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Functional unresponsiveness and replicative senescence of myeloid leukemia antigen-specific CD8⁺ T cells after allogeneic stem cell transplantation

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Abstract

Purpose—The therapeutic effect of allogeneic hematopoietic stem cell transplantation (HSCT) for patients with myeloid malignancies has been attributed in part to a graft-versus-leukemia effect that is dependent on donor T lymphocytes. CD8⁺ T cell responses to MHC class I restricted tumor epitopes, not just allogeneic antigens, may help mediate anti-leukemia effects after HSCT, but the specificity and function of such cells are not completely understood.

Experimental Design—We examined the diversity, phenotype, and functional potential of leukemia-associated antigen-specific CD8⁺ T cells in patients with myeloid leukemia following allogeneic HSCT. Screening for antigen-specific T cells was accomplished with a peptide/MHC tetramer library.

Results—Patients with AML or CML in remission following HSCT exhibited significant numbers of peripheral blood CD8⁺ T cells that recognized varying combinations of epitopes derived from leukemia-associated antigens. However, these cells failed to proliferate, release cytokines, or degranulate in response to antigen-specific stimuli. As early as two months after HSCT, CD8⁺ T cells from patients were predominantly CD28^{neg} CD57⁺ and had relatively short telomeres, consistent with cellular senescence.

Conclusions—Circulating leukemia-specific CD8⁺ T cells are prominent in myeloid leukemia patients after HSCT, but such cells are largely functionally unresponsive, most likely due to replicative senescence. These findings carry important implications for the understanding of the graft-versus-leukemia effect and for the rationale design of immunotherapeutic strategies for patients with myeloid leukemias.

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Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

Introduction

The curative potential of allogeneic hematopoietic stem cell transplantation (HSCT) for myeloid leukemias has been attributed in part to a graft-versus-leukemia (GVL) effect independent of the conditioning regimen and dependent on donor T cells (1). Recent studies demonstrate the existence of leukemia-associated antigens capable of eliciting specific CD8⁺ T cell responses against leukemia cells (2,3), suggesting that targets of graft-versus-leukemia effects may be distinct at least in part from graft-versus-host antigens. Proteinase 3 is an important prototype of such non-polymorphic antigens that are naturally processed and presented by tumor cells in the groove of MHC (4). Proteinase-3-specific CD8⁺ T cells are readily observed in CML patients after allogeneic HSCT (2,5,6) and strongly correlate with clinical outcome (2). Similarly, MHC-restricted CD8⁺ T cells specific for peptides derived from other leukemia antigens such as WT1 and hTERT have also been observed in CML patients after HSCT (5,6). Overall, these observations raise the hypothesis that naturally occurring anti-leukemic cellular immune responses specific for leukemia-associated antigens may be critical for the killing of residual tumor cells in the establishment of minimal residual disease (2,7).

Clinically, the concept of leukemia-specific T cells has been exploited by the use of donor lymphocyte infusions (DLI), particularly for myeloid leukemia (8). Anti-leukemia CD8⁺ T cell responses observed after DLI can be specific for MHC class I restricted epitopes derived from leukemia-associated, non-polymorphic self-antigens, and not just major or minor histocompatibility antigens (8). More recently, clinical trials targeting some of these leukemia-associated antigens have been initiated in patients with myeloid leukemias (3), but the functional and replicative capacity of the antigen specific T cell precursors being targeted in these approaches, particularly after HSCT, is incompletely understood.

In this study, we constructed a library of peptide/MHC tetramers to investigate the development of leukemia-associated antigen-specific CD8⁺ T cells in patients with myeloid leukemia following HSCT. We explored the diversity of CD8⁺ T-cells that recognize an array of leukemia-associated antigenic epitopes and evaluated the function of these cells by testing their ability to proliferate, degranulate, and secrete cytokines in response to cognate peptide stimulation. Our results demonstrate that leukemia antigen-specific T cells are readily observed following allogeneic HSCT but belong to a phenotypic niche consistent with replicative senescence.

Materials and Methods

Blood samples and cell lines

Blood samples were obtained from adult normal donors and patients with myeloid leukemia after institutional review board (IRB) approval and written informed consent. Detailed characteristics of 13 patients who underwent HSCT are shown in Supplementary Table 1. Indications for HSCT were AML (n = 5), C M L (n = 7) and CMML (n = 1) Two of the 13 patients underwent nonmyeloablative HSCT, and one had received donor lymphocyte infusion. One patient was already known to have a molecular relapse at time of sampling but the other 12 patients were in complete remission at the time of sample collection. Three of these patients subsequently relapsed. Eight patients were HLA-A*0201 (HLA-A2) positive. Controls consisted of blood samples obtained from both HLA-A2-positive and -negative normal donors and HLA-A2-negative (non-HSCT) patients with myeloid leukemia. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll centrifugation. Samples were cryopreserved in liquid nitrogen until use. The HLA-A2⁺ transporter associated with antigen processing (TAP)-deficient T2 cell line was obtained from American Tissue Culture Collection (Manassas, VA).

Peptide binding assay

T2 cells were washed in serum free medium, and 10^6 cells were pulsed with 50 $\mu\text{g/mL}$ peptide (New England Peptide, Gardner, MA) in the presence of 2.5 $\mu\text{g/mL}$ $\beta 2$ -microglobulin (Sigma-Aldrich) for 18 h at 37C. Cells were then washed in serum-free medium and incubated for 0 or 6 hrs at 37C to assess the rate of peptide dissociation from HLA-A2. Cells were stained for 30 min on ice with FITC-conjugated HLA-A2 specific Ab or isotype control (Becton-Dickinson) or FITC-conjugated control IgG Ab. HLA-A2 expression was measured using a FACSCalibur flow cytometer and the mean fluorescence intensity (MFI) was recorded. The fluorescence index (FI) was calculated for each peptide at 0 and 6 hours using $\text{FI} = (\text{MFI with peptide} - \text{MFI without peptide}) / (\text{MFI without peptide})$.

MHC class I tetramer analysis, antibodies and flow cytometry

Soluble HLA-A2 tetramers were prepared with peptides and $\beta 2$ -microglobulin as described (9) in the Abramson Cancer Center Human Immunology Core, conjugated to phycoerythrin (PE) or allophycocyanin (APC) or purchased from Beckman Coulter (San Diego, CA). Cells were incubated with PE- or APC-conjugated tetramers at 37C for 30 min in the dark and then with mAbs CD8-FITC, CD8-PE or CD8-APC, CD4-PerCP, and CD14-PerCP for 30 min at 4C. Antibodies against CD4 (Clone SK3), CD14 (Clone M ϕ PD), PD-1 (Clone J116), CD45RA (Clone HI100), CCR7 (Clone 3D12), CD27 (Clone M-T271), CD28 (Clone CD28.2), and CD57 (Clone NK-1) were purchased from BD Biosciences (San Jose, CA). CD8-FITC, -PE, and -APC (IOTest, Clone B9.11) were purchased from Immunotech (Marseilles, France). Data were collected on a FACSCalibur or FACSCanto flow cytometer and analyzed using BD FACSDiva Software (BD Biosciences Immunocytotechnology, San Jose, CA). The percentage of tetramer-positive cells among all CD8⁺ CD14^{neg} CD19^{neg} cells was recorded for each peptide. For each sample, 300,000 events in the lymphocyte gate in the forward scatter/side scatter plot were collected and analyzed.

Intracellular cytokine and CD107a mobilization assay

The ability of antigen-specific CD8⁺ T cells to secrete IFN- γ and mobilize CD107a was determined sequentially by staining for CD107a followed by intracellular IFN- γ staining. PBMC (1×10^6) were cultured in the presence of PE-labeled tetramer at 37C for 20 min. Cells were then mixed with 20 μL of anti-CD107a antibody (Clone H4A3, BD Biosciences) in the presence or absence of 10 $\mu\text{g/mL}$ peptide. After 1 hour of antigen stimulation, BD GolgiStop (BD Biosciences) (4 μL per 6 mL of culture) was added to inhibit protein secretion and the culture was incubated for an additional 4 hours. Following culture, cells were harvested and washed. For detection of cytoplasmic cytokine expression, the cells were stained with PE-anti-CD8 mAb for 20 min on ice. The cells were then fixed and permeabilized with Cytofix/CytoPerm solution (BD Biosciences, San Jose, CA), and stained with FITC-conjugated anti-IFN- γ mAb (Clone B27, BD Biosciences) or isotype control mAb for 30 min on ice. The percentage of cells expressing cytoplasmic IFN- γ and mobilizing CD107a was determined by flow cytometry.

In vitro stimulation assay

PBMC from HLA-A2-positive normal donors and patients were cultured in 24-well plates (5×10^5 cells/well) in complete medium supplemented with 10% heat-inactivated human AB serum. Autologous PBMC were irradiated at 33 Gy and added to non-irradiated PBMC at a ratio of 1:1 in the presence of 1 $\mu\text{g/mL}$ peptide, 10 ng/mL IL-7 (Sigma-Aldrich) and 2.5 $\mu\text{g/mL}$ $\beta 2$ -microglobulin. Human rIL-2 (20 IU/mL) (Chiron Corporation, Emeryville, CA) was added on days 2 and 5. On day 7, cells were harvested and analyzed. For blocking the PD-1/PD-L1 interaction, blocking anti-PD-L1 antibody (clone MIH1, BD Biosciences) or control IgG was added to cell cultures at a concentration of 10 $\mu\text{g/mL}$.

Telomere terminal restriction fragment (TRF) assay

CD3⁺CD8⁺ T cells were isolated by negative selection from PBMC using the human CD8⁺ T cell Isolation Kit (Miltenyi Biotec). Total genomic DNA was prepared from purified cells using a standard proteinase K plus phenol-chloroform method; 1 µg of genomic DNA was digested with a cocktail of RsaI, HinfI, BamHI, and HhaI and analyzed by in-gel hybridization using an end-labeled telomere repeat oligonucleotide (CCCTAAA)₄ as described (10). Mean telomere length was calculated using the weighted average of $\Sigma(OD_i)/\Sigma(OD_i/L_i)$ where OD_i is the background-corrected signal intensity in an interval 'i' and L_i is the average length of telomeres in interval 'i'. Fragments below 2.5kb and above 36.7 kb were excluded to avoid inclusion of interstitial telomere repeats or incompletely digested DNA, respectively.

Results

Selection of peptides and generation of HLA-A2 tetramers

To investigate the repertoire of leukemia-associated antigen-specific CD8⁺ T cells in patients with myeloid leukemia following allogeneic HSCT, we constructed a library of peptide/HLA-A2 tetramers that recognize distinct peptides from a panel of leukemia-associated antigens. Seven distinct antigens were selected for analysis on the basis of prior reports demonstrating expression in myeloid leukemias (4,11–15). The selected antigens were proteinase 3, WT1, PRAME, Meis1, HoxA9, hTERT, and survivin (Supplementary Table 2). HLA-A2-restricted epitopes for each antigen were selected based on previous reports (4,16–22) as well as prediction analysis using peptide binding scores determined with the MHC class I ligand prediction computer-based programs SYFPEITHI (23) and BIMAS (24). Epitopes with the highest predicted likelihood of binding HLA-A2 were evaluated empirically using a conventional binding assay with T2 cells as previously described (18) (Supplementary Table 2). In addition to the previously described proteinase 3 derived peptides, PR1 and PR2 (4), we identified 6 additional epitopes within PR1 that stabilized HLA-A2 molecules. We also identified antigenic epitopes within Meis1 and HoxA9 as well as two new epitopes within WT1. HLA-A2-binding epitopes derived from viral antigens were selected as controls (Supplementary Table 2). For each of the peptides shown in Supplementary Table 2, soluble peptide/HLA-A2 tetramers were manufactured.

Frequency of tetramer-positive CD8⁺ T cells in myeloid leukemia patients following allogeneic stem-cell transplantation

HLA-A2-negative normal donors and HLA-A2-negative non-HSCT patients with myeloid leukemia were initially screened with the library of tetramers to rule out non-specific binding of the reagents. None of the HLA-A2-negative normal donors or HLA-A2-negative patients demonstrated tetramer-positive CD8⁺ T cells specific for any of the leukemia-associated or viral antigenic epitopes (in each case, <0.05% tetramer-positive CD8⁺ cells among all CD8⁺ T cells). We then analyzed whether specific CD8⁺ T cells were present in a cohort of HLA-A2-positive normal donors (n=6), and found that none of these normal donors demonstrated specific CD8⁺ T cells to leukemia-associated antigens (<0.05% tetramer-positive CD8⁺ T cells), but as expected, most normal donors demonstrated specific CD8⁺ T cells for Flu and/or CMV epitopes (range 0.10% to 0.83% tetramer-positive CD8⁺ T cells).

In contrast, for HLA-A2-positive patients with myeloid leukemia, we identified a diversity of tetramer-positive CD8⁺ T cells specific for leukemia-associated antigens after allogeneic HSCT. The frequencies of tetramer-positive CD8⁺ T cells are shown in Table 1 and representative examples are shown in Fig. 1. As previously observed (2,5), we found that 50% of HLA-A2-positive patients following allogeneic HSCT display PR1 specific CD8⁺ T cells (Table 1). We also identified the presence of CD8⁺ T cells that recognize other proteinase 3 epitopes as well as epitopes derived from WT1, PRAME, Meis1, HoxA9, hTERT and survivin

(Table 1 and Fig. 1). Each patient was found to display a unique signature of CD8⁺ T cells reactive to leukemia-associated antigenic epitopes. Among the antigenic epitopes we analyzed, a majority of patients displayed CD8⁺ T cells that recognized PR1 (4 of 8 patients), PR-QLP (6 of 8 patients), PR-GII (4 of 7 patients), and hTERT (6 of 8 patients).

Peptide-specific CD8⁺ T cells after *in vitro* stimulation of T cells

We analyzed whether the identified leukemia-associated antigen-specific CD8⁺ T cells possessed a capacity to proliferate *in vitro*. Patient PBMC were incubated in the presence of irradiated autologous PBMC loaded with peptide in the presence of IL-2 (20 IU/mL) and IL-7 (10 ng/mL) and evaluated by flow cytometry after 7 days. While CMV- and Flu-specific CD8⁺ T cells maintained the ability to proliferate *in vitro* (Fig. 2A), PR1 specific CD8⁺ T cells from 4 of 5 patients failed to proliferate (Fig. 2B and C). Similarly, in 7 of 8 patients tested, CD8⁺ T cells specific for leukemia-associated antigens other than PR1 also failed to proliferate *in vitro* (Fig. 2B and C).

Neither higher doses of IL-2 (1000 IU/mL) nor the addition of IL-15 were able to rescue peptide-induced proliferative capacity of leukemia antigen-specific CD8⁺ T cells detectable by tetramers in PBMC (data not shown). Furthermore, the removal of CD4⁺ cells, a fraction of which might have potentially exhibited suppressor effects, was also ineffective in restoring peptide-induced proliferation of leukemia antigen-specific CD8⁺ T cells (data not shown). Finally, leukemia antigen-specific CD8⁺ T cells were sorted from PBMC based on tetramer labeling and cultured with polyclonal stimulation using anti-CD3 mAb, IL-2, and irradiated allogeneic PBMC, as previously described (25). Although Flu-specific CD8⁺ T cells could be expanded with this method, leukemia antigen-specific CD8⁺ T cells could not (data not shown).

The only patient displaying leukemia-associated antigen-specific CD8⁺ T cells capable of peptide-induced proliferation was patient 009, who was notably the HLA-A2-positive patient furthest from the time of HSCT at 72 months.

Cytokine release and degranulation of CD8⁺ T cells after *in vitro* stimulation of peripheral blood T cells

To investigate the ability of leukemia antigen-specific CD8⁺ T cells to undergo degranulation as part of T-cell-mediated cytotoxicity, we measured CD107a mobilization *in vitro* in response to peptide stimulation. CD107a is mobilized from vesicles to the cell surface during degranulation and T-cell-mediated cytotoxicity (26,27). In normal donors, viral-specific CD8⁺ T cells, identified by peptide-MHC tetramer labeling, underwent degranulation as manifested by CD107a mobilization and released IFN- γ in response to peptide stimulation (Fig. 3A), confirming that in the conditions of our assay, tetramer labeling and functional analysis are compatible. However, in patients following allogeneic HSCT, leukemia antigen-specific CD8⁺ T cells showed a lack of CD107a mobilization and release of IFN- γ in response to peptide stimulation (Fig. 3B). High dose IL-2 (1000 U/mL) was unable to rescue an ability of these cells to release IFN- γ or degranulate (data not shown). Additional studies were performed to demonstrate that tetramer labeling *in vitro* does not down regulate TCR expression during the course of the assay (Supplementary Fig. 1). Moreover, we also stimulated patient CD8⁺ T cells with peptide alone (in the absence of tetramer) and measured intracellular cytokine staining for IFN- γ for both viral and leukemia antigens. We found that viral-specific T cells from normal donors were able to secrete IFN- γ , but leukemia antigen-specific T cells from our patients failed to do so (Supplementary Fig. 2).

Overexpression of PD-1 is not responsible for the hyporesponsiveness of antigen-specific CD8⁺ T cells found in allogeneic HSCT patients

Although we are able to identify a diversity of CD8⁺ T cells that recognize an array of leukemia-associated antigenic epitopes in patients following allogeneic HSCT, our findings indicate that in most patients these T cells are unable to proliferate, degranulate or secrete IFN- γ in response to cognate peptide. Similar findings have been reported for virus-specific CD8⁺ T cells under conditions of chronic antigen stimulation for which CD8⁺ T cells express elevated levels of PD-1 and display a reduced ability to produce cytokines and proliferate (28,29). Therefore, we tested whether leukemia antigen-specific CD8⁺ T cells from patients following allogeneic HSCT had an increased expression of PD-1. Using flow cytometric analysis, we compared PD-1 expression on CD8⁺ T cells from normal donors vs. patients following allogeneic HSCT. Comparison of normal donors vs. patients revealed similar percentages in the expression of PD-1 within the bulk CD8⁺ T cell population (Supplementary Fig. 3). In both groups, the percentage of cells expressing PD-1 among viral-associated antigen-specific CD8⁺ T cells was in general much higher than that observed within the bulk population. Similarly, the percentage of cells expressing PD-1 among leukemia-associated antigen-specific CD8⁺ T cells in patients was often much higher than that observed within the bulk CD8⁺ T cell population. We also found that the percentage of antigen-specific CD8⁺ T cells expressing PD-1 was highly variable from patient to patient, but no relationship was observed between the percentage of CD8⁺ T cells expressing PD-1 and the duration from transplantation.

In HIV patients, blocking the PD-1/PDL-1 interaction is capable of restoring CD8⁺ T cell function *in vitro* (28,29). Therefore, to investigate whether the observed PD-1 expression on leukemia-associated antigen-specific CD8⁺ T cells is associated with *in vitro* hyporesponsiveness in patients following allogeneic HSCT, we stimulated patient PBMC with peptide after blocking one of the PD-1 ligands, PD-L1, as previously described (29). We found no restoration of the proliferative capability of leukemia-associated antigen-specific CD8⁺ T cells with blocking the PD-1/PD-L1 interaction (data not shown).

Antigen-specific CD8⁺ T cells from allogeneic HSCT patients display a phenotype consistent with senescence

Based on these findings, we hypothesized that leukemia-associated antigen-specific CD8⁺ T cells observed in patients after allogeneic HSCT are senescent and therefore assessed for the expression of CD28 and CD57 on the bulk population of CD8⁺ T cells found in normal donors vs. patients following allogeneic HSCT. Low CD28 expression and high CD57 expression have been tightly linked with T cell senescence both *in vitro* and *in vivo* (30–33). We found that the majority of CD8⁺ T cells from 20 normal donors was CD28⁺ (mean 74% $\pm$ 10.2%) and CD57^{neg} (mean 16% $\pm$ 8.1%), but in patients evaluated following allogeneic HSCT, normal levels of CD28 and CD57 expression were observed in only 3 of 13 patients evaluated (Fig. 4A). Instead, an increased proportion of CD8⁺ T cells were CD28^{neg} CD57⁺ (Fig. 4A). Using peptide/MHC tetramers, we further analyzed 6 of these patients and found that leukemia-associated antigen-specific CD8⁺ T cells also expressed minimal CD28 while displaying increased CD57 expression (shown for two patients in Fig. 4B). In contrast, viral specific CD8⁺ T cells identified with tetramers from normal donors were largely CD28⁺ CD57^{neg}.

Furthermore, we found that the proportion of CD8⁺ T cells bearing a CD28^{neg} CD57⁺ phenotype was related to the duration since allogeneic HSCT (Fig. 4A). With the exception a patient analyzed one month after transplantation, patients analyzed within 5 years of HSCT displayed high frequencies of CD28^{neg} and CD57⁺ T cells, both within the bulk CD8⁺ T cell population and among tetramer-positive CD8⁺ T cells (Fig. 4A and B). For 2 of 3 patients analyzed more than 6 years after allogeneic HSCT, there was a restoration of CD28⁺

CD57^{neg} cells to the level of normal donors (Fig. 4), including among CD8⁺ T cells reactive with leukemia antigen tetramers (not shown).

Further analysis with antibodies against CCR7, CD45RA and CD27 demonstrated that the CD28^{neg} CD57⁺ population of T cells was CCR7^{neg} and primarily expressed CD45RA while lacking expression of CD27 (data not shown), consistent with terminally differentiated effector cells rather than memory CD8⁺ T cells (34,35).

Another important marker of cellular senescence is telomere shortening. As a consequence of extensive rounds of cellular division, cells with telomeres that become critically shortened can enter a non-proliferative yet viable state called replicative senescence (30,31,36). To determine whether telomeres are shortened in CD8⁺ T cells from patients after HSCT compared to normal donors, mean telomere length of purified CD8⁺ T cells was determined by TRF analysis. Consistent with a phenotype of replicative senescence, CD8⁺ T cells from patients were found to have markedly shortened telomeres compared to age-matched normal donors (Fig. 5). Furthermore, in one case for which paired samples from donor and recipient were obtained, TRF analysis of CD8⁺ T cells from the 62-year-old donor of patient 012 revealed a mean length of 6.4 kb that markedly contrasted with the mean length of 3.8 kb observed in CD8⁺ T cells derived from patient 012 90 days after allogeneic HSCT (represented as solid circles and squares, respectively, in Fig. 5). Overall, these findings suggest that leukemia-associated antigen-specific CD8⁺ T cells undergo replicative senescence as early as 2 months following HSCT.

Discussion

A critical issue for understanding cancer immune surveillance and designing potential immunotherapy is the function and replicative capacity of tumor-antigen specific T cells. In this study, we examined the diversity, phenotype, and functional potential of leukemia-associated antigen-specific CD8⁺ T cells that naturally arise in patients with myeloid leukemia following allogeneic HSCT. Using a library of peptide/HLA-A2 tetramers for 20 epitopes from 7 leukemia-associated antigens, we found that circulating leukemia-specific CD8⁺ T cells were prominent in myeloid leukemia patients after HSCT, with each patient displaying a unique signature of CD8⁺ T cell specificities. However, with few exceptions, leukemia-specific CD8⁺ T cells were functionally unresponsive *in vitro*, as evidenced by their inability to proliferate, degranulate, and secrete IFN- γ in response to cognate peptide stimulation. The use of IL-15 or high-dose IL-2, elimination of CD4⁺ regulatory T cells, and blockade of PD-L1 all failed to rescue responsiveness of these CD8⁺ T cells in these *in vitro* assays. Rather, the mechanism for CD8⁺ unresponsiveness after HSCT appeared to be replicative senescence. As soon as 2 months after HSCT and for up to 5 years, CD8⁺ T cells after HSCT were predominantly CD28^{neg} CD57⁺ and exhibited significantly shortened telomeres. It should be noted that our patient cohort was relatively small (13 patients total of which 8 were HLA-A2⁺); if our results are confirmed in a larger series of patients, these findings would suggest the induction of replicative senescence among leukemia antigen-specific CD8⁺ T cells following allogeneic HSCT.

Originally appreciated in cultured fibroblasts, replicative senescence is considered a fundamental feature of normal somatic cells, including T cells (30,31). Following extensive rounds of cellular division, telomeres become critically shortened and cells enter a non-proliferative yet viable and apoptotic-resistant state called replicative senescence (30,31,36). T cell replicative senescence has been particularly well-characterized as a function of aging and chronic antigenic stimulation, such as from persistent HIV infection (37,38). Senescent CD8⁺ T cells fail to proliferate or secrete cytokines in response to cognate peptide even in the presence of exogenous IL-2 or IL-15 (33). Particularly for CD8⁺ T cells, replicative senescence

is tightly linked to a lack of CD28 expression (32,39). More recently, senescent T cells have also been shown to express high levels of CD57 (33). Although precise cell biological mechanisms of T cell senescence are not fully understood, a critical pathway is clearly telomere length erosion (36,40,41). Gradual shortening of telomere length in dividing cells eventually reaches a critical deficit of length or structural quality that mediates chromosomal abnormalities and cell death or senescence. Overall, senescent CD8⁺ T cells in humans are functionally unresponsive, express CD57 but not CD28, and display shortened telomeres. Here, we found that leukemia antigen specific T cells from patients were largely CD57⁺ but CD28-negative in contrast to viral-specific T cells from normal donors that exhibited the opposite CD57/CD28 phenotype.

There are multiple sources of chronic stimulation following HSCT that may contribute to potential T cell senescence, including graft-versus-host disease, graft-versus-leukemia activity, infection, and homeostatic proliferation to repopulate the T cell pool. For leukemia-specific CD8⁺ T cells, the level of residual leukemia present at the time of transplantation may incite an additional state of chronic antigenic stimulation resulting in the continuous proliferation of leukemia-associated antigen-specific CD8⁺ T cells that eventually senesce and accumulate. Slow reconstitution of CD4⁺ T cells after HSCT (42,43) may aggravate these effects, as CD4⁺ T cells play a critical role in the development and maintenance of functional memory CD8⁺ T cell responses (44–46). In the absence of sufficient CD4⁺ T cell help, CD8⁺ T cells after HSCT may proceed more quickly along a pathway toward replicative senescence in the setting of chronic stimulation.

Various other clinical factors may also be involved in the process of T cell unresponsiveness and senescence after HSCT. Population dynamics of the T cell pool after transplant may be influenced by the intensity of the conditioning regimen prior to transplant, T cell dose within the graft, immunosuppressive therapy, and the use of donor lymphocyte infusions. For example, 10 of the 13 patients we studied were on some type of immunosuppression at the time of sampling (Supplementary Table 1); interestingly, one HLA-A2⁺ patient (patient 006) was not on any immunosuppressives but nevertheless still exhibited leukemia antigen specific CD8⁺ T cells with functional unresponsiveness and a senescence phenotype demonstrated for HLA-A2⁺ patients on immunosuppression. With regard to concomitant graft versus host disease (GVHD), we do not have the sense that the rate of GVHD was higher in this cohort compared to comparably treated patients at our and other centers or rates reported in the literature. Acute GVHD develops in 40%–50% of these type of patients and therefore finding acute GVHD in 7/13 patients seems within this expectation. Chronic GVHD develops in 50%–60% of patients; the diagnosis of chronic GVHD in 10/13 patients here may be higher than expected but given the small numbers does not seem out of range.

These factors and many others play a role in the repopulation of the T cell compartment. In our study, patients had undergone a variety of treatment regimens for transplantation (Supplementary Table 1), and yet our observations of unresponsive leukemia-specific CD8⁺ T cells were uniformly observed. Nevertheless, it remains possible that variations in treatment and other details of the HSCT may impact the function observed for leukemia-specific CD8⁺ T cells in this setting. For example, for a cohort of CML patients studied after myeloablative allogeneic HSCT, investigators at the INSERM reported the induction of tetramer-positive but functionally hyporesponsive CD8⁺ T cells, similar to our observations (5). In contrast, five out of six CML patients studied at the NIH after HSCT exhibited CD8⁺ T cells that generated IFN- γ mRNA after *in vitro* stimulation with at least one leukemia antigen peptide (6). Interestingly, four of the responding patients in the NIH study had received DLI, compared to one such patient in our study.

Our findings also suggest that the predominance of unresponsive leukemia-specific CD8⁺ T cells with a senescent phenotype may be lost over time. For two CML patients in remission 73 and 120 months after HSCT, we observed CD8⁺ T cells with normal levels of CD28 and CD57 expression. Neither of these patients were HLA-A2-positive, limiting our ability to evaluate T cell responses, but a third CML patient, in remission 72 months after HSCT, displayed leukemia-associated antigen-specific CD8⁺ T cells capable of peptide-induced proliferation, unique among the HLA-A2 patients we evaluated. In each of these 3 patients (but not others who were studied within 5 years of HSCT), a prominent population of CCR7⁺ CD45RA⁺ cells among the CD8⁺ population was observed (not shown), suggesting recovery in the naïve CD8⁺ T cell pool.

Finally, our findings have implications for the development of tumor antigen-specific therapies for patients with myeloid leukemia. The efficacy of T cell vaccines requires not only the presence of targetable leukemia-associated antigens but also the availability of functional and responsive antigen-specific T cell precursors. Although the early post-transplant period may be an ideal setting for immunotherapy given the reduction of tumor burden and the induction of homeostatic proliferation, antigen-specific CD8⁺ T cells during this time point may be at risk for rapid induction of senescence. Clinical methods to preserve and maintain a competent pool of CD8⁺ T cell precursors after allogeneic HSCT, potentially such as DLI, represent important areas of ongoing and future studies.

Translational relevance

The curative potential of allogeneic hematopoietic stem cell transplantation (HSCT) for myeloid leukemia has been attributed in part to a graft-versus-leukemia (GVL) effect dependent on donor T cells. Here, we found that circulating CD8⁺ T cells specific for peptides derived from leukemia-associated antigens are prominent in patients with myeloid leukemia after HSCT, with each patient displaying a unique signature of CD8⁺ T cell specificities. However, such cells are functionally unresponsive and display features of replicative senescence. Although the early post-transplant period may be an ideal setting for immunotherapy given the reduction of tumor burden and the induction of homeostatic proliferation, antigen-specific CD8⁺ T cells during this time point may be at risk for rapid induction of senescence. Clinical methods to preserve and maintain a competent pool of CD8⁺ T cell precursors after allogeneic HSCT represent critical areas of ongoing and future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Viral and leukemia-associated antigen-specific CD8⁺ T cells in myeloid leukemia patients following allogeneic HSCT. PBMC from myeloid leukemia patients following allogeneic HSCT were labeled with anti-CD8, anti-CD14, anti-CD4, and peptide/HLA-A2 tetramers and analyzed. Cells were gated on CD8⁺ CD4^{neg} CD14^{neg} lymphocytes. Representative examples of CD8⁺ T cells recognizing various viral and leukemia-associated antigenic epitopes are shown for 6 patients compared to the HTLV-1 negative control tetramer. Patient identification numbers are shown to the left of each plot. The percentage of tetramer-specific CD8⁺ cells out of CD8⁺ CD4^{neg} CD14^{neg} lymphocytes is shown on each plot.

A

B

C

	Patient	001	002	003	004	005	006	009	011
	Disease	AML	AML	CML	AML	CMML	CML	CML	AML
Viral	Flu							●	
	CMV	●	●	●	●	●	●		●
Proteinase 3	PR1		×	×	×	×		●	
	PR2				×				×
	PR-QLP		×	×			×	×	×
	PR-TLI	×					×		
WT1	WT126	×							
	WT187				×				
	WT235	×							
PRAME	PRA100		×			×			
Other	Hox-TLD	×	×		×	×		●	×
	MEI-AIY	×							
	hTERT			×				×	×

Fig. 2.

Leukemia-associated antigen-specific CD8⁺ T cells lack proliferative capacity. CD8⁺ CD4^{neg} CD14^{neg} cells were analyzed from uncultured (fresh) PBMC for binding to HLA-A2-specific tetramers. PBMC were then subjected to *in vitro* stimulation with peptide and CD8⁺ CD4^{neg} CD14^{neg} cells were analyzed for binding to tetramer after 7 days. (A) Viral specific CD8⁺ T cells specific for either Flu or CMV show response to peptide stimulation. (B) Leukemia-associated antigen-specific CD8⁺ T cells show lack of proliferative capacity in the majority of cases. (C) Summary of results for 8 patients in whom PBMC were analyzed for *in vitro* stimulated (IVS) proliferation of viral and leukemia-associated antigen-specific CD8⁺ T cells. Evidence for proliferation was determined by observing a greater than two fold increase in the percentage of antigen-specific CD8⁺ T cells at analysis following IVS. ×, no proliferation. ●, proliferation. No symbol, not done.

A

B **Fig. 3.**

Leukemia-associated antigen-specific CD8⁺ T cells do not degranulate or release cytokines following cognate-peptide interaction. PBMC were stimulated *in vitro* with peptide for 4 hours. CD8⁺ CD4^{neg} CD14^{neg} T cells were gated on tetramer-positive cells and analyzed by flow cytometry for the ability of antigen-specific CD8⁺ T cells to mobilize CD107a and for cytoplasmic expression of IFN-γ following antigenic stimulation. (A) Flu-specific CD8⁺ T cells from normal donors (ND) stimulated with either negative control peptide or Flu peptide. (B) PR1-specific and PRA142-specific CD8⁺ T cells from three representative patients.

A

B **Fig. 4.**

Phenotype characterization of leukemia-associated antigen-specific CD8⁺ T cells. PBMC were labeled with anti-CD8, anti-CD14, anti-CD4, and HLA-A2 tetramers and analyzed for expression of CD28 and CD57. (A) CD28 and CD57 expression on bulk CD8⁺ T cells is shown separately for each of the 13 patients (one symbol per patient) and plotted versus the duration since HSCT. Mean frequency and standard deviation of CD28 and CD57 expression on bulk CD8⁺ T cells from 20 normal donors is presented on the right for comparison (filled square shown as mean $\pm$ 1 SD). (B) Representative examples of the expression of CD28 and CD57 on leukemia-associated antigen-specific CD8⁺ T cells from two patients following allogeneic HSCT, shown in comparison to viral-specific CD8⁺ T cells from 3 normal donors. Plots are shown after gating on tetramer-positive CD8⁺ CD4^{neg} CD14^{neg} lymphocytes.

Fig. 5.

Telomere shortening in CD8⁺ T cells following allogeneic HSCT. Whole genomic DNA from purified CD8⁺ T cells obtained from the peripheral blood of patients (□; 002, 004, 005, 012) and normal donors (○; 20–40 yo and 40–60 yo) was analyzed by southern blot for mean telomere length. The donor age for each patient was as follows: 002 (65 yo), 004 (51 yo), 005 (32 yo), and 012 (62 yo). Each symbol indicates an individual patient or normal donor. The filled circle(●) indicates the normal donor (62 yo, female) for patient 012, filled square (■). Solid lines indicate the mean for each group. p-values for the comparisons indicated were calculated using Student's t test.

Table 1
Frequency of tetramer-positive CD8⁺ T-cells in HLA-A2⁺ patients with myeloid leukemia following allogeneic HSCT*

Patient	001	002	003	004	005	006 [†]	009	011
Disease	AML	AML	CML	AML	CMMML	CML	CML	AML
Flu	0.08	0.02	0.09	0.01	0.03	0.05	0.03	0.27
CMV	<u>0.12</u>	<u>1.28</u>	<u>0.20</u>	0.04	0.04	<u>9.86</u>	0.03	<u>0.14</u>
PR1	0.01	0.20	0.08	0.23	0.04	0.01	1.73	0.05
PR2	0.00	0.10	ND	0.02	0.10	0.01	0.01	0.08
PR-QLP	0.02	0.45	0.03	0.10	3.00	2.86	0.71	0.20
PR-NLS	0.02	0.05	ND	0.00	0.04	0.01	0.04	0.04
PR-GII	0.19	0.05	ND	0.04	0.89	1.02	0.05	0.10
PR-ALA9	0.00	0.01	ND	0.00	0.00	0.00	0.01	<u>0.11</u>
PR-KLN	0.10	0.08	ND	0.02	0.02	0.01	0.02	0.01
PR-TLI	0.09	0.01	ND	0.01	<u>0.37</u>	<u>0.12</u>	0.05	0.05
WT10	0.00	0.02	ND	0.03	0.02	0.04	0.04	0.01
WT126	0.06	<u>0.11</u>	ND	0.01	0.02	0.02	0.01	0.03
WT187	0.00	0.02	ND	0.02	0.00	0.00	0.01	0.01
WT235	0.03	0.07	ND	0.00	<u>0.11</u>	0.01	0.01	0.02
PRA100	0.00	0.08	0.03	0.05	0.10	0.00	0.06	0.01
PRA142	0.01	<u>0.21</u>	ND	0.05	0.07	0.07	0.00	0.01
PRA300	0.00	0.04	ND	0.00	0.02	0.06	0.00	0.00
PRA425	0.00	0.05	ND	0.02	0.05	0.01	0.00	0.04
Hox-TLD	0.01	<u>1.19</u>	0.00	0.05	0.17	0.01	0.03	0.31
MEL-AYI	0.03	0.02	0.00	0.01	<u>0.29</u>	0.01	0.00	0.00
hTERT	<u>0.15</u>	0.02	<u>0.14</u>	0.01	0.52	<u>0.18</u>	0.07	0.41
SurIM2	0.00	0.06	0.00	0.00	0.00	0.01	0.02	<u>0.87</u>

* Frequencies are expressed as the percentage of tetramer-positive CD8⁺ cells among all CD8⁺ cells, with 0.05% used as the cutoff for positivity. Positive values are shown in boldface type. Frequencies that are greater than twice the cutoff value are also underlined.

[†] Patient 006 was sampled after she completed therapy for CMV reactivation.

ND, not determined