The Wilms’ Tumor Antigen Is a Novel Target for Human CD4+ Regulatory T Cells: Implications for Immunotherapy


Introduction

The Wilms’ tumor (WT1) gene exerts an oncogenic function in various types of leukemias (1). It is also overexpressed in several solid tumors (2), and therefore, it has been considered as an attractive target for cancer immunotherapy. Specific anti-WT1 immune responses have been described in which CD8+ cytotoxic T cells (3, 4) have been generated in vitro. However, a recent study showed that such response can be largely affected by the presence of CD4+CD25+ regulatory T cells (Treg) in which depletion of this T-cell population was necessary for the generation of an effective WT1-specific cytotoxic response (3).

It has been shown recently that Tregs directly suppress the antitumor immune responses in cancer patients (5, 6), and depletion of this T-cell population resulted in an enhancement of vaccine-mediated antitumor immunity in cancer patients (7). This highlights the role of Tregs in modulating both natural and adoptive immune responses in cancer patients. Tregs may directly modulate the CD8+ T-cell response (8) or alternatively promote tolerization of CD8+ T cells by preventing the licensing of antigen-presenting cells (APC) by CD4+ T helper cells (9). It has become clear that CD8+ T cells generated in the absence of CD4+ T cells help may have a normal primary response; however, their cytotoxic memory response is severely weakened (10). A recent study by Greiner and colleagues (11) investigated the influence of the expression levels of several leukemia-associated antigens (LAA) on the clinical outcome of patients with acute myeloid leukemia (AML). High expression of three LAAs, which was found to be associated with favorable clinical outcome, induced strong CD8+ T-cell responses. However, there was no correlation with the clinical outcome nor induction of natural detectable anti-WT1 CD8+ T-cell responses in these patients (11). In line with this, a recent study has shown the existence of tumor-specific Tregs, which actively suppress antigen-specific antitumor immunity in cancer patients (12). Furthermore, fully functional Treg specific for LAGE1 (13) and ARTC1 (14) were shown in melanoma patients. Another study by Nadal and colleagues (15) suggested that Tregs exert an inhibitory effect on graft versus leukemia and this was associated with relapse after allogeneic stem cell transplantation.

To this end, we asked whether an anti-WT1 Treg population exist in leukemia patients, which may contribute to the impairment of anti-WT1 responses. We have identified a human HLA-DRB1*0402–restricted CD4+ Treg population and showed that the WT1 is a novel target for leukemia-specific CD4+ Treg.

Materials and Methods

Patients and donors. Peripheral blood mononuclear cells (PBMC) from leukemia patients and healthy donors were isolated by density gradient centrifugation. This study was conducted in accordance with Helsinki Declaration and all patients and donors signed a consent form approved by the Research Ethics Committee of King Faisal Specialist Hospital and Research Center (KFSH&RC). The study was approved by the KFSH&RC Research Advisory Council (RAC#2030006). Patient and donor information is listed in Supplementary Data 1.
Peptides. A pool of 110 peptides derived from the WT1 protein designated as WT1-Pepmix and a microscale WT1 peptide set containing each peptide in a single well were obtained from JPT Peptide Technologies (Jerini AG, GmbH; Supplementary Data 2). The WT1-333-347 (RYPFKSLHQLMH5SRKH), WT1-84, HPV37-38 (ASDLRTIQQLLMGT) HLA-D8R0402–restricted peptide (16), and the WT1-dh126 (RMFPNAPYL) HLA-A0201–restricted peptide (4) were synthesized and high-performance liquid chromatography purified to ≥98% purity by Alta Bioscience.

Cell lines. B-lymphoblastoid cell lines (LCL) were established by transformation of B cells using EBV using standard techniques (17). K562 and T2 cell lines were purchased from the American Type Culture Collection. All cell lines were maintained in RPMI 1640 (Sigma) supplemented with 10% FCS (Cambrex Bio Science), 2 mmol/L L-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin (Sigma).

Generation of autologous monocyte-derived dendritic cells. CD14+ cells were isolated from PBMCs of three healthy donors, designated as BC-29, BC-52, and BC-62, using MACS Monocytes Isolation kit (Miltenyi Biotec) according to the manufacturer's instructions. CD14+ cells were adhered and cultured in 24-well plates in X-VIVO 15 medium supplemented with 2 mmol/L 1-glutamine [dendritic cell (DC) medium] in the presence of 30 ng/mL recombinant human interleukin-4 (rIL-4) and 100 ng/mL recombinant human granulocyte macrophage colony-stimulating factor (GM-CSF; R&D Systems). Recombinant human tumor necrosis factor-α (20 ng/mL; R&D Systems), 10 ng/mL rIL-1β (eBioscience), and 25 μg/mL polynosinic acid-polycytidine (Sigma) were added on day 0 and mature DCs were harvested on day 8.

Generation of autologous WT1-specific Treg clones and clones. Irradiated DCs (3,000 rads) were pulsed with 10 μg/mL WT1-Pepmix in DC medium and incubated for 4 h at 37°C. 5% CO2. DCs were cocultured with autologous PBMCs at a 10:1 T-cell to DC ratio. DC medium containing 10 ng/mL rIL-7, 20 pg/mL rIL-12 (R&D Systems), and 10% heat-inactivated human serum (Sigma) was used. T cells were restimulated at weekly intervals and 260 IU/mL rIL-2 was added 2 d after restimulation. Three of three anti-WT1-Pepmix-specific T-cell lines were obtained after the fourth restimulation. Anti–WT1-Pepmix T-cell clones were generated as described before (18). T cells were screened for their peptide specificity against the microscale WT1 peptide set.

Proliferation and 3H-Thymidine cytotoxicity assay. T-cell proliferation was assessed by 3H-Thymidine incorporation as described before (18). Briefly, T cells were cocultured with X-irradiated PBMCs, LCLs (Wallac). For cDNA synthesis, 1 μg total RNA was reverse transcribed using the human IFN-γ, IL-4, IL-5, and GM-CSF ELISA kits (Mabtech) and transforming growth factor (TGF)-β1 and TGF-β2 matched antibody pairs (R&D Systems) was performed according to the manufacturer's instructions.

Flow cytometry. T cells were phenotypically analyzed using the human Treg staining kit (eBioscience) according to the manufacturer's instructions and also stained with CTLA-4-PE (Dako Corp.), CD127-RPE, and GFP-APC (eBioscience). The T-cell receptor (TCR) Vβ profile was determined using the I0Test Beta Mark, TCR Vβ Repertoire kit (Beckman Coulter). Cells were analyzed using FACScan (Becton Dickinson).

Immunostaining. Immunostaining on cytopsins was carried out as described previously (19). Anti-Foxp3 antibody (eBioscience), anti-CD3 antibody (Dako), and anti-WT1 antibody (Dako) were used. Isotype-matched controls for all antibodies were used. The staining was evaluated by two independent scientists.

Reverse transcription-PCR for Foxp3 expression. Total RNA was isolated using RNeasy Micro kit (Qiagen). The forward primer CAGCTGCCCACACTGCGCTTAG and the reverse primer CAGTGCATTATTTCCAGCAG were used for Foxp3. The PCR conditions were 95°C/3 min, 35 cycles of 95°C/1 min, 67°C/40 s, and 72°C/1 min. The β-actin forward primer ATCGTCGACCACTCTTCTCATTGACGTC and the reverse primer GCTGATACCTGCCTGGTGTACCTGACTTGC were used as a control. PCR products were separated by electrophoresis on a 1% agarose gel (Sigma).

Quantitative real-time reverse transcription-PCR for WT1 expression. Total RNA was isolated using TRIzol (Invitrogen). For cDNA synthesis, 1 μg RNA using oligo(dT) primers and Moloney murine leukemia virus reverse transcriptase (Invitrogen) was used in a total volume of 25 μL. The WT1 mRNA expression was quantified using LightCycler FastStart DNA Master SYBR Green 1 kit (Roche) in a LightCycler (Roche). Porphobilinogen deaminase (PBGD) was used as housekeeping gene. cDNA (2 μL) from K562 was used to generate standard curves (20). Amplification was performed in a total volume of 20 μL for 40 cycles/10 s at 94°C, 4 s/64°C, and 35 s/72°C. The forward primer TTATCACAAGGAGGCAGCAGG and the reverse primer GTGCGAGGCGCTGTTGTA were used for WT1. For PBGD, the forward primer CATGTCTGGTAACGGCCAATG and the reverse primer TCTTCTCCAGGGCATGGTCAA were used.

Treg suppression assays and Transwell experiments. To examine the suppressive effect of the Treg clones on the induction of T helper alloresponses, a standard mixed lymphocyte reaction (MLR) was carried out as described before (21). Briefly, fresh BC-29 PBMCs (105 per well, responders) were cultured in DC medium containing 10% human serum for 5 d with 1 × 10^5 DCs (10% human serum) and with different concentrations of Treg clones + autologous LCL ± WT1-84 peptide. The proliferative activity was determined as before. For some experiments, neutralizing antibodies against TGF-β1/2, IL-4, IL-5 (R&D Systems), IL-10 (Bio source), and GM-CSF (Biolegend) were added in the assay at an optimum final concentration of 5 μg/mL. The effect of Treg inhibition on nonirradiated autologous LCL was also examined. Transwell experiments were performed in 24-well plates with pore size of 0.4 μm (Corning Costar). BC-29 PBMCs (0.75 × 10^5 per well, responders) were cultured in the outer wells of 24-well plates in DC medium containing 10% human serum and XR, 3,000 rads allogeneic (BC-8) PBMCs (1.5 × 10^5 per well, stimulators). Treg clones (0.15 × 10^6 cells per well) were added into the inner wells of autologous LCL ± WT1-84 peptide. After 4 d in culture, the cells in the outer wells were harvested and transferred to 96-well plates and the proliferative activity was determined as before.

The suppressive effect of Treg clones on the induction of alloreactive and anti-WT1-specific cytotoxic responses was examined. For alloreactive CTLS, BC-29 PBMCs (2.5 × 10^5 per well, responders) were cultured in DC medium containing 10% human serum for 7 d with XR, 7,500 rads allogeneic LCL (0.5 × 10^6 cells per well, stimulators) in 24-well plates. Cells were tested with well TCC 29B4.42, T2 clone + WT1-84 peptide. This procedure was used for the generation of anti-WT1 CTLS, except that autologous DC pulsed with the WT1-126 (RMFPNPAPYL) HLA-A0201–restricted peptide (4) was used. IL-2 (25 units/mL) was added on day 4 and a similar stimulation was repeated after 7 d. Several stimulations were carried out without addition of the Treg every 7 d. Flow cytometry analysis was used to determine the CD8, CD56, and CD8/CD56 cell population in the culture. CD8 T cells were purified using MACS human CD8-negative selection kit (Miltenyi Biotec). Cytotoxic and IFN-γ production activity were determined by 51Cr release and enzyme-linked immunospot (ELISPOT) assays, respectively.

ELISPOT assay. ELISPOT assay was performed using IFN-γ, granzyme B, and perforin kits (Mabtech). Autologous LCLs or AML cells were used as stimulators. Treg clones (10^5 cells per well) and stimulators (2 × 10^5 cells per well) were seeded in Multiscreen 96-well plates (Millipore) precoated with catching antibody. After 40 h of incubation, cell harvest was performed, and spots were counted using an automated ELISPOT reader (AID). Antigen-specific T-cell frequencies were considered to be increased when they were at least 2-fold higher than in the control wells.

Apoptosis assay. An apoptosis assay was carried out using Vybrant Apoptosis Assay Kit #2 (Invitrogen). Treg clones (2 × 10^5) and target cells (2 × 10^5) were incubated in DC medium + 10% human serum for 24 h. Targets were
gated after staining with monoclonal antibodies (mAb) to CD19-PE, CD40-PE, and CD33-PE (BD Biosciences) for LCL, DCs, and AML, respectively. Apoptosis was measured by flow cytometry assessment of phosphatidylserine externalization cells stained with Alexa Fluor 488 Annexin V in combination with propidium iodide.

Intracellular cytokine staining and determination of anti–WT1-84/Foxp3+ Treg frequencies in AML patients. PBMCs (2 × 10^6/mL) were cultured in DC medium + 10% human serum + 125 units IL-2 for 48 h with WT1-84 peptide. Brefeldin A (eBioscience) was added at 5 μg/mL in the last 12 h of culture. Cells were surface stained with anti-CD4 antibody (BD Biosciences) followed by fixation/permeabilization using eBioscience buffer. Cells were intracellularly stained with anti–granzyme B, anti–IL-5 antibodies (BD Biosciences), and anti–Foxp3 antibody (eBioscience) and analyzed using FACSscan as above.

Results

Generation of T-cell lines and clones against the WT1-Pepmix and analysis of their HLA restriction. PBMCs from three healthy individuals, BC-29, BC-52, and BC-62 (Supplementary Data 1), sharing the HLA-DRB1*0402 molecule, were stimulated repeatedly with autologous DCs pulsed with WT1-Pepmix. Corresponding T-cell lines designated TCL 29, TCL 52, and TCL 62 with specific proliferative activity against autologous WT1-Pepmix–pulsed LCLs were generated (Fig. 1A). The TCL 29 line, which gave high proliferative activity, was cloned by limiting dilution, and 48 clones were obtained and screened by proliferation assay against autologous LCL ± WT1-Pepmix. Eight clones showed specific proliferative activity against the WT1-pepmix. Four clones (TCC 29.B.9, TCC 29.B.16, TCC 29.B.19, and TCC 29.B.42), which showed the highest and most stable proliferative activity (Fig. 1A), were selected and expanded for further analysis. TCL 29 was then screened by proliferation assay against the individual 110 WT1-Pepmix peptides. A peptide designated WT1-84 with the WT1 333-347 RYFKLHQLQHRSRKH sequence induced the strongest specific proliferative activity of TCL 29 (Fig. 1B). Interestingly, both TCL 52 and TCL 62 lines also showed specific proliferative activity against the WT1-84 peptide (data not shown). The rest of the peptides did not induce significant proliferative activity, indicating the
immunodominance of this epitope. All clones had specific proliferative activity to the WT1-84 peptide and a dose-dependent response was shown in all clones with the optimal concentration at 40 μmol/L (data not shown). The HLA restriction of the clones was determined in which anti–HLA-DR mAb was found to significantly inhibit their proliferative activity, whereas their corresponding isotypes had no inhibition effect (Fig. 1C). To define more precisely the HLA-DR restriction, two HLA-DR–matched allogeneic LCLs sharing the DRB1*0402 (LCL-19) or DRB1*0701 (LCL-18) with the TCC 29.B.42 clone were used. LCL-18 failed to present the WT1-84 peptide, ruling out the involvement of the DRB1*0701 in this presentation. However, LCL-19 sharing the DRB1*0402 with the clone induced a specific proliferative activity against the WT1-84 peptide, ruling out the involvement of the DRB1*0701 in this presentation. However, LCL-19 sharing the DRB1*0402 with the clone induced a specific proliferative activity against the WT1-84 peptide, showing the requirement of DRB1*0402 restriction in this process (Fig. 1D). No proliferative response was recorded when LCL pulsed with a negative control HPV33-87, HLA-DRB1*0402–restricted peptide was used (data not shown). All clones were CD4+ (data not shown). Because CD4+ T cells can exert a cytotoxic effect (22), we used 51Cr release assay to examine cytotoxic effect against autologous LCL ± WT1-84. No lytic activity was detected (data not shown), ruling out this possibility.

Evaluation of the cytokine profile of the T-cell clones. Cytokines generated during an immune response dictate the outcome of this response. Hence, we evaluated the cytokine profile generated by the anti–WT1-84 clones. Various cytokines released were determined from cell culture supernatants of T cells cultured for 48 h with irradiated autologous PBMCs or LCL ± WT1-84 or ± WT1-Pepmix. All clones secreted very high amounts of IL-5 and GM-CSF, and high IL-4, specifically to WT1-84 and WT1-Pepmix, but little or no IL-10, TGF-β1/β2, or IFN-γ (Supplementary Data 3). This clearly shows a T helper 2 (Th2)-polarized immune response.

Characterization of the phenotypic profile and TCR usage of the T-cell clones. The cytokine profile of the clones suggested that they represent a Th2 phenotype. It has been recently shown (23) that human Th2 cells exert a Treg phenotype in which a generated Th2 clone also expressed Foxp3, a specific marker for Treg lineages (24, 25). Foxp3 is also expressed transiently in activated non-Tregs (26). Therefore, we tested whether TCL 29 and clones express Foxp3 after long-term culture. Figure 2 shows the presence of Foxp3 mRNA in TCL 29 and two other clones. Because Treg phenotype after more than 1 mo in culture.
woman as a positive control. Foxp3 expression was further confirmed at the protein level (Fig. 2B). We further confirmed the protein expression in these clones by fluorescence-activated cell sorting (FACS) analysis and found to exert a CD4+CD25+Foxp3+ Treg phenotype (Fig. 2C). Finally, we examined the clones for the expression of other Treg-related markers. GITR (28, 29) and CTLA-4 (30) molecules are constitutively expressed at high levels in Tregs. All clones expressed GITR but were negative for CTLA-4 and CD127, which has been recently used to discriminate between human regulatory CD127+ and activated T cells (Fig. 2D; ref. 31).

Altogether, these data indicate that the current anti-WT1 clones are phenotypically Tregs.

The TCR profile of the generated T-cell line and clones was determined using a TCR Vβ kit, which is used to detect the 24 most common human TCR Vβ molecules. All clones shared the same TCR Vβ8 chain (Supplementary Data 4A). We also determined the frequency of TCR Vβ8 in TCI 29 and PBMC-29 from the same donor. The TCR Vβ profile of PBMC-29 scattered between 1% and 8% in which 5% of T cells were TCR Vβ8 (Supplementary Data 4B). The frequency of other two T-cell populations with unknown

Figure 3. Expression of the WT1 antigen by leukemic cells and proliferative activity of the anti-WT1 Treg to different HLA-DR4–matched leukemias. A, cytospin immunocytochemical staining of three representative PBMCs from AML patients (AML-25, AML-27, and AML-28) showing the nuclear expression of the WT1 protein in the leukemic blasts (red). The K562 cell line was used as a positive control, and LCL-29 and PBMC-29, from which the TCL 29 and clones were generated, were used as negative controls. Cells were counterstained in instant hematoxylin. Magnification, ×520. B, proliferative activity of a representative T-cell clone (TCC 29.B.42) against the HLA-DR04 suballelic positive LCLs (LCL-06, LCL-11, LCL-10, LCL-25, and LCL-38) in the absence (gray columns) or presence (black columns) of the WT1-84 peptide. C, proliferative activity of the TCC 29.B.42 Treg clone against PBMCs from DR0402-matched (AML-13 and AML-23), nonmatched (AML-27 and AML-28), and HLA-DR04 suballelic positive AML (AML-21, AML-25, and AML-29) patients. T cells (5 × 10⁴ per well) were challenged for 72 h with irradiated (3,000 rads, XR) PBMCs (10⁵ per well) from AML patients, incubated for additional 18 h with [³H]thymidine, and harvested. T cells challenged with irradiated autologous PBMC-29 in the absence or presence of the WT1-84 peptide served as negative and positive control, respectively.
specificities was increased (V_{h2}, 8–21%; V_{h3}, 7–12%) after seven rounds of stimulation (Supplementary Data 4C). Interestingly, the TCR V_{h8} T-cell frequency was enriched to 40% in TCL 29 after seven rounds of stimulation, indicating the immunodominance of anti–WT1-84 TCR V_{h8} T-cell population in TCL 29.

Evaluation of WT1 expression and proliferative activity of anti-WT1 Tregs against DR4-matched healthy and leukemic cells. We examined the expression of the WT1 mRNA in fresh AML cells using quantitative reverse transcription-PCR (RT-PCR). WT1 protein expression was also evaluated by immunostaining and scored by two independent scientists. K562 cells, known to express high levels of WT1 (20), were used as a positive control. PBMCs and LCLs from normal donors served as negative controls. Higher levels of WT1 expression were recorded in AML samples (Supplementary Data 5). Figure 3A shows immunostaining of PBMCs from three AML patients indicating different levels of WT1 expression.

Because HLA-DR was the restriction element for the clones (Fig. 1C), it was important to evaluate its expression in the AML samples. All AML samples expressed HLA-DR, although at different levels (Supplementary Data 5). The proliferative activity of a selective clone was then measured against PBMCs from two DR0402-matched (AML-13 and AML-23) and two non-DR0402 (AML-27 and AML-28) AML patients. Specific proliferative activity was recorded for the clone against AML-13 and AML-23 cells expressing the DR0402 molecule (Fig. 3B). However, this clone failed to recognize non-DR0402 AML-27 and AML-28 cells, although they express high levels of WT1 (Fig. 3C). Percentages of leukemic blasts used in this study are shown in Supplementary Data 4, column 2. T cells challenged with PBMC-29 ± WT1-84

### Figure 4.

**A.** top, the proliferative activity of allo-MLR was strongly suppressed by a representative clone TCC 29.B.42 at different clone to allo-PBMCs ratios in which specific activation of the clones with WT1-84 peptide was required for such suppressive activity; middle, Transwell experiments showing that Tregs cultured in inner wells were able to significantly suppress the proliferative activity of the allo-MLR cells cultured in the outer wells (ratio was at 1:5); bottom, blocking antibodies to TGF-β1/2, IL-4, IL-5, IL-10, or GM-CSF did not restore the allo-MLR (ratio was at 1:20). **B.** top, the suppressive effect of the TCC 29.B.42 Treg clone on the expansion of alloreactive cytotoxic lymphocytes (inhibition effect of the Tregs on the expansion of NK (CD56), NK T (CD8/CD56), and CD8 cells after second and fifth stimulation; middle, the cytotoxic activity of purified CD8+ alloreactive T lymphocytes generated in the presence or absence of Tregs and tested against allogeneic LCL-5 and K562 cells; bottom, the cytotoxic activity of mixed alloreactive T lymphocytes generated in the presence or absence of Tregs and tested against allogeneic LCL-5 and K562 cells. **C.** top, the suppressive effect of the TCC 29.B.42 Treg clone on the induction of anti–WT1-126 IFN-γ–producing T lymphocytes. CTLs were challenged with irradiated T2 cells in the absence (TC[T2]) or presence (TC[T2+126 peptide]) of peptide. T cells only (TC) were used as negative control.
Evaluation of the inhibition effect of the Treg clones on alloreactive T helper/cytotoxic lymphocytes and induction of anti-WT1 CTL responses. To evaluate whether the Treg clones exert a suppressor function on alloreactive T helper lymphocytes, we used an allo-MLR (32) as a functional readout. All clones significantly suppressed the proliferative activity of the allo-MLR and showed a strong inhibition effect even at 1:100 T-cell to allo-PBMC ratio (Fig. 4A, top). Specific activation of the clones with WT1-84 peptide was required for such suppressive activity, as stimulation with LCL alone did not have a significant effect. To test whether cell-cell contact was required for such inhibition, we carried out experiments in Transwell plates. All clones cultured in inner wells were able to significantly suppress the proliferative activity of the allo-MLR cells cultured in the outer wells (Fig. 4A, middle), showing that cell-cell contact was not required for such inhibition. This inhibition effect was not mediated with TGF-β1/β2, IL-4, IL-5, IL-10, or GM-CSF, as mAbs to these cytokines did not restore the allo-MLR (Fig. 4A, bottom). The clones inhibited also the proliferation of autologous LCL (data not shown), suggesting the involvement of other soluble factors in such inhibition.

We next examined the suppressive effect of TCC 29.B.42 Treg clone on the expansion and function of alloreactive cytotoxic lymphocytes. The presence of Treg had a strong inhibition effect on the expansion of both natural killer (NK; CD56) and NK T (CD8/CD56) cells in early cultures, whereas a strong inhibition effect on the expansion of CD8 was recorded in late cultures (Fig. 4B, top). However, the cytotoxic activity of alloreactive CD8 T cells generated in the presence of Treg was comparable with that generated in the absence of Treg when allogeneic LCL-5 cells were used as a target (Fig. 4B, middle). Interestingly, NK activity against K562 was recorded only in mixed alloreactive cells generated in the absence of Treg (Fig. 4B, bottom).

Finally, we examined the suppressive effect of TCC 29.B.42 Treg clone on the induction of anti-WT1 CTL responses. The presence of Treg had a strong inhibition effect on the induction of anti-WT1-126 CD8 T cell responses as evidenced by both very low cytotoxic activity (Fig. 4C, top) and IFN-γ production (Fig. 4C, bottom) recorded for the CTL-126 Treg clone after challenge with the HLA-A0201+ T2 cell line as a target.

Production of granzyme B and induction of apoptosis by the Treg clones. We next sought to determine what other soluble factors are involved in such inhibition. Granzyme B is produced by Treg and induces apoptosis in target cells in a perforin-independent (33) and perforin-dependent (34) manners. Therefore, we examined our Treg clones for granzyme B and perforin production using ELISPOT and intracellular staining assays. Granzyme B was specifically produced in response to autologous LCL in the presence of the WT1-84 peptide and also to stimulation with DR4-matched AML-25 cells but not to non-DR4-matched AML-27 cells (Fig. 5A). Perforin was not detected by either assays (data not shown). We further examined the apoptotic effect of these clones. A specific apoptosis was induced in autologous DCs and LCL pulsed with WT1-84 peptide but no apoptotic effect was detected for either DR4-matched AML-25 or non-DR4-matched AML-27 cells (Fig. 5B). These data show the capability of Treg to selectively induce apoptosis in APCs and not in AML cells.

Determination of anti-WT1-84 Treg frequencies in AML patients. Our generated Treg clones exerted a CD4+Foxp3+ phenotype and specifically produced IL-5 and granulocyte in response to the WT1-84 peptide. Therefore, we have used intracellular cytokine staining to test for specific granulocyte B and IL-5 production and Foxp3 expression by CD4+ T cells in AML patients. Of eight HLA-DR4+ patients (Supplementary Data 1)
tested, five patients showed significant increase in granzyme B/Foxp3 expression and IL-5 production in the presence of the WT1-84 peptide (Fig. 6). Non–HLA-DR-4 (AML-27 and AML-28) and HLA-DR-4+ donors (BC-29 and BC-62; Supplementary Data 1) were negative (data not shown). These findings support our in vitro data and show the amplification of tumor-specific anti–WT1-84 Tregs in AML patients in HLA-DR4–restricted manner.

**Discussion**

In this study, we identified novel human anti-WT1 Treg and clones using a pool of 110 15-mer overlapping peptides across the entire WT1 protein. These T-cell lines and clones specifically recognized a WT1333-347 (RYFKLHQLMH5SH8K) peptide designated WT1-84 in an HLA-DRB1*0402–restricted manner. The T-cell clones produced GM-CSF, IL-4, and IL-5 in response to the WT1-84 peptide exerting a Th2 cytokine profile. During the course of our study, Fujiki and colleagues (35) described an anti-WT1 T helper 1 (Th1) T-cell clone restricted to HLA-DRB1*0405 and specific to a 16-mer WT1332-347 peptide that has an additional amino acid compared with the 15-mer WT1333-347 epitope described in our study. It has been shown that the affinity of the antigen for TCR and the antigen dose can influence the differentiation of Th1 and Th2 cells (36). Th2 cells are generated only in the presence of a low dose of an antigen, and a weak TCR signal is needed for their generation (37). Fujiki and colleagues used high dose (50 μg/mL) of the WT1332-347 as a single peptide to prime T cells, whereas only 10 μg/mL of the 110 Pepmix including the WT1333-347 peptide were used in the current study. Therefore, the antigen dose and peptide competition in the case of the WT1-Pepmix may contribute to the generation of two different T helper cell types. Finally, we used high concentration of IL-2 (260 IU/mL) for the T-cell generation, whereas Fujiki and colleagues used only 20 IU/mL in their study. High dose of IL-2 has been shown to increase the frequency of Treg in cancer patients (38) and selectively up-regulate the in vitro expression of Foxp3 in Tregs. It is possible that the presence of high dose of IL-2 in our T-cell cultures played a role in the polarization of T cells to Th2 phenotype as it has been described in other systems (39) and up-regulated the expression of Foxp3 in this T-cell population.

A recent study by Durinovic-Bello and colleagues (23) showed that human Th2 cells that exert a down-regulatory Treg phenotype also express Foxp3. However, Foxp3 has also been shown to be expressed transiently in activated non-Treg, whereas...
it is stably expressed in T<sub>reg</sub> (26). We have shown that the generated T cell clones were stably expressing Foxp3 and exerted a CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup> T<sub>reg</sub> phenotype. Furthermore, the T-cell clones were examined for the expression of other T<sub>reg</sub>-related markers (GITR, CTLA-4, and CD127) and confirmed to be CD4<sup>+</sup>CD25<sup>-</sup>Foxp3<sup>+</sup>GITR<sup>-</sup>CD127<sup>-</sup> antigen-specific T<sub>reg</sub>. The lack of CD127 expression rules out the activation-induced Foxp3 expression. These clones recognize the WT1-84 epitope through their TCR V<sub>β</sub>8 chain. The immunodominance of the anti-WT1-84/TCR V<sub>β</sub>8<sup>+</sup> T-cell population in the generated T-cell line may have had a down-regulatory effect on the generation of Th1 T cells against other epitopes in the WT1-Pepmix. Importantly, these T<sub>reg</sub> clones recognized HLA-DR<sup>+</sup>-matched leukemic cells expressing the WT1 antigen, showing the natural processing and presentation of the WT1-84 epitope. The WT1-84 peptide described in this study seems to be a promiscuous HLA class II-restricted T-cell epitope as it has been reported to be recognized by both HLA-DP<sup>+</sup>-restricted cytotoxic (22) and HLA-DR0405-restricted T helper (35) CD4<sup>+</sup> T cells.

Demonstration of functional T<sub>reg</sub> will reside eventually on their ability to inhibit immune responses in functional assays (40). Several molecular and cellular events have been attributed to the suppressive effect of T<sub>reg</sub> (41). The T<sub>reg</sub> clones described in the present study were able to significantly inhibit the proliferative activity of allogeneic T cells independently of cell contact. Moreover, priming in the presence of anti-WT1-84 T<sub>reg</sub> had a strong inhibition effect on the expansion and cytotoxic activity of NK and NK T-cell populations. This agrees with the recent study showing a direct inhibitory effect of T<sub>reg</sub> on the generation and NK and NK T-cell populations. This agrees with the recent study showing a direct inhibitory effect of T<sub>reg</sub> on the generation and function of NK cells (42). However, the presence of anti-WT1-84 T<sub>reg</sub> had a strong inhibition effect on the expansion of allosreactive CD8<sup>+</sup> T cells but no effect on their cytotoxic activity. The animal model study by Edinger and colleagues (43) showing that T<sub>reg</sub> suppress alloantigen-driven expansion of CD8<sup>+</sup> T cells without inhibition of their cytotoxic effect supports our observation. More importantly, we have shown that the presence of T<sub>reg</sub> strongly inhibited the induction of anti-WT1-126 CD8<sup>+</sup> CTL responses. The existence of such tumor-specific T<sub>reg</sub> can actively suppress the WT1-specific antitumor immunity in cancer patients as it has been shown in other systems (12).

Furthermore, the present T<sub>reg</sub> inhibited the proliferation of LCL, which does not depend on cytokines in their proliferation, suggesting the involvement of other soluble factors. It has been shown that production of granzyme B by T<sub>reg</sub> results in the induction of apoptosis in T and B cells in a perforin-independent fashion (33). The current T<sub>reg</sub> specifically produced granzyme B, but not perforin, in response to the WT1-84 peptide and induced apoptosis in LCL and DCs, suggesting a direct effect of granzyme B on the suppressive activity of these T<sub>reg</sub>. However, they failed to induce apoptosis in HLA-matched AML cells, although granzyme B was produced in response to these cells. It is known that leukemic cells use several antiapoptotic signals to escape killing (44). Apoptosis in response to granzyme B involves activation of caspase-dependent cell death pathways (45). Blockade in caspase activation pathways is a common feature in leukemia (46), and this may explain the current resistance of AML cells to apoptosis. Our findings raise the possibility that leukemic cells may function as APC to preferentially induce anti-WT1-specific T<sub>reg</sub>, and hence down-regulate the patients’ immune response through induction of apoptosis in APCs, such as DCs and B cells (34), and/or inhibition of specific and nonspecific anti-leukemia immune responses. Another important finding is the detection of anti-WT1-84 IL-5/granzyme B/Foxp3<sup>+</sup> CD4<sup>+</sup> T<sub>reg</sub> in five of eight HLA-DR<sup>+</sup> AML patients. The recent demonstration by Zhou and colleagues (47) that tumor-specific T<sub>reg</sub> are amplified in vivo following cancer vaccination supports our data.

Current interests in cancer immunotherapy should be focused on determining the therapeutic implications of T<sub>reg</sub>, regulating the regulators.” Interestingly, elimination of T<sub>reg</sub> by IL-2 conjugated to diphtheria toxin (ONTAK) enhanced vaccine-mediated antitumor immunity in cancer patients (7). However, targeting the CD25 molecule may eliminate T<sub>reg</sub> leading to an increase in the susceptibility to autoimmunity, and it can also deplete activated CD25<sup>+</sup> effector cells, which may be important for clearance of cancer and infection (48). Therefore, studies should be tailored toward developing selective depletion strategies directed against T<sub>reg</sub>-related markers. Alternatively, triggering of TLR8 or OX40, and potentially blocking adenosine, might improve the chances of neutralizing T<sub>reg</sub> immunosuppression in cancer patients (49). In conclusion, our findings should open opportunity for the clinical manipulation of anti-WT1 T<sub>reg</sub> for the treatment of leukemia patients.

Disclosure of Potential Conflicts of Interest
All authors read and approved the final manuscript. The authors declare that they have no financial competing interests.

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