Peripheral blood lymphocyte responses to cytomegalovirus seropositivity after allogeneic-hematopoietic stem cell transplantation

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Background: The main purpose of this study was to investigate the relationship among cytomegalovirus (CMV) viremia, peripheral immune cells alternations, and leukemia prognosis.

Patients and methods: We studied 90 leukemia patients who underwent allogeneic hematopoietic stem cell transplantation (allo-HSCT) from 2008 to 2015. Their complete clinical laboratory data were collected until 1 year after transplantation.

Results: All patients were serum CMV negative before allo-HSCT. After transplantation, the CMV reactivation group showed increased peripheral CD8+ T cells and decreased CD4+ T cells and B cells. However, CD8/CD4 ratio and B cells restored by control of CMV infection due to 2 months maximum course of ganciclovir treatment. CMV seropositivity was positively related to leukemia-free survival (LFS) of all recruited leukemia types.

Conclusion: In summary, CMV drives immune cell post-transplantation fluctuation, which also favors LFS of leukemia partly resulted from CD8+ T cells.

Keywords: cytomegalovirus, leukemia, allo-HSCT, CD8+ T cells, leukemia-free survival

Introduction

Cytomegalovirus (CMV) is a common double-stranded DNA herpes virus. Infection by CMV induces few, if any, clinical symptoms in healthy people.1 This virus may not ever be reactivated except for immune suppression circumstances, such as stem cell transplantation or human immunodeficiency virus infection. CMV can be detected from serum of leukemia patients early after transplantation, which are susceptible to CMV infection, and develop CMV pneumonia with highly mortality.2-5

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) has been widely applied as a recommended first-line therapy for leukemia. Before transplantation, patients receive chemotherapy to destroy tumor cells as well as immune system to make room for new stem cells for restoring hematopoiesis. To prevent CMV infection during therapy, patients are simultaneously designated antiviral drug, such as ganciclovir (GCV). Nevertheless, CMV still emerges in serum as early as a few days after transplantation. Moreover, some studies indicated that pre-surgery chemotherapy and GCV administration will impel patients more frail to CMV infection due to its side effects of myelosuppression, and postpone reconstitution of CMV-specific cellular immunity by suppressing proliferation of T cells as well as CMV-specific T-cell precursors.6,7

Immune system plays a significant role in controlling virus infection, survival, and proliferation. For example, CD4+ T-helper (Th) cells secrete interferon-γ, tumor necrosis factor-α, and interleukin-2 to exert a Th1-type immune reaction to eliminate
CMV seropositivity alters peripheral immune cell distribution

We analyzed percentages of peripheral CD4+ T cells, CD8+ T cells, NK cells, and B cells in the CMV+ and CMV− groups, respectively. As shown in Figure 1, CD4+ T cells were significantly downregulated and CD8+ T cells were highly upregulated in the CMV+ group, which led to obviously upregulated CD8/CD4 ratio. B cells were lower in the CMV+ group compared with the CMV− group. No difference was observed for NK cells. The CMV− group had a higher CD4/CD8 ratio compared to the CMV+ group. The CMV+ and CMV− groups had higher NK cell percentages compared to the CMV− group. These findings suggest that CMV seropositivity after allo-HSCT may alter the distribution of peripheral immune cells.
found in NK cells. We further analyzed the data of AML patients and found similar trends, as shown in Figure 2. These results suggest that CMV affects immune cells post-transplantation distribution, and that CD8+ T cells dominate in peripheral blood.

Figure 1 CMV seropositivity after allo-HSCT alters peripheral immune cells distribution.

Notes: Serum CMV DNA load was detected via real-time PCR 1 week after allo-HSCT. Patients suffered from CMV seropositivity defined as CMV+ group, otherwise CMV− group. The percentages of peripheral CD4+ T cells (A), CD8+ T cells (B), ratio of CD8/CD4 (C), NK cells (D), and B cells (E) of leukemia patients in the CMV+ and CMV− groups. Graphs show mean ± SEM. *P < 0.05; **P < 0.01; ****P < 0.0001.

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; CMV, cytomegalovirus; NK, natural killer.

Control of CMV infection induces peripheral immune cell re-distribution

Severe CMV infection often occurred within the first 100 days after allo-HSCT. The median time of CMV seropositivity in our leukemia patients was 38.5 days (range: 2–102 days).

Table 1 Clinical characteristics of 90 leukemia patients in the CMV+ and CMV− groups

| Variables                          | Patients | CMV+ group | CMV− group | P-value |
|-----------------------------------|----------|------------|------------|---------|
| Patients                          | 90       | 54 (60%)   | 36 (40%)   |         |
| Sex                               |          |            |            | 0.7     |
| Female/male                       | 46/44 (51%) | 27/27 (50%) | 19/17 (53%) |         |
| Age                               | 35.5 (6–65) years | 35 (6–60) years | 41 (9–65) years | 0.9     |
| Diagnosis                         |          |            |            |         |
| AML                               | 36 (40%) | 22 (40.7%) | 14 (38.9%) |         |
| ALL                               | 14 (15%) | 10 (18.5%) | 4 (11.1%)   |         |
| CML                               | 8 (9%)   | 4 (7.4%)   | 4 (11.1%)   |         |
| RBC leukemia                      | 6 (7%)   | 4 (7.4%)   | 2 (5.5%)    |         |
| Lymphoma                          | 14 (16%) | 5 (9.3%)   | 9 (25%)     |         |
| CLL                               | 2 (2%)   | 2 (3.7%)   | 0 (0%)      |         |
| MDS                               | 9 (10%)  | 7 (13%)    | 2 (5.6%)    |         |
| Others                            | 1 (1%)   | 0 (0%)     | 1 (2.8%)    |         |
| Conditioning regimen              |          |            |            | 0.1     |
| MAC                               | 68 (75.5%) | 45 (83.3%) | 23 (63.9%) |         |
| RIC                               | 15 (16.7%) | 7 (13%)    | 8 (22.2%)  |         |
| Others                            | 7 (7.8%) | 2 (3.7%)   | 5 (13.9%)   |         |
| Donors                            |          |            |            | 0.003   |
| HLA-matched siblings              | 62 (68.9%) | 33 (61.1%) | 29 (80.5%) |         |
| Unrelated matched                 | 20 (22.2%) | 18 (33.3%) | 2 (5.6%)   |         |
| Unknown                           | 8 (8.9%) | 3 (5.6%)   | 5 (13.9%)   |         |
| Stem cell source                  |          |            |            | 0.143   |
| PB + CB                           | 10 (11%) | 7 (12.9%)  | 3 (8.3%)    |         |
| PB                                | 76 (84%) | 42 (77.8%) | 33 (91.7%) |         |
| CB                                | 3 (3%)   | 3 (5.6%)   | 0 (0%)      |         |
| Unknown                           | 2 (2%)   | 2 (3.7%)   | 0 (0%)      |         |
| GVHD prophylaxis                  |          |            |            | 0.03    |
| CSA/MTX                           | 82 (91.1%) | 52 (96.3%) | 30 (83.3%) |         |
| ATG or other                      | 8 (8.9%) | 2 (3.7%)   | 6 (16.7%)   |         |
| GVHD                              |          |            |            | 0.009   |
| Acute GVHD                        | 43 (47.8%) | 34 (62.9%) | 9 (25%)    |         |
| Chronic GVHD                      | 16 (17.8%) | 7 (12.9%)  | 9 (25%)    |         |
| Status                            |          |            |            | 0.19    |
| Relapse                           | 13 (14.4%) | 7 (7.8%)  | 6 (6.7%)   |         |
| Dead                              | 16 (17.8%) | 7 (7.8%)  | 9 (6.7%)   |         |
| Missing                           | 24 (26.7%) | 16 (17.8%) | 8 (8.9%)   |         |

Abbreviations: AML, acute lymphoblastic leukemia; ALL, acute myeloid leukemia; AML, acute myeloid leukemia; ATG, Anti-human Thymocyte Globulin; CB, stem cells from cord blood; CLL, chronic lymphocytic leukemia; CML, chronic granulocytic leukemia; CMV, cytomegalovirus; CSA, Cyclosporin A; GVHD, graft-versus-host disease; HLA, human lymphocyte antigen; MAC, Myeloablative stem cell transplantation; MDS, myelodysplastic syndrome; MTX, Methotrexate; PB, stem cells from peripheral blood; RIC, Reduce-intensity conditioning.
within the CMV+ group. We weekly monitored peripheral CMV genome copies of these patients after allo-HSCT and documented the first date of CMV seropositivity and 2 months after the date, respectively, to investigate immune cell responses to GCV administration. We successively collected results of 18 patients in the CMV+ group. As shown in Figure 3, CD8+ T cells, CD4+ T cells, and B cells were restored when serum CMV was negatively converted, and the CD8/CD4 ratio dropped back to the baseline.

Next, we extended the follow-up period to 12 months after allo-HSCT and analyzed flow cytometry data of all patients in the CMV+ group. Their serum CMV all turned negative (if infection occurred) within 2 months after courses of GCV administration. Alteration of CD4+ T cells, CD8+ T cells, and B cells significantly occurred from 1 month to 3 months post-surgery (Figure 4). However, these differences between the CMV+ and CMV− groups became less pronounced with elongated follow-up time. Distribution of peripheral immune cells restored to pre-transplantation as early as 6 months. These results indicated that CMV drives immune cells’ post-transplantation fluctuation.

CMV seropositivity affects leukemia prognosis after allo-HSCT

Totally, 13 patients suffered leukemia relapse after allo-HSCT. The median relapse time was 262 days (8.7 months).

As shown in Table 2, both prophylactic GVHD treatment and occurrence of aGVHD were risk factors of LFS. As reported, CMV reactivation protected AML patients from relapse within 100 days post-transplantation. Therefore, we excluded the data of AML patients and further investigated the influence of CMV on other leukemia types. As shown in Table 3, CMV seropositivity was positively correlated with their LFS, indicating the favorable effect of CMV on leukemia overall prognosis.

CMV seropositivity, as well as stem cell sources, was positively correlated with LFS, although the overall survival of the CMV+ group was approximately to that of the CMV− group (53.10 months vs 59.21 months; $P = 0.24$; Figure 5). It indicated that CMV seropositivity may rescue leukemia patients, but it cannot extend their length of survival probably due to its control failure of peripheral immune system in the long-term.

Discussion

CMV often “hides” in hematopoietic progenitors. Along with the differentiation and maturation of these progenitors, CMV amplifies. Some suggested that latent virus can be reactivated by the allogeneic monocyte-derived macrophages. Therefore, peripheral blood mononuclear cells are believed to serve as CMV carriers. In this study, we elaborately collected data of 90 leukemia patients who were diagnosed...
Peripheral immune cell responses to CMV seropositivity after allo-HSCT

Figure 4 Distribution of peripheral immune cells during 12 months’ follow-up in all 90 leukemia patients after allo-HSCT.

Notes: We recorded their serum CMV load and correspondent flow cytometry data. Those with incomplete data were removed from the pool. Lymphocyte distribution was described in the time points of pre-BMT, 1–3 months (CMV seropositivity), 3–6 months (CMV negatively converted by gCV), 6–9 months, and 9–12 months. Representative dot plots of the percentages with circulating CD4+ T cells (A), CD8+ T cells (B), CD8/CD4 ratio (C), NK cells (D), and B cells (E) in CMV+ group and CMV− group. All the statistical graphs show mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001.

Abbreviations: allo-HSCT, allogeneic hematopoietic stem cell transplantation; BMT, bone marrow transplantation; CMV, cytomegalovirus; gCV, Ganciclovir; NK, natural killer.

Table 2 Clinical parameters related to LFS in 65 leukemia patients by multivariate variable Cox regression

| Parameters                  | Hazard ratio | 95% CI       | P-value |
|-----------------------------|--------------|--------------|---------|
| CMV seropositivity          | 2.293        | 1.136–4.629  | 0.021   |
| Age                         | 1.196        | 0.662–2.300  | 0.591   |
| Sex                         | 0.627        | 0.348–1.130  | 0.120   |
| Diagnosis                   | 1.214        | 0.976–1.510  | 0.081   |
| Conditioning regimen        | 0.810        | 0.456–2.736  | 0.810   |
| Stem cell sources           | 2.006        | 1.253–3.211  | 0.004   |
| GVHD prophylactic           | 0.097        | 0.011–0.884  | 0.038   |
| aGVHD                       | 0.526        | 0.280–0.991  | 0.047   |

Abbreviations: aGVHD, acute GVHD; CMV, cytomegalovirus; GVHD, graft-versus-host disease; LFS, leukemia-free survival.

Table 3 Clinical parameters associated with LFS in other leukemia patients, except for AML, by multivariate Cox regression analysis

| Parameters                  | Hazard ratio | 95% CI       | P-value |
|-----------------------------|--------------|--------------|---------|
| CMV seropositivity          | 3.384        | 1.196–9.575  | 0.022   |
| Age                         | 1.123        | 0.411–3.072  | 0.821   |
| Sex                         | 1.083        | 0.424–2.765  | 0.868   |
| Conditioning regimen        | 0.754        | 0.240–2.367  | 0.629   |
| Stem cell sources           | 2.550        | 0.816–7.969  | 0.108   |
| GVHD prophylactic           | 0.126        | 0.006–2.535  | 0.176   |
| aGVHD                       | 0.367        | 0.132–1.021  | 0.055   |

Abbreviations: aGVHD, acute GVHD; AML, acute myeloid leukemia; CMV, cytomegalovirus; GVHD, graft-versus-host disease; LFS, leukemia-free survival.
with AML, ALL, CML, CLL, MDS, and other undefined leukemia and had been determined CMV-free to standardize comparison criteria before allo-HSCT. Our data showed that the number of CD8+ T cells and the CD8/CD4 ratio were statistically higher in the CMV+ group, consistent with other study referring to CMV reactivation after transplantation, suggesting that post-surgery serum CMV status is closely related to peripheral immune cell alternation. CD8+ T-cell and CD4+ T-cell restoration after subsequent CMV seroconversion by GCV administration further confirms that CMV had impact on the human peripheral immune system in our study.

Another important finding in our study is that CMV seropositivity was positively related to LFS of patients regardless of leukemia types. It was supposed to be related to functional CD8+ T cells for exerting cytotoxic effects in resisting chronic virus invasion and expansion of the renewed clone, and likewise for its peripheral domination after CMV seropositivity in our study. Higher CD8+ T cells have been reported, but with increased CD4+ T cells in CLL patients. CMV-specific CD8+ T cells express relatively lower inhibitory markers PD-1 and CD160, and they maintain conventional virus-specific cytotoxicity by CD45RA+CD27+ phenotype. CMV-specific CD4+ T cells promote cytotoxic T-cell activity though Th1 cytokines and activation of antigen-presenting cells. They also play an important role in CMV-specific CD8+ cytotoxic T-lymphocyte differentiation and expansion via dendritic cell stimulation. Considering CD4+ T cells were not numerically increased in the CMV+ group in this study, it is worth exploring whether ratios of each subtype of CD4+ T cells alter correspondingly. Recently, Scheper et al found that gamma delta T cells (γδT) initiated by CMV infection after allo-HSCT cross-recognized both CMV-infected cells and primary leukemia blasts, indicating that CMV is a warrior against leukemia. The interplay between γδT cells and CMV infection seems to be a plausible explanation for the advantageous clinical prognosis. Mycophenolate mofetil affects viral replication by altering cytokine profiles and modulating adhesion-related molecules mediated by CMV, but it impairs both T and B cells’ proliferation. Still, fewer CD4+ T cells can induce T–B collaboration and reduce chronic GVHD (cGVHD) incidence. Moreover, CMV seropositivity increases the expression of leukocyte fixation antigen-3 on CMV-infected leukemia blasts and enhances NK cell-mediated blasts lysis without an increase in amount. Our data that CD19+ B cells reconstituted until 6 months after transplantation in the CMV+ group are consistent with the opinion that both aGVHD and cGVHD inhibit B cell reconstitution and delay B-cell immune recovery.

Leukemia patients who receive peripheral blood stem cells will acquire donor-derived passive immunity, whereas cord blood stem cells are immunological naïve, and upon activation, donor-derived T cells will aim at CMV lesion and directly exert cytotoxic effects towards leukemia blasts expressing CMV-related antigens. Unfortunately, the wide use of ATG in leukemia patients who are ready to receive cord blood stem cell transplantation messes with the recipient-derived immune cells, and this may be vital to post-surgery immune response especially encountering CMV infection.

Conclusion
Here, we reported the alternations of peripheral immune cells, including CD4+ T cells, CD8+ T cells, NK cells, and B cells, responses to CMV seropositivity of leukemia patients after allo-HSCT. Leukemia patients may benefit from CMV seropositivity on LFS aspect, probably due to the immune constitution of peripheral CD8+ T cells. Further, how to maintain functional and long-run peripheral CD8+ T cells after allo-HSCT may be a promising alternative therapeutic regimen in individualized prognosis.

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Disclosure
The authors report no conflicts of interest in this work.

References
1. Rafailidis PI, Mourtzoukou EG, Varbogitis IC, Falagas ME. Severe cytomegalovirus infection in apparently immunocompetent patients: a systematic review. *Virolo J.* 2008;5:47.
2. Nakamura R, Battiwalla M, Solomon S, et al. Persisting postransplantation cytomegalovirus antigenemia correlates with poor lymphocyte proliferation to cytomegalovirus antigen and predicts for increased late relapse and treatment failure. *Biol Blood Marrow Transplant.* 2004;10(1):49–57.
3. Nichols WG, Corey L, Gooley T, Davis C, Boeckh M. High risk of death due to bacterial and fungal infection among cytomegalovirus (CMV)-seronegative recipients of stem cell transplants from seropositive donors: evidence for indirect effects of primary CMV infection. *J Infect Dis.* 2002;185(3):273–282.
4. Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol Blood Marrow Transplant.* 2003;9(9):543–558.
5. Matsumura T, Narimatsu H, Kami M, et al. Cytomegalovirus infections following umbilical cord blood transplantation using reduced intensity conditioning regimens for adult patients. *Biol Blood Marrow Transplant.* 2007;13(5):577–583.
6. Foster AE, Gottlieb DJ, Sartor M, Hertzberg MS, Bradstock KF. Cytomegalovirus-specific CD4+ and CD8+ T-cells follow a similar reconstitution pattern after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant.* 2002;8(9):501–511.
7. Li CR, Greenberg PD, Gilbert MJ, Goodrich JM, Riddell SR. Recovery of HLA-restricted cytomegalovirus (CMV)-specific T-cell responses after allogeneic bone marrow transplant: correlation with CMV disease and effect of ganciclovir prophylaxis. *Blood.* 1994;83(7):1971–1979.
8. Pourgheysari B, Bruton R, Parry H, et al. The number of cytomegalovirus-specific CD4+ T cells is markedly expanded in patients with B-cell chronic lymphocytic leukemia and determines the total CD4+ T-cell repertoire. *Blood.* 2010;116(16):2968–2974.
9. te Raa GD, Pascutti MF, Garcia-Vallejo JJ, et al. CMV-specific CD8+T-cell function is not impaired in chronic lymphocytic leukemia. *Blood.* 2014;123(5):717–724.
10. Shedlock DJ, Shen H. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science.* 2003;300(5617):337–339.
11. Lancevaccia A, Saltusto F. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science.* 2000;290(5489):92–97.
12. Ridge JP, Di Rosa F, Matzinger P. A conditioned dendritic cell can be a temporal bridge between a CD4+ T-helper and a T-killer cell. *Nature.* 1998;393(6684):474–478.
13. Schoenberger SP, Toes RE, van der Voort EL, Offringa R, Melief CJ. T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions. *Nature.* 1998;393(6684):480–483.
14. 1193–1194.
15. 2014;123(5):717–724.
16. 2013;191(5):2708–2716.
17. 2011;118(5):1193–1194.
18. 53(2):393–400.
