Clonal predominance of cytomegalovirus-specific CD8+ cytotoxic T lymphocytes in bone marrow recipients

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Summary:

After lymphocyte-depleted BMT, CD8+ T cells have been expanded to or above normal levels in 45% of the recipients within 3 months. The mechanisms underlying proliferation of donor-derived CD8+ T cells after BMT are still unclear. We investigated whether these CD8+ T cells proliferate in response to specific antigens by determination of TCR clonality and whether these cells exert specific cytotoxicity. PCR analysis of TCR-γ gene rearrangements showed a marked clonal predominance in CD8+ T cells of recipients with a high number of these cells. Strong association between expansion of CD8+ T cells and CMV infection suggests involvement of CMV antigens. Therefore, we examined CMV-specific cytotoxicity of freshly isolated CD8+ T cells of two BMT recipients with clonal expansion after the onset of CMV infection. CD8+ T cells exerted HL-A-restricted cytotoxicity directed against CMV-infected fibroblasts indicating that CMV stimulates proliferation. The majority of CD8+ T cells in these recipients expressed CD57. We demonstrated that TCR clonality was irrespective of CD57 expression. Both CD8+CD57- and CD8+CD57+ T cells showed significant HLA-restricted CMV-specific cytotoxicity. These studies strongly suggest that CMV antigens can induce expansion of clonal CD8+ T cells after BMT.

Keywords: BMT; CD8+ T cell expansion; CMV; TCR clonality; cytotoxicity

Allogeneic BMT is an effective treatment for patients with leukemia. The restoration of both hematopoiesis and immune function is essential for the long-term success of this treatment. Donor-derived T cells have to repopulate the recipient to restore T-cell immunity. T-cell numbers are variable in the peripheral blood of BMT recipients and T cell phenotypes and functions remain impaired for a prolonged period of time.1

Previously, we found that CD8+ T cells were expanded to or above normal levels in 45% of recipients of lymphocyte-depleted bone marrow allografts within 3 months.2 The signals responsible for proliferation of these CD8+ T cells are still unclear. GVHD and virus infections may be involved in an antigen-driven expansion of CD8+ T cells. The presence of several predominant TCR transcripts after BMT supports this hypothesis.3,4 However, so far no specific antigens inducing expansion of CD8+ T cells and TCR clonality after BMT have been identified.

Expansion of CD8+ T cells after BMT was accompanied by CD57 expression.5 CD8+CD57+ T cells appear to mediate non-MHC-restricted cytotoxicity after activation with IL-2 or CD3 antibodies.6 In addition, cytotoxicity of allospecific CTL or natural killer cells as well as mitogen-induced B and T cell proliferation can be inhibited by CD8+CD57+ T cells.6,7 However, in vitro stimulation of CD8+CD57- T cells in secondary mixed lymphocyte reaction or with IL-2 induces expression of CD57.8-10 Therefore, it is not clear whether CD8+CD57+ T cells are a distinct lymphocyte subpopulation with unique functional properties or are derived from CD8+CD57 T cells by differentiation or activation.

The increase of CD8+ T cells in BMT recipients seems to be the result of stimulation by viral antigens and minor histocompatibility antigens.2,8-10 We, and others, have described that CD8+ T cell expansion after BMT is significantly correlated with CMV infection but independent of GVHD.2,6,10,11 This suggests that CMV antigens are involved in proliferation of these CD8+ T cells. Therefore, we studied CMV-specific cytotoxicity and clonal expansion of CD8+ T cells in BMT recipients. Our data strongly suggest that proliferation of CD8+ T cells by CMV antigens after BMT results in a clonal expansion of donor-derived CD8+ T cells, of which the majority express CD57.

Materials and methods

Patient description and cell isolation

For this study eight BMT recipients with an expansion of CD8+ T cells and three control BMT recipients with low numbers of CD8+ T cells were selected.3 All patients received allogeneic bone marrow, lymphocyte-depleted by counterflow centrifugation, from an HLA-identical, MLC negative sibling.2 All patients received orally acyclovir (4 x 400 mg/day) from days -9 to 60 as prophylaxis of herpes virus infection, CMV infection prophylaxis with ganciclovir or hyperimmune globulin was not given. GVHD status, CMV status and T cell phenotype data of the BMT recipients studied are given in Table 1. Peripheral blood and bone marrow samples were collected from
Table 1  Patient characteristics  

<table>
<thead>
<tr>
<th>Patient</th>
<th>UPN</th>
<th>GVHD</th>
<th>CMV status</th>
<th>CD4⁺ PBL (×10⁶/μl)</th>
<th>CD8⁺ PBL (×10⁶/μl)</th>
<th>CD8⁺CD57⁺ PBL (%)</th>
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<tr>
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<td><strong>Chronic</strong></td>
<td><strong>Serology D/R</strong></td>
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UPN = unique patient number; D = donor; R = recipient; PBL = peripheral blood lymphocytes.

*Acute and chronic GVHD: acute GVHD, stages 0 to IV; chronic GVHD, no (0), limited (L) or extensive (E).

**CMV status; CMV serology prior to BMT of donor and recipient; CMV infection defined by the presence of CMV antigen in blood and/or CMV in cultures of urine samples.

Absolute number of CD4⁺ and CD8⁺ PBL at time TCR clonality analysis was performed between 3 and 12 months after BMT. Normal range of analysis of peripheral blood of 20 normal individuals: CD4⁺ 0.57–0.97 × 10⁶/μl and CD8⁺ 0.28–0.56 × 10⁶/μl.

Donors and peripheral blood samples from BMT recipients between 3 and 12 months after BMT. PBMC and BMMC were isolated by using Ficoll-Paque (Pharmacia, Uppsala, Sweden) and washed twice in PBS before use. PBMC of all patients studied were of donor origin.

**CMV monitoring**

IgG, IgA and IgM antibodies to CMV were tested prior to BMT in the serum of both donor and recipient using an ELISA. An antibody titer of >10 was considered positive. The presence of CMV in urine samples was determined using a standard method. Briefly, the fibroblast cell line HEL was grown to confluence in flat-bottom tubes containing a coverslip. Tubes with cells were inoculated with urine specimens 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 the CMV antigen in blood was determined using a standard method. Briefly, granulocytes were isolated from citrate-anticoagulated blood by dextran. Cytosin preparations (1.5 × 10⁵ cells/cytospin) were fixed with acetone for 10 min at room temperature 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.

**Antibodies and immunofluorescence**

The following MoAb were used for phenotypic analysis: FITC- or PE-conjugated UCHT1 (CD3), MT310 (CD4), DK25 (CD8; Dakopatts, Glostrup, Denmark) and Leu7 (CD57; Becton Dickinson, Mountain View, CA, USA). Cells were incubated with the appropriate concentration of MoAb in PBS supplemented with 20% pooled human serum and 0.1% NaN₃ (4°C, 30 min). Cells were washed in PBS supplemented with 1% BSA and analyzed on an Epics XL flow cytometer (Coulter Electronics, Hialeah, FL, USA).

**Isolation of lymphocyte subpopulations**

CD4⁺ and CD8⁺ T cells were obtained by positive selection using immunomagnetic beads. Briefly, PBMC were washed twice and resuspended in PBS with 2% FBS (Integro, Zaandam, The Netherlands) at a concentration of 10 to 20 × 10⁶ cells/ml. Cells were incubated (4°C, 45 min) with either anti-CD4 or anti-CD8 immunomagnetic beads (Dynal, Oslo, Norway) on a rotating shaker. Cells bound to beads were isolated using a magnet. Beads were detached by Detachabead antibody solution (Dynal) on a rotating shaker (RT, 1 h). Cells were collected, washed and tested for purity by immunofluorescence analysis. Purity was more than 98%.

CD3⁺, CD8⁺CD57⁺ and CD8⁺CD57⁻ T cells were isolated by cell sorting with an Epics Elite flow cytometer (Coulter). Briefly, PBMC or isolated CD8⁺ T cells were incubated with the appropriate concentration of MoAb (4°C, 30 min). Cells were washed once with PBS and sorted cells were collected in IMDM (Gibco, Paisley, UK) containing 10% FBS. An aliquot of sorted cells was reanalyzed and purity was more than 95%.

**EBV transformation and CMV infection**

EBV-lymphoblastoid cell lines (EBV-LCL) were generated from PBMC by transformation with EBV of the EBV-shedding B95–8 cell line and 0.1 μg/ml cyclosporin A.
EBV-LCL were cultured in IMDM containing 10% FBS. Fibroblasts were isolated from bone marrow obtained from marrow donors as follows. BMMC were suspended at 2 x 10⁶/ml in IMDM with 20% FBS and were maintained in IMDM containing 10% FBS. Fibroblasts between passages three and 10 were used for CMV infection. CMV strain AD169 was grown in fibroblast cell line HEL infected at a multiplicity of infection (MOI) of 5. Infectious supernatant was harvested when 100% of the fibroblasts showed a cytopathic effect in order to prepare an AD169 stock with a titer of 3 x 10⁹/ml. AD169 stocks were stored at -70°C. Fibroblasts were infected with CMV strain AD169 at a MOI of 5 in 5 ml IMDM containing 10% FBS for 18 h.

Cytotoxicity assay

Cytotoxicity of isolated lymphocyte subpopulations was assayed by ⁵¹Cr-release of labeled target cells. EBV-LCL were labeled with 100 μCi ⁵¹Cr (Amersham, Bucks, UK) for 1.5–2 h. Fibroblast targets were preincubated before labeling with 100 U/ml IFN-γ (Boehringer Ingelheim, Zaandam, The Netherlands) for 2 days to increase the expression of MHC molecules which leads to an increased susceptibility of the fibroblasts for TCR-mediated cytotoxicity. Fibroblasts were infected and labeled simultaneously with 150 μCi ⁵¹Cr for 18 h. Labeled target cells were mixed in V-bottom microtiter plates (10⁵/well) with various numbers of effector cells in 150 μl IMDM with 10% FBS. Plates were centrifuged (50 g, 5 min) and incubated at 37°C for 4 h. After incubation 100 μl supernatant of each sample was collected and radioactivity was determined by a gamma counter. Spontaneous ⁵¹Cr release was determined by incubating target cells in medium alone and maximum ⁵¹Cr release by target cells incubated in 10% Triton X-100. The percentage specific lysis was calculated as: 100 x (experimental release - spontaneous release)/maximum release - spontaneous release). Spontaneous release in the absence of CTL was <25% of maximum release by detergent in all experiments.

TCR-γ PCR analysis

TCR clonality of isolated lymphocyte subpopulations was assayed by TCR-γ PCR analysis. Isolated cells were washed twice in PBS and 0.6 x 10⁶ cells were resuspended in 100 μl 1 x PCR buffer (50 mM KCl, 20 mM Tris (pH 8.4), 1.5 mM MgCl₂, 0.01% BSA) supplemented with 0.45% v/v Nonidet P40 and 0.45% v/v Tween. Cell lysate was incubated with proteinase K (0.1 mg/ml) at 55°C for 1 h. Finally, cell lysate was heated at 95°C for 10 min to denature proteinase K and cell lysate was stored at -80°C. The oligonucleotides C20/10 and T20/10 were used to prime the amplification of an approximately 400-bp region of rearranged TCR-γ locus involving Vγ1 subgroup genes and Jγ1/Jγ2 genes. Rearranged TCR-γ DNA of 10 μl cell lysate was amplified by PCR in 100 μl 1 x PCR buffer supplemented with 100 pmol each primer, 0.06 mM dNTPs, 2.5 U Taq polymerase (Life Technologies) and ³²P-dCTP (Amersham), for 30 cycles (94°C for 1 min, 55°C for 2 min, and 72°C for 3 min). Finally, samples were incubated at 72°C for 10 min and cooled to 4°C. TCR-γ clonality was determined by single-stranded conformational polymorphisms (SSCP). The SSCP technique is based on the conformation and electrophoretic mobility of single-stranded DNA in a non-denaturating polyacrylamide gel. Rearranged DNA products were diluted 1:4 with SSCP loading buffer (96% formamide, 20 mM EDTA, 20 mM NaOH, 0.05% xylene cyanol, 0.05% bromophenol blue), heated at 85°C for 3 min and cooled on ice for 10 min. A 3 μl sample was loaded onto a SSCP gel (5% polyacrylamide: acrylamide:N,N'-bis acrylamide = 49:1). 0.5 x TBE buffer, with or without 5% v/v glycerol). Single strand products were electrophoresed at 2 W for 6000 Volt hour in 0.5 x TBE buffer. Gels were autoradiographed by exposing to Kodak XAR films.

Results

TCR clonality in BMT recipients

Previously, we found that in 45% of recipients of lymphocyte-depleted bone marrow allografts CD8⁺ T cells expanded to or above normal levels within 3 months after BMT. In these recipients the mean number of CD8⁺ T cells at 3 months after BMT was significantly higher than in normal individuals (1.06 ± 0.92 x 10⁹/l, respectively; P < 0.001). The incidence of CMV infection after BMT was significantly higher in recipients with high numbers of CD8⁺ T cells (12/17) than in recipients with low numbers (2/21, P < 0.001). The median time of onset of CMV infection was 9 weeks (range 5–12 weeks). To determine whether the marked expansion of CD8⁺ T cells in these BMT recipients is either the result of a fast polyclonal repopulation or clonal proliferation, we determined TCR clonality of purified T cells and T cell subsets. Eight BMT recipients with high numbers of CD8⁺ T cells were compared with three BMT recipients with low numbers of CD8⁺ T cells (Table 1). Six out of the eight recipients with high numbers of CD8⁺ T cells had an active CMV infection after BMT (Table 1). Rearranged TCR Vγ-Jγ segments analyzed by the sensitive PCR-SSCP technique allows detection of in vivo clonal T cell expansions. Analysis of T cells isolated from BMT recipients with an expansion of CD8⁺ T cells showed predominant bands and relatively low polyclonal background in the TCR-γ products (Figure 1a). BMT recipients with low numbers of CD8⁺ T cells showed less predominant bands and more prominent polyclonal background in the TCR-γ products (Figure 1b). TCR-γ products of T cells of normal individuals are visualized by this PCR-SSCP analysis as a smear in contrast to clonal TCR-γ products of the T cell line Jurkat (Figure 1c). These data demonstrate that a number of distinct predominant T cell clones were present in BMT recipients with an expansion of CD8⁺ T cells. Predominance of rearranged TCR Vγ-Jγ segments was also found in CD4⁺ T cells of all recipients with predominant clonal CD8⁺ T cells (data not shown). However, less polyclonal background was found in CD8⁺ T cells.

We studied repopulation of clonal CD8⁺ T cells of BMT recipients before and after the onset of CMV infection. CD8⁺ T cells increased after the onset of CMV infection
T lymphocyte (BMT) clonality of CMV-specific CTL after BMT

H Dolstra et al

Figure 1  PCR-SSCP analysis of TCR-γ gene rearrangement in BMT recipients and normal individuals of sorted CD3⁺ T cells. (a) Five recipients with expansion of CD8⁺ T cells after BMT. (b) Three recipients with low numbers of CD8⁺ T cells after BMT. (c) Controls, normal individuals with a polyclonal pattern and T cell line Jurkat with a clonal pattern.

(Figure 2a). Simultaneously, predominant bands were detected in the TCR-γ PCR product of these CD8⁺ T cells (Figure 2b). High numbers of CD8⁺ T cells and TCR clonality persisted at least 1 year after BMT.

TCR clonality of CD8⁺ T cells in BMT recipients with high numbers of these cells suggests an antigen-induced proliferation.

CMV-specific cytotoxicity mediated by purified CD8⁺ T cells

Since the occurrence of CMV infection is more frequent in BMT recipients with a clonal expansion of CD8⁺ T cells (Table 1), one may speculate that some of the predominant clones represent CTL directed against MHC-class I-restricted CMV antigens. Freshly isolated CD8⁺ T cells of recipient UPN 247 and UPN 265 after the onset of CMV infection, both consisting of a high percentages of clonal T cells, lysed CMV-infected fibroblasts of the HLA-identical donor (DV and BT, respectively; Figure 3). In contrast, CMV-infected fibroblasts of HLA-mismatched donors (VG and HB, respectively) were not killed. Moreover, uninfected fibroblasts of both HLA-matched and -mismatched donors could not be lysed by these CTL (Figure 3).

CD8⁺ T cells isolated from two BMT recipients, with a clonal expansion coincidental with CMV infection, mediated HLA-restricted CMV-specific cytotoxicity without previous in vitro stimulation with CMV antigens. These results suggest that some of the clonally expanded CD8⁺ T cells specifically recognize and proliferate on CMV antigens.

Role of CD57 expression on CD8⁺ T cells in TCR clonality and CMV-specific cytotoxicity

The majority of CD8⁺ T cells in recipients with high numbers of these cells express CD57 while only a low percentage of the CD8⁺ T cells in recipients with low numbers coexpress CD57 (Table 1). It has been described that expression of CD57 is strongly correlated with T cell expansion and CMV infection after BMT. Therefore, we investigated whether expression of CD57 influence TCR clonality and CMV-specific cytotoxicity of CD8⁺ T cells. CD8⁺CD57⁺ and CD8⁺CD57⁻ T cell subsets showed the same predominant bands (Figure 4). SSCP analysis carried out in a gel without glycerol, to enhance reliability of the occurrence of the same TCR in both cell subsets, showed identical results (data not shown).

We investigated whether CD8⁺ T cells defined by CD57 expression differ in CMV-specific cytotoxicity. CD8⁺ T cells of recipient UPN 265 with significant CMV-specific cytotoxicity were sorted based on CD57 expression. CD8⁺ T cells, either CD57⁺ or CD57⁻, lysed HLA-identical CMV-infected fibroblasts, whereas uninfected and HLA-mismatched cells were not killed (Figure 5).

These results show that clonal CD8⁺ T cells can consist of both CD57⁺ and CD57⁻ cells. Moreover, CTL function regarding CMV-specific cytotoxicity is not affected by CD57 expression.

Discussion

In the present study we show that clonal predominance exists within CD8⁺ T cells of BMT recipients with an
TCR clonality of CMV-specific CTL after BMT

H Dolstra et al

Figure 2 Follow-up of the representative recipient UPN 257 after BMT. (a) Repopulation of CD8+ T cells in peripheral blood. Onset of CMV infection is 2 months after BMT. (b) PCR-SSCP analysis of TCR-γ gene rearrangement of CD8+ T cells.

Figure 3 CMV-specific cytotoxicity of freshly isolated CD8+ T cells of two BMT recipients 3 months after BMT. (a) CD8+ T cells from recipient UPN 247 were tested against uninfected and CMV-infected fibroblasts from the HLA-identical donor DV (DV F, -o-, and DV F-CMV, -•-, respectively), uninfected and CMV-infected fibroblasts from the HLA-mismatched donor VG (VG F, -o-, and VG F-CMV, -•-, respectively), and EBV-LCL from the HLA-identical donor DV (DV-EBV, -Δ-). (b) CD8+ T cells from recipient UPN 265 were tested against uninfected and CMV-infected fibroblasts from the HLA-identical donor BT (BT F, -o-, and BT F-CMV, -•-, respectively), uninfected and CMV-infected fibroblasts from the HLA-mismatched donor HB (HB F, -o-, and HB F-CMV, -•-, respectively), and EBV-LCL from the HLA-identical donor BT (BT-EBV, -Δ-).

expanded number of this T cell population. These CD8+ T cells appear to be donor-derived. Proliferation of these CD8+ T cells, present in approximately half of the recipients, could be the result of complex mechanisms. Expansion may be due to a better developing T cell system after BMT depending on the quality of the graft. CD8+ T cell expansion upon antigen stimulation by GVHD and virus infections might also be involved.

Clonal expansions within the CD8+ T cell population have been observed in BMT recipients.3-6 Patients with autoimmune diseases,20,21 patients with chronic B cell lymphocytic leukemia (B-CLL),22 but also in normal adults.23,24 Farace et al22 found a unique clonally expanded CD8+V/319+ T cell clone in a patient with a B-CLL that recognized specifically autologous tumor cells in vitro. These observations support the hypothesis that clonally expanded CD8+ T cells represent antigen-specific effector cells directed against antigens to which the immune system is exposed. In addition, Hingorani et al23 showed that exposure to antigen induces such a clonal response. After a booster with hepatitis B vaccine they could detect a predominant CD8+V/34+ T cell clone.

After BMT the composition of the T cell repertoire is correlated with GVHD and infection status.5 Dietrich et al24 have identified several recurrent TCR transcripts in
This suggests the presence of an adequate CMV-specific CTL response.

Expansion of donor-derived CD8+ T cells after BMT was accompanied by expression of the lineage-nonspecific glycoprotein CD57. Whether CD57 expression divides CD8+ T cells into distinct subsets with unique functional properties or reflect a differentiation or activation stage of these cells is not elucidated. In this study we show that CD8+CD57+ and CD8+CD57- T cells have the same dominant TCR-γ gene rearrangement. Our results confirm and extend the observation of Morley et al. They reported that in allogeneic BMT recipients CD8+CD57+ T cells, but not the CD57- cells, exhibited an oligoclonal pattern of TCR-γ gene rearrangement. In contrast to our data, they compared CD8+CD57+ T cells with all CD57- cells consisting of both CD8+ and CD4+ T cells. Depletion of CD4+ T cells results in less TCR variety in both subsets and therefore comparison of TCR clonality analysis between CD8+CD57+ and CD8+CD57- T cells is more conclusive. In addition, we show that within the CD8+ T cells both CD57+ and CD57- T cells exert HLA-restricted CMV-specific cytotoxicity. This indicates that CD8+CD57+ T cells besides their reported inhibitory effects and non-MHC-restricted cytotoxicity, also exert MHC-restricted cytotoxicity. From these results we conclude that TCR clonality and CMV-specific cytotoxicity is independent of CD57 expression.

In a previous study we described that the expansion of CD8+ T cells after BMT was associated with both a lower incidence of leukemic relapse and occurrence of CMV infection. The data presented in this manuscript confirmed that CMV-specific CTL response indeed exist in some BMT recipients. This suggests that a CMV-specific CTL response may prevent leukemic relapse. One hypothesis is that donor-derived CMV-specific CTL cross-react with antigens presented on leukemic cells. Viral infections appear to stimulate the generation of alloantigen-specific CTL coincident with the generation of virus-specific CTL. Burrows et al. isolated CTL clones with specificity for an HLA-B8 restricted EBV epitope and cross-reacting with the alloantigen HLA-B44,02. Cao et al. found CTL cross-reactivity between a H-2Kd restricted influenza epitope and an immunoglobulin Vh epitope. Furthermore, increased production of cytokines during a CMV-antigen stimulated immune response might induce proliferation of anti-leukemic CTL and enhance the susceptibility of leukemic cells to anti-leukemic CTL activity. In vitro TNF-α can enhance susceptibility of leukemic cells to specific CTL activity (unpublished observation).

In conclusion, we demonstrate that CMV antigens stimulate clonal proliferation of CD8+ CMV-specific CTL. The majority of these cells express CD57.

Acknowledgements

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with chronic graft-versus-host disease after allogeneic bone marrow transplantation. *Bone Marrow Transplant* 1990; 5: 295-300.