

# Terminally differentiated CD8<sup>+</sup> T cells and CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells are highly associated with the efficacy of immunotherapy using activated autologous lymphocytes

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**Abstract.** Treatment with activated autologous lymphocytes (AALs) has demonstrated mixed results for cancer treatment. Preliminary results revealed that the proportion of cluster of differentiation (CD)8<sup>+</sup>CD57<sup>+</sup> T cells is significantly increased in AALs, indicating that they are able to determine treatment outcome. Therefore, the role of CD8<sup>+</sup>CD57<sup>+</sup> T cells in AAL efficacy was investigated. T lymphocytes were isolated from 35 patients with stage IV gastric carcinomas (17 men and 18 women; aged 41-84 years) receiving immunotherapy using AALs (IAAL). Using fluorescence activated cell sorting, CD8, CD27, CD57, and forkhead box protein 3 (FOXP3) expression was investigated on CD8<sup>+</sup> T cell populations in CD8<sup>+</sup> T cell differentiation prior to and following *in vitro* culture. The association between these populations and progression-free survival (PFS) was analyzed using Cox univariate, and multivariate analyses and Kaplan-Meier survival analysis. CD57 expression was negative in early-differentiated CD8<sup>+</sup> T cells (CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>-</sup>), and positive in intermediate- (CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>+</sup>) and terminal- (CD27<sup>-</sup>CD8<sup>+</sup>CD57<sup>+</sup>) differentiated CD8<sup>+</sup> T cells. Univariate analysis revealed a significant association between terminal-CD8<sup>+</sup> T cells and longer PFS times (P=0.035), whereas CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were associated with shorter PFS times. Multivariate analysis revealed that CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells was an independent poor prognostic factor, whereas CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were not associated with PFS. Although IAAL increased the proportion of terminal-CD8<sup>+</sup> T cells relative to the pre-culture proportions, patients with a high

CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cell percentage exhibited repressed terminal-CD8<sup>+</sup> T cell induction, leading to poor patient prognosis. Terminally differentiated CD27<sup>-</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells were responsible for the effectiveness of AALs; however, CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells abrogated their efficacy, possibly by inhibiting their induction.

## Introduction

Cluster of differentiation 8 (CD8<sup>+</sup>) cytotoxic T lymphocytes (CTLs) typically differentiate linearly, from early-differentiated CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (early-CD8<sup>+</sup> T cells) through intermediate-differentiated CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells (intermediate-CD8<sup>+</sup> T cells) to terminally differentiated CD27<sup>-</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells (terminal-CD8<sup>+</sup> T cells) and end-differentiated CD27<sup>-</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells (end-CD8<sup>+</sup> T cells) (Fig. 1) (1). Terminal-CD8<sup>+</sup> T cells, which are the most mature effector (T<sub>MME</sub>) cells, have been recognized as the most potent effector cells. They express high levels of granzyme B (GB) and perforin, and may induce effective cytotoxicity against tumor cells (Fig. 1) (2-4). CD8<sup>+</sup> CTL differentiation is essential for maintaining a pool of mature CTLs in the blood (2-4). However, differentiation of early-CD8<sup>+</sup> T cells toward the T<sub>MME</sub> phenotype is inhibited by transforming growth factor-β, interleukin (IL)-10, and programmed cell death protein-1 in patients with cancer, in whom the accumulation of incompletely differentiated early- and intermediate-CD8<sup>+</sup> T cells leads to uncontrolled progression of malignant cells (2-4).

A previous study reported that patients with advanced gastric cancer with a high (>18%) CD57<sup>+</sup> T cell percentage in the peripheral blood had a shorter survival time compared with those with a low percentage (<18%) (5). In this study, it was demonstrated that a high CD57<sup>+</sup> T cell percentage is an independent poor prognostic factor in patients with advanced gastric carcinomas (5). A previous report revealed that patients with melanoma with a lower percentage (<23%) of CD8<sup>+</sup>CD57<sup>+</sup> T lymphocytes within the peripheral blood CD8<sup>+</sup> T-cell population prior to receiving adjuvant interferon (IFN)-α2b treatment survived for longer compared with patients with a higher percentage (>23%) of CD8<sup>+</sup>CD57<sup>+</sup> T cells (6); however, the opposite was observed in patients with advanced renal cell

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carcinoma (7). In these patients, adjuvant IFN- $\alpha$ 2b therapy significantly increased the survival time of patients with a higher percentage (>30%) of CD8<sup>+</sup>CD57<sup>+</sup> T cells, whereas no increase in survival was observed in those with a lower percentage (<30%) of CD8<sup>+</sup>CD57<sup>+</sup> T cells (7). Thus, in patients with a low pre-treatment percentage of CD8<sup>+</sup>CD57<sup>+</sup> T cells, IFN- $\alpha$  treatment appears to improve the level of CD8<sup>+</sup>CD57<sup>+</sup> T cells, as IFN- $\alpha$  promotes their expansion and survival. However, in patients with a higher pre-treatment CD8<sup>+</sup>CD57<sup>+</sup> T cell percentage, the level of these cells tends to decrease because IFN- $\alpha$  may eliminate over-activated T cells (8). These reports suggest that the cytotoxic subsets of CD8<sup>+</sup>CD57<sup>+</sup> T cells may predominate in patients with melanoma, while the expansion of the immunosuppressive subsets may prevail in patients with renal cell carcinoma. Wu *et al* (2) proposed that there are two types of CD8<sup>+</sup>CD57<sup>+</sup> T cells, based on the expression of the early effector-memory marker CD27: i) Incompletely differentiated CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells that are GB<sup>+</sup>perforin<sup>-/low</sup> (poorly cytotoxic); and ii) terminally-differentiated CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells that are GB<sup>high</sup>perforin<sup>high</sup> (highly cytotoxic), which may explain the aforementioned seemingly contradictory results.

FOXP3-expressing CD8<sup>+</sup> regulatory T cells (CD8<sup>+</sup> Tregs) have been reported to mediate immunosuppression in prostate, colorectal, hepatocellular and gastric cancer. This effect is similar to that of FOXP3<sup>+</sup>CD4<sup>+</sup> T cells, which share a phenotype, functional features and mechanisms of action with FOXP3<sup>+</sup>CD8<sup>+</sup> T cells (9-12). By contrast, during normal CD8<sup>+</sup> T cell differentiation, early-CD8<sup>+</sup> T cells (CD27<sup>+</sup>CD28<sup>+</sup>CD57<sup>+</sup> T cells) transiently express FOXP3 upon T-cell receptor stimulation *in vitro*, but do not necessarily acquire suppressive capabilities (13,14). Transient expression of FOXP3 during CD8<sup>+</sup> T cell activation may be a mechanism for limiting excessive immune activation and damage at the site of inflammation (15,16). Since these reports indicated certain roles for FOXP3 in CD8<sup>+</sup> T cell differentiation, the present study investigated the expression of FOXP3 in activated autologous lymphocytes (AALs).

As induction of T<sub>MME</sub> is the most important feature of immunotherapy using AALs (IAAL), the present study aimed to investigate the association between CD57-associated populations in the CD8<sup>+</sup> T cell differentiation pathway (early-CD8<sup>+</sup>, intermediate-CD8<sup>+</sup>, terminal-CD8<sup>+</sup>, end-CD8<sup>+</sup>, CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> and CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells) and IAAL efficacy in the present study.

## Patients and methods

**Patients.** All participants provided written informed consent prior to enrollment in the present study. Patients with Stage IV gastric carcinomas diagnosed according to the unified TNM classification (17) and with a performance status (Eastern Cooperative Oncology Group Performance Status) between 0 and 2 were eligible for the present study. The study protocol was approved by the Institutional Review Board at each participating center, Tamana Regional Health Medical Center (Tamana, Japan) and Kumamoto University (Kumamoto, Japan). All methods and procedures associated with the present study were conducted in accordance with the Good Clinical Practice guidelines and accorded ethically with the principles of the Declaration of Helsinki and local laws.

A total of 35 patients receiving IAAL at the Tamana Regional Health Medical Center and Hakuzandori Clinic (Tokyo, Japan) between July 2013 and March 2015 were enrolled in the current study. The patients with stage IV gastric carcinoma (17 men and 18 women) ranged between 41 and 84 years in age (mean  $\pm$  standard deviation, 59.4 $\pm$ 11.0 years).

**Sample collection and processing.** Peripheral blood samples (50 ml) were collected from all eligible patients prior to and following IAAL; patient eligibility was determined by the presence of histologically and clinically diagnosed carcinoma. Mononuclear cells were separated by Ficoll-Hypaque density gradient centrifugation as follows: With a sterile pipette, the Ficoll-Hypaque solution was placed into a 50-ml conical centrifuge tube, using 2 ml of Ficoll-Hypaque/1 ml blood; anticoagulated blood was mixed with an equal volume of PBS; the diluted blood was slowly layered over the Ficoll-Hypaque solution by gently pipetting the diluted blood down the side of the tube containing the Ficoll-Hypaque; samples were centrifuged for 40 min at 400  $\times$  g (22°C) with no brake; mononuclear cells, located at the interface between the plasma (upper layer) and the Ficoll-Hypaque (bottom) were carefully removed using a Pasteur pipette; the aspirated mononuclear cells were transferred to a 15-ml conical tube; mononuclear cells were cultured for 2 weeks with recombinant IL-2 (700 IU/ml; Shionogi & Co., Ltd., Osaka, Japan) and immobilized using anti-CD3 monoclonal antibody for 2 weeks at 37°C (MAb) (cat. no. 12277, 5  $\mu$ g/ml; Janssen-Kyowa; Johnson & Johnson, New Brunswick, NJ, USA). Sixteen-well cell cultures were performed for cells obtained from patients assigned to immunotherapy, preserving  $\sim 2 \times 10^{10}$  cells as the source for the second to the fifth infusions (18); however, cultures were discontinued for patients assigned to other treatments (18). Briefly, mononuclear cells are separated from approximately 30 ml of peripheral blood and cultured with immobilized OKT3 and IL-2 for 3-5 days (RPMI-1640+9 Sin and RPMI-1640 HSR Sin, Lymphotec Inc). This step was designated the activation step. The activation step was followed by additional culture in a medium containing only IL-2 for 9-11 days in gas permeable bags (CP-4 and backpack medium, Lymphotec Inc., Tokyo, Japan). This second step is designated the expansion step. Lymphocytes prior to and following the *in vitro* culture were phenotyped with MAbs against CD8 (RPA-T8; cat. no. 560917), CD27 (M-T271; cat. no. 557330), CD57 (NK-1; cat. no. 560844), and FOXP3 (259D/C7; cat. no. 560082) obtained from BD Biosciences (Franklin Lake, NJ, USA). These antibodies were diluted with IsoFlow (cat. no. 8599600) obtained from Beckman Coulter, Inc.

Samples were centrifuged at 652  $\times$  g at room temperature for 5 min to remove the supernatant, and then suspended in sheath solution. Antibodies (20  $\mu$ l) were added to tubes in accordance with combinations shown in Table I. A total of 1 ml of each sample was added to each tube. Staining was performed by keeping on ice for tubes I to VI and at room temperature for tubes I' and VII, for  $\sim 20$  min. To tubes I-VI, 2 ml sheath solution was added, and tubes were centrifuged at 652  $\times$  g at room temperature for 5 min. The supernatant was removed, and the pellet was suspended in sheath solution. Samples were evaluated using 3-color FACS analysis (Lymphotec, Inc., Tokyo, Japan) according to the manufacturers standard operating procedure.

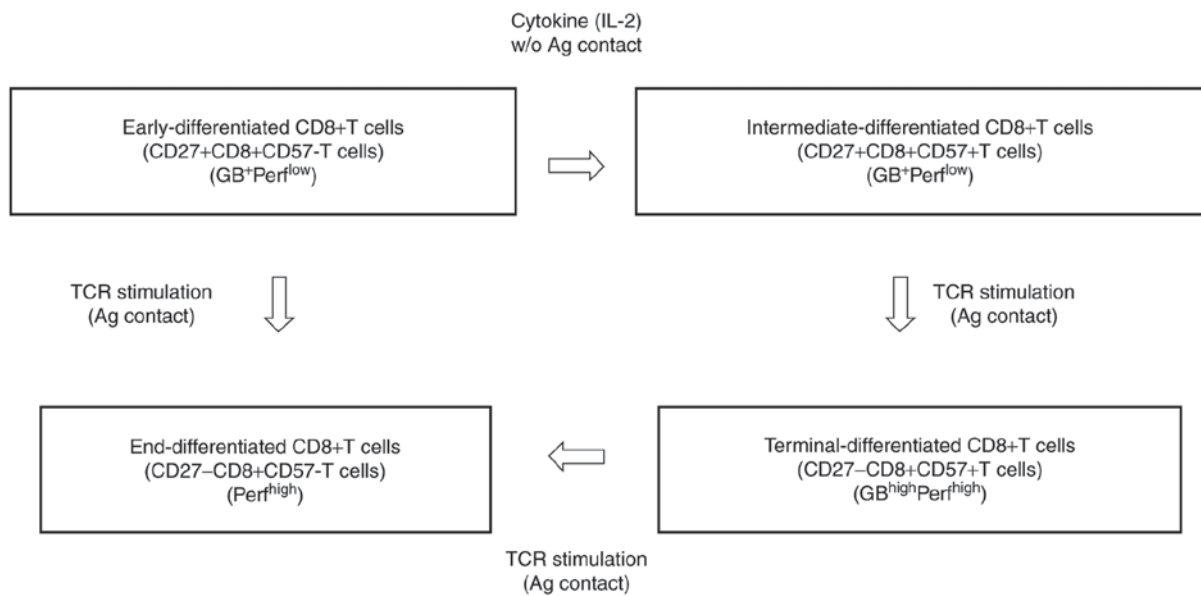


Figure 1. CD8<sup>+</sup> T cell differentiation pathway. IL-2, interleukin-2; CD8, cluster of differentiation 8; GB, granzyme B; Perf, perforin; TCR, T cell receptor; Ag, antigen.

The contents of tubes I' and VII were fixed by centrifuging at 250 x g for 10 min at room temperature with 2 ml of sheath solution. Buffer A (2 ml) was added and incubated in the dark at room temperature for 10 min. Tubes were centrifuged again at 500 x g for 10 min at room temperature, and the supernatant was removed.

The viability of the final cell products was determined using the dye exclusion test (trypan blue-exclusion test), and possible contamination by bacteria, fungi, and endotoxins was assessed twice (Ager medium method: Cell suspension (500  $\mu$ l) was seeded on soybean-casein digest agar medium (cat. no. P94505R200; Nikken Biomedical Laboratory Inc., Kyoto, Japan.).

In order to maintain safety in the laboratory, a closed system was maintained for the first 7 days of culture. The growth of bacteria was investigated twice. First, 2 days before culture ended, and second 2 days post-culture. Only when no growth of bacteria was recognized were the cell products provided for patients.

**Antibodies and fluorescence activated cell sorting (FACS).** The following reagents were used for FACS as described below: Anti-CD3-fluorescein isothiocyanate (FITC) (HIT3a; cat. no. 555339), anti-CD57-FITC (NK-1; cat. no. 555619), anti-CD27-phycoerythrin (PE) (M-T271; cat. no. 557330), anti-FOXP3-PE (259D/C7; cat. no. 560082), anti-CD56-PE (B159; cat. no. 561903), anti-CD57-PE (NK-1 cat. no. 560844), anti-CD4-PE-Cy7 (SK3; cat. no. 560909), and anti-CD8-PE-Cy7 (RPA-T8; cat. no. 560917) obtained from BD Biosciences. Cells were analyzed on a BD FACSCalibur 3A using BD FACS Express software (version 10.6.4; BD Biosciences). All antibodies with the exception of anti-FOXP3 were prediluted.

To each tube (1 ml) 2 ml sheath solution was added and tubes were centrifuged at 250 x g at room temperature for 10 min. The cell pellet was loosened by tapping the tubes, then 2 ml Buffer A was added and left to incubate in the dark at room temperature for 10 min. The tubes were centrifuged at

500 x g at room temperature for 10 min, the supernatant was removed, and the cell pellet loosened by tapping the tubes.

Buffer C (0.5 ml) was added to each tube, and incubated in the dark at room temperature for 30 min. Sheath solution (2 ml) was added and tubes were centrifuged at 500 x g for 10 min to remove the supernatant. An appropriate amount (300  $\mu$ l) of sample was added to each tube. Staining was performed by keeping at room temperature for tubes I' and VII, for about 20 min.

FoxP3(PE) antibody was added and incubated in the dark at room temperature for 30 min. Sheath solution (2 ml) was added and samples were centrifuged at 500 x g for 10 min to remove the supernatant. The samples were once again suspended in sheath solution and centrifuged as aforementioned, then suspended in 300  $\mu$ l sheath solution. Samples were evaluated using 3-color FACS analysis according to the manufacturers standard operating procedure

#### Flow cytometric analysis.

**Two-color flow cytometry.** Two- and three-color flow cytometric analyses were performed using the following MABs: Leu-7-FITC (cat. no. 555619) and anti-CD3-PE for CD57<sup>+</sup> T cells, anti-CD8-FITC and anti-CD57-PE for CD8<sup>+</sup>CD57<sup>+</sup> T cells, and anti-CD3-FITC and anti-CD56-PE for CD56<sup>+</sup> T cells (all BD Biosciences). Flow cytometric analysis was performed using a FACSCalibur flow cytometer (BD Biosciences). The proportions of CD56<sup>+</sup> T cells and CD57<sup>+</sup> T cells were expressed as a percentage of the total number of mononuclear cells.

**Three-color flow cytometry.** A total of 1x10<sup>6</sup> cell/ml of sample was dispensed into each tube and incubated with the aforementioned MABs for 20 min at 4°C (tubes I-VI) for cell surface immunostaining or at room temperature for intracellular staining (tubes I' and VII). A total of 2 ml IsoFlow sheath liquid (Beckman Coulter Inc., Brea, CA, USA) was added to tubes I-IV, and the samples were centrifuged at 2,700 x g for

Table I. Contents of each tube used in sample processing.

Tube no.	FITC	PE	PE-Cy7
I	Control (FITC/PE)		Control (PE-Cy7)
II	CD3	CD4	CD56
III	CD3	CD4	CD57
IV	CD3	CD8	CD56
V	CD3	CD8	CD57
VI	CD57	CD27	CD8
I'	Control (FITC/PE)		Control (PE-Cy7)
VII	CD57	FoxP3	CD8

FITC, fluorescein isothiocyanate; PE, phycoerythrin.

5 min at room temperature. Thereafter, the supernatant was removed and replaced with sheath liquid. For tubes I' and VII, 2 ml sheath liquid was added, and the samples were centrifuged at 250 x g for 10 min at room temperature. The pellets were resuspended in 2 ml Buffer A (10-fold dilution of Human FOXP3 Buffer A from Human FoxP3 Buffer set with water; BD Biosciences) after removing the supernatant and maintaining for 10 min at room temperature. These samples were analyzed using a FACSCalibur 3A flow cytometer and FACS Express software 10.6.4 (both BD Biosciences).

**Intracellular flow cytometry.** For intracellular staining, cells were fixed and permeabilized using the Human FoxP3 Buffer set, according to manufacturer's protocol. A total of 2 ml sheath liquid was added to each tube and centrifuged at 250 x g at room temperature for 10 min. The cell pellet was loosened by tapping the tubes, 2 ml Buffer A was added and the tubes were incubated in the dark at room temperature for 10 min. Tubes were centrifuged at 500 x g at room temperature for 10 min, the supernatant was removed and the cell pellet was loosened by tapping the tubes. Following the addition of 0.5 ml Buffer C (50-fold dilution of Human FOXP3 Buffer B with Buffer A) to each tube, the samples were incubated for 30 min at room temperature. Next, 2 ml sheath liquid was added, the samples were centrifuged at 500 x g for 10 min, and the supernatant was removed. PE-conjugated FOXP3 antibody (560082; BD Biosciences) was added and the tubes were incubated for 30 min at room temperature. Another 2 ml sheath liquid was added, the samples were centrifuged at 500 x g for 10 min, the supernatant was removed and the pellets were resuspended in 300  $\mu$ l sheath liquid. These samples were analyzed as aforementioned.

**Study endpoints and assessments.** The primary endpoint of this study was progression-free survival (PFS). PFS was measured as the duration between the date of randomization and the date of first recurrence or mortality regardless of the cause. The secondary endpoints included overall survival (OS), and cancer-specific survival and safety. OS was measured as the duration between the date of randomization and the date of mortality from any cause; and cancer-specific survival was measured between the date of randomization and the date

of mortality resulting from cancer. Tumor assessments were performed using dynamic computed tomography or magnetic resonance imaging every 3 months for 24 months, and every 3-6 months thereafter in the two groups. All scans at each site were reviewed by two independent, blinded radiologists, each with >5 years of experience. In cases of discordance, a third independent experienced radiologist reviewed the images to achieve a consensus. Adverse events (AEs) were classified and graded every 2 months according to the Common Terminology Criteria for Adverse Events (version 3.0; National Cancer Institute, Bethesda, MD, USA) (19) from the time that patient treatment was started until the end of the study or drop-out, continuing at least 30 days after the last dose of immunotherapy. Multiple events were counted once for each patient and the most severe event was summarized. The data collection cut-off date was November 29, 2015.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. Comparison of non-normally distributed variables between groups was performed using the Mann-Whitney U-test. Comparison of categorical variables between two groups was performed using the  $\chi^2$  test. The probability of survival was estimated using the Kaplan-Meier method, and differences in survival rates were evaluated by log-rank test. Multivariate analysis of prognostic factors was conducted using the Cox regression model. All statistical analyses were conducted using SPSS version 13.0 for Windows (SPSS Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Increased percentage of CD8<sup>+</sup>CD57<sup>+</sup> T cells in AALs.** The mean proportion of CD57<sup>+</sup> T cells (51.7 $\pm$ 2.6%), in particular the CD8<sup>+</sup>CD57<sup>+</sup> T cells (32.2 $\pm$ 2.7%), was markedly increased following the incubation of lymphocytes obtained from the peripheral blood with anti-CD3 MAbs and IL-2 when compared with the average proportions prior to culture (30.3 $\pm$ 2.9 and 15 $\pm$ 1.6%, respectively; Table II). As these results suggested that CD57<sup>+</sup> T cells, particularly CD8<sup>+</sup>CD57<sup>+</sup> T cells, mediate an important role in IAAL, all CD57-associated populations in the CD8<sup>+</sup> T cell differentiation pathway (early-, intermediate-, terminal-, and end-CD8<sup>+</sup> T cells) were investigated. In AALs, the proportion of early-CD8<sup>+</sup> T cells significantly decreased following culture (20.9 $\pm$ 2.9% vs. 41.5 $\pm$ 2.7% prior to culture;  $P < 0.0001$ ), whereas the proportion of terminal-CD8<sup>+</sup> T cells increased significantly following culture (35.7 $\pm$ 3.0% vs. 21.8 $\pm$ 2.5% prior to culture;  $P = 0.001$ ; Table II).

Previous reports revealed that FOXP3<sup>+</sup>CD8<sup>+</sup> T cells functioning as CD8<sup>+</sup> Tregs were the key prognostic factor in determining the clinical outcome of patients with cancer (8) and these cells do not express CD57 (16); thus, the expression of CD57 on FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were analyzed. In the present study, the percentage of CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells was significantly decreased in AALs following culture compared with their pre-culture percentage, whereas there were no significant differences in the proportion of CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells (Table II).

**Univariate and multivariate analysis of CD57-associated immune cells.** Cox proportional-hazards regression analysis



Table II. Comparison of CD57 T cells prior to and following culture.

T cell population	n	Mean $\pm$ standard deviation	P-value
CD57 <sup>+</sup> T			<0.0001
Pre	34	30.3 $\pm$ 2.9	
Post	35	51.7 $\pm$ 2.6	
CD8 <sup>+</sup> CD57 <sup>+</sup> T			<0.0001
Pre	34	15.0 $\pm$ 1.6	
Post	35	32.2 $\pm$ 2.7	
CD4 <sup>+</sup> CD57 <sup>+</sup> T			0.023
Pre	34	11.2 $\pm$ 1.7	
Post	35	18.1 $\pm$ 2.4	
Early-CD8 <sup>+</sup> T			<0.0001
Pre	34	41.5 $\pm$ 2.7	
Post	35	20.9 $\pm$ 2.9	
CD27 <sup>+</sup> CD8 <sup>+</sup> CD57 <sup>+</sup> T (terminal-CD8 <sup>+</sup> T)			0.001
Pre	34	21.8 $\pm$ 2.5	
Post	35	35.7 $\pm$ 3.0	
FOXP3 <sup>+</sup> CD8 <sup>+</sup> CD57 <sup>-</sup> T			0.202
Pre	34	16.6 $\pm$ 3.0	
Post	35	21.8 $\pm$ 2.7	
FOXP3 <sup>+</sup> CD8 <sup>+</sup> CD57 <sup>+</sup> T			0.005
Pre	34	31.8 $\pm$ 3.8	
Post	35	13.8 $\pm$ 2.4	

Pre, pre-culture; Post, post-culture; CD57, cluster of differentiation 57; FOXP3, forkhead box protein 3; Early-CD8<sup>+</sup> T cells, CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells; Terminal-CD8<sup>+</sup> T cells, CD27<sup>+</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells.

was conducted to determine the factors influencing the PFS rate of IAAL recipients (Table III). On the basis of the univariate analysis of eight variables, CD8<sup>+</sup>CD57<sup>+</sup> T cells [hazard ratio (HR), 0.948; 95% confidence interval (CI), 0.901-0.998; P=0.040], terminal-CD8<sup>+</sup> T cells (HR, 0.088; 95% CI, 0.010-0.744; P=0.026), and CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells (HR, 13.526; 95% CI, 1.678-109.0; P=0.014) were significantly associated with PFS (Table III).

To determine the independent value and the relative risk of these prognostic factors, multivariate analysis of the four potential determinants (CD8<sup>+</sup>CD57<sup>+</sup> T cells, early-CD8<sup>+</sup>, terminal-CD8<sup>+</sup>, and CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells) was performed using the Cox regression model. The results confirmed that CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were an independent PFS predictor for patients receiving IAAL (HR, 18.71; 95% CI, 1.922-182.1; P=0.012; Table III).

**Progression-free survival.** The results of univariate analysis demonstrated that the proportions of terminal-CD8<sup>+</sup> and CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells influenced the PFS rate. The patients receiving IAAL were divided into groups based on

Table III. Univariate and Multivariate analysis of CD57-associated CD8<sup>+</sup> T cells.

A, Univariate analysis			
Cells	HR	95% CI	P-value
CD57 <sup>+</sup> T			0.101
CD8 <sup>+</sup> CD57 <sup>+</sup> T	0.948	0.901-0.998	0.040
Early-CD8 <sup>+</sup> T			0.058
Intermediate-CD8 <sup>+</sup> T			0.209
Terminal-CD8 <sup>+</sup> T	0.088	0.010-0.744	0.026
End-CD8 <sup>+</sup> T			0.384
FOXP3 <sup>+</sup> CD8 <sup>+</sup> CD57 <sup>+</sup> T			0.288
FOXP3 <sup>+</sup> CD8 <sup>+</sup> CD57 <sup>-</sup> T	13.53	1.678-109.0	0.014
B, Multivariate analysis			
Cells	HR	95% CI	P-value
FOXP3 <sup>+</sup> CD8 <sup>+</sup> CD57 <sup>-</sup> T	18.71	1.922-182.1	0.012

CD57, cluster of differentiation 57; HR, hazard ratio; CI, confidence interval; FOXP3, forkhead box protein 3.

the proportions of terminal-CD8<sup>+</sup> and CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells with cutoff values of 33.7, and 21.8%, respectively as determined using a receiver operating characteristic curve (sensitivity, 68 and 88.9%; specificity, 100 and 75%; area under the curve, 0.788 and 0.819; P=0.009 and 0.005, respectively). A Kaplan-Meier survival curve revealed that patients with a high percentage of terminal-CD8<sup>+</sup> T cells had a significantly longer PFS duration compared with those with a low percentage of terminal-CD8<sup>+</sup> T cells (Fig. 2A; P=0.004). By contrast, patients with high percentages of CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells exhibited significantly shorter PFS durations compared with those with low percentage of these cells (Fig. 2B; P=0.001).

**Association between CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, CD27 expression, and PFS.** The presence of CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells was an independent poor prognostic factor as aforementioned, whereas CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were not associated with the PFS of IAAL recipients. These results indicated that CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells are the most likely candidates to be CD8<sup>+</sup> Tregs. Previous studies revealed that the CD8<sup>+</sup> Treg phenotype of early-CD8<sup>+</sup> T cells expressing FOXP3 is dependent on CD27 expression and the constitutive stimulation of CD70, a ligand of CD27 (20,21). To investigate the association between CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells and CD27 expression, the PFS rates of four patient subgroups were analyzed: FOXP3<sup>high</sup>/CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>+</sup>; FOXP3<sup>high</sup>/CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>+</sup>; FOXP3<sup>low</sup>/CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>+</sup>; and FOXP3<sup>low</sup>/CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells (Fig. 3). Among the subgroups, patients with FOXP3<sup>high</sup>/CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells exhibited the shortest PFS time, and those with FOXP3<sup>low</sup>/CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>+</sup> T

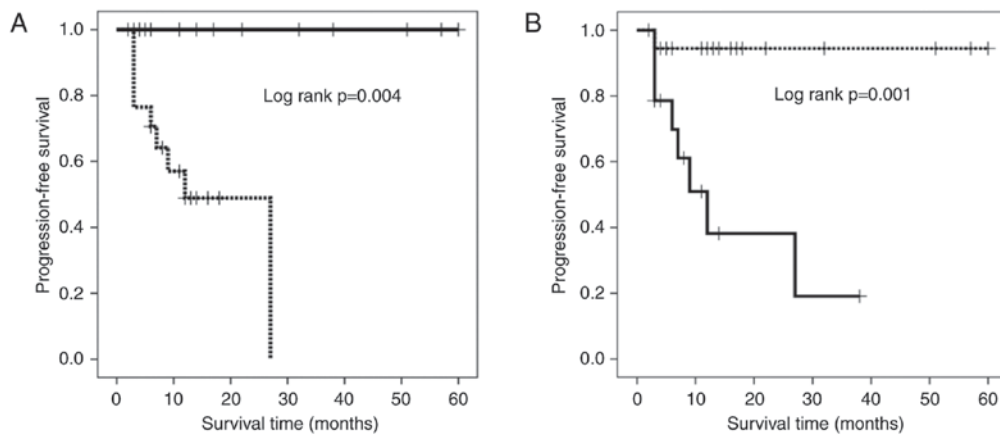


Figure 2. Comparison of progression-free survival between patients with high (bold line) and low (dotted line) percentages of (A) terminal-CD8<sup>+</sup> T cells and (B) CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells. Values indicate the cumulative progression-free survival rate. CD8, cluster of differentiation 8; FOXP3, foxhead box protein 3.

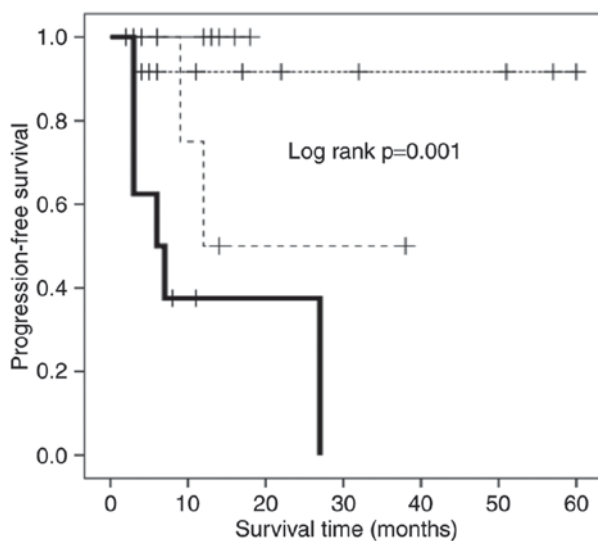


Figure 3. Comparison of progression-free survival among patients with FOXP3<sup>high</sup>CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (bold line), FOXP3<sup>high</sup>CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (broken line), FOXP3<sup>low</sup>CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (thin line), and FOXP3<sup>low</sup>CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (dotted line). Values indicate the cumulative progression-free survival rate. FOXP3, forkhead box protein 3; CD27, cluster of differentiation 27.

cells or FOXP3<sup>low</sup>/CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells exhibited the longest (Fig. 3); patients with FOXP3<sup>high</sup>/CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells exhibited an intermediate PFS time.

**High levels of CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells may impede CD8<sup>+</sup> T cell differentiation.** As CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells functioning as CD8<sup>+</sup> Tregs were previously reported to affect CD8<sup>+</sup> T cell differentiation (22), the terminal-CD8<sup>+</sup> T cell population of the CD8<sup>+</sup> T cell differentiation pathway was compared between patients with high and low percentages of CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells. Following culture, the level of terminal-CD8<sup>+</sup> T cells of patients with high levels of CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were significantly lower compared with those of patients with low CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, whereas there was no significant difference between them prior to culture (Fig. 4A). This decrease was more apparent when patients with high and low FOXP3<sup>high</sup>CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells were compared (Fig. 4B).

## Discussion

The AALs populations examined in the present study contained a high percentage of CD8<sup>+</sup>CD57<sup>+</sup> T cells, which was identified to be significantly associated with longer PFS times following univariate analysis. CD8<sup>+</sup>CD57<sup>+</sup> T cells are comprised of two different populations: Intermediate- and terminal-CD8<sup>+</sup> T cells, the latter of which are associated with longer PFS times. IAAL reduced the proportion of early- and intermediate-CD8<sup>+</sup> T cells, and increased that of cytotoxic terminal-CD8<sup>+</sup> T cells. These results indicated that IAAL promoted CD8<sup>+</sup> T cell differentiation to increase the proportion of cytotoxic terminal-CD8<sup>+</sup> T cells (CD27<sup>-</sup>CD8<sup>+</sup>CD57<sup>+</sup> T cells) as the T<sub>MME</sub>. However, data from the present study revealed that CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells are an independent poor prognostic factor and ultimately determine the efficacy of IAAL. In patients with a high percentage of CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, the proportion of cytotoxic terminal-CD8<sup>+</sup> T cells was significantly lower compared with those with a low percentage of CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, indicating that CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells are likely to function as CD8<sup>+</sup> Tregs, and may disrupt CD8<sup>+</sup> T cell differentiation to reduce the induction of terminal-CD8<sup>+</sup> T cells, leading to poor prognosis (22).

Cosmi *et al* (9) demonstrated the existence of FOXP3-expressing CD8<sup>+</sup> T cells with immunosuppression capability (CD8<sup>+</sup> Tregs), which was similarly identified in prostate, colorectal, hepatocellular and gastric cancer (10-12). However, it has been revealed that FOXP3 expression is not necessarily associated with regulatory functions in human CD4<sup>+</sup> and CD8<sup>+</sup> T cells (20). In the present study, CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were significantly reduced by IAAL and were not associated with the PFS of IAAL recipients. Anichini *et al* (23) reported the existence of FOXP3<sup>+</sup>CD8<sup>+</sup> T cells expressing an early effector profile (rather than a regulatory CD8<sup>+</sup> T cell phenotype) that differentiate into terminal-CD8<sup>+</sup> T cells through intermediate-CD8<sup>+</sup> T cells. The present study indicated that FOXP3 expression on CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells was transient in the absence of any associated regulatory function during the natural course of CD8<sup>+</sup> T cell differentiation (24,25). In contrast to CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, CD57FOXP3<sup>+</sup>CD8<sup>+</sup> T cells

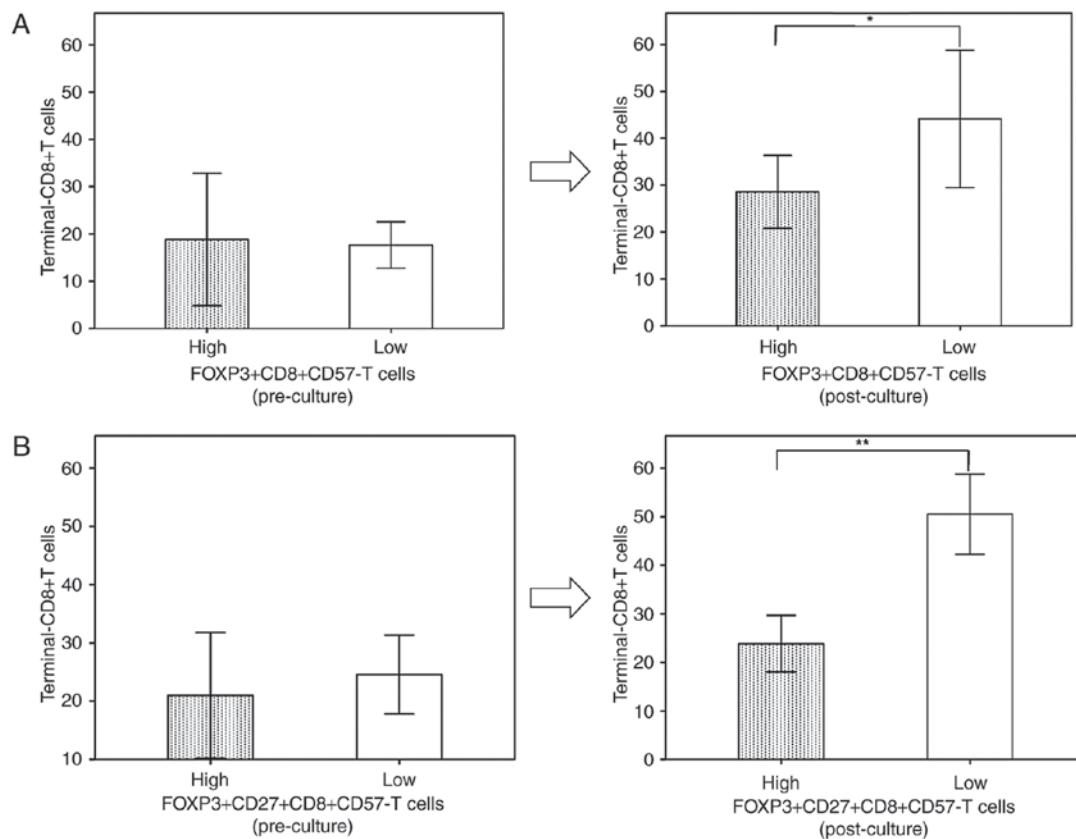


Figure 4. (A) Comparisons of terminal-CD8<sup>+</sup> T cell population between patients with high and low percentages of CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells. Prior to culture, there was no significant difference in the percentage of terminal-CD8<sup>+</sup> T cells between them but, following culture, there was a significant difference between them ( $P=0.040$ ). (B) Comparisons of terminal-CD8<sup>+</sup> T cell population between patients with FOXP3<sup>high</sup>CD27<sup>high</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (High) and FOXP3<sup>low</sup>CD27<sup>low</sup>CD8<sup>+</sup>CD57<sup>-</sup> T cells (Low) at pre- (left) and post-culture (right). There was a significant difference in the percentage of terminal-CD8<sup>+</sup> T cells between them following culture but not prior to culture. \* $P=0.040$ , \*\* $P=0.003$  using the Mann-Whitney U-test. CD8, cluster of differentiation 8; FOXP3, forkhead box protein 3.

were appropriately classified as CD8<sup>+</sup> Tregs in this study for the following reasons: i) CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were an independent poor prognostic factor in a multivariate analysis; ii) CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells inhibited CD8<sup>+</sup> T cell differentiation; and iii) like conventional CD8<sup>+</sup> Tregs, CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, which were identified as an independent poor prognostic factor in the present study, do not express CD57 (26). Taken together, it can be concluded that CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were CD8<sup>+</sup> Tregs, and CD57 expression on FOXP3<sup>+</sup>CD8<sup>+</sup> T cells may be an immunological marker for discriminating FOXP3<sup>+</sup>CD8<sup>+</sup> T cells with a regulatory function from those without.

An association between simultaneous expression of FOXP3 and CD27, and the poorest PFS of the four subgroups was also demonstrated in the present study, as CD27 expression was reported to discriminate regulatory from non-regulatory cells (27). CD27, a member of the tumor necrosis factor receptor superfamily, serves as a co-stimulatory factor, and CD27 signaling leads either to improved T cell function or to T cell dysfunction, depending on the level, duration, and timing of CD27 ligand (CD70) expression (21,28-30). Consequently, CD70 expression is tightly regulated, and CD70 is only transiently expressed on activated T and B cells, and on subsets of professional antigen-presenting dendritic cells and natural killer cells (21). By contrast, constitutive CD70 expression has been documented in various types of cancer, including

brain tumors (20), renal cell carcinomas (21) and certain lymphomas (31,32), and is often associated with FOXP3<sup>+</sup> Treg formation (31,32). Thus, CD27 signaling mediated by constitutive CD70 expression is essential for the induction of FOXP3<sup>+</sup> regulatory CD8<sup>+</sup> T cells. Therefore, we hypothesize that CD27 is expressed on CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells functioning as CD8<sup>+</sup> Tregs in the current study. Furthermore, the present study demonstrated that the proportion of CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells was unchanged following culture, when compared with the pre-culture proportion, indicating that once CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells (CD8<sup>+</sup> Tregs) adopt a regulatory function, their phenotype cannot be changed despite an improvement in the microenvironment induced by IAAL. Previous reports demonstrated that an anti-human CD27 agonist MAb was shown to induce T cell activation and tumor immunity in human CD27-transgenic mice (33) through a combination of co-stimulation, and Treg-depleting activities (34). These results indicated that a CD27-agonist MAb may deprive CD57-FOXP3<sup>+</sup>CD8<sup>+</sup> T cells of their regulatory function as CD8<sup>+</sup> Tregs and activate the co-stimulation signaling for CTLs to induce antitumor immunity. Thus, combinational immunotherapy using AALs and a CD27-agonist MAb may lead to more improved outcomes for IAAL.

In conclusion, the results of the present study demonstrated that terminal-CD8<sup>+</sup> T cells are the T<sub>MME</sub> of IAAL, and that IAAL may promote CD8<sup>+</sup> T cell differentiation to induce cytotoxic

terminal-CD8<sup>+</sup> T cells. In addition, CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells were revealed to be an independent poor prognostic factor for patients, to be CD8<sup>+</sup> Tregs and to inhibit CD8<sup>+</sup> T cell differentiation, which cannot be recovered by IAAL. Thus, CD57<sup>+</sup>FOXP3<sup>+</sup>CD8<sup>+</sup> T cells, as CD8<sup>+</sup> Tregs, are essential determinants of IAAL efficacy.

### Competing interests

The authors declare that they have no competing interests.

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