Impact of HLA A2 and cytomegalovirus serostatus on outcomes in patients with leukemia following matched-sibling myeloablative allogeneic hematopoietic cell transplantation

Veronique Erard
Katherine A. Guthrie
Stanley Riddell
Michael Boeckh

Background and Objectives. Donor cytomegalovirus seropositivity was reported to improve leukemia outcomes in HLA-A2 identical hematopoietic cell transplant (HCT) recipients, due to a possible cross-reactivity of donor HLA-A2-restricted CMV-specific T cells with minor histocompatibility (H) antigen of recipient cells. This study analyzed the role of donor CMV serostatus and HLA-A2 status on leukemia outcomes in a large population of HLA-identical HCT recipients.

Design and Methods. Leukemia patients transplanted between 1992 and 2003 at the Fred Hutchinson Cancer Research Center were categorized as standard risk [leukemia first remission, chronic myeloid leukemia in chronic phase (CML-CP)] and high risk (advanced disease) patients. Time-to-event analysis was used to evaluate the risk of relapse and death associated with HLA-A2 status and donor CMV serostatus.

Results. In standard risk patients, acute leukemia (p<0.001) and sex mismatch (female to male, p=0.004)) independently increased the risk of death, while acute leukemia increased the risk of relapse (p<0.001). In high risk patients acute leukemia (p=0.01), recipient age ≥40 (p=0.005) and herpes simplex virus (HSV) seropositivity (p<0.001) significantly increased the risk death; HSV seropositivity (p=0.006) increased the risk of relapse. Donor CMV serostatus had no significant effect on mortality or relapse in any HLA group.

Interpretation and Conclusion. This epidemiological study did not confirm the previously reported effect of donor CMV serostatus on the outcomes of leukemia in HLA-A2-identical HCT recipients. Addressing the question of cross-reactivity of HLA-A2-restricted CMV-specific T cells with minor H antigens in a clinical study would require knowledge of the patient's minor H antigen genotype. However, because of the unbalanced distribution of HLA-A2-restricted minor H antigens in the population and their incomplete identification, this question might be more appropriately evaluated in *in vitro* experiments than in a clinical study.

Key words: CMV–specific T cells, cross-reactivity, minor histocompatibility antigens, leukemia, relapse.

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From the Clinical Research Division Fred Hutchinson Cancer Research Center (VE, KAG, SR, MB); Department of Medicine, University of Washington, Seattle, WA, USA (MB).

Correspondence:
Michael Boeckh, MD, Fred Hutchinson Cancer Research Center, Program in Infectious Diseases, 1100 Fairview Avenue North D3-100, PO Box 19024 USA.
E-mail: mboeckh@fhcrc.org

The human leukocyte antigen (HLA) class I molecules (HLA-A, -B and -C) are ubiquitously expressed on the surface of most nucleated cells. HLA class I molecules present peptides derived from processed intracellular proteins and serve as ligands for the T-cell receptor (TCR) of CD8+ T cells, thereby enabling immune recognition of cells that may be infected with a pathogen or that have undergone malignant transformation.[1] $CD8^+$ T-cell responses to peptides derived from persistent viruses such as cytomegalovirus (CMV) are maintained at high frequency in immunocompetent individuals. In HLA-A2-positive individuals, a major component of the CMV-specific $CD8^+$ T-cell response is specific for CMV peptides presented by the HLA-A2 allele.[2] In the setting of allogeneic HLA-identical hematopoietic cell transplantation (HCT), donor T cells that recognize recipient minor histocompatibility (H) antigens play an important role in both graft vs leukemia (GVL) reactivity and graft-versus-host disease (GVHD).[3-6] Analysis of patients who have received donor-lymphocyte infusions as a treatment for relapsed disease has shown that expansion of HLA-A2-restricted T cells specific for HA-1 or HA-2, a subset of minor antigens selectively expressed by hematopoietic cells, correlates with an antileukemic effect.[7-10] A retrospective study by Nachbaur and colleagues analyzed the outcomes of 103 HLA-identical sibling HCT recipients in relation to the CMV serostatus of their donors, and observed that both overall and disease-free survival were significantly improved in HLA-A2-positive HCT recipients whose donors were CMV-seropositive.[11] These authors suggested that HLA-A2-restricted CMV-specific $CD8^+$ T cells might cross-react with leukemia-associated minor H antigens and contribute to a GVL effect. However, these results could not be confirmed by other investigators and concern about the validity of the statistical

analysis was raised in several letters to the editors.[12-14] Since these studies were relative small in size, a definitive study with appropriate sample size was suggested.[12] The purpose of this study was to analyze retrospectively the outcomes, in relation to donor CMV status, of a large cohort of leukemia patients who received a myeloablative HCT from HLA-identical siblings at our institution.

Design and Methods

The institutional review board of the Fred Hutchinson Cancer Research Center (FHCRC) approved this retrospective analysis. Informed consent to use information for future research was obtained from all patients according to the Declaration of Helsinki.

Study population characteristics

We included patients who received their first allogeneic HCT from an HLA-identical sibling donor for leukemia between July 1, 1992 and December 31, 2003 at the FHCRC. Patients were defined as *high-risk* and *standard risk* for post-transplant relapse on the basis of their disease status at the time of the HCT. This categorization was done in order to replicate the analysis of Nachbaur and colleagues.[11] Individuals with acute leukemia in first remission and chronic myeloid leukemia in its chronic phase (CML-CP) were defined as being at standard-risk, whereas patients with more advanced disease were considered at high-risk. Patients most commonly received conditioning regimen with cyclophosphamide (60 mg/kg/d for 2 consecutive days) plus total body irradiation (1200 cGy), or the combination of busulfan (4 mg/kg/d for 4 consecutive days) and cyclophosphamide (60 mg/kg/d for 2 consecutive days). All patients underwent an allogeneic T cell-replete transplant of bone marrow or granulocyte colony-stimulating factor in stimulated peripheral blood stem cells. The majority of patients received a cyclosporine-based GVHD prophylaxis regimen. Acute GVHD was diagnosed and graded according to previously published criteria.[15] Chronic GVHD was assessed in patients who survived at least 80 days after transplantation.[16] Standard care for the prevention of candidiasis and CMV disease included fluconazole until day 75 after transplant and ganciclovir based on monitoring for CMV pp65 antigenemia, respectively.[17-19] Until 1999, low-dose acyclovir prophylaxis was given until day 30 after transplantation for herpes simplex virus (HSV)-seropositive patients. Since 2000, low-dose acyclovir or valacyclovir prophylaxis was extended to at least 1 year after transplantation in varicella-zoster virus (VZV)-seropositive recipients.

Statistical analysis

Patients in the high and low risk groups were analyzed separately. Cox regression models were used to estimate the associations between several potential risk factors and the outcomes of interest, morphologic relapse and overall survival.[20] The candidate risk factors included age (<40 vs ≥40 years), disease group (acute lymphoid leukemia, acute myeloid leukemia, or chronic myeloid leukemia), cell source (peripheral blood stem cells vs bone marrow), recipient-donor sex match, conditioning regimen (busulphan/cyclophosphamide *vs* cyclophosphamide/total body irradiation *vs* other regimen), GVHD prophylaxis (cyclosporine A/methotrexate *vs* other), year of HCT, recipient HSV serostatus and concordant recipient/donor HLA-A2 status (HLA-A2: positive *vs* negative). After building the initial risk factor model, the predictive factors of interest, HLA-A2 status, recipient and donor CMV serostatus, were considered. The role of donor CMV-seropositivity on the HLA-A2 effect on the outcomes was evaluated by entering an interaction term of HLA-A2 by donor CMV-seropositivity into the model. Hazard ratios (HR) and corresponding 95% confidence intervals (CI) were estimated. *p*-values less than 0.05 were deemed statistically significant. Cumulative incidence curves were used to estimate the probabilities of relapse, acute GVHD and chronic GVHD. Death and second transplant were treated as competing risks for relapse; death and relapse were considered competing risks for acute and chronic GVHD. Relapse-free survival and overall survival were assessed using Kaplan-Meier estimates.[21] The log-rank test was used to assess the equality of survival estimates. A secondary analysis included only relapse-free survivors to day 100, to assess whether a possible delay in immunereconstitution effect changed the results of the analyses.

Results

Patients' characteristics

Seven hundred and sixty-eight HCT recipients were included in the study; 432 were categorized as at standard risk of relapse and 336 at high risk. Six hundred and twenty-nine patients survived without relapse to 100 days. The patients' characteristics and transplant modalities were retrieved from the FHCRC computerized database and are summarized in Table 1.

Standard risk patients

Risk of relapse. The probabilities of relapse among HLA-identical HCT recipients from CMV-seropositive and CMV-seronegative donors were not significantly different (p=0.70). When the analysis was restricted to the subset of HLA-A2-positive HCT recipients, the probability of relapse did not significantly differ accord-

Table 1. Patients' characteristics and transplant modalities divided according group of relapse-risk.

Characteristics	Risk of relapse: Standard		High	
Number of patients	432		336	
Median age of patients (y)	41	(1-61)	40	(1-67)
Median follow-up (m)	65	(0-156)	10	(0-149)
Diagnosis				
Acute lymphoblastic leukemia	38	9	79	24
Acute non-lymphoblastic leukemia	142	33	186	55
Chronic myeloid leukemia	252	58	71	21
Cell source				
Bone marrow	328	76	198	59
Peripheral blood cells	104	24	138	41
Donor/recipient(sex)				
Female/male	94	22	78	23
Male/female	100	23	51	15
Male/male	113	26	119	35
Female/male	125	29	88	26
CMV match (donor/recipient)				
-/-	140	32	90	27
-/+	74	17	70	21
+/+	150	35	134	40
+/-	67	16	42	13
Missing	1		0	
Conditioning regimen				
Busulphan/cyclophosphamide	304	70	62	18
Cyclophosphamide/TBI	78	18	226	67
Other	50	12	48	14
Various chemotherapy/TBI	87	20	257	76
GvHD prophylaxis				
Cyclosporine A/methotrexate	379	88	221	66
Cyclosporine A	13	3	32	10
Other	24	6	74	22
Missing	16	4	9	3
Transplant year				
1992-1995	146	34	127	38
1996-1999	172	40	144	43
2000-2003	114	26	65	19
HSV seropositivity	324	75	269	80
Missing	3		4	
HLA-A2 positive (donor/recipient)	196	46	143	43

Data represent number and percentage except for age and follow-up. CMV: cytomegalovirus; GvHD: graft-versus-host disease; HSV: herpes simplex virus 1 or 2.

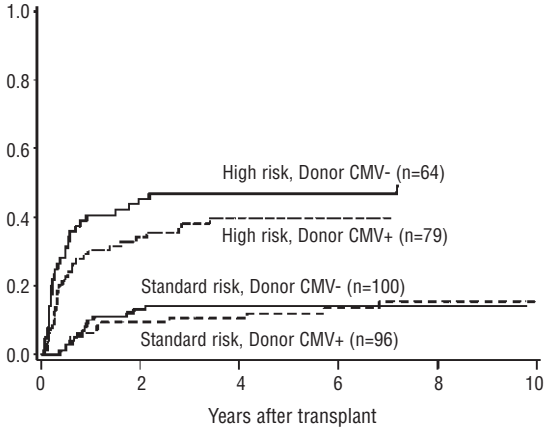


Figure 1. Probability of relapse in HLA-A2-positive identical HCT recipients stratified by the risk of relapse, in relation to donor CMV serostatus. The probability of relapse appears to be greater among patients receiving a transplantation from a CMV-seronegative donor than a CMV-seropositive donor in the high risk group but the difference was not statistically significant.

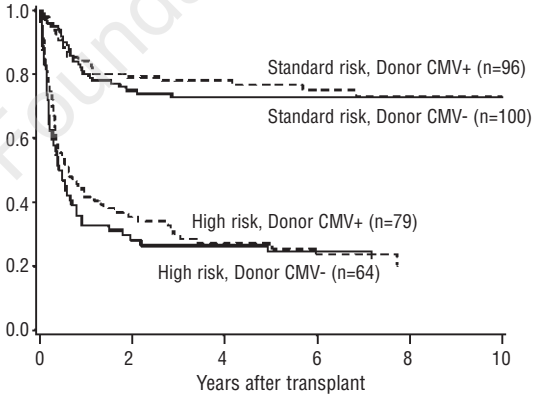


Figure 2. Relapse-free survival in HLA-A2-positive identical HCT recipients, stratified by risk of relapse, according to the donor CMV-serostatus. Relapse-free survival was comparable among recipients with a CMV-seronegative donor and among those with a CMV-seropositive donor.

ing to donor CMV serostatus (p=0.93, Figure 1). Similar findings resulted from analyses restricted to 2 and 5 years of follow-up (*results not shown*). Relapse-free survival was also similar in HLA-A2-positive identical HCT recipients whether from a CMV-seropositive or -seronegative donor (p=0.77, Figure 2). Comparable results were found in HLA-A2-negative HCT recipients.

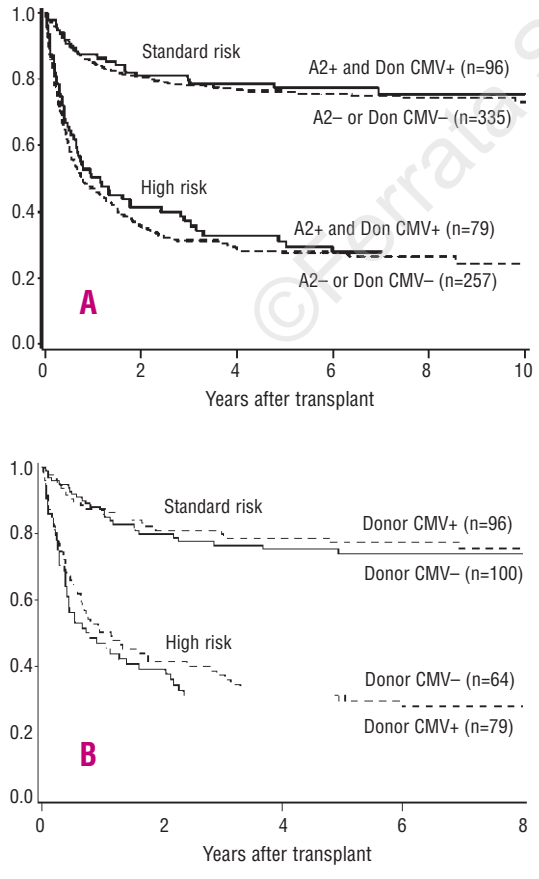
Multiple Cox regression analysis (Table 2) showed that acute non-lymphoid leukemia (HR 2.9, 95% CI 1.6-5.3, p<0.001) and acute lymphoid leukemia (HR 8.7, 95% CI 4.4-17.3, p<0.001) compared to chronic myeloid leukemia, and transplant years 1996-1999 compared to transplant years 1992-1995 (HR 0.4, 95% CI 0.2-0.8, p=0.02) were associated with the risk of relapse. After adjusting for these variables, neither HLA-A2 status nor donor CMV serostatus was associated with the risk of relapse. Furthermore, the interaction between HLA-A2 status and donor CMV serostatus did not significantly alter the risk of relapse (HR 0.9, 95% CI 0.3-2.5, p=0.85).

Risk of death. Overall survival was not superior among HLA-A2-positive identical HCT recipients with CMV-seropositive donors compared to that of recipients who were either HLA-A2-negative or had CMV-seronegative donors (p=0.69, Figure 3, panel A). When the analysis was restricted to HLA-A2-positive identical HCT recipients the overall survival of HCT recipients with CMV seropositive donor was not different from that of HCT recipients with a CMV seronegative donor (p=0.74, Figure 3, panel B). Multiple Cox regression analysis (Table 3) showed that acute non lymphoid leukemia (HR 3.5, 95% CI 1.9-6.2, p<0.001) or acute lymphoid leukemia (HR 2.0, 95% CI 1.3-3.0, p=0.001) compared

Table 2. Multivariable analysis of risk factors for relapse among standard and high relapse risk patients.

	Standard risk (n=431)			High risk (n=332)		
	HR	*95% CI*	*p value*	*HR*	*95% CI*	*p value*
Diagnosis						
Chronic myeloid leukemia	1.0			1.0		
Acute lymphoblastic leukemia	8.7	4.4-17.3	<0.001	1.5	1.0-2.4	0.06*
Acute non-lymphoblastic leukemia	2.9	1.6-5.3	<0.001			
Transplant year						
1992-1995	1.0					
1996-1999	0.4	0.2-0.8	0.02			
2000-2003	1.1	0.6-2.0	0.82			
Recipient HSV serostatus						
Negative				1.0		
Positive				1.9	1.2-3.0	0.006
Recipient HLA-A2						
Negative	1.0			1.0		
Positive	1.0	0.5-2.0	0.99	0.9	0.6-1.5	0.74
Recipent CMV serostatus						
Negative	1.0			1.0		
Positive	0.8	0.4-1.8	0.66	1.0	0.7-1.7	0.87
Donor CMV serostatus						
Negative	1.0			1.0		
Positive	0.9	0.3-2.1	0.73	1.0	0.5-1.8	0.91
HLA-A2 and donor CMV serostatus						
Either negative	1.0			1.0		
Both positive	0.9	0.3-2.5	0.85	1.0	0.5-1.9	0.92
Patient and donor CMV serostatus						
Either negative	1.0			1.0		
Both positive	1.2	0.4-3.6	0.76	0.8	0.4-1.6	0.58

*Cox regression analyis was used. HR: hazard ratio; CI: confidence interval. HSV: herpes simplex virus 1 or 2; CMV: cytomegalovirus. The impact of HSV serostatus in high risk patients remains unexplained. *Combined acute leukemia patients compared to chronic myeloid leukemia patients.*



to chronic myeloid leukemia, and female donor to male recipient (HR 1.8, 95% CI 1.2-2.7, p=0.004) compared to other sex matches were associated with a higher risk of death. After adjusting for these factors, neither HLA-A2 status nor donor CMV serostatus was associated with the risk of death. Furthermore, the interaction between HLA-A2 status and donor CMV serostatus did not alter the risk of death.

High risk patients

Risk of relapse. The probabilities of relapse among HCT recipients from CMV-seropositive and CMV-seronegative donors were not significantly different (p=0.15). When the analysis was restricted to HLA-A2 - positive HCT recipients, the probability of relapse in donor CMV-seropositive HCT recipients did not differ significantly according to donor CMV serostatus (p=0.25, Figure 1). Similar findings resulted from analyses restricted to 2 and 5 years of follow-up (*results not shown*). Relapse-free survival did not differ according to

Figure 3 (left). Overall survival according to HLA-status and donor CMV-serostatus in HLA-identical HCT recipients. HCT recipients who received their transplant from a CMV-seropositive donor do not appear to have a different survival from patients who are either HLA-A2 seronegative or had a CMV-seronegative donor (A). In HLA-A2-positive HCT recipients the same overall survival was observed in HCT recipients with a CMV-seropositive donor and in HCT recipients with a CMV-seronegative donor (B).

Table 3. Multivariable analysis of risk factors for death among standard and high relapse risk patients.

	Standard risk (n=431)			High risk (n=332)		
	HR	*95% CI*	*p value*	*HR*	*95% CI*	*p value*
Age at transplant (years)						
<40				1.0		
≥40				1.5	1.1-2.0	0.005
Diagnosis						
Chronic myeloid leukemia	1.0			1.0		
Acute lymphoblastic leukemia	3.5	1.9-6.2	<0.001	1.8	1.2-2.8	0.008
Acute non-lymphoblastic leukemia	2.0	1.3-3.0	0.001	1.5	1.1-2.2	0.01
Tranplant year						
1992-1995	1.0					
1996-1999	1.0	0.6-1.5	0.93			
2000-2003	0.6	0.3-1.0	0.05			
Donor/recipient sex						
Other	1.0					
Female/male	1.8	1.2-2.7	0.004			
Recipient HSV serostatus						
Negative				1.0		
Positive				2.2	1.5-3.3	<0.001
Recipient HLA-A2						
Negative	1.0			1.0		
Positive	1.4	0.8-2.4	0.26	0.8	0.6-1.2	0.35
Recipent CMV serostatus						
Negative	1.0			1.0		
Positive	1.2	0.7-2.1	0.59	1.0	0.7-1.5	0.83
Donor CMV serostatus						
Negative	1.0			1.0		
Positive	1.5	0.8-3.1	0.21	0.8	0.5-1.4	0.48
HLA-A2 and donor CMV serostatus						
Either negative	1.0			1.0		
Both positive	0.6	0.3-1.3	0.17	1.1	0.6-1.8	0.82
Patient and donor CMV serostatus						
Either negative	1.0			1.0		
Both positive	0.9	0.4-2.0	0.78	1.1	0.6-1.9	0.81

Cox regression analysis was used, HR: hazard ratio; CI: confidence interval; HSV: herpes simplex virus 1 or 2; CMV: cytomegalovirus. The impact of HSV serostatus on the risk of death in high risk patients remains unexplained.

donor CMV-seropositivity in either HLA-A2-negative (p=0.56) or HLA-A2-positive identical HCT recipients (p=0.83, Figure 2). In the multivariable analysis (Table 2), recipient HSV-seropositivity was associated with an increased risk of relapse, and there was a trend toward a higher risk of relapse among patients with acute leukemia (HR 1.5, 95% CI 1.0-2.4, p=0.06) than among patients with chronic leukemia. After adjusting for these factors, neither HLA-A2 status nor donor CMV serostatus was associated with the risk of relapse. Furthermore, the interaction between HLA-A2 status and donor CMV serostatus did not alter the risk of relapse (HR 1.0, 95 % CI 0.5-1.9, p=0.92).

Risk of death. Overall survival was not superior among HLA-A2-positive identical HCT recipients with CMV-seropositive donors compared to recipients who were either HLA-A2-negative or had CMV-seronegative donors (p=0.48, Figure 3, panel A). When the analysis was restricted to HLA-A2-positive identical HCT recipients the overall survival of HCT recipients with a CMV seropositive donor was not different than that of a HCT recipient with a CMV seronegative donor (p=0.72, Figure 3, panel B). Multiple Cox regression analysis (Table 3) showed that age 40 or greater (HR 1.5, 95% CI 1.1-2.0, p=0.005), acute lymphoid leukemia (HR 1.8, 95% CI 1.2-2.8, p=0.008) and acute non-lymphoid leukemia (HR 1.5, 95% CI 1.1-2.1, p=0.01) compared to chronic myeloid leukemia, and recipient HSV-seropositivity (HR 2.2, 95% CI 1.5-3.3, p<0.001) were associated with a higher risk of death. After adjusting for those variables, neither HLA-A2 status nor donor CMV serostatus was associated with the risk of death. Furthermore, the interaction between HLA-A2-status and donor CMV-serostatus did not alter the risk of death.

Risk of developing acute or chronic GVHD in standard and high risk patients. The probability of developing acute GVHD grade III-IV and chronic GVHD did not differ across strata of HLA-A2 status and donor CMV serostatus (Figure 4, panel A and B).

Patients surviving 100 days post HCT

In order to examine whether the immunity against leukemia is delayed after HCT, we restricted the analysis to patients who survived without leukemia relapse to day 100. We observed the same overall results that

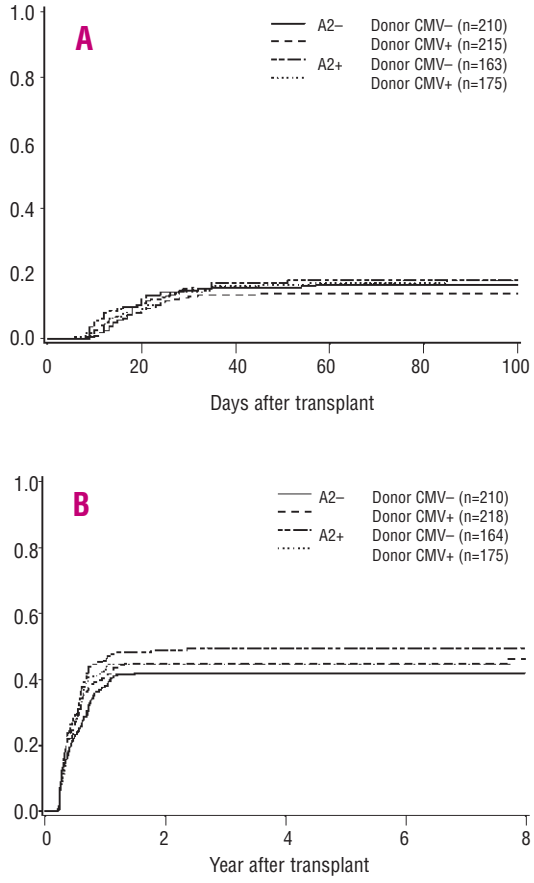


Figure 4. Probability of grade 3-4 acute GVHD (*panel A*) and chronic GVHD (*panel B*) in HLA identical HCT recipients. The probability of developing acute GVHD grade III-IV and chronic GVHD did not differ across strata of HLA-A2 status and donor CMV serostatus.

HLA-A2 status and donor CMV-seropositivity do not predict relapse or survival in this subgroup of HCT recipients (*data not shown*).

Discussion

Nachbaur and colleagues[11] reported that donor CMV-seropositivity is associated with improved survival and a reduced relapse rate in HLA-A2-positive sibling T-cell-replete HCT recipients. Nachbaur's study was conducted in a homogeneous population of patients with leukemia and HLA fully-matched related donors. To be able to compare results between studies, we used the same selection criteria as those reported by Nachbaur *et al*. Despite this, in a much larger study population and with appropriate statistical methodology, we were not able to confirm an association between donor CMV-seropositivity and a better outcome in terms of relapse or death in HLA-A2-identical HCT recipients with leukemia. Because the effect may have become apparent only late after transplantation, we also restricted the analysis to patients who survived until day 100 post-transplant, but again we were not able to document an effect.

Based on their findings, Nachbaur and colleagues suggested that donor HLA-A2-restricted CMV-specific CD8$^+$ T cells might cross-react with HLA-A2 minor H antigen complexes resulting in an improved GVL effect.[11] Multiple studies have shown that a significant degree of plasticity exists in the T-cell receptor, which allows this receptor to effectively recognize multiple distinct HLA-peptide complexes that have varying degrees of structural diversity.[22] Indeed, experimental data have demonstrated that virus-specific CD8$^+$ T cells can cross-react with major histocompatibility alloantigens.[23,24] More recent work in murine models and in humans have shown that virus-specific CD8$^+$ T cells can cross-react with distinct epitopes derived from other viruses and contribute to protective heterologous immunity and to immunopathology.[25,26] Although a recent study demonstrated that CD8$^+$ T cell alloreactivity directed against minor H antigens is predominantly contained in naïve T-cell and not in the memory T cells repertoire, which contains virus-specific CD8$^+$ T cells,[27] the degree to which virus-specific CD8$^+$ T cells cross-react with human minor H antigens is unknown. In HLA-matched stem cell transplantation, activation of alloreactive CD8$^+$ T cells specific for a minor H antigen will be initiated when minor H antigen-positive recipients undergo HCT from minor H antigen-negative donors. Therefore, a potential cross-reactivity between a donor's HLA-A2 restricted CMV-specific CD8$^+$ T cells and a specific recipient's minor H antigens would arise exclusively in the subset of HCT recipients who express the HLA-A2-restricted minor H antigen and have a donor negative for this particular minor H antigen.

Our study did not demonstrate an impact of donor CMV serostatus on the risk of relapse or of GVHD in HLA-A2-positive identical HCT recipients. However, the lack of knowledge on the minor H antigen genotype of both recipients and donors hampers us from supporting or refuting that donor HLA-A2-restricted CMV-specific CD8$^+$ T cells might cross-react with immunogenic minor H antigens expressed by the recipients. A potential minor H antigen cross-reactivity of CMV-specific CD8$^+$ T cells might be more appropriately evaluated in *in vitro* experiments than in a population-based study of leukemic HCT recipients. Indeed, because of the incomplete identification of minor H antigens, the chance of observing CMV-specific CD8$^+$ T-cell cross-reactivity directed against diverse minor H antigens in a population of HCT recipients might be impaired by the impossibility of selectively analyzing subgroups of recipients/donors who are mismatched for minor H antigens. On the other hand, the evaluation of CMV-specific CD8$^+$ T cell cross-reactivity directed against currently

known immunogenic HLA-A2-restricted minor H antigens,[10] such as HA-1 and HA-2, is constrained by the unbalanced representation of the HA-1 and HA-2 immunogenic peptides in the population.[28]

In conclusion our study did not provide evidence for an association between donor CMV serostatus and leukemia outcomes in HLA-A2 matched HCT recipients, suggesting that CMV-specific CD8+ T cells, contained in the memory T-cell subset, do not broadly cross-react with HLA-A2 -restricted minor H antigens. This suggestion is consistent with the recent demonstration that minor H antigen-specific CD8+ T cells are predominantly represented in the naïve T-cell repertoire. However, the appreciation of a potential CMV-specific T-cell cross-reactivity in a population-based study is limited by our partial knowledge of the diversity, the tissue representation, and the allelic distribution of minor H antigens.

VE analyzed the data and wrote the paper; KG analyzed data, and critically reviewed the paper; SR critically reviewed the paper; MB designed the study, supervised the data collection and analysis and had overall responsibility for the study.

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