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Tumor-Specific Human CD4+ Regulatory T Cells and Their Ligands: Implications for Immunotherapy

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Summary

Regulatory T cells play an important role in the maintenance of immunological self-tolerance by suppressing immune responses against autoimmune diseases and cancer. Little is known, however, about the nature of the physiological target antigens for CD4+ regulatory T (Treg) cells. Here we report the identification of the LAGE1 protein as a ligand for tumor-specific CD4+ Treg cell clones generated from the tumor-infiltrating lymphocytes (TILs) of cancer patients. Phenotypic and functional analyses demonstrated that they were antigen-specific CD4+ Treg cells expressing CD25 and GITR molecules and possessing suppressive activity on the proliferative response of naive CD4+ T cells to anti-CD3 antibody stimulation. Ligand-specific activation and cell-cell contact were required for TIL102 Treg cells to exert suppressive activity on CD4+ effector cells. These findings suggest that the presence of tumor-specific CD4+ Treg cells at tumor sites may have a profound effect on the inhibition of T cell responses against cancer.

Introduction

CD4+ effector and regulatory T cells recognize peptides presented by MHC class II molecules but play distinctly different roles in regulating host immune responses against cancer and other diseases (Germain, 1994; Rosenberg, 2001; Sakaguchi et al., 2001; Shevach, 2002). CD4+ effector (helper) T cells are required for the priming and maintenance of CD8+ T cells, thus enhancing the overall immune response (Houghton et al., 2001; Wang, 2001). Recently, CD4+ T cells were shown to be required for the subsequent expansion of memory CD8+ T cells (Janssen et al., 2003; Shedlock and Shen, 2003; Sun and Bevan, 2003). Thus, identification of MHC class II-restricted tumor antigens capable of stimulating CD4+ T cell responses is a critical step in the development of effective cancer vaccines (Wang, 2002).

Paradoxically, CD4+ T regulatory (Treg) cells can profoundly suppress host immune responses and induce self-tolerance (Roncarolo and Levings, 2000; Sakaguchi et al., 2001; Shevach, 2002). Their induction of self-tolerance extends to autoimmune diseases, allergy, and transplantation and antitumor immunity (Sakaguchi et al., 2001; Shevach, 2000). Besides naturally occurring CD4+ CD25+ Treg cells, other CD4+ Treg cells include Tr-1 cells secreting IFN-γ and IL-10, and Th3 cells secreting high levels of TGF-β, IL-4, and IL-10 (Francois Bach, 2003; Roncarolo and Levings, 2000; Weiner, 2001). Although expression of CD25 on T cells has been used as a useful marker of Treg cells, its expression is not necessarily associated with Treg cell function in that it is also expressed by activated, nonregulatory effector lymphocytes. Other molecules, including the TNF family molecule GITR and cytotoxic T lymphocyte antigen-4 (CTLA4) may serve as markers for Treg cells (Sakaguchi et al., 2001; Wood and Sakaguchi, 2003). Recently, several groups reported that foxp3 may serve as a potential marker of CD4+ Treg cells in mice (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003), but it is not clear whether the finding also applies to human CD4+ Treg cells. Therefore, Treg cells must be defined primarily according to their immunosuppressive function (von Herrath and Harrison, 2003). They inhibit other immune cell functions either directly through cell-cell contact or indirectly through the secretion of anti-inflammatory mediators such as IL-10, TGF-β, or IL-4 (Levings et al., 2002a; Shevach, 2002). Treg cells are demonstrated most convincingly by their ability to inhibit autoimmune disease development of transplant rejection in animal models, after de novo induction in vivo or after passive cotransfer with effector cells into suitable hosts (von Herrath and Harrison, 2003).

While most studies have been focused on the role of Treg cells in the prevention of various organ-specific autoimmune diseases (Sakaguchi et al., 1995; Shevach, 2000), several recent studies report an increased frequency of CD4+ Treg cells in cancer patients (Liyanage et al., 2002; Woo et al., 2001). In animal models, removal of CD4+CD25+ Treg cells enhances antitumor immune responses (Shimizu et al., 1999; Sutmuller et al., 2001), implying that these cells suppress immune responses against cancer cells. However, very little is known about the physiological target antigens recognized by CD4+ Treg cells in such settings. Our current knowledge of the antigen specificity of CD4+ Treg cells has come largely from studies with antigen-specific TCR transgenic animals (Apostolou et al., 2002; Curotto de Lafaille and Lafaille, 2002; Hori et al., 2002; Maloy and Powrie, 2001; Shevach, 2002). Because bulk CD4+ CD25+ T cell populations may display very diverse specificities for autoantigens (tissue-specific self-antigens) and tumor antigens, it has been difficult to identify the physiological ligands recognized by antigen-specific CD4+ Treg cells.

During the course of our work on MHC class II-restricted tumor antigens, we established tumor-specific CD4+ T cell clones from tumor-infiltrating lymphocytes (TILs) directly from fresh melanoma tumor samples. These CD4+ T cell clones were found to function as CD4+ Treg cells. In this report, we describe the generation and character-

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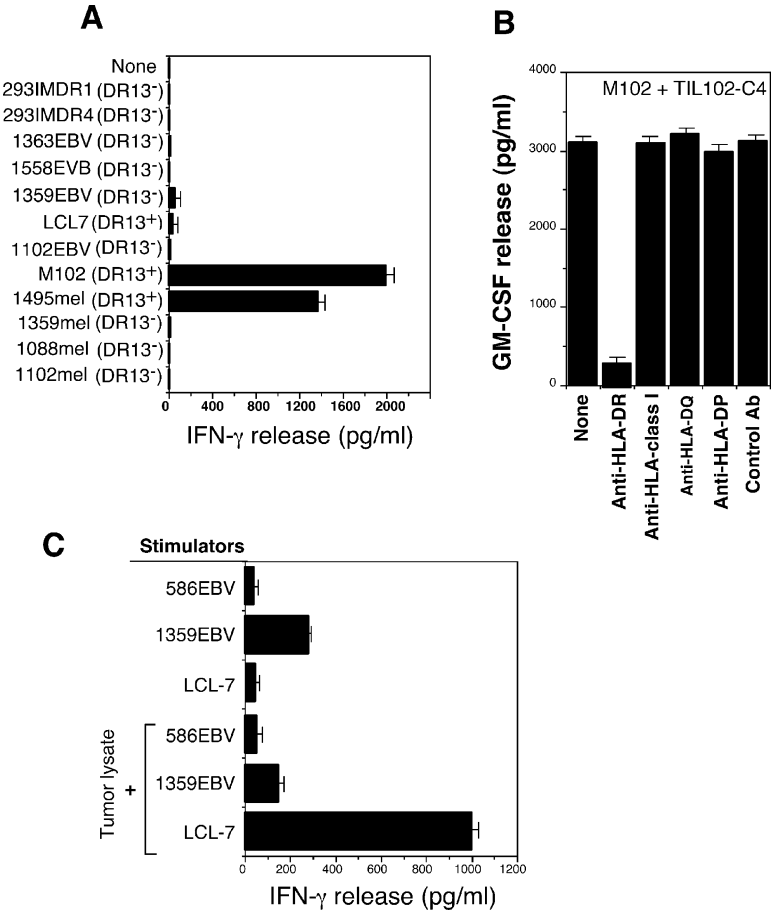

Figure 1. Specific Recognition of Tumor Cells by CD4+ TIL102 T Cells

(A) Recognition of HLA-DR13-positive M102mel and 1495mel cells, but not EBV-B cell lines and allogeneic melanoma cell lines or 293-derived cell lines. T cell recognition was evaluated by IFN-γ release from CD4+ TIL102-C4 cells.

(B) HLA restriction of T cell recognition. CD4+ TIL102-C4 cells were cocultured with autologous M102mel cells in the presence or absence of various anti-HLA antibodies. GM-CSF release was determined after an 18 hr incubation. T cell recognition of M102mel was specifically blocked by an anti-DR antibody, but not by anti-HLA class I, anti-HLA-DQ, or anti-DP antibodies.

(C) T cell recognition of HLA-DR13-positive LCL cells pulsed with tumor lysates. TIL102-C4 cells were cocultured with 586EBV (HLA-DR1+), 1359EBV (HLA-DR3+ and -DR4+), LCL-7 cells (HLA-DR13+) previously pulsed with or without tumor lysates of M102 overnight. IFN-γ release from CD4+ TIL102-C4 cells was measured in culture supernatants by ELISA. Similar results were obtained with the other 12 T cell clones.

ization of tumor-specific CD4+ Treg cells and identification of their natural ligands. Using these antigen-specific cells, we also show that cell-cell contact is required for Treg cell mediated immune suppression.

Results

Generation of Tumor-Specific CD4+ T Cell Clones

A tumor-reactive TIL line (TIL102) was first established from a fresh melanoma sample. After depletion of CD8+ T cells with a bead-coated anti-CD8 antibody, we established CD4+ T cell lines that recognized an autologous tumor cell line (M102) generated from the same tumor sample. To obtain tumor-reactive CD4+ T cell clones, we cloned T cells (0.3 cell/well) by a limiting dilution method in culture medium containing irradiated PBMCs (5×10^4 cells per well in a 96-well plate), anti-CD3, anti-CD28, and 30 IU/ml of IL-2 (Wang et al., 2002). After 14 days, the growing T cell clones were screened for tumor reactivity based on the secretion of GM-CSF, a cytokine released by different types of antigen-specific T cells, including CD4+ Th1, Th2, and CD8+ T cells. Thirteen tumor-reactive T cell clones were generated and further expanded. To determine the antigen specificity of T cell clones, we tested these T clones against various target cells, including HLA-DR3+ and DR13+ M102 tumor cells (the HLA typing for M102 is HLA-DR3 and -DR13). Representative data for one such CD4+ T cell clone (TIL102-C4) are shown in Figure 1A, while similar results were obtained with other T cell clones (data not shown). T cells responded to both HLA-DR13+ M102 and 1495mel tumor cell lines, but not to DR13+ EBV-transformed B cells or DR13− cell lines. T cell reactivity against M102 tumor cells could be specifically blocked by a monoclonal antibody against HLA-DR, but not by one against HLA-DQ, HLA-DP, or MHC class I molecules (Figure 1B). To further determine the restriction element, we tested whether CD4+ T cell clones could recognize DR3-positive or DR13-positive EBV-transformed B cells pulsed with M102 tumor lysates. As shown in Figure 1C, CD4+ T cells responded to DR13-positive LCL-7 cells pulsed with tumor cell lysates but recognize neither DR13-positve LCL-7 cells alone nor DR3-positive 1359EBV cells pulsed with the same tumor cell lysates. These studies suggest that CD4+ T cells recognize a tumor antigen in the context of an HLA-DR13 molecule.

Identification of a Gene Encoding a Nonmutated LAGE1 Protein

We next attempted to identify the target antigen recognized by tumor-specific CD4+ T cells. In previous studies, we devised a genetic targeting expression system and used it to identify several MHC class II-restricted tumor antigens (Wang et al., 1999a). For this system, we generated 293ECIIDR13 cells expressing Ii, DMA, DMB, and DR13 molecules as antigen-presenting cells for efficiently processing and presenting antigens to CD4+ T cells (Figure 2A), and constructed an Ii-fusion

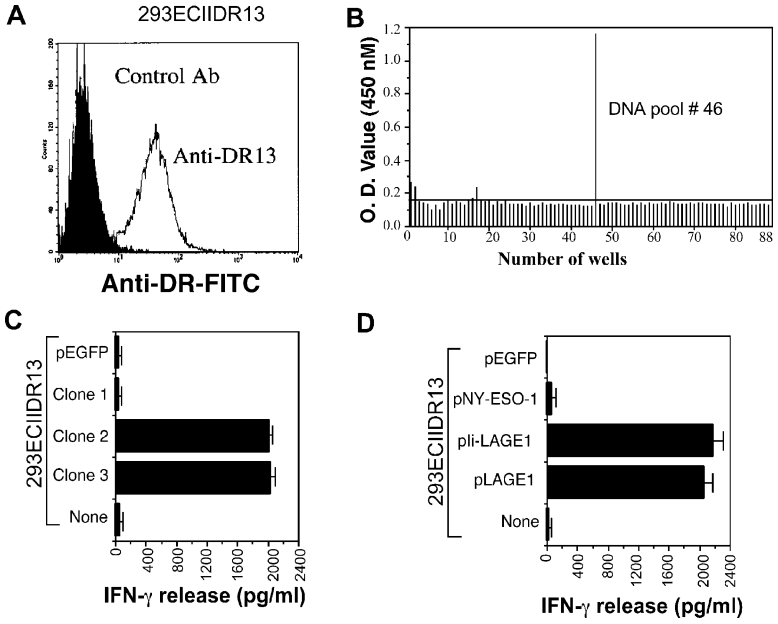

Figure 2. Identification of a cDNA Encoding LAGE1 by Screening an Ii-cDNA Library of M102mel

(A) Establishment of 293ECII cells expressing HLA-DR13 molecules. 293ECIIDR13 cells were stained with anti-HLA-DR13 or control antibodies. Stained cells were analyzed by FACS.

(B) Identification of a positive cDNA pool (no. 46) capable of stimulating increased secretion of IFN-γ from $CD4^+$ T cells. Optical density reading at a 450 nm wavelength revealed a single positive well in one of the 96-well plates.

(C) After rescreening the positive pool, two individual positive cDNA clones were identified on the basis of IFN-γ release from $CD4^+$ TIL102-C4 cells. $CD4^+$ TIL102-C4 cells recognized 293ECIIDR13 cells transfected with cDNA clones 2 and 3, but not with a control cDNA clone 1 or GFP.

(D) Recognition of both Ii fusion LAGE1 and the naive form of LAGE1 by T cells. 293ECIIDR13 cells were transfected with pIi-LAGE1, LAGE1, or NY-ESO-1 cDNA. T cell recognition was determined on the basis of IFN-γ release from $CD4^+$ TIL102-C4 cells.

cDNA library using mRNA isolated from M102 tumor cells. The quality of the cDNA library was evaluated by determining the size and percentage of cDNA inserts. DNA library pools were prepared in 96-well format plates and were transfected into 293ECIIDR13 cells. After screening the Ii-fusion cDNA library, we identified a positive pool that stimulated T cells for increased IFN-γ release (Figure 2B). The positive-pool DNA was then transformed into *E. coli*, and individual colonies were picked for preparation of plasmid DNA. After rescreening of individual plasmid DNAs, we identified single cDNA clones that could stimulate T cells for cytokine release. Figure 2C shows that cDNA clones 2 and 3 were capable of stimulating T cells, while cDNA clone 1 failed to activate T cells for the secretion of IFN-γ. DNA sequence analysis and database searches revealed that both positive cDNA clones encoded the LAGE1 protein, which shares 94% nucleotide sequence identity with NY-ESO-1 (Chen et al., 1997; Wang et al., 1998). LAGE1 was initially suggested as a tumor antigen on the basis of representational difference analysis (Lethe et al., 1998), but MHC class II-restricted T cell epitopes from this 180 amino acid protein have not been reported. Like NY-ESO-1, LAGE1 is expressed in cancer cells and normal testis, but not in other human normal tissues. When tested for their ability to recognize LAGE1 and NY-ESO-1, TIL102-C4 T cells responded to LAGE1-expressing HEK293ECIIDR13 cells, but not those expressing NY-ESO-1 (Figure 2D). Moreover, T cells recognized both the native and Ii-targeted forms of LAGE1, suggesting that the native form of LAGE1 is naturally processed and presented by HLA-DR13 molecules to $CD4^+$ T cells.

Identification of the T Cell Ligand/Epitope from LAGE1

To determine the proportion of T cell clones derived from TIL102 that recognize NY-ESO-1 and/or LAGE1, we transfected LAGE1 and NY-ESO-1 into 293ECIIDR13 cells. We added the T cell clones individually to wells containing 293ECIIDR13 cells transfected with LAGE1 or NY-ESO-1, and to wells seeded with M102 tumor cells. Following overnight incubation, IFN-γ release was determined from cell supernatants. As expected, all 13 of the T cell clones recognized M102 tumor cells, but none recognized 293ECIIDR13 cells transfected with NY-ESO-1 (Figure 3A). By contrast, 10 of the 13 $CD4^+$ T cell clones recognized LAGE1. These studies suggest that while the majority of tumor-reactive T cell clones recognize LAGE1 as the DR-13-restricted antigen, three T cell clones (numbers 3, 8, and 13) may recognize other unidentified tumor antigens presented by DR13 molecules. These data also imply that the ligand for TIL102-C4 T cells resides in a region of LAGE1 with different amino acid sequences from NY-ESO-1.

To identify T cell epitopes from LAGE1, we made two 13-mer peptides based on the DR13 peptide binding motif as well as amino acid differences between LAGE1 and NY-ESO-1, and tested their ability to stimulate T cells. Figure 3B shows that TIL102-C4 T cells recognized 293ECIIDR13 cells pulsed with LAGE-$P_{108\text{-}120}$, but not with a control peptide, LAGE-$P_{125\text{-}137}$, containing the HLA-DR13 peptide binding motif. T cell stimulation was observed at a peptide concentration of 0.1 μM for seven T cell clones tested (Figure 3C). While TIL102-C4 and C7 T cells showed a slightly low activity, other T cell clones exhibited similar activity and affinity for the LAGE-$P_{108\text{-}120}$ peptide.

TCR Usage and Cytokine Profiles of $CD4^+$ T Cell Clones

We next sought to determine whether all LAGE1-specific T cell clones are the same or different clones. Twenty-five pairs of TCR-Vβ-specific primers were synthesized and used for RT-PCR amplification of RNAs isolated from each T cell clone (McKee et al., 2000). We found that TIL102-C1, -C2, -C4, and -C7 T cell clones shared the same TCR-Vβ7 gene, while TIL102-C6 and TIL102-C12 shared the same TCR-Vβ6 gene. TIL102-C9, -C10,

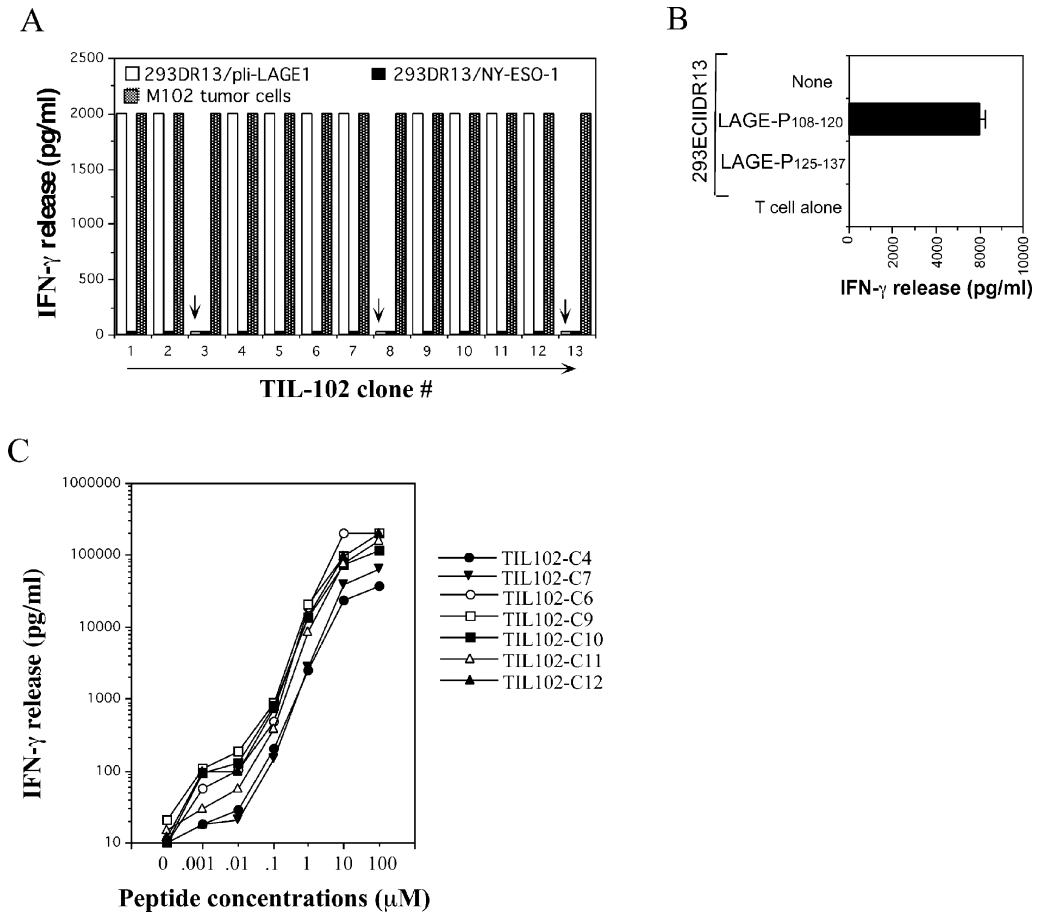

Figure 3. Identification of a T Cell Ligand for CD4$^+$ TIL102 T Cell Clones

(A) T cell recognition of LAGE1 presented by HLA-DR13 molecules. 293ECIIDR13 cells were transfected with LAGE1 or NY-ESO-1 cDNA. Thirteen T cell clones derived from TIL102 bulk T cell line were added to determine their ability to recognize LAGE1 or NY-ESO-1. M102 tumor cells were used as a positive control for T cell recognition. Ten of thirteen T cell clones recognized LAGE1 and tumor cells. Three recognized tumor cells, but not 293ECIIDR13 cells transfected with either LAGE1 or NY-ESO-1.

(B) Identification of a peptide recognized by T cell clones. TIL102-C4 responded to the LAGE-P$_{108-120}$ peptide, but not to the LAGE-P$_{125-137}$ control peptide.

(C) Titration of peptide concentrations for T cell recognition. To determine peptide concentrations required for T cell recognition, 293ECIIDR13 cells were incubated with different concentrations of the LAGE-P$_{108-120}$ peptide for 90 min and then washed three times with T cell assay medium. T cells from seven different T cell clones were added to peptide-pulsed 293ECIIDR13 cells overnight. IFN-γ release from T cells was determined with an ELISA kit. Similar results were obtained in three repeated experiments.

and -C11 T cells may differ in their TCR-Vβ usage because we failed to amplify any distinct band using 25 pairs of primers specific for Vβ genes, while the constant region was successfully amplified (data not shown). TIL102-C5 contained bands of Vβ6 and Vβ7 genes, suggesting that it represents a mixture of two T cell clones. Representative data from three T cell clones are shown in Figure 4A. The results of TCR profiling analysis led us to select TIL102-C4, -C6, and -C10 as representative T cell clones for further analysis.

To evaluate the secretion of IL-2, IL-4, IL-10, IFN-γ, and TGF-β by T cell clones, we measured cytokine release from T cell clones after coculturing with HLA-DR13-positive EBV-B or 293ECIIDR13 cells pulsed with the LAGE1-P$_{108-120}$ peptide for 18 hr. Representative data from TIL102-C4, -C6, and -C10 T cell clones are shown in Figure 4B. TIL102-C4 and -C6 T cells both secreted GM-CSF, IFN-γ, IL-4, and IL-10, but little or no IL-2 or TGF-β, while TIL102-C10 cells secreted GM-CSF, IFN-γ, and IL-10, but little or no IL-2, IL-4, or TGF-β. To compare this profile with results for other CD4$^+$ effector T cells, we generated antigen-specific CD4$^+$ T cell clones (TIL1363-C1 and -C2) from TIL1363 that recognized a fusion tumor antigen, LDFP (Wang et al., 1999b). We found that TIL1363-C1 and -C2 CD4$^+$ T cell clones secreted IL-2, GM-CSF, and IFN-γ, but no other cytokines after stimulation with the 1363mel target cells (Figure 4B). An antigen-unspecific CD4-C5 clone derived from PBMCs of a normal donor did not respond to a tumor mixture of M102 and 1363mel cells, and served as a specificity control.

CD4$^+$ T Cell Clones Phenotypically Resemble CD4$^+$ Treg Cells

The cytokine release profile of TIL102 CD4$^+$ T cell clones suggested that they may represent CD4$^+$ Treg cells

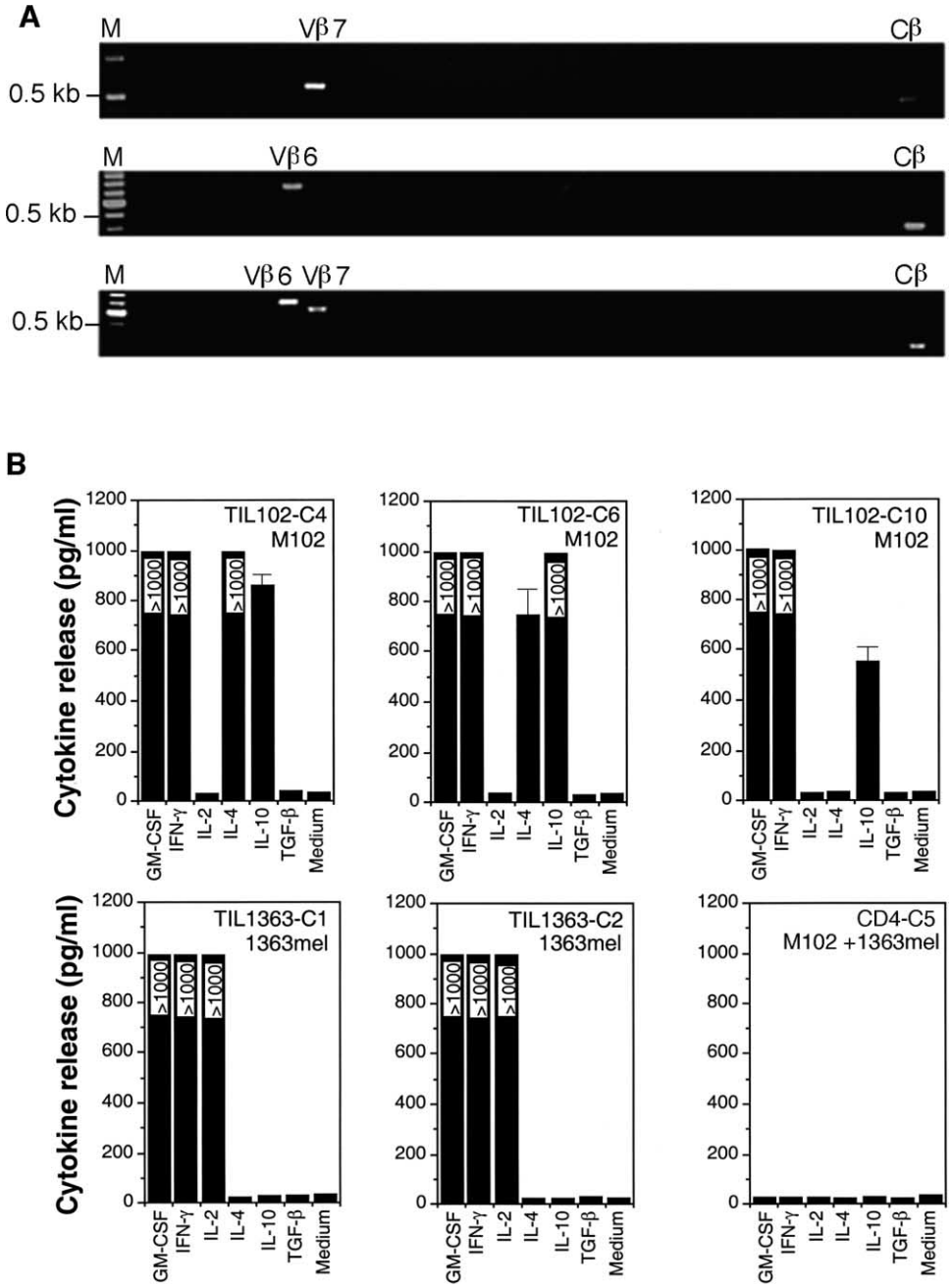

Figure 4. Characterization of Tumor-Reactive CD4$^+$ TIL102 T Cells

(A) Clonality of tumor-reactive TIL102 T cell clones by TCR profiling analysis. Total RNAs were isolated from individual TIL102 T cell clones and analyzed by RT-PCR using 25 pairs of Vβ chain-specific primers. The primers for the constant region Vβ chain were used as positive controls. Specific DNA bands amplified by PCR indicate the usage of TCR Vβ chains by TIL102 T cell clones and the purity of T cell clones. (B) Cytokine profiles of CD4$^+$ TIL102-C4, -C6, and -C10 T cells. GM-CSF, IFN-γ, IL-2, IL-4, IL-10, and TGF-β were determined from cell culture supernatants of T cells after coculturing with their corresponding tumor cells. TIL-1363-C1 and -C2 T cell clones were used as representatives of cytokine profiles of typical CD4$^+$ effector (Th1) T cells. CD4-C5 T cells derived from human PBMCs were not antigen specific and served as a specificity control. Bars with >1000 pg/ml indicate that optical readings at 450 nm were higher than that of the highest level of standards (1000 pg/ml).

rather than CD4$^+$ T helper cells. To test this possibility, we first examined the phenotypes of CD4$^+$ TIL102 clones together with a control CD4$^+$ T cell clone (CD4-C5) by FACS analysis. Figure 5A shows that CD4$^+$ TIL102 clones were positive for CD4$^+$, CD25, and GITR markers more than 1 month after T cell expansion, while the CD4-C5 T control grown in the same condition expressed few if any CD25 or GITR molecules. When intracellular stained T cells with an antibody against CTLA4, all T cell clones were uniformly positive, but no appreciable differences were noted among T cell clones (data not shown). Further testing of the expression levels of CD25

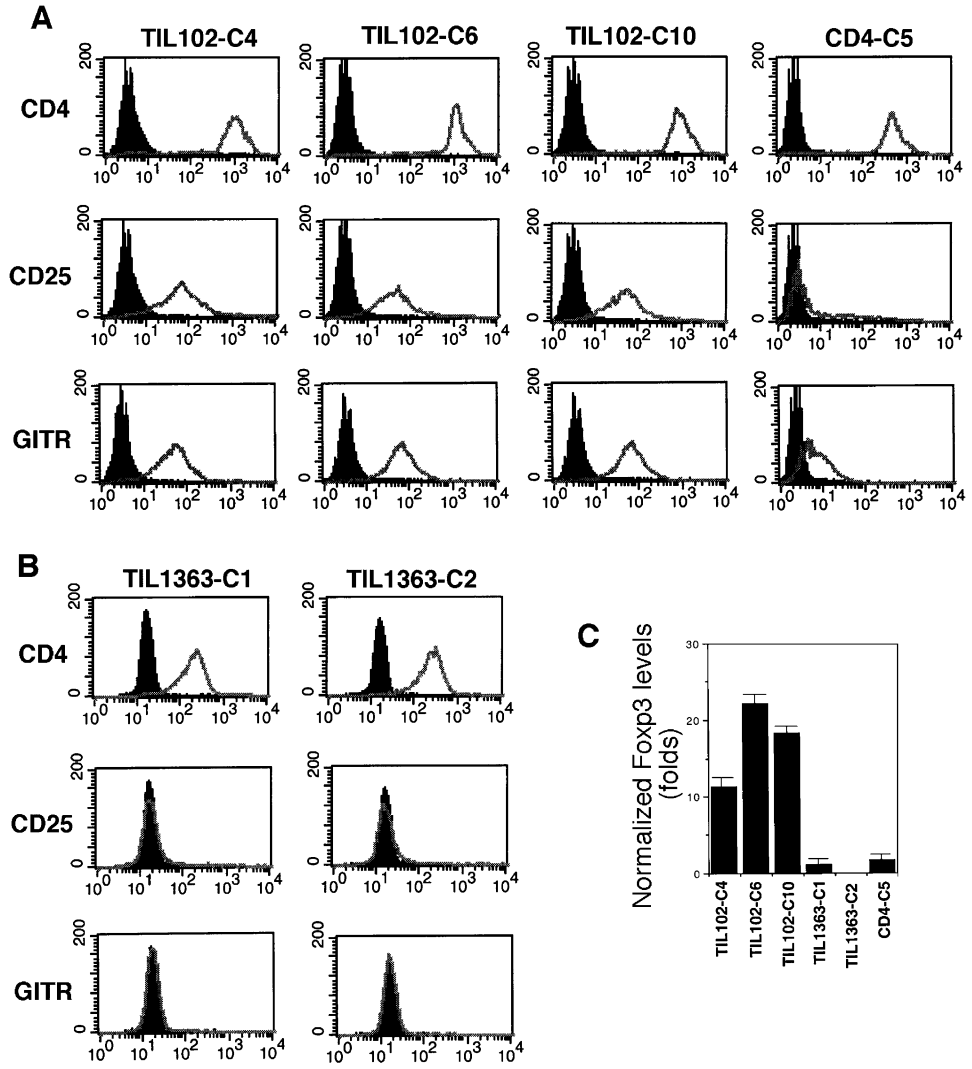

Figure 5. Phenotypic and Foxp3 Expression Analyses of CD4$^+$ TIL102 Treg Cells

(A) CD4$^+$ TIL102 Treg cells and a control CD4$^+$ T cell clones from human PBMCs were stained with phycoerythrin (PE)- or FITC-labeled mAbs to CD4, CD25, and GITR molecules. An isotype antibody served as the control. All three clones of TIL102-C4, -C6, and -C10 Treg cells expressed high levels of CD4, CD25, and GITR molecules, while the control CD4-C5 T cells expressed low levels of CD25 and GITR. All FACS analyses were conducted at more than 1 month after T cell expansion.

(B) Expression of CD4, CD25, and GITR molecules by antigen-specific TIL1363-C1 and -C2 T cells 2 weeks after T cell expansion.

(C) Foxp3 expression by TIL102 Treg cells and antigen-specific effector/control T cells. cDNA from each T cell clone was subjected to real-time quantitative PCR analysis using primers and an internal fluorescent probe for Foxp3 or HPRT (hypoxanthine-guanine phosphoribosyltransferase). The relative quantity of Foxp3 in each T cell sample was normalized to the relative quantity of HPRT. Foxp3 expression levels in TIL102-C4, -C6, and -C10 were much higher than those in TIL1363-C1, -C2, and CD4-C5 T cells. (A) through (C) each represent one of three independent experiments.

and GITR markers in antigen-specific TIL1363-C1 and -C2 T cells demonstrated that like CD4-C5, these CD4$^+$ effector T cells were negative for CD25 and GITR molecules (Figure 5B). All T cell clones were maintained in the same condition and growth medium containing a low level of IL-2 after expansion. Thus, it appears that the CD4$^+$ TIL102-C4, -C6, and -C10 T cells express the markers typically found on naturally occurring CD4$^+$ CD25$^+$ Treg cells (Shevach, 2002; Wood and Sakaguchi, 2003), while TIL1363 CD4$^+$ T cell clones display the markers of T helper cells.

Recently, the forkhead transcription factor Foxp3 was shown to be specifically expressed by CD4$^+$ Treg cells, thus affording a reliable marker for these cells (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Comparison of Foxp3 expression levels among tumor-specific CD4$^+$ TIL102 T cell clones revealed much higher levels of Foxp3 mRNA (10-fold or more) in CD4$^+$ TIL102-C4, -C6, and -C10 T cell clones than in CD4$^+$ TIL1363 and CD4-C5 T cell clones (Figure 5C). These results indicate that these antigen-specific CD4$^+$ TIL102 T cells are CD4$^+$ Treg cells.

$CD4^+$ T Cell Clones Suppress the Proliferative Activity of $CD4^+$ T Cells

Definitive evidence of $CD4^+$ Treg cells ultimately resides in the demonstration of their ability to suppress the proliferation of naive $CD4^+$ T cells in functional assays (von Herrath and Harrison, 2003). Hence, we purified naive $CD4^+$ T cells from human PBMCs as responding T cells and cocultured them with $CD4^+$ TIL102-C4, -C6, -C10, or TIL1363 T cell clones in growth medium containing purified APCs and anti-CD3 antibody. As shown in Figure 6A, the $CD4^+$ TIL102-C4, -C6, and -C10 T cells possessed only scant proliferative activity, whereas naive $CD4^+$ T cells proliferated activity strongly after stimulation with the anti-CD3 antibody. When cocultured with the purified $CD4^+$ T cells, $CD4^+$ TIL102 T cell clones significantly suppressed anti-CD3-induced proliferation in a dose-dependent manner. By contrast, we did not observe any suppressive activity by $CD4^+$ TIL1363 and a control $CD4^+$ T cell clone (Figure 6A), both of which enhanced the proliferative activity of naive $CD4^+$ T cells. Taken together, these results indicate that these tumor-specific $CD4^+$ TIL102 T cell clones can be functionally classified as $CD4^+$ Treg cells.

Suppressive Activity of TIL102 Treg Cells Requires Ligand-Specific Activation

We next addressed the question of whether suppressive effect of $CD4^+$ regulatory TIL102 T cell clones on $CD4^+$ effector TIL1363-C1 requires ligand-specific activation. $CD4^+$ Treg cells (TIL102-C4, -C6, and -C10 T cells) were cocultured with $CD4^+$ effector TIL1363-C1 cells in the presence of the LAGE-$P_{108-120}$ peptide-pulsed 293ECIIDR13 cells. IL-2 secretion from TIL1363-C1 was determined after exposure to 1363mel cells. As a control, we cocultured TIL102 $CD4^+$ Treg cells with $CD4^+$ effector TIL1363-C1 in the presence of the LAGE-$P_{125-137}$ control peptide-pulsed 293ECIIDR13 cells to determine the specificity and requirement for inhibition mediated by TIL102-C4, -C6, and -C10 T cells. As shown in Figure 6B, IL-2 secretion by TIL1363-C1 T cells was not inhibited by $CD4^+$ regulatory TIL102 T cells in the presence of the LAGE-$P_{125-137}$ control peptide. However, the ability of TIL1363-C1 T cells to secrete IL-2 was completely suppressed by $CD4^+$ TIL102-C4, -C6, and -C10 Treg cells activated by the LAGE-$P_{108-120}$ peptide. These data provide compelling evidence that suppressive activity of $CD4^+$ TIL102 Treg cells requires ligand-specific activation. Because HLA-DR1-restricted TIL1363-C1 cells recognized an LDFP antigen on 1363mel cells (Wang et al., 1999b), but not 293ECIIDR13/the LAGE-$P_{108-120}$ peptide, while $CD4^+$ Treg cells recognized 293ECIIDR13/the LAGE-$P_{108-120}$ peptide, but not 1363mel cells, we conclude that recognition of antigens on the same APCs by $CD4^+$ TIL102 Treg cells and effector T cells is not required for $CD4^+$ TIL102 Treg cells to exert its inhibitory effect. Thus, once activated, these $CD4^+$ Treg cells can inhibit other $CD4^+$ effector cells.

To test whether suppressive activity of $CD4^+$ Treg cells could be reversed by exogenous IL-2, we added 600 IU/ml final concentration of IL-2 to the culture medium during the suppressive assay. As shown in Figure 6C, there was no difference in suppressive activity of TIL102-C4 Treg cells in the presence or absence of IL-2, suggesting that exogenous IL-2 could not reverse the suppressive effect of TIL102-C4 Treg cells on the proliferative response of responding $CD4^+$ T cells.

Cell-Cell Contact Is Required for Suppressive Activity by $CD4^+$ Treg Cells

We next tested how $CD4^+$ TIL102 Treg cells inhibit other $CD4^+$ effector T cells. Several mechanisms have been proposed to explain how $CD4^+$ Treg cells inhibit $CD4^+$ effector T cells (Shevach, 2002). Some authors suggest that IL-10 and/or TGF-β are directly involved in T cell-mediated suppression, while others contend that cell-cell contact is required for suppression (Levings et al., 2002a; Shevach, 2002). These discrepancies may reflect the use of different systems or different $CD4^+$ Treg cell populations in these studies. We examined these possibilities by studying tumor-specific $CD4^+$ Treg cells in the presence of various antibodies against human IL-10 or TGF-β molecules. As shown in Figure 7A, neither anti-IL-10 nor anti-TGF-β antibody, nor both molecules together, could block the suppressive effect of $CD4^+$ TIL102-C4 Treg cells on naive $CD4^+$ T cells.

To test whether cell-cell contact is required for $CD4^+$ TIL102-C4 Treg cells to exert their suppressive activity, we performed transwell experiments. We first did coculture experiments using the same Treg cell clones (TIL102-C4, -C6, and -C10 clones) in parallel to make sure that these T cells possessed functional suppressive activity. As shown in Figure 7B, when $CD4^+$ TIL102-C4, -C6, and -C10 Treg cell clones were cultured together (1:1 ratio) with the naive $CD4^+$ T cells in the presence of anti-CD3 and the purified APCs, they inhibited the proliferative activity of the naive $CD4^+$ T cells. CD4-C5 T cells, by contrast, lacked the ability to inhibit the proliferative response of the naive $CD4^+$ T cells to anti-CD3 antibody stimulation, regardless of the coculturing or transwell conditions. However, in transwell experiments, $CD4^+$ TIL102-C4, -C6, and -C10 Treg cells, when cultured in the inner well containing medium with anti-CD3 and the purified APCs, did not proliferate by themselves and did not inhibit the proliferative activity of $CD4^+$ naive T cells cultured in the outer well containing the same medium with anti-CD3 and the purified APCs (Figure 7B). These results demonstrate that cell-cell contact is required for the T cell-mediated suppressive activity of $CD4^+$ Treg cells.

Discussion

In this study we demonstrate that antigen-specific $CD4^+$ Treg cell clones can be established from the TILs of patients with cancer by the limiting dilution method. After expansion in vitro, these T cells maintained their antigen specificity and suppressive function, most likely reflecting their tumor recognition and suppressive properties in vivo. Other studies have also shown increased proportions of tumor-associated $CD4^+$ $CD25^+$ Treg cells in the TILs of patients with cancer (Liyanage et al., 2002; Woo et al., 2002), but they provided no direct evidence of the antigen specificity of these T cells because of their reliance on bulk $CD4^+CD25^+$ T cell populations. A

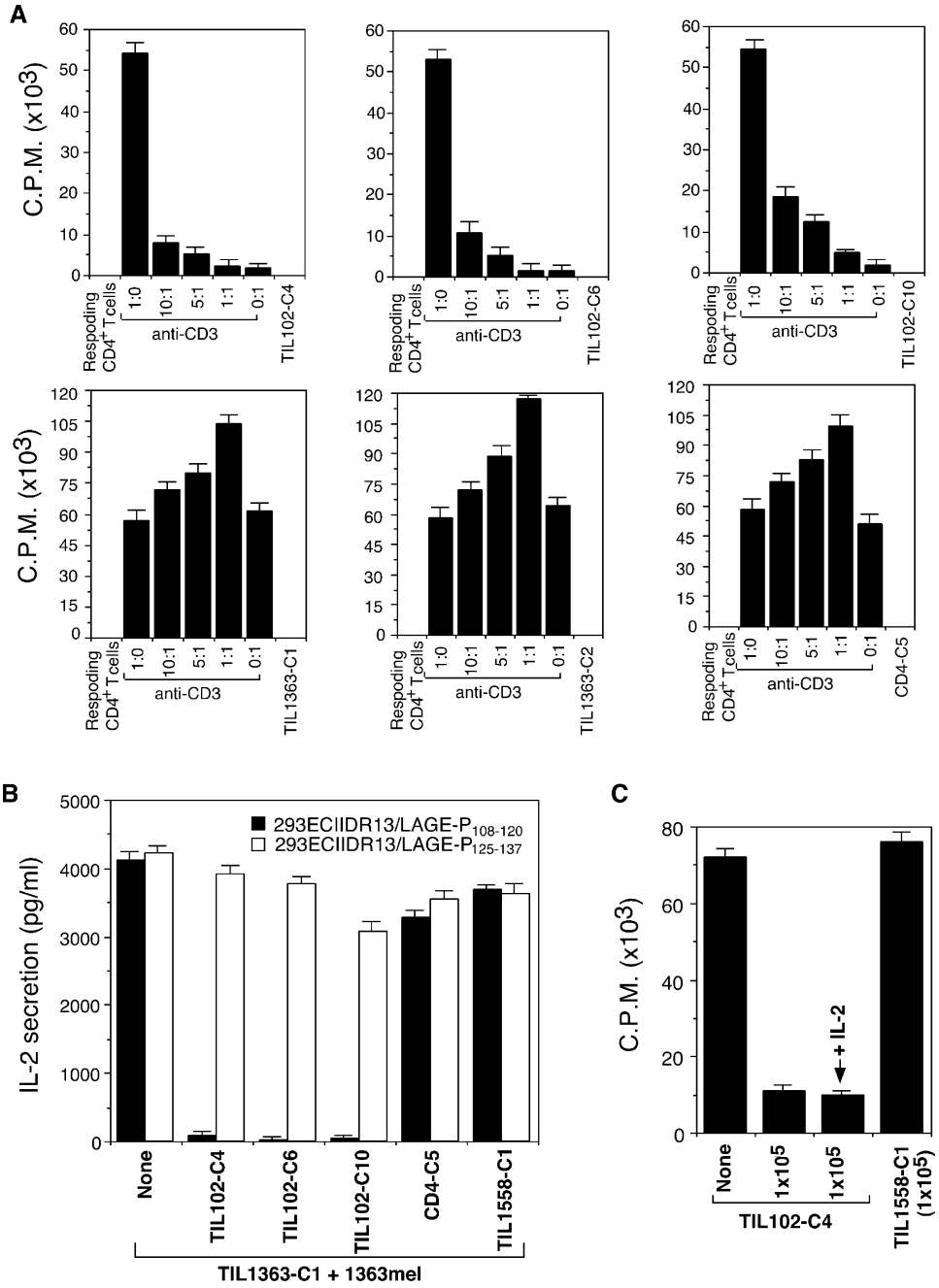

Figure 6. Functional Analysis of $CD4^+$ TIL102 Treg Cells

(A) Suppressive activity of TIL102 Treg cells. The proliferative activity of freshly prepared $CD4^+$ (responding) T cells (1×10^5) was inhibited by different numbers of TIL102-C4, -C6, and -C-10 Treg cells in the presence of anti-CD3 antibody. Proliferation of naive $CD4^+$ T cells was assayed by adding [^{3}H]thymidine during the last 12–16 hr of culture. By contrast, $CD4^+$ TIL1363-C1 and -C2 effector cells and CD4-C5 T cells enhanced rather than suppressed the proliferative activity of responding $CD4^+$ T cells.

(B) Suppression of the ability of antigen-specific $CD4^+$ effector cells to recognize tumor cells by $CD4^+$ TIL102 Treg cell clones. $CD4^+$ TIL1363-C1 T cells were coincubated with TIL102-C4, -C6, and -C10 cells, respectively, in the presence of LAGE-$P_{108-120}$ or LAGE-$P_{125-137}$ control peptides. Inhibition of the ability of TIL1363-C1 effector cells in response to 1363mel cells was determined by measuring IL-2 secretion after 18 hr coculture. TIL102-C4, -C6, and -C10 cells did not affect the ability of TIL1363-C1 to respond to 1363mel cells in the presence of a control peptide. By contrast, these $CD4^+$ Treg cell clones activated by the LAGE-$P_{108-120}$ peptide resulted in complete inhibition of the ability of 1363-C1 effector T cells to secret IL-2. Results in (A) and (B) are representative of three independent experiments.

(C) Exogenous IL-2 could not reverse the suppressive activity of TIL102-C4 Treg cells. Responding $CD4^+$ T cells were cocultured with TIL102-C4 Treg cells (1×10^5) in the presence or absence of exogenous IL-2 (600 IU/ml). Suppressive assays were performed as above. TIL1558-C1 cells served as a control for effector T cells.

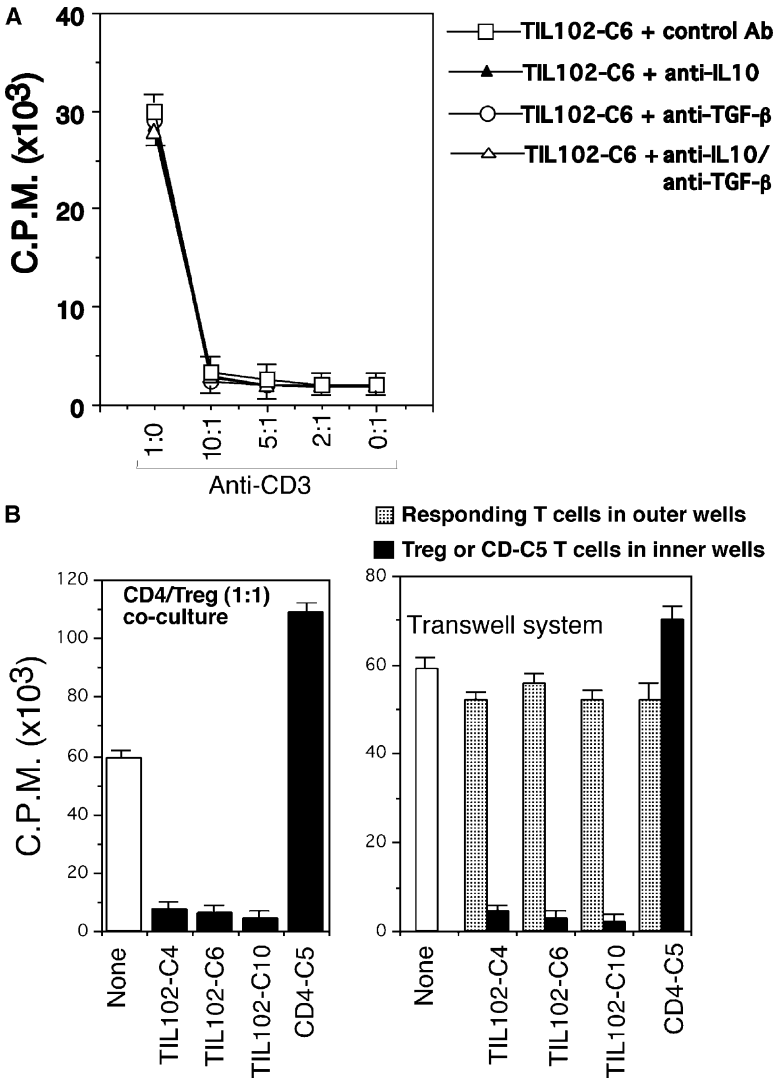

Figure 7. Cell-Cell Contact Is Required for T Cell Suppression by TIL102 Treg Cells

(A) Anti-IL-10, anti-TGF-β, or both antibodies failed to block the suppressive activity of TIL102-C6 Treg cells. The suppression of proliferative activity was conducted in the presence of anti-IL-10, anti-TGF-β, or both antibodies. The culture conditions were identical to that in Figure 6 except for the addition of antibodies to the culture.

(B) Cell-cell contact is required for T cell suppression. Equal numbers of $CD4^+$ responding T cells were cultured in outer wells; TIL102 Treg T cells or CD4-C5 T cells were cultured in inner wells. Otherwise, culture conditions were identical between the inner and outer wells. To ensure that the TIL102 T cells used had suppressive activity, we used the coculture system as our positive control for the suppressive activity of TIL102 Treg cells. Once the cells were separated with the responding $CD4^+$ T cells in the transwell system, there was no detectable suppressive activity of the responding $CD4^+$ T cells in the outer wells, regardless of the presence of TIL102 Treg, CD4-C5 T cells, or none in the inner wells. Results represent one of three independent experiments.

recent report demonstrated the generation of $CD4^+CD25^+$ Treg clones from human PBMCs, but their antigen specificity was not defined (Levings et al., 2002b). Hence, the $CD4^+$ T cell clones presented here are true tumor-specific $CD4^+$ Treg cells.

Tumor antigen-specific regulatory T cell clones that we have isolated possess an unusual phenotype. In many respects they resemble the Tr1 clones described by Roncarolo et al. in that they secrete both IFN-γ and IL-10 (Groux et al., 1997). On the other hand, they suppress activation of naive T cells by a cell contact-dependent mechanism and express FoxP3, thus resembling $CD4^+CD25^+$ naturally occurring suppressor cells (Wood and Sakaguchi, 2003). Another striking feature of these clones is that they maintained high level expression of CD25 and GITR when cultured in medium containing a low level of IL-2, in contrast to effector type clones cultured in the same condition which downregulated expression of these antigens (Figure 5). The biological significance of this observation is not clear, as very little data are available on the regulation of GITR expression on human $CD4^+$ T cells during the course of T cell activation. Furthermore, naturally occurring $CD4^+CD25^+$ cells do not produce effector cytokines during short-term culture in vitro, while all the LAGE1-specific clones produced large amounts of IFN-γ, IL-10, and IL-4 (Figure 4). The cellular origin of these regulatory T cell clones will therefore require further study. It is even possible that these clones were generated from $CD25^-$ T cells in vivo following interaction with naturally occurring $CD4^+CD25^+$ T cells as previously described in vitro (Dieckmann et al., 2002; Jonuleit et al., 2002). Regardless of the origin of these $CD4^+$ Treg cell clones, they functionally suppressed the proliferation and IL-2 secretion of CD4 effector cells (Figure 6).

Very little information is available on the target antigen recognized by naturally occurring $CD4^+CD25^+$ T cells. Most studies in humans and experimental animals have focused on bulk $CD4^+$ Treg cells, which may display very diverse specificity. Our results suggest that it is essential to generate antigen-specific $CD4^+$ Treg clones/lines as the first step for the identification of the target ligands for $CD4^+$ Treg cells. This strategy should be applied to identification of ligands for antigen-specific $CD4^+$ Treg cells derived from autoimmune diseases as well as the infected hosts. Although $CD4^+$ effector T cells

might recognize the same LAGE1 T cell epitope as $CD4^+$ Treg cells, several $CD4^+$ T cell clones we generated were exclusively $CD4^+$ Treg cells. LAGE1 belongs to an expanding list of cancer-testis antigens, which are expressed in cancer cells and normal testis, but not in other normal tissues (Lethe et al., 1998). While T cell epitopes have been identified from the CAMEL (an alternative open reading frame) (Mandic et al., 2003; Slager et al., 2003), $CD4^+$ T cell ligand within the LAGE1 primary open reading frame identified in this study has not been reported. Its identification as a natural ligand for $CD4^+$ Treg cells is of particular interest because many tumor immunologists believe that cancer-testis antigens are ideal targets for cancer immunotherapy. However, our data show that ligand-specific activation of $CD4^+$ Treg cells is required for subsequent suppressive effect on other effector cells (Figure 6B), suggesting that tumor-specific, nonmutated self-antigens such as LAGE1 may stimulate and expand $CD4^+$ Treg cells at the tumor sites, thus inducing immune tolerance.

This prediction appears relevant to the results of recent clinical trials using MHC class I-restricted tumor antigens. Although some evidence for a therapeutic effect on tumor growth was obtained in these studies, immune responses were weak and transient in most vaccinated patients. Among the many factors that may have contributed to this failure, the presence of tumor-specific $CD4^+$ Treg cells at tumor sites could have played a significant role in the suppression of antitumor immunity. Indeed, the removal of $CD4^+CD25^+$ T cells by an anti-CD25 antibody in animal models enhanced antitumor responses (Sakaguchi et al., 2001; Sutmuller et al., 2001). These observations suggest that the success of peptide-based immunotherapy for cancer and autoimmune diseases may ultimately depend on the balance between regulatory and effector T cells (Powrie and Maloy, 2003). New strategies that simultaneously stimulate $CD4^+$ effector T cells while inhibiting or depleting $CD4^+$ Treg cells are therefore needed to shift this dynamic equilibrium toward effector T cells in the treatment of cancer patients. The opposite approach, favoring expansion of the $CD4^+$ Treg population, might improve the treatment of autoimmune diseases.

In summary, we have generated and characterized tumor-specific $CD4^+$ T cell clones derived from the TILs of a melanoma patient. Their phenotypic and functional properties were indicative of antigen-specific $CD4^+$ Treg cells, and they recognized LAGE1 peptide as a natural ligand. Cell-cell contact was required for T cell-mediated immune suppression, but other aspects of the molecular mechanism remain to be defined in future studies. Such information should open opportunities for the manipulation of antigen-specific immune responses directed to cancer as well as autoimmune and infectious diseases.

Experimental Procedures

Cell Lines

$CD4^+$ tumor-infiltrating lymphocytes (TIL102) were cultured from a fresh tumor sample surgically removed from a melanoma patient. All TILs and T cell clones were grown in RPMI 1640 medium containing 10% human AB serum and recombinant IL-2 (300 IU/ml). Melanoma cell lines and Epstein-Barr virus (EBV)-transformed B cell lines were maintained in RPMI 1640 with 10% fetal calf serum (FCS).

T Cell Cloning and Expansion

T cell clones were generated from TIL102 and TIL1359 by limiting dilution methods (at 0.3 cell/well) as previously described (Wang et al., 2002). On day 0, T cells were stimulated in RPMI1640 medium containing anti-CD3 antibody (30 ng/ml) and 5×10^4 allogeneic peripheral blood mononuclear cells (PBMCs) as feeder cells and 10% human AB sera. On day 1, 300 IU/ml of IL-2 was added. Culture medium was changed every 3 days with fresh RPMI 1640 medium containing 10% human AB sera, glutamine, β-mercaptoethnol, and 300 IU/ml of IL-2. On day 14, T cell clones were picked and tested for their activities against M102 tumor cells. T cells in the absence of tumor cells served as a control. To obtain optimal expansion, we used the OKT3 expansion method as previously described (Wang et al., 1999a). T cell clones were maintained at a low IL-2 concentration (30 IU/ml). Melanoma cell lines and EBV-transformed B cell lines used in this study were cultured in RPMI 1640 medium containing 10% FCS. 293ECIIDR13 cells were established by transfecting plasmid DNA encoding DRB1*1301 cDNA into 293ECII cells as described (Wang et al., 1999a) and were selected with RPMI 1640/10% FCS containing Zeocin (250 μg/ml). HLA-DR13-positive cells were sorted by FACS after staining with anti-HLA-DR13-specific antibodies.

cDNA Library Construction

Total RNA was extracted from M102 cells using Trizol reagent (Invitrogen, Inc., San Diego, CA). Poly(A) RNA was purified from total RNA by the polyATract system (Promega, Madison, WI) and converted to cDNA using a cDNA construction kit (GIBCO BRL) with an oligo-dT primer. The cDNA inserts were then ligated to a pTSX vector containing an Ii fragment (amino acids 1–80) (Wang et al., 1999a), and cDNA libraries were electroporated into DH10B cells. Plasmid DNA for cDNA library pools was prepared from bacteria, each consisting of approximately 100 cDNA clones.

Screening of cDNA Library

DNA transfection and GM-CSF assays were performed as previously described (Wang et al., 1999a). In brief, 200 ng of cDNA pools were mixed with 2 μl of Lipofectamine in 100 μl of serum-free DMEM for 15–45 min. The DNA/Lipofectamine mixture was then added to the 293ECIIDR13 cells (5×10^4) and incubated overnight. The following day, cells were washed twice with AIM-V medium. $CD4^+$ T cells were then added at a concentration of 5×10^4 cells/well in AIM-V medium containing 120 IU/ml of IL-2. After 18–24 hr of incubation, 100 μl of supernatant was collected and IFN-γ concentrations were measured in a standard ELISA assay.

Peptides Synthesis and T Cell Epitopes

The peptides were synthesized by a solid-phase method using a peptide synthesizer (Model AMS 422, Gilson Co., Inc., Worthington, OH). Some peptides were purified by HPLC and had greater than 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 (Wang et al., 1999a).

FACS Analysis

Expression of GITR was determined after staining T cells with an anti-GITR antibody (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 weeks before FACS analysis. Analysis of the expression of intracellular CTLA-4 was performed as previously described (Shimizu et al., 2002). To determine the expression of CD4, CD25, CCR5, CCR7, and CD45RA, we stained T cells with the respective antibodies (BD Biosciences) conjugated to either PE or FITC. After washing, cells were analyzed by FACScan.

Antibody Blocking and Cytokine Release Assays

To determine whether T cell recognition could be blocked by specific antibodies, we measured T cell activity in the absence or presence of various antibodies, as previously described (Wang et al., 1999a). These antibodies, 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. 2×10^4 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 antibody (200 μg/ ml) for 30 min. 2×10^4 T cells in 100 μl 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, Rockford, IL).

DNA transfection and cytokine release assays were performed as previously described (Wang et al., 1999a). After transfection of HEK293ECIIDR13 cells with NY-ESO-1 or LAGE1 cDNA, $CD4^+$ T cells were added at a concentration of 5×10^4 cells/well in RPMI 1640 medium containing 10% human serum and 12 IU/ml of IL-2. After 18–24 hr of incubation, 50 μl of supernatant was collected, and the concentration of IL-2, IL-4, IL-10, TGF-β, and IFN-γ was measured in a standard ELISA assay. For tests of peptide recognition, 293ECIIDR13 cells or HLA-DR13-positive EBV B cells were incubated with peptides at 37°C for 90 min, and then washed three times with RPMI 1640 medium containing 10% human serum and 12 IU/ml of IL-2. T cells were added and incubated for an additional 18–24 hr before cytokine release from T cells was determined.

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. $CD4^+$ TIL102-C4, -C6, or -C10 Treg cells were cocultured (1:1 ratio) with TIL1363-C1 CD4 T helper cells, respectively, in the presence of 293ECIIDR13 pulsed with LAGE-$P_{108\text{-}120}$ or a control LAGE-$P_{125\text{-}137}$ peptides. IL-2 secretion in the culture supernatants was determined by ELISA after 18 hr incubation of the mixture of T cells (Treg and TIL1363-C1) with 1363mel cells. CD4-C5 T cells were used a control for TIL102-C4, -C6, and -C10 Treg cells.

Real-Time Quantitative PCR Analysis

Total RNA was extracted from 1×10^7 T cells using Trizol reagent (Invitrogen, Inc., San Diego, CA). A SuperScript II RT kit (Invitrogen, Inc.) was used in reverse transcription. Twenty microliters reverse transcription mixture contained 2 μg of total RNA and was incubated at 42°C for 1 hr. Foxp3 mRNA levels were quantified by real-time PCR using ABI/PRISM7000 sequence detection system (PE Applied Biosystems, Foster City, CA). PCR reaction was performed using primers; an internal fluorescent TaqMan probe specific to Foxp3 or HPRT was 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.

Proliferation Assays

$CD4^+CD25^-$ T cells (2×10^5) purified from human PBMCs by antibody-coated beads (Dynal, Inc.) were cultured for 60 hr in U-bottomed 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 [^{3}H]thymidine for the last 16 hr of culture, as previously described (Levings et al., 2002b). Cells were harvested and the radioactivity counted in a scintillation counter. For some experiment, antibodies against IL-10, TGF-β, and anti-GITR (R&D Systems, Minneapolis, MN) were added in the assay at a final concentration of 10 μg/ml. All experiments were performed in triplicate.

Transwell experiments were performed in 24-well plates with pore size 0.4 μm (Corning Costar, Cambridge, MA). 2×10^5 freshly purified naive $CD4^+$ T cells were cultured in the outer wells of 24-well plates in medium containing 0.5 μg/ml anti-CD3 antibody and 2×10^5 APCs. Equal numbers of regulatory T cells or nonregulatory CD4-C5 cells were added into the inner wells in the same medium containing 0.5 μg/ml anti-CD3 antibody and 2×10^5 APCs. After 56 hr culture, the cells in the outer and inner wells were harvested separately and transferred to 96-well plates. [^{3}H]thymidine was added, and the cells were cultured for an additional 16 hr before being harvested for counting the radioactivity with a liquid scintillation counter.

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