Ag tested, indicating that they may recognize an unidentified ligand.

A mutation in ARTC1 results in the production of an antigenic T cell ligand

DNA sequence analysis and database searches revealed that both positive cDNA clones were identical to a partial cDNA of a previously unknown gene except for a mutation (A→G at position 971). A putative open-reading frame was predicted on the basis of an in frame fusion with the invariant chain (Fig. 4). After comparing DNA sequences between a 3-kb cDNA sequence obtained from 164mel cells by using the 5’ and 3’ race method and cDNA sequences in databases, we identified two additional mutations at the nucleotide positions 236 (T→C) and 917 (C→T) compared with DNA sequences in databases. However, these two nucleotide changes may result from DNA polymorphism instead of mutations.
because the same nucleotide changes were found from 164fibroblasts isolated from the same patient. TIL164 Treg cell clones recognized 164mel, but not 164fibroblasts, suggesting that mutations at positions 236 (T$^C$) and 917 (C$^T$) did not contribute to T cell recognition. In contrast, the mutation at position 971 (ATA$^G$ATG) in 164mel cells resulted in a new start codon (Fig. 4), which could translate a 69-aa protein. The wild-type ARTC1 gene identified in the normal 164fibroblasts could not produce the 69-aa protein because of the lack of an initial start codon. This finding may explain why TIL164 T cell clones recognized 164mel tumor cells only. Interestingly, despite the 3-kb cDNA sequence of this gene, we could not find a long open reading frame, but instead only a few short open reading frames were identified. There was no intron sequence in ARTC1 by comparison with genomic sequences available in databases. Southern blot analysis of genomic DNAs isolated from several different cell lines revealed that the DNA band pattern detected in 164mel cells differed from those in 164fibroblasts and other tumor cell lines (Fig. 5A), indicating that besides the mutations identified within ARTC1, other genetic alterations also occurred in the 164mel tumor cells.

In addition, we found that the majority of EST sequences in the human EST databases were derived from libraries constructed from a variety of tumor cell lines, suggesting preferential expression of ARTC1 in cancer cells. To demonstrate the expression pattern of ARTC1, we performed Northern blot analysis and showed that the ARTC1 gene was indeed predominantly expressed in tumor cell lines and normal testis, with little or no expression in other normal human tissues (Fig. 5B), a pattern reminiscent of cancer-testis Ags such as LAGE1 (32). Thus, ARTC1 is a putative member of the increasing list of cancer-testis family genes, while its biological function remains unknown.

Identification of a peptide ligand from ARTC1 recognized by CD4$^+$ Treg cells

To identify the T cell ligand, we made two 15-mer synthetic peptides on the basis of an HLA-DR1 peptide binding motif and the predicted amino acid sequence: ARTC-PEP1 (YSVYFNLPADTITYN) and ARTC-PEP2 (ASSREKVVAPYILK). These peptides were pulsed onto 293ECIIDR1 cells, and tested for their ability to stimulate T cells. As shown in Fig. 6A, both TIL164-C1 and -C4 T cells recognized ARTC-PEP1-pulsed target cells, but not those pulsed with the ARTC-PEP2 peptide. The minimal peptide concentration required for T cell activity, determined by peptide titration experiments, was 1 $\mu$M (Fig. 6B). We then asked whether other T cell clones derived from TIL164 could recognize the same ARTC-PEP1 peptide. After coculturing other T cell clones with ARTC1-PEP1-pulsed target cells overnight, we measured IFN-γ release from cell supernatants, showing that all ARTC1-reactive TIL164 T cell clones recognized the same peptide (data not shown), suggesting that the ligand recognized by CD4$^+$ Treg cells is a dominant peptide derived from the mutated ARTC1 gene.

Peptide-specific activated CD4$^+$ Treg cells inhibit the function of CD4$^+$ effector T cells

To induce tumor-specific immune suppression, CD4$^+$ Treg cells may function at a local rather than systemic level. If so, they would need to be recruited and localized to the sites of Ag recognition (i.e., tumor sites); hence their specific suppression would depend on the availability of peptide ligand-MHC complexes on the cell surface. We tested whether peptide-specific activation of CD4$^+$ Treg cells is required for the suppression of immune responses. To test this possibility, we activated CD4$^+$ TIL164 Treg cells by...
293ECIDRI pulsed with the ARTC-PEP1 peptide as APCs. Activation of TIL164 Treg cells by plate-coated anti-CD3 Ab served as a positive control, while 293ECIDRI pulsed with the control ARTC-PEP2 peptide as a negative control for T cell activation. The activated or inactivated TIL164-C1 and C4 cells were then cocultured with the CD4\(^+\) effector TIL1363-C10 (as responding cells) capable of recognizing 1363mel target cells for IL-2 secretion. IL-2 secretion from the TIL1363-C10 clone was determined by ELISA. As shown in Fig. 7A, TIL164-C1 and -C4 T cells strongly inhibited IL-2 secretion from the TIL1363-C10 effector T cells in the presence of the ARTC-PEP1 peptide. However, in the presence of the ARTC-PEP2 control peptide, neither CD4\(^+\) TIL164 Treg clones nor CD4\(^+\) non-Treg cell clones (CD4-CT and TIL1558-C3) inhibited IL-2 secretion by TIL1363-C10 T cells. These results suggest that peptide-specific activation of Treg cells is required for their suppressive function.

**Direct presentation of the ARTC1 peptide by tumor cells to CD4\(^+\) Treg cells**

We next sought to determine how tumor-specific CD4\(^+\) Treg cells are activated and maintained at tumor sites. Beside direct presentation of an Ag to T cells by tumor cells as previously suggested (33), cross-presentation of Ags by B cells or DCs has also been documented for the activation of both CD4\(^+\) and CD8\(^+\) effector cells (34). In previous studies, we noted that TIL1359 and TIL1087 cells can recognize 1359mel and 1087mel tumor cells respectively, but not autologous tumor lysates presented by EBV B cells (25, 35), whereas TIL1363 and TIL1558 effector T cells recognize both whole tumor cells and tumor lysates presented by EBV B cells (27, 28). In experiments to determine whether CD4\(^+\) TIL164 Treg cells could recognize tumor cell lysates pulsed on B cells, we found that 164mel tumor cells expressing the ARTC1 ligand could specifically activate the corresponding CD4\(^+\) Treg cells, but exogenous presentation of 164mel tumor cell lysate by the MHC class II-matched EBV-B cells lacked any stimulating effect on TIL164-C1 cells (Fig. 7B). In contrast, TIL1363 T cell clones could respond to whole tumor cells as well as cell lysate-pulsed B cell targets. HLA-DR1-matched DCs pulsed with tumor lysate also failed to activate TIL164 CD4\(^+\) Treg cells, but ARTC1 peptide-pulsed DCs strongly stimulate T cell response (data not shown), suggesting that the expression level of ARTC1 in tumor cells may be too low to be efficiently processed and presented to T cells by B cells or DCs. Taken together, these results suggest that the unique presentation of a self-ligand from the ARTC1 Ag by tumor cells is a major pathway for Ag presentation, while cross-presentation by EBV-B cells is ineffective in activating CD4\(^+\) Treg cells.

**Discussion**

Although the evidence for the presence of CD4\(^+\)CD25\(^+\) Treg cells in tumor sites as well as metastatic melanoma lymph nodes has recently been documented (22, 23), very little is known about their Ag specificity and suppression mechanisms. Our recent study demonstrated the existence of LAGE1-specific CD4\(^+\) Treg cells in TIL102 derived from a cancer patient (21). Thus, it is likely that both tumor-specific and -nonspecific CD4\(^+\)CD25\(^+\) Treg cells infiltrate in tumor sites. Although Ag-nonspecific or self-Ag specific CD4\(^+\) Treg cells play an important and general role in the induction of immune tolerance, it is not known how they are activated and maintained at tumor sites. In contrast, tumor-specific CD4\(^+\) Treg cells are activated upon encounter with Ags expressed in tumor cells, and then suppress local immune responses at tumor sites. For these reasons, we focused our attention on tumor-specific CD4\(^+\) Treg cells derived from different cancer patients. In this study we demonstrate that an Ag-specific CD4\(^+\) bulk T cell line established from a melanoma patient after screening many TILs from cancer patients can suppress the proliferation of naive CD4\(^+\) T cells through the activity of CD4\(^+\) Treg cells. To further determine their Ag specificity, we established a panel of tumor-specific Treg cell clones. Although the cytokine secretion profile (IFN-\(\gamma\) and IL-10) of these tumor-specific Treg cells resembles that of human Tr1 cells (36), a cell contact-dependent mechanism appears essential for their suppressive activity, similar to naturally occurring CD4\(^+\)CD25\(^+\) Treg population (37). Therefore, it appears that...
these Ag-specific CD4+ Treg cells along with the recently described LAGE1-specific CD4+ Treg cells (21) are a unique subset of Treg cells at tumor sites that functionally suppress the proliferation as well as IL-2 secretion of effector cells in an Ag-specific manner.

To understand how CD4+ Treg cells are activated and maintained at tumor sites, we identified the ARTC1 gene, which encodes a T cell ligand that serves as an activation signal for TIL164 CD4+ Treg cells. Interestingly, DNA sequence encoding T cell epitopes is not mutated, but the mutation at position 971 creates a new translational start codon for a short polypeptide, indicating that the ARTC1 peptide is tumor-specific. Although both LAGE1 and ARTC1 are preferentially expressed in cancer cells and normal testis, LAGE1 is a nonmutated Ag, while the gene product of ARTC1 is generated through mutations. Furthermore, it should be noted that like tumor-specific CD4+ effector T cells, these CD4+ Treg cells recognize peptide ligands preferentially presented by tumor cells, raising a possibility that these CD4+ Treg cells may arise from Ag experienced CD4+CD25+ T cells in the suppressive cytokine milieu of tumor sites (9, 26) or after interaction with rather than inhibited the proliferation of naive CD4+CD25+ T cells. However, all ARTC1-reactive T cell clones analyzed to date recognize the LDFP peptide were all CD4+ effector T cells, suggesting that like LAGE1-reactive Treg cell clones, the ARTC1 peptide may preferentially stimulate CD4+ Treg cells. In contrast, over 200 T cell clones from TIL1363 that recognized the LDFP peptide were all CD4+ effector cells (Th) cells because they secreted IFN-γ, GM-CSF, and IL-2, and enhanced rather than inhibited the proliferation of naive CD4+ T cells in response to anti-CD3 Ab (data not shown). These results suggest that different Ags may differentially stimulate CD4+ Treg or effector T cells depending on their expression levels and their avidity for T cell recognition. This notion is supported by results obtained in transgenic models showing that the level or dose of self-peptide stimulation affects the peripheral generation and expansion of CD4+CD25+ Treg cells, and thus direct the selection and accumulation of such cells at sites where the self-peptide is expressed (42–44). Furthermore, it has been suggested that tumor-specific peptides when presented by tumor cells in subimmunogenic form may lead to generation of Ag-specific CD4+ Treg cells and the suppression of antitumor response (45). Although many tumor cell lines used in animal models are negative for MHC class II molecules, the expression of MHC class II molecules on human tumor cells has been documented in many tumor types, including melanoma, colorectal, renal, and breast cancers, and can be up-regulated following exposure to IFN-γ, IFN-α, TNF-α, and IL-2 secreted by NK, NKT, CD8+, and CD4+ T cells (46, 47), implying that MHC class II-positive tumor cells are actively engaged in the recruitment, activation and maintenance of Ag-specific CD4+ Treg cells. Tumor cells lacking expression of costimulatory molecules such as B7-1 and B7-2 have been suggested to induce tolerance in accord with the two-signal model for T cell activation (34). Although tumor cells may express other costimulatory molecules that are required for activation of Treg cells, FACS analysis of 164mel cells stained with anti-B7h1 and anti-B7h4 Abs are negative (data not shown).

Given the fact that CD4+ effector T cells are not only required for the priming and maintenance of CD8+ T cells, thus enhancing the immune response against cancer (4, 5), but also essential for enhancing the expansion of memory CD8+ T cells (6–8), it is of particular interest to know whether MHC class II-restricted peptides preferentially activate CD4+ Treg or effector cells. For example, a SEREX-defined autoantigen, Dna J-like 2, activates CD4+CD25+ Treg cells and suppresses antitumor immune response in mice (48). It appears that some antigenic ligands may predominantly induce CD4+ Treg cells, while others favor the activation of CD4+ effector T cells. The solution to this problem may rely in further identification and characterization of antigenic ligands recognized by CD4+ Treg cells or effector cells. Tumor-induced Treg cell response has been reported in both animal tumor models and human cancer patients (49–51). Ag doses, the mode of Ag presentation (i.e., by tumor cells, immature DC or mature DCs) and cytokine environment may ultimately determine the outcome of immune responses. One critical issue for developing effective cancer vaccines is how to block the suppressive activity of CD4+ Treg cells or to convert CD4+ Treg cells into effector cells. Although several reports have recently documented the conversion of naive CD4+CD25+ T cells to CD4+CD25+ Treg cells (40, 41), very little is known about Ag-specific CD4+CD25+ T cells could be converted to Ag-specific CD4+CD25+ effector cells, or whether the suppressive function of CD4+ Treg cells could be reversed. New strategies that allow CD4+ regulatory and effector T cells to be stimulated in different ways could be used to shift the balance toward Treg cells (for the treatment of autoimmune diseases) or toward effector T cells (for the treatment of cancer and infectious diseases). Such manipulation will undoubtedly require additional insight into the mechanisms by which CD4+ Treg cells suppress immune responses to self-tissues and organs. Further identification and classification of natural ligands for CD4+ Treg cells would significantly advance the mechanistic appreciation of T cell-mediated immune suppression and accelerate clinical efforts to achieve satisfactory immune responses against cancer and other diseases.

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