Figure legends

Figure 1. Overall survival according to donor type and risk of disease

Overall survival after transplantation from a related donor with an HLA-1-antigen mismatch in the graft-versus-host direction (RD/1AG-MM-GVH), HLA 8/8-allele-matched unrelated donor (8/8 MUD), and HLA-matched related donor (MRD) in patients with both-risk (A), standard-risk (B), or high-risk diseases (C). Survival rates in the 8/8 MUD and RD/1AG-MM-GVH groups were compared by the log-rank test.

Figure 2. Overall survival in patients with both-risk (A, B), standard-risk (C, D) or high-risk diseases (E, F) according to the locus of HLA mismatch in the GVH direction, and the number of mismatches in the HVG direction

Survival rates in patients with HLA-A, -B, and -DR mismatch in the GVH direction were compared by the log-rank test (A, C, E). Survival rates in patients with HLA 0-1 mismatch and 2-3 mismatches in the HVG direction were compared by the log-rank test (B, D, F). Survival rate of 8/8 MUD group are shown for visual comparison. 8/8 MUD, HLA 8/8-allele-matched unrelated donor.

Figure 3. Cumulative incidence of treatment-related mortality (A) and relapse (B) according to the locus of HLA mismatch in the GVH direction in patients with standard-risk diseases

Cumulative incidences in groups of related transplantation were compared by Gray's test. 8/8 MUD, HLA 8/8-allele-matched unrelated donor.

Figure 1.

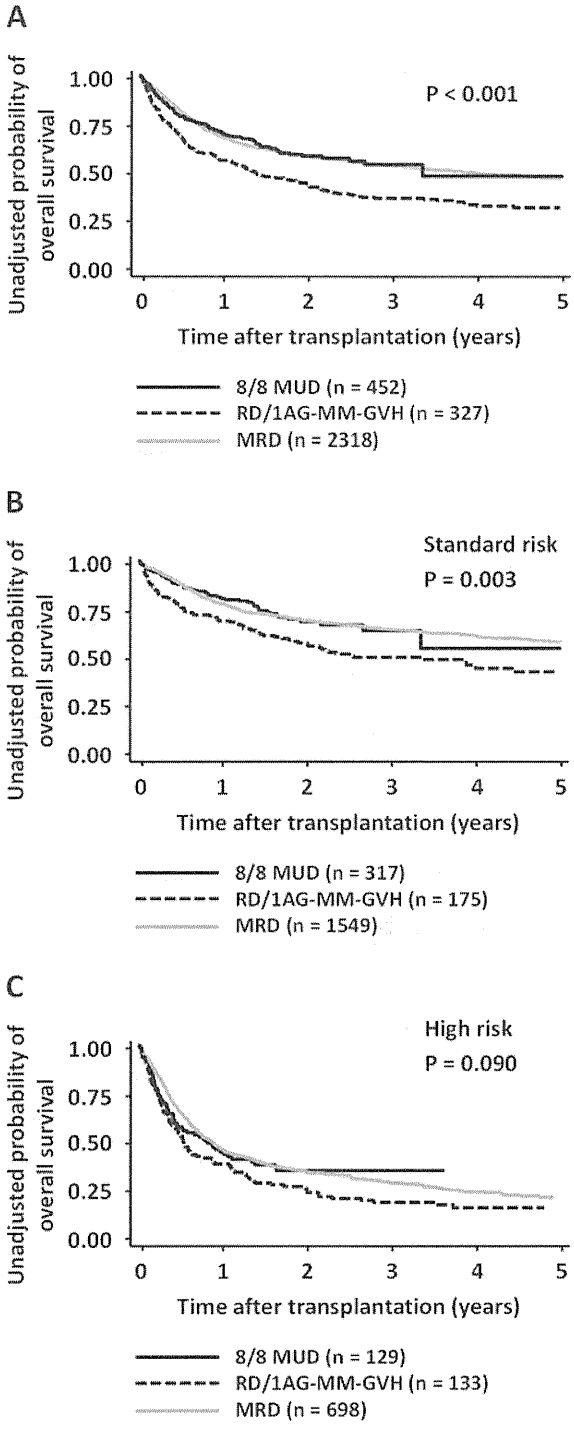


Figure 2.

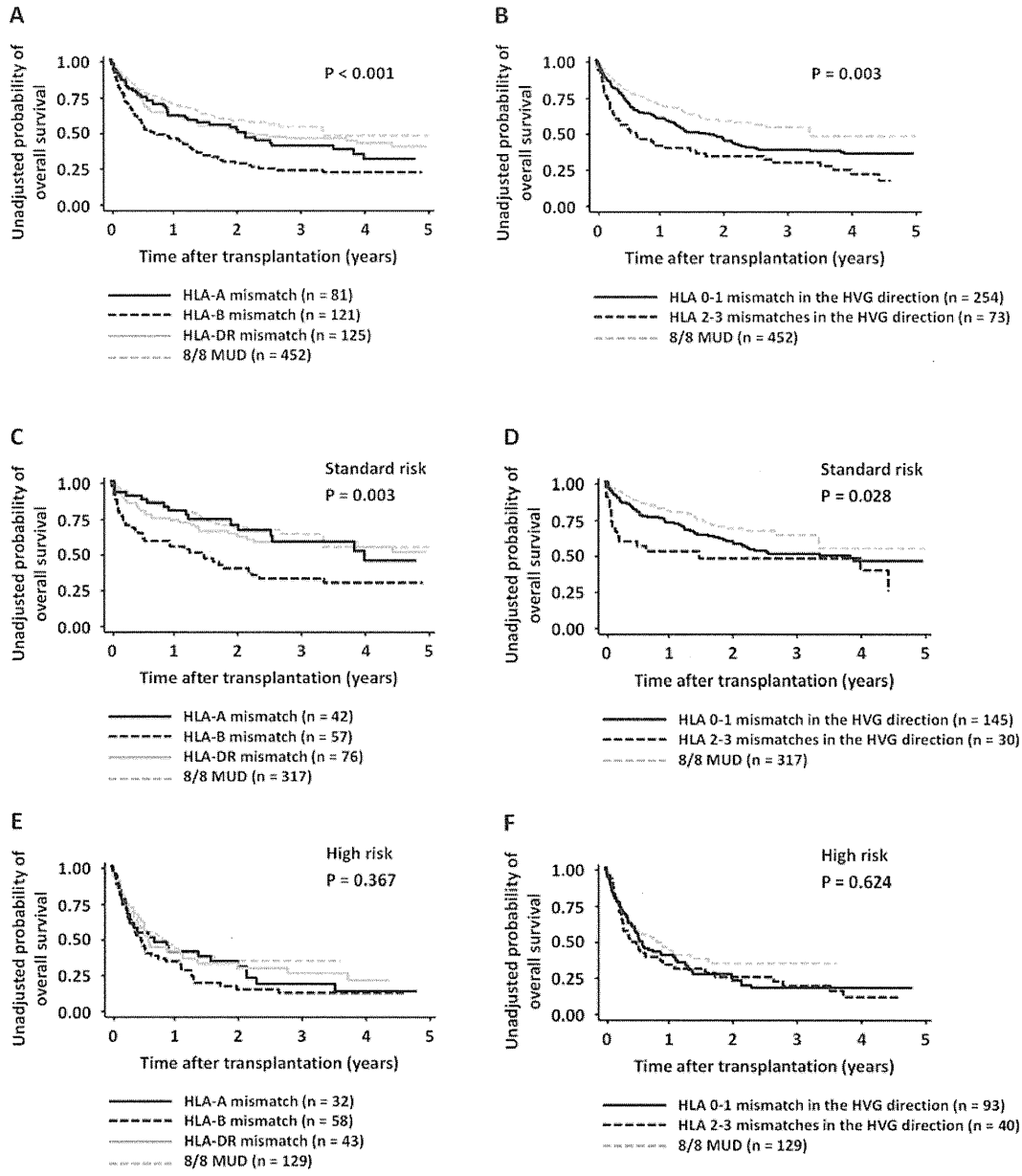
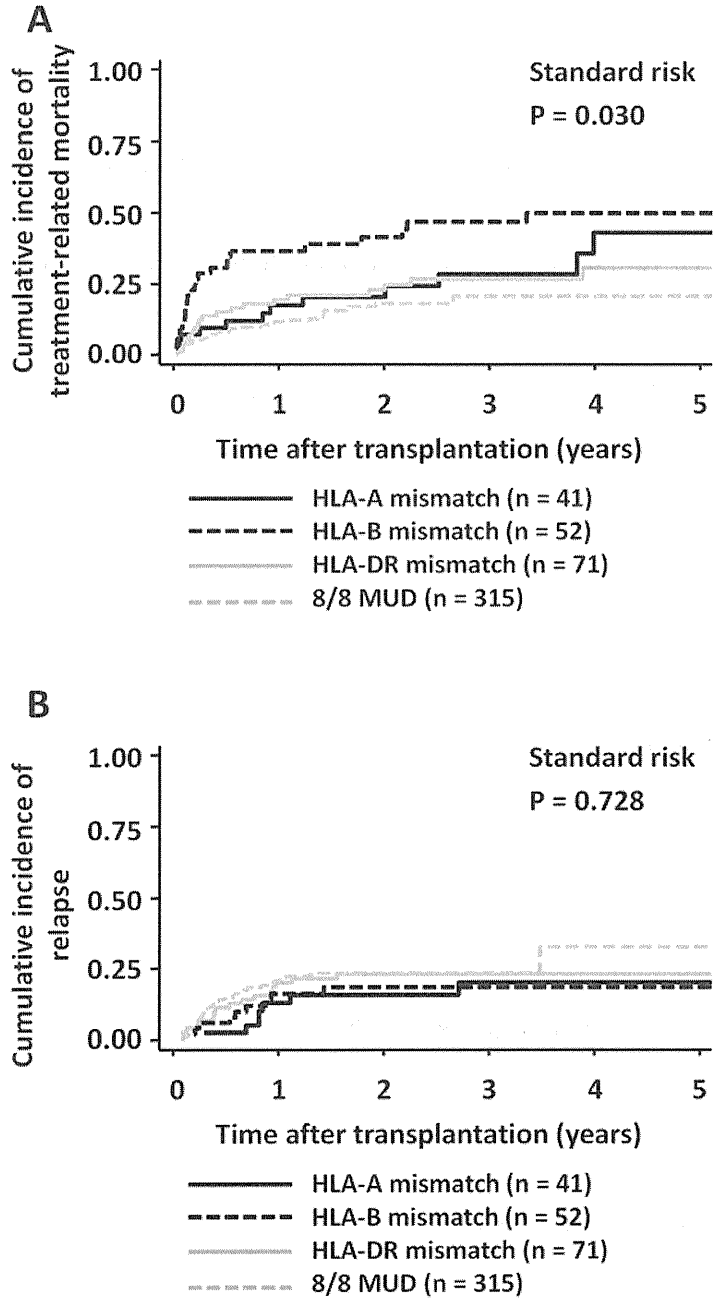


Figure 3.



L-index as a novel index to evaluate both the intensity and duration of lymphopenia after allogeneic h ematopoietic stem cell transplantation

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Abstract : We retrospectively investigated L-index, which evaluates both the intensity and duration of lymphopenia after allogeneic hematopoietic stem cell transplantation (HSCT) (n = 50). L-index was defined as the area over the lymphocyte curve during lymphopenia (absolute lymphocyte count <700/μl). We calculated the L-index from the start of conditioning to day 30 (L-index(30)) and to day 100 (L-index(100)) after HSCT. Multivariate analysis revealed that human leukocyte antigen mismatched donor, female sex, and non-lymphoid disease were significantly associated with high L-index(30). Grade III–IV acute graft versus host disease, alemtuzumab-containing regimen, and non-lymphoid disease were identified as independent significant factors for high L-index(100). Cytomegalovirus (CMV) antigenemia was detected more than 3 cells/2 slides by C10/11 method in 30 patients (CMV-AG ≥ 3 group) and was not detected in 20 patients (CMV-AG < 3 group). Although there was no significant difference in absolute lymphocyte count on day 30 between the 2 groups, the L-index(30) was significantly higher in CMV-AG ≥ 3 group than in CMV-AG < 3 group (P = 0.050). L-index(30) was identified as an independent factor on CMV reactivation in multivariate analysis when it was dealt as dichotomous variable with a cut-off value of 22,318 determined by receiver operating characteristic curve analysis. In conclusion, both the intensity and duration of lymphopenia in early phase after HSCT evaluated on the basis of L-index(30) showed significant association with CMV reactivation.

Keywords: L-index; lymphopenia; absolute lymphocyte count; allogeneic hematopoietic stem cell transplantation; cytomegalovirus antigenemia

Lymphocyte reconstitution plays an important role in preventing opportunistic infections and attacking residual tumor cells after allogeneic hematopoietic stem cell transplantation (HSCT) (1). Early lymphocyte recovery after allogeneic HSCT is associated with low risk of opportunistic infections, treatment-related mortality, and relapse (2-8). Further, low absolute lymphocyte count (ALC) impairs T cell tolerance and contributes to the development of graft-versus-host disease (GVHD) (3, 4). In clinical settings, lymphocyte reconstitution is based on ALC and lymphocyte subset analysis by flow cytometry (9). However, the duration of lymphopenia is not considered in these analyses as the parameters are measured from the sample obtained at a single point.

We developed the L-index as a novel parameter to evaluate lymphopenia after allogeneic HSCT. The L-index is based on a graph of ALC after starting conditioning regimen, and it is calculated as the area over the lymphocyte curve during lymphopenia (Fig. 1). The L-index can be used to evaluate the dynamics of lymphopenia, including its intensity and duration. Furthermore, the L-index can be calculated based only on serial ALC, which is determined in daily clinical practice. We calculated the L-index(30) and L-index(100), i.e., the cumulative L-indexes from the beginning of conditioning regimen until day 30 and day 100 after HSCT, respectively, and investigated the clinical and epidemiological factors that influenced the L-index. In addition, we analyzed the association between the L-index and

cytomegalovirus (CMV) reactivation after allogeneic HSCT. Lymphopenia is known as a risk factor for CMV infection (2, 10, 11). We compared the L-index with other lymphopenia parameters such as ALC, $CD4^+$ cell count, and $CD8^+$ cell count and determined its effect on CMV reactivation.

Patients and methods

Patients

We retrospectively reviewed the charts of consecutive patients who underwent allogeneic HSCT at our center between July 2007 and May 2009. Patients who died within 100 days after transplantation were excluded. Among 54 patients who underwent allogeneic HSCT during this period, 4 were excluded due to early death. Finally, 50 patients were included in this study. The clinical and epidemiological characteristics of the patients are shown in Table 1.

L-index and other lymphopenia indexes

The L-index was calculated based on a graph of ALC from the beginning of conditioning to 100 days after HSCT and a horizontal straight line at the cut off value of lymphopenia (ALC 700/μl) (Fig.1). We defined lymphopenia as ALC < 700/μl because the median number of ALC at day 90 was 754/μl in this study; therefore, more than half of the patients achieved ALC ≥ 700/μl by day 100 after HSCT. The L-index (Ae – Ao) was calculated as the difference between the observed area under curve (Ao), which was calculated by the trapezoidal method, and the expected lymphocyte area (Ae; 700/μl × days with lymphopenia) if the patient did not develop lymphopenia. We calculated L-index from the start of conditioning

regimen until day 30 [L-index(30)] and day 100 [L-index(100)] after transplantation. Because the L-index is calculated in the fixed period, it is inversely correlated with the area under the lymphocyte curve. However, when ALC was maintained at more than 700/μl for a certain period after conditioning or during early lymphocyte recovery where ALC of more than 700/μl was achieved, the L-index and area under the lymphocyte curve will be apart. Since we thought it was important to evaluate lymphocyte deficit, we did not use the area under the lymphocyte curve and the L-index was calculated by using the area over the lymphocyte curve. We investigated the clinical and epidemiological factors that could have influenced the L-index(30) and L-index(100). We also evaluated ALC, $CD4^+$ cell count, and $CD8^+$ cell count at 30, 60, and 90 days after HSCT as lymphopenia indexes and compared these parameters with the L-index to determine their influence on CMV reactivation.

Transplantation procedure

Myeloablative conditioning was mainly a combination of cyclophosphamide therapy and either total body irradiation (TBI) (n = 28) or busulfan treatment (n = 1) (12). A combination of high-dose cytarabine and TBI was used in 1 patient. Fludarabine-based reduced-intensity regimens, such as fludarabine combined with busulfan (13) or melphalan (14), were used in elderly or clinically infirm patients (n = 13). Patients with severe aplastic anemia received

fludarabine, cyclophosphamide, and anti-thymoglobulin (ATG), with or without low-dose TBI at 2 Gy (n = 7) (15). Alemtuzumab-containing regimens used in HSCT were obtained from a 2- or 3-antigen-mismatched donor (n = 4) (16).

GVHD prophylaxis consisted of continuous infusion of cyclosporine A (CsA) or tacrolimus (FK506) combined with short-term methotrexate (10–15 mg/m^2 on day 1, 7–10 mg/m^2 on days 3 and 6, and optional dose on day 11) (17). The dose of CsA was adjusted to maintain the blood CsA concentration between 450 and 550 ng/ml (CsA500) in standard-risk patients (n = 30) or between 250 and 350 ng/ml (CsA300) in high-risk patients (n = 19) (17). FK506 used in HSCT was from an unrelated donor (n = 1) and the target concentration was 15 ng/ml. Acute GVHD was graded as previously described (18).

Prophylaxis against bacterial, fungal, and Pneumocystis jiroveci infection consisted of fluoroquinolones; fluconazole, or itraconazole; and sulfamethoxazole/trimethoprim or inhalation of pentamidine, respectively. As a prophylactic measure against herpes simplex virus infection, acyclovir was administered from days –7, followed by the long-term low-dose administration of acyclovir for varicella zoster reactivation (19).

CMV antigenemia assay and p re-emptive therapy for CMV i nfection

CMV antigenemia assay was performed by C10/11 method (20). A randomized controlled

trial in Japan showed that C10/11 antigenemia assay with a cut-off value of 3 cells per 2 slides showed higher sensitivity than CMV plasma real-time PCR with a cut-off value of 300 copies /ml in bone marrow transplant recipients from an unrelated donor (21).

Pre-emptive therapy with ganciclovir was administered for CMV infection and CMV antigenemia was monitored every week after leukocyte recovery (22, 23). To start ganciclovir at an induction dose of 5 mg/kg, we established that the threshold of CMV antigenemia was 20 cells/2 slides in HSCT from an HLA -matched related donor and 3 cells/2 slides in HSCT from an HLA-mismatched related donor, an unrelated donor, and umbilical cord blood; however, 10 mg/kg/day ganciclovir was started when HSCT patients had received alemtuzumab-containing regimen and CMV antigenemia was detected in more than 1 cell/2 slides. The dose of ganciclovir was adjusted according to the renal function (24).

Statistical considerations

Dichotomous variables were compared using Fisher's exact test, and continuous variables were compared using the Mann-Whitney U test. The influence of clinical and epidemiological factors on the L-index and the association between various factors and CMV reactivation were first analyzed by univariate analysis, and then factors with at least

borderline significance ($P < 0.15$) were subjected to multivariate analysis by multiple regression modeling or logistic regression analysis. To assess the ability of the lymphopenia indexes to predict CMV reactivation, we performed a receiver operating characteristic (ROC) curve analysis. The cut off P-value was set at 0.05.

Results

Epidemiological and clinical factors that influence the L-index

The median values of the L-index(30) and the L-index(100) were 21,081 (range: 8,757–26,512) and 29,987 (range: 8,757–65,789), respectively. The epidemiological and clinical factors associated with high L-index(30) and the L-index(100) values are shown in Table 2. Univariate analysis revealed that female sex, non-lymphoid disease, alemtuzumab-containing regimen, unrelated donor, HLA-mismatched donor, and bone marrow transplantation (BMT) influenced the L-index(30) with at least borderline significance. Multivariate analysis revealed that among the above-mentioned factors, HLA-mismatched donor, female sex, and non-lymphoid disease were the independent significant factors ($P = 0.010$, $P = 0.019$, and $P = 0.042$, respectively). Further, univariate analysis revealed that female sex, non-lymphoid disease, ATG-containing regimen, alemtuzumab-containing regimen, HLA-mismatched donor, BMT, and grade III–IV acute GVHD were associated with higher value of the L-index(100) with at least borderline significance. Multivariate analysis revealed that grade III–IV acute GVHD, alemtuzumab-containing regimen, and non-lymphoid disease were the independent significant factors ($P = 0.003$, $P = 0.002$, and $P = 0.003$, respectively).

CMV reactivation

We divided patients into 2 groups and established the threshold of CMV antigenemia-positive cells as 3 cells/2 slides to eliminate the influence of pre-emptive therapy with ganciclovir. Four patients who were conditioned with alemtuzumab-containing regimen received intensive therapy, i.e., ganciclovir was started if CMV antigenemia was detected in more than 1 cell/2 slides. However, in all these 4 patients, antigenemia-positive cells exceeded 3 cells/2 slides.

Among 50 patients, 30 patients (CMV-AG ≥ 3 group) showed CMV antigenemia in more than 3 cells/2 slides and 20 patients (CMV-AG < 3 group) did not show CMV antigenemia (Table 1). The median number of days between HSCT and the day when antigenemia-positive cells exceeded 3 cells/2 slides was 29 (range: 13–61). The median CMV load when CMV antigenemia was detected in more than 3 cells/2 slides for the first time was 13 (range: 3–3,468). On the other hand, the median number of days between HSCT and the day when CMV load reached the maximum level was 51.5 (range: 17– 100). The median maximum load of antigenemia-positive cells was 20.5 (range: 3–3,468). In univariate analyses, the percentage of unrelated donors was significantly higher in CMV-AG ≥ 3 than CMV-AG < 3 group (P = 0.047). With regard to GVHD prophylaxis, CsA300 was more extensively used in CMV-AG ≥ 3 group than in CMV-AG < 3 group (P = 0.032). The

median age tended to be higher in CMV-AG ≥ 3 group than in CMV-AG < 3 group (P = 0.062). When age was dealt as dichotomous variable with the use of the median age of 41 as threshold, the difference between the 2 groups became statistically significant (P = 0.043). Alemtuzumab-containing regimen was tended to be used more frequently in CMV-AG ≥ 3 group than in CMV-AG < 3 group (P = 0.083). There was no significant difference in the development of acute and chronic GVHD or corticosteroid use between the 2 groups.

Evaluation of lymphopenia indexes and CMV reactivation

Association between lymphopenia indexes and CMV reactivation is shown in Table 3. Although there was no significant difference in ALC between the 2 groups at day 30, the L-index(30) was significantly higher in CMV-AG ≥ 3 group than in CMV-AG < 3 group (median 22,030 vs. 19,038; P = 0.050). As for the area under the lymphocyte curve from the conditioning regimen to day 30, there was no significant difference between the 2 groups (median 3,749 vs. 5,741; P = 0.166). Lymphocyte subset analysis showed that $CD4^+$ cell count at day 30 was significantly lower in CMV-AG ≥ 3 group than in CMV-AG < 3 group (P = 0.023), and $CD8^+$ cell count at day 90 was significantly higher in CMV-AG ≥ 3 group than in CMV-AG < 3 group (P = 0.041). With regard to the L-index(100) and other lymphopenia indexes, there was no significant difference between the 2 groups.

We performed ROC curve analyses on L-index(30) and $CD4^+$ cell count at day 30, which revealed that the L-index(30) and $CD4^+$ cell count at day 30 showed similar ability to predict CMV reactivation (Fig.2). The area under the ROC curve was 0.68 and 0.71 for the L-index(30) and $CD4^+$ cell count at day 30, respectively. The sum of the sensitivity and specificity reached the maximum when the thresholds for L-index(30) and $CD4^+$ cell count at day 30 were 22,318 and 59, respectively. With the use of these cut-off values, the sensitivity and specificity for predicting CMV infection were 50.0% and 85.0%, and 47.8% and 88.9%, respectively.

Factors ass ociated with CMV reactivation

Univariate and multivariate analyses as to possible factors associated with CMV infection are shown in Table 4. There were significantly larger number of patients with L-index(30) ≥ 22,318 in CMV-AG ≥ 3 group than in CMV-AG < 3 group in univariate analysis (P = 0.016). In multivariate analysis, L-index(30) and patients' age were identified as independent significant factors (odds ratio; 6.71 and 4.45, P = 0.0141 and 0.0263, respectively). The use of alemtuzumab was excluded in this multivariate analysis, because it was closely correlated with L-index.

Discussion

A tool to measure both the intensity and duration was first proposed to evaluate neutropenia (24). The D-index calculated as the area over the neutrophil curve during neutropenia was reported to be useful for predicting invasive mold infection in acute myelogenous leukemia patients who received induction chemotherapy (25), and in HSCT recipients who developed pulmonary infection (26). Similar to the D-index, we developed the L-index to measure both the intensity and duration of lymphopenia after allogeneic HSCT. In order to evaluate the lymphopenia during early and middle period after HSCT, respectively, we calculated the L-index from the beginning of conditioning to 30 days and to 100 days after transplantation.

Among the factors that affected the L-index, non-lymphoid disease was significantly associated with high values of both the L-index(30) and L-index(100). In other words, lymphocyte recovery was better in patients with lymphoid malignancy than in those with non-lymphoid disease. Savani et al. also reported that ALC at day 30 after HSCT was higher in patients with acute lymphoblastic leukemia compared to that in patients with acute myeloblastic leukemia or chronic myelogenous leukemia and in T cell-depleted allogeneic HSCT patients (3). The plausible explanation of this phenomenon was that patients with lymphoid malignancy had received intensive treatment that suppressed host lymphocytes before HSCT, which allowed the easy expansion of donor lymphocytes after HSCT. However,

whether this meant that lymphocyte immune function was better in patients with lymphoid malignancy than in those with non-lymphoid disease was unclear. Other factors associated with high L-index(30) values included HLA-mismatched donor and female sex. The former suggested that HLA incompatibility might have an inhibitory effect on lymphocyte recovery in early phase after HSCT. Although multivariate analysis suggested that the use of alemtuzumab was not a significant factor, its use in HSCT from a 2- or 3-antigen-mismatched donor might lead in part to a high L-index(30) value in HLA-mismatched donor. As for the effect of female sex on L-index(30), the reason or mechanism was undetermined. Further, grade III–IV acute GVHD was associated with high value of the L-index(100). Acute GVHD inhibits T cell recovery through T-cell apoptosis due to overexpression of death receptors and underexpression of prosurvival protein and through direct damage to thymic epithelium and stroma (1, 27). In addition to the effect of GVHD, corticosteroid use for the treatment of GVHD may also affect lymphocyte recovery (27). Our result showed that grade III–IV acute GVHD was a significant independent risk factor for high L-index(100) values, which suggested that severe acute GVHD itself delayed lymphocyte recovery. Although delayed or early lymphocyte recovery was reported to be an independent factor associated with more acute GVHD (3, 4), no association between the L-index(30) or ALC at day 30 and acute GVHD was found in the present study. The higher