LEUKEMIA

Transferred WT1-Reactive CD8$^+$ T Cells Can Mediate Antileukemic Activity and Persist in Post-Transplant Patients

Aude G. Chapuis,[1]* Gunnar B. Ragnarsson,[1]*† Hieu N. Nguyen,[1] Colette N. Chaney,[1] Jeffrey S. Pufnock,[1]‡ Thomas M. Schmitt,[1] Natalie Duerkopp,[1] Ilana M. Roberts,[1]§ Galina L. Pogosov,[2] William Y. Ho,[1]¶ Sebastian Ochsenreither,[1]‖ Matthias Wölfl,[1]** Merav Bar,[2] Jerald P. Radich,[2] Cassian Yee,[1]††‡‡ Philip D. Greenberg[1,3]‡‡

Relapse remains a leading cause of death after allogeneic hematopoietic cell transplantation (HCT) for patients with high-risk leukemias. The potentially beneficial donor T cell–mediated graft-versus-leukemia (GVL) effect is often mitigated by concurrent graft-versus-host disease (GVHD). Providing T cells that can selectively target Wilms tumor antigen 1 (WT1), a transcription factor overexpressed in leukemias that contributes to the malignant phenotype, represents an opportunity to promote antileukemic activity without inducing GVHD. HLA-A*0201–restricted WT1-specific donor-derived CD8$^+$ cytotoxic T cell (CTL) clones were administered after HCT to 11 relapsed or high-risk leukemia patients without evidence of on-target toxicity. The last four treated patients received CTL clones generated with exposure to interleukin-21 (IL-21) to prolong in vivo CTL survival, because IL-21 can limit terminal differentiation of antigen-specific T cells generated in vitro. Transferred cells exhibited direct evidence of antileukemic activity in two patients: a transient response in one patient with advanced progressive disease and the induction of a prolonged remission in a patient with minimal residual disease (MRD). Additionally, three treated patients at high risk for relapse after HCT survive without leukemia relapse, GVHD, or additional antileukemic treatment. CTLs generated in the presence of IL-21, which were transferred in these latter three patients and the patient with MRD, all remained detectable long term and maintained or acquired in vivo phenotypic and functional characteristics associated with long-lived memory CD8$^+$ T cells. This study supports expanding efforts to immunologically target WT1 and provides insights into the requirements necessary to establish potent persistent T cell responses.

INTRODUCTION

Leukemic relapse after hematopoietic cell transplantation (HCT) remains a major cause of treatment failure in high-risk patients who enter HCT with poor prognostic characteristics. Patients who develop graft-versus-host disease (GVHD) have reduced relapse rates, suggesting that lymphocytes present in engrafted cells can mediate a concurrent therapeutic graft-versus-leukemia (GVL) effect (*1*, *2*). However, because graft T cells have not been selected for specificity for leukemia antigens, and commonly recognize proteins expressed by many other host tissues, substantial morbidity and mortality from GVHD can occur.

One strategy to enhance the GVL effect without promoting GVHD in post-HCT patients is to target leukemia-associated antigens with purified antigen-specific CD8$^+$ cytotoxic T cells (CTLs). In this approach, CD8$^+$ CTLs are isolated and cloned from donor peripheral blood mononuclear cells (PBMCs) on the basis of antigen-specific T cell–mediated lysis of target cells, and the highest-avidity clone was selected from each patient-donor pair and expanded for infusion. Limiting adoptively transferred CD8$^+$ T cells to a homogeneous well-characterized product allows for tracking the antileukemia response, facilitating analyses to help define parameters for immune-mediated eradication and long-term control of leukemic relapse. The most ideal target antigens are unique mutated proteins that are also obligate for the leukemic phenotype. However, T cell responses to common mutations such as epitopes created by *TEL-AML1* or *BCR-abl* fusions have been hampered, in part due to limited processing and/or few unique epitopes that bind to human leukocyte antigen (HLA) alleles (*3*, *4*). Alternatively, nonpolymorphic proteins overexpressed by leukemic cells that contain many potential epitopes can be attractive candidate targets for CTLs (*5*). The zinc finger transcription factor Wilms tumor antigen 1 (WT1) is expressed at 10- to 1000-fold higher concentrations in leukemic cells compared to normal CD34$^+$ cells, and the magnitude of expression correlates with clinical aggressiveness of acute myeloid leukemia (AML), myelodysplastic syndromes (MDSs), and acute lymphoid leukemia (ALL) (*6*–*8*). Because WT1 promotes proliferation and oncogenicity, loss of its expression is disadvantageous for the tumor, making outgrowth of antigen-loss variants less likely (*9*). Although essential during embryogenesis, WT1 expression after birth is limited to low levels predominantly in kidney podocytes and CD34$^+$ hematopoietic stem cells (*10*–*12*). WT1-specific CD8$^+$ T lymphocytes can distinguish overexpressing targets from

[1]Program in Immunology, Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA 98109, USA. [2]Clinical Research Division, FHCRC, Seattle, WA 98109, USA. [3]Department of Immunology, University of Washington, Seattle, WA 98195, USA.
*These authors contributed equally to this work.
†Present address: Landspítali Háskólasjúkrahús, 101 Reykjavík, Iceland.
‡Present address: Seattle Children's Research Institute, Seattle, WA 98101, USA.
§Present address: Surgery Branch, National Institutes of Health, Bethesda, MD 20892, USA.
¶Present address: Igenica Inc., Burlingame, CA 94010, USA.
‖Present address: Department of Hematology, Charité, 10117 Berlin, Germany.
**Present address: University Children's Hospital, 97070 Würzburg, Germany.
††Present address: Department of Immunology, MD Anderson Cancer Center, Houston, TX 77030, USA.
‡‡To whom correspondence should be addressed. E-mail: pgreen@u.washington.edu (P.D.G.); cyee@fhcrc.org (C.Y.)

normal cells and have been demonstrated to inhibit the growth of and to lyse leukemic but not normal CD34$^+$ cells (*13*). Although vaccines targeting WT1 have resulted in clear antitumor responses in some patients, most patients have failed to benefit clinically, potentially reflecting the induction of weak responses because of the limited immunogenicity of vaccine regimens, the presence/generation of WT1-specific CD4 regulatory T cells (T_{regs}), and/or compromised patient immune systems or T cell repertoires (*14*).

Adoptive transfer of donor-derived ex vivo–expanded WT1-specific CD8$^+$ CTLs in post-transplant patients can potentially bypass the limitations encountered during vaccination by increasing the number and quality of T cells targeting tumor-associated antigens. An analogous strategy has proven effective in reducing tumor burdens in melanoma patients, with clinical responses tightly correlated with the duration of in vivo persistence of transferred T cells (*15*–*18*). Reinfusion of CD8$^+$ CTLs derived from less terminally differentiated populations such as central memory T cells (T_{cm}), which have the ability to self-renew and maintain robust responses over time, has been shown to establish prolonged responses (*19*–*21*). Increased persistence has also been observed with murine CD8$^+$ CTLs derived from the naïve pool when these cells were primed in the presence of the γ_c chain cytokine interleukin-21 (IL-21) (*22*), which promotes in vitro expansion of responding T cells that phenotypically appear less terminally differentiated (*23*). Because CTL clones for this study were generated from the repertoire of healthy donors and likely derived from the naïve cell population, we used IL-21 after it became available for clinical use in a subset of patients on this trial, hypothesizing that generating WT1-specific CTL clones in the presence of IL-21 might confer an increased ability for these cells to survive and persist in vivo after transfer.

Our results show that adoptive transfer to post-HCT patients of donor-derived WT1-specific CTLs followed by low-dose subcutaneous IL-2 is safe and results in direct evidence of antileukemic activity. Furthermore, all patients who received WT1-specific CTLs generated in the presence of IL-21 [three patients at high risk of relapse after HCT and one patient with minimal residual disease (MRD) at the time of treatment] have survived in the absence of leukemia relapse for >30 months, with no other antileukemic treatment or GVHD. In these patients only, transferred T cells remained detectable long term and maintained/acquired phenotypic and functional characteristics associated with long-lived memory CD8$^+$ T cells.

RESULTS

WT1-specific CD8$^+$ CTL clonal populations primed in the presence of IL-21 have higher fractions of cells expressing CD27, CD28, and CD127

WT1-specific CD8$^+$ T cells were generated from each donor after stimulation with WT1 peptide, and expanded and cloned as described in Materials and Methods. The clones for each patient-donor pair were tested for specific recognition and lysis of WT1$^+$ targets in a ^{51}Cr-release assay with HLA-A*0201, transporter for antigen presentation (TAP)–deficient, B lymphoblastoid cells (T2 B-LCL) pulsed with titrating doses of the HLA-A*0201–restricted $WT1_{126–134}$ (RMFPNAPYL) epitope, and the clones that recognized targets pulsed with the lowest concentration of the peptide were selected for infusions (Fig. 1, A and B). Immediately before infusion, all clones, whether generated in the presence or absence of IL-21 supplementation, expressed CD3, CD8, and CD45RO, but not CD4, CD16, CD19, or CD45RA, consistent with an antigen-experienced but not terminally differentiated phenotype. However, as we reported previously (*15*, *24*), the expanded cell populations, despite being clonal and derived from a single cell, do contain cells expressing different amounts of costimulatory and differentiation markers. In the WT1-specific clonal populations generated with exposure to IL-21, the median frequencies of cells expressing CD27, CD28, or CD127 were 32.5, 64, and 38%, respectively, compared to 2, 7, and 14% within clonal populations generated in the absence of IL-21. Median fluorescence intensities of CD27, CD28, and CD127 were also significantly higher ($P = 0.01$, 0.02, and 0.008, respectively) on clones generated in the presence of IL-21 (Fig. 1, C to E). However, previous exposure to IL-21 did not appear to affect the avidity or cytolytic function of the CTL clones generated, with no significant difference detected in the mean effective peptide concentrations required to induce 50% lysis (EC_{50}) of WT1-pulsed T2 B-LCL (mean EC_{50} of CTL clones generated without IL-21 was $10^{-2.59}$ ng/ml compared to mean EC_{50} with IL-21 of $10^{-3.77}$ ng/ml) or the percent maximal lysis at an E:T ratio of 10:1 (the mean for CTL clones generated without IL-21 was 90.46% compared to the mean with IL-21 of 94.55%) (Fig. 1, F and G).

Adoptive transfer of escalating doses of donor-derived WT1-specific CTLs does not injure normal tissues expressing low physiologic levels of WT1

Eleven patients with AML, MDS, or ALL received a total of 36 escalating doses of CTL clones after HCT specific for the peptide RMFNAPYL, an HLA-A*0201–restricted epitope of WT1 (Table 1 and fig. S1). Overall, 13 of 36 doses administered represented the maximum target dose of 1×10^{10} WT1-specific CTLs/m^2, and 11 of 36 doses (3.3×10^9 or 1×10^{10}/m^2) were followed by low-dose subcutaneous IL-2, which was administered to enhance the survival of transferred T cells (*25*). With the exception of expected transient side effects associated with activation of large numbers of antigen-specific CTLs transferred into patients with targets expressing the antigen and/or low-dose subcutaneous IL-2, the infusions were well tolerated. Specifically, fevers (≥38.3°C) ± chills resolving without specific treatment within 24 hours, a temporary drop in total lymphocyte counts that returned to preinfusion levels within 7 to 11 days in patients with no evidence of relapsed disease, and transient mild injection site reactions accompanying low-dose subcutaneous IL-2 occurred in 25, 77, and 82% of cases, respectively (table S1). No toxicities to the hematopoietic or renal systems, reflecting potential WT1$^+$ targets, were detected during the monitoring period, nor was there any evidence of new-onset GVHD. This absence of toxic effects or GVHD was also observed for the four patients in whom the T cell clones remained detectable in the blood for ≥7 to 14 months after infusions (Fig. 2). Thus, infusion of the most avid WT1-specific CTL clone derived from each patient's donor, with or without low-dose subcutaneous IL-2, was well tolerated and safe at doses up to 10^{10} cells/m^2.

Transferred WT1-specific CTL clones can persist after infusion into patients with MRD or undetectable leukemic burden

Analysis of patients for the presence of preexisting WT1-specific multimer$^+$ T cells in PBMCs (range, 0 to 0.06%) and bone marrow (BM) (range, 0 to 0.13%) revealed median frequencies of 0% at both sites, attesting to the essentially undetectable frequency of endogenous WT1-specific CD8$^+$ T cells in most patients. The first seven infused patients received escalating doses of WT1-specific CTL clones generated

in the absence of IL-21. Five had detectable leukemia during all or some infusions, and two were in complete remission (CR) after salvage therapy for relapse after HCT at least 60 days before the first infusion and remained in CR throughout the infusions (patients 6 and 17) (Fig. 2, A and B). In this subset of patients, none of the clones persisted beyond 14 days in blood or BM (range, 0 to 14; median, 1 day). In contrast, the last four patients received WT1-specific CTL clones generated in the presence of IL-21. Three were in CR at least 60 days before the first infusion, and one (patient 27) had MRD (B cell clonal population detected in the BM). For this subset of patients, the infused CTL clones persisted in all recipients at levels >0.05% well beyond 14 days; at ≥430 days in patients 21, 24, and 27; and at ≥230 days in patient 28 (Fig. 2C).

For patients who had received the maximal dose of 10^{10} CTLs/m^2 (irrespective of exposure to IL-21), direct intrapatient comparisons revealed that the median peak CTL frequency, achieved 24 to 72 hours after infusions, appeared to be higher if followed by IL-2 than without IL-2 (3.1% multimer$^+$CD8$^+$ T cells compared to 1.5%), as did the frequencies sustained at day 14 (1.5% with IL-2 compared to 0.6% without IL-2) (fig. S2, A and B). Although the difference, with the limited numbers of infusions being analyzed, did not achieve statistical significance, the benefits of IL-2 administration after T cell transfer have been previously reported (*25*). T_{regs} can be sensitive to exogenous IL-2 and could negatively affect the persistence and function of transferred CTLs. Therefore, we assessed T_{reg} numbers after all infusions based on expression of the surrogate markers CD4 and CD25 and the absence of CD127. T_{reg} frequencies increased after infusions followed by low-dose IL-2 from baseline levels at 7 ($P = 0.04$) and 14 days ($P = 0.01$), and returned to near-baseline levels by day 28 ($P = 0.26$) (fig. S3). Changes in T_{reg}

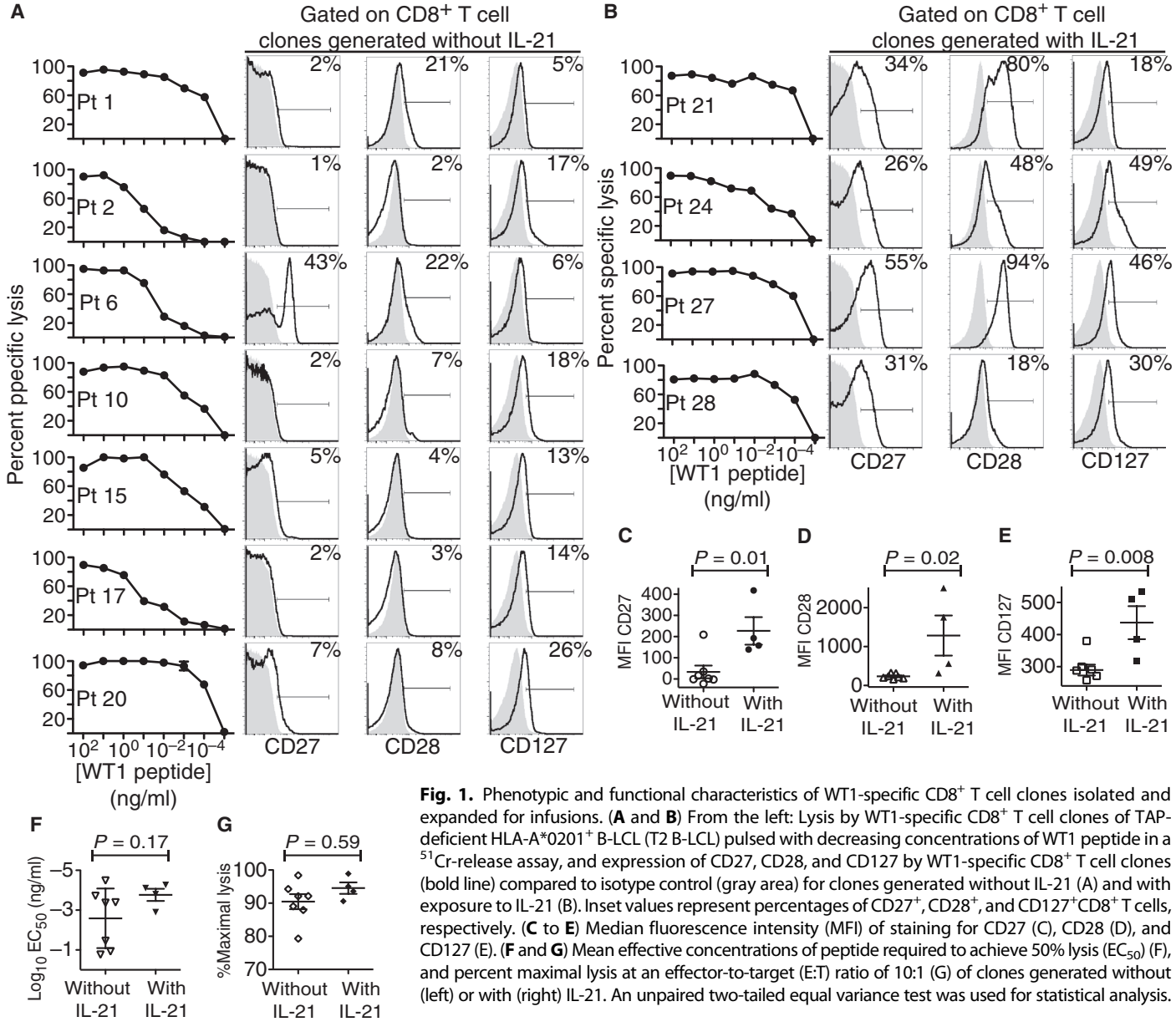

Fig. 1. Phenotypic and functional characteristics of WT1-specific CD8$^+$ T cell clones isolated and expanded for infusions. (**A** and **B**) From the left: Lysis by WT1-specific CD8$^+$ T cell clones of TAP-deficient HLA-A*0201$^+$ B-LCL (T2 B-LCL) pulsed with decreasing concentrations of WT1 peptide in a ^{51}Cr-release assay, and expression of CD27, CD28, and CD127 by WT1-specific CD8$^+$ T cell clones (bold line) compared to isotype control (gray area) for clones generated without IL-21 (A) and with exposure to IL-21 (B). Inset values represent percentages of CD27$^+$, CD28$^+$, and CD127$^+$CD8$^+$ T cells, respectively. (**C** to **E**) Median fluorescence intensity (MFI) of staining for CD27 (C), CD28 (D), and CD127 (E). (**F** and **G**) Mean effective concentrations of peptide required to achieve 50% lysis (EC_{50}) (F), and percent maximal lysis at an effector-to-target (E:T) ratio of 10:1 (G) of clones generated without (left) or with (right) IL-21. An unpaired two-tailed equal variance test was used for statistical analysis.

Table 1. Patient characteristics. HSCT, hematopoietic stem cell transplantation; Ara-C, arabinosylcytosine; TBI, total body irradiation; CR, complete remission; MA, myeloablative; NMA, nonmyeloablative; RI, reduced intensity.

Patient	M/F	Age	Disease	Disease characteristics at HCT	Conditioning regimen	Days between HSCT and first CTL infusion	Disease characteristics between HSCT and first CTL infusion	Disease characteristics at first CTL infusion
1	M	29	ALL; normal cytogenetics	Chemorefractory after two inductions (Ara-C/idarubicin), 12% BM blasts entering HCT	MA: CY/12 Gy TBI	193	1.7% BM blasts D28 after HCT; received two reinductions (Mylotarg then mitoxantrone, Ara-C) resulting in persistent disease	20% BM blasts 21 days before
2	F	59	AML with multilineage dysplasia; normal cytogenetics	Hematopoietic aplasia after two inductions (Ara-C/idarubicin)	NMA: fludarabine/ 2 Gy TBI	302	Relapse D156 after HCT; received two courses of Mylotarg resulting in progression (30% BM blasts); received mitoxantrone, VP-16, 44 days before CTL infusion	Hypocellular BM no blasts, persistent peripheral pancytopenia 1 day before
6	F	42	ALL; BCR-ABL$^+$	Hematologic remission after induction (Ara-C/idarubicin); molecular relapse BCR-ABL$^+$ by PCR	MA: 12 Gy TBI/CY	455	Persistent BCR-ABL$^+$ D35 after HCT; PCR for BCR-ABL resulted negative with increased imatinib and taper of immunosuppression	Molecular CR (BCR-ABL$^-$) 260 days before; on imatinib 600 mg daily
10	F	51	AML secondary to breast cancer treatment; t(8;21), del (19q)	CR after induction (Ara-C/idarubicin)	MA: busulfan/CY	127	Relapse D86 after HCT (43% BM blasts); received mitoxantrone, VP-16, Ara-C resulting in CR	Reinduction 44 days before CTL; CR 5 days before
15	F	51	AML secondary to MDS; complex cytogenetics including monosomy 7	Chemorefractory after two inductions (Ara-C/idarubicin, clofarabine/Ara-C), 3.4% BM blasts entering HCT	RI: ^{131}I-BC8 (anti-CD45 antibody) 24 Gy targeted/fludarabine/ 2 Gy TBI	192	Persistent monosomy 7 after HCT (1.8% D28, 4.5% D45); GVHD/infectious complications delaying CTL start	Relapse by flow (6.3% BM blasts, 11.8% PB blasts), morphology and cytogenetics, 2 days before
17	M	65	AML; normal cytogenetics	Second CR (Ara-C/idarubicin, mitoxantrone/VP-16/ Ara-C)	RI: ^{131}I-BC8 (anti-CD45 antibody) 24 Gy targeted/fludarabine/ 2 Gy TBI	876 (2.4 years)	Relapse 2 years after HCT (85% BM blasts); received mitoxantrone/ Ara-C resulting in morphological CR	Morphologic CR 62 days before CTL; CR (morphology, flow, cytogenetics) 1 day before
20	F	52	AML; normal cytogenetics	Third relapse (Ara-C/ idarubicin, cytosine/ arabinoside/idarubicin/ pravastatin) entering HCT (4.6% BM blasts, 0.9% PB blasts)	RI: treosulfan/ fludarabine	419	Relapse D383 after HCT with (0.01% PB and BM blasts); patient elected to receive WT1-specific CTL	Relapse (1.6% BM blasts, 0.3% PB blasts) by flow 1 day before
21	F	59	AML secondary to MDS; normal cytogenetics	Chemorefractory after four inductions (Ara-C/idarubicin, clofarabine/Ara-C × 2, Vidaza/Mylotarg), 47% BM blasts entering HCT	MA: 12 Gy TB/CY	168	CR	CR

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Patient	M/F	Age	Disease	Disease characteristics at HCT	Conditioning regimen	Days between HSCT and first CTL infusion	Disease characteristics between HSCT and first CTL infusion	Disease characteristics at first CTL infusion
24	F	42	AML; t(8;21)	Chemorefractory after two inductions (fludarabine/ Mylotarg/Ara-C, clofarabine/idarubicin/ Ara-C), 4.4% BM blasts, 3.5% PB blasts entering HSCT	RI: treosulfan/ fludarabine	159	Persistent MRD after HCT; received azacitidine/Mylotarg D66 after HCT resulting in CR D83 after HCT	CR since 76 days before
27	F	32	B-ALL; complex cytogenetics, BCR-ABL$^-$	Relapse after first HCT (12 Gy TB, CY); achieved CR with hyper-CVAD	MA: CY/busulfan	64 (after second HCT)	Persistent MRD (clonal B cell population)	Morphologic and flow cytometric CR; clonal B cell population detected by PCR 8 days before
28	M	26	AML, normal cytogenetics	CR after two inductions (Ara-C/idarubicin/ Mylotarg, Ara-C/ idarubicin)	RI: treosulfan/ fludarabine/ 2 Gy TBI	594 (1.6 years)	MRD (2% BM blasts) 1 year after HCT responding to taper of immunosuppression	CR since 182 days before

frequencies never achieved statistical significance after infusion of T cells without IL-2, and at day 28, the T_{reg} frequencies after infusions with or without IL-2 were nearly identical.

Transferred WT1-specific CD8 CTLs preferentially localize to the BM compartment

We next assessed whether transferred CTL clones reached the BM, the main site of accumulation of leukemic cells and the most common site of relapse. All patients consented to BM assessments before CTL infusions, 1 day after the second infusion, and 1 day after the last dose of subcutaneous IL-2. The median frequencies of WT1-specific CTLs in PBMCs and BM from all patients and at all time points in which transferred cells could be detected at either site were 0.45 and 1.31%, respectively ($P < 0.001$), suggesting preferential accumulation of the transferred cells in the BM (Fig. 3A). For the subset of patients who received WT1-specific CTLs generated with exposure to IL-21, which coincided with patients who demonstrated T cell persistence beyond 14 days, the median WT1-specific CTL frequencies in PBMCs and BM over multiple time points were 0.83 and 2.21%, respectively ($P = 0.001$) (Fig. 3B).

CTL clones generated with exposure to IL-21 maintain/acquire in vivo characteristics associated with long-lived memory

Patients who received CTL clones generated without IL-21 had absent or low expression of CD27, CD28, and CD127 (Fig. 1A), and all these clones persisted ≤14 days in vivo. Consequently, the in vivo phenotype could only be assessed for the brief period that the T cells persisted (≤14 days), but no change in expression of CD45RO, CD27, CD28, CD127, CD62L, or CCR7 was observed (Fig. 4A and fig. S4). In contrast, as previously described, CTL clones generated with exposure to IL-21 expressed higher levels of CD27, CD28, and CD127 before being infused (Fig. 1B) and demonstrated long-term in vivo survival after infusion. On the basis of gating multimer$^+$CD8$^+$ T cells at days 280, 250, 160, and 84 for patients 21, 24, 27, and 28, respectively (Fig. 4B), subpopulations of the infused cells maintained or up-regulated phenotypic markers associated with long-lived memory CD8 T cells (CD27, CD28, CD127, CD62L, and CCR7) (fig. S4).

The functional profile of persistent infused cloned T cells was determined by gating on interferon-γ (IFN-γ)–producing cells. Before infusions, all CTL clones secreted IFN-γ and tumor necrosis factor–α (TNF-α) in response to WT1-pulsed T2 B-LCL. Clones expressing CD28 secreted the highest levels of IL-2, consistent with the known contribution of a costimulatory signal via CD28 (*26*, *27*). Consistent with the results obtained by multimer staining, which revealed a low to undetectable frequency of endogenous WT1-reactive cells, no IFN-γ–producing cells could be detected in any patient before infusions. In patients who received CTL clones generated without IL-21, no IFN-γ–producing cells could be detected in vivo after infusions, despite the transient persistence of transferred T cells revealed by multimer staining (Fig. 5A). In contrast, clones generated with exposure to IL-21 secreted IFN-γ, TNF-α, and IL-2 both before infusion (consistent with the expression of CD28) and in vivo after adoptive transfer for the entire period the cells could be detected by multimer staining (Fig. 5B).

Because cells having the potential to divide might also persist better, we investigated whether infused CTL clones expressed Ki-67 in vivo, a marker of recent proliferation (*28*). For clones generated without/with exposure to IL-21 and harvested for infusion on day 14 of the stimulation cycle, a median of 37 and 37.5% of cells, respectively, expressed Ki-67 (Fig. 5C, left columns). Early after transfer (day 1), most infused multimer$^+$ clonal CTL populations entered the cell cycle, with >55% of cells expressing Ki-67. Cloned CTL populations generated without exposure to IL-21 lost Ki-67 expression by day 4 (Fig. 5C, dashed black line), but CTL clones generated with exposure to IL-21 expressed Ki-67 on a fraction of cells until day 21 after transfer (Fig. 5C, solid black line). Infusions of WT1-specific CTL clones had no impact on

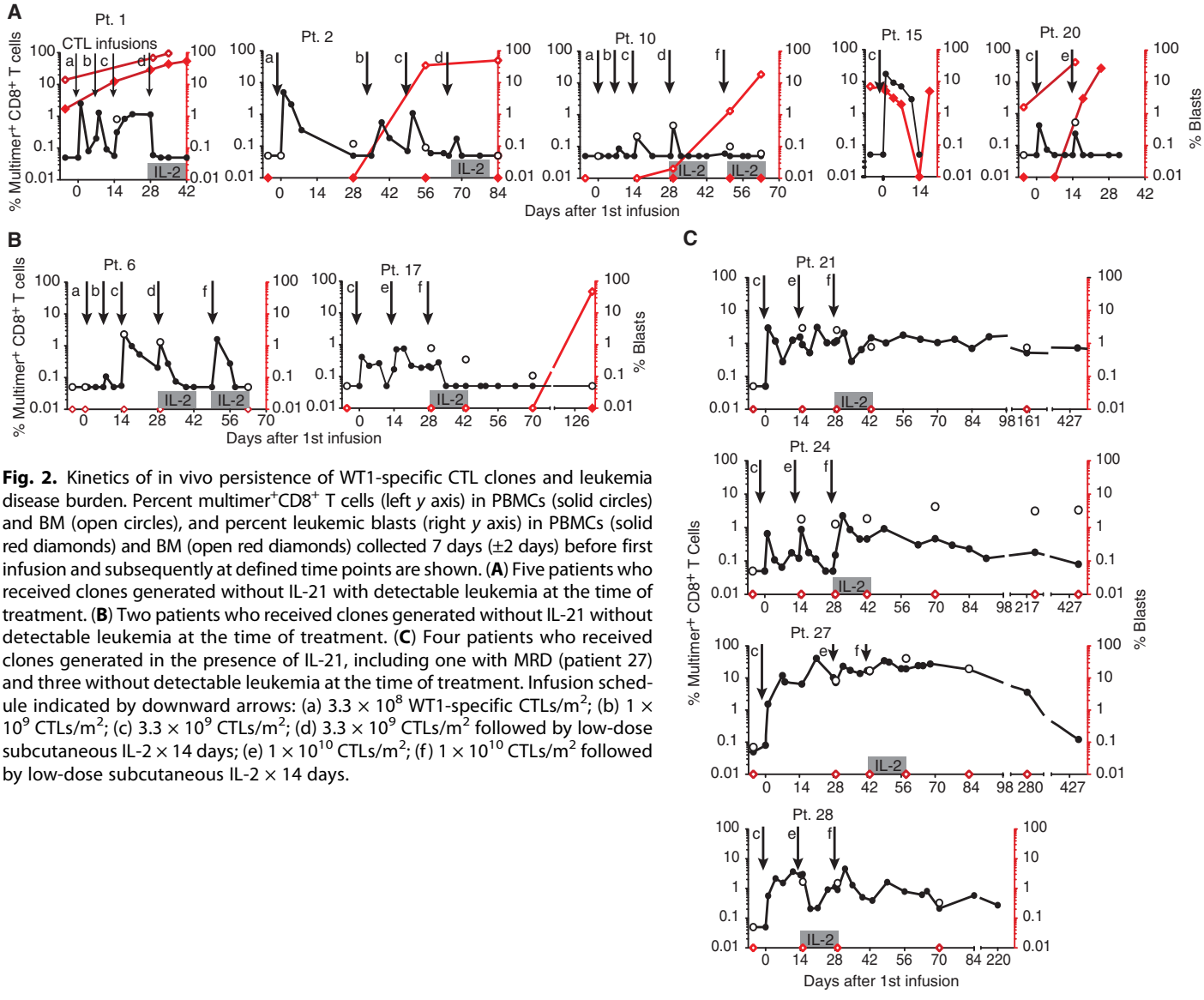

Fig. 2. Kinetics of in vivo persistence of WT1-specific CTL clones and leukemia disease burden. Percent multimer$^+$CD8$^+$ T cells (left *y* axis) in PBMCs (solid circles) and BM (open circles), and percent leukemic blasts (right *y* axis) in PBMCs (solid red diamonds) and BM (open red diamonds) collected 7 days (±2 days) before first infusion and subsequently at defined time points are shown. (**A**) Five patients who received clones generated without IL-21 with detectable leukemia at the time of treatment. (**B**) Two patients who received clones generated without IL-21 without detectable leukemia at the time of treatment. (**C**) Four patients who received clones generated in the presence of IL-21, including one with MRD (patient 27) and three without detectable leukemia at the time of treatment. Infusion schedule indicated by downward arrows: (a) 3.3×10^8 WT1-specific CTLs/m^2; (b) 1×10^9 CTLs/m^2; (c) 3.3×10^9 CTLs/m^2; (d) 3.3×10^9 CTLs/m^2 followed by low-dose subcutaneous IL-2 × 14 days; (e) 1×10^{10} CTLs/m^2; (f) 1×10^{10} CTLs/m^2 followed by low-dose subcutaneous IL-2 × 14 days.

the proliferation of host multimer$^-$CD8$^+$ T cells (Fig. 5C, dotted gray line). The in vivo proliferation of CTL clones after infusions followed by low-dose subcutaneous IL-2 (irrespective of the use of IL-21), as reflected by Ki-67 expression, tended to increase 4 days after transfer (mean with IL-2 was 65.8% compared to mean without IL-2 of 30.7%), but this difference did not reach statistical significance (fig. S5). Furthermore, administration of low-dose subcutaneous IL-2 did not affect the proliferation of host multimer$^-$CD8$^+$ populations (fig. S5). Thus, the CTL clones generated with exposure to IL-21 and infused into patients with MRD or undetectable leukemia exhibited phenotypic and functional properties associated with long-lived CD8$^+$ T cells capable of proliferating independent of CD4 T helper cells, and a fraction of these CD8$^+$ T cells sustained a proliferative state until day 21 after transfer.

WT1-specific CD8 CTLs show evidence of antileukemic activity

Eleven patients with high-risk disease were treated on this study (Table 1). High-risk features included poor prognostic indicators conferred by the cytogenetics of the primary leukemia (*29*), AML secondary to MDS or previous chemotherapy treatments (*30*), refractoriness to induction therapy before HCT (*31*), entering HCT with detectable blasts (*32*), or relapse after HCT (*32*, *33*). Patient 21 entered HCT with refractory disease and had a BM containing 40% blasts. Because of the heterogeneity of the patients treated, a ranking of their relative prognosis at study entry is not reliable (*34*), and neither is a comparison of prognosis between the patients who received clones generated with or without IL-21, but all had at least two high-risk features (table S2). The patients who received clones generated without IL-21 had all relapsed after HCT [associated with >95% mortality at 2 years (*33*)], and five of seven had detectable leukemia at the time they received WT1-specific T cells. Of the patients who received clones generated with IL-21, three of four had relapsed after HCT and one patient entered HCT with >40% blasts, which is associated with a <5% disease-free survival 1 year after HCT (*32*). However, only one patient (patient 27) had detectable disease at the time of WT1-specific T cell infusions. Thus, although all patients

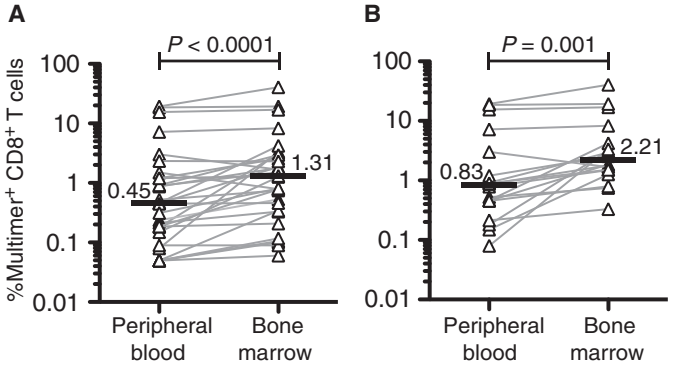

Fig. 3. Localization of adoptively transferred WT1-specific CTLs to the BM. (**A**) Percent multimer$^+$CD8$^+$ cells in blood (left) and BM (right) at all time points from the 11 patients in whom blood and BM were analyzed at the same time. (**B**) Same analysis as above performed at all time points on the four patients who received CTL clones that were generated with exposure to IL-21 and persisted long term in vivo. Only samples in which at least one site showed detectable transferred cells are shown. Horizontal bars indicate medians. A two-tailed paired signed-rank test was used for statistical analysis.

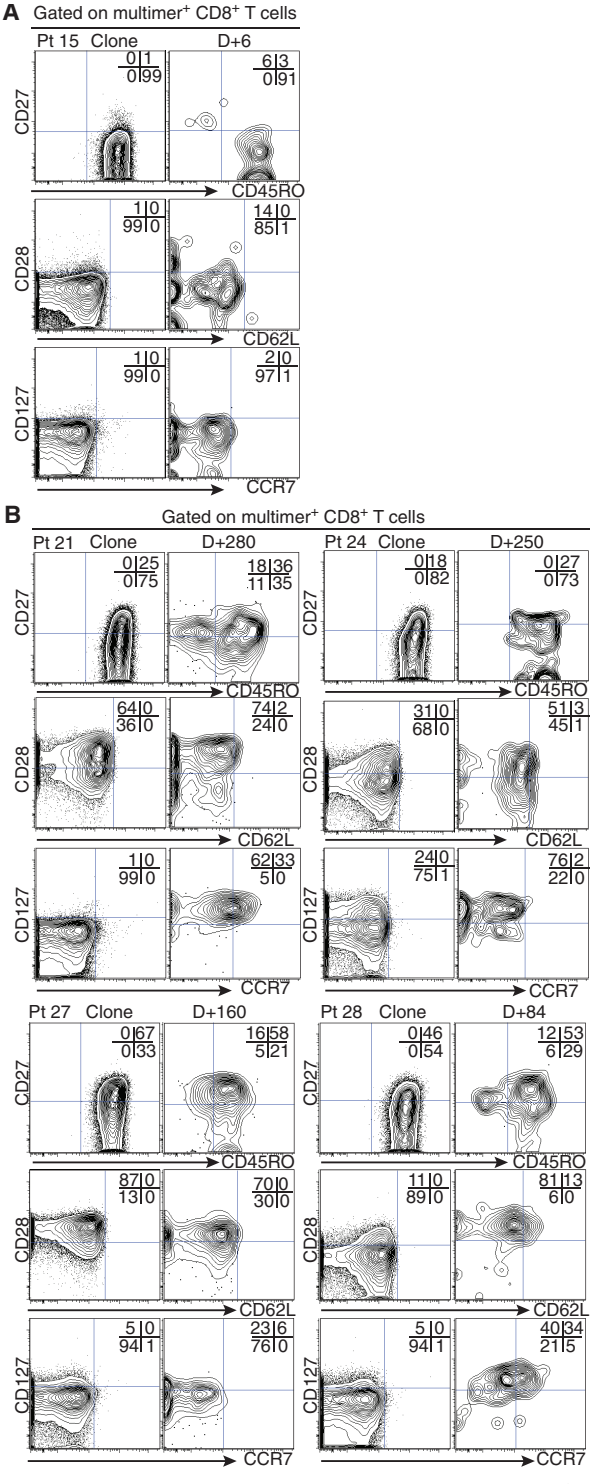

Fig. 4. Phenotypic characteristics of transferred WT1-specific CD8$^+$ T cells persisting in vivo. (**A**) Expression of CD27 (*y* axis) and CD45RO (*x* axis) (upper plots), CD28 (*y* axis) and CD62L (*x* axis) (middle plots), and CD127 (*y* axis) and CCR7 (*x* axis) (lower plots) on gated multimer$^+$ cells for CD8$^+$ CTL clones generated without IL-21 for patient 15 (representative) immediately before infusion and after 6 days in vivo. (**B**) CTL clones generated with exposure to IL-21 for patients 21, 24, 27, and 28 immediately before infusions, and 280, 250, 160, and 84 days, respectively, in vivo after the first infusion.

had high-risk disease, patients who received clones generated with IL-21 had MRD or no detectable leukemia at the time of infusions and therefore had better disease characteristics compared to patients who received WT1-specific clones generated without IL-21.

Table 2 summarizes the clinical outcomes of each patient. One patient (patient 15) with detectable leukemic blasts in the blood [7% of total white blood cells (WBCs)] exhibited a rapid reduction in the percentage of blasts after the infusion of 3.3×10^9 WT1-specific CTLs/m^2, with blasts declining to 0% of total WBCs after 14 days (Fig. 6A). The decrease in detectable leukemic blasts was associated with increasing absolute numbers of circulating normal WBCs and coincided with the presence of infused WT1-specific CTLs in the blood. The patient developed toxicity from previous antileukemic therapy that precluded qualifying for subsequent T cell infusions, and died with progressive disease after disappearance of the infused T cells. Another patient (patient 27) had no detectable leukemic blasts but presented with MRD before infusions as revealed by detection of a clonal B cell population and an abnormal karyotype in the BM. After the infusions of WT1-specific CTLs were completed, which resulted in percentages of WT1-specific multimer$^+$CD8$^+$ T cells maintained at 6.6 and 3.6% 168 and 280 days, respectively, from the time infusions began, the patient exhibited clearing of the clonal B cell population, and cytogenetic analysis no longer revealed any abnormalities (Fig. 6B).

The avidities of the clones infused for patients 15 and 27, who had detectable disease, and patients 21, 24, and 28, who were treated with undetectable disease, were similar to the highest avidities obtained in this study, as measured by the ability to lyse T2 B-LCL pulsed with titrated peptide doses (Fig. 6C). All four patients who received CTL clones generated in the presence of IL-21 demonstrated long-term CTL persistence and were in CR without additional chemotherapy or the development of GVHD at 22, 33, 35, and 38 months after HCT, despite historical probabilities of relapse estimated at 90 to 95% after 1 year and 95 to 97% after 2 years (table S3). Among the seven patients who received CTL clones generated in the absence of IL-21, all of whom demonstrated only short-term CTL persistence, five had

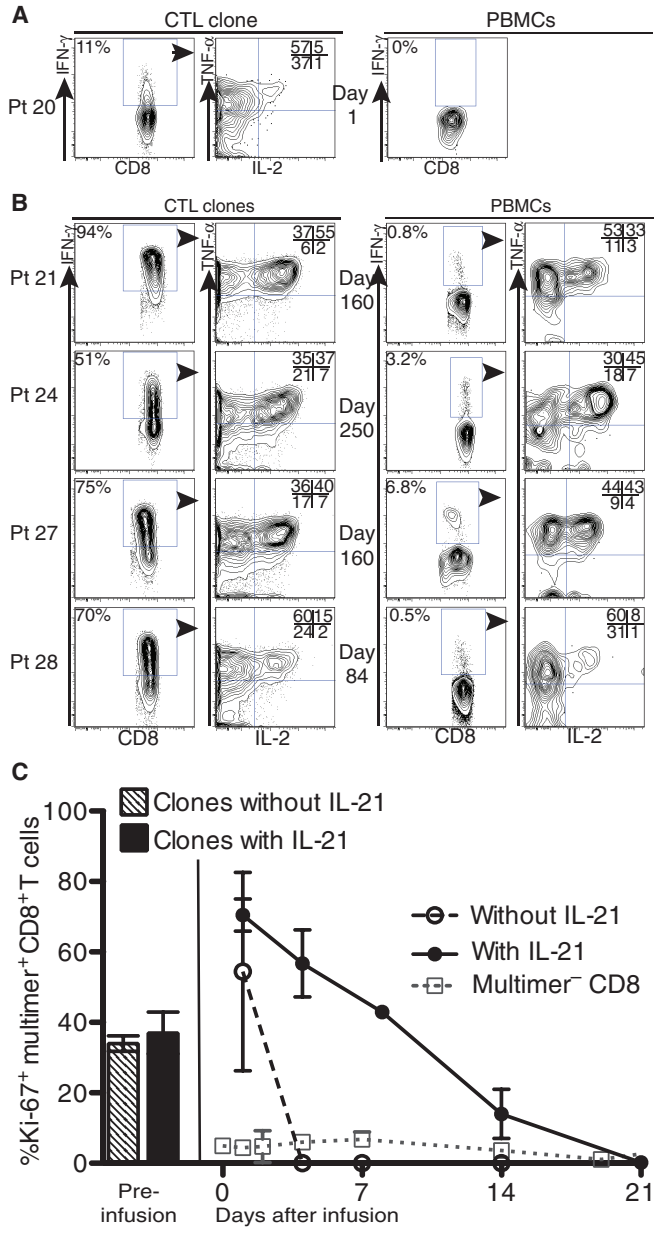

Fig. 5. Functional characteristics of persisting transferred WT1-specific CTLs. (**A**) Left plot: Percent cells producing IFN-γ by the CTL clone generated without IL-21 for patient 20 (representative) in response to WT1-pulsed T2 B-LCL at an E:T ratio of 10:1; middle plot: TNF-α (*y* axis) and IL-2 (*x* axis) production of IFN-γ^+ cells; right: the same analysis performed on PBMCs 1 day after transfer in vivo. (**B**) Plots to the left: Percent IFN-γ production for CTL clones generated with exposure to IL-21 (patients 21, 24, 27, and 28), and the respective TNF-α and IL-2 production. Plots to the right: The same analysis performed on PBMCs obtained after 160, 250, 160, and 84 days, respectively, in vivo. (**C**) Intranuclear Ki-67 expression on preinfusion CTL clones harvested on day 14 of the ex vivo expansion cycle generated without (striped/gray column) or in the presence of IL-21 (solid column). Intranuclear Ki-67 expression on post-infusion multimer$^+$CD8$^+$ cells averaged from PBMCs of patients who received CTL clones generated without (open circles, dashed lines) or in the presence of IL-21 (solid circles, solid lines). Open squares and gray dotted lines represent average Ki-67 expression on patient endogenous multimer$^-$CD8$^+$ cells for all patients combined.

detectable disease, and two (patients 6 and 17) had no detectable disease during infusions. However, patient 6 was receiving concurrent immunosuppressive therapy for preexisting GVHD, which included mycophenolate mofetil, which may have contributed to poor T cell persistence, especially in the presence of concurrent administration of exogenous IL-2, which likely promoted entry into the cell cycle. Patient 17 relapsed after clearance of the infused CTLs.

In 10 of 11 patients, leukemia cells were available for analysis, and expression of WT1 in the leukemic cells was confirmed. WT1 expression in both blood and BM was assessed longitudinally by PCR in all patients. Except for patient 17, whose leukemic cells expressed WT1 before therapy and who relapsed with 40% blasts in the BM 138 days after the first infusion with no increase in detectable levels of WT1 expression, the presence of leukemic blasts by morphology and/or flow cytometry correlated with levels of WT1. These results are consistent with continued expression of the pro-oncogenic WT1 protein in leukemia blasts independent of targeting with T cells in most patients (fig. S6).

DISCUSSION

Establishing robust T cell–mediated antileukemic activity after HCT without inducing accompanying GVHD remains a major challenge. WT1 is an antigen that is overexpressed in leukemic cells, which contributes to the leukemic phenotype and has been shown to be capable of eliciting T cell responses (*9*). Therefore, we have examined the potential to achieve a selective antileukemic effect by the adoptive transfer of homogeneous WT1-specific CD8$^+$ CTL clones. Clones were generated from the repertoire of each patient's HLA-matched donor that was not compromised by previous chemotherapy, and the clone exhibiting the highest avidity for targets expressing WT1 was selected for infusion. Infusions were well tolerated, nontoxic, and not associated with new-onset GVHD after doses of $\leq 10^{10}$ cells/m^2, including when cell infusions were followed by administration of low-dose subcutaneous IL-2. This contrasts with previous reports of adoptive transfer of unselected T cells containing potentially chronic myeloid leukemia (CML)–reactive CTLs, in which the incidence of GVHD doubled compared to control patients transplanted with CD34$^+$-selected cells who did not receive T cell infusions (30 versus 14%) (*35*, *36*).

The establishment of a persistent functional population of antigen-specific CTLs capable of eliminating cells responsible for late leukemic recurrences will likely be necessary to consistently derive long-term benefit from infused CTLs. Studies of transferred CD8$^+$ T cells for murine lymphocytic choriomeningitis virus (LCMV) infection revealed that transferred T_{cm} provided enhanced protective immunity from in vivo challenge compared to effector memory (T_{em}) (*21*). Furthermore, studies in nonhuman primates have shown that expanded CD8$^+$ T cell clones derived from T_{cm} exhibit greater replicative potential in response to antigen and prolonged in vivo persistence compared to CD8$^+$ T cell clones derived from T_{em}. Despite all cells displaying a differentiated T_{em} phenotype after in vitro expansion, a fraction of the transferred CD8$^+$ T cell clones derived from T_{cm} ultimately revert back to a T_{cm} phenotype in vivo (*20*). However, because spontaneous memory responses to WT1 are rare/nonexistent in healthy individuals, and were absent in the HLA-matched donors in our study, the CTL clones for therapy were most likely derived from the naïve repertoire rather than from a memory population (*37*). When used with IL-15 during the priming of antigen-specific CD8$^+$ T cells, the γ_c cytokine IL-21 has been shown to drive

Table 2. Assessment of clinical outcomes.

Patient	IL-21	Leukemia WT1 expression	Leukemia burden at time of CTL infusions	CTL persistence (days)	Outcome*	GVHD after CTL?	Additional treatment after CTL?	Alive/dead
1	No	Yes†	Present	14	Progressed while receiving CTL	No	Yes	Dead
2	No	Yes†	Present	14	Relapsed while receiving CTL	No	Yes	Dead
6	No	Unavailable	Absent	14	CR/chronic GVHD 4.9 years after CTL, 6 years after HCT	Yes (preexisting)	No	Alive
10	No	Yes†	Present	14	Relapsed while receiving CTL	No	Yes	Dead
15	No	Yes†	Present	14	Progressed after responding to first CTL infusion	No	No	Dead
17	No	Yes†	Absent	7	Relapsed D+132 after CTL	No	No	Dead
20	No	Yes†	Present	5	Progressed while receiving CTL	No	Yes	Dead
21	Yes	Yes‡	Absent	430	CR 30 months after first CTL, 35 months after HCT	No	No	Alive
24	Yes	Yes‡	Absent	430+	CR 28 months after first CTL, 33 months after HCT	No	No	Alive
27	Yes	Yes†	Present	430+	CR 19 months after first CTL, 22 months after HCT	No	No	Alive
28	Yes	Yes‡	Absent	230+	CR 18 months after first CTL, 38 months after HCT	No	No	Alive

*As of August 15, 2012. †Assessed by polymerase chain reaction (PCR). ‡Assessed by immunohistochemistry.

further expansion and prevent apoptosis of the cells responding to antigen stimulation, and leads to in vitro generation of $CD8^+$ T cells with a $CD28^{hi}$, less terminally differentiated phenotype in both murine and human studies (*23*, *38*). Here, the WT1-reactive CTL clones that had been primed in the presence of IL-21 expressed significantly higher levels of CD27, CD28, and CD127 before infusions, consistent with a less differentiated phenotype, compared to CTL clones generated without IL-21. Similar to previous observations in a murine model (*22*), the less differentiated phenotype induced by IL-21 gave the CTL clones enhanced capacities for in vivo persistence and proliferation compared to CTL clones generated in the absence of IL-21. The latter expressed a fully differentiated effector phenotype before infusion, stopped proliferating shortly after transfer, and exhibited only brief in vivo survival (≤14 days). Furthermore, as previously shown in the murine model of LCMV infection, the persisting cells generated with exposure to IL-21 displayed phenotypic and functional characteristics of T_{cm} (*39*).

Although the number of patients treated is insufficient to allow definitive conclusions, the data suggest that CTL clones derived from healthy donors and primed in vitro in the presence of IL-21 may provide a method to establish long-lived memory responses, bypassing the alternative strategy of generating CTL populations for transfer from T_{cm} reactive with tumor antigens that must already exist in vivo, which may often not be feasible. The high antigenic burdens of persistent/recurrent leukemia in most of the patients who received CTL clones generated in the absence of IL-21 may have contributed to their short survival (*40*). However, leukemic blasts were not detected in two patients for whom the CTL clones were generated without exposure to IL-21, and even in this setting, long-term T cell survival was not observed in either patient.

Direct evidence of antileukemic activity was observed in patient 15, but the response was short-lived because leukemic blasts rapidly repopulated the peripheral blood after the transferred CTLs were cleared. The patient was not eligible for subsequent T cell infusions because of toxicity from extensive previous cytotoxic therapies that fulfilled exclusion criteria. Exposure to IL-21 during priming may have been able to endow the cells transferred into this patient with sufficient survival and proliferative ability to maintain a more prolonged antileukemic effect, but could not be assessed because patient 15 was treated before IL-21 became available for this study. However, direct evidence of antileukemic activity was observed in patient 27, who had MRD at the time of T cell therapy and received a CTL clone generated with exposure to IL-21. In this patient, the long-term persistence of the infused T cells was associated with disappearance of the leukemic cells and a sustained

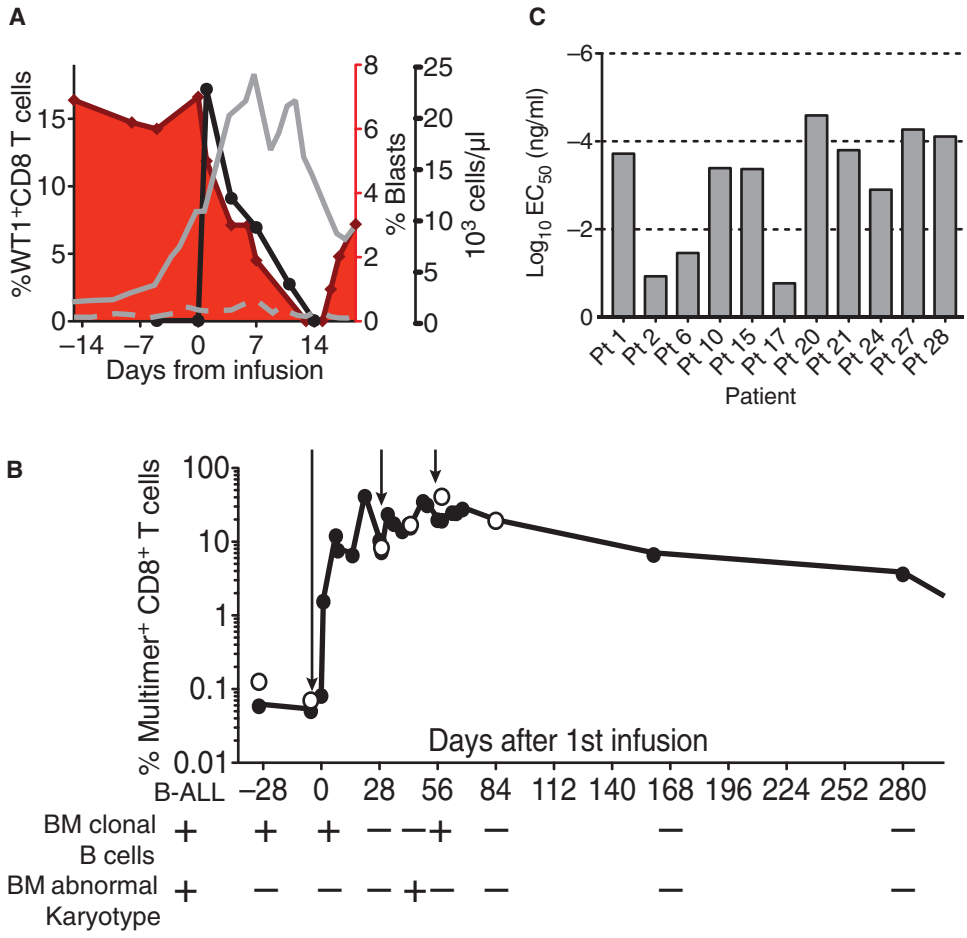

Fig. 6. Evidence of antileukemic activity. (**A**) Percent WT1-specific multimer$^+$ cells detected in PBMCs (solid black circles, black line) of patient 15 after infusion of 3.3×10^9 cells/m^2 (left *y* axis) and concurrent percent leukemic blasts in the blood (red area) (inner right *y* axis). Total WBCs (solid gray line) and lymphocytes (dashed gray line) (outer right *y* axis) in samples collected before and after the infusion (*x* axis). (**B**) Percent multimer$^+$CD8$^+$ T cells (*y* axis) among PBMCs (solid circles) and in BM (open circles) collected before and after infusions for patient 27. Arrows indicate time of infusions. Below the graph are characteristics of the patient's primary B-ALL at diagnosis (left column, below B-ALL) and at time points at which clonal B cells or the abnormal karyotype were analyzed and detected (+) in BM. (**C**) Mean effective concentrations of peptide required to achieve 50% lysis (EC_{50}) of pulsed T2 B-LCL at an E:T ratio of 10:1 by the CTL clones infused for each patient.

CR. The other three patients who received CTL clones generated in the presence of IL-21 had no measurable disease at the time of T cell infusions, but all had exceptionally high risks of relapse (table S1). All are surviving in CR without GVHD or additional antileukemic therapy. The absence of leukemic relapse in these patients cannot be definitively attributed to the continued presence of WT1-specific CTLs because of the lack of a comparative group, but these promising results warrant further study. Additionally, both the achieved frequency and persistence of functional transferred WT1-specific T cells generated with exposure to IL-21 (four of four) are markedly better than results obtained by vaccination to WT1 (*14*).

The results of our study suggest that targeting WT1 with T cells is safe and can lead to antileukemic activity, but in its current format, transferred T cells may not be sufficient to achieve a clinical benefit in all treated patients. Because WT1 overexpression is not restricted to leukemias but rather is present in many tumor types (*41*), the safety observed in this study also supports expanding efforts to treat other malignancies, particularly using strategies described here for establishing persistent WT1-specific responses. Although the most avid CTL clone generated for each patient-donor pair was selected for infusion on the basis of the ability to lyse targets pulsed with titrated doses of WT1 peptide, the avidities of the CTL clones obtained were variable. More reproducible clinical results might be achieved with T cell therapy if the infused CD8$^+$ T cells exhibited consistent comparably high avidities. Strategies to accomplish this are becoming increasingly available, including such methods as transducing patient T cells with a characterized high-affinity WT1-specific T cell receptor (TCR) that imparts high avidity for leukemic targets, a technology already being used to target other antigens (*42*, *43*). The use of such TCRs, in concert with expanding the transduced T cells in the presence of IL-21 and/or directly transducing cells derived from a CD8$^+$ T_{cm} pool, may predictably provide patients with potent persistent responses for the treatment of leukemia (before or after HCT) as well as solid tumors.

MATERIALS AND METHODS

Clinical protocol and patient characteristics

All clinical investigations were conducted according to the Declaration of Helsinki principles. Protocol 1655 was approved by the Fred Hutchinson Cancer Research Center (FHCRC) Institutional Review Board (IRB) and the U.S. Food and Drug Administration (FDA). The trial was registered at clinicaltrials.org as NCT00052520. Enrolled HLA-A*0201 patient-donor pairs provided written informed consent before receiving an HLA-matched allogeneic HCT for AML, ALL, MDS, or CML defined as high-risk, comprising MDS RAEB or RAEB-T, CML beyond chronic phase, AML beyond first remission, Philadelphia chromosome (*BCR-abl*)–positive ALL at any stage, any ALL beyond first remission, primary refractory AML or ALL, and secondary AML (*33*, *44*–*46*). Patients with molecular, cytogenetic, or morphologic evidence of relapse after HCT (treatment of active disease), or after recovery of hematopoiesis after HCT if transplanted with >5% blasts in the pre-HCT BM because of the greater risk of early relapse (prophylactic therapy), were eligible to receive escalating doses of WT1-specific CTLs generated by leukapheresis from the patient's matched donor. Between March 2006 and August 2010, 37 patient-donor pairs were enrolled, WT1-specific CTL clones were generated for 24 patients, and 11 patients received CTL infusions (fig. S7).

Treatment plan

The first two treated patients (patients 1 and 2) received escalating doses of WT1-specific CTLs, starting with 3.3×10^8 CTLs/m^2 on day 0, then 1.0×10^9 CTLs/m^2 on day 7, 3.3×10^9 CTLs/m^2 on day 14, and again on day 28, with the last cell dose followed by low-dose subcutaneous IL-2 (250,000 IU/m^2 twice daily) × 14 days. The next two treated patients (patients 6 and 10) received the same regimen with an additional dose of 1.0×10^{10} CTLs/m^2, followed by low-dose subcutaneous IL-2 × 14 days on day 49 (21 days after the last infusion). No major toxicities were observed, so the protocol was modified to increase the likelihood of delivering adequate cell doses to patients before disease progression, and all remaining patients received 3.3×10^9 CTLs/m^2 on day 0, 1.0×10^{10} CTLs/m^2 on day 14, and 1.0×10^{10} CTLs/m^2 on day 28 followed by low-dose subcutaneous IL-2 × 14 days (fig. S1). All the described modifications were reviewed by the FHCRC IRB and the FDA. Patients were monitored for toxicities on the basis of Common Toxicity Criteria v4.0 (*47*). BM aspirates were obtained for analysis within 14 days of the first CTL infusion, 1 day after the second infusion, and 1 day after the last dose of subcutaneous IL-2, and then as clinically indicated.

Isolation and expansion of WT1-specific CTL clones

All ex vivo manipulations involving processing of products destined for infusion were performed in the current good manufacturing practice Cell Processing Facility of the FHCRC. Donor PBMCs were obtained by leukapheresis, and $CD8^+$ T cells were bead-selected (Miltenyi Biotec Inc.) and stimulated up to three times for 7- to 10-day cycles with autologous dendritic cells (DCs) pulsed with the A*0201-restricted $WT1_{126–134}$ (RMFPNAPYL) peptide (AnaSpec) at a DC-to-effector ratio of 1:2 to 1:10 to obtain sufficient frequencies (>5%) of WT1-reactive $CD8^+$ T cells. On day 2 of each stimulation, the γ_c chain cytokines IL-2 (12.5 IU/ml), IL-7 (5 ng/ml), and IL-15 (1 ng/ml) were added. For patients 21, 24, 27, and 28, IL-21 (30 ng/ml) was also added once on day 0 of each stimulation cycle before limiting dilution cloning. Clones were screened for binding to the $WT1_{126–134}$ peptide–major histocompatibility complex multimer, and the most avid clones (based on lysis of T2 B-LCL pulsed with decreasing concentrations of the $WT1_{126–134}$ peptide) were further selected for expansion (*25*, *37*). CTL clones were analyzed for surface expression of CD3, CD8, CD4, CD45RO, CD27, CD28, CD127, CD62L, and CCR7, and for cytotoxicity. Most selected clones were tested for monoclonality by analysis of TCR-Vβ usage (table S4). Briefly, DNA was isolated from WT1-specific T cell clones, and the TCRβ chains were amplified by RACE PCR (Clontech). For each clone, only a single band was detected from $\geq 5 \times 10^5$ cells, which was then amplified and sequenced. The TCRβ chain and the complementarity determining region 3 sequence were determined with the IMGT program (http://www.imgt.org).

SUPPLEMENTARY MATERIALS

www.sciencetranslationalmedicine.org/cgi/content/full/5/174/174ra27/DC1
Materials and Methods
Fig. S1. Treatment plan.
Fig. S2. Effect of low-dose subcutaneous IL-2 on post-infusion WT1-specific CTL frequencies.
Fig. S3. Dynamics of $CD4^+$ T_{regs} after WT1-specific CTL infusions and exogenous low-dose subcutaneous IL-2.
Fig. S4. Phenotypic changes of WT1-specific CTLs before and after infusions.
Fig. S5. Effect of low-dose subcutaneous IL-2 on post-infusion WT1-specific CTL proliferation 4 days after transfer.
Fig. S6. Correlation of WT1 expression by PCR with blast percentages in blood and BM.
Fig. S7. Flow diagram of patients enrolled in the clinical study.
Fig. S8. FoxP3 expression by $CD4^+CD25^+CD127^-$ T cells.
Table S1. List of adverse events related to treatment.
Table S2. Adverse prognostic factors of treated patients.
Table S3. Prognosis of the patients who demonstrated long-term persistence of transferred WT1-specific CTL clones.
Table S4. Monoclonality of WT1-specific CTL clones.

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Transferred WT1-Reactive CD8$^+$ T Cells Can Mediate Antileukemic Activity and Persist in Post-Transplant Patients

Aude G. Chapuis, Gunnar B. Ragnarsson, Hieu N. Nguyen, Colette N. Chaney, Jeffrey S. Pufnock, Thomas M. Schmitt, Natalie Duerkopp, Ilana M. Roberts, Galina L. Pogosov, William Y. Ho, Sebastian Ochsenreither, Matthias Wölfl, Merav Bar, Jerald P. Radich, Cassian Yee and Philip D. Greenberg

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Targeting Leukemic Cells for Destruction

After a patient is diagnosed with leukemia, the first line of treatment is generally chemotherapy. If it doesn't work, the patient can get a bone marrow transplant, which can sometimes cure otherwise untreatable leukemia. However, for patients who have already relapsed after a transplant, or whose cancer has particularly unfavorable characteristics, few options remain. Such patients' prognosis is generally very poor, with a high risk of relapse and death from leukemia. Immune cells derived from the donor bone marrow can help fight the cancer by attacking malignant cells inside the graft recipient. At the same time, however, they often attack the recipient's healthy cells as well and cause graft-versus-host disease (GVHD), which can itself be lethal. Now, Chapuis and coauthors report a way to harness the power of the donors' immune cells against some leukemias, without triggering GVHD in the bone marrow recipients.

In this pilot trial, the authors enrolled 11 patients who had leukemia with poor prognostic characteristics and who had each undergone a bone marrow transplant. The patients then received T cells that were derived from t heir respective donors and selected for their ability to recognize Wilms tumor antigen 1 (WT1). Very small amounts of WT1 are present in some healthy cells, but its expression in malignant cells is much greater and corresponds t o the aggressiveness of the cancer. In the first seven patients, the authors found that T cells that recognized WT1 c ould suppress the leukemia temporarily, but the new cells disappeared within 14 days, and the cancer rebounded. In the last four patients, the authors tried a modified protocol, pretreating the cells with interleukin-21 (IL-21) before infusion into the recipients. T cells pretreated with IL-21 developed characteristics of memory cells, including a g reatly extended life span. At this time, all four patients who received IL-21–treated T cells are still alive, with no recurrence of their cancer up to 30 months after the T cell infusion. Notably, none of the patients who received WT1-specific T cells in this study have developed GVHD, supporting the idea that WT1 targeting is specific to the tumor cells and safe for patient use.

The study by Chapuis *et al.* is a small pilot trial, and its results must be replicated with larger groups of patients before this protocol can become standard practice. Nevertheless, the combination of targeting WT1 in leukemia and pretreating leukemia-targeting T cells with IL-21 to extend their life span looks very promising and offers a potential safe and effective treatment for patients who have few other options.