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 donality 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 HLA-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 donality 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 donality; 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 donality 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.$^7,9$ 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.$^{10,11}$ However, in vitro stimulation of CD8$^+$CD57$^-$ T cells in secondary mixed lymphocyte reaction or with IL-2 induces expression of CD57.$^{12}$ 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.$^{2,11,13}$ We, and others, have described that CD8$^+$ T cell expansion after BMT is significantly correlated with CMV infection but independent of GVHD.$^{2,10,11}$ 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.$^5$ 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
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. Cytospin preparations (1.5 x 10⁵ cells/cytospin) 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 immunoalkaline 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, Zaanstad, The Netherlands) at a concentration of 10 to 20 x 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 XL 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 10^5/ml in IMDM with 20% FBS and were maintained in IMDM containing 10% FBS. Fibroblasts between passages three and ten 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 10^8/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 cytoxicity. Fibroblasts were infected and labeled simultaneously with 150 μCi ^51Cr for 18 h. Labeled target cells were mixed in V-bottom microtiter plates (10^5/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 10^6 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 lystate was heated at 95°C for 10 min to denature proteinase K and cell lystate 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.15 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.16,17 The PCR products were diluted 1:4 with SSCP loading buffer (96% formamide, 20 mM EDTA, 20 mM NaOH, 0.05% xylene cyanol, 0.05% bromphenolblue), 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 10^9/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 CD8+ 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.
TCR 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
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+Vβ19+ 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+Vβ34+ T cell clone.

After BMT the composition of the T cell repertoire is correlated with GVHD and infection status. Dietrich et al3,4 have identified several recurrent TCR transcripts in
Furthermore, CD8+CD57+ T cells play an important role in the immunological control of CMV and provide protection against the development of CMV disease. None of the BMT recipients in this study with a CMV infection coincidental with clonally expanded CD8+ T cells exhibited an oligoclonal pattern of TCR-γ gene rearrangement. In contrast to our data, they compared CD8+CD57+ T cells with CD8+CD57- 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 are 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 coincidental 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. 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-nonrestricted glycoprotein CD57. Whether CD57 expression divides CD8+ T cells in 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 24 who showed that the same T cell clone is present in both CD8+CD57+ and CD8+CD57- T cells, but differ from a study of Gorochov 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 are independent of CD57 expression.

skin and blood at the time of acute GVHD suggesting that minor histocompatibility antigens induce clonal expansions. Our observation that occurrence of CMV infection is more frequent in BMT recipients with clonally expanded CD8+ T cells suggests that CMV antigens might also be a stimulatory signal. Additionally, this study shows that CD8+ T cells from BMT recipients with an expansion of these cells exert HLA-restricted CMV-specific cytotoxicity. CMV-specific CTL which are stimulated during CMV infection after BMT play an important role in the immunological control of CMV and provide protection against the development of CMV disease. None of the BMT recipients in this study with a CMV infection coincidental with clonally expanded CD8+ T cells developed clinical CMV disease.

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