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Coccomyxa sp.KJ extract affects the fate of T cells stimulated by toxic shock syndrome toxin-1, a superantigen secreted by *Staphylococcus aureus*

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Abstract

T cell stimulation by bacterial superantigens induces a cytokine storm. After T cell activation and inflammatory cytokine secretion, regulatory T cells (Treg) are produced to suppress the immune response. *Coccomyxa* sp.KJ (IPOD FERM BP-22254), a green alga, is reported to regulate immune reactions. Therefore, we examined the effects of *Coccomyxa* sp.KJ extract (CE) on the superantigen-induced immune response. When human peripheral blood mononuclear cells (PBMCs) were stimulated with toxic shock syndrome-1 (TSST-1) in the presence of CE, the number of activated T cells decreased moderately. Purified T cells stimulated in the presence of CE comprised more non-proliferating cells than those stimulated in the absence of CE, whereas some T cells proliferated more quickly. The levels of activation markers on the stimulated T cells increased in the presence of CE. Most of the inflammatory cytokines did not change but IL-1β, IL-17, IL-4, and IL-13 secretion increased, whereas that of IL-2, TNF-α, and IL-18 decreased. IL-10 secretion was also decreased by CE treatment, suggesting that the immune response was not suppressed by Treg cells. CE enhanced the expression of stem cell-like memory cell markers in T cells. These results suggest that CE can regulate the fate of T cells and can help to ameliorate superantigen-induced T cell hyperactivation and immune suppression.

KEYWORDS

activation marker, *Coccomyxa*, cytokine, memory T cells, toxic shock syndrome toxin-1

Abbreviations: CE, *Coccomyxa* sp.KJ extract; CFSE, 5-(6)-carboxyfluorescein diacetate succinimidyl ester; FCM, flow cytometry; HD, healthy donor; iT_{SCM}, induced T_{SCM}; MFI, mean fluorescence intensity; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; RT, room temperature; Tc, cytotoxic T cell; TCR, T cell receptor; Treg, regulatory T cell; T_{SCM}, stem cell-like memory T cell; TSST-1, toxic shock syndrome-1.

NTRODUCTION

Superantigens induce T cell receptor (TCR) signal transduction by crosslinking multiple TCR V beta chains with major histocompatibility complex (MHC) antigens.[1] In the absence of antigen specificity, selective T cell activation cannot be implemented causing mass activation of T cells followed by a cytokine storm. Complete T cell activation requires TCR-MHC crosslinking and CD28-B7 interactions. However, superantigens produced by *Staphylococcus aureus* (*S. aureus*) solely induce TCRMHC crosslinking, causing T cells to enter an anergic state.[2] Additionally, immune stimulation by superantigens induces the differentiation of regulatory T cells (Tregs) resulting in immune system exhaustion and immune suppression.[3] To overcome superantigen-mediated immune suppression, it is crucial to induce antigen-specific T cell activation to prevent cytokine storms and immune exhaustion in infected patients.

Coccomyxa sp.KJ (IPOD FERM BP-22254) is a green alga that can accommodate large amounts of fat in its cytoplasm.[4] It has recently gained recognition for inducing neuroprotective effects,[5] enhancing learning and memory,[6] and inhibiting benign prostate hyperplasia.[7] Its ability to regulate the immune system has also been reported. Monogalactosyl diacylglyceride isolated from *Coccomyxa* sp.KJ was shown to suppress viral replication in the genital cavity of herpes simplex virus type-2-infected mice.[8] Crude polysaccharides isolated from *Coccomyxa* sp.KJ were reported to modulate immune responses in chickens.[9] *Coccomyxa* polysaccharides also suppress inflammatory responses in a macrophage cell line, RAW 264.7, post-lipopolysaccharide stimulation.[10] Considering the immune-modulatory effects of *Coccomyxa* sp.KJ towards viral and bacterial pathogens, we hypothesized that *Coccomyxa* sp.KJ extract (CE) could regulate T cell activation and differentiation into effector/memory/anergic T cells in response to immunological challenge by *S. aureus* superantigen.

Following complete activation in the presence of TCR-MHC crosslinking and CD28-B7 interactions, activated T cells differentiate into effector T cells or memory T cells. Memory T cells are further categorized into central memory and effector memory cells.[11] Central memory T cells are characterized by two typical migration markers, CD62L and CCR7, which mediate the localization of T cells to peripheral lymphoid organs, such as the lymph nodes and spleen. In contrast, effector memory T cells down-regulate the expression of these markers and localize to peripheral tissues, where they differentiate into effector T cells to fight pathogens. Recently, a subset of memory T cells termed stem cell-like memory T cells (T_{SCM}) that form before the central memory T cells was identified in mice[12] and in humans.[13] These cells express a naïve T cell marker, CD45RA, in addition to CCR7 and CD62L. There are also several other markers to distinguish these cells, such as CD127 and CXCR3. T_{SCM} cells differentiate from naïve T cells and are highly proliferative. With their self-renewal capacity and multipotent nature, these cells can fully repopulate differentiated effector T cells.[14] Moreover, a T cell stage associated with a phenotype similar to that of T_{SCM} cells was reported, which could develop from activated effector T cells, and it was named induced T_{SCM} (iT_{SCM}).[15]

In this study, we investigated the influence of CE on T cell differentiation into effector/memory T cells post-TCR stimulation by *S. aureus* toxic shock syndrome-1 (TSST-1) using multicolor flow cytometry (FCM).

MATERIALS AND METHODS

Ethical approval

Human peripheral blood mononuclear cells (PBMCs) were derived from healthy donors (HDs) upon receiving written informed consent from the subjects and approval by the Institutional Review Board, the Tokai University Human Research Committee (approval no. 20R051, 21R059). The studies were conducted in accordance with the guidelines of the Declaration of Helsinki and the Japanese federal regulations outlined for the protection of human subjects. Healthy donors without a history of malignant diseases were selected to obtain blood samples for the study.

Preparation of human PBMCs

RPMI 1640 medium and supplements were purchased from NISSUI; 50 mL of peripheral blood (PB) was collected from each healthy donor in the morning using Vacutainer ACD tubes (NIPRO Corporation) containing heparin. The collected blood was immediately transferred to 10 mL of density gradient medium Ficoll-Hypaque (Sigma-Aldrich), and centrifuged (500 × g, 30 min, 20°C) to isolate mononuclear cells. The remaining erythrocytes were removed through osmotic lysis. The cells were washed with phosphate-buffered saline (PBS) for 5 min at 300 × g, 4°C, and the cell number was estimated.

Preparation of *Coccomyxa* sp.KJ crude extracts

Lyophilized *Coccomyxa* sp.KJ (2.5 g; IPOD FERM BP-22254) was added to 25 mL of distilled water and incubated with shaking at 37°C, 100 rev/min for 6 hr. The suspension was centrifuged at 3600 × g for 10 min. The supernatant was collected and lyophilized.

Culture of human PBMCs

The cells were seeded in six-well plates and cultured at a density of 1×10^6 cells/mL in RPMI 1640 medium (Nissui Co. Ltd) containing 10% FCS (Sigma Aldrich) and

antibiotics (streptomycin 0.1 mg/mL, penicillin 100 U/mL; Meiji Seika) in the presence of 1 μg/mL TSST-1 (Toxin Tec.) at 37°C and 5% CO_2. The cells were incubated with varying concentrations of CE in the culture medium and collected at 72 hr, followed by washing with PBS and staining with fluorochrome-labeled mAbs for analysis using FCM.

Restimulation of purified T cells

The Pan T Cell Isolation kit (Miltenyi Biotec) was used for T cell sorting. The PBMCs were cultured as described for 72 hr. Briefly, the cells were collected, washed, and incubated with the Pan T cell biotin-antibody cocktail at 4°C for 5 min. After adding 40 μL of wash buffer, 20 μL of Pan T cell micro bead cocktail was added and incubated at 4°C for 10 min. The T cells were sorted using the Automacs system (program: depletion; Miltenyi Biotec) and labeled using CellTrace™ Cell proliferation kits (Thermo Fisher) following the manufacturer's instructions. 5-(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) powder was reconstituted with dimethyl sulfoxide (Wako) at a concentration of 5 mM. The cells were incubated with CFSE (final concentration, 5 μM) at 37°C for 20 min in the dark, washed with PBS, and resuspended in RPMI 1640 medium. The cells were stimulated with Dynabeads™ Human T-Activator CD3/CD28 (Thermo Fisher) or with an anti-CD3 coated microplate. The cells were collected, and the cell cycle was analyzed using FCM for 3 days, described as follows.

Analysis of immune cell composition by FCM

Mononuclear cells were collected from each well, quantified, and stained with appropriate dilutions of fluorochrome-labeled mAbs for 15 min at 4°C, followed by washing with 1% (w/v) bovine serum albumin (Sigma Aldrich) in PBS. The cells were analyzed for the surface expression of differentiation antigens using the BD LSRFortessa™ flow cytometer (BD Bioscience). For each analysis, the living white blood cells or lymphocytes were gated for propidium iodide and analyzed with FlowJo software v10.3 (BD Bioscience). The mAbs used for staining are summarized in Table S1. CFSE was analyzed using the BD FACSVerse™ Flow Cytometer (BD Bioscience).

Quantification of cytokines secreted by cultured PBMCs

Supernatants of the cultured cells were collected for cytokine quantitation using the bead-based multiplex LEGENDplex (BioLegend) according to the manufacturer's instructions. Briefly, 25 μL of supernatant was mixed with 25 μL of capture beads and incubated for 2 hr at room temperature (RT). The beads were washed, mixed with detection antibodies, and incubated for 1 hr at RT. Subsequently, streptavidin-phycoerythrin was added, and the mixture was incubated for 30 min at RT. Finally, the beads were washed and analyzed using FCM. The cytokines IL-1β, IFN-α, IFN-γ, TNF-α, MCP-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-17F, IL-18, IL-22, IL-23, and IL-33 were quantified. Analysis was performed using the BD FACSVerse™ Flow Cytometer (BD Biosciences). The data were analyzed in pg/mL using LEGENDPlex™ V8.0 (BioLegend).

Statistics

Statistical analyses were performed by using one-way repeated ANOVA and paired Student's *t*-test (Microsoft Excel) (Microsoft). The data are presented as mean ± standard deviation.

RESULTS

CE regulates the number of activated T cells

First, PBMCs were stimulated with TSST-1 in the presence of various concentrations of CE. As shown in Figure 1a, the total number of cells was not significantly changed post TSST-1 stimulation. However, in the presence of CE, a significant decrease was observed in the cell numbers in a concentration-dependent manner (one-way repeated ANOVA results; $P = 0.006246$). Specifically, the proportion of the cells in the large gate indicating activated lymphocytes decreased significantly ($P = 1.56E{-}05$) (Figure 1b). Moreover, the aggregated clusters were enlarged in the 3000 μg/mL CE culture (Figure 1c). We conducted the flow cytometry to analyze the profile of the lymphocytes. The definition of lymphocytes is shown in Table 1. The proportion of Th cells slightly, but significantly, increased and that of the cytotoxic T cells (Tc), B cells, and NK cells decreased significantly, whereas the proportion of NKT cells remained constant (Figure S1). These results suggest that CE affects PBMC activation post-TSST-1 stimulation.

CE regulates T cell proliferation

As CE decreased the PBMC number and changed the morphology of aggregation, we tried to determine whether the influence of CE on the T cell proliferation rate was responsible for this phenomenon. The T cells were purified after a 72 hr stimulation with TSST-1 in the presence or absence of 3000 μg/mL CE, labeled with CFSE, and stimulated with CD3 mAb or anti-CD3 and anti-CD28 mAb-conjugated beads. After 24–72 hr, the cells were collected, and their proliferation was measured by assessing CFSE attenuation. The T cells of the small and large lymphocyte gates showed different levels of CFSE intensity

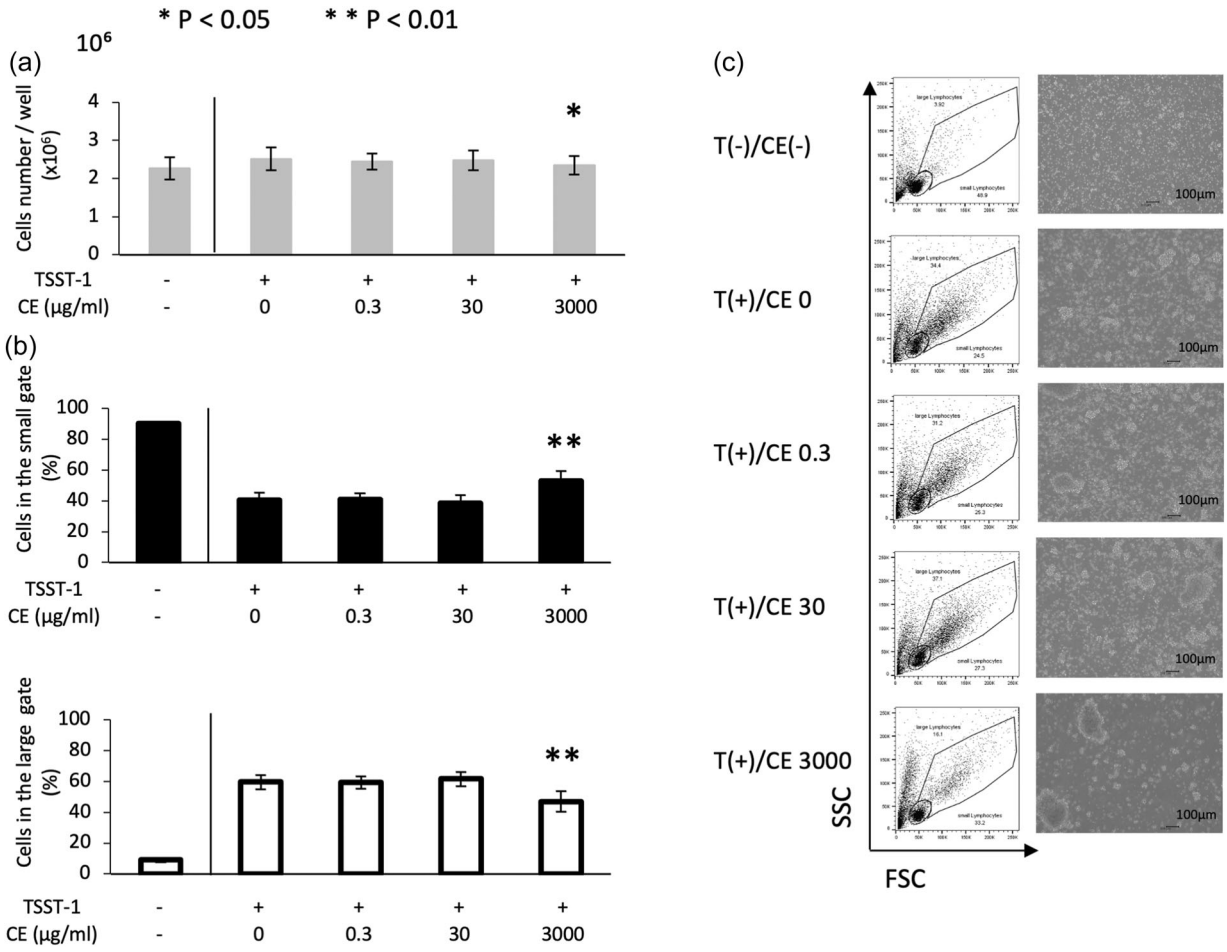

FIGURE 1 *Coccomyxa* sp.KJ extract (CE) affects activated peripheral blood mononuclear cell (PBMC) numbers and morphology. (a) PBMC numbers are indicated by gray bars. (b) Ratio of cells in small lymphocyte gate (upper panel; black bars) and large lymphocyte gate (lower panel; open bars) at different concentrations of CE (0 to 3000 μg/mL) 72 hr after toxic shock syndrome-1 (TSST-1) stimulation. Left bars divided by solid lines represent T cells without TSST-1 stimulation. One-way repeated ANOVA and paired Student's *t*-test (Microsoft Excel) were performed. $*P < 0.05$; $**P < 0.01$. (c) Small and large lymphocyte gates of each sample are indicated in the FSC/SSC panels of flow cytometry (FCM) left panel. Morphological observation of PBMCs (KEYENCE BZ-X710 phase difference) (right panels). The sample number of experiment is $n = 4$ for TSST-1(−), $n = 5$ for TSST-1(+), CE0.3, CE 30 and CE3000

after 24 hr, at which timing no proliferation started on stimulated naïve T cells in the small-lymphocyte gate as shown in the upper panels of Figure S2. As shown in Figure 2, the CFSE level of small lymphocyte-gated cells are lower than large lymphocyte-gated cells, because the surface area is much larger in activated cells than in resting cells. However, after 48 hr, a high-intensity peak of large lymphocyte gate was not observed, suggesting that the cells had divided, resulting in a left-shifted peak. The T cells cultured in the presence of 3000 μg/mL CE without CD3 stimulation proliferated early (48 hr) compared with cells cultured without CE, as shown in Figure 2, whereas a significant number of T cells remained in a nonproliferative state after 72 hr. Among TSST-1-stimulated cell fractions, most of the cells started proliferation after CD3- or CD3/CD28-stimulation without CE, regardless of the pre-treatment of CE for 72 hr (Figure S2, 72 hr lower right four panels).

These results suggest that some part of the T cells proliferated more quickly, whereas a large proportion of T cells in the small lymphocyte gate remained in the resting state when they were stimulated in the presence of CE.

CE enhances effector T cell differentiation

As some T cells proliferated more quickly, this indicated that CE induced effector T cell differentiation. Therefore, we analyzed the surface activation markers of stimulated T cells. Because the expression of CD25 and PD-1 reaches maximum and the proliferation starts, we selected the time point as 72 hr to analyze T cell activation markers. In the large lymphocyte gate, CD25 and programmed death-1 (PD-1) expression in both CD4 and CD8 T cells increased 72 hr after TSST-1 stimulation. In the presence of CE, CD25 expression was increased significantly for both CD4 and

TABLE 1 Definition of surface marker and immune cells by surface markers

Cell type	Markers						
T cells	$CD3^+$						
Th cell	$CD3^+$	CD4 +					
Tc cell	$CD3^+$	$CD8^+$					
B cells	$CD19^+$						
NK cell	$CD3^-$	$CD56^+$					
NKT cell	$CD3^+$	$CD56^+$					
Activated T cells	$CD3^+$	CD25 +	PD-1^-				
Exhausted T cells	$CD3^+$	$CD25^+/^-$	PD-1^+				
Th1	$CD3^+$	$CD4^+$	$CD25^-$	$CCR6^-$	$CXCR3^+$	$CCR7^-$	$CCR4^-$
Th2	$CD3^+$	$CD4^+$	$CD25^-$	$CCR6^-$	$CXCR3^-$	$CCR7^-$	$CCR4^+$
Th17	$CD3^+$	$CD4^+$	$CD25^-$	$CCR6^+$	$CXCR3^-$	$CCR7^-$	$CCR4^+$
Naïve T cell	$CCR7^+$	$CD45RA^+$	CD45RO-	$CD95^-$	$CD127^+$		
TSCM cells	$CCR7^+$	$CD45RA^+$	CD45RO-	$CD95^+$	$CD127^+$		
Effector T cell	$CCR7^-$	CD45RA-	$CD45RO^+$	$CD95^+$	CD127int		

Tc, cytotoxic T cell; TSCM, stem cell-like memory T cell.

CD8 T cells (CD4 small gate; $P = 0.00689$, large gate $P = 0.000259$, CD8 small gate; $P = 0.00127$, large gate $P = 0.000105$), whereas PD-1 expression was decreased in the CD4 T cells in the large lymphocyte gate (CD4 small gate $P = 0.997$, large gate $P = 0.00486$, CD8 small gate $P = 0.963$, large gate $P = 0.301$) (Figures 3a,b, S3). Since CD25 and PD-1 can be used to characterize Treg cells, in addition to serving as activation markers, we examined the secretion of IL-10, a cytokine known to be secreted by Treg cells to suppress the immune system. As hypothesized, IL-10 secretion was decreased significantly ($P = 0.00584$) (Figure 3c), suggesting that CE did not enhance Treg differentiation and thereby immune exhaustion, but enhanced the number of activated effector T cells.

We also examined Th1, Th2, and Th17 cell-specific surface markers following the instructions of He et al.[16] The population of the Th1 subset was significantly increased in the small lymphocyte gate in the presence of CE in a concentration-dependent manner ($P = 0.00076$) (Figure 3d,e). The Th2 and Th17 subsets also exhibited a significant increase (Th2: $P = 0.003$ and Th17: $P = 0.009$), although the increase did not affect the cell number because the proportion of effector T cells was not high (Figures 3d,e, S4). We also examined the effects of CE on cytokine secretion. Most inflammatory cytokines were increased after TSST-1 stimulation, but CE did not alter this cytokine production. However, IL-1β and IL-17A, IL4 and IL-13 (IL-1β: $P = 7.06E{-}05$, IL-17A: $P = 1.87E{-}05$, IL-4: $P = 0.0111$, IL-13: $P = 0.0125$) showed a significant increase in the presence of CE (Figure S5). In contrast, the TNF-α, IL-18, and IL-2 levels were decreased significantly (TNF-α: $P = 0.00224$; IL-18: $P = 0.00238$; IL-2: $P = 0.00324$). These results suggest that CE significantly induced effector T cell differentiation and changed the cytokine secretion profile. Th1 cytokines tended to decrease, but the ratio of Th1 cells to Th17 and Th2 cells was increased in the presence of CE.

CE enhances memory T cell differentiation

Since TSST-1-stimulated T cells in the presence of CE contained both non-proliferative and proliferative cells, we determined the phenotype of the stimulated T cells 72 hr after the stimulation. We observed that the proportion of T cell fractions expressing both CD45RA and CD62L increased in the large lymphocyte gate in the presence of CE in a concentration-dependent manner ($P = 4.59E{-}07$) (Figure 4a,b). Since the CD45RA and CD62L double-positive cells are indicative of naïve T cells, we further analyzed the expression level of naïve and memory T cell markers CCR7, CD127, CD95, and CXCR3, by quantifying their mean fluorescence intensities (MFIs). The double positive cells cultured with 3000 μg/mL CE increased, maintaining these markers, and especially CD95, suggesting that the cells involved were developed into T_{SCM} cells. However, most CD4 T cells also expressed CD45RO (Figures 4c, S6), indicating that the cells were not typical T_{SCM} cells but were iT_{SCM} cells.

DISCUSSION

We demonstrated the regulatory effect of CE on human T cells activated by TSST-1. CE enhanced the differentiation of effector T cells and induced the formation of early memory T cells with a phenotype similar to that of T_{SCM}

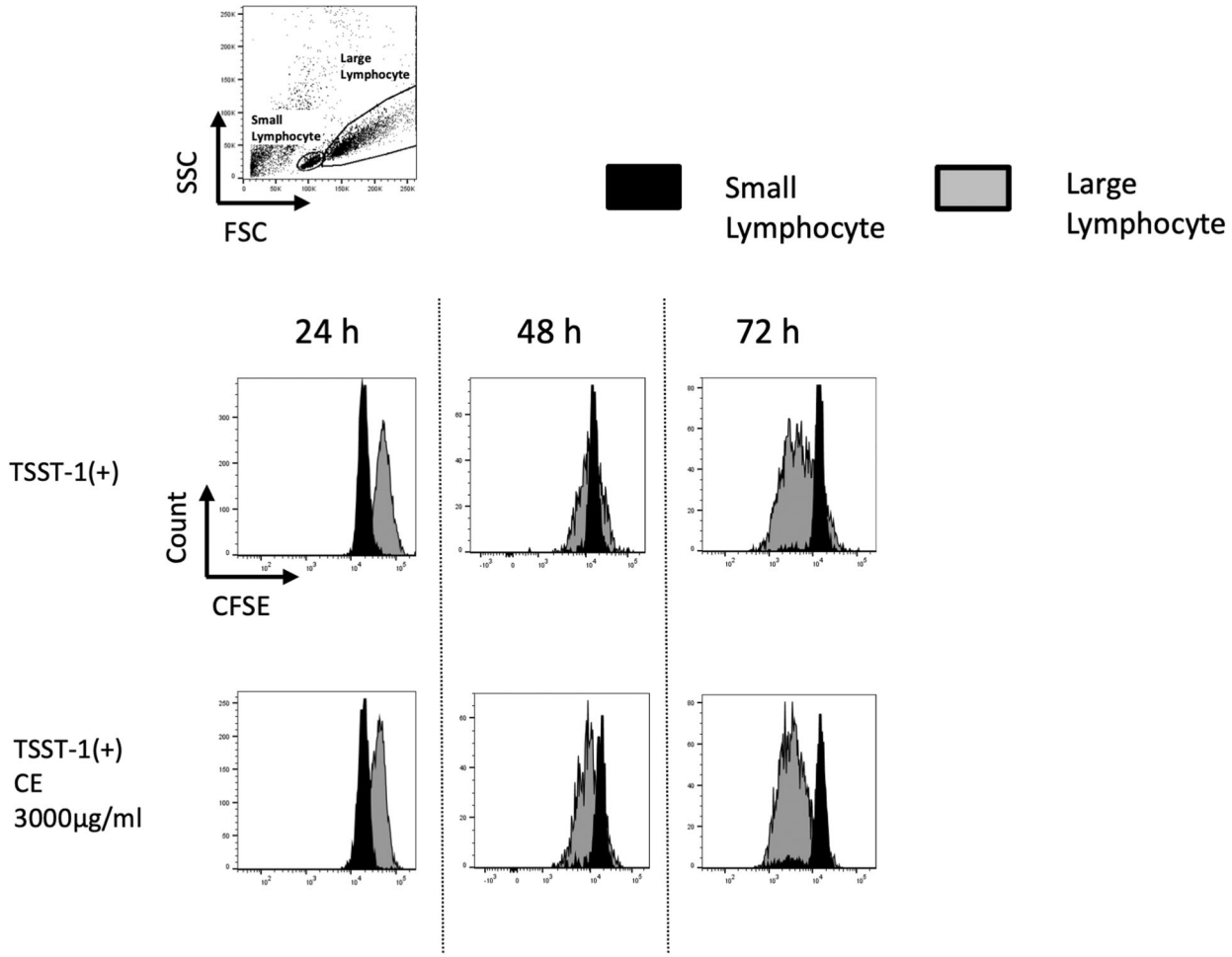

FIGURE 2 *Coccomyxa* sp.KJ extract (CE) affects activated T cell proliferation kinetics. Proliferation analysis of purified T cells stimulated with plate-coated anti-CD3 mAb or anti-CD3/CD28 microbeads. Upper panel: gating of small lymphocyte and large lymphocyte. Lower panels: Left two panels: 24 hr; middle two panels: 48 hr; right two panels: 72 hr; upper panels: T cells post-toxic shock syndrome-1 (TSST-1) stimulation; lower panels: T cells with TSST-1 stimulation in the presence of 3000 μg/mL CE. These panels represent cells without additive T cell receptor (TCR) stimulation. The horizontal axis represents the 5-(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) fluorescence intensity, and the peaks indicate the rate of cell division with the patterns representing three independent experiments. The sample number of experiment is $n = 3$ for all experiments

cells. The effect was unique, because CE could enhance both the activation of effector T cells and the differentiation of early memory T cells while simultaneously maintaining the resting cells.

The concentration effective to T cell activation is significantly high (3000 μg/mL). However, the concentration was similar to the results of Dai et al. using RAW264.7, as they used the fraction of *Coccomyxa gloeobotrydiformis* polysaccharide (2–4 mg/mL) and found that the viability of the cell line decreased significantly.[10] As antigen-presenting cells (APCs) such as macrophages affect the T cell activation, our result is comparable to their report. On the other hand, Guo et al. reported that acidic polysaccharide isolated from *Coccomyxa gloeobotrydiformis* modulated avian PBMCs at a concentration around 100 μg/mL,[9] suggesting some of the fractions such as acidic polysaccharide of CE might have the effect if it is concentrated.

As shown in Figure 1c, we observed aggregated clusters in stimulated T cell culture. The clusters were largest in the 3000 μg/mL CE treated cells. The clusters are usually formed by APCs and T cells, which are tightly bound by immunological synapses.[17] Therefore, the cluster is larger if the synapse is larger or stronger. Therefore, we speculate that the CE component might affect the reaction of APC and T cells, which may change the fate of T cells.

The activated T cells proliferated more quickly in the presence of a high concentration of CE. The proportion of effector T cells, Th1, Th2, and Th17 cells, showed an increasing trend among PBMCs. CD25 expression was increased, suggesting that some T cells were activated extensively. Previously, Guo et al. reported that AEX, a component of *Coccomyxa*, enhances the secretion of inflammatory cytokines such as IFN-β, IL-1β, IL-6, and TNF-α.[9] Moreover, they reported that IL-10 and IL-12p70 expression was also increased. In contrast, Dai et al. reported that the expression of IL-1β, IL-6, and TNF-α was suppressed in the macrophage cell line RAW 264.7 in the presence of *Coccomyxa* polysaccharide.[10]

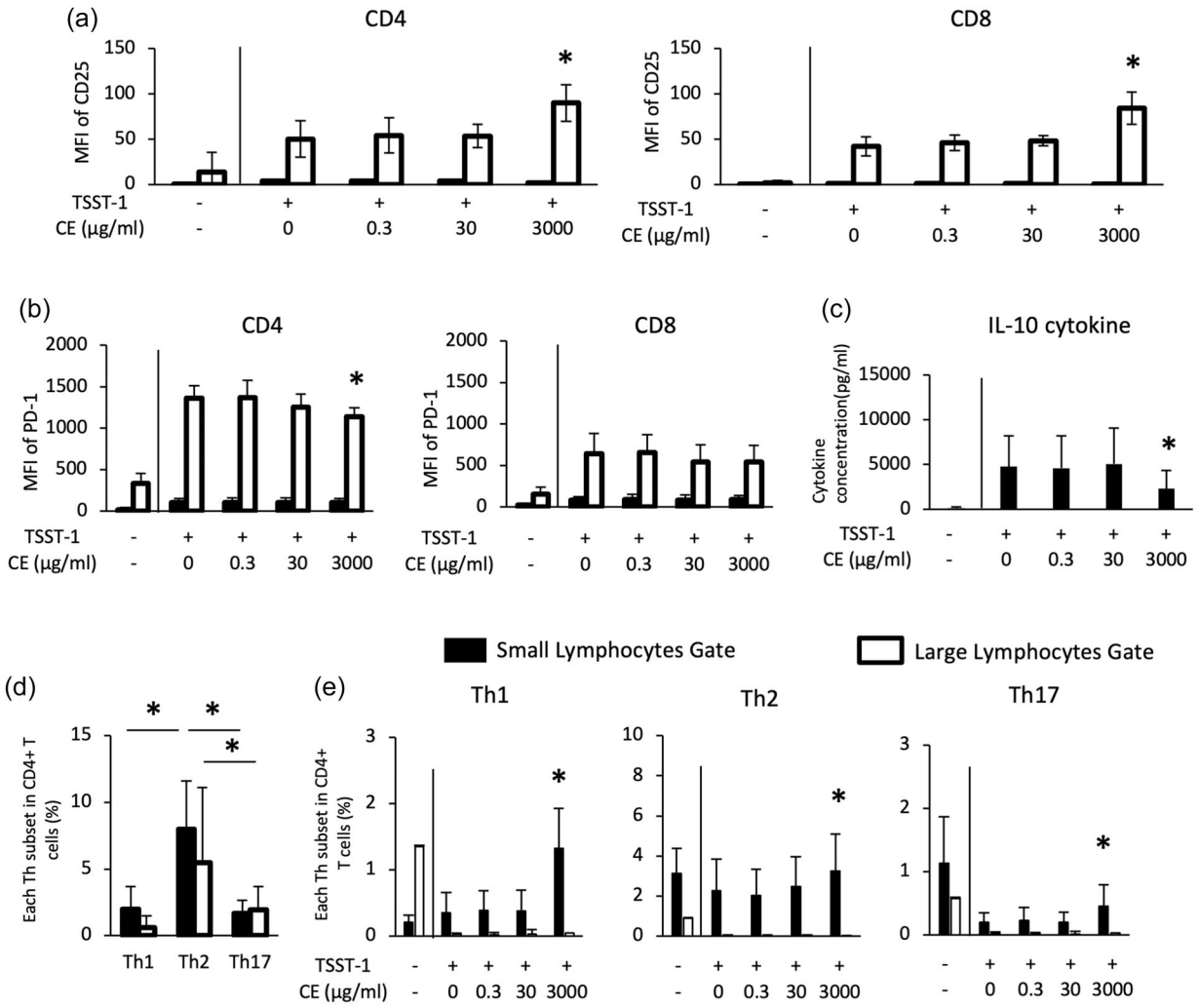

FIGURE 3 *Coccomyxa* sp.KJ extract (CE) affects effector T cell differentiation. (a) Mean fluorescent intensity (MFI) of CD25 in CD4 gate (Th cells) and CD8 gate (Tc cells). Unstimulated T cells (TSST-1[−]) and T cells stimulated with 0 to 3000 μg/mL CE are shown. (b) MFIs of PD-1. (c) IL-10 concentration in the culture supernatants. The vertical axis shows the concentration of IL-10 (pg/mL). (d) Th1, Th2, and Th17 cells (%) developed in the presence of 3000 μg/mL CE, defined by surface markers. Left panel: day 0; right panels: day 3 (72 hr). (e) Th1, Th2, and Th17 cells (%) developed in the presence of varying CE concentrations. Closed bars: small lymphocyte gate; open bars: large lymphocyte gate. One way repeated ANOVA and paired Student's *t*-tests were performed. $^*P < 0.05$. The sample number of experiment is $n = 4$ for TSST-1(−), $n = 5$ for TSST-1(+), CE0.3, CE 30, and CE3000

In this study, we observed a significant increase in IL-1β, but the levels of TNF-α and IL-10 were decreased significantly. Since the stimulation protocol, species, and cells were different among the three studies, we cannot directly compare the differences of the cytokine profiles. However, in each case, *Coccomyxa component* modulated the inflammatory cytokine profiles of lymphocytes and/or innate immune cells.

One of our new findings is that activated T cells do not increase PD-1 expression. PD-1 is a late-activation marker and PD-1-expressing cells induce apoptosis via PD-L1/PD-1 signaling.[18] The decrease in PD-1 suggests that T cell exhaustion is suppressed in the presence of CE. Therefore, the activated T cells quickly proliferated in the presence of CE, as shown in Figure 2. If this were to occur immediately after infection, it might be beneficial to counteract the superantigen-secreting pathogens.

In contrast, in the large cell gate, the proportion of $CD45RA^+$ $CD62L^+$ (DP) T cells increased (up to 90%). The DP T cells exhibited increased expression of other naïve markers such as CCR7, whereas CD95, a memory marker, was also enhanced compared with levels in the CE 0 μg/mL fraction, indicating the formation of T_{SCM} cells (Figure 4).[13] T_{SCM} cells have been reported to proliferate extensively, which is in accordance with the extensive proliferation of large-gate cells shown in Figure 2. Most of the CD4 T cells also expressed CD45RO, which might not be expressed on the conventional T_{SCM} cells but is expressed on the induced T_{SCM} cells,[19] central memory T cells, and effector memory T cells. Therefore, CE might regulate the differentiation of TSST-1-stimulated T cells by inducing a signal mediating development into not only central and effector memory T cells but also into early memory T cells such as T_{SCM} cells. Related to this, several reports suggest that Notch signaling

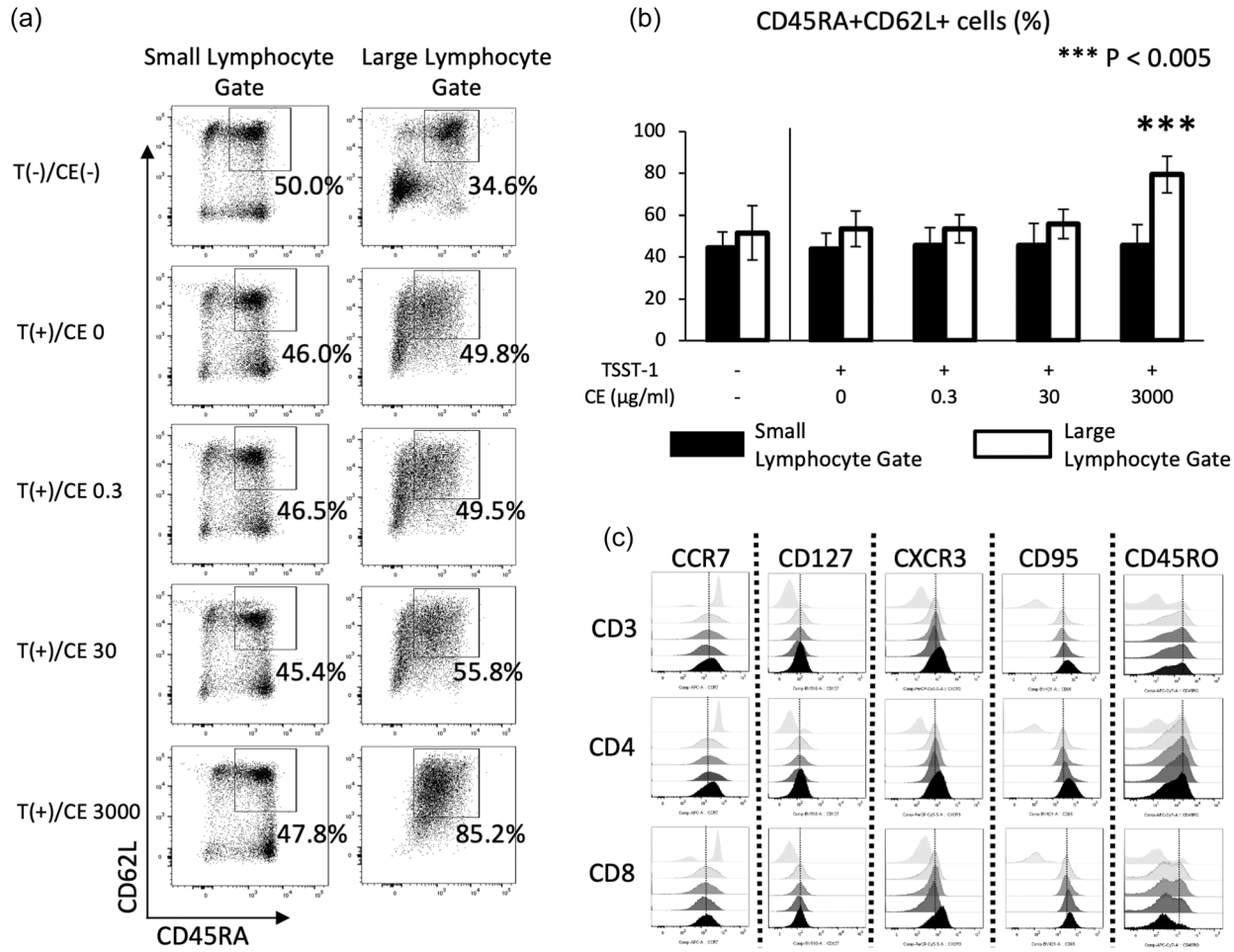

FIGURE 4 *Coccomyxa* sp.KJ extract (CE) affects memory T cell differentiation. The expression of naïve/memory T cell markers on unstimulated (TSST-1[−]) and stimulated peripheral blood mononuclear cell (PBMC) (0–3000 μg/mL) is shown. (a) Left panels: flow cytometry (FCM) patterns of CD45RA and CD62L expression in cultured PBMC. (b) Proportion of $CD45RA^+$ $CD62L^+$ cells; One way repeated ANOVA and paired Student's *t*-test were performed. ***$P < 0.005$. (c) Histograms of CCR7, CD127, CXCR3, and CD95 in the $CD45RA^+$ $CD62L^+$ cell gate, and histograms of CD45RO in the $CD3^+$ cell gate. Each panel shows the histogram of unstimulated (top) and PBMC stimulated with CE (bottom). The order is the same as (a). Reference line was set based on CE0 peak. The sample number of experiment is $n = 4$ for TSST-1(−), $n = 5$ for TSST-1(+), CE0.3, CE 30, and CE3000

can induce stemness in T cells.[20] As Notch molecule glycosylation regulates signal transduction,[21] sugars similar to Notch-decorating sugars might be involved in the CE components, substituting for the role of the signals.

In our results, T cell proliferation was suppressed in the presence of CE and CD3/CD28 compared with that with CE and CD3 (Figure 2). Moreover, the proportion of large CD8 T cells decreased (Figure S1), in parallel with a decrease in IL-2 production (Figure S5). This phenomenon might reflect the nested antagonistic feedback circuits reported by Zenke et al.[22] In the circuit, clustering of CD8 T cells by ICAM-1 induces CTLA-4, which enables regulation of the balance of cells between proliferation and apoptosis to guarantee the robustness of population dynamics. CE might accelerate the feedback circuits to maintain the immune system of bacteria-infected patients. Since we used the CE in this study, we need to further elucidate whether the two functions are simultaneously induced by the same factor or if different components play a role in each phenomenon in future studies.

In conclusion, we demonstrate that CE regulates the fate of TSST-1-stimulated T cells via two pathways. CE induces extensive proliferation of a fraction of T cells, and the activated T cells develop into effector T cells without a significant shift to Th1, Th2, Th17, or Treg cells. In parallel, CE enhances the expression of T_{SCM} and iT_{SCM}-like T cell markers, which might preserve memory T cells and prevent the exhaustion and apoptosis of T cells. Further analysis might reveal the effect of CE on preventing cytokine storms and subsequent immune suppression.

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DISCLOSURE

As employees of Denso Corporation, the following people are paid by Denso: Komatsu S., Kanno A., and Kuno H. Other authors declare no conflict of interest. Denso Corporation provided *Coccomyxa* sp.KJ extract and financial support for data collection, analysis, and manuscript publication.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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