Cancer Therapy: Preclinical

Generation of Tumor Antigen-Specific T Cell Lines from Pediatric Patients with Acute Lymphoblastic Leukemia—Implications for Immunotherapy

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Abstract

Purpose: Although modern cure rates for childhood acute lymphoblastic leukemia (ALL) exceed 80%, the outlook remains poor in patients with high-risk disease and those who relapse, especially when allogeneic hematopoietic stem cell transplantation is not feasible. Strategies to improve outcome and prevent relapse are therefore required. Immunotherapy with antigen-specific T cells can have antileukemic activity without the toxicities seen with intensive chemotherapy, and therefore represents an attractive strategy to improve the outcome of high-risk patients with ALL. We explored the feasibility of generating tumor antigen-specific T cells ex vivo from the peripheral blood of 50 patients with ALL [26 National Cancer Institute (NCI) high-risk and 24 standard-risk] receiving maintenance therapy.

Experimental Design: Peripheral blood mononuclear cells were stimulated with autologous dendritic cells pulsed with complete peptide libraries of WT1, Survivin, MAGE-A3, and PRAME, antigens frequently expressed on ALL blasts.

Results: T-cell lines were successfully expanded from all patients, despite low lymphocyte counts and irrespective of NCI risk group. Antigen-specificity was observed in more than 50% of patients after the initial stimulation and increased to more than 90% after three stimulations as assessed in IFN-γ enzyme-linked immunospot (ELISpot) and 51Cr-release assays. Moreover, tumor-specific responses were observed by reduction of autologous leukemia blasts in short- and long-term coculture experiments.

Conclusion: This study supports the use of immunotherapy with adoptively transferred autologous tumor antigen-specific T cells to prevent relapse and improve the prognosis of patients with high-risk ALL.

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Introduction

The outcome of patients with acute lymphoblastic leukemia (ALL), the most common childhood leukemia, has significantly improved with the advent of intensive chemotherapy regimens that can achieve cure rates of more than 90%. However, for patients classified as high-risk, according to National Cancer Institute (NCI) criteria, or those who relapse, the best chance of cure is allogeneic hematopoietic stem cell transplantation (HSCT; ref. 1). Not all patients are eligible for HSCT and over a third of transplanted patients still relapse after HSCT for ALL (1, 2). Therefore, strategies to prevent relapse in patients with ALL, classified as high-risk, are needed. Immunotherapy is an effective tool for the treatment of leukemia. First shown in patients with chronic myelogenous leukemia (CML), the infusion of unselected donor lymphocytes (DLI) has been shown to stably eradicate minimal residual disease (3, 4). In ALL, a high absolute lymphocyte count at the end of induction therapy is associated with improved survival, suggesting the importance of the host immune response in controlling disease (5). In addition, in the transplant setting, there is strong evidence for a T cell–mediated graft-versus-leukemia effect (6). However, DLI rarely induces responses in patients relapsing after HSCT (7–9), suggesting that the antileukemic effects of T cells may require augmentation to prevent or treat relapsed ALL. Recently, T cells genetically modified with chimeric antigen receptors (CARs) targeting a CD19 epitope were shown to induce prolonged remissions in patients with relapsed ALL (10, 11). However, relapse can occur by selection of a CD19 negative leukemia cell population (12). We have shown that T cells with native tumor-specificity can be activated and expanded using single epitopes.
of tumor antigens as stimulus in vitro for adoptive cell transfer or in vivo in response to vaccines (13, 14). Furthermore, we have shown that, in healthy donors, it is possible to induce T cells specific for multiple tumor antigens which can target and kill acute myeloid leukemia cells (15).

We therefore chose to evaluate the feasibility of generating and expanding tumor antigen-specific T-cell lines from patients with ALL as a potential adoptive immunotherapeutic strategy to prevent relapse in high-risk patients or patients not eligible for allogeneic HSCT. ALL cells express a number of tumor-associated antigens (TAAs). We selected WT1 (16), Survivin (17, 18), MAGE-A3 (19), and PRAME (6, 20) as target antigens for the generation of tumor antigen-specific T cells with the aim of broadening the applicability of T-cell therapy to the majority of patients with high-risk ALL.

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In this study, we have developed a novel strategy to activate autologous T cells targeting multiple tumor antigens. We showed that by using both autologous dendritic cells and phytohemagglutinin (PHA)-blasts as antigen-presenting cells (APC), we can successfully expand TAA-specific T-cell lines from 50 patients with ALL during maintenance therapy, irrespective of NCI risk status or lymphocyte count.

**Materials and Methods**

**Patient samples**

Peripheral blood was obtained from 50 pediatric patients with ALL, receiving maintenance chemotherapy at the Texas Children’s Cancer Center (Houston, TX). All families had provided written informed consent on treatment protocols approved by the Baylor College of Medicine Institutional Review Board in accordance with the Declaration of Helsinki. Approximately, 40 mL of blood was collected from 50 patients with ALL, 24 standard-risk and 26 high-risk patients, according to criteria (ref. 21; Supplementary Table S1). Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation and cryopreserved.

**Generation of antigen-presenting cells**

Monocyte-derived dendritic cells were generated by plate adherence of PBMC. PBMC were incubated for 2 hours in dendritic cell media (CellGro DC media; CellGenix) supplemented with 2 mmol/L Glutamax (Invitrogen). Non-adherent cells were collected and washed. Adherent cells were cultured in dendritic cell media in the presence of interleukin (IL)-4 (1,000 U/mL) and granulocyte macrophage colony-stimulating factor (GM-CSF; 800 U/mL; both R&D). On day 5, immature dendritic cells were matured in dendritic cell media with a cytokine cocktail consisting of IL-4 (1,000 U/mL), GM-CSF (800 U/mL), IL-6 (10 ng/mL), TNF-α (10 ng/mL), IL-1β (10 ng/mL; all R&D), and PGE2 (1 µg/mL; Sigma-Aldrich), and were harvested after 48 hours of maturation for use as APC.

For PHA-blast generation, PBMC were stimulated with the mitogen PHA-P (5 µg/mL; Sigma-Aldrich) in presence of IL-2 to promote blast formation (PHA-blasts). PHA-blasts were cultured in RPMI-1640 supplemented with 5% human serum (GemCell; Gemini Bio-Products), 2 mmol/L Glutamax, and IL-2 (100 U/mL; Teceleukin; Chiron Therapeutics).

**Generation of TAA-specific T-cell lines**

TAA-specific T-cell lines were generated from total PBMC. Matured dendritic cells were harvested and used as APC and peptide-pulsed with a mix of four peptide libraries (PepMix; JPT Peptide Technologies): WT1, Survivin, MAGE-A3, and PRAME. Dendritic cells were used at a stimulator-to-effector ratio of 1:10. T cells were cultured in RPMI-1640 supplemented with 45% Clicks media (Irvine Scientific), 5% human AB serum, and 2 mmol/L Glutamax. For initial stimulation, a cytokine mix containing IL-7 (10 ng/mL), IL-12 (10 ng/mL), IL-15 (5 ng/mL) (all Peprotech), and IL-6 (100 ng/mL) was added (15, 22). T cells were restimulated with peptide-pulsed autologous irradiated (30 Gy) PHA-blasts at a ratio of 1:1 on day 10 to 12 and cultures were maintained in IL-2 (50 U/mL)—supplemented media and restimulated every 7 days as described previously for a minimum of four stimulation cycles, but could be kept in culture up to eight restimulations without loss of specificity. No further selection or enrichment of T cells was carried out at any point throughout the sensitization and expansion period.

**IFN-γ enzyme-linked immunospot assay**

Peptide recognition was tested in an IFN-γ enzyme-linked immunospot (ELISpot) assay (23). Recognition of the pooled TAs as well as single antigens was tested as
compared with no-peptide (media) control (labeled as control in all graphs) and irrelevant peptide (NY-Eso-1), which was not used for T-cell generation.

Millipore Multi Screen HTS filter plates (Millipore) were coated with IFN-γ capture antibody (Mabtech) at a concentration of 10 μg/mL overnight at 4°C. Plates were washed with PBS and blocked for 1 hour at 37°C to rule out nonspecific protein binding. T cells were washed and resuspended and stimulated with PepMix or single peptides at a concentration of 0.1 mg/mL. The plates were incubated for 16 to 20 hours at 37°C. For development, plates were washed in PBS/0.05% Tween 20 and incubated with biotinylated IFN-γ detection antibody (0.5 μg/mL; Mabtech) for 2 hours at 37°C, followed by incubation with streptavidin-coupled alkaline phosphatase complex (Vectastain; Vector Laboratories) for 1 hour at room temperature and spots were developed by incubation with 3-amino-9-ethylcarbazole substrate solution. Spot-forming cells (SFC) were counted and evaluated by Zellnet Consulting using an automated plate reader system (Karl Zeiss).

HLA-blocking experiments

HLA-restriction of antigen recognition was tested in IFN-γ-ELISpot using autologous PHA blasts pulsed with the relevant peptide (positive control) or without peptide (negative control) and blocking antibodies against HLA class I and II (both BD Biosciences). ELISpot plates were incubated and developed as described earlier.

51Chromium-release cytotoxicity assay

Cytolytic activity of T cells was assessed in a 51Chromium (51Cr)-release cytotoxicity assay. Autologous PHA blasts were peptide-pulsed and labeled with 51Cr for 1 hour at 37°C, washed, and resuspended. Effector cells were used at effector-to-target (E:T) ratios of 40:1 to 1.25:1 and tested against peptide-pulsed target cells [specific and irrelevant peptide (NY-Eso-1) not used for T-cell generation] and unpulsed target cells and incubated for 4 to 6 hours. Spontaneous lysis was determined by measuring 51Cr-release into the supernatant on a gamma-counter. Spontaneous release was assessed by incubating target cells alone, maximum lysis by addition of 1% Triton X-100 (Sigma-Aldrich). Specific lysis was calculated as follows:

\[
\text{Specific lysis (\%)} = 100 - \left(\frac{\text{experimental release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}}\right)
\]

Phenotyping of T-cell lines

T-cell lines were phenotyped by extracellular antibody staining with anti-CD3, CD4, CD8, CD45RA and RO, CCR7, CD62L, CD56, and CD19 (all BD Biosciences) and analyzed on a BD FACSCalibur Flow Cytometer. Control samples labeled with appropriate isotype-matched antibodies were included in each experiment. Data were analyzed using FlowJo Flow Cytometry software (TreeStar).

Coculture with autologous blasts

To test recognition of autologous blasts, TAA-specific T-cell lines were cocultured with each patient’s autologous leukemic bone marrow sample, which had been cryopreserved at diagnosis. Coculture experiments were carried out at ratios of 10:1 or 5:1 (T cells:leukemic blasts) for 3 days in presence of IL-2 (50 U/mL). Nonspecific autologous T cells were used as negative controls. Leukemia cells were quantified by costaining with anti-CD10 and CD19 antibodies and samples were acquired on a BD FACSCalibur Flow Cytometer using Count Bright absolute counting beads (Molecular Probes) for quantification of absolute cell counts.

ELISA

Detection of cytokine release in the supernatants of cocultured T cells was analyzed by an ELISA specific for IFN-γ and IL-4 (both R&D). For detection of IFN-γ, a 96-well microplate was coated with anti-IFN-γ capture antibody and incubated overnight. Cell culture supernatants were incubated and specific binding of IFN-γ was detected by incubation with a biotinylated detection antibody, streptavidin-coupled horseradish peroxidase and developed by incubation with substrate solution. Optical density of plates was read at 450 nm with a wavelength correction at 570 nm. For IL-4, the same principle was applied and optical density was determined at 490 nm. Cytokine release was quantified in comparison with a specific standard; the assay was run in duplicates.

Colony-forming unit assay

A colony-forming unit (CFU) assay was carried out to show blast elimination in a long-term coculture. T cells were cocultured with autologous leukemic blasts at a 10:1 ratio in Iscove’s modified Dulbecco’s medium (IMDM; Invitrogen) substituted with 10% PBS and 2 mmol/L GlutaMax for 6 hours. After this preincubation, wells were collected and incubated in MethoCult methylcellulose media (STEM-CELL Technologies) in duplicates. Colonies were counted on day 14 and relative inhibition of colony formation calculated in comparison with nonspecific T cells as follows:

\[
\text{Relative inhibition (\%)} = 100 - \left(\frac{\text{CFU}_{\text{specific T cells}}}{\text{CFU}_{\text{nonspecific T cells}}}\right) \times 100.
\]

Statistical analysis

Data are summarized as mean ± SD or mean ± SE, as noted in the text or figure legends. Student t test was used to determine whether there was a statistically significant difference between samples, with two-tailed P values less than 0.05 indicating a significant difference.

Results

TAA-specific T-cell lines can be reliably expanded from patients with ALL on maintenance therapy, irrespective of their NCI risk status or absolute lymphocyte count.

TAA-specific T-cell lines were generated from peripheral blood obtained from 50 patients with ALL, 26 designated as...
high-risk and as 24 standard-risk according to NCI criteria, during the maintenance phase of chemotherapy. Blood samples were collected at least 3 months after the start of maintenance therapy to minimize effects of prior, more intensive chemotherapy and were used for the generation of dendritic cells, PHA-blasts, and T-cell lines. Stimulation of PBMC was carried out with antigen–pulsed dendritic cells using four complete peptide libraries spanning the entire amino acid sequence of the TAAs WT1, Survivin, MAGE-A3, and PRAME.

Generation of dendritic cells as well as T-cell lines was possible from all samples obtained, despite 76% of patients having low absolute lymphocyte counts of less than 1,000/μL (Supplementary Table S1). At least a minimal expansion of 1.2-fold could be achieved in all patients with a maximum 31.4-fold expansion (mean 6.4-fold) after three stimulations (Fig. 1A). Phenotyping of the ex vivo–expanded T-cell lines showed a mean CD3+ content of 97.2% (range, 80.3%–99.9%) and varying distribution of CD4+ (mean, 38.4%; range, 8.3%–89.4%) and CD8+ (mean, 42.6%; range, 7.9%–82.1%) T cells, few natural killer cells (mean, 1.3%; range, 0%–10.9%) and rare residual B cells (mean, 0.2%; range, 0%–5.8%; Fig. 1B). The majority of both CD4+ (mean, 51.2%; range, 12.0%–93.0%) and CD8+ (mean, 49.3%; range, 4.0%–91.0%) T cells were composed of CD45RO+/CD62L−/CCR7− T cells in accordance with an effector-memory phenotype. Very few naïve CD45RA+/CD62L+/CCR7+ cells (CD4+: mean, 1.0%; range, 0%–8%; CD8+: mean, 0.2%; range, 0%–3.0%) and central-memory CD45RO+ /CD62L−/CCR7+ cells (CD4+: mean, 5.2%; range, 0.1%–33.0%; CD8+: mean, 2.9%; range, 0%–13.0%) were present after three restimulations (Fig. 1C and D).

Ex vivo–expanded TAA-specific T cells recognize multiple target antigens with broad epitope specificity

Antigen specificity of the ex vivo–expanded T-cell lines was evaluated weekly in response to the mix of TAAs and individual antigens in IFN-γ-ELISpot assay. Specific reactivity against the pooled TAAs after the initial stimulation could be seen in 54% of standard-risk and 65% of high-risk patients (data not shown). After three stimulations, 92% of standard-risk and 96% of high-risk patients showed specific responses against at least one antigen. There was almost equal distribution of standard-risk versus high-risk patients showing reactivity against one (21% standard-risk; 19% high-risk), two (21% standard-risk; 23% high-risk), three (21% standard-risk; 23% high-risk), or all four antigens (29% standard-risk; 19% high-risk; Fig. 2A). The majority of the expanded TAA-specific T-cell lines elicited responses against PRAME (79% standard-risk vs. 69% high-risk), followed by WT1 (71% standard-risk vs. 54% high-risk) and MAGE-A3 (58% standard-risk vs. 57% high-risk). In contrast, T-cell responses to Survivin were seen in a minority of patients (42% standard-risk vs. 27% high-risk; Fig. 2B). Similarly, highest specificity as measured by spot counts in IFN-γ-ELISpot assays, was seen in response to the pooled TAAs (standard-risk: mean, 312, range, 31–970; high-risk: mean, 258, range, 17–777) and PRAME (standard-risk: mean, 230, range, 0–851; high-risk: mean, 173, range, 2–681), followed by MAGE-A3 (standard-risk: mean, 168, range, 0–1,324; high-risk: mean, 127, range, 0–562). WT1 (standard-risk: mean, 121, range, 2–534; high-risk: mean, 145, range, 2–642), and least against Survivin (standard-risk: mean, 109, range, 0–541; high-risk: mean, 84, range, 2–498; Fig. 2C).

Figure 1. Expansion and phenotype of T-cell lines. A, n-fold (mean ± SD) expansion of antigen-specific T-cell lines generated from patients with ALL during maintenance therapy. Cell counts were assessed at the end of each restimulation cycle on days 10, 18, and 25 of culture (n = 50). B, phenotype of T-cell lines after the third stimulation; gated on live lymphocytes, C, T-cell subsets within the CD3+/CD4− population, D, T-cell subsets within the CD3+/CD8− population.
Cytolytic activity of T-cell lines was tested in a standard $^{51}$Cr-release assay against peptide-pulsed autologous PHA-blasts. Mean lysis of target cells pulsed with the pooled TAs at an E:T ratio of 20:1 was 27% for T-cell lines generated from the standard-risk patients (range, 0%–77%) versus 31% for the high-risk patients (range, 0%–90%). Killing of targets pulsed with the individual antigens for the standard-risk patients showed: mean, 13% (range, 0%–66%) for WT1; mean, 10% (range, 0%–58%) for Survivin; mean, 12% (range, 0%–70%) for MAGE-A3; and mean, 26% (range, 0%–74%) for PRAME. No appreciable differences in cytolytic responses were seen in T-cell lines generated from high-risk patients: mean, 15% (range, 0%–100%) for WT1; mean, 8% (range, 0%–58%) for Survivin; mean, 16% (range, 0%–68%) for MAGE-A3; and mean, 21% (range, 0%–55%) for PRAME (Fig. 2D). No killing ($<60\%$) of unpulsed target cells was observed.

**Polyclonal MHC class I- and II–mediated T-cell responses can be elicited in vitro from patients on treatment of ALL**

To compare the epitope-specific responses in T-cell lines generated from patients with ALL to those previously described in healthy donors (15, 24–28), we conducted epitope mapping for WT1 in 11 T-cell lines. Reactivity against minipools of 15mer peptides overlapping by 11 amino acids spanning the whole sequence of WT1 was tested, as previously described (29). Subsequently, confirmation of single-peptide recognition and HLA-restriction was conducted (15).

Results of the epitope-mapping experiments of a representative TAA-specific T-cell line are shown in Fig. 3. As seen in Fig. 3A, IFN-$\gamma$-ELISpot assay showed 70 SFC/10^5 cells in response to pooled TAs after the initial stimulation, which increased to 200 SFC/10^5 cells after three in vitro stimulations. The expanded T-cell line recognized both WT1 and PRAME (Fig. 3B). Epitope mapping was then conducted for WT1, which showed reactivity against two regions within WT1, peptides #9/10 (QWAPVLDFAAPGASAYGSL) and peptides #71/72 (LCGAQYRHHTGVRGIQD). Using HLA class I- and class II–blocking antibodies, the overlapping sequence of peptides #71/72 was confirmed to contain an HLA class I–restricted epitope previously published by Wolfl and colleagues (28; Fig. 3C; Table 1). In contrast, recognition of peptides #9/10 was HLA class II–restricted, as confirmed by blocking experiments. This 15mer peptide is known to contain both HLA class I- and II–restricted epitopes, however, different from the HLA-restrictions seen in our patient (ref. 24; Fig. 3D; Table 1). In all, analysis of the WT1-specific T-cell responses in 11 patient-derived T-cell lines identified a total of 11 15mer class I- and II–restricted peptides, as shown in Table 1. The majority of these peptides have previously been described and identified to be immunogenic in healthy volunteers (15, 24–28). Hence, these results show that we can successfully generate antigen-specific T-cell lines from patients with ALL, and the spectrum of the specificity in these lines is similar to that of healthy donors with a range of class I- and II–restricted epitopes (15, 24). Furthermore, the polyfunctionality of the expanded tumor antigen-specific T cells is shown in Fig. 3E where a T-cell line with specificity against all the targeted antigens released both IFN-$\gamma$ and TNF-$\alpha$ in response to stimulation.

**Antigen recognition and antileukemic activity against autologous bone marrow blast samples**

We next investigated whether TAA-specific T cells generated from patients with ALL were able to recognize and kill
autologous leukemia cells in vitro. Coculture experiments were set up for 8 patient-derived TAA-specific T-cell lines using, as targets, autologous ALL blasts obtained from bone marrow aspirates that had been cryopreserved at diagnosis. Antigen-specificity as well as short- and long-term cocultures were evaluated in IFN-γ-ELISpot, flow-based, and CFU assays. In addition, the coculture supernatants were evaluated by IFN-γ and IL-4 ELISA assays. Nonspecific T cells derived from the same patient were used as controls. In ELISpot assays at a 1:1 ratio of leukemia cells to T cells, the
Table 1. Immunogenic WT1 peptides identified by epitope mapping of WT1-specific T cells derived from patients with ALL

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<th>HLA-B</th>
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Abbreviations: n.d., not determined; AA, amino acid.
TAA-specific T cells showed increased IFN-γ-release (mean, 300 SFC/10^5 cells; range, 8–1,701) compared with control T cells (<10 SFC/10^5 cells; Fig. 4A). No cytokine secretion by leukemia cells alone was observed. Coculture of T cells at an E:T ratio of 10:1 in the presence of IL-2 (50 U/mL) showed a variable elimination of autologous leukemia blasts in vitro. After 3 days of coculture with TAA-specific T cells there were 13.6% residual blasts remaining compared with 46.8% in the controls (calculated relative to the initial leukemia cell counts on day 0; P = 0.04; Fig. 4B). Analysis of IFN-γ and IL-4 levels in the coculture supernatants showed high concentrations of IFN-γ by TAA-specific T cells after 3 days of coculture with autologous blasts (mean, 630 ± 364 pg/10^6 cells/mL) compared with control T cells (mean, 0 pg/10^6 cells/mL; P = 0.04). Lower levels of IL-4 were secreted by TAA-specific T cells (mean, 19.6 ± 3.3 pg/10^6 cells/mL; control T cells mean 7.6 ± 1.1 pg/10^6 cells/mL) after coculture with autologous blasts (P = 0.017; Fig. 4C). Furthermore, a specific inhibition of colony formation was observed in CFU assays after 14 days when leukemia blasts were cocultured with TAA-specific T cells compared with control T cells. As shown in Fig. 4D, a mean relative inhibition of 42.3% of colony formation was observed (P = 0.012).

Representative examples for the leukemia-specific activity of TAA-specific T cells are shown in Fig. 5 and supplementary Figs. S2–S8 respectively. In patient ID#1032 designated as standard-risk, peptide reactivity against the pooled TAs in ELISpot assay after initial (Fig. 5A) and three (Fig. 5B) stimulations showed recognition of WT1 and PRAME. Furthermore, cytolytic activity against these antigens was shown in a ^51^Cr-release assay using peptide-pulsed autologous PHA-blasts as targets (Fig. 5C).

Coculture of TAA-specific T cells with autologous blasts showed reduction of autologous leukemia cells to 13% of the initial cell count after 1 day and 4.8% after 3 days. In contrast, when nonspecific T cells derived from the same patient were incubated with autologous blasts, 23% of leukemia blasts remained on day 1 and 10% on day 3 of coculture (Fig. 5D). Reduction of leukemia cell numbers was also apparent when applying absolute

Figure 4. Reactivity of tumor antigen-specific T-cell lines against autologous leukemia cells. Summary of coculture experiments of eight patient-derived tumor antigen-specific T-cell lines against their autologous bone marrow leukemia blast samples cryopreserved at the time of diagnosis. Non-specific T cells or unstimulated cells of the same patient were used as controls in all experiments. A, summary of the IFN-γ-ELISpot coculture results of T-cell lines (n = 8; CTL, black bar) versus non-specific T cells (n = 8; control, white bar; mean ± SE) at an E:T ratio of 1:1. B, in a 3-day coculture at an E:T ratio of 10:1 leukemia cell counts were assessed by FACS analysis and compared with the absolute leukemia cell count obtained on day 0. Leukemia blasts were quantified by anti-CD10 and CD19 costaining; T cells were quantified by LDS positivity. Absolute cell numbers were assessed by quantification with FACS counting beads and the percentage of remaining leukemia cells was calculated compared with cell numbers on day 0 (nonspecific T cells; control, white bars; specific T cell lines, CTL, black bars; mean ± SD). C, IFN-γ and IL-4 ELISA of the coculture supernatants after 24 hours (mean ± SE; control, n = 8; white bars; CTLs, n = 8, black bars). D, relative inhibition of colony formation of leukemic cells in CFU assays when cocultured with specific T-cell lines (n = 8; CTL, black bar) versus nonspecific cells (n = 8; control, white bar; E:T ratio 10:1; mean ± SE).
quantification with fluorescence-activated cell sorting (FACS) counting beads. After 3 days, elimination of leukemia cells was observed when blasts were cocultured with autologous TAA-specific T cells but not control T cells (Fig. 5E). Activation of TAA-specific T cells by the autologous blasts was further shown in IFN-γ-ELISpot assay,
where the T cells showed increased IFN-γ production (mean, 252 SFC/10^5 cells) compared with the control (mean, 128 SFC/10^5 cells; Fig. 5F). To evaluate the expression of the targeted antigens on the ALL blasts, cytospins were stained by immunohistochemistry and showed weak positivity for MAGE-A3 and higher for Survivin (Fig. 5G). Similarly, in Supplementary Figs. S2–S8 the results of the coculture experiments with autologous blasts of the remaining patients are shown, including the 3-day coculture and ELISpot assays as well as the immunohistochemistry of the leukemia blasts. There was no correlation between the generation of effective T-cell lines and leukemia antigen expression in the blasts in the 8 patients where leukemia blasts and peripheral blood samples were available.

Discussion

Patients with ALL who have high-risk disease or who relapse have a high rate of mortality and the best chance of cure is the allogeneic HSCT. There is a need for more effective treatment options for patients not eligible for allogeneic transplant and immunotherapy may be most effective for preventing relapse in high-risk patients after chemotherapy. Therefore, we sought to develop a strategy to generate autologous T cells that target multiple TAAs and show specific antileukemic activity for use as adoptive T-cell immunotherapy to prevent relapse in pediatric ALL. Our rationale for obtaining samples during maintenance therapy was to show feasibility of collection at this time point for potential use as a first-line strategy, to augment therapy in high-risk patients in first remission. We show here that TAA-specific T cells can be generated from patients with ALL during maintenance therapy regardless of NCI risk group and despite low lymphocyte counts at the time of sample acquisition. Patients with high-risk ALL might have more compromised immune systems, correlating with their poor outcome. Nevertheless, we observed no difference in the quality of TAA-specific T cells that could be generated from high- and standard-risk patients. None of the commonly known risk factors for relapse, including age and gender, affected our ability to ex vivo expand TAA-specific T cells from these patients and extensive in vivo testing showed antileukemic activity of the expanded products against autologous ALL blasts in long- and short-term coculture experiments.

Antigen-specific responses were detectable within 7 to 10 days after just a single stimulation in more than half of the tested patients, indicating that the immunosuppression due to the chemotherapeutic drugs administered during maintenance therapy did not preclude the expansion of TAA-specific T cells in vitro. Moreover, upon subsequent restimulation, TAA-specific T cells were expanded from more than 90% of patients, suggesting that the in vivo immunosuppressive environment could be overcome in vitro. At least a minimal 1.2-fold expansion of the autologous T-cell lines was possible in all cases, using methods approved for good manufacturing practices (GMP). Nevertheless, for a clinical scale product, the conditions need to be adjusted to achieve cell numbers necessary for infusion, for example, through use of gas-permeable cell culture flasks (30).

After 3 weeks in culture, the T-cell lines expanded from patients with ALL displayed predominantly an effector-memory phenotype, although small populations of T cells with central memory and naïve phenotypes were also detected. Effector-memory T cells have been shown to have effective antitumor and antiviral efficacy, but are short-lived in vivo, leading to tumor outgrowth after loss of the adoptively transferred cells (31). In contrast, it has been shown that naïve T cells or naïve-derived effector T cells exert the most effective antitumor effects as well as having better in vivo expansion and persistence than effector cells derived from memory subsets (31, 32). We and others previously showed that virus- and TAA-specific T cells can be expanded from naïve T cells derived from cord blood as well as from healthy donors (15, 23, 28, 33, 34). In contrast to virus-specific T cells where memory T cells are the main source of expanded T cells, naïve T cells seem to be an important source of TAA-specific T cells (15, 34). The lymphopenia following high-dose chemotherapy may favor the in vivo generation of T cells from naïve cell populations. However, further experiments will be needed to define the origin of the TAA-specific T-cell lines. In addition to the ability to generate T-cell lines, the adaptive transfer of in vitro–primed T cells into a lymphopenic milieu may favor the persistence and expansion of the transferred T cells, as has been shown by the infusion of predominantly effector-memory T cells in the transplantation setting (35–37).

The antigens we selected for T-cell generation are expressed in a wide variety of hematologic disorders as well as solid tumors. Immunohistochemistry of the available patient samples showed only low levels of antigen expression in the leukemia cells, which might limit the antileukemic effects of the T cells in vivo, despite their high specificity. However, antigen expression in leukemic cells can be enhanced by epigenetically modifying drugs (38) and clinical trials in adults and pediatric patients with acute leukemia are currently under investigation. Furthermore, epigenetic modulation has been shown to render malignant cells more susceptible to T-cell recognition without impairing expansion or function of in vitro–generated T cells (38–40). These observations suggest the feasibility of combining epigenetic modification and adoptive T-cell transfer.

Immunotherapeutic approaches for patients with acute leukemia have included vaccines, which generally target a single-peptide epitope and clinical responses have been observed that seem to correlate with expansion of epitope-specific T cells in vivo. More recently, CD19-specific antibodies (41, 42) and CD19CAR gene-modified T cells targeting the CD19-receptor on B cell malignancies have shown remarkable success for CD19+ malignancies including pediatric ALL (10–12). Our strategy offers a synergistic approach to these strategies. First, our technique covers multiple immunogenic epitopes. This offers the potential for a combined immunotherapeutic approach with vaccines to rechallenge the patients with the antigens used
for T-cell generation and boosts the adoptively transferred T cells in vivo. This may prolong their persistence and increase their potency. Second, our approach permits T-cell generation from all patients without limitation to certain HLA-types, unlike single-peptide vaccine studies (14, 27, 43). Finally, the polyfunctionality and simultaneous targeting of multiple antigens may decrease the risk of tumor immune escape, such as the downregulation of antigen expression, elimination of clones expressing the targeted epitope, or outgrowth of antigen-negative populations, already observed with CD19-directed therapy (12). Therefore, to increase the breadth of the tumor-specific activity by T cells, TAA-specific T cells could be administered in combination with CD19 antibodies or could be genetically modified to express a CD19CAR.

Previous studies have shown the importance of CD4+ T-helper cells for the strength and persistence of in vivo immune responses against viral and tumor antigens (44, 45). In this study, tumor-specific responses were seen in both the CD4+ and CD8+ T-cell populations and immunogenic peptides restricted by MHC class I and II were detected by epitope mapping for WT1. The combination of CD4+–restricted epitopes with the commonly used CD8+ epitopes for T-cell stimulation can improve the survival of the generated T-cell lines and vaccination studies using a mix of MHC class I- and II–restricted peptides show a sustained activation and better survival of specific T cells (44–46). Furthermore, CD4+ T cells can also have a direct cytolytic effect on tumor cells (47, 48). Thus, the infusion of TAA-specific T cells containing antigen-specific CD4+ and CD8+ T cells may facilitate a better antileukemic effect in vivo.

Although it is encouraging that our TAA-specific T cells reduced autologous leukemia blasts in coculture experiments, it remains to be determined whether TAA-specific T cells have therapeutic efficacy in patients with ALL. It would be possible to generate a severe combined immunodeficient (SCID)/hu mouse model of ALL to further test the antileukemic effect of TAA-specific T cells. However, while such a model, using autologous T cells to prevent or treat ALL, might yield positive results, it would not guarantee success in man. Conversely, were the TAA-specific T cells ineffective in the murine model, we would not wish to abandon evaluating TAA-specific T cells in a carefully designed clinical trial, where we can track the fate and function of in vitro–generated TAA-specific T cells and evaluate antileukemic efficacy by measuring residual disease. Given the safety of infused TAA-specific T cells generated in healthy donors (13) and the lack of relevance of animal models for establishing safety, our next step will be to generate GMP grade TAA-specific T cells for a phase I clinical trial.

This study shows that the generation of TAA-specific T-cell lines from the peripheral blood of patients with ALL on maintenance therapy irrespective of NCI risk group is feasible, and that these T cells show antileukemic activity against autologous blasts in vitro. Therefore, adoptive immunotherapy with autologous TAA-specific T cells to boost antileukemic immune responses and prevent relapse seems to be a promising approach and may improve outcomes in high-risk ALL both in the first-line setting, and as a new approach to treat relapsed patients not eligible for allogeneic stem cell transplant in combination with other therapeutic options.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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