

## Anti-host cytotoxic T cells of bone marrow transplant recipients with or without graft-versus-host disease are equally sensitive to cyclosporin A

LM Liem, JT van Noort and E Goulmy

Department of Immunohematology and Blood Bank Leiden University Hospital The Netherlands

### Summary:

It is generally accepted that cytotoxic T cells (CTLs) play an important role in the pathogenesis of graft-versus-host disease (GVHD). Nonetheless, anti-host CTLs can be observed in the peripheral blood of patients both with and without clinical signs of GVHD following HLA genotypically identical bone marrow transplantation. Thus we questioned whether a qualitative difference, such as a differential *in vitro* sensitivity to cyclosporin A (CsA), may exist between the anti-host CTLs, generated from these groups of patients post-bone marrow transplantation (BMT). We analyzed anti-host CTL precursors (CTLp) of patients without clinical signs of GVHD, of patients suffering from acute GVHD (grade II-IV) and of patients suffering from acute GVHD followed by chronic GVHD for their *in vitro* sensitivity to the inhibitory effects of CsA. The results obtained in a total of 20 patients revealed anti-host CTLp frequencies (CTLpf) post-BMT in all three groups of patients and addition of CsA in the tests resulted in approximately 60% inhibition of the CTLpf independent of the GVHD status of the patients. Thus, in view of the *in vitro* CsA sensitivity, no difference exists between the anti-host CTLs in patients with or without GVHD.

**Keywords:** CTL, CsA, LDA, GVHD

significant Th cell activity *in vitro* correlated with clinical acute GVHD. Studies on the phenotype and function of T lymphocytes infiltrating the skin during GVHD following allogeneic HLA-identical BMT revealed CD8 cells,<sup>9</sup> but predominantly CD4 cells.<sup>10,11</sup> Most recent observations support the notion that mHag specific, IL-2 producing Th cells are likely to play a role in the pathogenesis of acute GVHD.<sup>12,13</sup>

Several reports demonstrated the presence of anti host mHag-specific cytotoxic T cells (CTLs) in patients suffering from GVHD after HLA genotypically identical BMT.<sup>14-19</sup> However, the role of anti-host CTLs in the development of GVHD is still controversial. In our earlier studies, we demonstrated the presence of anti host mHag-specific CTLs in the blood of patients undergoing GVHD. Although patients with chronic GVHD tended to develop higher and more persistent levels of anti-host CTL activity than those without GVHD, this finding was not statistically significant.<sup>16</sup> Subsequent analyses at the quantitative level, ie determination of the anti-host specific CTL precursor frequency (CTLpf) revealed high frequencies of mHag specific CTLs early after BMT irrespective of the GVHD status of the patient.<sup>20</sup> *In vitro* data in support of a role of CTLs in GVHD in man were provided by Kaminski *et al*.<sup>21</sup> The frequency of recipient-reactive CTLp present in donor PBL before BMT was found to predict the severity of GVHD after BMT. However, the donor-recipient pairs in the latter study were unrelated HLA-matched, MLC-nonreactive individuals.

These results urged us to re-analyze the role of the anti host CTLs in the development of GVHD. Earlier studies in kidney grafting showed that the *in vitro* resistance of recipients' lymphocytes for the immunosuppressive drug cyclosporin A (CsA) was correlated with a higher rate of graft loss.<sup>22</sup> We therefore set out to investigate whether we could find differences in the *in vitro* CsA sensitivity of anti host CTLs in patients with and without GVHD. Here we present the results obtained in 20 recipients analyzed at the anti-host CTLp level at different times after HLA genotypically identical BMT.

The results of clinical bone marrow transplantation (BMT) show that the selection of MHC identical sibling bone marrow donors does not guarantee avoidance of graft-versus-host disease (GVHD), graft failure or disease-free survival.<sup>1</sup> It is believed that disparities of minor histocompatibility antigens (mHag) between donor and recipient constitute a potential risk factor for GVHD or graft failure.<sup>2-4</sup> Over the last few years, evidence has accumulated that mHag specific helper T cells (Th) could be relevant to the pathogenesis of GVHD. *In vitro* studies reporting on host directed Th cells have been described in patients with GVHD.<sup>5-7</sup> Van Els *et al*<sup>8</sup> reported on the long-term kinetics of Th cells in response to host mHag in 16 patients and demonstrated that

Correspondence: Dr LM Liem, Department of Immunohematology and Blood Bank, Leiden University Hospital, PO Box 9600, 2300 RC Leiden, The Netherlands.

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## Material and methods

### Patients

Twenty HLA genotypically identical donor–recipient pairs are included in this study. All except one patient (UPN 11) received non T cell-depleted bone marrow. All patients received cyclophosphamide (60 mg/kg/day  $\times$  2) and total body irradiation (8 Gy) as pretransplant conditioning regimen, except UPN 9 who received  $4 \times 50$  ng/kg cyclophosphamide,  $5 \times 1$  mg/kg busulphan and five treatments with Campath and UPN 11 who received  $4 \times 150$  mg cyclophosphamide,  $4 \times 75$  mg busulphan and five treatments with Campath. Methotrexate (MTX) and/or CsA (and in one case methylprednisone) was given as GVHD prophylaxis. The relevant clinical information is summarized in Table 1. Patients are divided into groups according to their GVHD status. The 'no GVHD' group consists of patients without GVHD or with acute GVHD grade I ( $n = 7$ ). The 'acute GVHD' group consists of patients with acute GVHD grade II–IV ( $n = 7$ ) and the 'chronic GVHD' group consists of patients who suffered from chronic GVHD which was preceded by acute GVHD grade I or more ( $n = 6$ ).

### Blood samples

Heparinized blood samples were taken from the recipients before and at regular intervals after BMT and from their bone marrow donors. Peripheral blood leukocytes (PBL) were isolated by Ficoll-amidotrizoate density gradient centrifugation and cryopreserved in liquid nitrogen. A total of 61 samples post-BMT was used for analysis.

### Cyclosporin A

CsA was a kind gift from Sandoz, Basel, Switzerland. It was dissolved at 1 mg/ml in ethanol. CsA was used in a final dilution of 50 ng/ml culture medium, corresponding to therapeutical levels, and was present during the whole culture period.

### Limiting dilution analysis

Responder cells (RC) were PBL from the donor and PBL from the recipient at several dates after BMT. Seven concentrations of RC (range: 40 000–625 cells per well) were set up in round-bottom microtiter plates in the presence of 5000 irradiated (5 Gy) stimulator cells (SC) in a total volume of 200  $\mu$ l RPMI 1640 medium supplemented with 10% pooled human serum and 20 U/ml rIL-2. Stimulator cells were Epstein–Barr virus transformed B cell lines (EBV-BLCL) of the recipient before BMT or of an unrelated individual. EBV-BLCL were used as SC because of the limited number of PBL of the recipients before BMT.<sup>20</sup> Target cells were PBL stimulated with 1% PHA for 3 days and restimulated with irradiated (3 Gy) feeder cells once every week for 1 or 2 weeks. In this way a T cell line of each target was made to ensure sufficient target cells. Of each dilution, 18 wells were set up in two series: one series with and one without CsA in the culture medium. All responder–stimulator combinations of each patient were set up simultaneously and tested on the same day in the same experiment. After 5 days, 100  $\mu$ l culture medium containing 20 U/ml rIL-2 was refreshed. At day 7, each well was tested for cytotoxicity against 5000 Europium-labelled<sup>23</sup>

**Table 1** Relevant clinical data of the patients included in this study

UPN	Sex (P/D)	Diagnosis	Conditioning	Prophylaxis	aGVHD (day) <sup>a</sup>	cGVHD	Survival (days) <sup>b</sup>
2	F/M	AML-M4	Cy/TBI	MTX	0	0	alive
3	M/M	AML-M1	Cy/TBI	MTX	0	0	alive
4	M/F	AML-M2	Cy/TBI	MTX	0	0	alive
5	M/F	AML-M3	Cy/TBI	MTX	0	0	alive
18	F/F	ALL	Cy/TBI	MTX	0	0	alive
16	F/M	AML-M4	Cy/TBI	CsA	I (+42)	0	alive
23	F/F	ALL	Cy/TBI	CsA + MTX	I (+8)	0	alive
6	F/M	AML-M4	Cy/TBI	CsA	II (+14)	0	+175
20	M/F	AA	Cy/TBI	MTX	II (+34)	0	+147
7	F/F	AML-M2	Cy/TBI	CsA	II–III (+12)	0	alive
8	F/M	AML-M3	Cy/TBI	MTX	III (+54)	0	alive
9	M/M	NHL/AML-M4	CBE	CsA	III (+17)	0	+107
17	M/M	AML-M1	Cy/TBI	CsA	III (+11)	0	+137
22	M/M	AML-M4	Cy/TBI	MTX	III (+47)	0	+158
10	M/M	AML-M2	Cy/TBI	MP	II (+27)	severe	alive
11	M/F	CML	CBC	CsA	II (+37)	severe	+243
12	F/M	CML	Cy/TBI	CsA	I–II (+11)	mild	alive
13	F/M	AML-M2	Cy/TBI	MTX	IV (+28)	severe	+397
14	F/M	AML-M4	Cy/TBI	MTX	II (+35)	mild	alive
15	F/F	AML-M4	Cy/TBI	CsA	I (+17)	severe	alive

UPN = unique patient number, P/D = sex of patient (P) and donor (D), SAA = severe aplastic anemia, ALL = acute lymphoblastic leukemia, AML = acute myeloid leukemia with FAB classification, CML = chronic myeloid leukemia (chronic phase); NHL = non-Hodgkin lymphoma Cy/TBI = cyclophosphamide and total body irradiation, CBC = cyclophosphamide, busulphan, Campath and T cell depletion, CBE = cyclophosphamide, busulphan and Campath, CsA = cyclosporin A, MTX = methotrexate, MP = methylprednisone.

<sup>a</sup>Day of onset of acute GVHD

<sup>b</sup>Survival follow-up of patients still alive is  $\geq 1$  year post-BMT

target cells in the cell-mediated lympholysis (CML) test EBV BLCL were never used as target cells to exclude the contribution of EBV antigen specific CTLp to the value measured<sup>20,24</sup> Wells were considered positive, when fluorescence exceeded the mean spontaneous release (18 wells containing no RC) plus 3 standard deviations (s.d.)<sup>23</sup>

### Statistical analysis

Frequencies of responding CTLp, their 95% confidence interval and the goodness of fit were calculated using the Jackknifed maximum likelihood method<sup>25</sup> The Mann-Whitney *U* test was used to compare the effect of CsA in the different patient groups

### Results

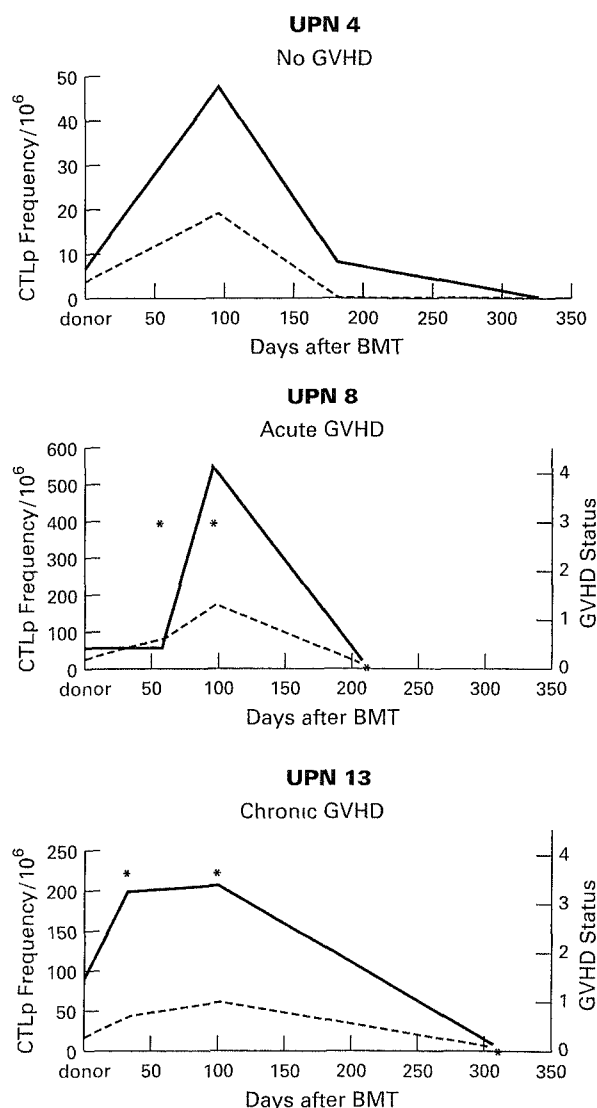
Twenty patients were divided into three groups according to their GVHD status PBLs were isolated before and at different dates after HLA genotypically identical BMT and used to determine host directed CTLpf of each patient in limiting dilution assays, with and without the addition of CsA to the test

Figure 1 demonstrates three representative experiments of the CTLpf with or without the addition of CsA measured in a patient without GVHD (UPN 4), in a patient with acute GVHD (UPN 8) and in a patient with acute GVHD followed by chronic GVHD (UPN 13) Independent of the occurrence of GVHD, the post-BMT anti-host CTLpf increase from day 30 post BMT onwards, reach maximum levels around day 100 and decrease gradually thereafter Figure 1 is also representative for our general finding that the CTLp are equally susceptible for the addition of CsA in the culture medium in the three groups of patients studied

Figure 2 demonstrates 61 anti host CTLpf measured, with and without CsA *in vitro*, in a total of 20 patients post BMT The mean CTLpf of the 'no GVHD' patients without addition of CsA in the medium is  $17.8/10^6$  PBL (range 0–62/ $10^6$  PBL) The addition of CsA leads to strong inhibition of most CTLpf (% inhibition = 56%, Table 2), although two out of seven patients still show a significant CTLpf in the presence of CsA ( $>25/10^6$  PBL) The mean CTLpf in the presence of CsA is  $7.8/10^6$  PBL (range 0–68/ $10^6$  PBL) Statistical analysis shows a significant inhibition of the CTLpf by CsA present in the medium ( $P = 0.0138$ , Table 2)

In the 'acute GVHD' group the mean CTLpf without CsA present is  $96/10^6$  PBL (range 0–992/ $10^6$  PBL) The CTLpf found in the presence of CsA are mostly very low or absent (mean CTLpf =  $31.4/10^6$  PBL, range 0–403/ $10^6$  PBL), but also here two out of seven patients still show a significant CTLpf Three out of seven 'acute GVHD' patients have a higher CTLpf ( $>100/10^6$  PBL) than 'no GVHD' patients at at least one time point post-BMT, whereas the others do not differ in their CTLpf Statistical analysis shows that CsA in the medium causes a significant inhibition of the CTLpf (% inhibition = 67%,  $P = 0.0187$ )

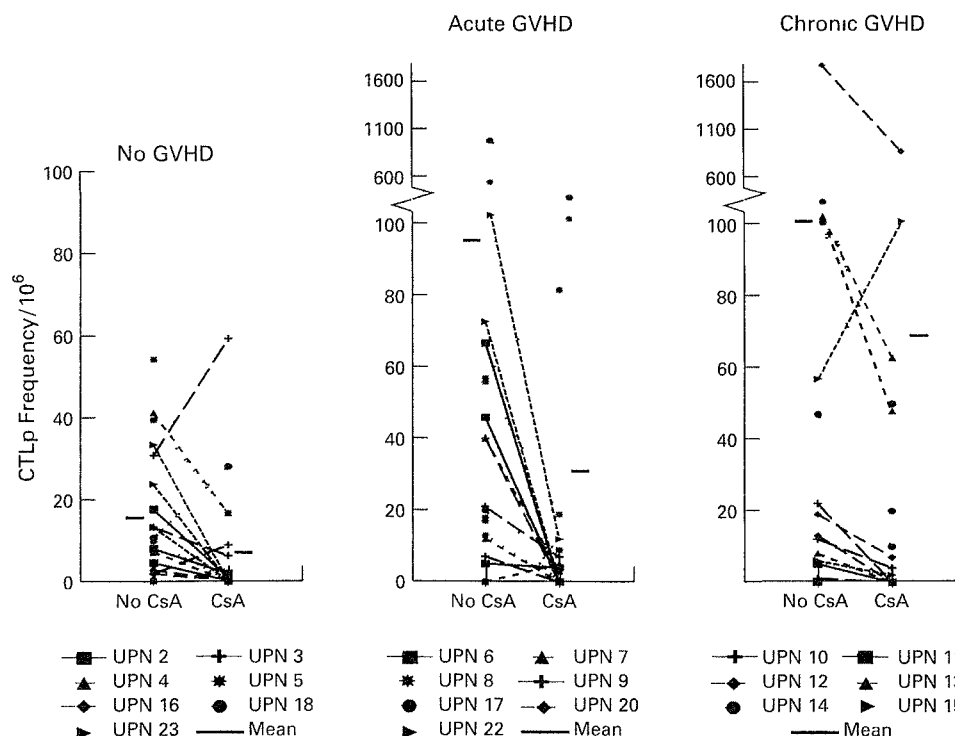
The mean CTLpf of chronic GVHD patients is the highest of the three groups (mean =  $161/10^6$  PBL, range 0–1801/ $10^6$  PBL) Three out of six 'chronic GVHD' patients



**Figure 1** Kinetics of CTLpf after BMT of a patient without GVHD (UPN 4) of a patient suffering from acute GVHD (UPN 8) and of a patient suffering from acute GVHD followed by chronic GVHD (UPN 13) GVHD status indicated with an asterisk is represented as grade I (1 = very mild) to IV (4 = severe) CTLpf without CsA are represented with a straight line CTLpf in the presence of CsA are represented with a dashed line

have higher CTLpf than 'no GVHD' patients at at least one time point post BMT A majority of chronic GVHD patients (four out of six) has also a relatively high CTLpf in the presence of CsA The mean CTLpf in the presence of CsA is  $69.5/10^6$  PBL (range 0–891/ $10^6$  PBL) The Mann-Whitney *U* test for comparison shows that CTLpf in the absence or presence of CsA are not significantly different (% inhibition = 57%,  $P = 0.1066$ ) Summarizing, our results suggest that CsA leads to a significant decrease in CTLpf in patients without GVHD and patients with acute GVHD, but not in patients with chronic GVHD A large variation is found in post-BMT CTLpf between patients with GVHD

The data are summarized in Table 2 The mean CTLpf observed in the three groups of patients without or with CsA added in the culture medium are indicated and is the



**Figure 2** Each line represents the CTLpf with and without CsA of one sample post BMT. All samples of one patient are represented with the same line and symbol.

**Table 2** CsA sensitivity of CTLpf in different patient groups

Patients	No. samples	CsA added	Mean CTLpf ( $/10^6$ )	s.d.	% Inhibition	Mann-Whitney
No GVHD $n = 7$	20	No	17.8	18.4	56	$P = 0.0138$
		Yes	7.9	16.7		
Acute GVHD $n = 7$	23	No	96.0	228.2	67	$P = 0.0187$
		Yes	31.4	89.8		
Chronic GVHD $n = 6$	18	No	161.0	421.2	57	$P = 0.1066$
		Yes	69.5	208.6		

Patients were divided into three groups according to GVHD status. Twenty post BMT samples of seven patients without GVHD or acute GVHD grade I, 23 samples of seven patients with acute GVHD II–IV and 18 samples of patients with chronic GVHD following acute GVHD grade I or more were tested. The percentage inhibition =  $(1 - (\text{mean CTLpf with CsA} / \text{mean CTLpf without CsA})) \times 100\%$ . s.d. = standard deviation.  $P$  values comparing CTLpf without CsA with CTLpf with CsA are calculated using the Mann–Whitney  $U$  test.

highest in the 'chronic GVHD' group. The effect of CsA presented as the percentage inhibition of the mean CTLpf is, although comparable, the strongest in the acute GVHD group.

## Discussion

More than a decade ago, it was established by *in vitro* experiments that CsA mediates suppression of primary CTL responses.<sup>26</sup> Studies by Hess *et al*<sup>27</sup> subsequently demonstrated the resistance to CsA of primed CTL. Similarly, on the CTLp level, the frequency of CTL grown out of primed mixed lymphocyte culture cells is not influenced by CsA. Yet CsA has a profound influence on the primary activation of alloreactive CTLp induced in MLC bulk cul-

tures.<sup>28,29</sup> Variability in CsA resistance seems to exist at the individual as well as at the clonal level.<sup>30</sup> The clinical significance of the differential *in vitro* CsA susceptibility is reflected in studies wherein the *in vitro* CsA resistance appeared to correlate with a high rate of kidney graft loss due to acute rejection.<sup>22</sup> Muluk *et al*<sup>31</sup> demonstrated that the CD4<sup>+</sup> Th cells reactive with alloantigen-derived peptides presented on self-antigen presenting cells (indirect pathway) are mainly responsible for kidney graft rejection, this population of cells appeared most sensitive to CsA as well.<sup>32</sup> Some patients awaiting kidney transplant form antibodies against HLA antigens due to previous transfusions, pregnancies or failed transplants. In these highly sensitized patients it is notable that CTL against HLA antigens toward which the patient has developed antibodies (not acceptable mismatches) were resistant to CsA *in vitro* whereas CTL

against HLA antigens toward which no antibodies were present (acceptable mismatches) were sensitive to CsA *in vitro*<sup>33</sup> Additional observations supporting the notion that a decreased *in vitro* CsA sensitivity of organ donor reactive CTLp is correlated with a higher risk of graft rejection was substantiated by studies in corneal grafting<sup>34</sup> and heart transplantation<sup>35</sup>

These results prompted us to investigate whether a decrease in CsA sensitivity of CTLp would correlate with an increased risk for GVHD after HLA genotypically identical BMT To test this assumption, we analyzed the *in vitro* CsA sensitivity of the anti host CTLpf in a 7-day limiting dilution assay Our results differ from those described for organ transplantation, but are in line with previous results where no differences in inhibitory effects of IL-2 production mediated by CsA were observed between patients with or without acute GVHD<sup>36</sup> We observed no clear difference between patients suffering from either acute or chronic GVHD and patients with no GVHD Neither was there a difference in CsA sensitivity of patients who used MTX or CsA as GVHD prophylaxis, nor did we notice an influence of disparities for the known minor histocompatibility antigens between bone marrow donor and recipient<sup>4</sup> CsA is able to inhibit the CTLpf *in vitro* in the three groups of patients, although an interindividual variability in the *in vitro* CsA sensitivity was noted, a phenomenon described before by others<sup>30,37</sup> The apparent tendency of enhanced resistance to CsA of the 'chronic GVHD' group (Mann-Whitney *U* test,  $P = 0.1066$ ) must therefore be interpreted with care in view of the interindividual CTLpf and CsA variability previously mentioned

Yet the increment of CsA-resistant CTL observed in the chronic GVHD patients may be indicative of a higher risk of development of chronic GVHD The limited patients' material did not allow us to evaluate whether higher concentrations of CsA would break this resistance The presence of this relatively resistant anti-host CTL population may also reflect the composition of a different CTL population as opposed to acute GVHD, related to the chronic form of GVHD

In chronic GVHD the continuous stimulation of GVHD related T cells might give rise to an increase in CD45RO<sup>+</sup> memory T cells Recent data suggests that CD45RO<sup>+</sup> T cells are less susceptible to the inhibitory effects of CsA than CD45RA<sup>+</sup> naive T cells<sup>38</sup> An increase in CsA resistant CD45RO<sup>+</sup> T cells will then decrease the overall CsA sensitivity of the total PBL pool

The aim of this study was to find a *raison d'être* for the anti-host CTL observed in patients without any clinical signs of GVHD Here to we investigated whether there may exist a differential susceptibility for CsA between anti host CTL observed in patients with or without GVHD Our data show however that in an HLA genotypically identical situation no correlation is found post-BMT between the *in vitro* sensitivity for CsA of anti-host CTLs and the occurrence of clinical GVHD

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