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Increased CD226 Expression on CD8+ T Cells Is Associated with Upregulated Cytokine Production and Endothelial Cell Injury in Patients with Systemic Sclerosis

Masahiro Ayano,* Hiroshi Tsukamoto,* Kentaro Kohno,† Naoyasu Ueda,‡ Atsushi Tanaka,* Hiroki Mitoma,* Mitsuteru Akahoshi,* Yojiro Arinobu,* Hiroaki Niiro,§ Takahiko Horiiuchi,¶ and Koichi Akashi*

Systemic sclerosis (SSc) is an autoimmune disease characterized by vascular damage and fibrosis of the skin and internal organs. Because activated and oligoclonally expanded CD8+ T cells can be detected in peripheral blood and lungs of SSc patients, effector memory CD8+ T cells may play a critical role for organ involvement in SSc; however, the pathogenic functions of effector memory CD8+ T cells remain incompletely understood. In this study, we performed DNA microarray analysis of the sort-purified effector memory CD8+ T cells from SSc patients and healthy controls, and showed that the expression of genes related to immune response and cell adhesion, including CD226 (also known as DNAX accessory molecule-1 [DNAM-1]), was significantly altered. Moreover, detailed analysis of CD226 revealed that CD226highCD8+ T cells were increased in SSc patients (mean, 50.7%) compared with healthy controls (32.9%) and were appreciably associated with the severity of skin sclerosis and interstitial lung disease. Furthermore, CD226+CD8+ T cells produced higher amount of various cytokines than CD226lowCD8+ T cells from SSc patients showed upregulated IL-13 production and positive correlation with the cytotoxic capacity of CD8+ T cells against HUVECs. Finally, the neutralization of CD226 in CD8+ T cells impaired costimulation, cytokine productions, and cytolysis against HUVECs. These findings indicate that upregulated CD226 expression on CD8+ T cells reflects disease severity and is involved in SSc pathogenesis via the production of various cytokines, including profibrotic IL-13 and endothelial cell injury, and that CD226 may be a useful target in the treatment of SSc. The Journal of Immunology, 2015, 195: 892–900.

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The microarray data presented in this article have been submitted to the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under accession number GSE63903.

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; CBA, cytometric beads array; CRTH2, chemotaxtractant receptor-homologous molecule on Th2 cells; DAVID, Database for Annotation, Visualization, and Integrated Discovery; FVC, forced vital capacity; GATA3, GATA binding protein 3; HCl, healthy control; HRCT, high-resolution computed tomography; ILD, interstitial lung disease; limma, linear models for microarray analysis; MRSS, modified Rodnan skin thickness score; SSc, systemic sclerosis.
patients and healthy controls (HCs). Based on the results of the microarray analysis, we investigated the characteristic functions of CD226+CD8+ T cells in SSc pathogenesis.

### Materials and Methods

**Patients**

We studied 50 Japanese patients (9 for microarray analysis and 41 for FACS analysis) who were treated for SSc at the Kyushu University Hospital and 31 HCs (5 and 26, respectively). We included SSc patients who fulfilled the 1980 classification criteria of the American College of Rheumatology for SSc (17) and had no other autoimmune diseases. This study was approved by the ethics committee of our institution, and the principles of the Helsinki Declaration were followed throughout the study. Informed consent was obtained from all participants.

We obtained the information from the medical records of the patients, including demographic data, clinical manifestations, laboratory findings, and medications. SSc patients were classified as having limited cutaneous or diffuse cutaneous disease according to the criteria of LeRoy et al. (18). The disease duration was calculated from the time of onset of the first non-Raynaud phenomenon. Skin sclerosis was evaluated with the modified Rodnan skin thickness score (MRSS) (19) and graded as mild (MRSS, 1–14) or moderate-to-severe (MRSS, 15–39) (20). SSc-related interstitial lung disease (ILD) was classified as extensive or limited disease based on combined evaluation with chest high-resolution computed tomography (HRCT) and pulmonary functional tests (21). In brief, extensive ILD was defined as HRCT extent >30%; or HRCT extent ranging 10–30% and forced vital capacity (FVC) <70%; limited ILD as HRCT extent <10% or HRCT extent ranging 10–30% and FVC ≥70%. FVC was expressed as a percentage of predicted values according to the prediction equations of the Japanese Respiratory Society (22).

**Cell sorting**

PBMCs were separated from the heparinized fresh blood using centrifugation on Lymphocyte Separation Medium gradients (MP Biomedicals, Santa Ana, CA). PBMCs obtained were stained with mAb mixtures in PBS containing 2% heat-inactivated FBS (Thermo Fisher Scientific, Waltham, MA) at 4°C for 30 min in the dark and sorted using the BD FACSaria flow cytometer (BD Biosciences, San Jose, CA). The sorted CD226+CD8+ or CD226−CD8+ T cells were suspended in serum-free AIM-V medium (Invitrogen, Carlsbad, CA) for RNA isolation.

**Microarray analysis**

Total RNA was extracted from the sort-purified effector memory CD8+ T cells (5000 cells) using TRIzol, and biotinylated cRNA was synthesized with two rounds of amplification steps using the MessageAmpII aRNA Amplification Kit and Illumina TotalPrep RNA Amplification Kit (both from Applied Biosystems, Foster City, CA). Next, 750 ng of cRNA from the sample was hybridized to the HumanHT-12 v4 Expression BeadChip (Illumina, San Diego, CA). After staining and washing, the BeadChip was scanned with an Illumina Bead Array reader, and the data were compiled using Bead Studio software (Illumina).

The raw signal intensities of all samples were log2-transformed and normalized using a quantile algorithm with a “preprocessCore” library package (23) of Bioconductor software (24). The genes flagged as present (detection p < 0.01) in all samples were selected and used for further analysis. The linear models for microarray analysis (limma) package (25) of the Bioconductor software was used to identify the differentially expressed genes. Functional annotation clustering analysis was performed with Database for Annotation, Visualization, and Integrated Discovery (DAVID) using default parameters on its Web site (http://david.abcc.ncifcrf.gov/home.jsp) (26, 27). The heat map was generated using MeV software (28). Hierarchical clustering was performed using the Pearson correlation metric and average linkage clustering.

**FACS analysis**

Freshly isolated PBMCs were stained with the following direct conjugated mAbs: FITC-conjugated anti-CD3 (UCHT1), anti-CD45RO (UCHL1), or anti-CD62L (DREG-56; Beckman Coulter); PE-conjugated anti-CD226 (DX11); PerCP-conjugated anti-CD8 (RPA-T8) or anti-CD19 (HIB19; both from BioLegend); PE-C5–conjugated anti-CD45RO (UCHL1); PE-Cy7–conjugated anti-CD3 (SK7); allopurinol-conjugated anti-CD4 (DREG-56); anti-CD56 (B159; all five from BD Biosciences), or anti-TCR γ/δ (B1; BioLegend); Alexa Fluor 647-conjugated anti-chemoattractant receptor-homologous molecule on Th2 cells (CRTH2; clone BM16; BioLegend); and allopurinol-conjugated-Cy7–conjugated anti-CD8 (SK1). Isotype control Abs (BD Biosciences) were used to determine the level of background staining. Samples were acquired with the BD FACSCalibur (BD Biosciences) or FACSaria flow cytometer. Data analysis was performed using FlowJo software (Tree Star, Ashland, OR).

### Cytometric beads array

The sorted CD226+CD8+ or CD226−CD8+ T cells (2 × 10⁵ cells) were stimulated with a combination of 50 ng/ml PMA (Sigma, St Louis, MO) and 1 μg/ml ionomycin (Sigma) for 6 h in serum-free AIM V medium. After centrifugation, the supernatants were collected and stored at −20°C until the analysis. The protein levels of IFN-γ, TNF-α, IL-4, IL-5, IL-10, IL-13, and granzyme B were measured using cytometric beads array (CBA; BD Biosciences) on the BD FACS Calibur flow cytometer according to the manufacturer’s instructions.

### Intracellular staining of IL-13

Heparinized whole blood (200 μl), diluted 1:1 with sterile RPMI 1640 (Wako, Osaka, Japan) medium, was stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 5 h in the presence of GolgiStop (BD Bio-

### Table I. Clinical characteristics of SSc patients

<table>
<thead>
<tr>
<th>Feature</th>
<th>Microarray (n = 9)</th>
<th>FACS (n = 41)</th>
<th>p Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD (y)</td>
<td>53.6 ± 12.3</td>
<td>57.9 ± 11.4</td>
<td>0.32</td>
</tr>
<tr>
<td>Female, n (%)</td>
<td>9 (100)</td>
<td>37 (90)</td>
<td>1</td>
</tr>
<tr>
<td>Disease duration, mean ± SD (y)</td>
<td>3.6 ± 3.3</td>
<td>7.0 ± 4.9</td>
<td>0.06</td>
</tr>
<tr>
<td>Diffuse cutaneous SSc, n (%)</td>
<td>6 (67)</td>
<td>27 (66)</td>
<td>1</td>
</tr>
<tr>
<td>MRSS, mean ± SD</td>
<td>16.4 ± 11.1</td>
<td>10.0 ± 6.7</td>
<td>0.19</td>
</tr>
<tr>
<td>ILD, n (%)</td>
<td>7 (78)</td>
<td>23 (56)</td>
<td>0.28</td>
</tr>
<tr>
<td>FVC, % predicted, mean ± SD</td>
<td>8.8 ± 10.3</td>
<td>8.59 ± 17.1</td>
<td>0.58</td>
</tr>
<tr>
<td>DLCO, % predicted, mean ± SD</td>
<td>50.0 ± 12.6</td>
<td>66.4 ± 21.0</td>
<td>0.10</td>
</tr>
<tr>
<td>Antinuclear Ab, n (%)</td>
<td>8 (89)</td>
<td>37 (90)</td>
<td>1</td>
</tr>
<tr>
<td>Anti-topoisomerase I Ab, n (%)</td>
<td>5 (56)</td>
<td>15 (37)</td>
<td>0.45</td>
</tr>
<tr>
<td>Anticentromere Ab, n (%)</td>
<td>1 (11)</td>
<td>8 (20)</td>
<td>0.66</td>
</tr>
<tr>
<td>Low-dose corticosteroids, n (%)</td>
<td>6 (67)</td>
<td>20 (49)</td>
<td>0.47</td>
</tr>
<tr>
<td>Immunosuppressive drug use, n (%)</td>
<td>2 (22)</td>
<td>9 (22)</td>
<td>1</td>
</tr>
</tbody>
</table>

DLCO, diffusing capacity for carbon monoxide; FVC, forced vital capacity.
The activated cultures were treated with 4 ml RBC Lysis Solution (Miltenyi Biotec) for 10 min. After centrifugation, the cells were suspended in PBS containing 2% FBS and stained with FITC-conjugated anti-CD3 (UCTH1), PerCP-conjugated anti-CD8 (RPA-T8), and PE-conjugated anti-CD226 (DX11). The cells were subsequently fixed and permeabilized using BD fixation-permeabilization solution (BD Biosciences) and stained with allophycocyanin-conjugated anti–IL-13 (JES10-5A2; BD Biosciences). The samples were analyzed using the BD FACSCalibur flow cytometer.

FIGURE 1. Gene expression profiles of effector memory CD8⁺ T cells from 9 SSc patients and 5 HCs. (A) The heat map generated by hierarchical clustering shows 55 significantly altered genes (limma \( p < 0.05; \) absolute fold change \( \text{FC} > 2 \)) between the samples from SSc patients and those from HCs. Enriched functional clusters of these differentially expressed genes are shown in detail in Supplemental Table I. *Genes annotated to cluster 1 (including immune response). †Genes annotated to cluster 2 (including cell adhesion). (B) Top five upregulated or downregulated genes related to both clusters.
Quantitative real-time PCR

The sorted CD226\(^{\text{high}}\)CD8\(^{+}\), CD226\(^{\text{low}}\)CD8\(^{+}\), or CD226\(^{-}\)CD8\(^{+}\) T cells (4 × 10\(^4\) cells) were stimulated with PMA (50 ng/ml) and ionomycin (1 \(\mu\)g/ml) for 4 h in serum-free AIM V medium. The activated cells were harvested using TRIzol reagent, total RNA was extracted, and cDNA was synthesized with QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantitative PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems) with TaqMan Gene Expression Master Mix (Applied Biosystems). The following primers were purchased from Applied Biosystems: GAPDH (Hs02758991_g1), IL-13 (Hs00174379_m1), and GATA binding protein 3 (GATA3; Hs00231122_m1). Relative gene expression quantification was calculated according to the comparative cycle threshold method using GAPDH as an endogenous control.

Isolation of CD8\(^{+}\) T cells

CD8\(^{+}\) T cells were negatively isolated from PBMCs using magnetic beads (CD8\(^{+}\) T Cell Isolation kit; Miltenyi Biotec). The purity of the CD8\(^{+}\) T cells was >95\%, as determined by flow cytometry. Freshly isolated cells were cultured in complete RPMI 1640 medium supplemented with 10% FBS, 50 IU/ml penicillin, and 20 \(\mu\)g/ml streptomycin (both from Life Technologies, Grand Island, NY).

Cell culture

HUVECs were purchased from Lonza (Basel, Switzerland) and cultured in fully supplemented endothelial growth medium (EGM-2; Lonza) at 37 \(^\circ\)C in a 5% CO\(_2\) humidified atmosphere. HUVECs were used up in passage 4–7.

Coculture of CD8\(^{+}\) T cells with HUVECs

HUVECs (1 × 10\(^5\) cells), which expressed high levels of CD155 (one of the CD226 ligands), were plated in 96-well plates and grown overnight to confluent monolayers in EGM-2 medium. After removing the medium, the cells were washed and subsequently cocultured with purified CD8\(^{+}\) T cells (1 × 10\(^5\) cells) in RPMI 1640 medium supplemented with 10% FBS in the presence of Anti-Biotin MACSiBead Particles loaded with CD3-Biotin (anti-CD3–coated beads; both from Miltenyi Biotec), and anti-CD226 blocking Ab (DX11; 10 \(\mu\)g/ml) or isotype control Ab (both from BD

**FIGURE 2.** CD226 expression on CD8\(^{+}\) T cells is upregulated in SSc patients compared with HCs. CD226 expression on lymphocyte subsets from 41 SSc patients and 23 HCs was analyzed using flow cytometry. (A) Representative histograms show the percentage of CD226\(^{+}\) cells on gated CD3\(^{+}\)CD8\(^{+}\) T cells from SSc patients and HCs (left panels). The proportion of CD226\(^{+}\)CD8\(^{+}\) T cells was compared between SSc patients and HCs (right panel). (B–F) The frequency of CD226-expressing cells in (B) CD3\(^{+}\)CD4\(^{+}\) T cells, (C) CD19\(^{+}\) B cells, (D) γ\(\delta\) cells, (E) NK cells, and (F) effector memory CD8\(^{+}\) T cells (CD8\(^{+}\)CD45RO\(^{+}\)CD62L\(^{-}\)) was compared between SSc patients and HCs. (G) Representative dot plots show CD45RO and CD62L expression on CD226\(^{\text{low}}\)CD8\(^{+}\), CD226\(^{\text{high}}\)CD8\(^{+}\), and CD226\(^{-}\)CD8\(^{+}\) T cells. Numbers indicate the proportion of each population. (H) The percentage of CD226\(^{\text{high}}\)CD8\(^{+}\) T cells was compared between SSc patients and HCs. Each data point represents a single subject. Horizontal lines show the mean. CM, central memory T cells; EM, effector memory T cells; EMRA, terminal differentiated effector memory T cells; naive, naive T cells.
To reveal the immune dysfunction of effector memory CD8+ patients compared with HCs

0.05 and absolute fold change, (fector memory CD8+ T cells from SSc patients and those from

CD8+ T cells, 2

frequency of CD226highCD8+ T cells was compared among

CD226highCD8+ T cells was compared between patients

Biosciences), and then the percentage of dead HUVECs (CFSE+7-AAD+)
cells were collected and labeled with 7-aminoactinomycin D (7-AAD; BD

show the mean.

Each data point represents a single subject. Horizontal lines

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CD226 expression by flow cytometry on CD8+ T cells from 41 SSc

patients

CD226 expression on CD8+ T cells is upregulated in SSc

CD226 expression on CD8+ T cells is upregulated in SSc patients

FIGURE 3. CD226highCD8+ T cells are increased in patients with diffuse cutaneous SSc (dcSSc) and are associated with the severity of skin and lung involvement. (A) The percentage of CD226highCD8+ T cells was compared between dcSSc patients (n = 27) and patients with limited cutaneous SSc (lcSSc; n = 14). (B) The proportion of CD226highCD8+ T cells was compared between patients with anti-topoisomerase I Abs (ATA; n = 15) and those with anticientromere Abs (ACA; n = 8). (C) The percentage of CD226highCD8+ T cells was compared between patients with mild skin sclerosis (MRSS, 1–14; n = 31) and those with moderate to severe (MRSS, 15–39; n = 10). (D) The frequency of CD226highCD8+ T cells was compared among SSc patients without ILD (ILD--; n = 18), those with limited ILD (n = 18), and those with extensive ILD (n = 5).

Each data point represents a single subject. Horizontal lines show the mean.

CD226 expression on CD8+ T cells in SSc patients

To validate the results of the microarray analysis, we investigated CD226 expression by flow cytometry on CD8+ T cells from 41 SSc patients (mean age, 57.8 y; female, 37) and 23 HCs (mean age, 55.7 y; female, 19). No significant differences were found between SSc patients and HCs in terms of age and sex. As described in Table I, the clinical characteristics of the SSc patients investigated were generally similar between two analysis groups.

CD226 was constitutively expressed on CD8+ T cells (Fig. 2A). CD226 expression had no association with age, sex, or medication (data not shown). The proportion of CD226+CD8+ T cells was significantly higher in SSc patients than in HCs (Fig. 2A), whereas CD226 expression on CD4+ T cells, CD19+ B cells, NK cells, and γδ cells was almost the same between two groups (Fig. 2B–E).

In this study, we attempted to validate CD226 expression at a protein level and to investigate the characteristic functions of CD226 on CD8+ T cells in SSc pathogenesis, because CD226 has been reported as a genetic risk factor for the susceptibility to SSc in genome-wide association studies (30).

CD226 expression on CD8+ T cells is upregulated in SSc patients

Statistical analysis

The differences between two groups were analyzed using Student or Welch t test according to their distributions. Multiple comparisons were analyzed using Tukey-Kramer honestly significant difference test. Comparison between the same individual was performed using a paired t test. The relations between two continuous variables were analyzed using the Spearman rank correlation. All tests were two-tailed, and p values < 0.05 were considered significant. All analyses were performed using JMP statistical software (SAS Institute, Cary, NC).

Results

Genes related to immune response and cell adhesion are differently expressed in effector memory CD8+ T cells from SSc patients compared with HCs

To reveal the immune dysfunction of effector memory CD8+ T cells in SSc patients, we performed comprehensive gene expression analysis by cDNA microarray of the sort-purified effector memory CD8+ T cells from SSc patients (n = 9; Table I) and HCs (n = 5). We obtained 55 significantly altered genes (limma p < 0.05 and absolute fold change, > 2) between the samples of effector memory CD8+ T cells from SSc patients and those from HCs (Fig. 1A). We next completed DAVID functional annotation clustering analysis to explore the functional roles of the differentially expressed genes and found two significantly enriched functional clusters (defined as an Enrichment Score > 1.3; Supplemental Table I). One cluster contained eight genes associated with an immune response: CCL3-like 3 (CCL3L3); CCL4-like 1 (CCL4L1); IFN-γ; IFN-induced protein 44-like (IFI44L); phospholipase C, γ 2 (PLCG2); MHC class II DR α (HLA-DRA); IL-32; and complement factor D (adipsin) (CFD). The other cluster contained seven genes associated with cell adhesion: CCL4L1; G protein-coupled receptor 56 (GPR56); integrin, α M (complement component 3 receptor 3 subunit; ITGAM); IL-32; adhesion molecule, interacts with CXADR Ag 1 (AMICA1); ninjin 1 (NINJ1); and fasciulation and elongation protein ζ 1 (zygin I) (FEZ1). Because the results indicate that the functions of immune response and cell adhesion were affected in SSc patients and may be critical in SSc pathogenesis, we also focused on the genes related to both clusters. In the 61 genes selected, the genes with a relatively high fold change were CCL4L1; CD300a molecule (CD300A); CD226 molecule (CD226); VCAM1; and spondin 2, extracellular matrix protein (SPON2; Fig. 1B).

In genome-wide association studies (30).
For further validation, we also analyzed CD226 expression on effector memory CD8+ T cells and found that CD226-expressing cells were increased in SSc patients compared with HCs, as expected from the microarray results (Fig. 2F).

As shown in Fig. 2A and 2G, the CD226+CD8+ T cells were subdivided into two subpopulations on the basis of the intensity of expression of CD226 and differentiation markers (CD45RO and CD62L). CD8+ T cells with low intensity of CD226 (CD226low CD8+ T cells) mainly contained naive cells; in contrast, CD8+ T cells with high (CD226high CD8+ T cells) were heterogeneous and included memory and effector cells. In this line, further analysis with attention to CD226high CD8+ T cells, which may be more pathogenic, revealed that the percentage of the cells was markedly greater in SSc patients than in HCs (Fig. 2H).

**Increased CD226high CD8+ T cells are associated with the severity of skin and lung involvement**

To study the roles of upregulated CD226 expression on CD8+ T cells in SSc patients, we investigated the association between CD226high CD8+ T cells and clinical manifestations of SSc. The percentage of CD226high CD8+ T cells had no association with disease duration (data not shown). Concerning the subgroups of SSc, the expansion of CD226high CD8+ T cells was significantly larger in patients with diffuse cutaneous SSc than in those with limited cutaneous SSc (Fig. 3A). SSc patients with anti-topoisomerase I Abs also showed a significantly higher proportion of CD226high CD8+ T cells than those with anticientromere Abs did (Fig. 3B). The percentage of CD226high CD8+ T cells was significantly greater in patients with moderate to severe skin sclerosis (MRSS, 15–39) than in those with mild (MRSS, 1–14; Fig. 3C). When we studied the association of CD226 expression on CD8+ T cells with ILD, which is one of the major organ involvement in SSc, the frequency of CD226high CD8+ T cells was higher in patients with extensive ILD than in those with limited ILD and those without ILD (Fig. 3D). These results indicate that CD226high CD8+ T cells are associated with the severity of skin and lung involvement.

CD226high CD8+ T cells produce abundant cytokines via CD226/CD155 costimulatory signaling

To investigate the roles of the elevated CD226 expression level of CD8+ T cells in SSc pathogenesis, we examined the functional properties of CD226+CD8+ T cells. We first compared cytokine production after in vitro stimulation with PMA/ionomycin be-
tween CD226+CD8+ and CD226−CD8+ T cells using the CBA system. We confirmed that cytokine production was not affected by anti-CD226 Ab staining for cell sorting (data not shown). In HCs, the sort-purified CD226+CD8+ T cells showed higher levels of production of various cytokines (IFN-γ, IL-4, IL-5, and IL-13) than CD226−CD8+ T cells did (Fig. 4A). Interestingly, IL-4, IL-5, and IL-13 were almost exclusively produced by CD226+CD8+ T cells, although these type 2 cytokines were not produced as much as type 1 cytokines (Fig. 4A). We next analyzed cell surface expression of CRTH2, the most reliable marker for the cells producing these type 2 cytokines (Tc2) (31), on CD8+ T cells and found that CRTH2+ cells were enriched in CD226highCD8+ T cells (Fig. 4B).

We further performed a detailed analysis of IL-13, because it was reported as a profibrotic cytokine (32) and it had the highest levels among these type 2 cytokines. FACs analysis of cytokine production from CD8+ T cells also revealed that CD226high subpopulation was a major source of IL-13 in SSc patients and HCs (Fig. 4C). IL-13-producing CD226highCD8+ T cells were significantly increased in SSc patients compared with HCs (Fig. 4C). We then assessed whether CD226 was involved in the IL-13 production of CD8+ T cells. The mRNA expression analysis revealed that GATA3 (master transcription factor of Tc2) and IL-13 expression after in vitro stimulation with PMA/ionomycin were markedly higher in CD226highCD8+ T cells than in CD226lowCD8+ and CD226−CD8+ T cells (Fig. 4D), suggesting that CD226highCD8+ T cells had more potent capacity to differentiate into Tc2 and produce IL-13. Next, to clarify the importance of costimulatory signals through CD226, we cocultured purified CD8+ T cells with HUVECs, which expressed high levels of CD155 (one of the CD226 ligands), in the presence or absence of anti-CD226 blocking Ab. In this assay, the blocking costimulatory signals through CD226 significantly suppressed effector functions and impaired the IL-13 and IFN-γ production of CD8+ T cells, measured using the CBA system (Fig. 4E). These findings suggest that CD226highCD8+ T cells are involved in SSc pathogenesis by producing abundant cytokines including IL-13 via CD226/CD155 costimulatory signaling.

CD226 is involved in the upregulated cytotoxic capacity of CD8+ T cells in SSc patients

To evaluate the effect of CD226 expression on the cytotoxic capacity of CD8+ T cells, we analyzed granzyme B production after in vitro stimulation with PMA/ionomycin by the CBA system. In HCs, CD226+CD8+ T cells showed higher levels of granzyme B production than CD226−CD8+ T cells did, indicating that CD226+CD8+ T cells were highly cytotoxic populations (Fig. 5A). To confirm this phenotype, we performed a CD8+ T cell–mediated cytotoxicity assay. Because endothelial cell injury was considered as one of the earliest events of SSc (4), we selected HUVECs as the target of the CD8+ T cell–mediated cytotoxicity assay. The cytotoxic ability of CD8+ T cells was significantly upregulated in SSc patients compared with HCs at both E:T ratios tested (Fig. 5B). Furthermore, the cytotoxic capacity was positively correlated with the percentage of CD226highCD8+ T cells, and this correlation was remarkable in SSc patients (Fig. 5C). We next performed this assay in the presence or absence of anti-CD226 blocking Ab to establish whether CD226 was involved in HUVEC cytolysis. The cytotoxicity of anti-CD3–activated CD8+ T cells against HUVECs was significantly inhibited by CD226 neutralization (Fig. 5D). These data indicate that CD226 is involved in the upregulated cytotoxic capacity of CD8+ T cells in SSc patients.

Discussion

In this study, we showed that genes related to immune response and cell adhesion were differently expressed in effector memory CD8+ T cells and that the expression of CD226, which is associated with

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**FIGURE 5.** CD226 is involved in the upregulated cytotoxic capacity of CD8+ T cells in SSc patients. (A) The sort-purified CD226+CD8+ or CD226−CD8+ T cells were stimulated with PMA/ionomycin for 6 h. Granzyme B production was measured in the supernatants using the CBA system. Bars are the mean ± SEM of seven HCs. (B) Purified CD8+ T cells were cocultured with CFSE-labeled HUVECs for 6 h at the E:T ratio of 10:1 or 40:1. Dead HUVECs were defined as CFSE+7-AAD+ cells by flow cytometry. The cytotoxic capacity of CD8+ T cells against HUVECs was compared between SSc patients and HCs. (C) Correlation between the cytotoxic capacity and the percentage of CD226highCD8+ T cells in SSc patients and HCs (left, E:T = 10:1; right, 40:1). (D) Purified CD8+ T cells were activated with anti-CD3-coated beads and cocultured with CFSE-labeled HUVECs for 6 h in the presence of anti-CD226 blocking Ab or isotype control Ab. Each data point represents a single subject (two SSc patients and three HCs). Horizontal lines show the mean.
both functions, is higher on CD8+ T cells from SSc patients than from HCs. We also demonstrated that CD226highCD8+ T cells are associated with skin and lung involvements and may be involved in SSc pathogenesis by upregulated production of cytokines, such as IL-13, and by cytotoxic capacity against endothelial cells. CD226 is a member of the Ig-superfamily of receptors and is constitutively expressed on the cell surface of T cells, NK cells, monocytes, platelets, and a subset of B cells (33). It is also known as a costimulatory and adhesion molecule (34, 34) and mediates activation signals for cytotoxicity by CD8+ T cells and NK cells via its binding to CD155, CD112, or both on target cells (35, 36). The important function of CD226 is well known as the immune surveillance of tumors (37). Concerning autoimmune diseases such as SSc, the nonsynonymous rs763361 polymorphism in CD226 has been identified as a genetic risk factor in the Western white population (30, 38). More recently, Avouac et al. (39) reported that mice deficient for CD226 were protected from bleomycin-induced dermal fibrosis in animal models of SSc. In humans, however, the roles of CD226 in SSc pathogenesis remain unknown.

In this study, we showed that CD226highCD8+ T cells, especially CD226highCD8+ T cells, were expanded in SSc patients compared with HCs. The expansion was striking in patients with diffuse cutaneous SSc and in those with ILD. Furthermore, the expanded CD226highCD8+ T cells reflected the severity of skin and lung involvement. Recent reports about analysis of CD226 polymorphism have shown that the CD226 rs763361 T allele was associated with the diffuse cutaneous SSc subtype (30) and SSc-related fibrosing alveolitis subsets (30, 38). Although the level of CD226 expression was not influenced by the CD226 rs763361 genotype (30), this CD226 polymorphism substituted a glycine to a serine residue at amino acid position 307 in the intracellular domain and has a possibility to affect downstream signaling and cell functions (40). These findings suggest that the CD226 polymorphism is also associated with the characteristic functions of CD226highCD8+ T cells.

IL-13 is one of cytokines that mediate tissue fibrosis (32, 41) and plays a pivotal function in SSc pathogenesis (42, 43). In this study, we found that CD226highCD8+ T cells produced a large amount of cytokines including IL-13 and that the IL-13 production of CD226highCD8+ T cells was upregulated in SSc patients compared with HCs. The roles of CD226 in the functions of CD8+ T cells were well understood about type 1 cytokine production and cytotoxicity (33), but not about type 2 cytokine production. In the current study, CD226highCD8+ T cells produced higher amount of type 2 cytokines (IL-4, IL-5, and IL-13) as well as type 1 cytokines (IFN-γ and TNF-α) than CD226-negative ones. Furthermore, CD226highCD8+ T cells had potent capacity to differentiate into Te2, and the blockage of CD226 signaling inhibited IL-13 and IFN-γ production. These results indicate that CD226 is an important costimulator of CD8+ T cells in producing not only IFN-γ, but also IL-13. Recent studies about CD4+ T cells revealed that CD226 was involved in Th1 differentiation (44, 45), that IL-13 production did not correlate with CD226 expression (46), and that the blockage of CD226 signaling did not affect IL-13 production (46). Although this discrepancy might reflect the difference between CD8+ T cells and CD4+ T cells, further study is required to investigate the detailed mechanism of the IL-13 production of CD8+ T cells.

Endothelial cell apoptosis could be a primary event in the pathogenesis of SSc (4). NK cells (47) and γδ cells (48) were reported as the mediators of the cytotoxicity; however, it remains unclear whether CD8+ T cells are involved in endothelial cell injury. On this point, we observed that the cytotoxic capacity of CD8+ T cells against endothelial cells was upregulated in SSc patients. In addition, the cytotoxic capacity was correlated with CD226 expression on CD8+ T cells and partially suppressed by blocking CD226. In CD226-deficient mouse, activated CD226highCD8+ T cells showed impaired cytotoxic activity because of dysfunctional cell–cell contact required for the efficient killing of target cells (49), and CD8+ T cells required CD226 for costimulation when recognizing Ag presented by nonprofessional APCs (50). Our results and these findings indicate that interaction between CD226 on CD8+ T cells and its ligand CD155 on endothelial cells is critical for both cell–cell contact and costimulatory signals. Upregulated interaction of CD8+ T cells and endothelial cells was supported by the recent report that increased expression of CD226 on perivascular inflammatory cells (e.g., T cells) in the lesional skin of SSc patients (39).

The limitations of this study include small sample size, cross-sectional design, and race bias. Further investigation with larger sample size and various populations is needed to confirm the results of this study.

In conclusion, upregulated CD226 expression on CD8+ T cells is associated with skin and lung involvement, reflects disease severity, and may be involved in SSc pathogenesis via the production of cytokines such as profibrotic IL-13 and endothelial cell injury. Because blockage of CD226 impairs the IL-13 production and cytotoxic capacity of CD8+ T cells, CD226 may be a useful target in the treatment of SSc.

Disclosures
The authors have no financial conflicts of interest.

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