Immunological effect of Zeusion X

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Abstract

It is very important to understand CD8+ T-cell dynamics to determine the immune status of cancer patients: CD8+ T-cell populations, i.e., early-differentiated CD8+ T cells (CD27+CD8+CD57− T cells, hereafter referred to as early-CD8+ T cells), intermediate-differentiated CD8+ T cells (CD27+CD8+CD57+ T cells, hereafter referred to as intermediate-CD8+ T cells), and terminally differentiated CD8+ T cells (CD27−CD8+CD57+ T cells, , hereafter referred to as terminal-CD8+ T cells). We conducted univariate analysis of CD8+ T-cell populations related to CD8+ T-cell differentiation in a culture of activated self lymphocyte cells and peripheral blood of 100 stage IV cancer patients and reported that early-CD8+ T cells are involved in a poor prognosis and terminal-CD8+ T cells are involved in a favorable prognosis. In addition, we reported that there is an inverse correlation between early-CD8+ T cells and terminal-CD8+ T cells (Spearman correlation coefficient, 0.919; $p < 0.0001$). The CD57 ratio (terminal-CD8+ T cells/early-CD8+ T cells) was significantly more correlated with prognosis than early-CD8+ T cells and terminal-CD8+ T cells alone in multivariate analysis and was a good, independent prognostic factor. The CD57 ratio is a good indicator of the dynamic state of CD8+ T-cell differentiation, and we believe that it is

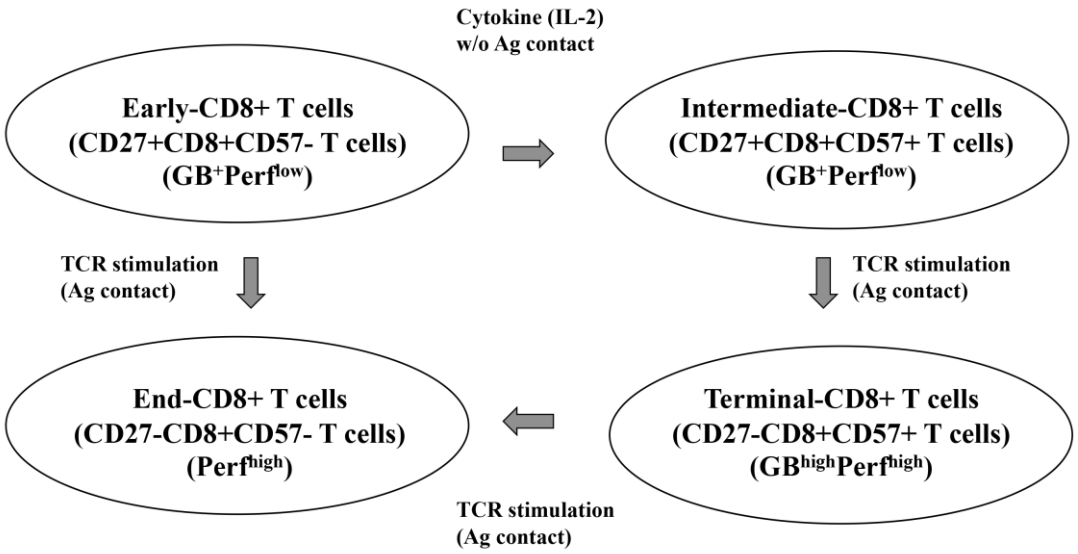
highly likely to be the best indicator of CD8+ T-cell differentiation. In the present study, we examined the immunological effect of Zeusion X, consisting of plant lactic acid bacteria, organic mineral fulvic acid, and golden oyster mushroom (β-1,3 D-glucan). Zeusion X was administered to 15 patients with progressive stage IV cancer for 3 months. Peripheral blood was collected before and 3 months after administration. Early-CD8+ T cells, intermediate-CD8+ T cells, terminal-CD8+ T cells, PD-1^+ terminal-CD8+ T cells, and the CD57 ratio were measured by flow cytometry and subjected to analysis. In 8 of 15 patients (53.3%), the CD57 ratio increased and PD-1^+ terminal-CD8+ T cells decreased. However, in all 4 patients with a CD57 ratio of >4.0, the CD57 ratio decreased and PD-1^+ terminal-CD8+ T cells increased, but all of these had a good prognosis. Conversely, 8 of 11 patients (72.7%) with a CD57 ratio of <4.0 showed an increase in the CD57 ratio, and 7 patients had a good prognosis. Five of the 6 patients in whom the CD57 ratio increased and PD-1^+ terminal-CD8+ T cells decreased had a good prognosis. Conversely, all 3 patients in whom the CD57 ratio decreased and PD-1^+ terminal-CD8+T cells increased had a poor prognosis. These findings suggest that Zeusion X regulates CD8+ T-cell immunity in both positive and negative directions by controlling the CD57 ratio and PD-1^+ terminal-CD8+ T cells.

Introduction

We have long focused on CD57+ T cells, particularly CD8+CD57+ T cells, as prognostic factors for cancer patients. CD57+ T cells are independent poor prognosis factors in advanced stomach cancer (HR = 3.136, 95% CI 1.142–8.16, p = 0.027), and the CD57+ T-cell elevation group (>18%) has a significantly poorer prognosis than the low value group (Kaplan–Meyer log rank p = 0.011) [1]. In melanoma patients, the group with low levels of CD8+CD57+ T cells in peripheral blood (<23%) before IFN-α administration has been found to have a better prognosis than the high-level group [2], but in advanced renal cancer patients, the outcome has been the opposite. That is, the prognosis in the group of patients with peripheral blood CD8+CD57+ T-cell elevation (>30%) before IFN-α administration has been found to be good [3]. IFN-α has the effect of increasing CD8+CD57+ T cells when they are low in numbers before administration. Conversely, IFN-α decreases CD8+CD57+ T cells when they are high in numbers before administration. In melanoma patients, the CD8+CD57+ T-cell level, which is low before administration, increases by IFN-α administration and the survival time is prolonged. Conversely, in advanced renal cancer patients, the CD8+CD57+ T-cell level, which is high before administration, is decreased by IFN-α administration and the

survival time is prolonged. This indicates that the characteristics of peripheral blood CD8+CD57+ T cells of melanoma patients and peripheral blood CD8+CD57+ T cells of advanced renal cancer patients are different. Cytotoxic CD8+CD57+ T cells are dominant in melanoma patients, and immunosuppressive CD8+CD57+ T cells are dominant in advanced renal cancer patients. We recently reported that CD8+CD57+ T cells (equivalent to terminally differentiated CD8+ T cells, hereafter referred to as terminal-CD8+ T cells) are involved in a good prognosis in activated autologous lymphocyte therapy [4]. Thus, both a cytotoxic population and an immunosuppressive population exist in CD8+CD57+ T cells in peripheral blood, and which population proliferates dominantly seems to depend on the cancer type.

CD8+CD57+ T cells appear in the pathway of differentiation of CD8+ T cells (Fig. 1)



At least the following 2 types of CD8+CD57+ T cells are present in the CD8+ T-cell differentiation pathway: intermediate-differentiated CD8+ T cells (CD27+CD8+CD57+ T cells, hereafter referred to as intermediate-CD8+ T cells) and terminal-CD8+ T cells (CD27−CD8+CD57+ T cells). The properties of these 2 CD8+CD57+ T cells are quite different. Intermediate-CD8+ T cells are CD27 positive, are GB+Per^{lo}, and have low cytotoxic activity. Terminal CD8+ T cells are CD 27 negative, are $GB^{high}Per^{high}$, and have high cytotoxic activity. CD57+ T cells (CD8+CD57+ T cells), which increased in peripheral blood of advanced stomach cancer and advanced renal cancer patients and

were involved in a poor prognosis, are thought to be intermediate-CD8+ T cells. Moreover, terminal-CD8+ T cells are thought to increase in melanoma patients and activate autologous lymphocyte cells. It has been reported that differentiation from early-differentiated CD8+ T cells (hereafter referred to as early-CD8+ T cells)/intermediate-CD8+ T cells to terminal-CD8+ T cells and cytotoxic activity are inhibited by TGF-β [5], IL-10, and PD-1 [6] in cancer patients. Because activated autologous lymphocyte therapy causes lymphocyte proliferation using CD3 antibody and IL-2 in vitro, this suppression of differentiation is lost and terminal-CD8+ T cells with high cytotoxic activity are increased. This is believed to contribute to a good prognosis [4]. Thus, it is considered that the prognosis of cancer patients is defined by which population increases during the differentiation of CD8+ T cells. Therefore, it is important in cancer treatment to always consider the stage of cytotoxic T lymphocyte (CTL) differentiation of CD8+ T cells. The CD57 ratio is a very important index because it accurately expresses the process of its differentiation.

We conducted univariate analysis of CD8+ T-cell populations related to CD8+ T-cell differentiation in a culture of activated self lymphocyte cells [4] and peripheral blood of

100 stage IV cancer patients [7,8] and reported that early-CD8+ T cells are involved in a poor prognosis and terminal-CD8+ T cells are involved in a favorable prognosis [7,8]. Furthermore, the CD57 ratio (terminal-CD8+ T cells/early-CD8+ T cells) calculated using early-CD8+ T cells and terminal-CD8+ T cells, which show an inverse correlation, was significantly more correlated with prognosis than early-CD8+ T cells and terminal-CD8+ T cells alone and was a good, independent prognostic factor [7,8]. The CD57 ratio is considered to be an important indicator of the dynamic state of differentiation of CD8+ T cells. When the CD57 ratio is high, the differentiation of CD8+ T cells is smooth and cytotoxic terminal-CD8+ T cells are sufficiently induced. However, when the CD57 ratio is low, there is some obstacle to the differentiation of CD8+ T cells, which is considered to be a state in which immunosuppressive early-CD8 + T cells are induced. In advanced cancer patients, this CD57 ratio declines in many patients, and we think that raising the CD57 ratio, i.e., activating the immunity of CD8+ T cells, is an important cornerstone of cancer treatment. When the CD57 ratio is low, i.e., when immunity is not sufficiently induced, treatment is not always effective. We were looking for a therapeutic agent capable of increasing the CD57 ratio and activating CD8+ T-cell immunity. We found Zeusion X in such a situation.

Zeusion X consists of plant lactic acid bacteria, organic mineral fulvic acid, and golden oyster mushroom (β-1,3 D-glucan). It has been reported that lactic acid bacteria have a tumor-suppressive effect in colon cancer patients [9] and breast cancer patients [10]. In our study, Zeusion X showed a tumoricidal effect equivalent to that of cisplatin at a concentration of 12.5 mg/ml in a colon-26 cultured cell line. Because Zeusion X contains plant lactic acid bacteria and golden oyster mushroom (β-1,3 D glucan), in addition to the direct tumor-killing effect, an immune-mediated mechanism is assumed to contribute to this tumor-suppressing effect.

In the present study, we administered Zeusion X to 15 stage IV progressive cancer patients for 3 months. Peripheral blood was collected before administration and 3 months after administration, and each cell fraction of the CD8+ T-cell differentiation process (early-CD8+ T cells, intermediate-CD8+ T cells, terminal-CD8+ T cells, and the CD57 ratio) was measured and analyzed.

Patients and Methods

We evaluated 100 patients (45 males, 55 females) diagnosed with stage IV cancer at the Tamana Regional Health and Medical Center from July 2013 to March 2016

(stomach cancer, 9; colorectal cancer, 23; pancreatic cancer, 10; liver cancer, 6; lung cancer, 14; breast cancer, 9; others, 29). In total, 10 ml of peripheral blood of these patients was collected, and FACScan was performed. The antibodies used were anti-CD3 FITC (HIT3a), anti-CD57 FITC (NK-1), anti-CD 27 PE (M-T 271), anti-Fox P3 PE (259 D/C7), anti-CD 57 PE (NK-1), and anti-CD8 PE-Cy7 (RPA-T8) (BD Pharmingen, San Diego, CA, USA). Lymphocytes were analyzed with BD FACSCalibur, and BD CellQuest software was used for data analysis. All these tests were conducted at SRL Inc. (Tokyo, Japan).

Fifteen patients took 8 ml of Zeusion X 4 times a day (morning, noon, night, and before bed) for 3 months. Peripheral blood was collected twice: before administration and after 3 months of administration. CD57+ T cells, CD8+CD57+ T cells, early-CD8+ T cells, intermediate-CD8+ T cells, and terminal-CD8+ T cells in peripheral blood were measured by FACScan. The cases were as follows: stomach cancer, 1; colon cancer, 5; breast cancer, 2; pancreatic cancer, 1; liver cancer, 1; lung cancer, 1; and other cancers, 4.

Statistical analysis was performed using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA). Mann–Whitney *U* test was used to compare the numerical values of the 2 groups, which were not normally distributed, and the $\chi 2$ test was used for comparison of categories between the 2 groups. The Kaplan–Meier method was used for analysis of the survival rate, and the log-rank test was used to test for significant difference in the survival rate. The Cox regression model was used for multivariate analysis.

Results

Univariate and multivariate analyses

Univariate and multivariate analyses were conducted to determine the association between progression-free survival (PFS) and early-CD8+ T cells, intermediate-CD8+ T cells, and terminal-CD8+ T cells, which are the groups of cells involved in the differentiation of CD8+ T cells. We found that CD57+ T cells, CD8+CD57+ T cells, and terminal-CD8+ T cells were associated with a favorable prognosis (Table 1).

Table 1 **Univariate and multivariate analyses**

(**Univariate analysis**)	HR	HR95%CI	P-value
CD57+ T cells	0.362	0.186~0.704	0.003
CD8+CD57+T cells	0.424	0.220~0.820	0.011
Early-CD8+ T cells (CD27+CD8+CD57− T cells)	5.937	2.589~13.6	<0.0001
Intermidiate-CD8+ T cells (CD27+CD8+CD57+ T cells)	2.325	1.216~4.447	0.011
Terminal-CD8+ T cells (CD27−CD8+CD57+ T cells)	0.154	0.072~0.329	<0.0001
End-CD8+ T cells (CD27−CD8+CD57+ T cells)			0.337
CD57− ratio	0.143	0.065~0.314	<0.0001

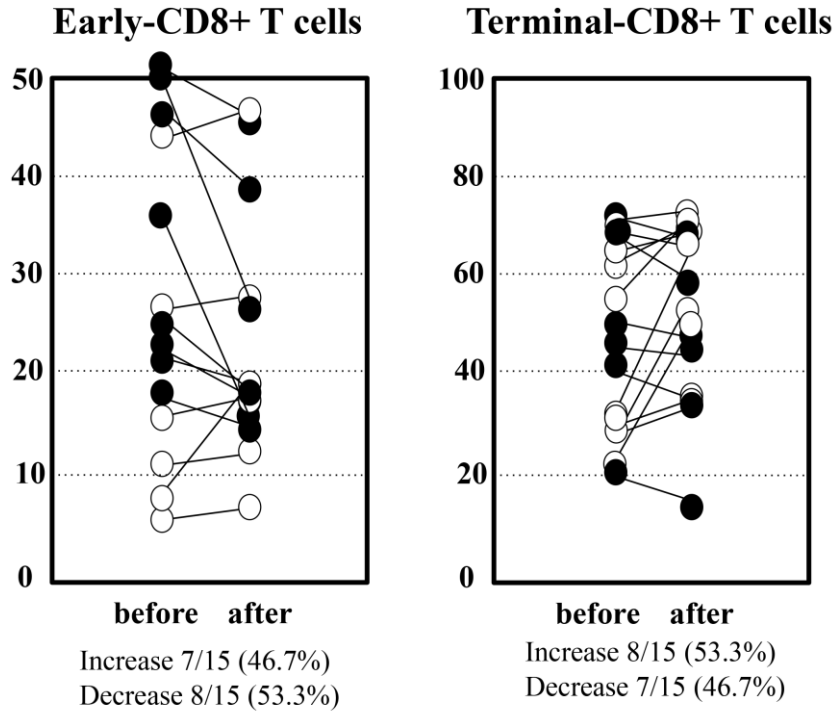
(multivariate analysis)	HR	HR95%CI	P-value
CD57− ratio	0.143	0.065~0.314	<0.0001

Early-CD8+ T cells and intermediate-CD8+ T cells were associated with a poor prognosis (Table 1). As reported previously [7, 8], early-CD8+ T cells and terminal-CD8+ T cells have a strong inverse correlation, and each has both poor and good prognostic factors. Therefore, in order to determine additional potent prognostic immunological parameters, the CD57 ratio (=terminal-CD8+ T cells/early-CD8+ T cells) was calculated. This is an immunological indicator that well reflects the differentiation status of CD8+ T cells. If it is high, CD8+ T-cell differentiation is smooth

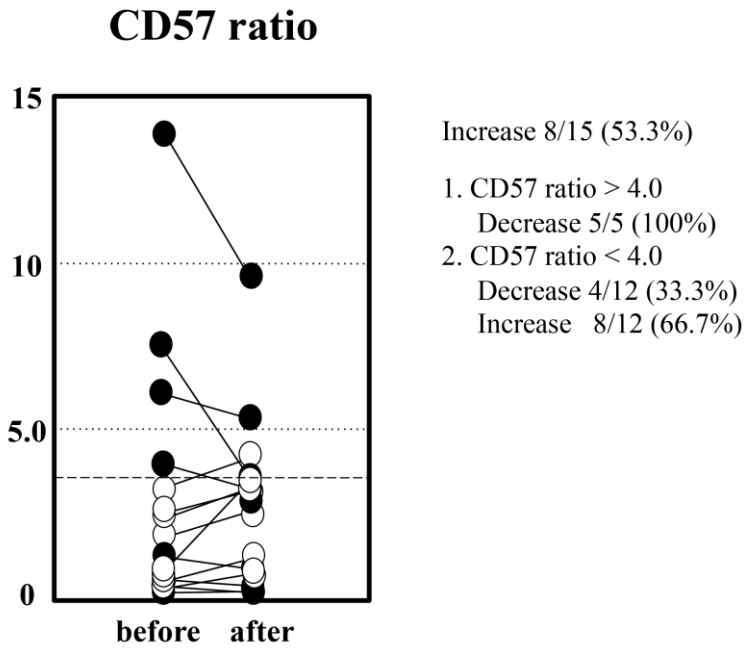
and terminal-CD8+ T cells, showing cytotoxic activity, are sufficiently induced. Conversely, if it is low, there is some obstacle to CD8+ T-cell differentiation, and terminal-CD8+ T cells are not sufficiently induced. In this state, early-CD8+ T cells are involved in immunosuppression [7, 8]. Results of multivariate analysis indicated that the CD57 ratio is an independent favorable prognostic factor that correlates more significantly with PFS than early-CD8+ T cells or terminal-CD8+ T cells alone. The CD57 ratio is considered to be an important parameter in ascertaining the immunological status of cancer patients.

Effect of Zeusion X on CD57 ratio

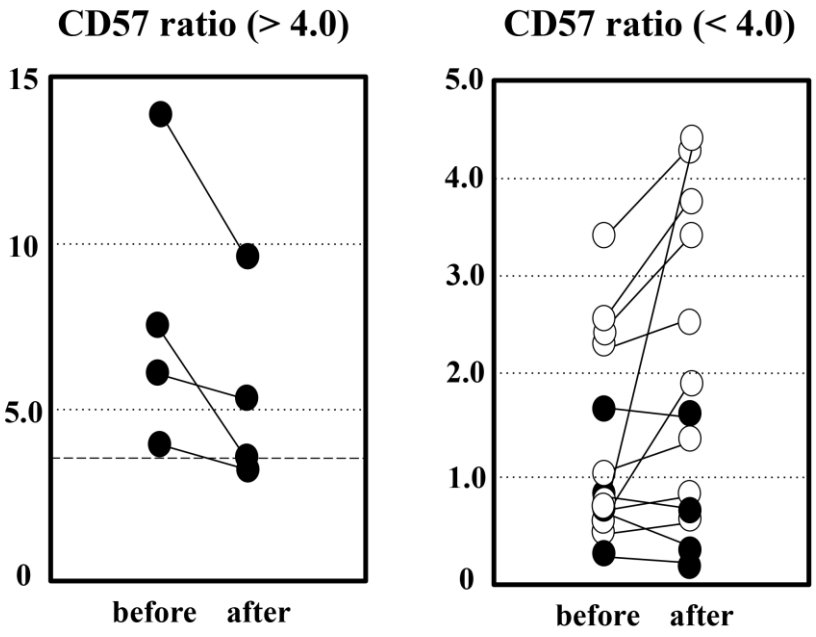
Early-CD8+ T cells decreased in 8 of 15 patients (53.3%), and terminal-CD8+ T cells increased in 8 of 15 patients (53.3%) after the administration of Zeusion X (Fig. 2).



The CD57 ratio increased in 8 of 15 patients (53.3%) (Fig. 3)



In approximately half of the patients, the administration of Zeusion X led to an increase in the CD57 ratio, i.e., a decrease in early-CD8+ T cells and an increase in terminal-CD8+ T cells. This suggests that Zeusion X can activate CD8+ T-cell differentiation and induce the production of terminal-CD8+ T cells, which are effector cells. Further detailed examination revealed that of patients with a CD57 ratio of >4.0, compared with before administration, all 4 patients (100%) showed a decrease in the CD57 ratio after the administration of Zeusion X. Conversely, of patients with a CD57 ratio of <4.0, compared with before administration, 8 of 11 patients (72.7%) showed an increase in the CD57 ratio after the administration of Zeusion X (Fig. 4).



Effect of Zeusion X on PD-1$^+$ terminal-CD8+ T cells

The proportion of PD-1$^+$ terminal-CD8+ T cells was decreased in 8 of 15 patients by the administration of Zeusion X (Table 2).

Table 2 The change in PD-1$^+$ terminal-CD8+ T cells before and after the administration of Zeusion X using the CD57 ratio

	CD57 ratio	
PD-1$^+$terminal-CD8+ T cells	**Increase**	**Decrease**
Increase	**2**	**5**
Decrease	**6**	**2**

Pearson χ^2 test p=0.030

Further, we examined the change in PD-1$^+$ terminal-CD8+ T cells before and after the administration of Zeusion X using the CD57 ratio. We found that PD-1$^+$ terminal-CD8+ T cells were decreased in 6 of 8 patients (75%) in whom the CD57 ratio was increased by the administration of Zeusion X (Table 2). In 5 of patients in whom the CD57 ratio was not increased by the administration of Zeusion X, PD-1$^+$ terminal-CD8 + T cells increased. Although the CD57 ratio decreased after the administration of Zeusion X and PD-1$^+$ terminal-CD8+ T cells increased in the 4 patients with a CD57 ratio of >4.0, the prognosis was good in all the patients. Of the 11 patients with a CD57 ratio of <4.0, the prognosis was good in 5 of 6 patients in whom the CD57 ratio increased and PD-1

decreased after the administration of Zeusion X. However, all 3 patients in whom the CD57 ratio decreased and PD-1 increased showed a poor prognosis (Table 3).

Table 3 The relationship between the change in CD57 ratio and PD-1^{+} terminal-CD8+ T cells after the administration of Zeusion and the prognosis

	No	**CD57 ratio**	**PD-1**	**prognosis**	
				better	**poorer**
CD57ratio >4.0	**4**	↓	↑	**4**	**0**
CD57ratio <4.0	**2**	↑	↑	**2**	**0**
	6	↑	↓	**5**	**1**
	3	↓	↑	**0**	**3**
	0	↓	↓	**0**	**0**

Discussion

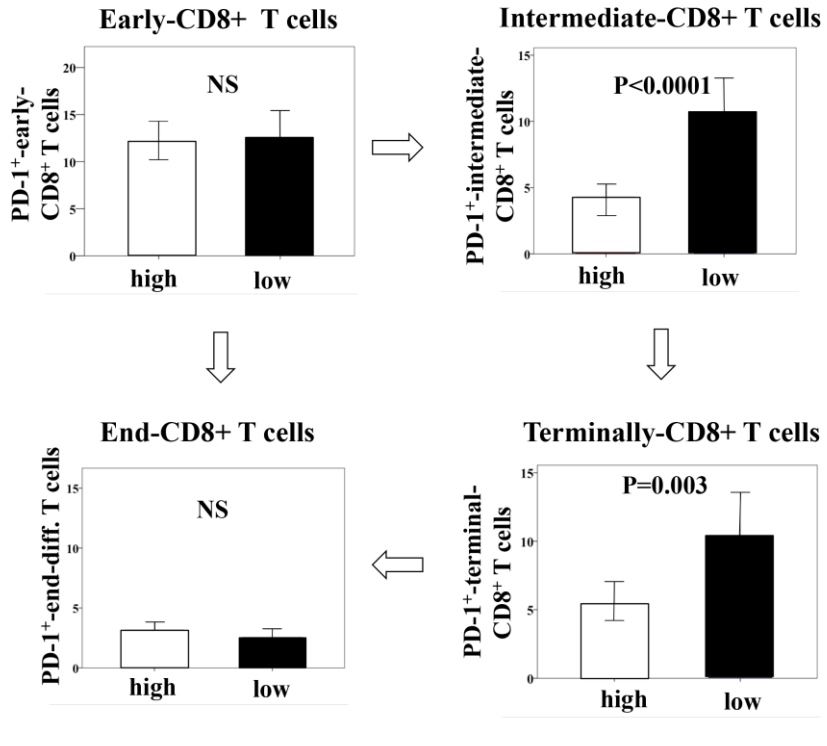
As reported to date, the results of univariate analysis confirmed that intermediate-CD8+ T cells in peripheral blood of cancer patients are involved in a poor prognosis. In addition, we found that early-CD8+ T cells (CD27+CD8+CD57− T cells) are involved in a poor prognosis (Table 1). Although it has been reported that intermediate-CD8+ T cells are involved in a poor prognosis, the fact that early-CD8+ T cells are involved in a poor prognosis is a new finding that has never been reported to date. Terminal-CD8+ T cells are involved in a good prognosis (Table 1). In addition,

because we showed a strong inverse correlation between early-CD8+ T cells and terminal-CD8+ T cells (Spearman correlation coefficient, 0.919; $p < 0.0001$) [7, 8], the CD57 ratio (=terminal-CD8+ T cells/early-CD8+ T cells) was calculated. This is thought to reflect the differentiation state of CD8+ T cells from early-CD8+ T cells to terminal-CD8+ T cells. When the CD57 ratio is low (early-CD8+ T cells are high and terminal-CD8+ T cells are low), it is suggested that CD8+ T-cell differentiation is suppressed. Conversely, when the CD57 ratio is high (early-CD8 + T cells are low and terminal-CD8+ T cells are high), it is suggested that the differentiation of CD8+ T cells is smooth and the production of effector terminal-CD8+ T cells is smooth. Therefore, this CD57 ratio is considered to be an indicator directly reflecting the dynamic state of the differentiation of CD8+ T cells, which is directly linked to the prognosis of cancer patients. As a result of multivariate analysis, we found that the CD57 ratio most strongly reflects a patient's prognosis among any cell groups in the CD8+ T-cell differentiation process (HR = 0.143, 95% CI 0.065–0.314, $p < 0.0001$) (Table 1). From the ROC curve, the cutoff value of the CD57 ratio was 1.45 (AUC 0.810, sensitivity 85.5%, specificity 78.9%, $p < 0.0001$) [7, 8]. The high value group in terms of the CD57 ratio (>1.45) had

a significantly better prognosis than the low value group (<1.45) (Kaplan–Meyer log rank $p < 0.0001$) [7, 8].

We investigated the immunological effect of Zeusion X using early-CD8+ T cells, terminal-CD8+ T cells, PD-1^+ terminal-CD8+ T cells, and the CD57 ratio. The results showed that Zeusion X increased the CD57 ratio in 8 of 15 patients (53.3%). In other words, it is thought that Zeusion X activated cytotoxic terminal-CD8+ T cells by activating the differentiation of CD8+ T cells in approximately half of the patients. Further, as shown in Fig. 3, the CD57 ratio decreased after the administration of Zeusion X in all 4 patients with a CD57 ratio of 4.0 or more. Conversely, the CD57 ratio increased in 8 of 11 patients (72.7%) with a CD57 ratio of <4.0 (Table 3). The prognosis was good in all 4 patients in whom the CD57 ratio was high (>4.0) before the administration of Zeusion X. Of 11 patients with a low CD57 ratio (<4.0) before the administration of Zeusion X, the CD57 ratio increased in 8 after administration, and their prognoses were good, except in 1 patient. However, 3 of 4 patients in whom the CD57 ratio decreased after administration had a poor prognosis (Table 3).

We also measured the expression of PD-1 in terminal-CD8+ T cells in the present study. The expression of PD-1 in CD8+ T cells is most common in early-CD8+ T cells and decreases as CD8+ T-cell differentiate into intermediate-CD8+ T cells and further into terminal-CD8+ T cells [11]. Normally, there is little expression of PD-1 in terminal-CD8+ T cells. However, it has been reported that in cancer patients, the expression of PD-1 in CD8+ T cells persists without decreasing, despite the progress of differentiation of CD8+ T cells [12,13]. In case of cancer patients with a high CD57 ratio (CD8+ T-cell differentiation is normal), the expression of PD-1 in CD8+ T cells decreased with differentiation and PD-1 in terminal-CD8+ T cells was almost undetectable(Fig.5).



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However, in patients with a low CD57 ratio (patients in whom CD8+ T-cell differentiation is compromised), PD-1 expression in CD8+ T cells did not decrease and PD-1 was highly expressed in the cells (Fig. 5). Terminal-CD8+ T cells expressing PD-1 are thought to be CD8+ T cells exhausted by continuous stimulation with cancer antigens. Exhausted CD8+ T cells have extremely low cytotoxic activity, and it is thought that the prognosis will be poor in cancer patients with increased exhausted CD8+ T cells. Therefore, by measuring PD-1^+ terminal-CD8+ T cells, it is possible to predict the prognosis of cancer patients. Although the CD57 ratio decreased and PD-1^+ terminal-CD8+ T cells increased with the administration of Zeusion X in patients with a

high CD57 ratio (>4.0), the prognosis was good in all the patients. This is considered to be a normal biological reaction when the immune reaction is excessive (CD57 ratio > 4.0). In case of a low CD57 ratio (<4.0), there is a good prognosis when the CD57 ratio increases and PD-1^+ terminal-CD8+ T cells decrease after the administration of Zeusion X. These cases are considered to be cases compatible with Zeusion X. Conversely, even when Zeusion X is administered, the CD57 ratio decreases and PD-1^+ terminal-CD8+ T cells increase in some cases. The prognosis of those cases is obviously poor. Therefore, these cases are considered to be incompatible with Zeusion X.

These results suggest that Zeusion X reduces the CD57 ratio and increases PD-1 in terminal-CD8+ T cells to suppress immunity in patients in whom immunity induction of CD8+ T cells is excessive, such as those with a CD57 ratio of > 4.0. On the other hand, in patients in whom CD8+ T-cell immunity induction is insufficient, such as those with a CD57 ratio of <4.0, Zeusion X is suggested to increase the CD57 ratio and decrease PD-1 in terminal-CD8+ T cells to activate immunity. We speculate that patients with a low CD57 ratio, which are often cancer patients, the administration of Zeusion X increases the CD57 ratio, decreases PD-1^+ terminal-CD8+ T cells, activates CD8+ T-cell

differentiation, and induces cytotoxic terminal-CD8+ T cells. However, in cancer patients with a high CD57 ratio, we speculate that Zeusion X decreases the CD57 ratio and increases the PD-1^{+} terminal-CD8+ T cells to adjust the immune system against acting excessively. However, in cancer patients in whom immunity cannot be controlled successfully, i.e., cancer patients who do not show any improvement with Zeusion X administration despite having a low CD57 ratio, cytotoxic terminal-CD8+ T cells may be insufficiently induced and PD-1^{+} terminal-CD8+ T cells that suppress cytotoxic activity may increase. Therefore, the prognosis is considered to be poor. Conversely, in patients in whom the CD57 ratio does not decrease and PD-1^{+} terminal-CD8+ T cells do not increase despite a high CD57 ratio, autoimmune disease may occur. Thus, Zeusion X suppresses the immunity induction of CD8+ T cells when it is excessive and activates the immunity induction of CD8+ T cells when it is deficient, suggesting the possibility of regulating CD8+ T-cell immunity. These findings suggest that Zeusion X could be effective not only in cancer patients but also in patients with autoimmune diseases. However, because the presence of cases that are incompatible with Zeusion X was also presumed in this study, we need to consider countermeasures against these cases in the future.

Conclusion

Our results suggest that Zeusion X increases the CD57 ratio by inducing terminal-CD8+ T cells and reducing PD-1+ terminal-CD8+ T cells so that overall immunity is activated in patients with a low CD57 ratio. On the other hand, when the CD57 ratio is high, it is suggested that Zeusion X decreases the CD57 ratio by decreasing the induction of terminal-CD8+ T cells and increasing PD-1^{+} terminal-CD8+ T cells so that overall immunity is suppressed. These results demonstrate that Zeusion X could improve various diseases by normalizing the body in a properly maintained immune state.

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