

Development of Tumor-Reactive T Cells After Nonmyeloablative Allogeneic Hematopoietic Stem Cell Transplant for Chronic Lymphocytic Leukemia

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Abstract Purpose: Allogeneic nonmyeloablative hematopoietic stem cell transplant (NM-HSCT) can result in durable remission of chronic lymphocytic leukemia (CLL). It is thought that the efficacy of NM-HSCT is mediated by recognition of tumor cells by T cells in the donor stem cell graft. We evaluated the development of CTLs specific for CLL after NM-HSCT to determine if their presence correlated with antitumor efficacy.

Experimental Design: Peripheral blood mononuclear cells obtained from 12 transplant recipients at intervals after NM-HSCT were stimulated *in vitro* with CLL cells. Polyclonal T-cell lines and CD8⁺ T-cell clones were derived from these cultures and evaluated for lysis of donor and recipient target cells including CLL. The presence and specificity of responses was correlated with clinical outcomes.

Results: Eight of the 12 patients achieved remission or a major antitumor response and all 8 developed CD8⁺ and CD4⁺ T cells specific for antigens expressed by CLL. A clonal analysis of the CD8⁺ T-cell response identified T cells specific for multiple minor histocompatibility (H) antigens expressed on CLL in six of the responding patients. A significant fraction of the CD8⁺ T-cell response in some patients was also directed against nonshared tumor-specific antigens. By contrast, CLL-reactive T cells were not detected in the four patients who had persistent CLL after NM-HSCT, despite the development of graft-versus-host disease.

Conclusions: The development of a diverse T-cell response specific for minor H and tumor-associated antigens expressed by CLL predicts an effective graft-versus-leukemia response after NM-HSCT.

Allogeneic hematopoietic stem cell transplantation (HSCT) can cure many hematologic malignancies, although graft-versus-host disease (GVHD) and relapse remain significant obstacles. The efficacy of HSCT results from cytotoxic conditioning and a graft-versus-leukemia (GVL) effect (1, 2). Myeloablative conditioning regimens that use total body irradiation and/or intensive chemotherapy exhibit potent antitumor activity but are limited to young patients due to nonhematopoietic toxicities. Allogeneic HSCT can be extended to older patients and those with comorbidities using reduced intensity nonmyeloablative conditioning regimens that provide less antitumor activity but immunosuppress the recipient

sufficiently to allow engraftment of donor hematopoietic cells and enable a GVL effect (3–7). Nonmyeloablative HSCT (NM-HSCT) leads to remission in a subset of patients with refractory indolent hematologic malignancies including chronic lymphocytic leukemia (CLL; refs. 8–17). The eradication of CLL after NM-HSCT is associated with GVHD, and presumed to be a consequence of T-cell recognition of alloantigens expressed by leukemic cells (18). However, many patients do not respond to NM-HSCT despite developing GVHD and others respond without significant GVHD. Thus, the basis for a successful GVL effect remains poorly defined in individual patients.

CLL is amenable to studies of the GVL effect because leukemia cells can be obtained from most patients, and induced to become efficient antigen-presenting cells (APC) by stimulation through CD40 (19–21). Here, we used recipient CD40L-stimulated CLL as APC to isolate donor T cells that were specific for CLL after NM-HSCT. CD8⁺ and CD4⁺ T cells that recognized multiple minor histocompatibility (H) antigens expressed on recipient CLL were isolated from all patients who achieved or maintained a complete remission (CR) after NM-HSCT. In addition, CD8⁺ T-cell clones that recognized recipient CLL but not Epstein-Barr virus (EBV) transformed B cells were isolated from responding patients, suggesting a component of the response is directed against tumor-specific determinants. Despite the development of GVHD and high levels of donor T-cell chimerism, CLL-specific T cells were not detected in recipients with persistent or progressive leukemia.

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Translational Relevance

Allogeneic nonmyeloablative hematopoietic stem cell transplant (NM-HSCT) can cure chemotherapy refractory chronic lymphocytic leukemia (CLL). A graft-versus-leukemia (GVL) effect is required for tumor eradication after NM-HSCT, but the mechanisms responsible are not well defined, and separating GVL from graft-versus-host disease (GVHD) is difficult. In a cohort of NM-HSCT recipients, we investigated whether CLL-reactive T cells could be detected after transplant and correlated their presence with antitumor activity. T cells specific for CLL developed with variable kinetics in all patients who achieved a complete remission but not in patients who failed to respond to NM-HSCT. CLL-reactive T cells were specific for recipient minor histocompatibility antigens expressed by CLL and other recipient cells but also antigens that were only expressed by the malignant B cells. The molecular identification of minor histocompatibility and tumor-associated antigens expressed on CLL may define targets for adoptive T-cell transfer or vaccination to augment GVL activity without GVHD.

These results show that the specificities of the T-cell responses that develop after allogeneic NM-HSCT are critical in determining antitumor efficacy, and illustrate the potential to manipulate T-cell reactivity to target antigens expressed selectively by tumor cells to improve outcome.

Materials and Methods

Patient and donor eligibility. Patients with CLL who failed to meet National Cancer Institute Working Group Criteria for complete or partial response (22) after therapy with a regimen containing fludarabine, or who relapsed within 12 mo after completing fludarabine, and had an HLA-A, HLA-B, HLA-C, HLA-DRB1, and HLA-DQB1 matched related or unrelated donor were eligible. Exclusion criteria included central nervous system involvement, history of malignancy other than CLL within the past 5 y, Karnofsky score of <60%, and severe cardiovascular, pulmonary, or hepatic dysfunction. Granulocyte colony-stimulating factor mobilized peripheral blood was the source of stem cells. All patients and their related donors signed consent forms approved by the Fred Hutchinson Cancer Research Center institutional review board. Informed consent was obtained from the unrelated donors according to National Marrow Donor Program regulations.

Treatment plan. Patients were conditioned for transplantation with fludarabine 30 mg/m²/d on days -4 to -2, and 200 cGy of total body irradiation on day 0. The patients received immunosuppression with cyclosporine or tacrolimus given orally every 12 h beginning on day -3; and mycophenolate mofetil given beginning on day 0 every 12 h to recipients with related donors, and every 8 h to recipients with unrelated donors (16). Analysis of donor/recipient chimerism in peripheral blood CD3⁺ and CD33⁺ cells was done after transplant on approximately day 28, 56, and 84; at 6, 12, 18 mo; and then annually.

Disease response and GVHD assessment. Disease response was assessed using National Cancer Institute Working Group Criteria (22). Patients underwent evaluation for minimal residual CLL by flow cytometry of peripheral blood and bone marrow. Diagnosis and grading of acute and chronic GVHD were done according to established criteria (23, 24).

Cell lines. EBV-transformed B cells (B-LCL) were generated from patients and donors as described (25). B-LCL and CLL from individuals of known HLA types were used as targets to determine

the HLA-restricting allele and the frequency of individual minor H or tumor-associated antigens. Fibroblast cell lines were generated and maintained as described (25).

Isolation of CLL-reactive T-cell lines and clones. Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation over Ficol-Hypaque and cryopreserved. NIH 3T3 cells transfected with human CD40L (tCD40L) were a gift of Dr. J. Schultze (Laboratory for Genomics and Immunoregulation, University of Bonn, Bonn, Germany) and used to generate CD40-activated CLL cells (CD40-CLL) as described (26). After 3 d of activation, an aliquot of cells was analyzed for expression of CD5, CD19, HLA-class I, HLA-class II, CD80, CD86, CD54, and CD58 to determine purity and confirm activation through CD40. A second aliquot of CD40-CLL was γ -irradiated and used as APC to stimulate posttransplant PBMC. When further expansion of CLL cells was required, CD40-CLL were transferred to fresh tCD40L every 3 to 4 d.

To generate T-cell lines, PBMC (3×10^6 /well) obtained from the recipient at intervals after transplant were stimulated with γ -irradiated (35 Gy) recipient CD40-CLL (0.75×10^6 /well) in RPMI, 10% human serum, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin. Cultures were restimulated after 7 d with γ -irradiated recipient CD40-CLL at a responder to stimulator ratio of 4:1. The medium was supplemented with interleukin (IL)-7 (10 ng/mL on day 0 of each stimulation) and IL-2 (2 U/mL) on day 3 after the first stimulation, and then 10 U/mL on days 1 and 4 of the second stimulation. After 2 wk, T-cell lines were tested for cytotoxic activity against recipient- and donor-derived B-LCL and recipient CD40-CLL.

CD8⁺ and CD4⁺ T cells that secreted IFN- γ after stimulation with CD40-CLL were sort purified using IFN- γ capture. An aliquot (1×10^6 cells) of each of the T-cell lines was cocultured with CD40-CLL ($1-2 \times 10^6$) in media containing 20 U/mL IL-2 at 37°C for 4 h and then processed as described (27, 28). CD4⁺ and CD8⁺ IFN- γ ⁺ cells were sorted and then expanded by stimulation with anti-CD3 monoclonal antibody or cloned at 0.5 cells per well in 96-well round-bottomed plates with anti-CD3 monoclonal antibody (30 ng/mL) as described (25, 29). In selected cases, γ -irradiated recipient CD40-CLL (2×10^3 cells per well) were used for stimulation in cloning cultures in place of anti-CD3 monoclonal antibody. After 12 to 14 d, cloning wells with growth were tested for cytotoxicity against recipient and donor B-LCL to identify T-cell clones specific for recipient minor H antigens. In selected cases, recipient CLL cells were included as target cells during screening to identify T-cell clones specific for putative tumor-associated antigens expressed by CLL but not nonmalignant B cells.

Cytotoxicity assays and ELISA. Target cells, which included B-LCL, CD40-CLL, primary CLL, and fibroblasts, were labeled with ⁵¹Cr for 2 h, washed twice, plated in triplicate at 1 to 2×10^3 cells per well with effector cells at various effector to target (E/T) ratios. When <75% of the recipient PBMC activated with CD40 expressed CD5 and CD19, the CD5⁺ CD19⁺ cells were sorted using a FACSVantage cell sorter (Becton Dickinson) before cytotoxicity assays. Fibroblasts were treated with 10 U/mL IFN- γ for 48 h before use as targets. In some experiments, Cr⁵¹-labeled target cells were pulsed with peptides (10 μ g/mL) for 30 min, and then washed before use in the assay. Supernatants were harvested for γ -counting after a 4-h incubation of effector and target cells, and specific lysis calculated using the standard formula.

For IFN- γ ELISA, target and effector cells were washed twice and plated in triplicate at an E/T ratio of 3:1. After 24 h of incubation, the supernatant was collected and analyzed for IFN- γ using a primary and a biotinylated secondary anti-human IFN- γ antibody (Pierce Biotechnologies) according to the manufacturer's instructions. Color development was done with streptavidin-conjugated horseradish peroxidase and TMP (Sigma-Aldrich), and absorbance determined using a Thermo Multiskan EX reader (Thermo Scientific). A standard curve was obtained with serial dilutions of recombinant human IFN- γ with a potency of 2.0×10^4 IU/ μ g (R&D Systems).

Flow cytometry. T cells, primary CLL, and CD40-CLL were stained with one or more of the following monoclonal antibodies conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE): CD3,

CD4, CD5, CD8, CD19, CD40, CD54, CD58, CD80, CD86, HLA-class I, HLA-DR, and isotype-matched control antibodies (BD Pharmingen). Flow cytometric analysis was done on a FACSCalibur, and data were analyzed using CellQuest software (Becton Dickinson).

Results

Patient characteristics and clinical outcomes after allogeneic NM-HSCT. The characteristics of 12 patients treated with NM-HSCT from HLA-matched donors are shown in Table 1. Eleven of the 12 patients had ≥50% bone marrow involvement with CLL at the time of NM-HSCT, and one patient (UPN 18802) was transplanted in CR after receiving chemotherapy for Richter’s transformation. Seven of the 12 patients achieved or maintained a CR 3 to 12 months after transplant. One of these seven patients (UPN 9661) only achieved a CR after receiving a donor leukocyte infusion (DLI) for the treatment of EBV-associated lymphoproliferative disease. One additional patient (UPN 29449) has an ongoing partial remission with a reduction in bone marrow tumor burden from 70% pretransplant to 5% at 6 months after transplant. Six of these eight patients with a major antitumor response developed acute GVHD and five developed extensive chronic GVHD. Four patients had persistent or progressive CLL after NM-HSCT, despite the administration of DLI to one of these patients (UPN 28196). All four nonresponding patients developed acute GVHD, and one of three that survived beyond day 100 developed chronic GVHD. One of the nonresponding patients remains alive with progressive CLL. There was no difference in the immunosuppressive therapy regimens used for GVHD prophylaxis between responding and nonresponding patients.

Generation of CLL-reactive T cells from posttransplant PBMC. We hypothesized that donor cell engraftment might result in the development of a T-cell response specific for recipient minor H or tumor-associated antigens on CLL and contribute to tumor regression. Aliquots of recipient CLL that were cryopreserved before transplant were used to stimulate T cells obtained from the recipient posttransplant after donor engraftment was established. CLL expresses class I and II MHC molecules and can be recognized by CD8⁺ and CD4⁺ T cells but has low levels of costimulatory and adhesion molecules (19, 20). Engagement of CD40 on CLL with CD40 ligand

(CD40L) up-regulates the expression of costimulatory (CD80 and CD86), adhesion (CD54 and CD58), and MHC molecules and makes the tumor cells more efficient APC (19, 20, 26). Therefore, we cocultured the pretransplant CLL from the 11 patients with a high tumor burden with tCD40L before their use *in vitro* for stimulating posttransplant T cells. The frequency of CLL cells in the cultures after CD40L activation was 30.1% to 97.4% (median 80%) as assessed by coexpression of CD5 and CD19. In the patient in morphologic CR before HSCT, we expanded CD5⁺ CD19⁺ CLL cells from bone marrow cells after multiple stimulations with tCD40L and used these cells as APC. CLL activated through CD40 expressed higher levels of costimulatory, adhesion, and MHC molecules as previously reported (data not shown; refs. 19, 20, 26).

PBMC were obtained from each recipient at intervals after HSCT, stimulated twice 1 week apart with γ -irradiated recipient CD40-CLL, and then evaluated for lysis of recipient CD40-CLL, and recipient and donor B-LCL. CD40-CLL rather than thawed primary CLL cells were used as target cells in these experiments because CD40-CLL were of higher purity and exhibited improved viability and uptake of ⁵¹Cr. CTLs that recognized both recipient CLL and B-LCL but not donor B-LCL were detected after two stimulations at one or more time points from all seven patients with a CR after HSCT or DLI, and from the one patient with a major partial response at 6 months after HSCT (Fig. 1A). Stimulation of PBMC obtained directly from each of the donors with recipient CD40-CLL under the same conditions did not elicit specific CTL activity against recipient CLL or B-LCL (data not shown), demonstrating the reactivity detected in posttransplant PBMC resulted from *in vivo* activation of T cells to recipient minor H and possibly tumor-associated antigens.

The antitumor activity of NM-HSCT is often delayed for several months after transplant, and we found the kinetics with which CLL-reactive T-cell responses developed after HSCT differed in individual patients. CLL-reactive T cells developed rapidly after transplant in some patients including UPN 22388 (Fig. 1A), who had a circulating lymphocyte count of 28,500 cells per microliter consisting of >90% CD5⁺ CD19⁺ tumor cells at the time of transplant. CLL-reactive T cells were detected in this patient in the PBMC sample obtained on day 35 after transplant, and coincided with a dramatic reduction in CD19⁺ CD5⁺ B cells. T-cell responses specific for CLL were not detected

Table 1. Characteristics and clinical outcome of recipients

UPN	Age	Sex (R/D)	Donor	Acute GVHD	Chronic GVHD	BM % CLL (Pre)	Disease response	Outcome
22388	59	M/F	MRD	None	E (skin, oral, GI)	80	CR at 12 mo	Alive, 47 mo
9661	55	F/F	MUD	Grade II (skin)	E (skin, eye, oral, liver)	>90	CR at 7 mo	Dead, GVHD (day 1,309)
21899	55	F/F	MRD	Grade II (skin)	E (skin, oral, GI)	50	CR at 6 mo	Alive, 29 mo
24487	45	M/F	MRD	None	E (liver, GI)	50	CR at 12 mo	Alive, 12 mo
23134	54	M/M	MRD	Grade II (GI)	None	79	Progressive	Dead, CLL (day 114)
19765	64	M/F	MRD	Grade III (skin, GI)	E (skin, oral, eye)	90	Progressive	Alive, >24 mo
19647	62	M/F	MUD	Grade II (skin)	NE	>90	Persistent CLL	Dead, sepsis and pneumonia (day 60)
28196	64	M/F	MRD	Grade I (skin)	None	92	Progressive	Dead, CLL (day 214)
18802	64	M/M	MUD	Grade II (GI)	Late acute GVHD (GI)	0	CR pretransplant	Dead, GVHD (day 227)
25843	55	M/M	MRD	Grade II (GI)	None	50	CR	Alive, 12 months
28736	57	M/M	MRD	Grade II (skin)	None	55	CR at 3 mo	Dead, Aspergillus (day 120)
29449	58	M/M	MUD	Grade II (skin, GI)	None	70	Partial response	Alive, 6 mo

Abbreviations: R, recipient; D, donor; M, male; F, female; MRD, matched related donor; GI, gastrointestinal tract; MUD, matched unrelated donor; E, extensive; NE, not evaluated.

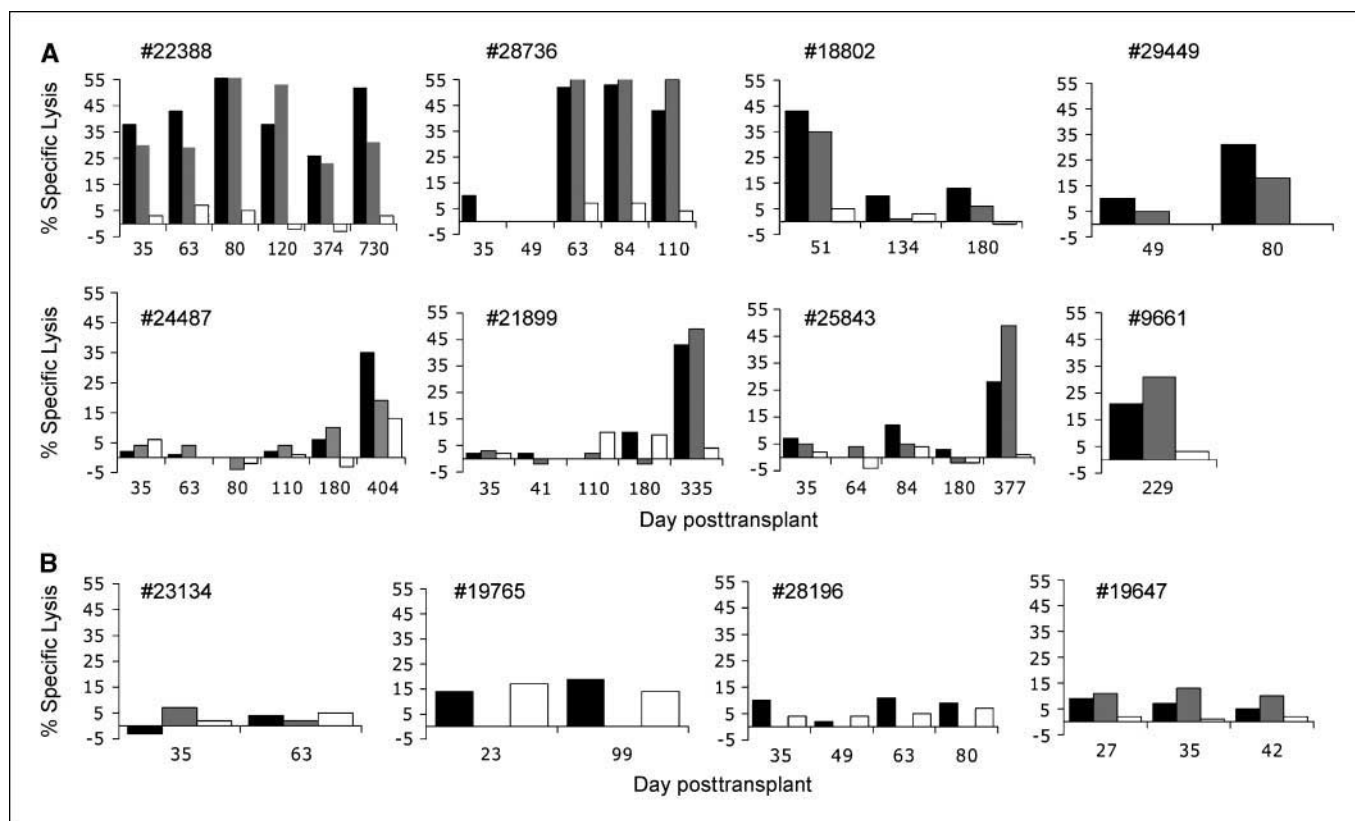


Fig. 1. Cytotoxicity of T-cell lines generated from NM-HSCT recipients by stimulation with recipient CD40-CLL. PBMC were obtained from recipients at the designated days after NM-HSCT, stimulated twice 1 wk apart with γ -irradiated recipient CD40-CLL, and tested in a cytotoxicity assay against ^{51}Cr -labeled recipient CD40-CLL (■), recipient B-LCL (■), and donor B-LCL (□). Data are shown for an effector to target ratio of 30:1 or 40:1. *A*, reactivity of T-cell lines generated from patients who had a major antitumor response after NM-HSCT. *B*, reactivity of T-cell lines generated from patients who had persistent or progressive disease after NM-HSCT.

in other patients until several months after transplant, after improvement in donor CD3 T-cell chimerism to >90% (UPN 24487; UPN 25843) or reduction in immunosuppressive drug therapy (UPN 21899; Fig. 1A). All four of the patients who did not achieve CR after transplantation lacked specific cytotoxicity for recipient CLL at any of the time points analyzed (Fig. 1B), despite the occurrence of GVHD in all patients and >90% donor CD3 T-cell chimerism in three of the four patients.

The development of GVHD in the nonresponding patients indicates that alloreactive T cells must be present in these patients, but our assays suggest the alloreactivity was not directed against antigens on malignant cells. An alternative explanation is that there was a defect in APC function of the CLL cells from the nonresponding patients. To address this possibility, we evaluated the ability of CD40-CLL from the four nonresponding patients and one responding patient to elicit alloreactive T cells in a mixed lymphocyte culture using T cells from an MHC-disparate unrelated donor. We found that the CLL from the nonresponding patients were equivalently effective APC (Fig. 2A and B). Additionally, a nonresponding patient (UPN 28196) and a responding patient (UPN 22388) were both HLA-A2⁺ males, and the CLL from each of these patients stimulated IFN- γ production from a T-cell clone specific for the SMCY minor H antigen (FIDSYICQV; Fig. 2C). These data show the failure to detect CLL-reactive T cells in nonresponders was not due to lack of APC function by the tumor cells, but rather suggest that the specificity of donor

alloreactive T cells that caused GVHD in the recipient was not directed against minor H antigens expressed on recipient CLL.

CD4⁺ and CD8⁺ T cells that recognize CLL develop in responding patients. Analysis of the phenotype of the CLL-reactive T-cell lines generated from each of the eight responding patients showed the presence of both CD4⁺ and CD8⁺ T cells in cultures from all of the eight patients. To determine whether both T-cell subsets recognized CLL, we stimulated an aliquot of the polyclonal cultures derived from each patient with recipient CD40-CLL, and evaluated IFN- γ production. As shown for two representative patients, a fraction of the CD4⁺ and CD8⁺ T cells from each patient produced IFN- γ after stimulation with recipient CLL and B-LCL, but not with donor B-LCL, demonstrating recipient minor H antigens on CLL were recognized by both CD4⁺ and CD8⁺ T cells (Fig. 3A and B). To determine whether both CD4⁺ and CD8⁺ T cells also lysed CLL, we sorted T cells that produced IFN- γ from two patients into CD4⁺ and CD8⁺ fractions and expanded each subset by stimulation with anti-CD3 monoclonal antibody. After a single cycle of expansion, the CD4⁺ IFN- γ ⁺ fraction contained 96.2% and 99.8% CD4⁺ T cells, respectively, and the CD8⁺ IFN- γ ⁺ fraction contained 74.1% and 96.2% CD8⁺ T cells, respectively (data not shown). We used these enriched populations as effector cells against recipient CD40-CLL, primary CLL, and recipient and donor B-LCL. The CD4⁺ T cells from each patient exhibited absent or weak lytic activity against recipient CD40-CLL and B-LCL, and failed to lyse primary CLL cells (Fig. 3C). By

contrast, the CD8⁺ T cells from each patient efficiently lysed both recipient CD40-stimulated and primary CLL (Fig. 3D), and lysis was inhibited by anti-class I monoclonal antibody (data not shown). Donor B-LCL and CD40-activated B cells served as controls and were not lysed by CD8⁺ T cells.

Clonal analysis of the CD8⁺ T-cell response to CLL reveals recognition of both minor histocompatibility and tumor-associated antigens. All of the patients who responded to NM-HSCT also developed GVHD, which is mediated by donor T cells specific for minor H antigens expressed on cells in the skin, gastrointestinal tract, and/or liver (30–32). Minor H antigens have previously been identified that are preferentially expressed on hematopoietic cells including B-lineage cells, and recognition of these antigens may be associated with less GVHD (30, 33–35). Thus, the isolation of T-cell clones from CLL-reactive T-cell lines derived from our patients would provide reagents to identify additional minor H antigens that could potentially be targeted to induce a GVL response without GVHD. We focused our efforts on analysis of the CD8⁺ T-cell response because the development of minor H antigen-specific CD8⁺ T cells after DLI has correlated with tumor regression in other settings (33, 36), and CD8⁺ T cells that produced IFN- γ in response to CD40-CLL lysed primary CLL cells more efficiently than CD4⁺ T cells (Fig. 3C and D).

We derived CD8⁺ T-cell clones from T-cell lines generated at time points that coincided with tumor regression from seven responding patients. In three patients where adequate numbers of cryopreserved CLL was available, γ -irradiated CLL rather than anti-CD3 monoclonal antibody was used for stimulation in the cloning cultures. In the four patients where tumor cells were limited, cloning wells positive for growth were screened for recognition of recipient and donor B-LCL to identify clones specific for minor H antigens. These T-cell clones were subsequently analyzed for recognition of recipient CD40-CLL and fibroblasts to identify minor H antigens that were expressed on tumor cells and preferentially on hematopoietic cells. In the three patients where we had sufficient tumor cells for cloning, we tested each positive well for recognition of recipient CLL and recipient and donor B-LCL to identify T-cell clones specific for either minor H or tumor-associated antigens.

Multiple CD8⁺ T-cell clones that recognized a minor H antigen presented by both recipient CD40-CLL and B-LCL, but not donor B-LCL, were isolated from all 7 patients (11 for UPN 22388, 10 for UPN 9661, 6 for UPN 21899, 2 for UPN 24487, 12 for UPN 18802, 6 for UPN 28736, and 13 for UPN 29449). We attempted to map the HLA-restricting allele for each minor H antigen-specific T-cell clone by testing recognition of a panel

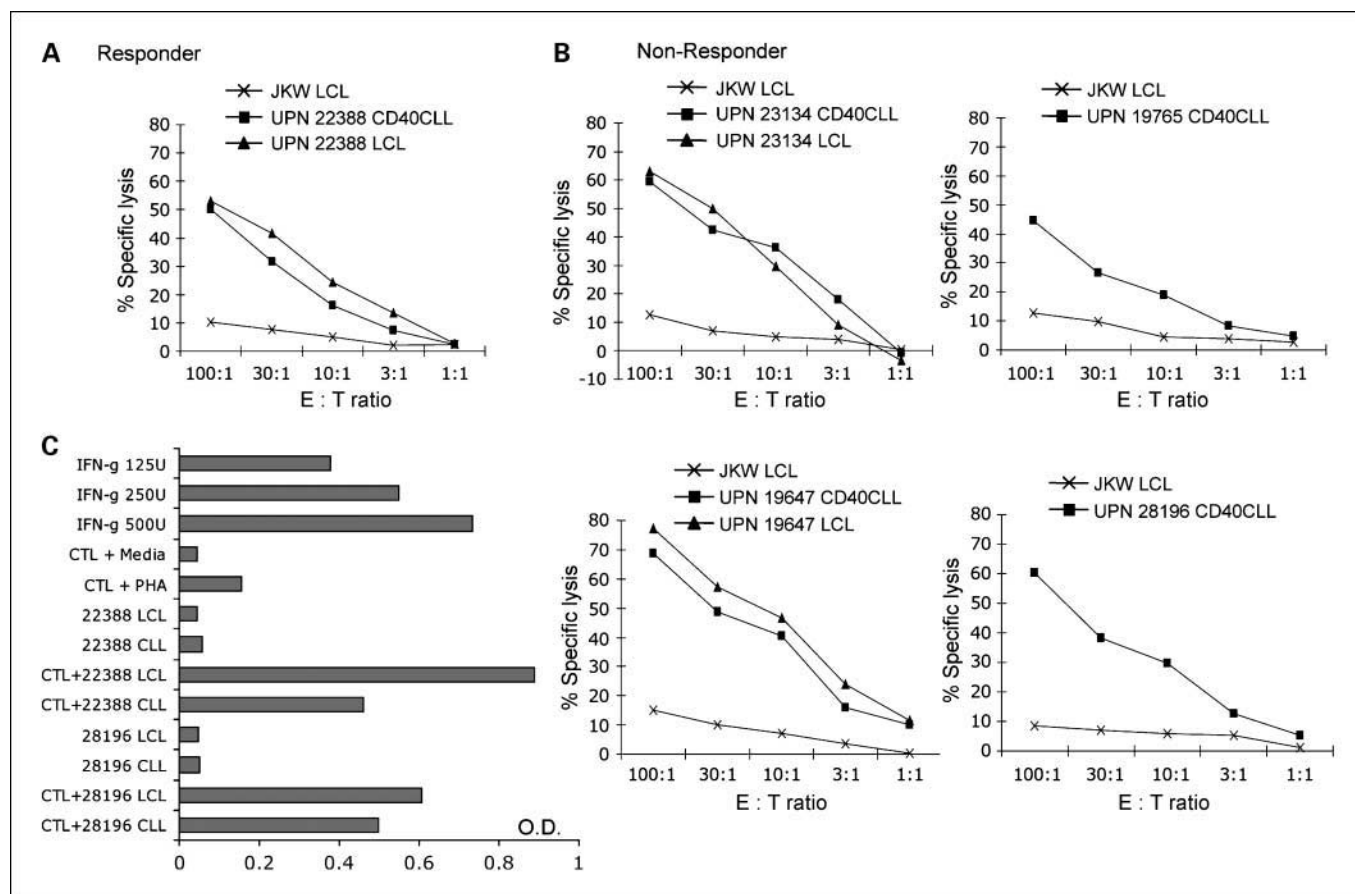


Fig. 2. CD40-CLL from patients who fail to respond to NM-HSCT are effective APCs. *A to B*, PBMC from an unrelated HLA-disparate individual (*JKW*) were stimulated in a mixed lymphocyte culture twice 1 wk apart with γ -irradiated CD40-CLL derived from a responding patient (UPN 22388) as a control (*A*), and with γ -irradiated CD40-CLL from each of the four transplant recipients who failed to achieve a CR after transplant (*B*). The T-cell lines were then assayed for cytotoxic activity against autologous (*JKW*) B-LCL, recipient CD40-CLL, and recipient B-LCL if available at effector to target ratios of 1:1–100:1. *C*, CLL cells and B-LCL from an HLA-A2⁺ male patients who had progressive CLL after NM-HSCT (28196) or who responded to NM-HSCT (22388) were tested for the ability to stimulate an SCMY-specific T-cell clone (CTL) to produce IFN- γ . T cells and stimulator cells were cocultured at a ratio of 3:1 for 24 h, supernatants were collected and IFN- γ was measured by ELISA.

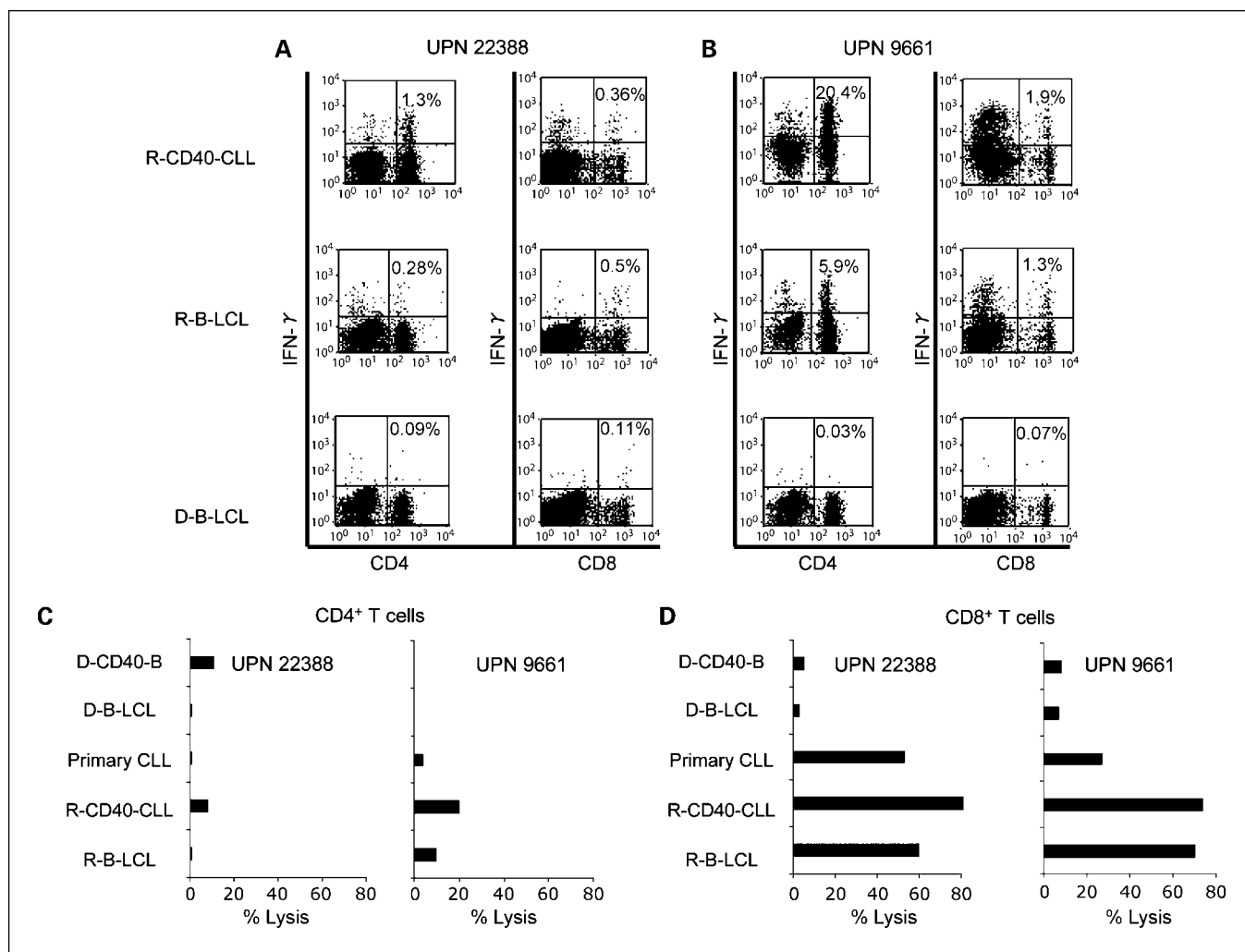


Fig. 3. CLL-reactive CD4⁺ and CD8⁺ T cells that produce IFN- γ and cytolytic CD8⁺ T cells are elicited after NM-HSCT in responding patients. *A* and *B*, T-cell lines from two representative patients (UPN 22388 and UPN 9661) were stimulated with recipient CD40-CLL (*top*) and B-LCL (*middle*), and with donor B-LCL (*bottom*) and stained with PE-conjugated IFN- γ detection reagent and either FITC-conjugated anti-CD4 or anti-CD8 monoclonal antibodies. *C* and *D*, CD4⁺ and CD8⁺ T cells were isolated by IFN- γ ⁺ capture after stimulation with recipient CD40-CLL, expanded with anti-CD3 monoclonal antibody and tested for lysis of recipient B-LCL, CD40-CLL, and primary CLL cells, and against donor B-LCL and CD40-activated B cells. Data are shown at an effector to target ratio of 30:1.

of B-LCL from unrelated donors that shared only a single HLA allele with the recipient. The restricting HLA allele and an estimate of the allele frequency was determined for nine minor H antigen-specific T-cell clones (Table 2A). Three of these T-cell clones also lysed IFN- γ -pretreated fibroblasts ($\geq 10\%$ lysis) demonstrating the expression of the minor H antigen recognized by these T cells was not restricted to hematopoietic cells. Five clones did not lyse fibroblasts and were specific for minor H antigens that are potentially selectively expressed on hematopoietic cells (Table 2A). We were unable to map the restricting HLA allele for many of the T-cell clones because the panel of partially matched unrelated B-LCL was not sufficient for conclusive assignment. However, based on differential recognition of B-LCL from unrelated donors, we could conclude that the T-cell lines from six of the seven patients targeted at least two distinct minor H antigens. Thus, at the time of tumor regression after NM-HSCT, the T-cell response was directed against multiple distinct minor H antigens expressed on CLL.

UPN 22388 had a dramatic reduction in circulating tumor cells early after transplant and did not develop GVHD in the first 80 days (Fig. 4A). Eleven T-cell clones were isolated from blood obtained on day 63 and all were restricted by HLA-B40 as shown for clone 2A2 (Table 2A). These T-cell clones did not recognize dermal fibroblasts consistent with the absence of clinical GVHD in this patient at day 63 after HSCT. The patient subsequently developed persistent chronic GVHD at day 120, suggesting that the T-cell response might have broadened to include recognition of additional minor H antigens. T-cell clones were generated from blood obtained at day 120 and 730 after transplant when the patient had active chronic GVHD, and analysis of the HLA-restricting allele for 9 of these T-cell clones revealed the recognition of additional distinct minor H antigens presented by HLA-A2, HLA-A31, HLA-B15, HLA-B40, and HLA-Cw3 (Table 2B). In contrast to the HLA-B40-restricted T-cell clone isolated at day 63 that did not recognize fibroblasts, the minor H antigens targeted by five of the T-cell clones isolated at these later time points were expressed both

by CD40-CLL and fibroblasts. One clone (13C6) isolated at day 120 only lysed B-LCL from male HLA-A2⁺ donors, suggesting it was specific for a minor H antigen encoded on the Y-chromosome (Table 3). Further studies revealed that 13C6 recognized a previously described epitope encoded by the SMCY gene (Fig. 4B), which has been associated with GVHD in prior studies (30). Of interest, SMCY-specific T cells were not detected in the T-cell lines generated at day 63 by tetramer analysis when the patient did not have GVHD, but tetramer binding cells were readily detected in the T-cell lines generated from day 120 and 374 samples (Fig. 4C). These data show that additional minor H antigen-specific T cells are recruited with time after NM-HSCT, analogous to epitope

spreading that has been described in autoimmunity and potentially induced by cytotoxic tumor cell death (37, 38). The T-cell clones that were specific for broadly expressed minor H antigens and isolated late after NM-HSCT, also recognized the recipient's CLL, and may contribute to sustaining the GVL effect (Table 2B).

The percentage of CD8⁺ T cells that produced INF-γ was higher after stimulation with CD40-CLL compared with recipient B-LCL in many patients, suggesting that T cells specific for minor H antigens or tumor-associated antigens that are selectively expressed on CLL may be present. In three patients in which sufficient tumor cells were available to use both as stimulator cells during cloning and as target cells in screening

Table 2. Characteristics of CD8+ CLL-reactive T-cell clones

A. T-cell clones isolated from responding patients at the time of tumor regression												
UPN	HLA			Days after HCT	T-cell clone	HLA restricting allele	Population frequency	% Specific lysis				
	A	B	C					R-B-LCL	R-CD40 CLL	D-B-LCL	Fibroblast	
9661	2/2	18/44	5/7	d+229	4F12	B44	2/12 (17%)	29	50	1	10	
					16B9	B44	6/12 (50%)	31	38	-1	4	
					20A5	A2	8/31 (26%)	37	43	2	4	
21899	2/24	8/18	7/12	d+355	2C2	B8	2/12 (17%)	46	39	10	7	
24487	2/33	7/15	3/7	d+404	2B10	A2	8/27 (30%)	63	20	2	20	
28736	1/3	7/8	7/7	d+110	9H11	A3	9/11 (81%)	42	71	-4	12	
					12C2	B7	6/11 (54%)	14	69	0	3	
29449	1/3	7/13	6/7	d+80	2A11	A3	4/9 (44%)	56	35	0	ND	
22388	2/31	15/40	3/3	d+63	2A2	B40	9/14 (64%)	73	64	-1	-1	
B. T-cell clones isolated from UPN 22388 when the patient had clinical GVHD												
22388	2/31	15/40	3/3	d+120	3C12	B40	10/14 (71%)	45	60	3	15	
					6F1	Cw3	6/13 (46%)	68	25	1	28	
					6F6	A31	2/4 (50%)	63	55	1	ND	
					13C6	A2	12/21 (57%)	63	60	1	25	
					16C4	B15	7/11 (63%)	70	65	0	22	
					14H10	A31	3/4 (75%)	14	71	0	ND	
					20G2	A31	3/4 (75%)	35	38	0	ND	
					d+730	5F4	B40	7/11 (63%)	79	72	0	71
					7B8	A2	2/11 (18%)	67	80	0	ND	
					C. T-cell clones that only recognized patient CLL but not B-LCL							
UPN	HLA			Days after HCT	T-cell clone	% Specific lysis						
	A	B	C			R-B-LCL	R-CD40 CLL	D-B-LCL				
28736	1/3	7/8	7/7	d+110	2A3	1	66	1				
					8A9	0	55	0				
					9C9	0	34	0				
					12D6	2	53	0				
29449	1/3	7/13	6/7	d+80	1A2	0	47	0				
					1B2	0	32	-1				
					2D2	-1	30	0				
					2E10	0	32	0				
					3G1	-1	54	-1				
22388	2/31	15/40	3/3	d+120	14D1	0	40	0				
					17C5	0	58	0				
					18E5	0	61	0				
				d+730	8A9	0	42	0				
					10A10	0	60	0				
					23H3	0	78	0				
					30B2	0	76	0				

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assays, multiple T-cell clones that recognized recipient CLL cells but not B-LCL were identified (Table 2C). The proportion of T-cell clones that lysed recipient CLL but not B-LCL varied between 30% and 79% of all T-cell clones isolated from these patients, suggesting that the tumor-specific response was a significant component of the overall T-cell response to CLL. We tested 16 clones obtained from the three patients against panels of primary and activated CLL from other patients sharing at least one HLA allele to determine if these T-cell clones might be directed against shared and/or nonshared tumor-associated antigens. Twelve of the 16 clones only lysed primary and/or activated CLL from the particular patient they were derived, but not CLL from other patients. Four clones generated from UPN 22388 (14D1, 8A9, 10A10, 30B2) recognized CLL from one or more unrelated patients; however, we did not have large enough panels of CLL to conclusively identify the HLA-restricting allele (data not shown). Nevertheless, our data show that T cells specific for antigens exclusively expressed on CLL but not on B-LCL are elicited after NM-HSCT. These antigens may include nonshared or shared tumor-specific antigens on CLL cells and/or minor H antigens that are only expressed on CLL.

Discussion

NM-HSCT can result in a durable remission for 40% to 70% of CLL patients who progress after fludarabine-based regimens (11, 15–17). The efficacy of HSCT in CLL does not correlate with factors that determine outcome after standard chemotherapy and is ascribed to a GVL effect mediated by immune cells in

the stem cell graft (9, 10, 13, 14). This study is the first to analyze the presence and kinetics of development of CLL-reactive T cells after NM-HSCT and to correlate T-cell responses with antitumor efficacy. The results show that T cells that specifically recognize recipient CLL and B-LCL, but not donor B-LCL, develop in all patients who achieved or maintained an antitumor response after NM-HSCT, but not in patients with persistent or progressive disease.

We used recipient CD40-CLL rather than PBMC as APC because our focus was to uncover T cells specific for antigens on the tumor cells, and the number of T cells in posttransplant samples was not sufficient to compare the alloreactivity that would be elicited using recipient APC derived from different cell types. In all responding patients, both CD8⁺ and CD4⁺ T cells that recognized recipient CLL and B-LCL, but not donor B-LCL were detected by IFN- γ production and/or cytotoxicity demonstrating that a component of the T-cell response that developed in responding patients is directed against minor H antigens on CLL. We used CD40-CLL cells as target cells in the initial assays to assess tumor cell recognition by the polyclonal T-cell lines generated from each patient. In subsequent experiments, CD4⁺ and CD8⁺ T cells were selectively enriched from the T-cell lines and assessed for recognition of primary CLL cells. CD8⁺ T-cells efficiently lysed primary CLL cells demonstrating that tumor cell recognition by this subset did not depend on CD40 activation. Isolation of individual CD8⁺ T-cell clones from the polyclonal T-cell lines confirmed that a significant component of the antitumor response was specific for minor H antigens, some of which were not broadly expressed on nonhematopoietic cells.

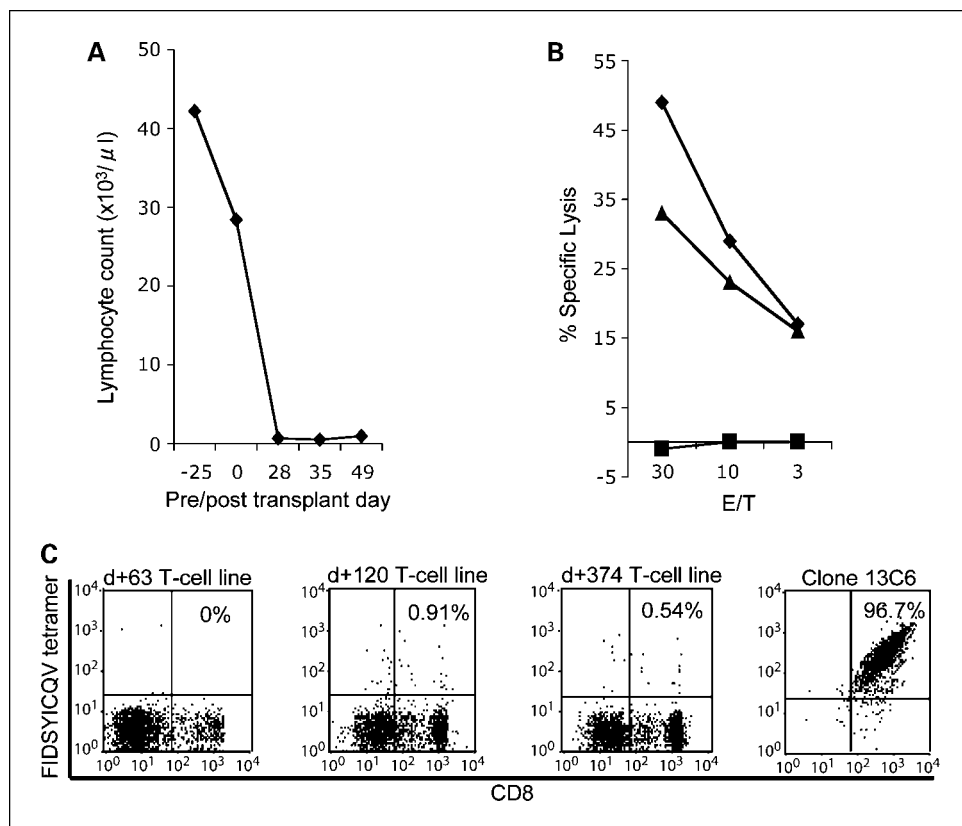


Fig. 4. CD8⁺ T cells reactive with the SMCY peptide FIDSYICQV develop late after NM-HSCT in UPN 22388. **A**, decline in total lymphocyte count in UPN 22388 in the first 28 d after transplant. **B**, recognition by SMCY-specific clone 13C6: recipient B-LCL (\blacklozenge); donor B-LCL either unpulsed (\blacksquare) or pulsed (\blacktriangle) with 10 $\mu\text{mol/L}$ FIDSYICQV and 3 $\mu\text{g/mL}$ human β -2 microglobulin. **C**, SMCY-specific T cells developed late after transplant in UPN 22388. The T-cell lines generated from UPN 22388 at day +63, +120, and +374 after transplant and the T-cell clone 13C6 were stained with PE-conjugated anti-CD3 and FITC-conjugated anti-CD8 monoclonal antibodies and an APC-conjugated HLA-A*0201 tetramer folded with FIDSYICQV.

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Table 3. Recognition of male and female B-LCL by clone 13C6

B-LCL	Sex	Lysis (%)
Recipient	Male	65
Donor	Female	-1
DEM	Male	83
DH	Male	89
RCB	Male	92
DMH	Male	80
VNA	Male	85
PGA	Male	73
RVC	Male	71
DMJ	Female	0
LMS	Female	-1
KSN	Female	-3

NOTE: The data are shown for an E/T ratio of 10:1.

Studies are in progress to clone the genes that encode minor H antigens recognized by these CLL-reactive CD8⁺ T cells.

A greater frequency of T cells produced IFN- γ in response to recipient CLL than to recipient B-LCL, suggesting that non-polymorphic tumor-associated antigens expressed only by CLL may be recognized. This was confirmed in a subset of three patients where sufficient tumor cells were available to use as APC in the cloning cultures. CD8⁺ T-cell clones that lysed recipient CLL but not B-LCL were isolated from all three patients. The tumor-associated antigens recognized by most of the T-cell clones were not shared by CLL cells from unrelated individuals, suggesting these antigens are derived from mutations or protein sequences that are unique to the CLL of the individual patient. Autologous CLL-reactive T cells have been described previously (39), and immunoglobulin idiotype has been identified as a tumor antigen in B-cell malignancies (40, 41). The possibility that idiotype is recognized by a component of the T-cell response elicited after NM-HSCT is currently being investigated.

The results of our analysis of CLL-reactive T cells after NM-HSCT provide insight into why the GVL effect may be so potent in eradication of this malignancy. Efforts to target malignancies by T-cell immunotherapy are commonly directed at a single tumor-associated antigen, and can fail because of the outgrowth of antigen loss tumor variants (42, 43). Our studies show that the CLL-reactive T-cell response after allogeneic NM-HSCT is composed of both CD8⁺ and CD4⁺ T cells, and commonly directed against multiple antigens. Moreover, analysis of the CD8⁺ T-cell response over time in a single patient illustrated the emergence of new T-cell clones specific

for minor H antigens expressed by CLL. This diversification of the alloreactive T-cell response capable of recognizing CLL may diminish the potential for outgrowth of tumor cell variants, and may explain why complete tumor regression is often delayed for several months.

Stimulation of posttransplant PBMC with recipient CD40-CLL did not elicit CLL-reactive T cells from any patient with persistent or progressive disease after transplant, despite GVHD in these patients. The failure of the GVL effect in these patients may be due to the absence of disparity between donors and recipients in the subset of minor H antigens that are expressed by CLL, or to a failure of T cells specific for disparate minor H antigens expressed on CLL to be activated and expand *in vivo*. CLL cells have been shown to induce defects in T cells in tumor-bearing patients and to be defective in immune synapse formation (44, 45), and it is possible that a large tumor burden at the time of transplant reduces the activation of donor T cells specific for antigens present on CLL.

Acute or chronic GVHD developed in all of the patients who achieved a CR, emphasizing the difficulty separating the potent GVL effect from GVHD with current approaches to NM-HSCT. Clonal analysis of CLL-reactive CD8⁺ T cells that developed in patients with GVHD showed recognition of minor H antigens that are broadly expressed on nonhematopoietic tissues (30, 32). Prior studies have identified minor H antigens such as PANE-1 and HB-1 that are selectively expressed on B-lineage cells (34, 35), but the allele frequency of these antigens is such that very few patients could benefit from targeted therapy to induce a GVL effect. Discovery of additional determinants with selective expression on recipient hematopoietic cells may allow targeting of leukemic cells by adoptive T-cell transfer or vaccination. Ideally, nonpolymorphic antigens expressed on CLL might be used as targets for immunotherapy. There is evidence that immunoglobulin framework, survivin, and fibromodulin peptides can be presented by CLL (46–48). Further studies to identify tumor-specific and minor H antigens that are recognized by T cells that develop after NM-HSCT might lead to more effective and selective targeting of malignant cells after NM-HSCT.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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