CANCER DIAGNOSIS & PROGNOSIS
4: 97-104 (2024) doi: 10.21873/cdp.10293

NK and T-lymphocyte Kinetics Predict Outcome in Myeloma Patients Treated With Elotuzumab, Lenalidomide Plus Dexamethasone

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Abstract. *Background/Aim: Elotuzumab, an anti-SLAMF7 monoclonal antibody, can enhance immune activity via elevated antibody-dependent cellular cytotoxicity and reduced SLAMF7$^+$CD8$^+$CD57$^+$ regulatory T-cells (Tregs). This multicenter observational study investigated the kinetics of lymphocytes in myeloma patients treated with elotuzumab, lenalidomide, and dexamethasone (ERd) by two-color flow cytometry using peripheral blood samples. Patients and Methods: Twenty-one patients were included in this study. The median duration of ERd was 22.6 months, and the cutoff time for long-duration ERd was two years. Results: The CD2$^+$CD16$^+$ and CD16$^+$CD57$^-$ NK cells were significantly increased over time in the long-duration ERd group compared to those in the short-duration ERd group (p=0.035 and p<0.001). The CD8$^+$ and CD16$^-$CD57$^+$ lymphocytes, identified as low-activity NK cells or SLAMF7$^+$ Tregs, were significantly increased in the patients whose ERd outcome was progressive disease (PD) compared to those in the non-PD group (p=0.023 and p<0.001). The mean CD4/CD8 ratio and CD19$^+$ lymphocyte counts in the long-duration ERd group were significantly lower than those in the short-duration ERd group, although the kinetics of them did not change over time (p=0.016 and p=0.011). When the cutoff value of CD4/CD8 ratio was 0.792 according to ROC curves, the two-year time to next treatment (TTNT) in the low CD4/CD8 group was significantly longer than that in the high CD4/CD8 group (80.0% vs. 15.0%, p=0.024). Conclusion: The change in NK cells and CD8$^+$ Tregs predicted long-duration ERd and PD, and maintaining low CD4/8 ratio predicted long TTNT, suggesting that these lymphocyte fractions might be biomarkers for a durable therapeutic effect of ERd in myeloma patients.*

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Key Words: Elotuzumab, multiple myeloma, natural killer cell, regulatory T cell, CD4/8.

Elotuzumab, an anti-SLAMF7 monoclonal antibody, and lenalidomide plus dexamethasone (ERd) is a standard salvage chemotherapy for patients with relapsed or refractory multiple myeloma (RRMM) (1). We previously reported that the efficacy and tolerability of monthly ERd were similar to those of conventional ERd, which is weekly ERd within the first two months followed by biweekly ERd after three cycles, in a Japanese multicenter observational study (2). Additionally, we investigated lymphocyte subsets in peripheral blood for the efficacy of ERd as a sub-analysis. The main mechanism of action for elotuzumab was antigen-dependent cellular cytotoxicity (ADCC) *in vivo* and *in vitro* (3, 4), suggesting that the activity of natural killer (NK) cells can improve and maintain the efficacy of ERd. Immunophenotypes regarding CD16 and CD57 represent NK-cell activity; for instance,

$CD57^+CD16^-$ NK cells show low activity (5). Additionally, $CD57^+CD16^-$ lymphocytes may not be identified as low-activity NK cells but mainly as $CD57^+CD8^+$ T-cells (5-7). Recently, regulatory T-cells (Tregs) were found to be increased in patients with high-risk smoldering myeloma compared to healthy individuals; reduced Tregs predicted long progression-free survival (8). $CD57^+CD8^+$ Tregs may be a new target for ERd *in vitro* (9). Without focusing on ADCC, the CD4/CD8 ratio predicted clinical outcomes in patients with myeloma (10, 11). In addition, high $CD19^+$ B-cells may show immune reconstitution and are associated with long survival in patients with myeloma (12). However, the prognostic value of the CD4/CD8 ratio and $CD19^+$ cell counts was unclear, forcing treatment of patients with ERd. This observational study aimed to investigate several lymphocyte subsets as biomarkers in patients with myeloma treated with ERd.

Patients and Methods

We reviewed the medical records of patients with RRMM treated with ERd at six institutes in Japan. This study was a sub-analysis of a previous multicenter retrospective study (2) and approved by the Independent Ethics Committee and Institutional Review Board of the Jikei University School of Medicine [31-027(9526)].

Patients. Patients were included if they were >18 years old and diagnosed as symptomatic myeloma, and had previously undergone one or more chemotherapy regimens. Briefly, we investigated the efficacy and safety of two schedules of ERd in seventy-five patients who were treated with four cycles of ERd or more in our previous study (2). Every 4 weeks of ERd *via* the planned interval extension of elotuzumab was defined as monthly ERd. The elotuzumab dose was 10 mg/kg in all patients. The doses of lenalidomide and dexamethasone were decided by the physicians. ERd was administered not only for RRMM but also non-RRMM for the purpose to improve therapeutic response. Relapse and refractory diseases were defined according to the International Myeloma Working Group criteria (13).

Identification of lymphocytes using flow cytometry (FCM). We investigated the kinetics of several lymphocytes, including $CD4^+$ and $CD8^+$ T-cells, $CD19^+$ B-cells, and NK cells by two-color FCM using peripheral blood samples collected on day 1 of every ERd cycle. The percentage of NK cells was analyzed using the $CD2^+CD16^+$ fraction. NK cell activity was evaluated by FCM using the CD16 and CD57 antigens. $CD16^+CD57^-$, $CD16^+CD57^+$, and $CD16^-CD57^+$ fractions show high, intermediate, and low NK activities, respectively, according to a previous report (6).

Statistical analysis. The kinetics of these lymphocyte subsets were analyzed in patients treated with conventional *vs*. monthly ERd, RRMM *vs*. non-RRMM, progressive disease (PD) *vs*. non-PD, complete response (CR) *vs*. non-CR as the best response, and short-duration *vs*. long-duration ERd. The cutoff time for long-duration ERd continuation was two years, considering the median duration of ERd of 22.6 months. The kinetics of several immune cell counts were analyzed using repeated-measures ANOVA. To address the ability of biomarkers to predict long-duration ERd, we analyzed their sensitivity and specificity using receiver operating characteristic (ROC) curves. We evaluated the area under the ROC curve (AUC) to assess the diagnostic accuracy of a test and compare its usefulness (14). Fisher's exact test was used to compare various parameters between the long- and short-duration ERd groups and the monthly and conventional ERd groups. Finally, the time-to-next treatment (TTNT) was analyzed using the cutoff value of the biomarker. TTNT was defined as the interval between the start of ERd and the start of the next chemotherapy, regardless of why the treatment was changed. Actuarial survival analysis was performed using the Kaplan–Meier method, and the resultant curves were compared using the log-rank test. All reported *p*-values were two-sided, and *p*-values <0.05 were considered statistically significant. All statistical analyses were performed using EZR (Saitama Medical Center, Jichi Medical University), a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria) (15). It is a modified version of R Commander that incorporates frequently used biostatistical functions.

Table I. *Patient characteristics*.

	n=21
Age	72 (46-85) years
Sex	
Male	16
Female	5
Prior line number	1 (1-3) lines
Prior lenalidomide exposure	
Yes	18
No	3
Lenalidomide refractoriness	
Yes	7
No	14
ASCT history	
Yes	9
No	12
RRMM	
Yes	5
No	16
Schedule of ERd	
Monthly	12
Conventional	9
Best response by ERd	
CR	12
Non-CR	9
Outcome of ERd	
PD	8
Non-PD	13

ERd: Elotuzumab, lenalidomide plus dexamethasone; ASCT: autologous stem cell transplantation; PD: progressive disease; CR: complete response.

Results

Patients. We investigated lymphocyte kinetics in 21 patients, including 12 in the monthly ERd group, 5 in the RRMM group, and 8 in the PD group. The median patient age was 72 years (range=46-85 years). The median number of prior chemotherapies was one (1-3). The number of patients in the

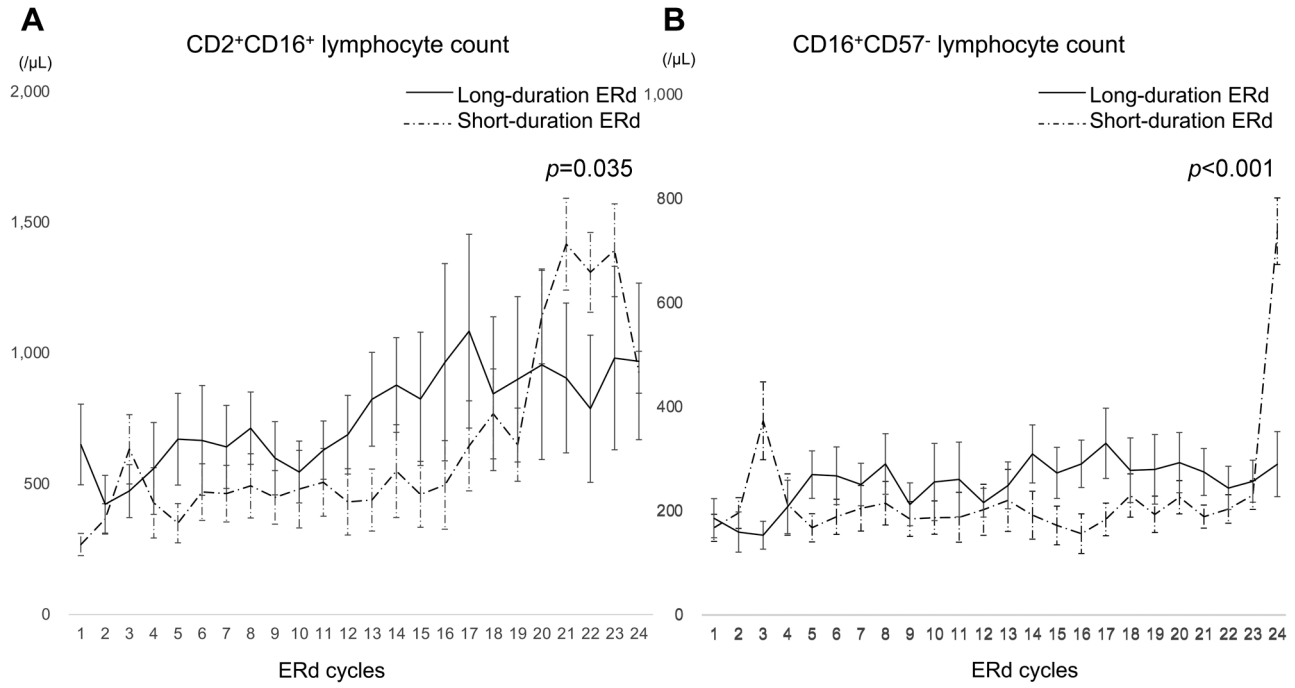

Figure 1. *The kinetics of CD2+CD16+ and CD16+CD57− NK cells predicted long-duration ERd. (A) The CD2+CD16+ (B) and CD16+CD57− NK cell counts were significantly increased over time in the long-duration ERd group compared to those in the short-duration ERd group (p=0.035 and <0.001).*

long and short-duration ERd groups were ten and 11, respectively. Table I shows the patient characteristics. The frequencies of monthly ERd and CR in the long-duration ERd group were significantly higher than those in the short-duration ERd group (p=0.024 and 0.008). The frequency of non-RRMM in the monthly ERd group was significantly higher than that in the conventional ERd group (p=0.048).

The kinetics of lymphocyte count predict clinical outcomes. The overall and $CD16^+CD57^-$ NK cell counts significantly increased over time in the long-duration ERd group compared to those in the short-duration ERd group (p=0.035, Figure 1A and <0.001, Figure 1B). There was no significant difference in $CD16^+CD57^+$ and $CD16^-CD57^+$ NK cells between the long and short-duration ERd groups (p=0.727 and 0.993). The $CD16^-CD57^+$ and $CD8^+$ lymphocyte counts were significantly increased in patients whose ERd outcome was PD independently from the best response compared to those in the non-PD group (p<0.001, Figure 2A and p=0.023, Figure 2B). There was no significant difference in $CD16^+CD57^-$ and $CD16^+CD57^+$ NK cell counts between the PD and non-PD groups (p=0.914 and 0.999). The mean $CD16^+CD57^-$ NK cell count was significantly higher and the mean $CD16^-CD57^+$ NK cell count was lower in the monthly ERd group compared to those in the conventional ERd group (p=0.037 and 0.006), although the kinetics of $CD16^+CD57^-$ and $CD16^-CD57^+$ NK cell counts were similar between the monthly and conventional ERd groups (p=0.991 and 0.538). Additionally, CR as the best response and RRMM did not affect the kinetics of NK cells. Finally, significant changes over time in $CD4^+$ and $CD19^+$ lymphocyte counts, and CD4/CD8 ratio were not observed during ERd treatment.

Meanwhile, the mean CD4/CD8 ratio and $CD19^+$ lymphocyte count in the long-duration ERd group were significantly lower than those in the short-duration ERd group, although the kinetics of CD4/CD8 ratio and $CD19^+$ lymphocyte count did not change over time (p=0.016 and 0.011; Figure 3A and B). Therefore, we considered the CD4/CD8 ratio might be a potential biomarker for long-duration ERd and determined that the cutoff value of CD4/CD8 ratio was 0.792 according to ROC curves (Figure 4A). The two-year TTNT in the low CD4/CD8 group was significantly longer than that in the high CD4/CD8 group (80.0% *vs.* 15.0%; HR=7.045, 95%CI=1.713-28.97; p=0.024; Figure 4B). The $CD19^+$ lymphocyte count did not predict long-duration ERd when the cutoff value was calculated using the ROC curve.

Discussion

We demonstrated that increased $CD2^+CD16^+$ NK cell and high-activity $CD16^+CD57^-$ NK cell counts predicted long-

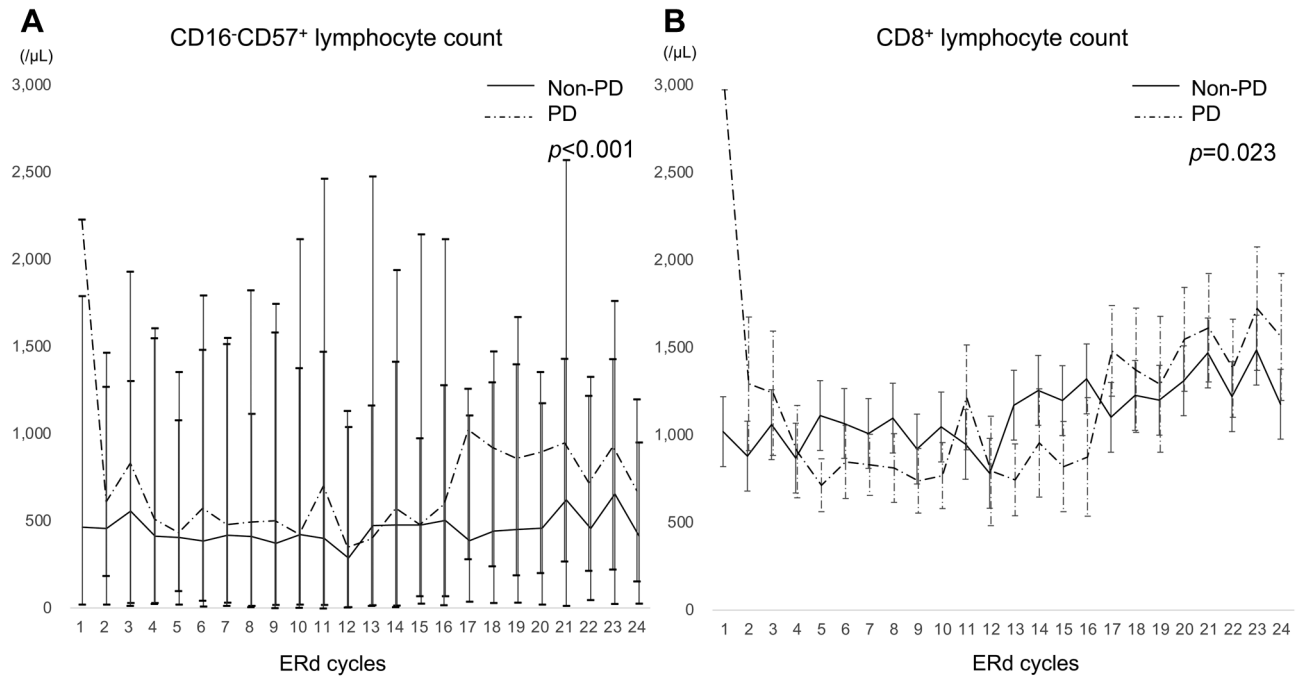

Figure 2. *The kinetics of CD16⁻CD57⁺ and CD8⁺ cells predicted the refractoriness of ERd. (A) The CD16⁻CD57⁺ and (B) CD8⁺ lymphocyte counts were significantly increased in the PD after ERd group than in the non-PD after ERd group (p<0.001 and 0.023).*

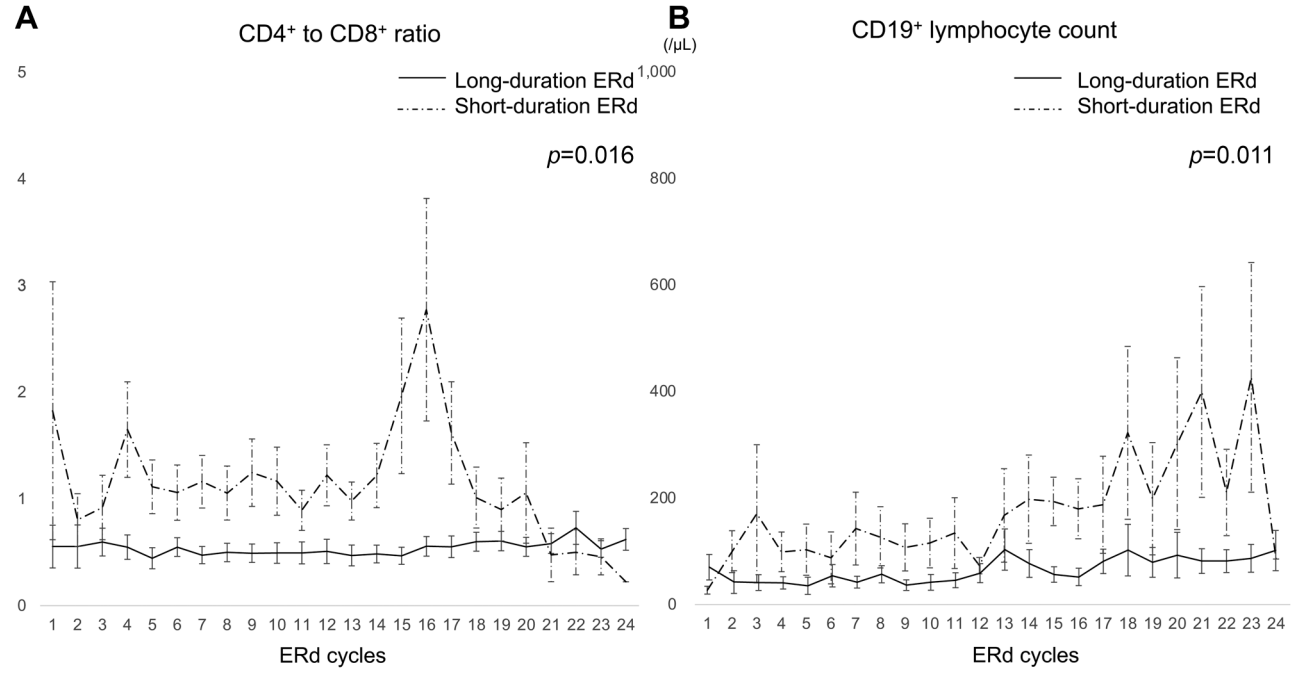

Figure 3. *The mean value of CD4 to CD8 ratio and CD19⁺ lymphocyte count predicted long-duration ERd. (A) The mean CD4 to CD8 ratio and (B) CD19⁺ lymphocyte count were significantly higher in the long-duration ERd group than in the short-duration ERd group (p=0.016 and 0.011).*

duration ERd, and CD16⁻CD57⁺ NK cell and CD8⁺ T-cell counts increased over time before ERd was refractory. A high CD4/CD8 ratio predicted long TTNT in the patients treated with ERd, while a change over time in the CD4/CD8 ratio was not observed. Thus, the immune system can predict clinical outcomes in patients treated with ERd.

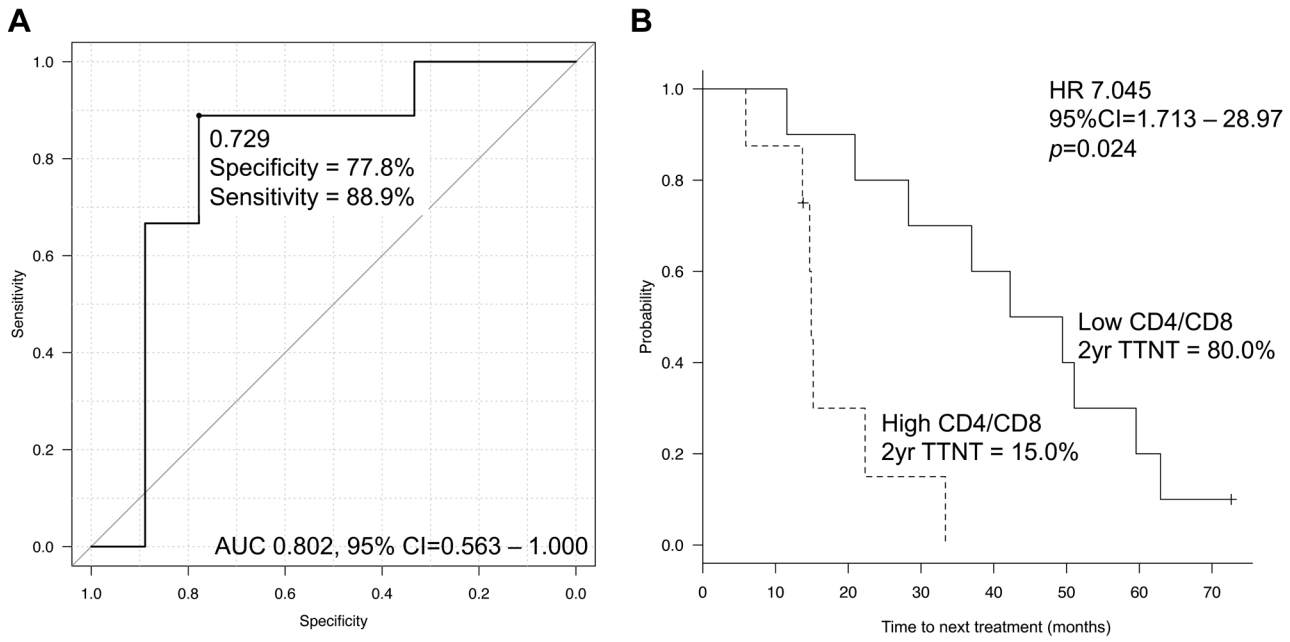

Figure 4. *Time to next treatment between high and low CD4 to CD8 ratio. (A) The receiver operating characteristic (ROC) curve revealed that the cutoff value of the CD4 to CD8 ratio was 0.792. (B) The two-year time-to-next treatment in the low CD4/CD8 group was significantly longer than that in the high CD4/CD8 group (80.0% vs. 15.0%; p=0.024).*

NK cells are generally a $CD2^+CD3^-CD16^+$ lymphocyte subset (16). Approximately 90% of NK cells, especially those circulating in peripheral blood, express CD16, while NK cells in lymph nodes do not express CD16 (17). Additionally, FcR-gamma IIIA, identified as CD16, is a key molecule for ADCC because monoclonal antibodies recognize CD16 in NK cells (18, 19). ADCC does not work in solid tumors because of the down-regulation of CD16 (20). $CD16^+$ NK cells contributed to the efficacy of elotuzumab plus lenalidomide *in vitro* because myeloma cell lysis was not observed in the setting of elotuzumab without NK cells and elotuzumab with NK cells and anti-CD16 monoclonal antibody (21). In addition, the therapeutic activity might depend on the phenotype of NK cells for myeloma patients treated with ERd. Balasa *et al*. demonstrated that IL-2 and TNF-α enhanced the ADCC of elotuzumab monotherapy or elotuzumab plus lenalidomide *in vitro* (22). CD25 expression was up-regulated in NK cells treated with elotuzumab plus lenalidomide, suggesting that $CD25^+$ NK cells contribute to high anti-myeloma activity by enhancing ADCC. However, the association between the clinical outcome and phenotype of NK cells using CD16 and CD57 antigens has never been investigated in patients treated with ERd.

There is little evidence regarding the phenotype of NK cells that function as ADCC, although the activity of NK cells can be classified using CD16 and CD57 antigens (6). CD57 is a terminally sulfated carbohydrate determinant (glycoepitope) and has been identified at various surface glycoproteins, proteoglycans, and glycolipids on subsets of NK and T-cells (23, 24). $CD57^+$ NK cells are terminal NK cells that produce interferon-gamma (INF-γ) and show potent lytic activity. Functional maturation of NK cells depends on the expression of CD57 on $CD56dimCD16^+$ NK cells, and $CD57^+$ NK cells induce cytolytic activity by stimulating CD16 but do not respond to IL-12 and IL-18 (25). Additionally, $CD57^+$ NK cells proliferate less than $CD57^-$ NK cells. Thus, $CD57^+CD16^-$ NK cells can be identified as having relatively low activity considering the less reaction due to cytokines and less proliferative activity. However, $CD16^+CD57^-$ NK cells secrete more INF-γ than $CD16^+CD57^+$ NK cells, which are identified as a mature NK cell subset with loss of proliferative reaction due to inflammatory cytokines (25). Thus, increasing the immature high-activity $CD16^+CD57^-$ NK cell count may enhance ADCC. Therefore, it is reasonable that increased $CD2^+CD16^+$ and high-activity $CD16^+CD57^-$ NK cell counts were observed in the long-duration ERd group in this study.

In our study, the $CD16^-CD57^+$ NK cell counts increased before the refractoriness of ERd. Increased $CD16^-CD57^+$ NK cell counts may indicate low-activity NK cells and relatively reduced high-activity NK cell counts, suggesting that ADCC may become weak in patients with increased $CD16^-CD57^+$ NK cell counts. Meanwhile, the expression of CD57 on $CD4^+$

and $CD8^+$ T-cells was increased in 42% of patients with untreated hematological malignancies compared to that in healthy individuals (26). CD57 expression was increased, and CD28, a co-stimulator for T-cell activation, was decreased during the reduced activity of $CD8^+$ T-cells, suggesting that $CD57^+CD8^+$ T-cells show low cytotoxic activity (27, 28). In addition, $CD57^+CD8^+$ T-cells have shorter telomeres, lower telomerase activity, and lower expression of cell cycle-associated genes than $CD57^-CD8^+$ T-cells (24). There was a positive correlation between $CD57^+CD28^-CD8^+$ T-cells and cancer survival according to investigations focused on untreated patients with myeloma (29) and patients treated with thalidomide for RRMM (30). Recently, according to an analysis of the Mayo Clinic Biospecimen database, $CD57^+$ T-cells were increased in cluster 2, mainly including RRMM, compared to cluster 1, mainly including newly diagnosed myeloma, suggesting that immunosenescent, terminally differentiated T-cells were increased in RRMM (31). Therefore, increasing CD16-$CD57^+$ NK cell counts could predict refractoriness for ERd by weakening ADCC and increasing immunosenescent $CD57^+CD8^+$ T-cells.

Generally, a decreased CD4/8 ratio is associated with poor prognosis because decreased $CD4^+$ T-cell and increased $CD8^+$ T-cell counts are related to poor outcomes (10, 11). Increased clonal $CD8^+$ T-cells are identified as effector memory T-cells with limited T-cell receptor (TCR) Vβ expression and are associated with persistent stimulation by myeloma-associated antigens (32). The count of clonal $CD8^+$ T-cells in peripheral blood was higher in patients with myeloma who survived for more than 10 years than in those who died in less than 10 years (33). However, our results were the opposite; a high CD4/CD8 ratio was associated with long-duration ERd in this study. Therefore, we considered that $CD8^+$ T-cells were not clonal effector memory T-cells but $CD8^+$ Tregs (7). Before commencing this observational study, we considered that the $CD16^-CD57^+$ lymphocytes were identified as low-activity NK cells using only two-color FCM using CD16 and CD57 for lymphocytes, but may include other $CD57^+$ lymphocytes, such as $CD8^+CD57^+$ Tregs (7). In this study, the $CD8^+$ cell count in the PD group was higher than that in the non-PD group over time. Considering these results and the increased $CD16^-CD57^+$ cells in the PD group, $CD16^-CD57^+$ cells may indicate an increase in $CD8^+CD57^+$ Tregs. Recently, Awwad *et al*. reported that elotuzumab targeted $SLAMF7^+CD8^+CD57^+$ Tregs and improved immunosuppressive conditions (9). Thus, these results suggest that $CD16^-CD57^+$ lymphocytes may increase over time if elotuzumab cannot suppress $SLAMF7^+CD8^+CD57^+$ Tregs before ERd is refractory.

The $CD19^+$ cell count in the long-duration ERd group was predominantly lower than that in the short-duration ERd group. SLAMF7 is also expressed in approximately 10% of normal B-cells and is associated with normal B-cell proliferation (34), suggesting that elotuzumab may decrease the number of $SLAMF7^+$ B-cells. Meanwhile, chemokine ligand type 20 (CCL200) and chemokine receptor type 6 interactions are associated with elotuzumab resistance (35). CCL20 is secreted by B-cells; therefore, high $CD19^+$ B-cell counts may be associated with elotuzumab resistance *via* CCL20 secretion.

Study limitations. First, the immune profiles of T-cells, B-cells, and Tregs were not well investigated because the immune profile analysis was performed using only two-color FCM for CD16 plus CD57 and CD2 plus CD16 antigens and single-color FCM for CD4, CD8, and CD19 antigens. For a better understanding, cytometry by time-of-flight (CyTOF) might be a suitable option. Second, we did not evaluate several cytokines, such as serum IL-2 and TNF-α, which are related to ADCC. The kinetics of cytokines may also be a biomarker for predicting the therapeutic efficacy of ERd. Finally, we analyzed only the patients treated with ERd, therefore, it was controversial whether these changes in immune profile depended on elotuzumab or lenalidomide.

Conclusion

Increased NK cells, especially the high-activity subset, predicted long-duration ERd, suggesting that ADCC may be a key to improve clinical outcomes for ERd. The CD16-$CD57^+$ lymphocyte percentage, which was identified as $CD8^+CD57^+$ Treg or low activated NK cell, was increased before PD, suggesting that the kinetics of these lymphocytes might be a biomarker for refractoriness for ERd. Finally, the CD4/CD8 ratio predicted TTNT in myeloma patients treated with ERd. However, this was a small-scale observational study. Therefore, we will conduct a larger study to investigate the immune profiles in ERd and lenalidomide plus dexamethasone (Rd) in the future.

Conflicts of Interest

K. Suzuki received personal fees from Takeda Pharmaceutical Company, Janssen Pharmaceutical K.K., Sanofi, Celgene, outside the submitted work; M. Matsumoto received honoraria from Bristol-Myers Squibb K.K., Janssen Pharmaceutical and Takeda Pharmaceutical, Ono Pharmaceutical, Sanofi K.K., outside the submitted work; K. Suzuki received honoraria from Takeda, ONO, Amgen, Novartis, Sanofi, Bristol-Myers Squibb, Abbvie and Janssen, consulted for Amgen, Takeda, and Bristol-Myers Squibb, and received research funding from Bristol-Myers Squibb. The other Authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization, K.S.; writing – original draft preparation, K.S.; writing – review and editing, M.M., Y.H., N.T., Y.T., and K.S. All Authors have read and agreed to the submitted version of the manuscript.

Acknowledgements

The Authors would like to thank the attending doctors and nurses at the Japanese Red Cross Medical Center, Shibukawa Medical Center, Japanese Red Cross Society Himeji Hospital, the Jikei University Kashiwa Hospital, National Hospital Organization Disaster Medical Center, and Shonan Kamakura General Hospital. The Authors would also like to specially thank the lymphoma patients and their families for their participation in our study.

Funding

This study was funded by Bristol-Myers Squibb K.K. All Authors had full access to all of the data in the study and were responsible for the decision to submit the manuscript for publication.

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Received December 6, 2023
Revised January 19, 2024
Accepted January 22, 2024