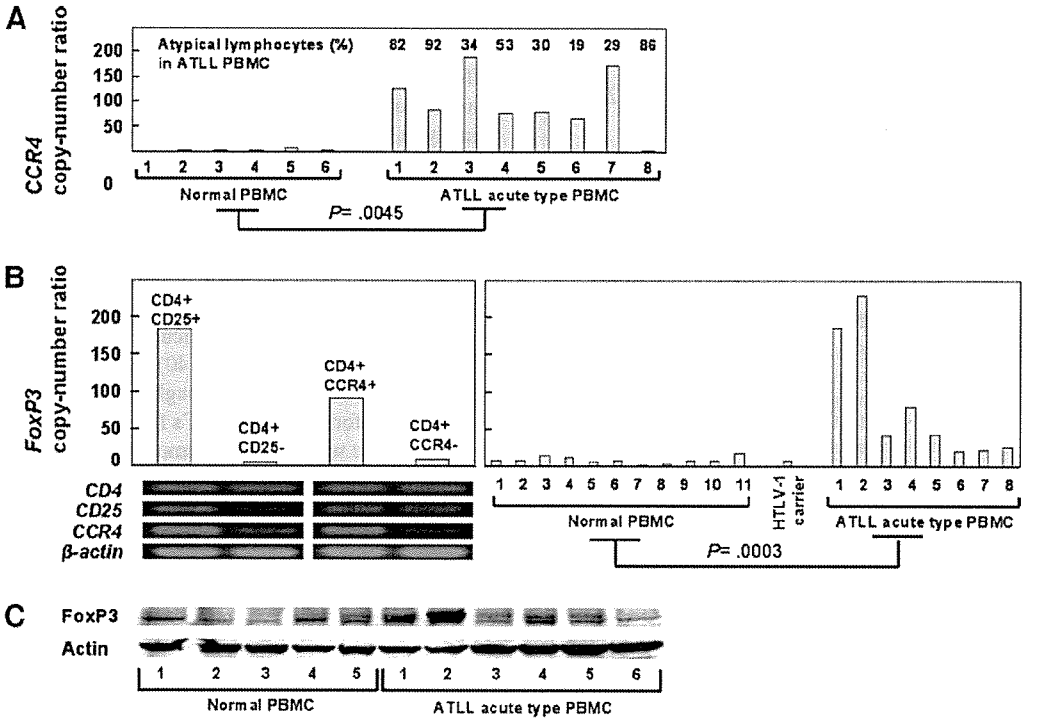

Fig. 5 Expression of CCR4 and FoxP3 in PBMCs from normal volunteers or patients with ATLL. *A*, quantification of the relative *CCR4* mRNA levels in PBMCs obtained from six normal volunteers and eight patients with acute-type ATLL. Serially diluted cDNA aliquots from these PBMCs were analyzed with primer sets for *CCR4* and β-*actin* in a 20-μL reaction mixture containing FastStart DNA master SYBR Green I with the aid of a LightCycler Quick System 330. The quantitative assessment of the mRNA of interest was done by dividing the *CCR4* expression level by that of β-*actin* and expressing the result as a copy-number ratio. *B*, expression of *FoxP3* in CD4+ T-cell subpopulations of PBMCs obtained from a normal volunteer. cDNA aliquots prepared from the purified $CD4^+CD25^+$, $CD4^+CD25^-$, $CD4^+CCR4^+$, and $CD4^+$ $CCR4^-$ subpopulations were subjected to conventional reverse transcription-PCR for *CD4*, *CD25*, *CCR4*, and β-*actin* (*left panel*). Quantification of the relative *FoxP3* mRNA levels was carried out for cDNA prepared from PBMCs of 11 normal volunteers, 1 HTLV-1 seropositive carrier, and 8 patients with acute-type ATLL (*top right panel*). *C*. Expression of FoxP3 protein was analyzed in PBMC extracts obtained from six patients with acute-type ATLL and 5 healthy adult volunteers by Western blot analysis.

antibody increased the CDC lysis of ATL102 irrespective of the presence of KM2760, indicating that ATL102 can be lysed with complement alone if CD55 is blocked functionally. In addition, KM2760 did not induce CDC activity against any of eight non-ATLL T-cell lines irrespective of the presence of CD55 and/or CD59 (data not shown).

Effect of KM2760 on Proliferation of ATLL Cell Lines and Fresh ATLL Cells. We investigated whether KM2760 could inhibit proliferation of both ATLL cell lines and fresh ATLL cells obtained from several acute type patients. No inhibitory effect induced by KM2760 on proliferation of ATLL cell lines or fresh ATLL cells was observed (data not shown). Addition of recombinant human IFN-α or recombinant human IFN-γ did not affect the proliferation in any of them, whereas IFN-α alone induced growth inhibition of MT-2 and all fresh ATLL cells (data not shown).

DISCUSSION

In the present study, we extended our previous observations of potent KM2760-induced ADCC activity against CCR4-positive non-ATLL T-cell lines (16) to ask whether tumor cells from patients with ATLL and PTCL also could be good targets in KM2760-based immunotherapy. Our data clearly showed the potent KM2760-induced ADCC against both ATLL cell lines and fresh ATLL cells obtained from patients. This KM2760-induced ADCC was completely dependent on the cell surface expression of CCR4 on the target cells. However, the observed ADCC activity differed in individual PBMCs used for the assay and in the cell lines tested. There are several potential explanations to account for these observations: CCR4 expression level, percentage of $CD16^+$ cells, and genotype of the *FCGR3A* polymorphism. Firstly, the cell surface expression level of CCR4 seemed to be an important factor for better ADCC activity as MT-2 cell line, which was stained the best with CCR4 antibody, showed the highest ADCC activity. Secondly, the percentage of $CD16^+$ cells among PBMCs is most likely critical because it was almost reproducibly correlated with ADCC activity, as reported in myeloma study with a plasma cell-specific antibody (36). However, it is of note that one of the normal donors (PBMC 3) who had only 4.2% of $CD16^+$ cells in PBMCs still showed sufficient ADCC activity against CCR4-positive ATLL cells, supporting our previous report that defucosylated chimeric anti-CCR4 monoclonal antibody needed much fewer effector cells to achieve the same cytotoxicity as

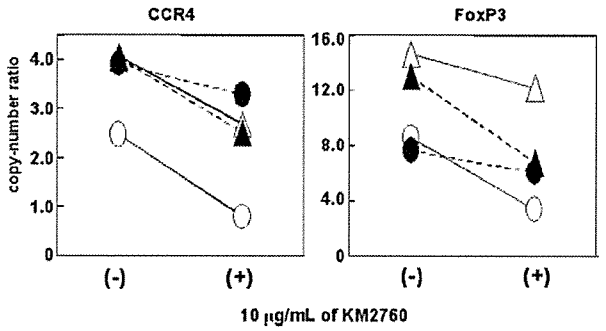

Fig. 6 Reduction of *CCR4* and *FoxP3* mRNA levels in PBMCs treated with KM2760. Fresh PBMCs from four normal volunteers were incubated in the presence (+) or absence (−) of 10 μg/mL KM2760 in RPMI 1640 supplemented with 10% of heat-inactivated pooled human serum. After 6 hours of incubation at 37°C, 5% CO_2, cDNA was prepared from harvested cells and assessed for the expression of *CCR4* and *FoxP3* by quantitative reverse transcription-PCR, and the copy number ratio for each was calculated. ○, PBMC 1; ●, PBMC 2; △, PBMC 3; and ▲, PBMC 4.

that shown by nondefucosylated antibody (16). This feature also should be therapeutically beneficial because the number of effector cells capable of penetrating into tumor masses could be much less than that of tumor cells in the clinical settings. Finally, it has been shown that human IgG1 binds more strongly to FcγRIIIa on NK cells homozygous for *FCGR3A-158V* allele than to those homozygous or heterozygous for *158F* alleles (37, 38). FcγRIIIa is expressed on both NK cells and monocytes, which are the most important natural cytotoxic effectors. Homozygosity for the *158V* allele is associated with better clinical and molecular responses to the chimeric anti-CD20 IgG1 monoclonal antibody rituximab in follicular lymphoma (39) but not in chronic lymphocytic leukemia (40). The *FCGR3A* genotype of the effector PBMCs used throughout the current study was examined. Unfortunately, PBMCs from donors homozygous for the *158V* allele were not found among our donors; nevertheless, a robust ADCC activity was observed irrespective of the absence of donors homozygous for the *158V* allele. We have recently shown that defucosylated chimeric IgG1 monoclonal

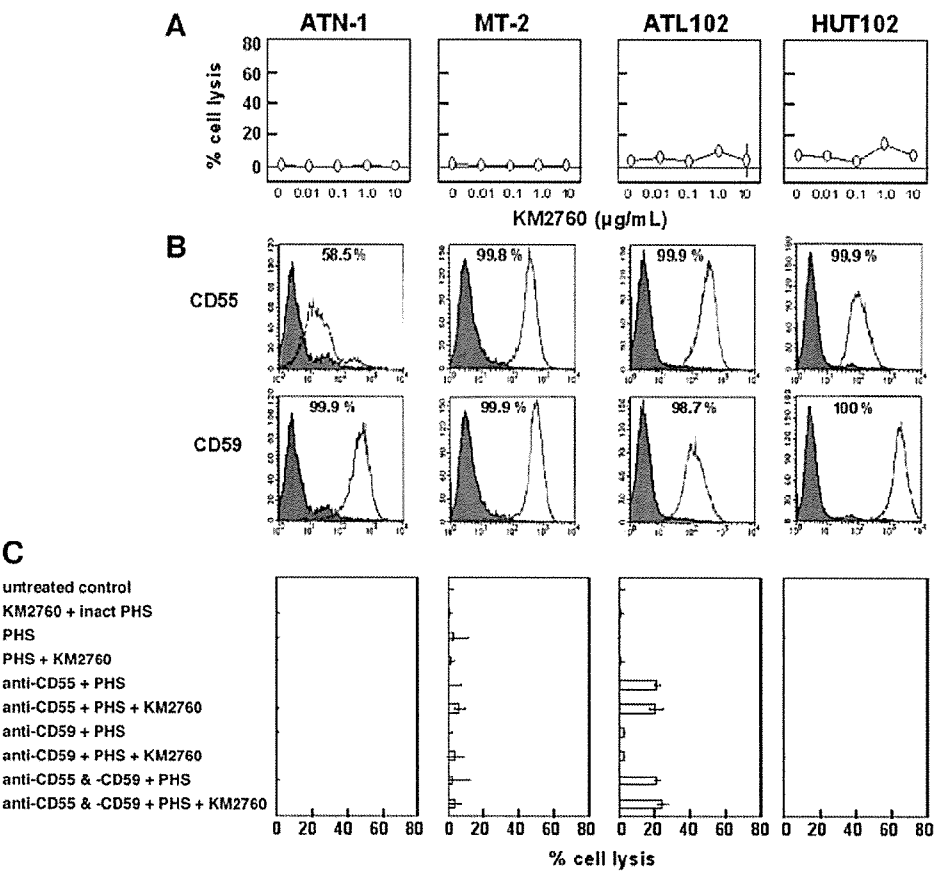

Fig. 7 CDC activity of KM2760 against ATLL cell lines and effect of CD55 and CD59 blocking. *A*. CDC activity against ATLL cells was measured by 1.5 hours of ^{51}Cr release assay. Cells were incubated with the indicated concentrations of KM2760 in RPMI 1640 supplemented with intact pooled human serum (PHS) at the final concentration of 20%. All experiments were done in triplicate, and the percent lysis is presented as the average ± SD. *B*. ATLL cell lines were stained with FITC-conjugated anti-CD55 or anti-CD59 monoclonal antibodies (blank histograms). Staining with isotype control monoclonal antibody is shown as filled histograms. The percentage of positive cells is indicated in each panel. *C*. Blocking antibodies against CD55 (1C6) and CD59 (1F5) were added to the CDC assay to block the function of the CD55 and CD59, individually or in combination. The concentration of KM2760, anti-CD55, and anti-CD59 monoclonal antibodies were fixed at 10 μg/mL. All experiments were done in triplicate, and the percent cell lysis is presented as the average ± SD. *A* and *C* each represents three independent experiments.

antibodies, anti-CD20 and anti-CCR4, can induce much stronger ADCC than nondefucosylated ones (15, 16). These findings can probably explain why the PBMCs, even from donors homozygous or heterozygous for the *158F* allele, induced remarkable antibody-dependent cellular cytotoxicity activity in the presence of defucosylated chimeric anti-CCR4 monoclonal antibody KM2760. This feature should be therapeutically beneficial because a comparable ADCC activity of KM2760 can be expected irrespective of *FCGR3A* genotype.

From the clinical point of view, KM2760 induced a potent ADCC activity at concentrations that are considered to be clinically attainable (1 to 10 μg/mL) against freshly isolated tumor cells in the presence of allogeneic PBMCs as effector cells. Antibody-dependent cellular cytotoxicity against freshly isolated ATLL cells mediated by autologous effector cells was generally lower than that mediated by allogeneic control ones. However, a robust activity, which was comparable with that mediated by allogeneic effector cells, was induced in two of the four cases. It has been shown that HTLV-1 can infect many human cell types other than CD4-positive T-lymphocytes such as NK cells but not hematopoietic progenitor cells (41–47). Previous reports have revealed that the NK cell activity was significantly decreased in patients with HTLV-1-associated myelopathy/tropical spastic paraparesis, although the underlying mechanism remains unknown (48, 49). If HTLV-1 integration into effector cells is the direct reason for their impaired effector function, newly generated cells from the hematopoietic progenitor cells would be free from the virus and expected to show normal function until they become infected. Because close cell-to-cell interaction is required to transmit viruses from HTLV-1-bearing cells to noninfected cells (50), and this process should be time-consuming, there must be an open-window period in which to repopulate normal effector cells. Alternatively, effector cells differentiated or remaining in an immunocompromised environment caused by the copresence of ATLL cells might have impaired cytolytic function (51). In any case, our observation, that one patient in partial remission after chemotherapy showed much better ADCC activity despite the lower percentage of autologous $CD16^+$ effector cells in the CD3-negative subset of PBMCs, indicates that chemotherapy before KM2760 administration could be an optimal choice for patients with large tumor burdens in peripheral blood. In addition, treatment with KM2760 for patients with lymphoma type ATLL or PTCL, the latter of which was shown in this study, could be clinically effective because these patients are free from peripheral blood involvement of the tumor cells.

In this report, we showed not only that *FoxP3* mRNA was expressed in the $CD4^+CCR4^+$ T-cell subset at a much higher level than that in the $CD4^+CCR4^-$ T-cell subset obtained from normal volunteers but also that FoxP3 was highly expressed in freshly isolated ATLL cells. It is known that the surface phenotype of ATLL cells is represented by positivity for CD4, CD25, and CCR4. Our novel findings suggest that ATLL cells might originate from $CD4^+CD25^+$ ($CCR4^+$) immunoregulatory T cells. Thus, it can be envisaged that *FoxP3*-expressing ATLL cells would give rise to a profound immunosuppressive environment around themselves so that they can escape from the host's immunosurveillance. Interestingly, all established ATLL cell lines expressed extremely low or no detectable FoxP3 when assessed by quantitative PCR and Western blot analysis. We surmise that they do not need to express FoxP3 because there exists no effector T cells that attack the ATLL cell lines in culture. In addition, the suppression of the host's normal effector T cells by these ATLL cells can result in a severe immunocompromised state, which is one of the clinical characteristics of patients with ATLL. A similar situation has been shown in Hodgkin's lymphoma (52). Moreover, it has been shown that tumor cells in Hodgkin's lymphoma express thymus and activation-regulated chemokine, which is one of the specific ligands for CCR4, and that reactive lymphocytes surrounding the tumor cells do express CCR4 (53). These studies support our hypothesis of the close relationship between FoxP3 and CCR4 expressions in ATLL cells. Because KM2760 reduced the *FoxP3* mRNA expression level, presumably by specifically killing FoxP3-coexpressing $CCR4^+$ T cells in PBMCs obtained from normal volunteers, KM2760 may be beneficial in reducing the immunosuppressive effect of not only FoxP3-expressing ATLL cells but also FoxP3-expressing normal immunoregulatory T cells and subsequently provoke effective tumor immunity or restore the host's profound immunosuppressive state. Collectively, our findings strongly suggest that KM2760 also can be used as a potential immunomodifier.

KM2760 showed no CDC activity against CCR4-positive target cells. ATLL cell lines showed high expression of complement inhibitors such as CD55 and CD59, whereas addition of blocking antibodies against CD55 or CD59 did not induce CDC activity by KM2760. Besides CDC, we did not detect any direct inhibitory effect on proliferation of the ATLL cell lines or fresh ATLL cells, even when combined with IFN-α or IFN-γ, which have been used for the treatment of several hematologic malignancies, including ATLL. Collectively, we conclude that the major antitumor activity of KM2760 is mediated by ADCC.

In conclusion, the present study shows a promising ADCC activity of the defucosylated chimeric anti-CCR4 monoclonal antibody, KM2760, against tumor cells of ATLL and PTCL, although the optimal conditions for obtaining maximal effector function of autologous effector cells from patients still need to be explored. Moreover, the ability of KM2760 to act as an immunomodifier and break tolerance to tumor cells is also encouraging. As rituximab, a chimeric anti-CD20 monoclonal antibody, has changed the standard therapy in elderly patients with diffuse large B-cell lymphoma (54), now KM2760 could be an ideal treatment modality against patients with ATLL and CCR4-positive PTCL.

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Recovery from and consequences of severe iatrogenic lymphopenia (induced to treat autoimmune diseases)

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Abstract

To ascertain the consequences of severe leukopenia and the tempo of recovery, we studied the immunity of 56 adult patients treated for multiple sclerosis or systemic sclerosis with autologous CD34 cell transplantation using extremely lymphoablative conditioning. NK cell, monocyte, and neutrophil counts recovered to normal by 1 month; dendritic cell and B cell counts by 6 months; and T cell counts by 2 years posttransplant, although CD4 T cell counts remained borderline low. Initial peripheral expansion was robust for CD8 T cells but only moderate for CD4 T cells. Subsequent thymopoiesis was slow, especially in older patients. Importantly, levels of antibodies, including autoantibodies, did not drop substantially. Infections were frequent during the first 6 months, when all immune cells were deficient, and surprisingly rare (0.21 per patient year) at 7–24 months posttransplant, when only T cells (particularly CD4 T cells) were deficient. In conclusion, peripheral expansion of CD8 but not CD4 T cells is highly efficient. Prolonged CD4 lymphopenia is associated with relatively few infections, possibly due to antibodies produced by persisting pretransplant plasma cells.

Keywords: Immunodeficiency; T lymphocytes; B lymphocytes; Autoimmunity

Introduction

Autoimmune diseases may be caused by a one time failure of negative selection leading to the generation of an autoreactive T or B cell clone. This hypothesis lead to the development of clinical trials of extremely lymphoablative therapy, typically with autologous CD34 cell transplantation to minimize hematological toxicity [1]. The aim was to eliminate the autoreactive T or B cell clone and hope that the error in negative selection would not be repeated. The trials have provided a unique opportunity to study the consequences of severe leukopenia (in particular, lymphopenia) and homeostatic recovery in humans. The conditioning used in our trials [2,3] consisted of total body irradiation and cyclophosphamide administered from day 5 to day 2 and anti-thymocyte globulin (ATG) administered from day 5 to day 5; this resulted in severe lymphopenia (significantly more severe than after autologous transplantation for cancer using

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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

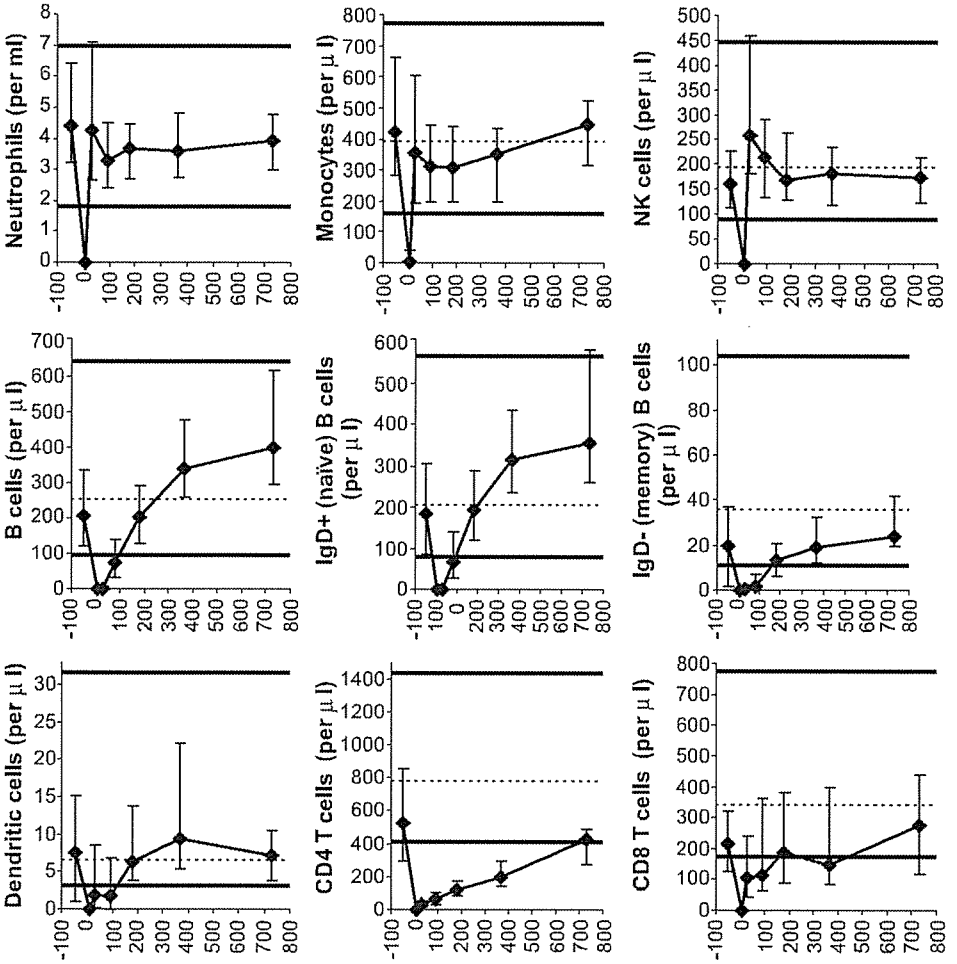


Fig. 1. Recovery of leukocyte subsets. All horizontal axes display days posttransplant. Patient medians (diamonds) and 25th–75th percentiles (error bars) are shown. Normal medians are indicated by the dashed horizontal lines (except for neutrophils—not available). The thick horizontal lines denote the 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

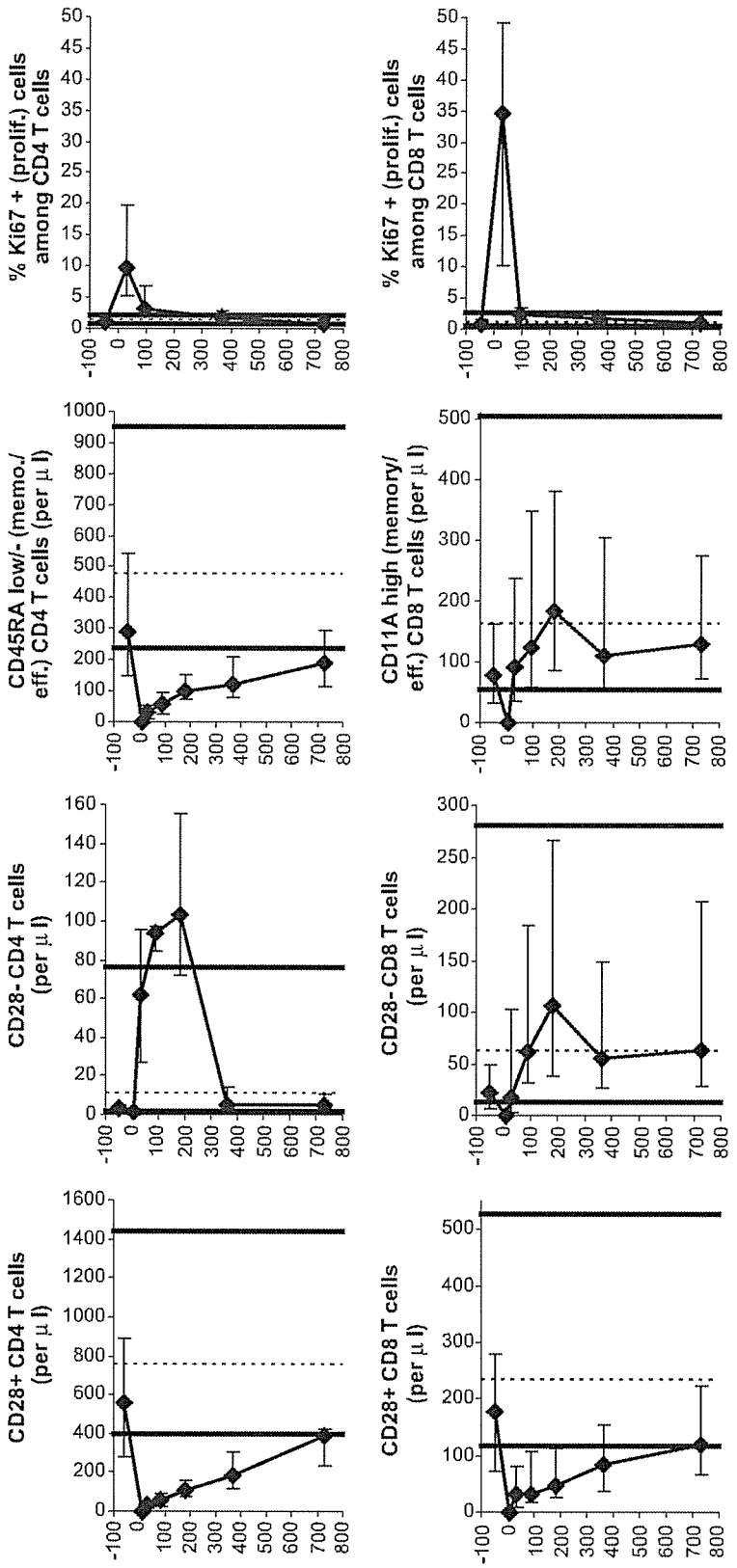


Fig. 2. Recovery of T cell subsets. For symbols and the numbers of patient samples analyzed, see Fig. 1 legend.

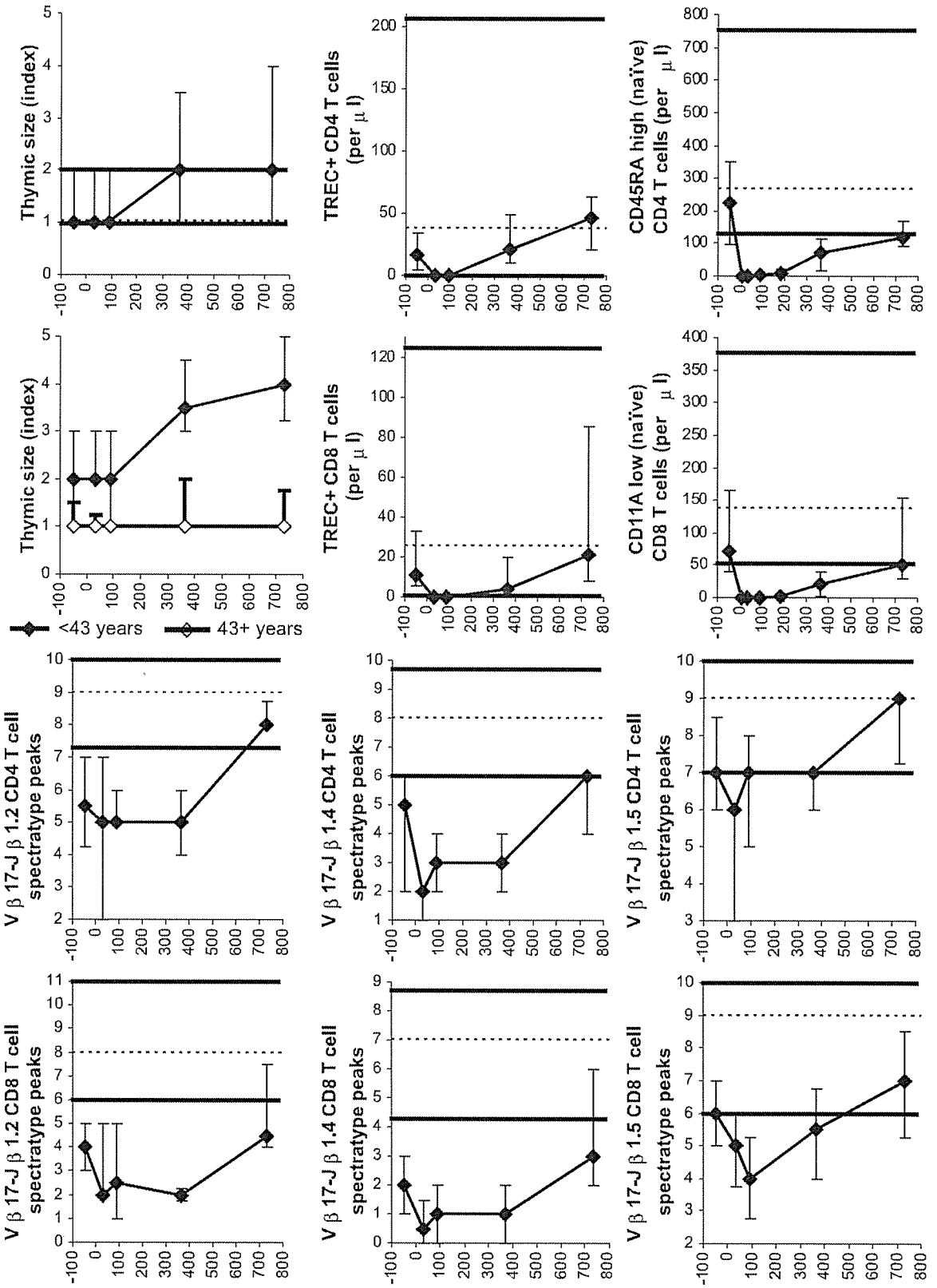
Vantage flow sorter (BD Biosciences). A total of 50,000 CD4 or CD8 T cells were sorted into an Eppendorf tube. After centrifugation, supernatant was removed and the "dry" cell pellet was stored frozen (−80°C). Real-time PCR of the αδ signal joint was performed directly from lysate of the cell pellet, 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 GCCAGCTG-CAGGGTTTAGG and probe FAM-ACACCTCTGG-TTTTTGTAAAGGTGCCCACT-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, $Vβ17^+CD4^+CD8^-$ and $Vβ17^+CD4^-CD8^+$ cells were first sorted to >95% purity from Ficoll-separated MNCs, using Vantage flow sorter (BD Biosciences). A total of 2000 $Vβ17^+$CD4 or $Vβ17^+$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 GGA-GAGTCGAGTCCCATCA (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).

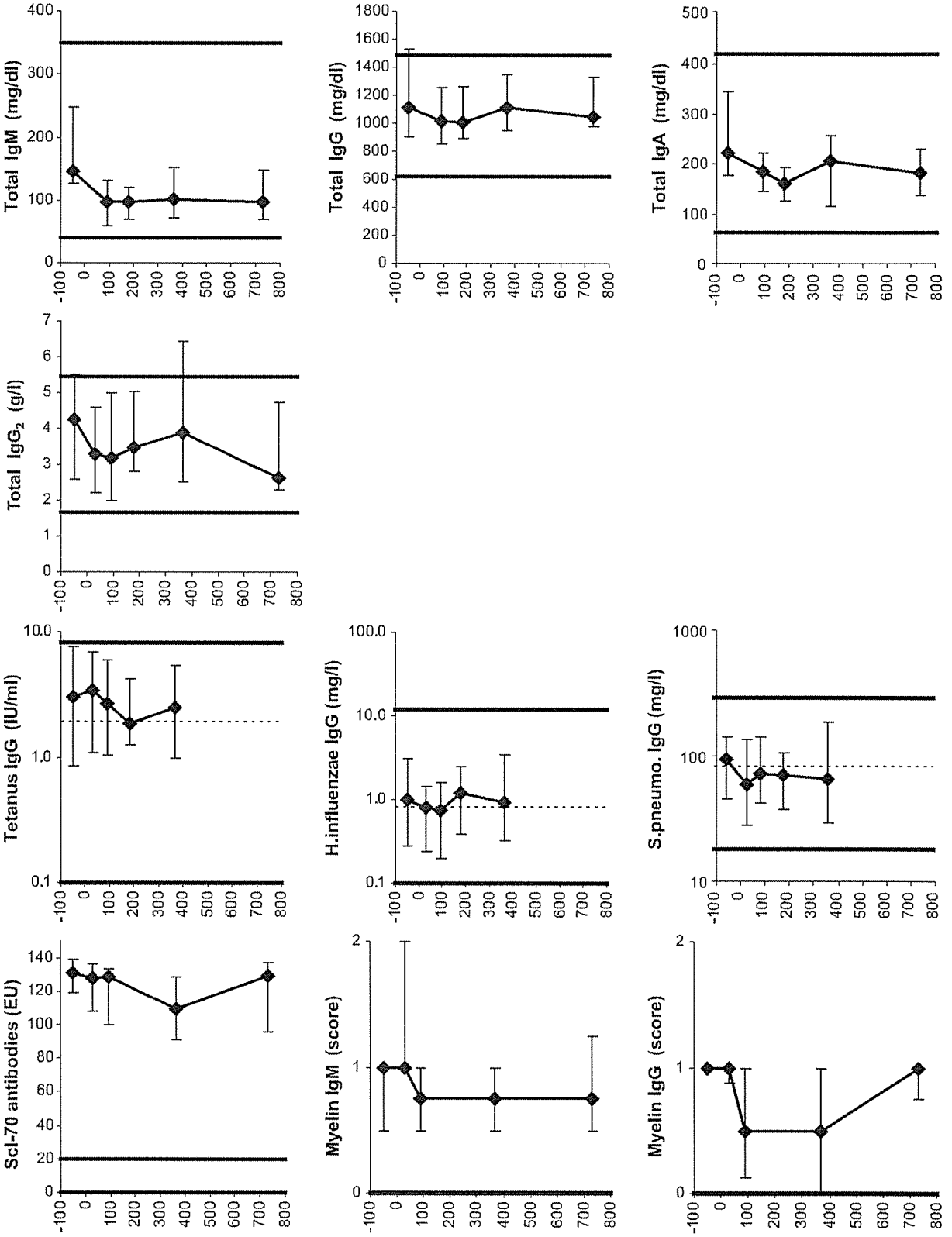

Total IgM (mg/dl)
Total IgG (mg/dl)
Total IgA (mg/dl)
Total IgG2 (g/l)
Tetanus IgG (IU/ml)
H.influenzae IgG (mg/l)
S.pneumo. IgG (mg/l)
Scl-70 antibodies (EU)
Myelin IgM (score)
Myelin IgG (score)

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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