radio/chemotherapy conditioning without ATG). In addition, contrary to other clinical settings used to study the homeostatic recovery of lymphocytes (e.g., in AIDS patients treated with antiretroviral drugs or allogeneic hematopoietic cell transplant recipients), the recovery from lymphopenia was only minimally influenced by factors altering the homeostatic recovery. In AIDS patients, T lymphopoieses might be hampered by HIV or antiretroviral drugs [4,5]. In allogeneic hematopoietic cell transplant recipients, T and B lymphopoiesis might be hampered by graft-vs.-host disease (GVHD) or its treatment with immunosuppressive drugs [6–8]. In contrast, the autologous transplant recipients presented here were HIV-negative, did not develop true GVHD by definition, and were treated typically (per protocol) with only low-dose prednisone ($\leq$0.5 mg kg^{-1} day^{-1}). As prednisone was typically discontinued by 2 months posttransplant, immune recovery after 2 months posttransplant should reflect "natural" homeostatic recovery.

Methods

Patients and donors

Fifty-six patients with diseases of presumed autoimmune etiology (30 patients with systemic sclerosis and 26 patients with multiple sclerosis) underwent autologous CD34 cell transplantation as described [2,3]. Median age at transplant was 43 years (range, 23–61 years). There were 22 males and 34 females. None of the patients had a history of splenectomy. Twenty-eight patients were CMV seropositive pretransplant, 26 were CMV seronegative, and CMV serostatus was unknown for two patients. Transplant conditioning consisted of cyclophosphamide (120 mg/kg), total body irradiation (8 Gy), and ATG (typically of equine origin, 90 mg/kg). The CD34 cell autografts contained median 261.3 $\times$ 10^6 CD34 cells, 10.5 $\times$ 10^6 monocytes, 1.0 $\times$ 10^6 NK cells, 0.1 $\times$ 10^6 dendritic cells, 2.0 $\times$ 10^6 CD4 T cells, 1.2 $\times$ 10^6 CD8 T cells, and 8.1 $\times$ 10^6 B cells (determined in 27 patients). Blood for immune assays was drawn pretransplant (before filgrastim treatment for CD34 cell harvest), on day 7, and at approximately 1, 3, 6, 12, and 24 months posttransplant. Patients were followed for the assessment of immunity (by laboratory parameters and infection rates) for 2 years or until death, disease progression/relapse/pulmonary toxicity or last contact, whatever occurred first. The follow-up ended at the time of disease progression/relapse or pulmonary toxicity because at that time patients typically started treatment with corticosteroids or other immunosuppressive drugs. Thirty-seven patients were followed for 2 years and 19 patients were followed for <2 years. The numbers of blood samples analyzed at each time point are given in the legends to Figs. 1–4. Posttransplant infection prophylaxis and prednisone were administered as described in Table 1. During the 2-year follow-up, patients were not treated with immunoglobulin.

For the determination of the normal reference ranges displayed in Figs. 1–4, for most assays, we studied blood from healthy adult volunteers (n = 104 for surface immunophenotyping, 27 for Ki67 intracellular immunophenotyping, 64 for TREC determination, 27 for spectratyping, and 65 for tetanus, *Hemophilus influenzae*, and *S. pneumoniae* IgG). Their median ages were similar to the median age of the patients (43 years for surface immunophenotyping, 43 for Ki67 intracellular immunophenotyping, 44 for TREC determination, 43 for spectratyping, and 43 for tetanus, *H. influenzae*, and *S. pneumoniae* IgG). For neutrophil counts and IgM, IgA, IgG, and IgG_2 levels, we displayed the normal adult 2.5th–97.5th percentile range determined by the manufacturer of the instrument or kit used. For autoantibody levels, see "Antibody levels". For normal thymic size (index), we used 22 adult patients of median age, 43 years, who had chest computer tomogram (CT) done for various reasons. They had no acute illness, congenital T cell deficiency, HIV disease, myasthenia gravis, hyperthyroidism, or malignancy, and were not treated with chemotherapy, radiation, or immunosuppressive drugs/systemic steroids. The rationale for displaying normal reference ranges in Figs. 1–4 in addition to patient pretransplant values is that the pretransplant values may be artificially low due to previous chemotherapy/immunosuppressive therapy [2,3]. The study was approved by the Institutional Review Board.

Immunophenotyping

Enumeration of mononuclear cell (MNC) subsets was performed as described [9]. Naïve CD4 T cells were defined as $CD45RA^{high}$ CD4 T cells because this subset contains thymic emigrants, and nearly all cord blood CD4 T cells are $CD45RA^{high}$ [10–12]. Naïve CD8 T cells were defined as $CD11a^{low}$ CD8 T cells because virtually all cord blood CD8 T cells are $CD11a^{low}$ and become $CD11a^{high}$ after activation [13,14]. Moreover, after hematopoietic cell transplantation, $CD45RA^{high}$ CD4 T cell counts correlate with $TREC^+$ CD4 T cell counts, and $CD11a^{low}$ CD8 T cell counts correlate with $TREC^+$ CD8 T cell counts [15]. Naïve B cells were defined as IgD^+ B cells as most IgD^+ B cells lack somatic mutations [16]. Monocytes were defined as $CD14^+$ MNCs. NK cells were defined as MNCs expressing CD16 or CD56 and not expressing CD3 or CD14. Dendritic cells were defined as $HLADR^{high}$ MNCs not expressing CD3, CD14, CD16, CD20, CD34, or CD56. For the enumeration of $Ki67^+$ CD4 or CD8 T cells, FACS Lysing Solution (BD Biosciences, San Jose, CA), 2.5 ml, was added to a pellet of up to 2 million blood MNCs (cryopreserved, as opposed to the above surface-only staining and flow cytometry performed on fresh MNCs). The cells were resuspended and incubated at room

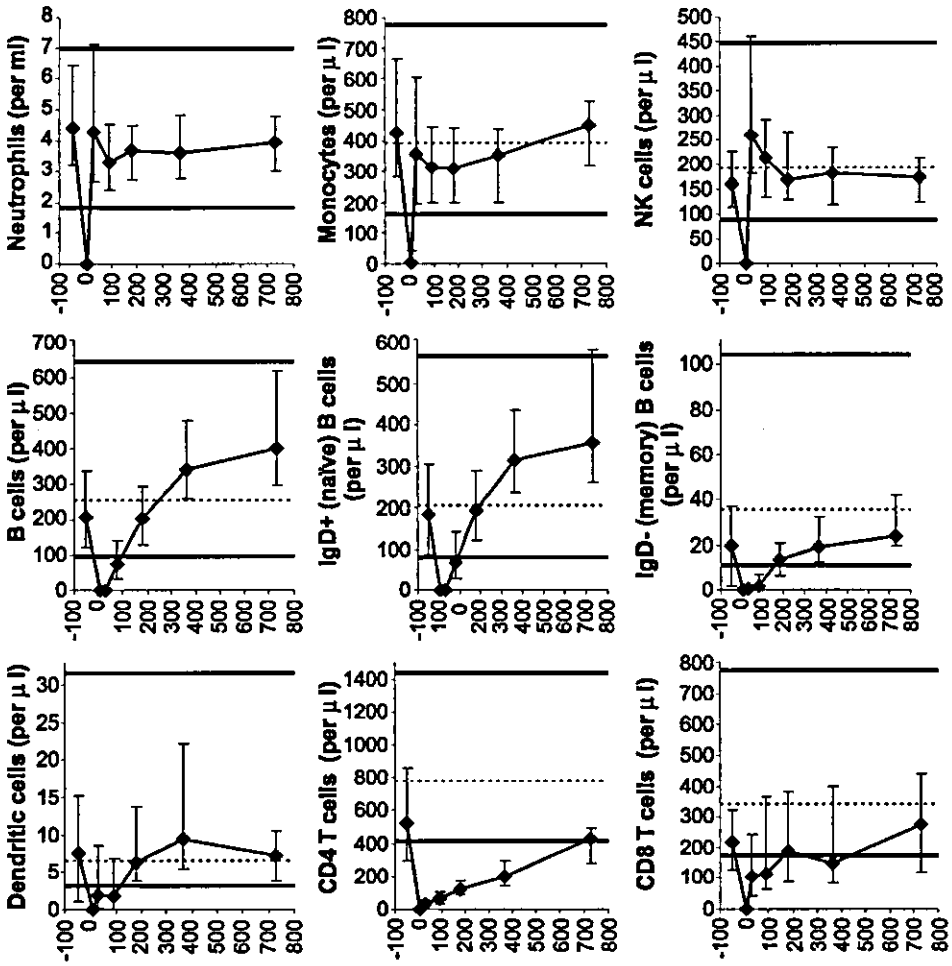


Fig. 1. Recovery of leukocyte subsets. All horizontal axes display days posttransplant. Patient medians (diamonds) and 25th–75th percentiles (error bars) are shown. Normal medians are indicated by the dashed horizontal lines (except for neutrophils—not available). The thick horizontal lines denote the normal 5th and 95th percentiles (except for neutrophils—2.5th and 97.5th percentiles). Pretransplant studies are arbitrarily shown as day −50 studies. The following numbers of patient blood samples were analyzed: for neutrophils (by an automated hematology analyzer), 56 pretransplant, 49 on day 7, 49 at 1 month, 50 at 3 months, 34 at 6 months, 40 at 12 months, and 26 at 24 months posttransplant; for all other leukocyte subsets (by immunophenotyping), 47 pretransplant, 41 on day 7, 48 at 1 month, 41 at 3 months, 33 at 6 months, 36 at 12 months, and 17 at 24 months posttransplant.

temperature for 10 min. After centrifugation, the cells were resuspended in 500 μl of 1× FACS Permeabilizing Solution and incubated at room temperature for 10 min. Cells were washed in flow cytometry buffer (PBS with 1% bovine serum albumin and 0.1% sodium azide). After centrifugation and removal of supernatant by tube inversion, the cells were resuspended in the residual buffer (approximately 100 μl) and incubated for 30 min at 4°C with the following monoclonal antibody–fluorochrome conjugates: CD3-FITC, Ki67-PE, CD11A-APC, CD8-APCCy7, CD4-PerCp5.5, and CD45RA-ECD, or CD3-FITC, isotype control-PE, CD11A-APC, CD8-APCCy7, CD4-PecCp 5.5, and CD45RA-ECD (negative control). After washing with flow cytometry buffer, analysis was done on LSR-II cytometer (BD Biosciences). A minor portion of the immunophenotyping results has been published (the counts of total CD4 and CD8 T cells, B cells, and NK cells) [2,3].

Thymic size

Patients with systemic sclerosis had chest CT performed routinely pretransplant and at 1, 3, 12 months and annually posttransplant. Thymic index (a semiquantitative determination of thymic size) was determined as described by McCune et al. [17] except that a scale of 1–5 was used (1 denotes 0 or 1 of McCune's scale). The determination was done by one radiologist (E.L.) blinded to patient demographic and clinical data. The numbers of CT studies analyzed were 17 pretransplant, 12 at 1 month, 20 at 3 months, 19 at 1 year, and 12 at 2 years posttransplant.

TREC assay

$CD3^+CD4^+CD8^-$and $CD3^+CD4^-CD8^+$ cells were sorted to >98% purity from Ficoll-separated MNCs, using

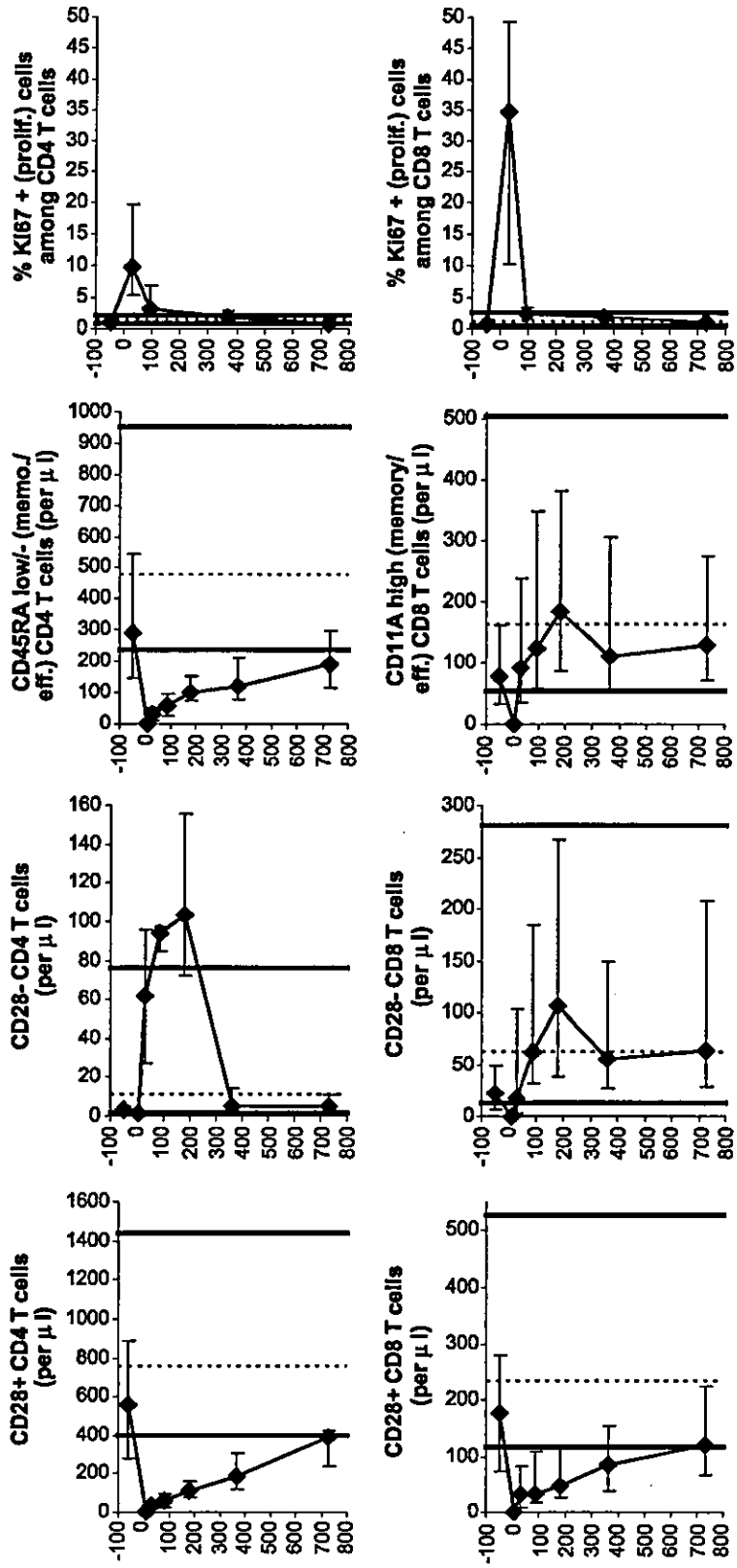


Fig. 2. Recovery of T cell subsets. For symbols and the numbers of patient samples analyzed, see Fig. 1 legend.

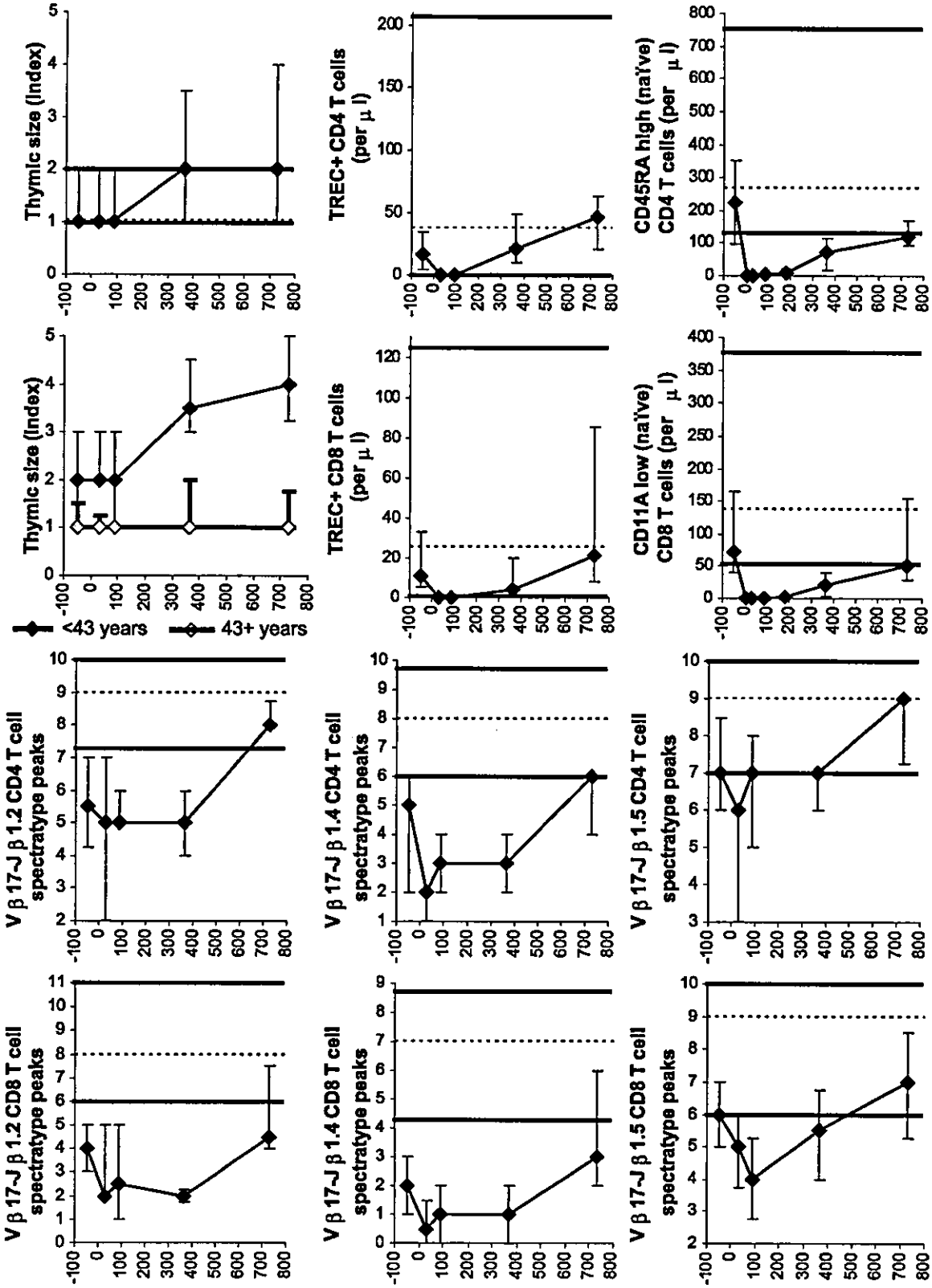
Vantage flow sorter (BD Biosciences). A total of 50,000 CD4 or CD8 T cells were sorted into an Eppendorf tube. After centrifugation, supernatant was removed and the "dry" cell pellet was stored frozen (−80°C). Real-time PCR of the αδ signal joint was performed directly from lysate of the cell pellet, which was lysed in 100 μg/ml proteinase K. The 5′-nuclease (Taqman) assay was performed on 5 μl of cell lysate (total volume of the lysate of 50,000 cells was 40 μl; thus, 5 μl of the lysate contained TRECs from 6250 cells), using primers CACATCCCTTTCAACCATGCT and GCCAGCTGCAGGGTTTAGG and probe FAM-ACACCTCTGGTTTTTGTAAAGGTGCCCACT-TAMRA (MegaBases, Chicago, IL). PCR reaction contained 0.5 U Taq polymerase, 3.5 mM $MgCl_2$, 0.2 mM dNTPs, 400 nM each primer, 200 nM probe, and Blue-636 reference (MegaBases). The reactions were run at 95°C/5 min, then at 95°C/30 s, and 60°C/60 s for 40 cycles, using ABI Prism 7700 Sequence Detector (PE Biosystems, Norwalk, CT). Samples were analyzed in triplicates. Plasmids containing the αδ signal joint region were used as standards. A standard curve was plotted and the TREC level (the number of TREC copies per 6250 CD4 or CD8 T cells) was calculated using the ABI7700 software. The absolute count of $TREC^+$ CD4 (CD8) T cells (per microliter of blood) was calculated as the TREC level (per 6250 cells) multiplied by the absolute CD4 (CD8) T cell count (per microliter) and divided by 6250.

Spectratyping

To maximize the likelihood of finding a low number of spectratype peaks in samples with low T cell diversity, Vβ-Jβ instead of Vβ-Cβ spectratyping was done. Because of the limited number of T cells available early posttransplant, we focused on only one Vβ family (arbitrarily Vβ17). To maximize sensitivity and specificity of the downstream PCR, $V\beta17^+CD4^+CD8^-$ and $V\beta17^+CD4^-CD8^+$ cells were first sorted to >95% purity from Ficoll-separated MNCs, using Vantage flow sorter (BD Biosciences). A total of 2000 $V\beta17^+$CD4 or $V\beta17^+$CD8 T cells were sorted into solution D, prepared by mixing 7 μl β-mercaptoethanol (14.2 M) with 100 μl Lysis Buffer from Absolutely RNA Nanoprep kit (Stratagene, La Jolla, CA). RNA was extracted using the Absolutely RNA Nanoprep kit, and cDNA was synthesized using Superscript II RNase H^- Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA) and $Oligo(dT)_{12-18}$ primers (Invitrogen). Each reverse transcription yielded 20 μl cDNA. Vβ17-Jβ1.2, Vβ17-Jβ1.4 or Vβ17-Jβ1.5 segments were separately amplified by PCR, using forward primer FAM-CAGAAAGGAGATATAGCTGAA (Vβ17) and reverse primer GTTAACCTGGTCCCCGAAC (Jβ1.2), AGACAGAGAGCTGGGTTCCA (Jβ1.4), or GGAGAGTCGAGTCCCATCA (Jβ1.5). PCR reactions were carried out in a 20-μl volume. Each reaction contained 5 μl cDNA, 1 U Platinum-Taq polymerase (Invitrogen), 2.5 mM $MgCl_2$, 500 μM dNTPs, and 500 nM each primer. The reactions were run at 95°C/9 min, then at 94°C/30 s, 58°C/30 s, and 72°C/30 s for 35 cycles, and finally at 72°C/9 min. The dye-labeled PCR products were detected on an ABI 3100 sequence analyzer and the number of peaks was determined visually using GeneScan software (Applied Biosystems, Foster City, CA). *Note:* per the ImMunoGeneTics nomenclature [18], Vβ17 is TRBV19, Jβ1.2 is TRBJ1-2, Jβ1.4 is TRBJ1-4, and Jβ1.5 is TRBJ1-5.

Antibody levels

Serum levels of total IgM, IgA, and IgG were determined in local clinical laboratories, typically by nephelometry (our clinical laboratory used a kit from Dade Behring, Marburg, Germany). Levels of total IgG_2 and IgG specific for tetanus toxoid, *H. influenzae* capsular polysaccharide and pneumococcal polysaccharides were determined in our laboratory as described [19], using ELISA kits from The Binding Site (Birmingham, U.K.). Tetanus, *H. influenzae*, and *S. pneumoniae* IgG levels were not analyzed at 2 years because some patients were vaccinated against tetanus, *H. influenzae* and *S. pneumoniae* between 1 and 2 years posttransplant, and data on the timing of vaccination and the number of vaccine doses were not available. Scl-70 antibodies were analyzed only in patients who had these antibodies pretransplant, or, if the pretransplant sample was missing, in the first 3 months posttransplant (n = 6 pretransplant, 7 at 1 month, 7 at 3 months, 7 at 12 months, and 3 at 24 months posttransplant). Scl-70 antibodies were determined by ELISA using a kit from BioRad/Helix Diagnostics (West Sacramento, CA). The normal reference range in Fig. 4 is displayed as 0–20 enzyme units (EU) as per the manufacturer of the kit; levels >20 EU are defined as abnormal. Myelin antibodies (against either myelin basic protein or myelin oligodendrocyte glycoprotein) were determined in patients with multiple sclerosis who had these antibodies pretransplant, or, if the pretransplant sample was missing, in the first 3 months posttransplant (n = 14/10 [IgM/IgG] pretransplant, 11/8 at 1 month, 14/10 at 3 months, 10/9 at 12 months, and 4/4 at 24 months posttransplant) by Western blot as described [20,21]. As controls, monoclonal antibodies to myelin basic protein (MAB381, Chemicon, Temecula, CA) and myelin oligodendrocyte glycoprotein (8.18-C5 [22]) and a positive and a negative human control sera were used. The levels were semiquantitatively scored by a blinded investigator as negative (0), marginally positive (0.5), weakly positive (1), moderately positive (2), or strongly positive (3). If a patient had antibodies against both myelin oligodendrocyte glycoprotein and myelin basic protein, the higher score (of the two antibodies) was used for analysis. The normal reference (5th–95th percentile) range displayed in Fig. 4 is 0–0, as 97% normal adults have undetectable myelin antibodies (Berger, unpublished).


Thymic size (Index)
TREC+ CD4 T cells (per μl)
CD45RA high (naïve) CD4 T cells (per μl)
Thymic size (Index)
TREC+ CD8 T cells (per μl)
CD11A low (naïve) CD8 T cells (per μl)
<43 years
43+ years
V β 17-J β 1.2 CD4 T cell spectratype peaks
V β 17-J β 1.4 CD4 T cell spectratype peaks
V β 17-J β 1.5 CD4 T cell spectratype peaks
V β 17-J β 1.2 CD8 T cell spectratype peaks
V β 17-J β 1.4 CD8 T cell spectratype peaks
V β 17-J β 1.5 CD8 T cell spectratype peaks

Infections

Documented infection was defined as an illness with symptoms and signs consistent with an infection and microbiological documentation of a pathogen, except for dermatomal zoster where the clinical diagnosis was considered sufficient. Microbiological documentation included isolation of the pathogen by culture from a sterile site or a nonsterile site (if from a nonsterile site, the organism had to be clinically judged as pathogenic) or histological/immunohistological evidence. Presence of a microorganism in blood by culture (not by antigen or nucleic acid detection) was counted as a documented infection even in the absence of symptoms or signs of infection. Clinical (presumed) infection (without an identified microorganism) was defined as illness with symptoms and signs consistent with an infection; however, presumed respiratory tract infections were discounted because they could not be reliably distinguished from allergy; hemorrhagic cystitis was also discounted because it could not be differentiated from conditioning regimen-induced cystitis. Fever of presumed infectious etiology was counted only if >38.5°C and if it responded to antibiotics within 3 days. A chronic infection was counted as one infection. A recurrent infection was counted as multiple infections only if the episodes were clearly separated by >4-week asymptomatic period. A polymicrobial infection of one organ or several adjacent organs was counted as one infection (due to the organism that was considered the major pathogen). Infections with one microorganism in two nonadjacent organs were counted as two infections. Respiratory tract was considered adjacent to paranasal sinuses and lungs. Lungs and paranasal sinuses were considered nonadjacent. An organ infection with viremia/bacteremia/fungemia was counted as one infection. Severe infections were defined as infections treated in a hospital. Nonsevere infections were treated in an outpatient setting.

Statistics

Significance of differences in laboratory parameters of immunity between healthy volunteers and patients at each time point were tested using Mann–Whitney–Wilcoxon rank sum test. In Figs. 1–4, whenever a patient median falls on or outside of the 5th–95th percentile range, the difference is significant ($P < 0.05$, two-tailed), except for thymic index pretransplant and at 1 and 3 months posttransplant ($P > 0.05$) and for neutrophil counts and IgM, IgG, IgA, and Scl-70 antibody levels (the significance was not tested because the individual reference group data were not available to us). The information on the significance of differences between patients and normals is not presented in Figs. 1–4 to avoid information congestion. Significance of differences in thymic size (index) between <43-year-old and ≥43-year-old patients at each time point was tested by the Mann–Whitney–Wilcoxon rank sum test. Significance of the difference in the percentage of $Ki67^+$ cells among CD4 vs. CD8 T cells was also tested by the Mann–Whitney–Wilcoxon rank sum test. Significance of correlation between patient age and a laboratory parameter of immunity was tested by Spearman test. Significance of difference between <43- and ≥43-year-old patients in the infection rates (tabulated as the number of days at risk with and without a newly diagnosed infection) was tested by the χ^2 test.

Results

Immune cells

Median leukocyte (WBC) count measured by an automated hematology analyzer reached a nadir of 20/μl on day 5. On day 7, it was 50/μl, and virtually all leukocytes were mononuclear cells (MNCs). By flow cytometry, median 75.6% of the MNCs were monocytes, 19.5% NK cells, 0.6% T cells, and 0.2% B cells.

Innate immune cell (neutrophil, monocyte, and NK cell) counts returned to the normal range by 1 month posttransplant. B cell counts recovered by 6 months posttransplant. Memory B cell counts recovered more slowly than naïve B cell counts. This may be because naïve B cells only differentiated into memory B cells after encountering their cognate antigens. Dendritic cell counts recovered by 6 months posttransplant. T cells showed the slowest recovery—CD8 T cell counts were borderline low at 6 and 12 months and returned back to normal by 2 years, and CD4 T cell counts reached borderline low normal levels only at 2 years posttransplant (Fig. 1).

T cell recovery was studied in detail (Figs. 2 and 3). Peripheral expansion, assessed by the percentage of $Ki67^+$ (proliferating) T cells, was robust early posttransplant, particularly for CD8 T cells. At 1 month posttransplant, median 10% CD4 T cells and 35% CD8 T cells were $Ki67^+$ (normal ≤ 3%). The difference in the percentage of $Ki67^+$ cells among CD4 vs. CD8 T cells at 1 month was statistically significant ($P = 0.01$). The difference was important as memory/effector CD8 T cell counts recovered to normal within 1 month posttransplant whereas

Fig. 3. Thymic size and recovery of T cell subsets and diversity. For symbols, see Fig. 1 legend. The numbers of patient CT studies and blood samples analyzed were as follows: for the thymic index, 17 pretransplant, 12 at 1 month, 20 at 3 months, 19 at 1 year, and 12 at 2 years posttransplant; for TRECs, 28 pretransplant, 33 at 1 month, 22 at 3 months, 23 at 12 months, and 9 at 24 months posttransplant; for phenotypically naïve T cells, 47 pretransplant, 41 on day 7, 48 at 1 month, 41 at 3 months, 33 at 6 months, 36 at 12 months, and 17 at 24 months posttransplant; and for spectratyping, 27 pretransplant, 21 at 1 month, 21 at 3 months, 17 at 12 months, and 10 at 24 months posttransplant. In the top left graph on thymic size, both the low normal limit (5th percentile) and the median are 1 (for clarity, the low normal limit is displayed as 0.97 and the median as 1.03).

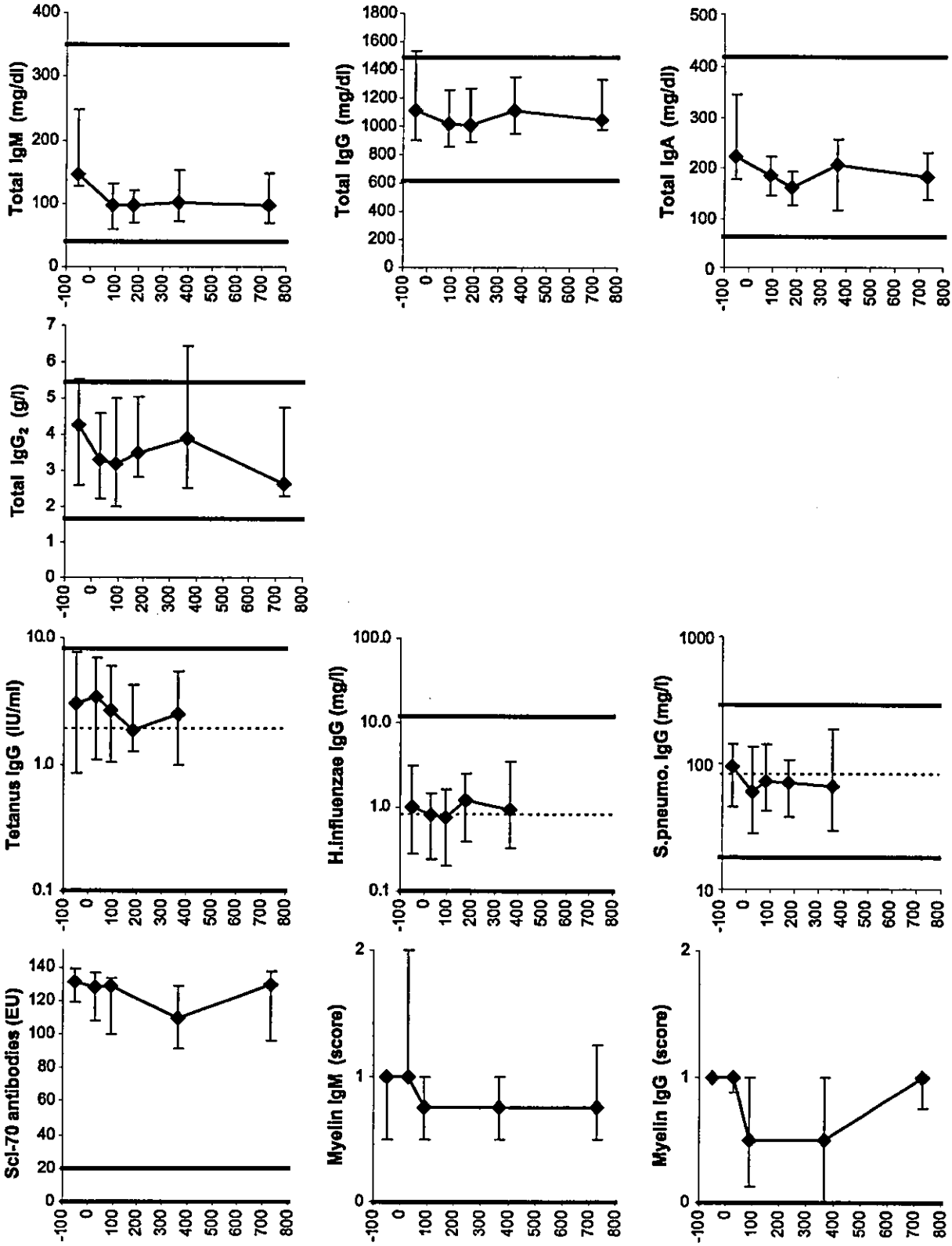

Total IgM (mg/dl)
Total IgG (mg/dl)
Total IgA (mg/dl)
Total IgG2 (g/l)
Tetanus IgG (IU/ml)
H.influenzae IgG (mg/l)
S.pneumo. IgG (mg/l)
Scl-70 antibodies (EU)
Myelin IgM (score)
Myelin IgG (score)

Table 1
Infection rates[a]

Time interval	Days 0–30	Days 31–180	Days 181–365	Days 366–730
Substantially deficient components of immunity	Neutrophils, monocytes, NK cells, enterocytes (presumed), dendritic cells, B cells, CD8 T cells, CD4 T cells (median 0 on d7 and 33/μl on d30)	Dendritic cells, B cells, CD8 T cells, CD4 T cells (median 33/μl on d30 and 118/μl on d180)	CD4 T cells (median 118/μl on d180 and 194/μl on d365)	CD4 T cells (median 194/μl on d365 and 429/μl on d730)
Infection prophylaxis	Antiviral[b], cephalosporin or Trim/sulfa[c], fluconazole[d]	Antiviral[b], Trim/sulfa[c], Fluconazole[d]	Antiviral[b], Trim/sulfa[c]	None
Immuno-suppressive drugs	Prednisone[e]	Prednisone/None[e]	None[e]	None
Rate of total infections	5.75	0.94	0.29	0.16
Rate of severe infections[f]	4.90	0.54	0.05	0.08
Rate of nonsevere infections[g]	0.85	0.40	0.24	0.08
Rate of documented infections[h]	4.90	0.74	0.05	0.11
Microorganisms causing the documented infections[i]	HSV (1) CMV (2) BKV (1) Gram+ (14) Gram-(5)	HSV (1) VZV (3) EBV (2) Parainfl 3 (1) Gram+ (5) Gram− (3)	VZV (1)	VZV (3) Gram+ (1)

[a] Each infection rate is expressed as the number of infections per 365 patient days, i.e., the number of infections in all patients occurring during the specified period divided by the number of days at risk and multiplied by 365. The numbers of days at risk (the sum of days at risk for each patient, i.e., the number of days from the beginning of the time period until the end of the time period or death, disease progression/relapse/pulmonary toxicity or loss to follow-up) were 1713 for days 0–30, 7407 for days 31–180, 7683 for days 181–365, and 13,514 for days 366–730. Presumed (clinical) respiratory tract infections were discounted (see Methods).

[b] Typically acyclovir 800 mg twice a day orally or valacyclovir 500 mg twice a day orally until day 365. In addition, CMV pp65 antigenemia or plasma CMV DNAemia was monitored weekly until day 100 and then every other week until day 365. Ganciclovir was given to two patients without documented CMV disease preemptively. Documented CMV disease occurred in two patients (one had gastritis and one had gastritis+esophagitis+pneumonia).

[c] Typically ceftazidime 2 g every 8 h intravenously during neutropenia <500/μl, and trimethoprim/sulfamethoxazole (Trim/sulfa) 160/800 mg once a day orally from neutrophil engraftment until day 365.

[d] Typically fluconazole 400 mg once a day orally until day 75.

[e] Patients with systemic sclerosis typically received 0.5 mg kg^{-1} day^{-1} between days −6 and 30 with subsequent taper to day 60. Patients with multiple sclerosis typically received 0.5 mg kg^{-1} day^{-1} between days 7 and 21 with subsequent taper to day 30. One patient had a prolonged prednisone taper, reaching 10 mg/day at 6 months posttransplant. One patient restarted prednisone 85 mg/day at 5 months posttransplant for lymphocytic gastritis, and subsequently tapered, reaching 10 mg/day at 15 months posttransplant. One patient restarted prednisone 20 mg/day at 9 months posttransplant for pericarditis, and subsequently tapered, reaching 10 mg/day at 10 months posttransplant.

[f] Treated inpatient.

[g] Treated outpatient.

[h] Infections with a known etiologic agent.

[i] Numbers in parentheses denote the number of infections. Abbreviations: HSV—herpes simplex virus; VZV—varicella-zoster virus; CMV—cytomegalovirus; EBV—Epstein–Barr virus; BKV—BK virus; Parainfl 3—parainfluenza 3 virus; Gram+—gram-positive bacteria; Gram− —gram negative bacteria.

memory/effector CD4 T cell counts remained low for at least 2 years (Fig. 2). The relative insufficiency of the peripheral expansion of CD4 T cells could be due not only to decreased proliferation (compared to that of CD8 T cells) but also shorter survival. Indirect evidence for a contribution of the latter mechanism is that abnormally high numbers of $CD28^-$ CD4 T cells were generated in the first several months posttransplant (Fig. 2). $CD28^-$ T cells have short telomeres and thus presumed short life span [23,24].

Fig. 4. Serum antibody levels. For symbols, see Fig. 1 legend. For normal reference ranges, see Methods. Normal medians were not available for total IgM, IgG, IgA, and IgG_2, and Scl-70 and myelin antibodies. The 24 months time point results are not displayed for the tetanus, *H. influenzae*, and *S. pneumoniae*-specific IgG levels because of inconsistent immunization between 12 and 24 months posttransplant. The numbers of patient serum samples analyzed were as follows: for total IgM, IgG and IgA, 23 pretransplant, 34 at 3 months, 18 at 6 months, 33 at 12 months, and 19 at 24 months posttransplant; for total IgG_2 and tetanus/*H. influenzae*/*S. pneumoniae*-specific IgG, 42 pretransplant, 36 at 1 month, 38 at 3 months, 27 at 6 months, 37 at 12 months, and 14 at 24 months posttransplant; for Scl-70 antibodies, 6 pretransplant, 7 at 1 month, 7 at 3 months, 7 at 12 months, and 3 at 24 months posttransplant; for myelin IgM, 14 pretransplant, 11 at 1 month, 14 at 3 months, 10 at 12 months, and 4 at 24 months posttransplant; and for myelin IgG, 10 pretransplant, 8 at 1 month, 10 at 3 months, 9 at 12 months, and 4 at 24 months posttransplant.

De novo generation (thymopoiesis) contributed significantly to increasing T cell counts at >3 months posttransplant. An increase in thymic size from baseline and an increase in $TREC^+$ T cells from a median of zero were first detected between 3 and 12 months, and $TREC^+$ T cell counts continued to rise between 1 and 2 years posttransplant (Fig. 3). This coincided with increasing numbers of phenotypically naïve T cells and increasing T cell diversity assessed by the number of T cell receptor β-chain spectratyping peaks (Fig. 3). De novo generation was age-dependent. Thymic hypertrophy was detectable by CT at 1 and 2 years posttransplant in<43-year-old but not in ≥ 43-year-old patients [Fig. 3; the difference in thymic size was statistically significant at 1 year ($P = 0.01$) and 2 years ($P = 0.03$) posttransplant]. There were also trends toward an inverse correlation between patient age and the counts of $TREC^+$ CD4 and CD8 T cells, phenotypically naïve CD4 and CD8 T cells, and total CD4 (but not CD8) T cells at 1 and 2 years posttransplant and the number of spectratyping peaks at 2 years posttransplant (data not shown). Statistical significance of the inverse correlation was reached for phenotypically naïve CD4 ($R = -0.45$, $P = 0.007$) and phenotypically naïve CD8 ($R = -0.38$, $P = 0.02$) T cells at 1 year posttransplant and for Vβ17-Jβ1.5 CD8 spectratyping peaks at 2 years posttransplant ($R = -0.71$, $P = 0.02$). Interestingly, although thymic hypertrophy was not detected by CT in most ≥ 43-year-old patients, an increase in $TREC^+$ CD4 and CD8 T cell counts, though smaller than in <43-year-old patients, occurred after 3 months posttransplant in all ≥43-year-old patients measured (data not shown).

Antibodies

Even though B cell and CD4 T cell counts were very low in the first 3 months posttransplant, median serum levels of total IgM, IgA, IgG, IgG_2 as well as IgG specific for tetanus toxoid, *H. influenzae*, and *S. pneumoniae* remained normal (Fig. 4). Median levels of autoantibodies against Scl-70 and myelin continued to be abnormally high throughout the 2-year follow-up (Fig. 4).

Infections

As expected, infections were frequent in the first month, when multiple components of immunity were abnormal. Surprisingly, infections between day 31 and 730 were infrequent in spite of the profound initial lymphopenia (in particular CD4 lymphopenia) (Table 1). Two fatal infections occurred, both due to Epstein–Barr virus (EBV)-associated lymphoproliferation at approximately 2 months posttransplant. These occurred in two patients who at 1 month posttransplant had undetectable T cells (at that time point, 4/48 patients analyzed by immunophenotyping had undetectable T cells whereas the remaining 44 patients had detectable T cells). Details on these two patients were previously reported [25].

Day 181–730 infections attributable to T-lymphopenia (as counts of other immune cells have recovered by day180) occurred with a low frequency of 0.21 total infections per patient year (0.07 severe infections and 0.09 documented infections per patient year). The day 181–730 total infections were less frequent in <43-year-old patients than in ≥43-year-old patients (0.07 vs. 0.36 per patient year, $P = 0.03$). There were no statistically significant differences in the day 181–730 total infection rates between patients with above vs. below median values of CD4 or CD8 T cell counts, naïve CD4 or CD8 T cell counts, $TREC^+$ CD4 or CD8 T cell counts, or numbers of CD4 or CD8 spectratype peaks (average of the 3 families measured) at 1 year posttransplant. However, the power to detect these differences at a statistically significant level was lower than the power to detect the difference in infection rates between the <43- and ≥43-year-old patients—the laboratory parameters at 1 year posttransplant were available for only ≤36 patients, whereas the data on infections and age were available for all 44 patients surviving without relapse/disease progression/pulmonary toxicity beyond day 180.

Discussion

In agreement with published reports on recovery from moderate leukopenia (reviewed in Refs. [26–29]), we found that following severe leukopenia (particularly lymphopenia) induced by cyclophosphamide, total body irradiation, and ATG, cells of innate immunity recovered first, followed by B cells, and then T cells. T cell recovery was biphasic. Peripheral expansion dominated in the first 3 months, whereas thymopoiesis contributed to increasing T cell counts and diversity later posttransplant. The novel findings of this study (discussed below) are that the severe lymphopenia was not associated with a substantial decline in antibody levels, and that infections attributable to CD4 T lymphopenia were rare.

This study highlighted an important difference between CD4 and CD8 T cells in their ability to undergo homeostatic peripheral expansion. For CD8 T cells, the expansion was so robust that normal memory/effector CD8 T cell counts were reached by 1 month. For CD4 T cells, the expansion was only moderate, and normal memory/effector CD4 T cell counts were not reached by 2 years. The fact that CD8 T cell counts recover faster than CD4 T cell counts has been known [26,27,29]; the novel finding of our study is that the faster CD8 T cell recovery is due to the robust peripheral expansion. Viral infections typically result in a far greater expansion of CD8 than CD4 T cells [30]. Perhaps, reactivating endogenous pathogens (e.g., herpesviruses) or exogenous pathogens stimulate the preferential expansion of CD8 cells. Consistent with that, in â2microglobulin-deficient mice whose CD8 T cells cannot be stimulated by cognate antigens (peptides), expansion of CD8 T cells after radiation-induced lymphopenia did not occur [31].

The peripherally expanded T cells early posttransplant likely originated from the few pretransplant T cells that survived the conditioning or were infused with the graft rather than from de novo generation. Though low in number, T cells were typically detectable in the blood on day 7 (median 0.3/μl), indicating that some T cells survived or were reinfused. T cells generated de novo from the grafted CD34 cells were not expected to exist on day 7 as thymocyte precursor to T cell differentiation takes at least 12 days [32]. Moreover, at 1 month posttransplant, 82% patients had undetectable TRECs.

Our study more closely reflects the "natural" homeostatic recovery of lymphocytes. Unlike in other studies, our patients were not influenced by HIV, cancer, GVHD, or drugs potentially influencing the immune recovery (except for low-dose prednisone in the first 2 months posttransplant). However, it is theoretically possible that our patients had an autoimmune disease-associated defect of a lymphopoietic organ. Such a defect has not been described in humans with multiple sclerosis or systemic sclerosis, but thymic histological abnormalities have been described in an avian model of systemic sclerosis [33]. To minimize the potential impact of the underlying disease on the immune recovery, we censored our patients at the time of disease progression/relapse. Moreover, there was no significant difference in the counts of $TREC^+$ CD4 or CD8 T cells at 1 or 2 years posttransplant between patients with multiple sclerosis and those with systemic sclerosis (median 14 vs. 38/il at 1 year and 21 vs. 59 at 2 years for CD4 cells, and 3 vs. 7/il at 1 year and 7 vs. 23 at 2 years for CD8 cells), suggesting against a thymic defect in patients with systemic sclerosis. Also, a substantial impact of the underlying disease in our study is unlikely as the tempo of recovery of lymphocyte subsets in our patients recovering from extreme (radiochemotherapy and ATG-induced) lymphopenia was similar to the tempo of recovery in patients recovering from moderate (radio/chemotherapy-induced) lymphopenia [26,27,29,34–36].

Surprisingly, antibody levels did not drop substantially, in spite of the severe CD4 T and B lymphopenia during the first several months posttransplant. Consistent with that, oligoclonal immunoglobulins in the cerebrospinal fluid of most multiple sclerosis patients treated with autologous transplantation did not disappear [3,37,38]. As the half life of IgM and IgA is only ~5 days and that of IgG only ~23 days [39], the antibodies detected in the sera of our patients were continuously produced, presumably by plasma cells generated pretransplant. Plasma cells are radiation-resistant and long-lived [40–42]. The persistent antibody production does not appear to require posttransplant exposure of patients to the cognate antigens. Tetanus IgG levels in the first posttransplant year remained stable, even though the patients were not vaccinated in the first year and natural exposure to tetanus toxin in developed countries is extremely unlikely [43]. However, it is unclear whether this applies to other autologous transplant settings. In three studies presenting tetanus antibody levels after autologous transplantation for malignancies, the levels appeared to drop between pretransplant and 1 year posttransplant [44–46].

From the infectious disease point of view, the persistent production of antibodies by plasma cells generated before the autologous transplantation may be beneficial, as most frequent pathogens are likely encountered pretransplant. Unfortunately, from the point of view of autoimmune diseases caused by autoantibodies like pemphigus (anti-desmoglein) or Lambert–Eaton myasthenic syndrome (anti-voltage gated calcium channel), the persistent production of autoantibodies may be deleterious. This may not apply to systemic sclerosis or multiple sclerosis in which the role of autoantibodies is uncertain. It is unlikely that the diseases caused by autoantibodies would be cured by autologous transplantation. In contrast, allogeneic transplantation might cure such diseases as it is associated with graft-vs.-host plasma cell reaction [47–49]. However, attempts to treat such diseases with autologous transplantation may still be warranted for the following two reasons: First, contrary to our observation, in five of six systemic lupus patients, double-stranded DNA antibodies became undetectable after autologous transplantation [50]. Second, clinical improvement may occur even if the autoantibody thought to cause the disease persists. In two of two myasthenia gravis patients, symptoms markedly improved after high-dose chemotherapy in spite of persisting acetylcholine receptor antibodies [51].

Clinical manifestations of the lymphopenia following the extreme lymphoablation were surprisingly mild, when compared to other settings of a similar degree of lymphopenia. In the first 6 months after transplant, when median CD4 T cell counts ranged from <1 to 118/μl, infections that were not covered by prophylactic antimicrobial drugs and would be expected to occur in AIDS patients with a similar degree of lymphopenia (cryptosporidiosis, Kaposi sarcoma, mycobacteriosis [52]) did not occur. Between 1 and 2 years posttransplant, when no prophylactic antimicrobial drugs were given, the incidence of infections (excluding presumed respiratory tract infections) was 16% (Table 1), whereas in HIV-seropositive individuals with similar CD4 T cell counts (200–450/μl) not receiving antimicrobial drugs, the annual incidence of oral candidiasis alone was 16–26% and that of other infections (excluding respiratory tract infections) was 16–19% [53–55]. This may be attributed to the fact that HIV infects not only CD4 T cells but also other immune cells (e.g., monocytes/macrophages, dendritic cells) [56]. The infection rates in the autologous transplant recipients presented here were also markedly lower than in allogeneic transplant recipients. Between day 30 and 365 after allogeneic marrow transplantation, when median CD4 counts ranged between 76 and 185/μl, the infection rate was 3.17 per patient year [9] compared to 0.94 per patient year between day 31 and 180 (median CD4 counts 33–118/μl) or 0.29 per patient year between day 181 and

365 (median CD4 counts 118–193/μl) in the autologous transplant recipients (Table 1), using similar definitions of infections and similar infection prophylaxis. This may be because after allogeneic transplantation T and B cells are not only quantitatively deficient but also dysfunctional due to GVHD or its treatment [57]. Congenital severe T cell deficiency with or without B cell deficiency is fatal (unless cured with allogeneic transplantation or gene therapy) [58], whereas only 2 of the 56 autologous transplant recipients died due to an infection (EBV lymphoproliferation). This may be because in contrast to the patients with congenital severe lymphopenia, in our autologous transplant recipients, the severe lymphopenia was only transient and that the patients had normal levels of antibodies against recall antigens (presumably against most frequent pathogens, which they had encountered before transplantation). Collectively, iatrogenic transient severe lymphopenia is relatively well tolerated (using the prophylactic antimicrobial strategy described in Table 1). Thus, attempts to treat autoimmune diseases with therapies causing short-term severe lymphoablation are relatively safe, at least from the infectious disease point of view. In the future, the severe lymphopenia may be even better tolerated if EBV DNAemia and EBV-specific T cell counts are monitored and rituximab is given preemptively (however, antibody responses to bacteria encountered posttransplant may be impaired in the patients treated with rituximab) [59,60].

In conclusion, peripheral expansion is highly efficient for CD8 but not CD4 T cells. The prolonged CD4 lymphopenia is associated with few infections, possibly due to antibodies produced by plasma cells persisting from pretransplant, suggesting that autologous transplantation using extremely lymphoablative conditioning is relatively safe. The persistent antibody production raises a concern that autoimmune diseases caused by autoantibodies may not be cured by autologous transplantation.

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Graft-versus-tumor effects

Expansion and activation of minor histocompatibility antigen HY-specific T cells associated with graft-versus-leukemia response

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Summary:

The immune system of females is capable of recognizing and reacting against the male-specific minor histocompatibility antigen (mHA), HY. Thus, cytotoxic T-lymphocytes (CTLs) recognizing this antigen may be useful in eradicating leukemic cells of a male patient if they can be generated *in vivo* or *in vitro* from a human leukocyte antigen (HLA)-identical female donor. The HLA-A*0201-restricted HY antigen, FIDSYICQV, is a male-specific mHA. Using HLA-A2/HY peptide tetrameric complexes, we reveal a close association between the emergence of HY peptide-specific CD8$^+$ T cells in peripheral blood and molecular remission of relapsed BCR/ABL$^+$ chronic myelogenous leukemia in lymphoid blast crisis in a patient who underwent female-to-male transplantation. Assessment of intracellular cytokine levels identified T cells that produce interferon-γ in response to the HY peptide during the presence of HY tetramer-positive T cells. These results indicate that transplant with allogeneic HY-specific CTLs has therapeutic potential for relapsed leukemia, and that expansion of such T cells may be involved in the development of a graft-versus-leukemia response against lymphoblastic leukemia cells.

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Minor histocompatibility antigens (mHAs) are immunogenic peptides derived from polymorphic cellular proteins.[1] These peptides bind to human leukocyte antigen (HLA) and are recognized by allogeneic T cells. Following stem cell transplantation (SCT) with HLA-matched donor cells, graft-versus-host disease (GVHD) can arise through disparities in mHAs between the donor and the recipient. Using tetrameric HLA class I–mHA complexes, Mutis *et al*[2] demonstrated that a limited number of mHA-specific T cells expand in peripheral blood (PB) in parallel with increasing GVHD severity. It is believed that donor-derived T cells specific for mHAs play a significant role in the development of graft-versus-leukemia (GVL) effect as well as GVHD after allogeneic SCT. However, there is only limited evidence for the killing of leukemic cells by mHA-specific T cells.

Kern *et al*[3] reported that human cytomegalovirus peptide-specific CD8$^+$ T cells can be detected by flow cytometry in samples whose HLA is known. Kuzushima *et al*[4] showed that the frequency Epstein–Barr virus-specific CD8$^+$ T-cell frequencies are detectable irrespective of HLA typing when PB lymphocytes are incubated with an autologous lymphoblastic cell line (LCL). Both methods are based on multiparameter flow cytometric assays that detect rapid intracellular accumulation of interferon (IFN)γ after *in vitro* antigen stimulation in the presence of an intracellular transport blocker. By assessing the frequency of IFNγ-producing cells, the presence of functional T cells reactive with target antigens and target cells can be detected.

One male-specific mHA is the HLA-A*0201-restricted peptide, FIDSYICQV, from the male-specific antigen HY.[5] Cytotoxic T-lymphocytes (CTLs) recognizing this peptide may be useful in eradicating leukemic cells of a male patient with HLA-A2 if they can be generated *in vivo* or *in vitro* from an HLA-identical female donor. By HLA-A2/HY peptide tetramer staining and intracellular IFNγ assessment, we provide the first evidence that the emergence and activation of transferred HY-specific female CTLs contributes to molecular remission of chronic myelogenous leukemia (CML) in a male patient in lymphoid crisis.

To date, a small number of studies describe a clear dominance in T-cell receptor (TCR) variable (V)-gene segment usage in the recognition of certain HLA class I/peptide complexes in humans after SCT.[6,7] We examined PB CD8$^+$ T cells producing intracellular IFNγ of a male patient with CML in lymphoid crisis for the emergence of clonal T-cell proliferation by analyzing the T-cell repertoire

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as well as *in vitro*-generated HY-specific CTLs. TCR BV spectratyping showed a similar peak with same size in some BV family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific $CD8^+$ T cells. These findings suggest the circulation of a functional T-cell clone capable of eradicating lymphoblastic leukemia cells.

Materials and methods

Case report

A patient was a 15-year-old Japanese male (HLA-A*0201-positive) with chronic-phase CML. He had experienced no GVHD after receiving busulfan- and cyclophosphamide-conditioned bone marrow from his HLA-identical sister. At 40 months after bone marrow transfer, lymphoid blast crisis suddenly developed. After treatment with cyclophosphamide, adriamycin, vincristin and prednisolone, the hematologic relapse persisted. At 2 months after chemotherapy, the patient underwent PB stem cell transplantation (PBSCT) from the same donor following conditioning with cytarabine, cyclophosphamide and total body irradiation. An unmanipulated PB stem cell graft including a total of 5.5×10^6 CD34-positive cells/kg was infused. Cyclosporine, which is used to prevent GVHD, was withdrawn on day 21 to induce GVL effect. At 3 weeks after PBSCT, conversion to full donor chimerism was obtained. The patient developed grade II acute GVHD on day 29 after PBSCT, and the disease progressed to extensive chronic GVHD on day 80. At 20 weeks after PBSCT, no BCR/ABL transcripts were detected, and he remained in molecular remission until a relapse at 13 months after PBSCT. The patient died of veno-occlusive disease shortly after the third transplantation with cytarabine- and idarubicin-conditioned bone marrow from the same donor.

Peptide

An HLA-A201-restricted HY peptide was synthesized using a semiautomatic multiple peptide synthesizer based on the reported sequence.[5] The purity of the peptide was checked by reverse-phase high-pressure liquid chromatography.

Cell preparation

Cells were obtained from the post transplant patient and his stem cell donor. Peripheral blood mononuclear cells (PBMC) were prepared using density-gradient centrifugation. For the establishment of Epstein–Barr virus (EBV)-transformed LCLs, PBMC from the donor were depleted of T cells using the rosette formation method. A total of $2–3 \times 10^6$ non-T cells were incubated in RPMI 1640 (GIBCO, Grand Island, NY, USA) medium containing 10% fetal calf serum (FCS; GIBCO) containing 10% culture medium from an EBV-producing cell line, B95-8, at 37°C for 2 h. The EBV-infected cells were cultured for 3 weeks until transformed LCL cells grew. LCL cells were maintained by changing the medium every 4 to 5 days.

Tetrameric HLA-A2/mHA HY peptide complexes

The generation of HLA-A2/mHA HY tetramers and tetramer labeling of HY-specific T cells was performed as described previously.[8]

Generation of dendritic cells (DCs) from monocytes

PB monocyte-derived DCs were generated as described previously.[9] Briefly, monocytes were isolated by adherence of donor PBMCs to plastic for 2 h. Monocytes were cultured in RPMI 1640 medium supplemented with 10% pooled AB serum, 10 ng/ml recombinant human interleukin-4 (IL-4) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (a gift from Kirin Brewery, Tokyo, Japan). On day 5, 100 U/ml recombinant human tumor necrosis factor (TNF)α was added. On day 8 or 9, the cells were harvested for use as monocyte-derived DCs for antigen presentation. Cultured cells expressed DC-associated antigens, such as CD1a, CD80, CD83, CD86 and HLA class I and class II.

Induction of HY peptide-specific CTLs

DCs were pulsed with an HY peptide for 90 min at 37°C in serum-free RPMI 1640. After washing, 1.0×10^6 peptide-(40 nM) pulsed DCs and 1.0×10^7 donor-derived (autologous) PBMC were cultured together in 24-well culture plates. The culture medium was RPMI 1640 supplemented with 2 mM L-glutamine, minimal essential amino acids, sodium pyruvate and ampicillin (all from GIBCO) plus 10% autologous plasma. The cells were kept at 37°C in a humidified, 5% CO_2–air mixture. At days 7, 14 and 21, responder cells were restimulated with peptide-pulsed autologous DCs. From day 21, cultured T cells were suspended in 100 U/ml IL-2- (a gift from Shionogi, Osaka, Japan) containing culture medium and were restimulated weekly with peptide-pulsed autologous monocytes or DCs. T cells were harvested at day 35 and used for the cytotoxicity assay and RNA extraction for T-cell repertoire analysis.

Cytotoxicity (^{51}Cr release) assays

Donor-derived LCL cells and DCs as well as fibroblasts and leukemic cells of the patient were used as target cells in the standard 4-h ^{51}Cr release assay.[10] Specific lysis was calculated using the following formula: 100 × (cpm experimental release–cpm spontaneous release)/(cpm maximal release–cpm spontaneous release).

Preparation of target cells

Patient fibroblasts were isolated from a biopsy specimen and cultured in RPMI 1640 plus 10% FCS for 4 weeks. Single-cell suspensions were prepared by trypsinization. Donor-derived LCL cells and DCs and the patient's fibroblasts were suspended in 100 μl of ^{51}Cr solution containing an HY peptide at a concentration of 4 nM. In some experiments, concentrations of the peptide were changed as noted. Bone marrow mononuclear cells containing 98% BCR/ABL^+ cells were obtained from the patient

just before the second transplantation and were cryopreserved for use as leukemic cells. Thawed leukemic cells were cultured in RPMI 1640 plus 10% pooled AB serum for 24 h before use as a target in the CTL assay.

Blocking of cytotoxicity by monoclonal antibodies (MoAbs)

Polyclonal antibodies (control) or purified MoAbs were added to cultures of HY peptide- (4 nM) pulsed DCs in 96-well plates at a concentration of 10 μg/ml, and CTLs were immediately added to each well. MoAbs were HU-4 (anti-HLA-DR; kindly provided by Dr Akemi Wakisaka, Hokkaido University, Japan) and W6/32 (anti-HLA class I; American Type Culture Collection, Rockville, MD, USA).

Identification and isolation of IFNγ-producing CD8$^+$ T cells by flow cytometry

To detect circulating CD8$^+$ T-lymphocytes that recognize HY peptide, intracellular IFNγ was assessed by flow cytometry as described previously with slight modifications.[3,4] Briefly, donor-derived LCL cells were incubated for an hour with or without HY peptide. PBMCs were taken from the patient 12 weeks after the second transplantation. The CD8$^+$ T-lymphocytes were isolated from PBMCs using magnetic beads coated with an anti-CD8 monoclonal antibody (mAb) according to the manufacturer's instructions (Dynal AS, Oslo, Norway). A total of 10^6 CD8$^+$ cells were mixed with 10^6 autologous LCL cells in a culture tube in RPMI 1640 medium and cultured in a humidified 5% CO_2 incubator at 37°C for 1 h. Brefeldin A (Sigma, St Louis, MO, USA) was added at a final concentration of 10 μg/ml and the cells were cultured for an additional 5 h. After incubation, the cells were fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with phosphate-buffered saline, cells were permeabilized with IC Perm (Biosourse International, Camarillo, CA, USA) and stained with PE-labeled anti-CD8 (Coulter, Miami, FL, USA) and FITC-labeled anti-human IFNγ (Biosourse International) MoAbs. Stained cells were analyzed and sorted on a FACScan (Becton Dickinson, San Jose, CA, USA).

RNA extraction and cDNA preparation

Total RNA was extracted from PBMCs or CTLs using a technique described elsewhere,[11] and was reverse transcribed into cDNA in a reaction primed with oligo(dT) using SuperScript II reverse transcriptase as recommended by the manufacturer (BRL, Bethesda, MD, USA).

Spectratyping of complementarity-determining region 3 (CDR3)

Conditions for the generation of the CDR3 size spectratyping have been reported previously.[6] Briefly, cDNA was polymerase chain reaction (PCR) amplified through 35 cycles (95°C for 1 min, 55°C for 1 min and 72°C for 1 min) with a fluorescent BC primer and primers specific to 24 different BV subfamilies. Analyses of the pseudogenes BV10 and BV19 were excluded from this study.[12] A measure of 1 μl of each amplified products was mixed with 2.0 l 100% formamide, heated at 90°C for 3 min and electrophoresed on a 6.75% denaturing polyacrylamide gel. The distribution of CDR3 size within the amplified product of each BV subfamily was analyzed with an automatic sequencer (Applied Biosystems Division, Foster City, CA, USA) equipped with a computer program allowing the determination of the fluorescence intensity of each band. The results are given as peaks corresponding to the intensity of the fluorescence. Expansion of a limited number of T cells was judged when a prominent peak appeared in the CDR3 pattern with or without a reduced number of peaks (five peaks). Given the BV-NDN-BJ sequence of the identical CDR3 size between in HY-specific CD8$^+$ cells in PB and the CTLs, a more specific primer covering CDR3 and different BJ subfamilies[13] was designed specifically to amplify cDNA of the BV22$^+$ T-cell clone in both CTLs.

Direct sequencing of PCR products

BV22-BJ PCR products were purified and sequenced as described previously.[14]

Results

Cytolytic activity of in vitro*-generated HY-specific CTLs*

Cultured T female donor cells stimulated with autologous HY peptide-pulsed DCs pulsed were able to lyse HY peptide-pulsed autologous DCs and patient fibroblasts, but could not lyse untreated DCs (Figure 1a). Of note, the CTLs lysed nonpeptide pulsed leukemic cells of the patient more efficiently than his fibroblasts. CTLs showed cytotoxicity to HY peptide-loaded autologous DCs, in a peptide concentration-dependent manner (Figure 1b). Cytotoxicity mediated by the CTLs against HY peptide-pulsed autologous DCs was blocked to a similar degree by the addition of MoAb either against anti-class I or anti-CD3, but was not affected by the addition of anti-HLA-DR (Figure 1c). In addition, the CTLs could effectively lyse LCL cells of unrelated males who shared HLA-A*0201. In contrast, apparent cytotoxic activity was neither observed against allogeneic LCL cells that did not possess HLA-A2 nor against LCL cells of unrelated females (Figure 1d).

Tetramer staining of HY-specific CTLs

The patient in lymphoid crisis with CML relapse developed acute GVHD shortly after the second transplant, which progressed to extensive chronic GVHD. He achieved a molecular remission at 4 months after PBSCT despite the fact that leukemic cells accounted for more than 98% of his bone marrow cells when the conditioning regimen was started. This unusual clinical course appeared to suggest the induction of GVL reactions associated with GVHD.

Other than the HY antigens, there was no disparity in the minor histocompatibility alleles, including HA-1, HA-2,

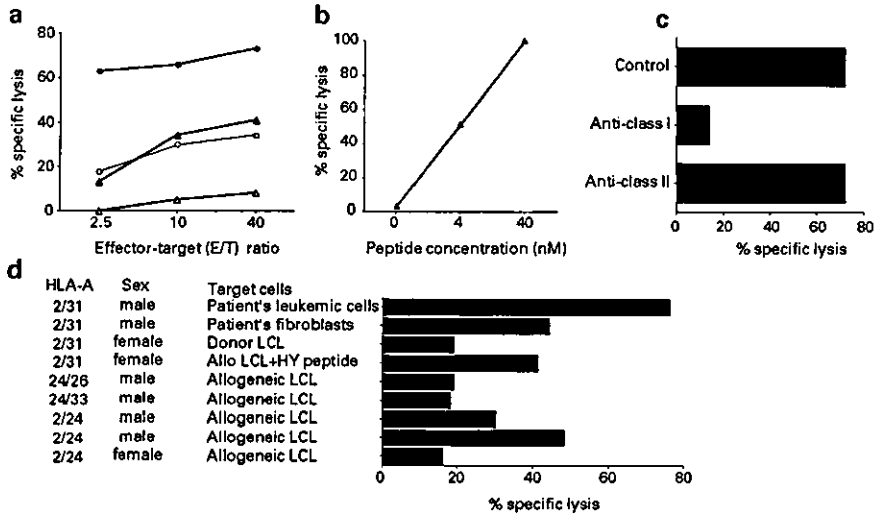

Figure 1 Cytotoxic activity of *in vitro*-generated HY-specific CTLs. (a) Cytotoxicity of cultured T cells stimulated by autologous DCs pulsed with HY peptide. The amount of peptide utilized in (a, b and c) was 4 nM. Target cells: autologous DCs without HY peptide (Δ); autologous DCs pulsed with HY peptide (▲); fibroblasts of the patient (○); leukemic cells of the patient (•). In (b, c, and d), cytotoxicity was determined at an E/T ratio of 10:1. (b) Effect of concentration of HY peptide on cytotoxicity of CTLs. Cytotoxicity of CTLs to autologous DCs loaded with various concentration of HY peptide was tested. (c) Antibody blockade of cytotoxicity against autologous DCs pulsed with HY peptide. Polyclonal antibodies (control), anti-class I MoAb or an anti-class II MoAb were added to cultures for testing blockade of cytotoxicity. (d) Cytotoxicity of HY peptide for 6 h. HY-specific CTLs against LCL cells of unrelated males who share HLA-A2 (A*0201).

CD31, CD49b and CD62L,[15,16] between the donor and the recipient. Tetramer staining demonstrated the expansion of HY peptide-specific T cells from undetectable prior to PBSCT to 15.9% of the circulating CD8$^+$ T cells 12 weeks after PBSCT (Figure 2a). Thereafter, frequencies of HY tetramer-positive T cells declined and disappeared from the PB, coinciding with molecular relapse. The *in vitro*-generated HY-specific CTLs were 90% HY tetramer-positive CD8$^+$ T cells (Figure 2b).

Detection of HY-reactive CD8$^+$ T cells by intracellular cytokine assessment

To demonstrate the HY peptide-driven expansion of CD8$^+$ T cells, we assessed intracellular accumulation of IFNγ in PB CD8$^+$ T cells by flow cytometry. At 12 weeks after PBSCT, 6.8% of PB CD8$^+$ T cells produced intracellular IFNγ in response to HY peptide-pulsed autologous LCL cells (Figure 3a), while IFNγ production was negligible in CD8$^+$ T cells in response to autologous LCL cells without the HY peptide (Figure 3b). These findings indicate that *in vivo* expansion and activation of HY peptide-reactive T cells occurred after the second transplantation. At that time, the proportion of circulating CD8$^+$ cells positive for HY-tetramer staining was 15.9% as show in Figure 2a. Although inducibility of IFNγ in HY-tetramer-positive CD8$^+$ T cells was not examined, there could be overlapping between CD8$^+$ T cells producing intracellular IFNγ in response to HY peptide and CD8$^+$ T cells stained with HY tetramer, because HY-tetramer staining must detect functional T cells reactive with HY peptide.

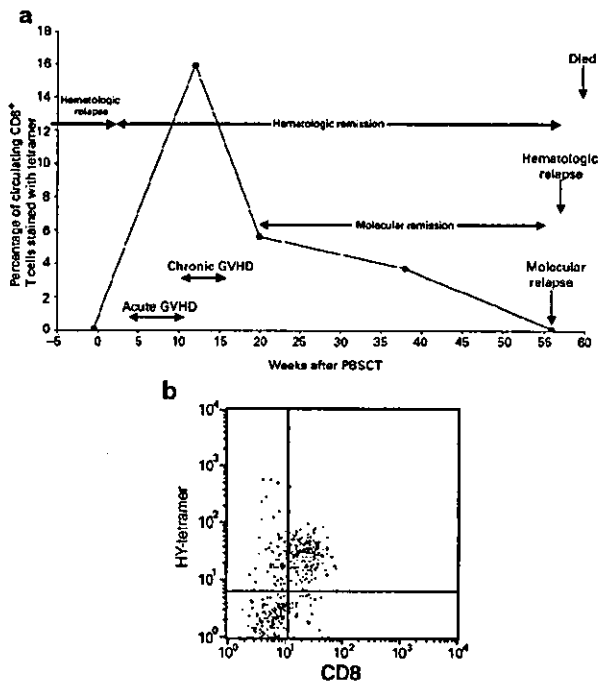

Figure 2 Monitoring and generation of HY-specific CD8$^+$ T cells. (a) Correlation between frequency of circulating HY-tetramer-positive CD8$^+$ T cells and clinical events. (b) HY-tetramer staining of the *in vitro*-generated HY-specific T cells, showing FITC-conjugated anti-CD8 antibody (*x*-axis) and PE-conjugated HY-tetramers (*y*-axis). Appropriate gates were set on vital lymphocytes according to their typical forward- and side-scattering characteristics.

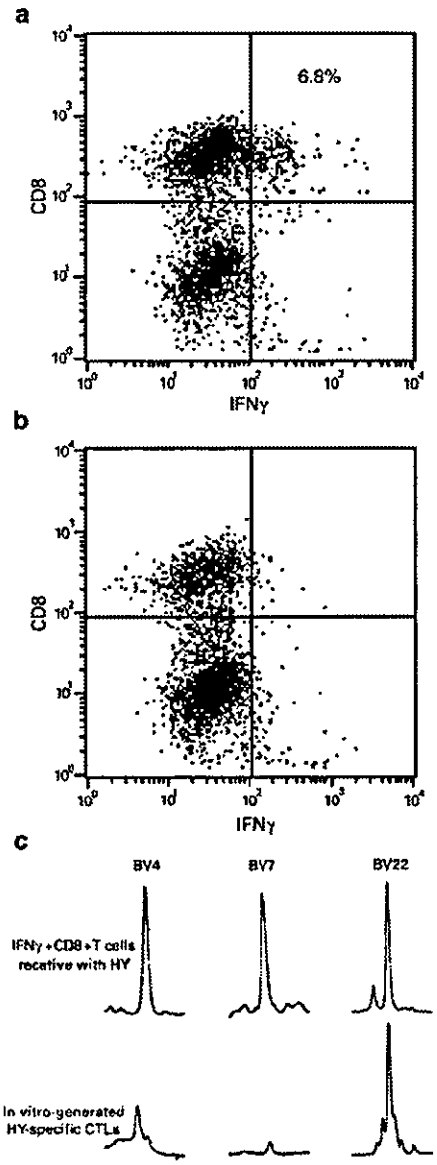

Figure 3 HY-specific IFNγ-producing CD8$^+$ T cells in PB. (**a**) PB CD8$^+$ T cells were incubated with autologous LCL cells pulsed with after fixation and permeabilization, the cells were stained for CD8 and IFNγ. The frequency of CD8$^+$ T cells producing IFNγ in response to HY peptide is shown as a percentage of total CD8$^+$ cells. (**b**) IFNγ production was negligible in CD8$^+$ T cells stimulated by autologous LCL cells not pulsed with the HY peptide. (**c**) CD8$^+$ IFNγ$^+$ T cells detected in Figure 3a were selected by fluorescence-activated cell sorting, and spectratyping was performed. CDR3 sizes of TCR BV subfamilies from CD8$^+$ IFNγ$^+$ T cells are shown together with the data from *in vitro*-generated HY-specific CTLs.

CDR3 size distribution of TCR BV cDNA of HY-specific T cells

Spectratyping of the TCR BV region was performed on *in vitro*-generated HY-specific CTLs and PB CD8$^+$ T cells that were stained with intracellular IFNγ MoAb in response to HY peptide stimulation and sorted as shown in Figure 3a. PB HY-reactive CD8$^+$ cells showed prominent skewing within BV4, BV7, BV22 and BV24, and the *in vitro*-generated HY-specific CTLs showed skewing within BV4, BV7, BV12, BV16 and BV22. The two T-cell populations shared the usage of BV4, BV7 and BV22 (Figure 3c), but only the BV22$^+$ T cells from *in vitro*-generated HY-specific CTLs and HY-responsive PB CD8$^+$ T cells had a similar peak with the same CDR3 size distribution.

Deduced amino-acid sequence of CDR3 of BV22 cDNA

To determine if HY-specific CTLs circulate in the patient, we subcloned the amplified cDNA of the *in vitro*-generated BV22$^+$ CTLs and BV22$^+$ T cells that produced intracellular IFNγ in response to HY peptide stimulation as shown in Figure 3a and determined the CDR3 sequence (Table 1). One of three N-D-N sequences of HY-specific CD8$^+$ cells from PB was identical to one of those of the CTLs. These findings indicate that the same cells isolated from the donor are expanding *in vivo*, and suggest that HY-specific CD8$^+$ T cells in PB of the patient have cytotoxic activity against leukemic cells.

Discussion

In mHAs HA-1- and HA-2-matched stem cell transfer between a female donor and a male recipient, we have observed the emergence of HY peptide-specific CTLs that result in the durable remission of relapsed leukemia. The present study utilized tetramer staining to show that HLA-A*0201-restricted HY peptide-specific CD8$^+$ T cells were present in the PB after the development of GVHD and led to the eradication of BCR/ABL transcript-positive leukemic cells. Further, these cells disappeared upon molecular relapse of disease. Assaying for the frequency IFNγ-producing cells by intracellular cytokine staining, we demonstrated the emergence of functional T cells in PB that were reactive with the HY peptide during the presence of HY tetramer$^+$ T cells. The data imply that HY-specific CTLs may have therapeutic potential as adoptive immunotherapy for relapsed leukemia after allogeneic SCT.

In this patient, leukemic relapse had occurred as full-blown disease of CML in lymphoid blast crisis. Although a beneficial effect of donor immunity was expected in the control of leukemic relapse, it would take months to start to work. Thus, we had chosen multiple transplants instead of donor leukocyte infusion (DLI) to reduce leukemic cells sufficiently.

Recently in mHAs HA-1 and/or HA-2 incompatible donor–recipient pairs an association between the emergence of HA-1 or HA-2 tetramer-positive CTLs and the complete disappearance of BCR/ABL$^+$ cells or of myeloma cells was reported.[17] Of the three reported patients, one who underwent female-to-male transplantation experienced an increase in HLA-B7-restricted HY-specific T cells as well as an increase in HA-2-specific T cells, but not that of

Table 1 Junctional amino-acid sequence of TCR BV22 of HY-specific T cells

V	*N-D-N*	*J*	*BJ family*
(a) BV22$^+$ T cells from in vitro-generated HY-specific CTLs			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	REGGRS	GYTFCSGTKLTV	1.2
CASS	KQKGNPPPI	SPLHFGNGTRLTVT	1.6
(b) Functional BV22$^+$ T cells from HY-reactive CTLs circulating in PB			
CASS	GGTGTV	YTEAFFGQGTRLT	1.1
CAS	RQSQGS	GYTFCSGTKLTV	1.2
CASS	RQGRGVSEF	SPLHFGNGTRLTVT	1.6

HLA-A2-restricted HY-specific T cells. However, the cell population(s) contributing to the GVL effect were unable to be identified. It is worth noting that this reported case with lymphoid blast crisis of BCR/ABL transcript-positive CML obtained molecular remission after the development of GVHD and the emergence of HY-specific CTLs as seen in the present case.

The *in vitro*-generated HY peptide-specific CTLs efficiently lysed leukemic cells of the patient, and were also to a lesser extent cytotoxic to the nonhematopoietic cells such as fibroblasts of the patient. Gratwohl *et al*[18] reported that male recipients with CML of female blood or marrow stem cell grafts are at a high risk of GVHD, but benefit from reduced incidence of disease recurrence. These findings provide evidence that HY-specific CTLs may be commonly induced in male patients given a stem cell graft from a female donor, leading to the development of GVL reactions and GVHD. This implies that the availability of a female blood or marrow graft may be beneficial to a leukemic male recipient at high risk of relapse.

In contrast to the ubiquitous expression of HY, HA-1 and HA-2 are exclusively expressed on hematopoietic cells.[1] *In vitro*-generated HA-1- and HA-2-specific CTLs specifically lyse leukemic cells, but not nonhematopoietic cells in a ^{51}Cr release assay.[1,19] Thus, upon HA-1- or HA-2-mismatched SCT and adoptive immunotherapy such as DLI, a low risk of GVHD would be expected. However, HA-1 disparity between a patient and a donor has been associated with the development of GVHD without reducing a rate of relapse.[15,20] Marijt *et al*[17] demonstrated the emergence of HA-1- and HA-2-specific CD8$^+$ T cells in PB of three patients after DLI preceding complete remission of relapsed leukemia. Relapse was associated with the development of GVHD in all three patients. A recent report showed that GVHD does not require alloantigen expression on host epithelium, and its development is primarily mediated by inflammatory cytokines such as TNFα and IL-1.[21] This may account for discrepancies between *in vitro* behavior of HA-1- and HA-2-specific CTLs and clinical observations. Based on these findings, we believe that in mHA-oriented allogeneic immunotherapy the ability of mHAs to induce powerful immune reactions is more important than restriction of mHAs to hematopoietic tissue, and so far it appears that GVHD is an inevitable consequence. In the future, selective blockade of cytokines mediating GVHD[21] may be a strategy to preserve GVL, while reducing toxicity of GVHD after mHA-oriented immunotherapy.

TCR BV spectratyping showed a similar peak with same size in a BV22$^+$ family between *in vitro*-generated HY-specific CTLs and *in vivo*-activated HY-specific CD8$^+$ T cells, and one shared N-D-N sequence. These findings suggest the expansion of a functional T-cell clone that participates in eradicating lymphoblastic leukemia cells positive for BCR/ABL transcripts, although we were not able to provide direct evidence demonstrating antileukemic activity of HY-specific CD8$^+$ T cells taken from PB of the patient. It would have been beneficial to sort the HY-tetramer-positive cells detectable in PB of the patient, to expand these cells using HY peptide-pulsed LCL cells, and to test their cytotoxicity against the leukemic targets. Restricted TCR BV usage for HA-1-specific CTLs has also been described.[7] Spectratyping could be beneficial in monitoring HY-specific CTLs *in vivo*, because spectratype analysis is more sensitive than tetramer analyses, and can be performed using as little as 500 cells.[6]

Compared with tetramer staining, a flow cytometric assay assessing intracellular IFNγ levels can be used to screen a large number of allogeneic peptides with a relatively little effort. In allogeneic SCT, this approach should be useful for initial screening of candidates for mHAs derived from polymorphic cellular proteins. Moreover, intracellular IFNγ assessment between CD8$^+$ T cells during the GVL effect or GVHD as a responder and hematopoietic cells or nonhematopoietic cells of a host as a stimulator may enable the detection of undefined mHAs-specific CTLs. As the IFNγ capture assay enables isolation of live T cells stained for surface-associated IFNγ,[22] further studies with regard to the function of responding effector T cells could elucidate their putative target antigens.

Another advantage of the flow cytometric cytokine production assay is that it is possible to assess the production of multiple cytokines on an antigen-specific, single-cell basis. It has already been demonstrated by Nazaruk *et al*[23] that a subset of EBV-specific CD8$^+$ T-cell lines produce IL-4 or IL-13 in addition to IFNγ upon stimulation with phorbol myristate acetate and ionomycin. Such a technique could be utilized in determining the cytokine production capabilities of mHA-specific CTLs in PBSCT.

In conclusion, the present data provide evidence that the emergence and activation of HY-specific CD8$^+$ T cells may participate in eradicating lymphoblastic leukemic cells. This implies that *in vitro*-generated HY-specific CTLs may have therapeutic potential for relapsed leukemia after allogeneic SCT.

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