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Expansion of CD8+CD57+ T cells after allogeneic BMT is related with a low incidence of relapse and with cytomegalovirus infection

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Summary. Peripheral blood lymphocytes of 46 recipients of lymphocyte-depleted bone marrow allografts were phenotypically analysed over a period of 1 year. We investigated the repopulation of lymphocyte subpopulations and their relation with clinical parameters such as graft-versus-host disease (GVHD), graft-versus-leukaemia and cytomegalovirus (CMV) infection. The number of repopulated T cells varied strongly between the blood samples of the recipients. In 45% of the recipients the number of T cells recovered to or above normal levels within 3 months after bone marrow transplantation (BMT), whereas the other recipients remained below normal up to 1 year after BMT. In recipients with a high repopulation, the CD8+ T-cell subset contributed more to this high repopulation than the CD4+ T-cell subset. We showed that the majority of T cells of these recipients expressed the αβ T-cell receptor, CD8, CD57 and CD11b. HLA-DR was also highly expressed reflecting the activation stage of T cells in these recipients. BMT recipients with a high repopulation of CD8+ T cells showed a lower incidence of leukaemic relapse than recipients with a low repopulation. The 3-year probability of relapse was 19% versus 64% (P = 0.03), respectively. The relative high number of CD8+ T cells at 3 months after BMT was not associated with the incidence of GVHD. In contrast, occurrence of CMV infection after BMT was significantly higher in these recipients. Our results indicate that CD8+ T cells, predominantly CD57+, of BMT recipients with an expansion of these cells represent an in vivo activated cell population. This CD8+ T-cell population may consist partially of cytotoxic cells with anti-leukaemic activity as suggested by a low relapse rate. The signal for the strong expansion of these CD8+CD57+ T cells after BMT is still unclear, but association with CMV infection suggests that viral antigens are involved.

Keywords: BMT, T-cell subsets, CD57, graft-versus-leukaemia, cytomegalovirus.

Allogeneic bone marrow transplantation (BMT) is an effective treatment for patients with leukaemia that has been associated with graft-versus-leukaemia (GVL) activity (Horowitz et al, 1990). Donor-derived cytotoxic effector cells appear to be involved in the elimination of residual leukaemic cells after BMT. Graft-versus-host disease (GVHD) is an adverse complication of allogeneic BMT, but is associated with this GVL activity (Horowitz et al, 1990). T-cell depletion of the graft reduces the incidence and severity of acute GVHD (De Witte et al, 1984), but unfortunately results in higher relapse rate (Horowitz et al, 1990). Cytomegalovirus (CMV) infection has also been suggested to be associated with decreased incidence of leukaemic relapse (Ringdén, 1992). These data indicate that alloantigens and viral antigens may induce cell populations that mediate GVL activity after BMT.

CMV is frequently reactivated after allogeneic BMT and the onset of CMV infection is during the first 3 months. CMV profoundly stimulates CD8+ T cells which represent the majority of T cells in the blood of BMT recipients (Leroy et al, 1986; Wursch et al, 1985; Yabe et al, 1990). Polyclonal stimulation of CD8+ cytotoxic T lymphocytes (CTL) occurs during viral infections (Nahill & Welsh, 1993). Reconstitution of CD8+ CMV-specific CTL response, during CMV infection after BMT, provides protection from the development of CMV disease (Reussner et al, 1991). Most CD8+ T cells in BMT recipients express the CD57 antigen, whereas in

In this study we investigated reconstitution of peripheral blood lymphocyte (PBL) subpopulations in recipients of lymphocyte-depleted bone marrow allografts. We observed that the absolute number of T cells, of which the majority express TCRαβ, CD8 and CD57, returned to normal levels in approximately half of the recipients within 3 months after BMT. In contrast, T-cell numbers in the other recipients remained below normal for at least 1 year. The high repopulation of CD8⁺ T cells in BMT recipients was associated with a low relapse rate and with CMV infection. Therefore we suggest that the CD8⁺ T-cell population, predominantly CD57⁺, contains cytotoxic cells that obtain GVL activity during CMV infection after BMT.

MATERIALS AND METHODS

Patient characteristics. 46 patients (28 males and 18 females) with a median age of 40 years (range 18–55) were transplanted for haematological diseases in the period July 1990 to October 1993. Indications for transplantation were acute myeloid leukaemia (AML) in 17 patients (15 in first complete remission [CR1]), acute lymphoblastic leukaemia (ALL) in nine patients (eight in CR1), chronic myeloid leukaemia (CML) in 11 patients (all in first chronic phase [CP1]), non-Hodgkin's lymphoma (NHL) in four patients (all in CR1), multiple myeloma (MM) in three patients and severe aplastic anaemia (SAA), refractory anaemia (RA) in one patient each. All patients received an allogeneic bone marrow graft from an HLA-identical, mixed lymphocyte culture negative sibling. Donor marrow was depleted of 98% of lymphocytes by counterflow centrifugation using a four-chamber elutriator rotor in the Curamé 3000 centrifuge (Heraeus Separationstechnik, Ostenrode, Germany) (Plas et al, 1988). The absolute number of T cells infused ranged from 0·7 to 1·3 (median 0·9) x 10⁹/kg body weight. Patients transplanted for haematological malignancies were conditioned for BMT with cyclophosphamide (60 mg/kg body weight, days −6 and −5) and fractionated total body irradiation in two equal fractions of 4·5 Gy each on days −2 and −1. In 23 patients idarubicin (42 mg/m²) was added to the conditioning regimen by continuous intravenous (i.v.) infusion during 48 h. The patients transplanted for SAA were conditioned with total lymphoid irradiation to a total dose of 12 Gy on days −9, −8 and −7 followed by cyclophosphamide (50 mg/kg body weight) on days −5 to −2. Cyclosporin A (CsA; 3 mg/kg body weight/d) was given by continuous i.v. infusion from days −1 to 14, followed by 2 mg/kg body weight/d continuous i.v. on day 21. From day 21 onward, CsA was given orally in a dose of 6 mg/kg body weight/d to 12 weeks after BMT, followed by a gradual tapering off and discontinuation after 16 weeks post-grafting (Schattenberg et al, 1990). All patients received orally acyclovir (4 x 400 mg/d) from days −9 to 60 as prophylaxis of herpes virus infection. CMV infection prophylaxis with hyperimmune globulin was not given.

CMV monitoring. IgG, IgA and IgM antibodies to CMV were tested prior to BMT in serum of both donor and recipient using an ELISA. An antibody titre of >10 was considered positive. The presence of CMV in urine samples was determined using a standard method. Briefly, fibroblast cell line HBL was grown to confluency in flat-bottom tubes containing a coverslip. Tubes with cells were inoculated with urine specimen by centrifugation for 45 min at 37°C. After 48 h, cells were fixed with methanol for 20 min at 4°C and examined for detection of immediate-early antigen with the MoAb NEA-9221 (Dupont, Dreieich, Germany) by indirect immunofluorescence. The presence of CMV antigen in blood was determined using a standard method. Briefly, granulocytes were isolated from citrate-anticoagulated blood by dextran. Cells were collected and cytospin preparations (1·5 x 10⁵ cells/cytospin) were made. Cytospin preparations were fixed with acetone for 10 min at room temperature (RT) and examined for detection of pp65 antigen with the MoAbs CMV-C10 and CMV-C11 (Biotest, Dreieich, Germany) by indirect immuno-alkaline phosphatase staining. CMV infection was defined by the presence of CMV antigen in blood and/or CMV in cultures of urine samples. CMV disease was defined by evidence of CMV in bronchoalveolar lavage fluid, in tissue sections of the liver or by gastrointestinal endoscopy plus evidence of CMV in biopsies associated with clinical signs and symptoms.

Blood sampling and haematological parameters. Peripheral blood samples were obtained from donors and patients at 2 weeks before BMT and from patients at 1, 3, 6, 9 and 12 months after BMT. The haematological parameters including leucocyte differential counts were measured by a Technicon H1 haematology analyser (Technicon Instrument Co., Tarrytown, N.Y.). Full blood was immediately used for phenotypic analysis or stored in liquid nitrogen after isolation of PBL.

Peripheral blood lymphocytes. PBL were isolated from heparinized blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden). Cells were collected and washed twice in glucose phosphate buffered saline (GPBS) and finally suspended in Iscove's medium supplemented with 5% heat-inactivated fetal bovine serum (FBS; Gibco, Paisley, Scotland) and 10% dimethylsulfoxide (Merck, Darmstadt, Germany). Cells were cryopreserved and stored in liquid nitrogen until use.

Phenotypic analysis. The following MoAbs were used: UCHT1 (CD3), MT310 (CD4), DK25 (CD8), HD37 (CD19), ACT1 (CD25), UCHL1 (CD45RO), CR3/43 (HLA-DR) purchased from Dakopatts, Glostrup, Denmark; OKM1 (CD11b) purchased from Ortho, Raritan, N.Y.; Leu19 (CD56), Leu7 (CD57) purchased from Becton Dickinson, Mountain View, Calif.; and WT31 (TCRαβ) kindly provided by Dr W. Tax, Division of Nephrology, University Hospital Nijmegen, The Netherlands. Direct immunophenotyping was performed using a standard method. Briefly, 100 µl of heparinized full blood was incubated with the appropriate concentration of FITC-conjugated and PE-conjugated MoAbs at RT for 15 min. Bythocytes were subsequently
lysed in FACS lysing reagent (Becton Dickinson) at RT for 10 min. After lysis, cells were washed in phosphate-buffered saline (PBS) and finally fixed in 1% paraformaldehyde solution. Cells were analysed on an Epics Elite flow cytometer (Coulter Corporation, Hialeah, Fla.). Indirect immunophenotyping was performed as follows. PBL were suspended in PBS supplemented with 20% pooled human serum (PHS) and 0-1% NaN₃. Cell suspensions of 100 µl (10⁶ cells) were incubated (20 min, 4°C) with the appropriate concentration of MoAb. Cells were washed in PBS supplemented with 0.5% bovine serum albumin and subsequently incubated (20 min, 4°C) with FITC-conjugated F(ab')₂ fragment goat-anti-mouse IgG (American Qualex International, La Mirada, Calif.) diluted in PBS supplemented with 20% PHS and 0-1% NaN₃. After washing, the cells were incubated with 10% normal mouse serum in PBS supplemented with 0.5% bovine serum albumin and subsequently incubated (20 min, 4°C) with FITC-conjugated F(ab')₂ fragment goat-anti-mouse IgG (American Qualex International, La Mirada, Calif.) diluted in PBS supplemented with 20% PHS and 0-1% NaN₃. After washing, the cells were incubated with UCHT1-PE (CD3) for 20 min at 4°C. Cells were washed and analysed by flow cytometry (FCM).

Statistical analysis. Absolute numbers of lymphocyte subpopulations of BMT recipients and normal individuals were compared using the non-parametric Mann-Whitney U-test. Correlations between absolute numbers of lymphocyte subpopulations at different time periods after BMT were analysed with the Spearman's rank test. Relapse and survival analysis was performed using the Kaplan-Meier product-limit method. Actuarial curves were calculated for relapse, survival and leukaemia-free survival (LFS) and the effect of high repopulation of T cells on the curves was compared using the log-rank test. The Fisher's exact test was used to assess differences in clinical parameters. For all tests P value <0.05 was considered significant.

RESULTS

Phenotypic analysis of lymphocytes after BMT

PBL of BMT recipients were analysed by FCM at several time periods until 1 year postgrafting. Fig. 1 shows the absolute number of repopulated lymphocyte subpopulations in comparison with lymphocyte numbers of normal individuals. The median number of both NK cells and T cells were below the normal range for at least 1 year. The median number of CD8+ T cells was also below normal at 1 month, but increased to normal within 3 months. CD4+ T cells repopulated very slowly and the median number remained below normal during the whole follow-up period. Almost all CD4+ cells coexpressed CD45RO corresponding with a helper and memory phenotype (data not shown). The percentage of NK cells was higher than normal at 1 month after BMT, but by increasing numbers of T cells this returned gradually to normal during the subsequent months.
As shown in Fig 1, the number of T cells varied strongly between the blood samples of BMT recipients. T-cell numbers of some patients increased towards normal values between 1 and 3 months after BMT. Statistical analysis revealed that the number of circulating T cells was strongly correlated with the number of CD8+ T cells at 3 months after BMT (r = 0.99, P = 0.001). Although the number of CD4+ T cells was also significantly correlated with the number of T cells at 3 months after BMT (r = 0.62, P = 0.001), the CD8+ T-cell subset contributed more to the number of T cells than the CD4+ T-cell subset, as shown by the correlation coefficients.

The absolute number of T cells at 3 months after BMT was strongly correlated with coexpression of the CD57 antigen on these cells (Fig 2; r = 0.70, P < 0.001). To determine coexpression of other antigens on T cells, PBL of five BMT recipients with a high repopulation of T cells were additionally characterized (Fig 3). The majority of these T cells expressed both CD8 and CD57 (68–88%). 90% of T cells expressed the αβ T-cell receptor (87–98%). In all five patients studied, <30% of T cells expressed CD4. Furthermore, we studied the expression on these cells of IL-2 receptor α-chain (CD25) and HLA-DR because both antigens are associated with T-cell activation. A small percentage of T cells expressed CD25 (1–16%), whereas HLA-DR was expressed on 48–76% of T cells. CD11b which is frequently coexpressed with CD57 on lymphocytes after BMT (Yabe et al, 1990), was expressed on 33–88% of T cells. These data show that in BMT recipients with a high repopulation of T cells the majority of these cells express TCRαβ, CD8, CD57, CD11b and HLA-DR.

<table>
<thead>
<tr>
<th>Table I. Clinical data of patients transplanted in CR1/CP1 from group 1 with low numbers of CD8+ T cells and patients from group 2 with relatively high numbers of CD8+ T cells.</th>
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<td><strong>Group 1</strong></td>
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<td>No. of patients</td>
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| Median age* | 37 | 40 | NS  
| Male/female | 16/5 | 8/9 | NS  
| Underlying disease |  |  |  
| AML | 8 (38%) | 7 (41%) | NS  
| ALL | 4 (19%) | 4 (23%) | NS  
| CML | 8 (38%) | 3 (18%) | NS  
| NHL | 1 (5%) | 3 (18%) | NS  
| Conditioning regimen with idarubicin | 13 (62%) | 9 (53%) | NS  
| T cells x 10^9/kg transplanted | 0.9 ± 0.2 | 0.9 ± 0.2 | NS  
| Probability of relapse‡ | (64%) | (19%) | 0.03  
| Probability of LFS | (34%) | (60%) | NS  
| Probability of survival | (61%) | (63%) | NS  
| Transplant-related mortality | 2 (10%) | 3 (18%) | NS  
| Acute GVHD grade 2 | 4 (19%) | 4 (23%) | NS  
| Chronic GVHD |  |  |  
| Limited | 8 (38%) | 4 (24%) | NS  
| Extensive | 1 (5%) | 4 (24%) | NS  
| CMV serology pre-BMT |  |  |  
| Recipient negative/donor negative | 16 (76%) | 2 (12%) | 0.001  
| Recipient negative/donor positive | 1 (5%) | 2 (12%) | NS  
| Recipient positive/donor negative | 3 (14%) | 3 (17%) | NS  
| Recipient positive/donor positive | 1 (5%) | 10 (59%) | 0.001  
| CMV infection after BMT | 2 (10%) | 12 (71%) | 0.001  

* Age at time of transplantation in years.  
‡ Projected 3-year probability.
On the basis of the difference in repopulation of CD8+ T cells, patients were divided in two groups. The median number of CD8+ T cells at 3 months after BMT was $0.28 \times 10^9/l$ (range $0.02 - 3.87 \times 10^9/l$). This value was chosen retrospectively as a cut-off point to divide the patients in two equally sized groups. The mean number of CD8+ T cells at 3 months after BMT in group 1 (24/46 patients) was strongly below normal CD8+ T-cell numbers ($0.14 \pm 0.08 \times 10^9/l$ and $0.42 \pm 0.14 \times 10^9/l$, respectively; $P < 0.001$; Fig 1, closed circles). The mean number of CD8+ T cells at 3 months after BMT in group 2 (22/46 patients) was above normal levels ($1.06 \pm 0.92 \times 10^9/l$ and $0.42 \pm 0.14 \times 10^9/l$, respectively; $P < 0.001$; Fig 1, open circles).

These data demonstrate that in 45% of the recipients the number of CD8+ T cells recovered above normal levels within 3 months after BMT, whereas the other recipients remained strongly below normal levels.

**Relationship between repopulation of CD8+ T cells and clinical data**

To determine the contribution of CD8+ T-cell repopulation to the clinical outcome, clinical data of both patient groups were evaluated (Table I). Patients with MM, SAA and RA as underlying disease and patients not in CR1 or CP1 were not included in this evaluation. Both groups were not significantly different with regard to age, sex, underlying disease, conditioning therapy and T cells/kg body weight transplanted. However, a significant difference could be found in the projected 3-year probability of relapse between patients transplanted in CR1/CP1 in groups 1 and 2, 64% versus 19% ($P = 0.03$, Fig 4A). The probability of survival was not significantly different between the two groups (Table I). The projected 3-year probability of survival for group 1 was 61% and for group 2 63% ($P = 0.64$). No difference was found in transplant-related mortality (TRM) between groups 1 and 2, 10% versus 18% ($P = 0.40$), respectively. However, a trend was observed towards a longer leukemia-free survival (LFS) for recipients in group 2 with a projected 3-year probability of LFS of 60% versus 34% for group 1 ($P = 0.15$, Fig 4B).
The projected 3-year probability of relapse, survival and LFS for all patients studied was 43%, 62% and 46%, respectively.

No significant differences in acute and chronic GVHD were found between the two groups (Table I). A positive CMV serologic determination of both recipient and donor prior to BMT was more frequently detected in group 2 than in group 1 (P = 0.001). Moreover, a negative CMV serologic determination of both recipient and donor prior to BMT was more frequently detected in group 1 than in group 2 (P = 0.001). The incidence of CMV infection after BMT was significantly higher in group 2 (12/17) than in group 1 (2/21, P < 0.001). The median time of onset of CMV infection was 9 weeks (range 5–12 weeks). None of the patients studied developed CMV disease.

Although the probability of relapse and LFS for patients with CMV infection (n = 14) versus patients without CMV infection (n = 24) showed differences, it was not statistically significant. The projected 3-year probability of relapse for patients with CMV infection was 23% and for patients without CMV infection 47% (P = 0.40). The projected 3-year probability of LFS for patients with CMV infection was 56% and for patients without CMV infection 45% (P = 0.92).

These data indicate that high repopulation of CD8+ T cells, predominantly CD57+, is related to a low incidence of leukemic relapse. Moreover, high repopulation of CD8+ T cells is correlated with the occurrence of CMV, suggesting that viral antigens are involved.

DISCUSSION

Donor-derived cytotoxic cells of recipients of bone marrow allografts appear to be involved in the elimination of residual leukemic cells that survive the pretransplant conditioning regimen. Clinical data support such GVL activity by both GVHD-dependent and GVHD-independent effector mechanisms (Horowitz et al, 1990). We studied repopulation of PBL in recipients of lymphocyte-depleted bone marrow allografts to investigate the relation of recovery of lymphocyte subsets and GVIL activity.

Between 1 and 3 months after BMT the number of T cells repopulated to or above normal levels in 45% of the BMT recipients studied. This high repopulation of T cells was the result of an increase of both CD4+ and CD8+ T cells but the CD8+ T-cell subset contributed more to the high repopulation of T cells than the CD4+ T-cell subset.

Expansion of CD8+ T cells between 1 and 3 months after BMT was accompanied by coexpression of the CD57 antigen on these cells. CD57 is a lineage-nonrestricted glycoprotein that normally is expressed on a subpopulation of NK cells and a minority of T cells. The function of this antigen recognized by CD57 MoAbs is still unknown. Kruse et al (1984) have shown that a carbohydrate structure of neural cell adhesion molecule (NCAM) and myelin-associated glycoprotein (MAG) is recognized by CD57 MoAbs. Lalier et al (1992) have identified an α1β1 integrin on avian neural crest cells that bears the CD57 epitope and is involved in adhesion to laminin. Therefore the CD57 antigen expressed on lymphocytes after stimulation in certain pathological conditions may be involved in adhesion. CD8+CD57+ T cells mediate in vitro IL-2- and anti-CD3-induced cytotoxicity (Phillips & Lanier, 1986; Leroy et al, 1990; Madariaga et al, 1990). However, it has also been described that these cells inhibit cytotoxicity of allospecific CTL or NK cells as well as mitogen-induced B- and T-cell proliferation (Leroy et al, 1986; Autran et al, 1991).

HLA-DR was expressed on most T cells of BMT recipients with a high repopulation of these cells. This suggests that these cells has been activated in vivo. However, <15% of these cells expressed the IL-2 receptor α-chain (CD25).

Expression of CD11b the α-subunit of integrin Mac-1 (CD11b/CD18), also associated with lymphocyte activation, was found on 33–88% of these T cells. Normally CD11b is only expressed on the surface of a small subset of T cells and on all neutrophils, eosinophils, monocytes, and most NK cells and plays a role in adhesion (Schleiffenbaum et al, 1989). McFarland et al (1992) found that CD11b is expressed on a subset of CD8+ T cells that include active virus-specific CTL. Therefore Mac-1 might be involved in cellular interactions resulting in activation and cytolytic activity of CD8+CD57+ T cells.

The increase of CD8+CD57+ T cells in BMT recipients seems to be the result of stimulation by alloantigens and/or viral antigens (Leroy et al, 1986; Wursch et al, 1985; Yabe et al, 1990; Izquierdo et al, 1990; Fuku et al, 1994). It has been described that increased numbers of CD8+CD57+ T cells correlate with the incidence of GVHD (Yabe et al, 1990; Fuku et al, 1994). In contrast to these investigators, we found that the increase of CD8+CD57+ T cells is not correlated with the incidence of GVHD, but our results confirm and extend the observation that the increase of CD8+CD57+ T cells is strongly correlated with a positive CMV serology of both recipient and donor prior to BMT (Wursch et al, 1985; Izquierdo et al, 1990). Moreover, occurrence of a CMV infection after BMT, defined by the presence of CMV antigen in blood and/or CMV in cultures of urine samples, was significantly higher in patients with increased numbers of CD8+CD57+ T cells. However, none of the BMT recipients in this study developed CMV disease although no prophylactic or therapeutic treatment was given with ganciclovir or foscarnet. CMV infection has also been associated with an increased risk of GVHD (Ringdén 1992), but the incidence of GVHD in this study was low due to lymphocyte depletion of the graft by counterflow centrifugation (De Witte et al, 1984; Schattenberg et al, 1990).

We observed a lower relapse rate in leukemia patients transplanted in CR1 or CP1 with a relatively high number of CD8+ T cells 3 months after BMT than in patients with a low number of CD8+ T cells (19% v 64%; P = 0.03). This observation suggests that an increase of CD8+ T cells after BMT associated with CMV infection prevents leukemic relapse independent of GVHD. Ringdén et al (1988) and Lönnqvist et al (1986) showed that patients with a CMV infection after BMT had a reduced risk of recurrent leukemia. Moreover, transplantation of bone marrow from CMV seropositive donors resulted in lower incidence of leukemic relapse (Jacobsen et al, 1986). Therefore we
hypothesize that donor-derived CTL become activated during CMV infection which induces GVIL activity. Prophylactic treatment with ganciclovir may lead to a delay in recovery of CMV-specific immunoresponsiveness because of the absence of CMV infection (Li et al., 1994). This prophylactic treatment for CMV infection could increase the incidence of leukemic relapse.

Viral infections appear to stimulate the generation of alloantigen-specific CTL coincidental with the generation of virus-specific CTL (Nahill & Welsh, 1993; Yang et al., 1989; Tomkinson et al., 1989). Nahill & Welsh (1993) reported that a high frequency of these CTL were cross-reactive for allogeneic and virus-infected syngeneic target cells. Moreover, CTL clones have been isolated specific for a HLA-B8 restricted Epstein-Barr virus epitope and cross-reactive with the alloantigen HLA-B44.02 (Burrows et al., 1994).

In conclusion, in this report we demonstrate that high repopulation of CD8+ T cells after lymphocyte-depleted allogeneic BMT is related with a lower relapse rate. Relation between CMV infection and high numbers of CD8+ T cells in BMT recipients suggests that CMV can be the signal for expansion of these cells. To elucidate the trigger for expansion of CD8+ T cells after BMT and the role of CD57, CD11b and HLA-DR antigens on these cells further studies are needed. Investigations whether donor-derived CTL show cross-reactivity against the recipient’s leukemic cells upon stimulation during CMV infection and determination of the specificity of these cells are in progress.

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