

Original Article

The clinical impact of absolute lymphocyte count in peripheral blood among patients with methotrexate - associated lymphoproliferative disorders

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Regressive lymphoproliferative disorders (R-LPD) after methotrexate (MTX) withdrawal are one of the specific features of methotrexate - associated lymphoproliferative disorders (MTX-LPD). Although the impact of the absolute lymphocyte count (ALC) on the pathogenesis of R-LPD has been recently emphasized, understanding relapse/regrowth events (RRE) and differences among LPD subtypes is necessary. In this study, we confirmed ALC recovery in the regressive group (R-G; R-LPD without RRE) and relapse/regrowth group (R/R-G; R-LPD with RRE). The increase in ALC lasted at least 2 years in R-G, whereas it decreased within 3 years in R/R-G, supporting the better overall survival (OS) in R-G, as previously reported. In addition, our study suggested that an ALC of 1000/ μ L at the time of development of LPD is a significant predictor for treatment-free survival (TFS). Furthermore, an ALC of 1000/ μ L at 6 months after MTX withdrawal was found to be a significant indicator of TFS and OS for R-G and R/R-G. The ALC decreased gradually before LPD development in R/R-G, whereas it decreased 6 months before LPD development in R-G, confirming the important role of ALC in the pathogenesis of MTX-LPD such as regressive events and RRE. In addition to ALC, other predictive factors, such as serum C-reactive protein and soluble interleukin-2 receptors, may be helpful in the management of MTX-LPD, including the decision making for an additional chemotherapy for regressive LPD after MTX withdrawal.

Keywords: methotrexate, lymphoproliferative disorders, rheumatoid arthritis, relapse/regrowth, absolute leukocyte count

INTRODUCTION

Methotrexate-associated lymphoproliferative disorders (MTX-LPD), which are LPD developing upon MTX administration, have been investigated for over 20 years,¹⁻¹⁹ although the 2017 World Health Organization (WHO) classification of tumors groups these with other iatrogenic immunodeficiency-associated LPD.¹ Low-dose MTX is known to induce LPD in patients with autoimmune disorders (AID), especially those with rheumatoid arthritis (RA),¹⁻¹⁹ which has been described as a MTX-LPD in RA since the first report of regression phenomenon in MTX-LPD in 1993.² Previous reports suggested that the complex pathogenesis of MTX-LPD


is mediated by factors such as chronic inflammation with basal AID,^{10,20} immunosuppressive state caused by anti-AID drugs²¹⁻²³ and Epstein-Barr virus (EBV) impairment,^{24,25} and genetic factors.¹¹ Although MTX-LPD have complex pathogenesis mechanisms, two-third of patients with MTX-LPD exhibit LPD regression after MTX withdrawal (regressive-LPD, R-LPD). Several recent studies suggested the important role of lymphocytes in the pathogenesis of R-LPD:¹⁵⁻¹⁸ the recovery of lymphocytes in peripheral blood (PB) may be related to the R-LPD phenomenon. We previously proposed three different clinical courses according to the characteristics of R-LPD: the regressive group (R-G), in which R-LPD develops without relapse-regrowth events (RREs); relapse-regrowth

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group (R/R-G), in which RRE develop after R-LPD; and persistent group (P-G), in which LPD is persistent after MTX withdrawal without R-LPD^{5,7,11} (Figure 1). Thus, R-LPD is observed in R-G and R/R-G. In the clinical setting, the decision making for an additional chemotherapy is an important issue when LPD regresses after MTX-LPD: patients in R-G do not need therapy, whereas patients in R/R-G require chemotherapy with careful follow-up after LPD regression. In this retrospective study, we analyzed the clinical data in terms of the changes in absolute lymphocyte count (ALC) in PB according to these three clinical patterns and different LPD associated with MTX. As previous studies analyzed the changes in ALC in both R-G and R/R-G as an R-LPD,¹⁵⁻¹⁸ this investigation focused on the three clinical patterns to reveal the pathogenesis of MTX-LPD development and RRE, in addition to the treatment strategy.

PATIENTS AND METHODS

Patients

Data were collected from 33 patients with RA who developed LPD and were treated at our institution between 2004 and 2017. All patients were Japanese and received MTX. We thus described them as having MTX-LPD in this study. We categorized the patients into three clinical patterns after MTX withdrawal (R-G, R/R-G, and P-G, as indicated in Figure 1). In this study, the patients with an observation period after MTX withdrawal of 2 or more years were

selected in R-G to exclude the possibility of RRE, based on the findings that RRE commonly develop within 1-2 years after LPD regression due to MTX withdrawal.^{5,7-9,11,12}

Immunohistochemistry and in situ hybridization

All diagnoses of LPD were confirmed by immunohistochemistry performed on paraffin-embedded tissue sections using a panel of monoclonal antibodies, as we reported previously.^{7,11} Tissue sections were tested for the presence of EBV by in situ hybridization for Epstein-Barr virus-encoded small RNA -1 (EBER-1) using the REMBRANDT Detection Kit (Zymed, San Francisco, CA). All pathological samples were reviewed by two pathologists among the authors, and were classified and diagnosed according to the 2017 WHO classification.¹ Six patients exhibited a non-specific pattern without polyclonal or monoclonal expansion features and were described as having LPD. As the test for EBER-1 was unable to be performed for one patient as it was a consulted case, the data for this patient were combined into diffuse large B-cell lymphoma (DLBCL)—not otherwise specified (NOS) in this analysis.

White blood cell count and ALC during the clinical course

We analyzed the white blood cell (WBC) count and ALC in the PB of 33 patients during the clinical course. In all patients, the ALC was measured at the time of MTX withdrawal due to LPD development (0M) and 1 month after MTX withdrawal (1M). For R-G, the time points of 6 months after MTX withdrawal (6M) and 24 months after

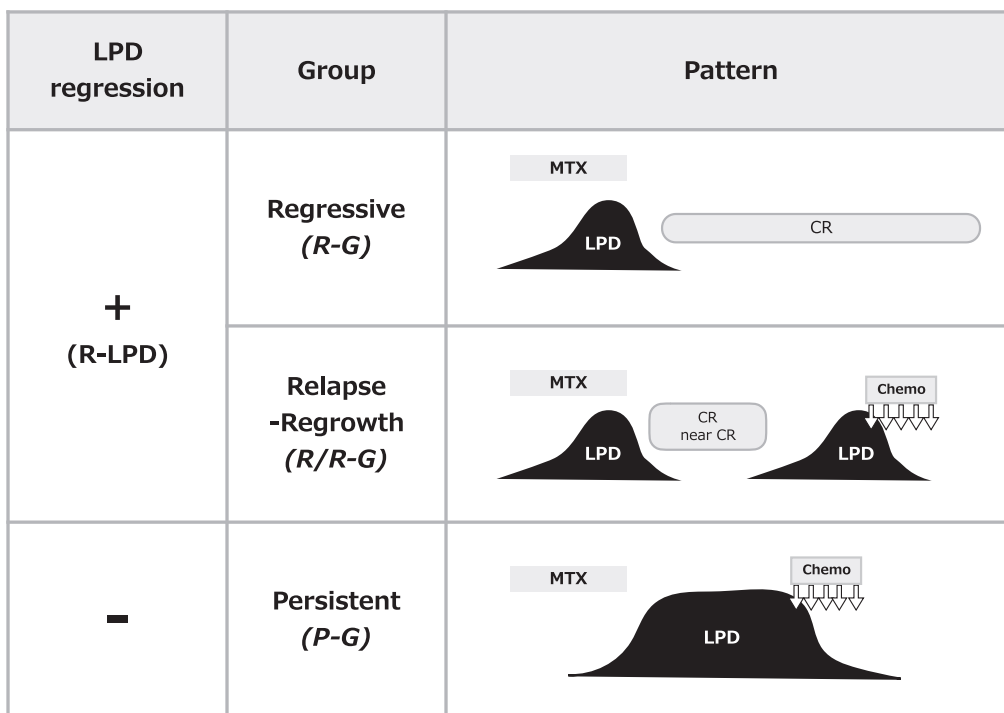


Fig. 1. Three clinical courses of methotrexate-associated lymphoproliferative disorders (MTX-LPD). Three clinical courses are observed in patients with MTX-LPD after MTX withdrawal: regressive LPD (R-LPD) without relapse/regrowth events (RRE, regressive group, R-G), R-LPD with RRE (R/R-G), and persistent LPD (P-G). R-LPD is observed in R-G and R/R-G. CR, complete response; Chemo, chemotherapy.

MTX withdrawal (24M) were added. For R/R-G, the time points of 6M and RRE were added. Furthermore, the changes in the WBC count and ALC were assessed by collecting the data of 15 patients before the development of LPD. The ALC was obtained at five time points before LPD development: 3, 6, 12, and 24 months before LPD development (-3M, -6M, -12M, and -24 M, respectively), and at the time of starting follow-up at our institution at least 36 months before LPD development (ST).

Statistical analysis

The duration of underlying RA was defined as the day of diagnosis of RA to the time of MTX withdrawal. Treatment-free survival (TFS) was defined as the interval between 0M and the time of treatment initiation for LPD or the last follow-up without therapy for LPD. Overall survival (OS) was defined as the interval between 0M and death from any cause or the last follow-up. The WBC count and ALC were compared by the Wilcoxon signed rank test, Friedman test, Mann-Whitney U test, and Kruskal-Wallis test using EZR software (Tokyo, Japan).²⁶ Survival analysis and comparisons between groups were performed using the Kaplan-Meier procedure with EZR software.

RESULTS

Clinical background of MTX-LPD

All patients developed LPD upon MTX administration. The median age of patients at the time of LPD diagnosis was 65 (range, 36–86) years. In total, 13 men and 20 women were included in this study. The basal disease of all patients was RA. The median durations of RA and MTX treatment were 10.7 (1.3–39.0) and 6.9 (0.8–33) years, respectively. Histopathological analysis of the LPD revealed 19 cases of DLBCL (including 9 that were EBV+ and 10 classified as –NOS), 9 cases of classic Hodgkin lymphoma (cHL), and 5 cases of LPD.

Details of each LPD and the three clinical patterns

The features of each LPD are shown in Table 1. Different populations were associated with different clinical

courses. For example, EBV+DLBCL was associated with a higher percentage of R-G (56%) relative to other populations (22%, R/R-G and P-G). The highest percentages among DLBCL—NOS patients were R-G and P-G (40% each), whereas R/R-G was 20%. In contrast, the majority of cHL patients were R/R-G (89%), with one patient in P-G (11%). All LPD patients were R-G. Among the 33 patients, the percentages of R-G, R/R-G, and P-G were 41%, 35%, and 24%, respectively. The median TFS in each group (R-G, R/R-G, and P-G) was 5.2 (2.7–8.9), 1.2 (0.7–3.4), and 0.2 (0.0–1.6) years, respectively, and the median OS was 5.2 (2.7–8.9), 3.4 (0.8–8.8), and 1.9 (0.5–4.6) years, respectively.

The survival rate among all patients was 73% (24/33). All patients in R-G were alive without RRE. Among the 9 patients who died, 6 and 3 patients belonged to R/R-G and P-G, respectively. Regarding LPD subtypes, 7 had cHL, and 1 each had EBV+DLBCL and DLBCL—NOS.

WBC count and ALC at 0M and 1M in the three clinical patterns

The comparison of WBC count and ALC in each clinical pattern at 0M and 1M is shown in Figure 2. The median WBC count at 0M in R-G, R/R-G, and P-G was 5950/ μ L (range, 3600–11170/ μ L), 7100/ μ L (range, 3800–11900/ μ L), and 6250/ μ L (range, 1700–12770/ μ L), respectively. No significant difference was detected among the three groups (R-G vs R/R-G, $p=0.517$; R-G vs P-G, $p=0.949$; P-G vs R/R-G, $p=0.999$). Conversely, the median ALC at 0M in R-G, R/R-G, and P-G was 1146/ μ L (range, 517–1647/ μ L), 570/ μ L (range, 248–902/ μ L), and 780/ μ L (range, 324–1491/ μ L), respectively, with a significant difference between R-G and R/R-G ($p=0.0002$), but not between R-G and P-G ($p=0.298$) or P-G and R/R-G ($p=0.430$).

Regarding the WBC count and ALC at 1M, significant differences were not detected among the three groups. The WBC count at 1M in R-G, P-G, and R/R-G was 6600/ μ L (range, 4400–10400/ μ L), 6200/ μ L (range, 3500–10300/ μ L), and 7000/ μ L (range, 2200–15000/ μ L), respectively (R-G vs P-G, $p=0.979$; R-G vs R/R-G, $p=0.732$; P-G vs R/R-G, $p=0.786$). The ALC at 1M in R-G, P-G, and R/R-G was 1516/ μ L (range, 713–2860/ μ L), 1174/ μ L (range, 730–1495/ μ L), and 1090/ μ L (range, 490–2100/ μ L), respectively (R-G vs P-G,

Table 1. The clinical outcome of LPD subtypes with three clinical patterns

Subtype	N	Clinical Pattern			Outcome		
		Regressive (R-G)	Regressive/Relapse (R/R-G)	Persistent (P-G)	Outcome		
		N (ratio)	N (ratio)	N (ratio)	Alive	Dead	
				N (ratio)	N (ratio)	Details	
EBV+DLBCL	9	5 (56%)	2 (22%)	2 (22%)	8 (89%)	1 (11%)	1 of P-G
DLBCL–NOS	10	4 (40%)	2 (20%)	4 (40%)	9 (90%)	1 (10%)	1 of P-G
cHL	9	0 (0%)	7 (89%)	2 (11%)	2 (22%)	7 (78%)	1 of P-G, 6 of R/R-G
LPD	5	5 (100%)	0 (0%)	0 (0%)	5 (100%)	0 (0%)	
Total	33	14 (41%)	11 (35%)	8 (24%)	24 (73%)	9 (23%)	6 of R/R-G, 3 of P-G

EBV, Epstein–Barr virus; DLBCL, diffuse large B-cell lymphoma; NOS, not otherwise specified; cHL, classical Hodgkin lymphoma; LPD, lymphoproliferative disorder.

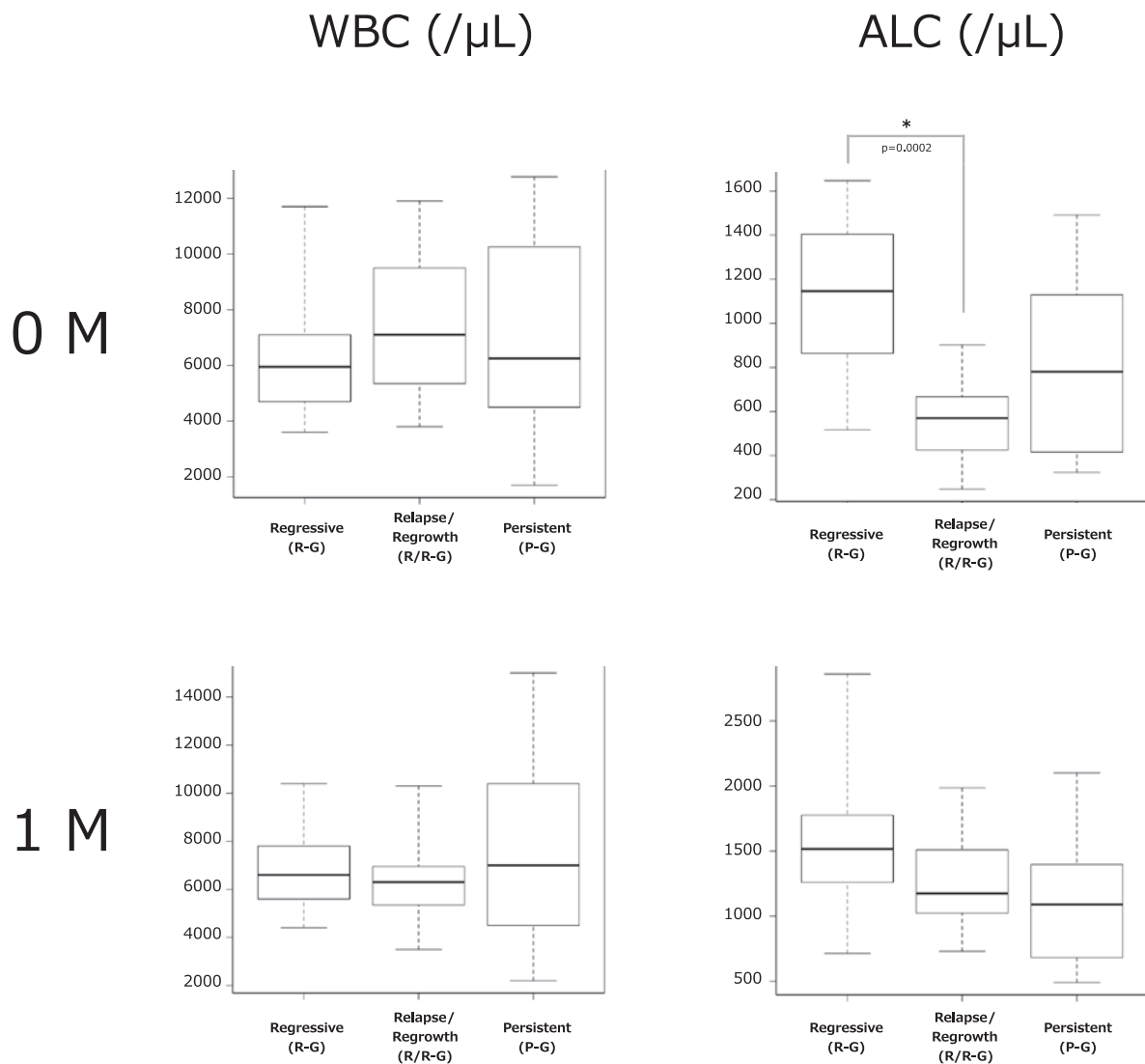


Fig. 2. The relationship between the three clinical patterns and WBC count/absolute leukocyte count (ALC) Regarding the WBC count at the time of methotrexate withdrawal due to the development of lymphoproliferative disorders (LPD, 0M), no significant difference was observed among the regressive group (R-G), relapsed/regrowth group (R/R-G), and persistent group (P-G). In contrast, the ALC in R/R-G was significantly lower than that in R-G ($p=0.0002$). The WBC count and ALC at 1 month after 0M (1M) were not significantly different among the groups.

$p=0.084$; R-G vs R/R-G, $p=0.230$; P-G vs R/R-G, $p=0.786$).

Changes in the WBC count and ALC in the three clinical patterns

Next, we analyzed the changes in the WBC count and ALC over the course of treatment among patients exhibiting the three clinical patterns (Figure 3A, B, and C). No significant increase or decrease in the WBC count was observed for any clinical pattern; the WBC count in R-G at 0M, 1M, 6M, and 24M was $6250 \pm 2021/\mu\text{L}$, $6800 \pm 1608/\mu\text{L}$, $6641 \pm 2105/\mu\text{L}$, and $6250 \pm 1630/\mu\text{L}$, mean \pm SD, respectively (p -values for all comparisons between 2 points are 1) (Figure 3A); the WBC count in R/R-G at 0M, 1M, 6M, and RRE was $7346 \pm 2682/\mu\text{L}$, $6581 \pm 2072/\mu\text{L}$, $6418 \pm 2030/\mu\text{L}$, and $6590 \pm 1992/\mu\text{L}$, respectively (p -values for all comparisons between 2 points were 1) (Figure 3B); the WBC count in P-G at 0M and 1M was $7059 \pm 3747/\mu\text{L}$ and $7625 \pm 4324/\mu\text{L}$,

respectively, $p=1$) (Figure 3C).

In contrast, ALC trends among the three clinical patterns were different. In R-G, the ALC gradually increased over the clinical course; the ALC at 0M, 1M, 6M, and 24M was $1103 \pm 353/\mu\text{L}$, $1575 \pm 565/\mu\text{L}$, $1780 \pm 496/\mu\text{L}$, and $1794 \pm 614/\mu\text{L}$ (significant differences were observed between 0M and 1M, $p=0.0023$, and between 1M and 6M, $p=0.0023$) (Figure 3A). ALC recovery persisted until 24M in R-G. The ALC in R/R-G demonstrated a different pattern from that in R-G (Figure 3B). The ALC at 0M was markedly lower ($559 \pm 196/\mu\text{L}$) and was significantly recovered by 1M ($1249 \pm 398/\mu\text{L}$, $p=0.0059$). Unlike in R-G, the recovered ALC decreased after 1M. The ALC at 6M and RRE was $946 \pm 412/\mu\text{L}$ and $682 \pm 320/\mu\text{L}$, respectively. Although the ALC remained significantly higher at 6M than at 0M ($p=0.015$), it slightly decreased from 1M ($p=0.084$). This decreasing trend continued until the development of RRE,

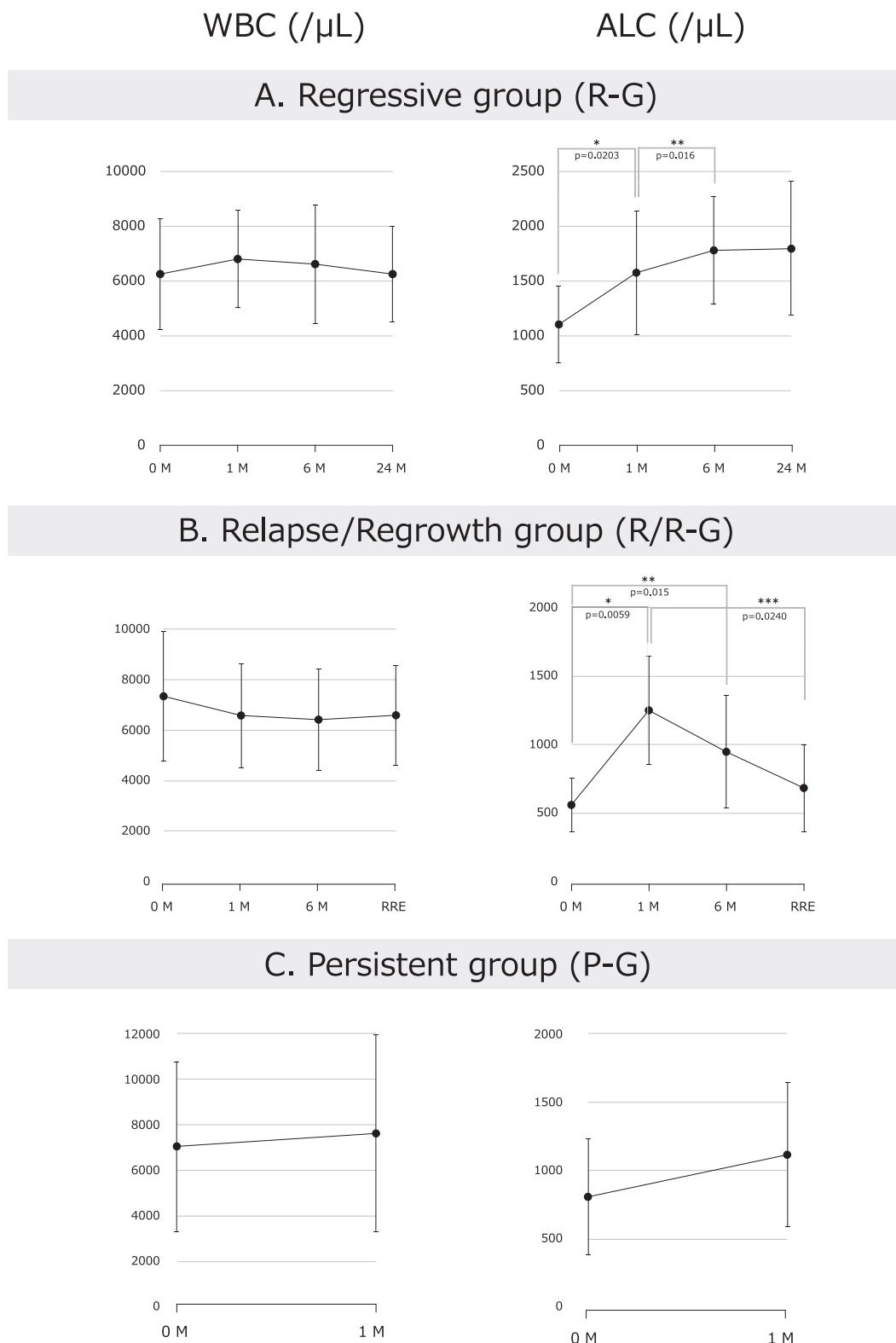


Fig. 3. The changes in WBC count/absolute leukocyte count (ALC) over time among the three clinical patterns. The changes in WBC count and ALC were analyzed among the three clinical patterns in the regressive (R-G), relapsed/regrowth (R/R-G), and persistent groups (P-G). **(A)** R-G: The WBC count was not significantly different among the time points of 0M, 1M, 6M, and 24M. In contrast, the ALC was significantly increased at 1M and 6M compared with the previous time point ($p=0.0203$ and 0.016 , respectively). In addition, the ALC continued to increase until 24M. **(B)** R/R-G: The WBC count was also not significantly different among the time points of 0M, 1M, 6M, and RRE. Although the ALC significantly increased at 1M from that at 0M ($p=0.0059$), it began to decrease after 1M. Similarly, although the ALC at 6M was significantly higher than that at 0M ($p=0.015$), it significantly decreased at RRE compared with at 1M ($p=0.0240$). **(C)** P-G: The WBC count and ALC were not significantly different between 0M and 1M, although the ALC slightly increased ($p=0.1$).

reaching a significantly different ALC from that at 1M ($p=0.0240$). In P-G, the ALC increased at 1M from that at 0M ($1115\pm 531/\mu\text{L}$ and $808\pm 425/\mu\text{L}$, respectively) without a significant difference ($p=0.195$) (Figure 3C).

WBC count and ALC in each pathological phenotype LPD

Regarding the differences among each LPD, the WBC count and ALC at 0M and 1M were compared. The WBC counts in DLBCL—NOS, EBV+DLBCL, cHL, and LPD were not significantly different at 0M ($5740\pm 2781/\mu\text{L}$, $7455\pm 2482/\mu\text{L}$, $7819\pm 3139/\mu\text{L}$, and $5900\pm 1251/\mu\text{L}$, mean \pm SD, respectively). Similarly, the ALC at 0M was not significantly different between each LPD ($986\pm 427/\mu\text{L}$, $966\pm 424/\mu\text{L}$, $531\pm 218/\mu\text{L}$, and $900\pm 299/\mu\text{L}$, respectively), although that in cHL was slightly lower than that in DLBCL—NOS ($p=0.068$). At 1M, the WBC count and ALC were not different among each LPD: WBC, $7800\pm 4138/\mu\text{L}$, $6767\pm 665/\mu\text{L}$, $6011\pm 1111/\mu\text{L}$, and $7500\pm 1898/\mu\text{L}$, respectively; ALC, $1511\pm 689/\mu\text{L}$, $1451\pm 545/\mu\text{L}$, $1142\pm 341/\mu\text{L}$, and

$2016\pm 1897/\mu\text{L}$, respectively). Regarding the changes in WBC count and ALC between 0M and 1M among each LPD, a significant difference was only detected in the ALC of cHL ($p=0.00391$) and the ALC of DLBCL—NOS slightly increased ($p=0.0645$), whereas other comparisons produced no significant values (data not shown).

ALC and TFS/OS

As ALC in PB is one of the predictive factors of R-LPD, whether ALC in PB can be used as a clinical indicator for TFS and OS was investigated. Regarding TFS, 13 patients were treatment-free, whereas 20 patients received therapies for LPD. Thus, the 5-year TFS rate was 36.1% (Figure 4A). Next, the efficacy of the ALC cut-off level of $1000/\mu\text{L}$ toward TFS was analyzed. The TFS of patients with an ALC of less than $1000/\mu\text{L}$ at 0M ($N=23$) was significantly poorer than that of patients with an ALC greater than $1000/\mu\text{L}$ at 0M ($N=10$, $p=0.0157$, Figure 4B), although the TFS of the patients with an ALC less than $1000/\mu\text{L}$ at 1M ($N=9$) was not

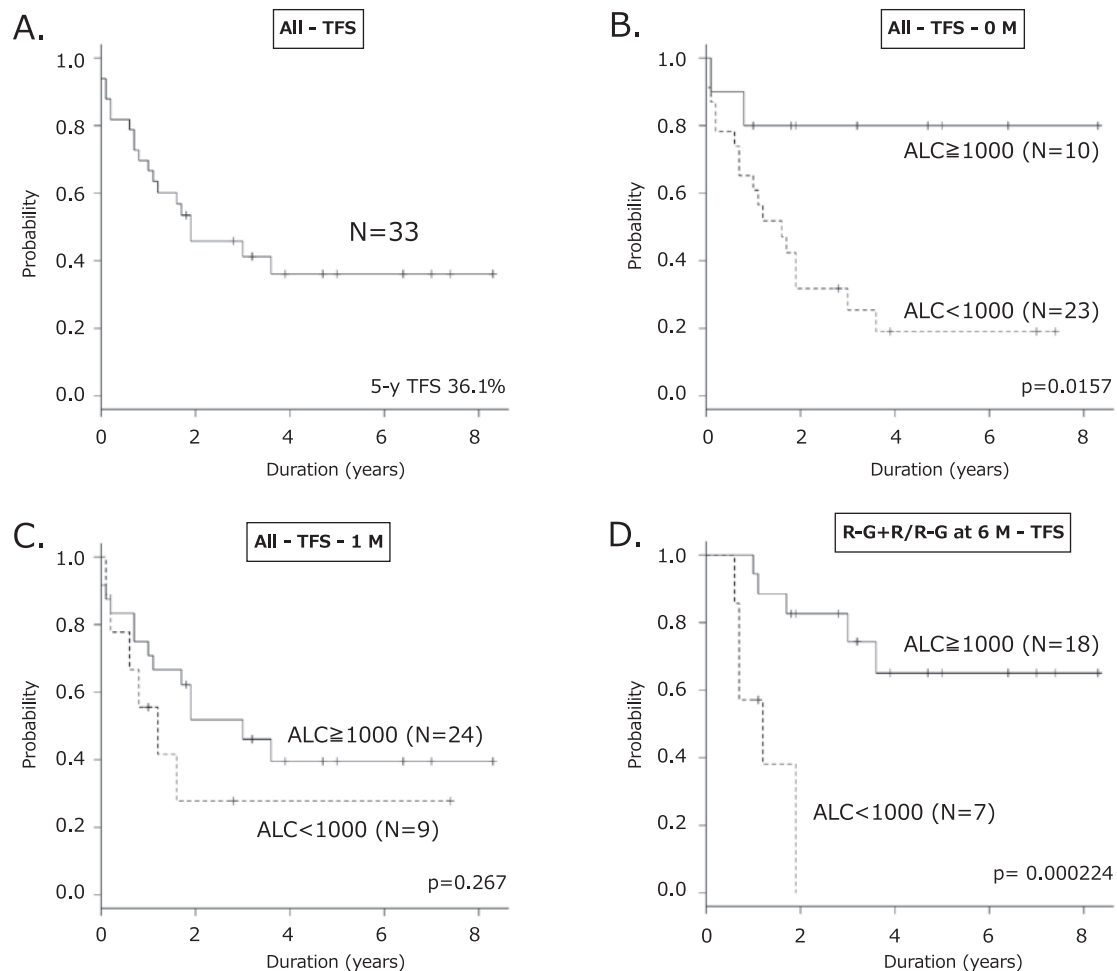


Fig. 4. Impact of the absolute leukocyte count (ALC) on treatment-free survival (TFS) (A) The five-year TFS of all patients ($N=33$) was 36.1%. (B, C) The TFS of patients with an ALC of less than $1000/\mu\text{L}$ at 0M was significantly lower than that of patients with an ALC greater than $1000/\mu\text{L}$ at 0M ($p=0.0157$), although the TFS at 1M was not significantly different between the 2 groups ($p=0.267$). (D) Among the patients with regressive phenomena (regressive group (R-G) and relapse/regrowth group (R/R-G)), those with an ALC less than $1000/\mu\text{L}$ at 6M had a significantly poorer TFS than those with an ALC greater than $1000/\mu\text{L}$ at 6M ($p=0.000224$).

significantly different from that of patients with an ALC greater than $1000/\mu\text{L}$ at 1M ($N=24$, $p=0.267$, Figure 4C). Focusing on the influence of the ALC at 6M, we analyzed the patients with R-LPD in R-G and R/R-G (Figure 4D). The TFS of patients with an ALC of less than $1000/\mu\text{L}$ at 6M ($N=7$) was significantly poorer than that of patients with an ALC greater than $1000/\mu\text{L}$ at 0M ($N=18$, $p=0.000224$).

Regarding OS, 9 patients died and 24 patients survived. The 5-year OS was 64% (Figure 5A). In terms of the ALC cut-off level of $1000/\mu\text{L}$, significant differences in OS were not detected at 0M and 1M among patients with an ALC of less than or greater than $1000/\mu\text{L}$ ($p=0.241$ and $p=0.125$, respectively), although the OS was poorer for patients with an ALC of less than $1000/\mu\text{L}$ at both time points (Figure 5B and 5C). In contrast, an ALC less than $1000/\mu\text{L}$ at 6M influenced the OS of patients with R-LPD in R-G and R/R-G (Figure 5D, $p=0.00116$).

ALC changes before LPD development

For the subsequent analysis, the WBC count and ALC before MTX development were investigated. Fifteen patients in whom the WBC count and ALC were able to be measured from the time of MTX administration over 3 years were selected. The median age, disease duration, and MTX administration duration were 64 years (range, 52–84), 12.1 years (range, 3.8–32.4), and 6.9 years (range, 3.7–21.3), respectively. Eight patients in R-G and 7 patients in R/R-G were included. Regarding the WBC count, no significant changes were noted among the 5 time points (ST, -24M, -12M, -6M, -3M, and 0M) in both groups. The ALC in R-G and R/R-G were almost similar at ST (Figure 6, $1349\pm 221/\mu\text{L}$ and $1323\pm 509/\mu\text{L}$, $p=0.898$, respectively). On the other hand, the changes in ALC were different between the 2 groups. The ALC in R/R-G decreased gradually toward 0M, and a significant difference was detected at 0M compared with that at ST ($p=0.0313$). However, the ALC in R-G was not significantly different between each time point, although

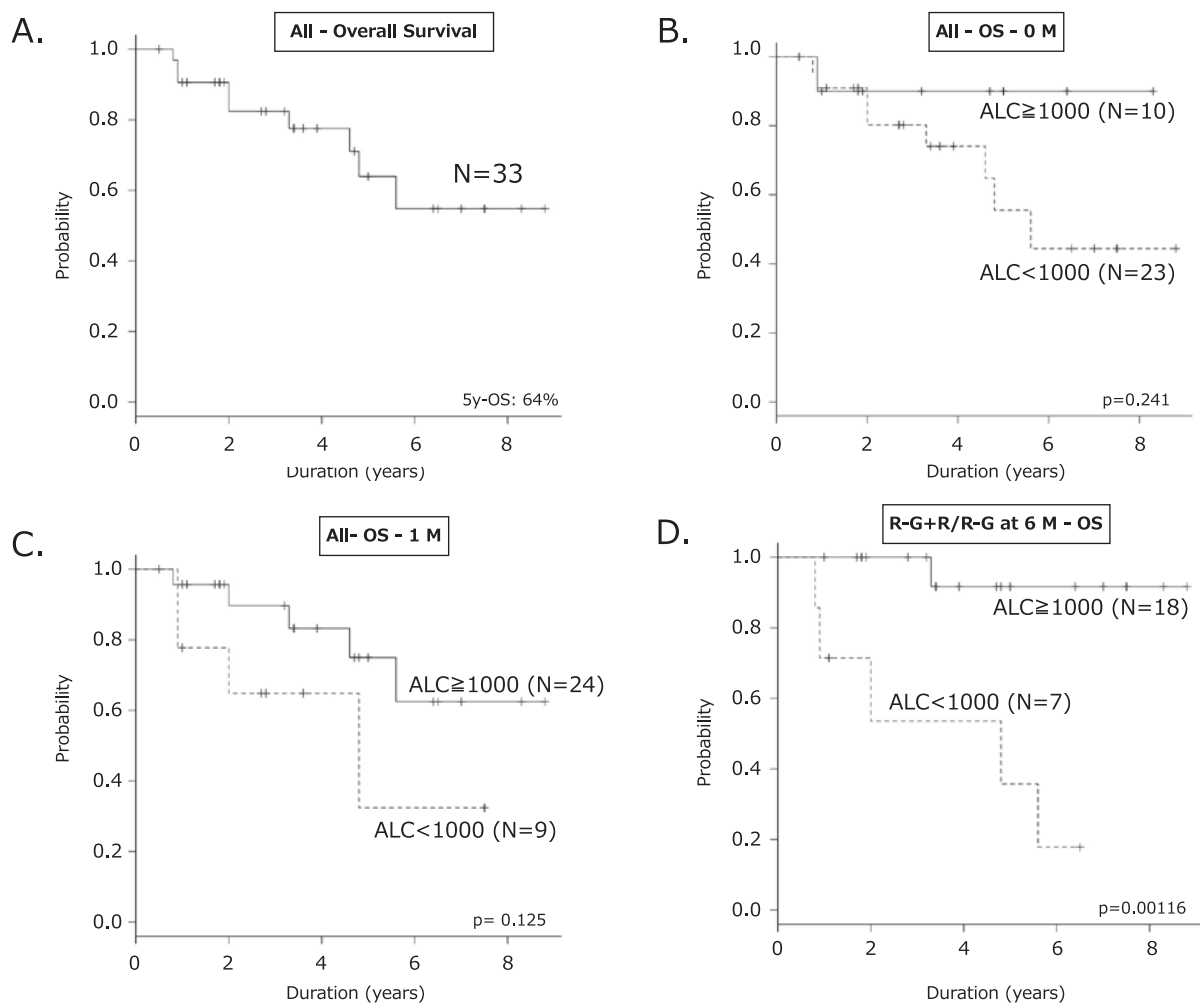


Fig. 5. Impact of the absolute leukocyte count (ALC) on overall survival (OS) (A) The five-year OS of all patients ($N=33$) was 64%. (B, C) Although an ALC of less than $1000/\mu\text{L}$ at 0M and 1M negatively impacted the OS ($p=0.241$ and $p=0.125$, respectively), no significant differences were noted. (D) In contrast, patients with an ALC of less than $1000/\mu\text{L}$ at 6M had a significantly poorer OS than those with an ALC greater than $1000/\mu\text{L}$ at 6M among the patients with regressive phenomena (regressive group (R-G) and relapse/regrowth group (R/R-G), $p=0.00116$).

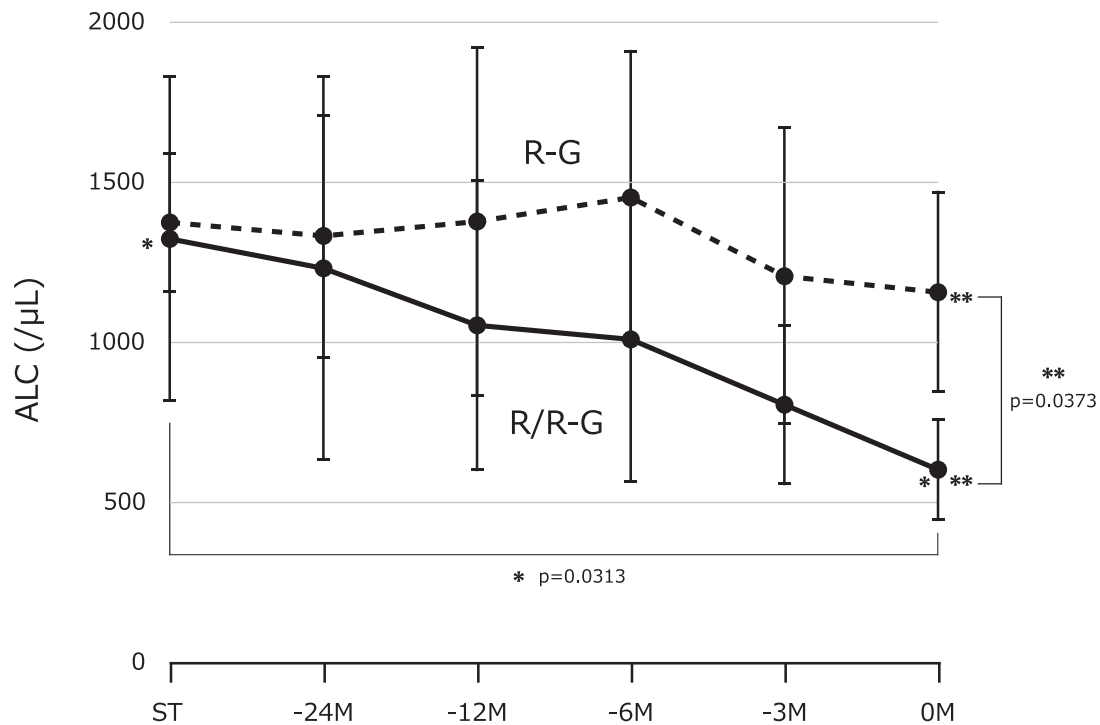


Fig. 6. The absolute leukocyte count (ALC) changes from the time of methotrexate (MTX) initiation. Of the 15 patients whose WBC count/ALC were able to be obtained over 3 years before lymphoproliferative disorder (LPD) development, the ALC was analyzed at times of MTX withdrawal due to LPD development (0M), 3 months before 0M (-3M), 6 months before 0M (-6M), 12 months before 0M (-12M), 24 months before 0M (-24M), and the first measurement at our institution (starting time, ST). The change in ALC in the regressive group (R-G, N=8) exhibited a decreasing trend, and the ALC at 0M was significantly lower than that at ST ($p=0.0313$). On the other hand, no significant difference was detected among each point in R-G. At 0M, the ALC in relapse/regrowth group (R/R-G, N=7) was significantly lower than that in R-G ($p=0.0373$).

it slightly decreased after -6M. The ALC at each point between the 2 groups was not significantly different, except at 0M ($1131\pm 316/\mu\text{L}$ and $602\pm 191/\mu\text{L}$ respectively, $p=0.0373$).

Other influencing factors

Other factors that influenced OS or TFS were investigated by assessing several factors such as age, MTX duration, EBV positivity, LPD subtypes, international prognosis index (IPI), the three clinical patterns, ALC at 0M, serum C-reactive protein (CRP) level in serum at 0M, and soluble interleukin-2 receptor (sIL-2R) level in serum at 0M. In terms of TFS, cHL, IPI (≥ 3 , ALC $< 1000/\mu\text{L}$, CRP > 5 mg/dL, and sIL-2R > 4000 mg/dL were significant on the log-rank test ($p=0.00486$, $p=0.00192$, $p=0.00157$, $p=0.000458$, and $p=0.000534$, respectively). The multivariate analysis with Cox's proportional hazard regression model was performed for these parameters. Thus, 2 parameters of ALC $< 1000/\mu\text{L}$ and sIL-2R > 4000 mg/dL significantly influenced TFS ($p=0.020$ and $p=0.0013$, respectively). Regarding OS, a similar analysis was performed, and 5 parameters were demonstrated to be significant by the log-rank test: age (> 70 -year, $p=0.0478$), IPI (≥ 3 , $p=0.00408$), R/R-G or P-G ($p=0.00495$), CRP (> 5 mg/dL, $p=0.0016$), and sIL-2R (> 4000 mg/dL, $p=0.0275$). However, multivariate analysis using these parameters revealed no significant differences

($p=0.1721$, $p=0.1349$, $p=0.9986$, $p=0.3219$, and $p=0.7091$, respectively).

DISCUSSION

In this retrospective study, several aspects of MTX-LPD, especially focusing on ALC, were investigated. First, the differences between R-G and R/R-G regarding the changes in ALC after MTX withdrawal were assessed. Previous studies confirmed the important role of ALC recovery in PB after MTX withdrawal in patients with a regressive event.¹⁵⁻¹⁸ For example, Inui *et al.* demonstrated a significant difference in ALC between regressive and non-regressive LPD,¹⁵ which was also confirmed by Saito *et al.*¹⁶ However, these previous studies did not separately analyze R-G and R/R-G. Thus, this study investigated the difference regarding the changes in ALC among the three clinical patterns. There were several clinical findings: (1) ALC recovery was confirmed in patients with R-LPD in R-G and R/R-G; (2) at 0M, the ALC in R/R-G was significantly lower than that in R-G (Figure 2); and (3) the ALC patterns differed between R-G and R/R-G (Figure 3). The recovery of ALC gradually decreased after 1M toward the development of RRE in R/R-G, although it lasted for at least 2 years in R-G, supporting the clinical reports in which the regressive phenomenon in R-G continued for years. Taken together, the differences in

ALC may influence the pathogenesis of R-LPD and RRE in MTX-LPD. Furthermore, the ALC in P-G increased at 1M, although not significantly (Figure 3). As several patients exhibited rapidly progressing LPD in this study, the balance between ALC effects and tumor activity may be important to understand the impact of ALC on the pathogenesis of regression following MTX withdrawal.

Second, the ALC was investigated in the clinical setting when the critical threshold was met. Thus, an ALC level of less than 1000/ μ L was found to be a predictor for RRE. In all patients, an ALC \geq 1000/ μ L at 0M suggested relapse-free survival during the clinical course (Figure 4B). In clinical management, the prediction of RRE during clinical observation after MTX withdrawal in patients with R-LPD is important. Thus, ALC<1000/ μ L at 6M is a significant predictor for RRE in patients with R-LPD (R-G+R/R-G) under carefully monitored conditions (Figure 4D). Furthermore, an ALC of 1000/ μ L affects the OS. For example, an ALC of less than 1000/ μ L at 6M may be considered a predictive marker for OS among patients with R-LPD in R-G+R/R-G (Figure 5D).

Third, no significant differences were noted among each LPD of DLBCL–NOS, EBV+DLBCL, cHL, and LPD regarding the ALC level. Although the ALC in cHL at 0M was lower than that in other LPD, the value was not significant. Thus, our study suggests that the impact of ALC depends on the three clinical courses in MTX-LPD. Recent attention has been focused on immunity impairment around malignancies, including several immune checkpoints.^{27,28} In addition to this, many factors, such as the decrease in anti-tumor cells, including cytotoxic T cells and natural killer/T (NK/T) cells, lead to the impairment causing LPD.¹⁷ Indeed, ALC is one of the seven prognostic factors in advanced cHL.²⁹ In addition, recent studies investigated the impact of lymphocytes as anti-tumor factors against several LPD such as cHL, DLBCL, and nasal-type or extranodal NK/T-cell lymphoma.³⁰⁻³²

Fourth, ALC changes before MTX-LPD development were investigated. The duration from the time of MTX administration to the time of LPD development is known to widely vary from several months to over 30 years.¹⁻¹⁹ The pathogenesis of MTX-LPD is thought to be complex with many related factors, as mentioned previously. Among these factors, Saito *et al.* reported that the ALC gradually decreases in patients with R-LPD during LPD development, whereas that in patients without R-LPD and control patients without LPD did not change,¹⁶ suggesting the important role of ALC in the pathogenesis of R-LPD. In this study, we investigated the differences in ALC between R-G and R/R-G (Figure 6). Although ALC gradually decreased in R/R-G, it did not decrease until -6M in R-G. In addition, no significant differences were noted in the ALC at -6M, -3M, or 0M, although a decreasing trend was observed. Indeed, the ALC at 0M was significantly different between R-G and R/R-G ($p=0.00373$). These differences in ALC changes between R-G and R/R-G may affect the pathogenesis of MTX-LPD, including RRE.

In summary, this study revealed the impact of ALC on the

pathogenesis of MTX-LPD. Different ALC patterns were noted after MTX withdrawal among R-G, R/R-G, and P-G. In addition, the value of 1000/ μ L was found to be a cut-off point for regressive and relapse/regrowth events after MTX withdrawal, and the clinical outcome, including OS. In addition to ALC, other predictive factors, such as CRP and sIL-2R, may be helpful to manage MTX-LPD, including the decision making for an additional chemotherapy for regressive LPD after MTX withdrawal. However, the results of this study are from a small-sized retrospective study. In addition, the patients in R-G may relapse during further observation, although we selected patients in R-G with an observation period longer than 2 years after MTX withdrawal to exclude the possibility of RRE. Taken together, further investigation is required to confirm our findings.

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CONFLICTS OF INTEREST

M.T received honoraria from Ezai Co., Ltd, Ayumi Pharmaceutical Cor., Bristol-Myers K.K, Mitsubishi Tanabe Pharma Co., and Chugai Pharmaceutical Co., Ltd. K.A received honoraria from Bristol-Myers K.K, Pfizer Japan Inc, and Pfizer Japan Inc, and Astellas Pharma Inc. TJ received honoraria from Takeda Pharmaceutical Company Limited, and a grant from Nichirei Bioscience Inc. The remaining authors declare no competing financial interests.

ETHICAL APPROVAL

All procedures performed were in accordance with the ethical standards of each institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This study was approved by the ethics committee of Saitama Medical Center, Saitama Medical University (approved No.1808).

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