STIMULUS REPORT

blood advances

The association of CMV with NK-cell reconstitution depends on graft source: results from BMT CTN-0201 samples

Armin Rashidi,[1] Xianghua Luo,[2] Sarah Cooley,[1] Claudio Anasetti,[3] Edmund K. Waller,[4] Claudio G. Brunstein,[1] Frank Cichocki,[1] Daniel J. Weisdorf,[1] and Jeffrey S. Miller[1]

[1]Division of Hematology, Oncology, and Transplantation, Department of Medicine, and [2]Masonic Cancer Center Biostatistics Core, University of Minnesota, Minneapolis, MN; [3]Moffitt Cancer Center, Tampa, FL; and [4]Department of Hematology and Medical Oncology, Emory University School of Medicine, Atlanta, GA

Key Points

- CMV reactivation was associated with the maturation of reconstituting NK cells from BM, but not PB, unrelated donor grafts.
- CMV reactivation was associated with CD8$^+$, but not CD4$^+$, T-cell recovery, more so after BM than PB unrelated donor grafts.

Introduction

Delayed immune reconstitution after allogeneic hematopoietic cell transplantation (HCT) increases the risk of relapse,[1] infection,[2] and secondary malignancy.[3] Natural killer (NK) cells, the earliest reconstituting immune cells, reach normal levels within weeks after HCT[4,5] and, with T cells, contribute to the graft-versus-tumor effect.[6] Understanding NK cell reconstitution can help predict risks of relapse and identify novel therapeutic interventions.

Cytomegalovirus (CMV) exposure results in expansion of a unique subset of NK cells (termed adaptive) that exhibit properties of immune memory.[7] This terminally differentiated CD56^{dim}NKG2C$^+$CD57$^+$ NK-cell subset has potent antibody-dependent cytolytic function and persistence long after resolution of infection.[7-9] Adaptive NK cells have been associated with protection against leukemia relapse[10-12] and improved disease-free survival[13] after HCT.

We asked whether the effect of CMV reactivation on NK- and T-cell reconstitution after HCT is influenced by graft source. Using unique samples from a large randomized study of peripheral blood (PB) vs bone marrow (BM) unrelated donor HCT, we demonstrate that CMV reactivation is associated with more mature NK cell reconstitution after HCT with BM, but not PB grafts.

Methods

Clinical data and PB mononuclear cell samples were provided by the Blood and Marrow Clinical Trials Network (BMT CTN). BMT CTN-0201 was a phase 3 randomized multicenter trial (2004-2009, 551 patients) comparing unrelated donor BM vs PB HCTs, with 2-year survival as the primary end point.[14] The trial demonstrated similar rates of acute graft-versus-host disease (aGVHD) but higher rates of chronic GVHD among PB graft recipients. An analysis of infections[15] showed the BM group with a higher 2-year cumulative incidence of all infections and bacterial infections but similar 2-year cumulative incidence of CMV and fungal infections.

We included all patients with a day 100 PB mononuclear cell sample without relapse by day 100 ($n = 259$) (Table 1). We classified patients into 3 CMV groups: (1) patients who reactivated CMV by day 100 (CMVr), (2) CMV-seropositive patients who did not reactivate CMV by day 100 (CMV+), and (3) CMV-seronegative patients who did not reactivate CMV (CMV−). We compared the frequency of day 100 immune cell subsets among these 3 CMV groups.

Fresh blood samples (without cryopreservation) were stained fresh within 30 hours of collection for analysis. The Translational Therapy Laboratory (Masonic Cancer Center, University of Minnesota) processed specimens, stained and acquired cells on an LSRII flow cytometer (BD Biosciences), and analyzed data using FlowJo (supplemental Methods). CD4$^+$ and CD8$^+$ T-cell subsets and CD56^{bright}, CD56^{dim}, and KIR$^+$NKG2A$^-$ NK-cell subsets were enumerated. We used a cocktail of 3 KIR antibodies with the same fluorochrome (CD158a, CD158b, and NKB1) to measure the KIR$^+$ population. We used KIR$^+$NKG2A$^-$ expression on CD56^{dim} NK cells to estimate the most mature NK-cell subset (as NKG2C expression was not available). NKG2C and NKG2A expression are generally mutually exclusive.

Submitted 14 April 2019; accepted 21 July 2019. DOI 10.1182/bloodadvances.2019000298.

The full-text version of this article contains a data supplement.

Table 1. Patient- and transplant-related characteristics

Variable	Overall (n = 259)	BM (n = 126)	PB (n = 133)	P
Sex				0.31
Male	152 (59)	78 (62)	74 (56)	
Female	107 (41)	48 (38)	59 (44)	
Age at transplantation, y				0.90
Median (range)	44 (0.4-67)	42 (5-67)	41 (0.4-67)	
Race				0.20
Nonwhite	20 (8)	7 (6)	13 (10)	
White	239 (92)	119 (94)	120 (90)	
CMV group				0.11
CMVr	34 (13)	22 (18)	12 (9)	
CMV+	110 (43)	53 (42)	57 (43)	
CMV−	115 (44)	51 (41)	64 (48)	
Disease group				0.43
Acute myeloid leukemia	127 (49)	59 (47)	68 (51)	
Acute lymphoblastic leukemia	59 (23)	30 (24)	29 (22)	
Chronic myeloid leukemia	25 (10)	13 (10)	12 (9)	
Myelodysplastic syndrome	40 (15)	22 (18)	18 (14)	
Chronic myelomonocytic leukemia	4 (2)	2 (2)	2 (2)	
Myelofibrosis	4 (2)	0 (0)	4 (3%)	
HLA match degree				0.07
5/6	26 (10)	17 (14)	9 (7)	
6/6	233 (90)	109 (87)	124 (93)	
Acute GVHD grade II to IV by day 100				0.52
No	143 (55)	67 (53)	76 (57)	
Yes	116 (45)	59 (47)	57 (43)	

Data are presented as n (%), unless otherwise indicated.

CMVr, patients who reactivated CMV by day 100; CMV+, CMV-seropositive patients who did not reactivate CMV by day 100; CMV−, CMV-seronegative patients who did not reactivate CMV.

Additionally, Malmberg et al showed that mature adaptive NK cells express increased KIR and low NKG2A.[16] While the KIR^+NKG2A^- phenotype of $CD56^{dim}$ NK cells is an estimate, true adaptive NK cells would be proportional to this phenotype.

Statistical analysis

Patient- and treatment-related variables were compared between the treatment groups using a Student *t* test, χ^2 test, or Fisher's exact test as appropriate. The percentage of cell subsets was compared between groups using the Wilcoxon rank sum test. We used linear regression for each cell type by including graft source, CMV group, and their interaction as covariates. Because aGVHD can influence NK cell reconstitution,[17] models adjusted or unadjusted for grade II to IV aGVHD by day 100 were compared to evaluate whether our results are influenced by aGVHD.

Results and discussion

In univariate analysis considering graft source as the only independent variable (supplemental Table 1), day 100 $CD4^+$ T cells were significantly higher in the PB group ($P < .0001$), while total NK cells and their $CD56^{bright}$ subset were significantly higher in the BM group ($P < .001$).

The kinetics of lymphocyte reconstitution at 100 days is shown in Figure 1A-C. In both BM and PB subgroups, day 100 $CD8^+$ T cells were higher in CMVr and CMV+ groups than the CMV− group ($P < .001$ in BM and $P < .01$ in PB). In both BM and PB subgroups, day 100 $CD4^+$ T cells were reciprocally slightly lower in the CMVr group than the CMV+ and CMV− groups (not statistically significant). In regression modeling using an interaction term for graft source and CMV group, the interaction was significant for $CD8^+$ T cells ($P = .04$), where the association of CMV reactivation with $CD8^+$ T-cell recovery was more pronounced with BM. No significant interaction was found for $CD4^+$ T cells ($P = .53$). These results suggest expansion of $CD8^+$ T cells due to CMV reactivation (more prominently in BM recipients), consistent with potent immune imprinting of CMV on these cells.[18,19] Specifically, CMV reactivation results in clonal expansion of effector memory $CD8^+$ T cells with a compromised T-cell receptor repertoire, a linked global contraction of $CD4^+$ and $CD8^+$ naïve T cells,[20] and possibly also a lower fraction of CMV-specific $CD8^+$ T cells with robust cytokine response.[21]

In the BM subgroup, CMV reactivation was associated with fewer total ($P < .05$) and $CD56^{bright}$ ($P < .01$) NK cells compared with the CMV− group (Figure 1C-D). However, more mature $CD56^{dim}$ NK cells expanded in the CMVr group than the CMV− group ($P < .01$; Figure 1E). The frequency of KIR^+NKG2A^- NK cells was highest in the CMVr group, followed by the CMV+ ($P < .05$) and CMV− groups ($P < .05$) (Figure 1F). The results for absolute counts (supplemental Figure 1) were consistent with our main findings.

These findings are consistent with previous reports of NK-cell maturation following CMV reactivation.[9,11] In the PB subgroup, there was an increase in mature KIR^+NKG2A^- NK cells in the CMV+ group, but not the CMVr group, compared with the CMV− group ($P < .01$). In regression modeling using an interaction term for graft source and CMV group, the interaction was significant for KIR^+NKG2A^- NK cells, where the effect of CMV reactivation on KIR^+NKG2A^- NK-cell recovery was more pronounced with BM ($P = .03$). No significant graft source interaction was found for total ($P = .48$), $CD56^{bright}$ ($P = .30$), or $CD56^{dim}$ NK cells ($P = .30$). CMV group was not associated with relapse or nonrelapse mortality in either the BM or PB group. Since the results for the graft source × CMV group interaction in regression models adjusted or unadjusted for aGVHD were very similar, grade II to IV aGVHD by day 100 did not confound our main findings (supplemental Table 2).

The association of CMV reactivation with NK and other lymphoid cell immune reconstitution depends on graft source, with a stronger association in BM recipients compared with PB. This association is CMV dose dependent in recipients of BM, but not PB grafts, where detectable CMV reactivation had the greatest association compared with latent CMV persistence in CMV-seropositive patients without detectable reactivation. Why the association of CMV reactivation with NK-cell maturation is graft-source dependent may have several explanations. Since the CMV reservoir is thought to occur in myeloid cells, this latent pool may differ between BM and PB grafts either directly from the graft source or perhaps acting through host cells. Graft source compositional differences in T cells

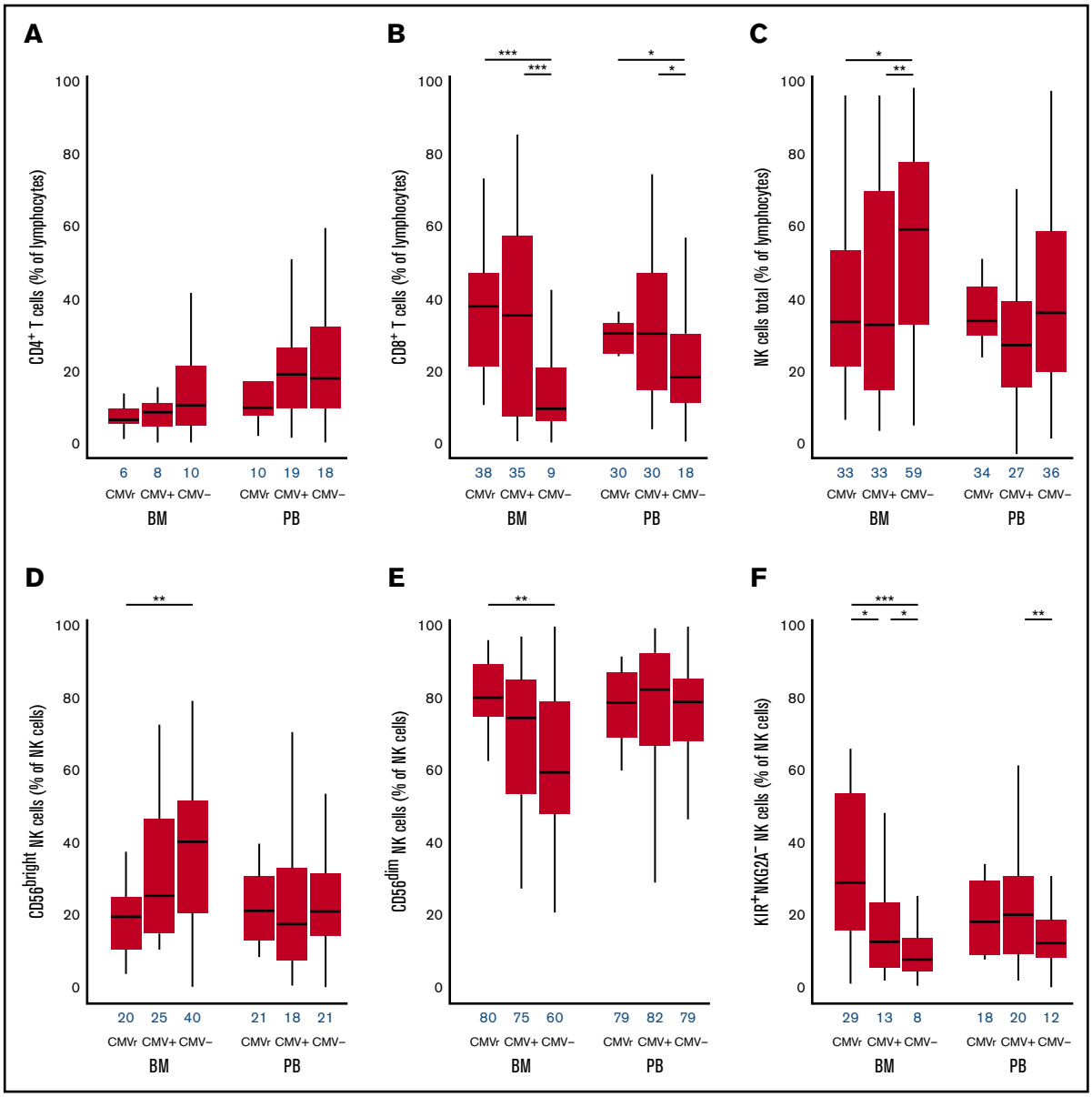

Figure 1. Association of CMV reactivation with T- and NK-cell reconstitution at 100 days in unrelated donor allografts using BM vs PB as a graft source. The frequency of $CD4^+$ T cells (A), $CD8^+$ T cells (B), and total NK cells (C) were determined based on a lymphocyte gate determined by forward and side scatter. The NK cells ($CD56^+/CD56^-$ gate) were subsetted into $CD56^{bright}$ (D), $CD56^{dim}$ (E), and KIR^+NKG2A^- (F) NK cells in 3 groups based on CMV reactivation status and in BM vs PB groups separately. Blue numbers on the x-axis show medians. CMVr, patients who reactivated CMV by day 100; CMV+, CMV-seropositive patients who did not reactivate CMV by day 100; and CMV−, CMV-seronegative patients who did not reactivate CMV by day 100. *$P < .05$; **$P < .01$; ***$P < .001$.

(frequency, differentiation status, and CMV specificity), dendritic cells, and their subsets may also contribute.[22] It is also plausible that BM is the source for certain subsets of NK cells. Our findings could suggest that a CMV-responsive precursor may be enriched in BM harvests and less so in granulocyte-colony stimulating factor–mobilized PB collections. Tissue-resident lymphocytes and their precursors may differ between graft sources. While it is believed that $CD56^{bright}$ NK cells develop into $CD56^{dim}$ NK cells with maturation that is linear and stage specific,[23] this remains uncertain and has not been well studied in cells from BM vs PB. Nonhuman primate data suggest parallel lineages rather than sequential.[24] Other possibilities include blunting of the CMV response with a Th2-biased effect induced by granulocyte-colony stimulating factor mobilization in PB grafts[25] and higher levels of homeostatic cytokines in BM recipients as a key driver of the immune effects. Lastly, it is possible that the immune reconstitution associations observed are indirect and attributable to a higher incidence of CMV reactivation by day 100 (18% in BM and 9% in PB patients in our analysis, $P = .04$) as the dominant driver of immune differences.

The analysis performed here was on fresh samples obtained from the clinical trial, and higher resolution NK immunophenotypic panels were not available at the time of the initial trial design. Nonetheless, this is the first report on CMV-induced NK-cell immune

reconstitution that differs between BM and PB graft sources. Further studies are needed to understand the mechanism and manipulate it for therapeutic purposes.

Acknowledgments

The authors thank the BMT CTN for providing the samples.

Research reported in this publication was supported by the National Institutes of Health, National Cancer Institute (grant P30CA077598) utilizing the Biostatistics and Bioinformatics Core and the Biospecimen Repository in the Translational Therapy Laboratory Shared Resource of the Masonic Cancer Center, University of Minnesota and by the National Center for Advancing Translational Sciences of the National Institutes of Health (award number UL1-TR002494). National Institutes of Health, National Cancer Institute grants P01 CA111412 and P01 CA65493 provided partial support. BMT CTN is supported by the National Institutes of Health, National Heart, Lung, and Blood Institute and National Cancer Institute (grant U10HL069294). The Department of the Navy, Office of Navy Research, and the National Marrow Donor Program also provided support for the BMT CTN-0201 study. Enrollment support was provided by DKMS (Deutsche KnochenMarkSpenderdatei) Germany.

Any views, opinions, findings, conclusions or recommendations expressed in this material are those of the authors and do not reflect the views of the official policy or position of the above-mentioned parties.

Authorship

Contribution: A.R., S.C., D.J.W., and J.S.M. designed the study and critically evaluated the results; X.L. analyzed the data; A.R., D.J.W., and J.S.M. wrote the manuscript; and S.C., C.A., E.K.W., C.G.B., and F.C. critically reviewed and edited the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

ORCID profiles: A.R., 0000-0002-9384-272X; E.K.W., 0000-0003-0816-6729; D.J.W., 0000-0001-8078-8579; J.S.M., 0000-0002-0339-4944.

Correspondence: Jeffrey S. Miller, Division of Hematology, Oncology, and Transplantation, Department of Medicine, University of Minnesota, 420 Delaware St SE, MMC 480, Minneapolis, MN 55455; e-mail: mille011@umn.edu.

References

1. Maraninchi D, Gluckman E, Blaise D, et al. Impact of T-cell depletion on outcome of allogeneic bone-marrow transplantation for standard-risk leukaemias. *Lancet*. 1987;2(8552):175-178.
2. Storek J, Gooley T, Witherspoon RP, Sullivan KM, Storb R. Infectious morbidity in long-term survivors of allogeneic marrow transplantation is associated with low CD4 T cell counts. *Am J Hematol*. 1997;54(2):131-138.
3. Curtis RE, Rowlings PA, Deeg HJ, et al. Solid cancers after bone marrow transplantation. *N Engl J Med*. 1997;336(13):897-904.
4. Ottinger HD, Beelen DW, Scheulen B, Schaefer UW, Grosse-Wilde H. Improved immune reconstitution after allotransplantation of peripheral blood stem cells instead of bone marrow. *Blood*. 1996;88(7):2775-2779.
5. Komanduri KV, St John LS, de Lima M, et al. Delayed immune reconstitution after cord blood transplantation is characterized by impaired thymopoiesis and late memory T-cell skewing. *Blood*. 2007;110(13):4543-4551.
6. Farag SS, Fehniger TA, Ruggeri L, Velardi A, Caligiuri MA. Natural killer cell receptors: new biology and insights into the graft-versus-leukemia effect. *Blood*. 2002;100(6):1935-1947.
7. Gumá M, Angulo A, Vilches C, Gómez-Lozano N, Malats N, López-Botet M. Imprint of human cytomegalovirus infection on the NK cell receptor repertoire. *Blood*. 2004;104(12):3664-3671.
8. Cooley S, Parham P, Miller JS. Strategies to activate NK cells to prevent relapse and induce remission following hematopoietic stem cell transplantation. *Blood*. 2018;131(10):1053-1062.
9. Foley B, Cooley S, Verneris MR, et al. Human cytomegalovirus (CMV)-induced memory-like NKG2C(+) NK cells are transplantable and expand in vivo in response to recipient CMV antigen. *J Immunol*. 2012;189(10):5082-5088.
10. Davis ZB, Cooley SA, Cichocki F, et al. Adaptive natural killer cell and killer cell immunoglobulin-like receptor-expressing T cell responses are induced by cytomegalovirus and are associated with protection against cytomegalovirus reactivation after allogeneic donor hematopoietic cell transplantation. *Biol Blood Marrow Transplant*. 2015;21(9):1653-1662.
11. Foley B, Cooley S, Verneris MR, et al. Cytomegalovirus reactivation after allogeneic transplantation promotes a lasting increase in educated NKG2C+ natural killer cells with potent function. *Blood*. 2012;119(11):2665-2674.
12. Cichocki F, Cooley S, Davis Z, et al. CD56dimCD57+NKG2C+ NK cell expansion is associated with reduced leukemia relapse after reduced intensity HCT. *Leukemia*. 2016;30(2):456-463.
13. Cichocki F, Taras E, Chiuppesi F, et al. Adaptive NK cell reconstitution is associated with better clinical outcomes. *JCI Insight*. 2019;4(2):125553.
14. Anasetti C, Logan BR, Lee SJ, et al; Blood and Marrow Transplant Clinical Trials Network. Peripheral-blood stem cells versus bone marrow from unrelated donors. *N Engl J Med*. 2012;367(16):1487-1496.
15. Young JH, Logan BR, Wu J, et al; Blood and Marrow Transplant Clinical Trials Network Trial 0201. Infections after transplantation of bone marrow or peripheral blood stem cells from unrelated donors. *Biol Blood Marrow Transplant*. 2016;22(2):359-370.
16. Béziat V, Liu LL, Malmberg J-A, et al. NK cell responses to cytomegalovirus infection lead to stable imprints in the human KIR repertoire and involve activating KIRs. *Blood*. 2013;121(14):2678-2688.

17. Ullrich E, Salzmann-Manrique E, Bakhtiar S, et al. Relation between acute GVHD and NK cell subset reconstitution following allogeneic stem cell transplantation. *Front Immunol*. 2016;7:595.

18. Sylwester AW, Mitchell BL, Edgar JB, et al. Broadly targeted human cytomegalovirus-specific CD4+ and CD8+ T cells dominate the memory compartments of exposed subjects. *J Exp Med*. 2005;202(5):673-685.

19. Lugthart G, van Ostaijen-Ten Dam MM, Jol-van der Zijde CM, et al. Early cytomegalovirus reactivation leaves a specific and dynamic imprint on the reconstituting T cell compartment long-term after hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant*. 2014;20(5):655-661.

20. Suessmuth Y, Mukherjee R, Watkins B, et al. CMV reactivation drives posttransplant T-cell reconstitution and results in defects in the underlying TCRβ repertoire. *Blood*. 2015;125(25):3835-3850.

21. Ozdemir E, St John LS, Gillespie G, et al. Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. *Blood*. 2002;100(10):3690-3697.

22. Waller EK, Logan BR, Harris WAC, et al. Improved survival after transplantation of more donor plasmacytoid dendritic or naïve T cells from unrelated-donor marrow grafts: results from BMTCTN 0201. *J Clin Oncol*. 2014;32(22):2365-2372.

23. Freud AG, Yokohama A, Becknell B, et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. *J Exp Med*. 2006;203(4):1033-1043.

24. Wu C, Espinoza DA, Koelle SJ, et al. Clonal expansion and compartmentalized maintenance of rhesus macaque NK cell subsets. *Sci Immunol*. 2018;3(29):eaat9781.

25. Pan L, Delmonte J Jr, Jalonen CK, Ferrara JL. Pretreatment of donor mice with granulocyte colony-stimulating factor polarizes donor T lymphocytes toward type-2 cytokine production and reduces severity of experimental graft-versus-host disease. *Blood*. 1995;86(12):4422-4429.