Augmented ICOS expression in patients with early diffuse cutaneous systemic sclerosis

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Abstract

Objective. Inducible costimulator (ICOS), expressed on activated T cells, and its ligand, ICOS ligand (ICOSL), expressed on antigen-presenting cells, have been considered a single receptor-ligand pair. Here we investigated the expression of ICOS and ICOSL in patients with SSc.

Methods. ICOS expression on peripheral blood T cells, and ICOSL expression on B cells and macrophages was determined by flow cytometry. Expression of ICOS and ICOSL was assessed by immunohistological staining and real-time PCR of lesional skin.

Results. ICOS expression levels were specifically increased on both peripheral blood memory T cells and Tregs from early dcSSc patients compared with those from healthy controls. Mean ICOSL expression on B cells or macrophages was comparable between SSc patients and healthy controls. ICOS-expressing T cells, ICOSL-expressing macrophages and mRNA levels of ICOS and ICOSL were increased in the lesional skin of patients with early dcSSc. In vitro ICOS costimulation enhanced production of IFN-γ, IL-4 and IL-17A from T cells in SSc patients vs normal controls. Soluble ICOS levels were significantly increased in SSc patients and were negatively associated with the presence of ACAs and positively associated with CRP values. Serum levels of soluble ICOS were more closely associated with clinical features compared with levels of soluble IL-2 receptor.

Conclusion. Augmented ICOS signalling may contribute to the pathogenesis of SSc during early progressive disease. Soluble ICOS levels may be used as a serum marker for the activity and severity of SSc.

Key words: systemic sclerosis, scleroderma, ICOS, ICOSL, T cell, B cell, macrophage, cytokine, biomarker, sIL-2R.

Introduction

SSc is a CTD characterized by tissue fibrosis in the skin and internal organs. A growing body of evidence suggests that overproduction of extracellular matrix components by activated fibroblasts results from complex interactions between endothelial cells, leucocytes and fibroblasts via a number of mediators, including cytokines [1–4].

Inducible costimulator (ICOS) is the third member of the CD28 family of costimulatory molecules, and is induced on the cell surface following T-cell activation [5–7]. ICOS ligand (ICOSL) (also called B7-H2, B7h, B7RP-1, LICOS and GL50), the unique ligand of ICOS, is weakly expressed on antigen-presenting cells in the steady state and is up-regulated after activation of these cells [6, 8]. Originally it was reported that the ICOS–ICOSL pathway promotes T-cell activation, differentiation and effector responses, and T-cell-dependent B-cell responses. ICOS-mediated costimulation of T cells leads predominantly to the production of effector cytokines such as IL-4 and IL-10, and to a lesser extent, IL-2, IFN-γ and TNF-α [9], thereby playing a more important role in Th2 responses than Th1 responses [5, 10–12]. However, recent studies have demonstrated that ICOS influences the expansion of follicular T cells, Th17 cells and Tregs [13–15]. Therefore it has been suggested that ICOS–ICOSL signalling is most critical for the survival or expansion of T cells rather than T-cell differentiation.
There are numerous reports regarding the roles of ICOS-ICOSL signalling in various animal disease models, including autoimmunity, allergy, infectious diseases, tumour immunity and transplantation [16-19]. Overexpression of ICOS on CD4+ and CD8+ T cells has been reported in patients with SLE [20, 21]. In addition, we reported previously that ICOS and ICOSL affect inflammation and fibrosis in bleomycin-induced skin and lung fibrosis, a model of SSc [22]. However, there were no reports regarding ICOS or ICOSL in patients with SSc. In this study we addressed this deficiency by investigating the expression of ICOS and ICOSL in patients with SSc.

Materials and methods

Patients and clinical assessments

All patients fulfilled the SSC criteria proposed by the ACR [23]. Patients were grouped into lcSSc and dcSSc according to the degree of skin involvement, based on the classification system proposed by LeRoy et al. [24]. The dcSSc patients were further divided into early dcSSc (disease duration <3 years) and intermediate/late dcSSc (disease duration >3 years), as proposed by Medsger [25].

The presence of disease-specific autoantibodies was determined by immunoprecipitation. Complete medical histories, physical examinations and laboratory tests were conducted on all patients. The degree of skin involvement was determined according to the modified Rodnan skin thickness score, as described elsewhere [26]. Organ system involvement was defined as described previously [27]. The study was approved by the Ethical Review Board of Kanazawa University. Informed consent was obtained from study participants.

Measurement of serum soluble ICOS levels

Serum levels of soluble ICOS (sICOS) were measured in duplicate with a specific sandwich ELISA kit (Uscn Life Science Inc., Wuhan, China), according to the manufacturer’s protocol.

Measurement of serum soluble IL-2 receptor levels

Serum levels of soluble IL-2 receptor (sIL-2R) were measured in duplicate with a specific sandwich ELISA kit (BioVendor Inc., Karasek, Czech Republic), according to the manufacturer’s protocol.

Flow cytometric analysis

Heparinized blood samples were collected. Three- or four-colour analysis was performed with a combination of APC-Cy7, FITC, Pacific blue, PE, PE-Cy7 or PerCP-conjugated anti-ICOS antibody (Ab) (Biolegend, San Diego, CA, USA), anti-ICOSL Ab (Biolegend), anti-CD4 Ab (BD Biosciences, San Jose, CA, USA), anti-CD8 Ab (BD Biosciences), anti-CD14 Ab (BD Biosciences), anti-CD19 Ab (BD Biosciences), anti-CD25 Abs (Beckman Coulter, Brea, CA, USA), anti-CD45RO Abs (BD Biosciences), anti-CD69 Abs (Beckman Coulter), anti-CD127 Abs (BD Biosciences) or anti-HLA-DR Abs (Beckman Coulter), as reported previously [22]. FoxP3 staining was performed using intracellular fixation and staining procedures according to the manufacturer’s protocol (eBioscience, San Diego, CA, USA).

Immunohistochemical staining of ICOS and ICOSL

ICOS and ICOSL expression in the skin were determined by immunohistochemical staining, as reported previously [22]. The serial tissues of skin were stained with rat anti-CD3 (AbD Serotec, Kidlington, OX, UK), mouse anti-CD20 (Dako Cytomation A/S, Tokyo, Japan) and mouse anti-CD68 (Dako Cytomation A/S) Abs to identify the leucocyte subsets of cells expressing ICOS or ICOSL.

RNA isolation and real-time PCR

Total RNA was isolated from the frozen tissue of skin lesions using Qiagen RNeasy spin columns (Qiagen Ltd, Crawley, SXW, UK). Total RNA from each sample was reverse-transcribed into cDNA. Expression of ICOS and ICOSL was analysed using a real-time PCR quantification method (Applied Biosystems, Foster City, CA, USA), as reported previously [22].

T-cell costimulation

Purified CD4+ (2 × 10^5/well) T cells from each SSc patient and healthy subject were cultured in 96-well, flat-bottomed plates pre-coated with anti-CD3 Ab (12-F6, 3 µg/ml; eBioscience) plus anti-CD28 (CD28.6, 5 µg/ml; eBioscience), anti-ICOS (C398.4A, 5 µg/ml; eBioscience) or hamster lgG (isotype control, 5 µg/ml; eBioscience) for 48 h at 37°C. Each specimen was stimulated in triplicate wells. The supernatants were used for cytometric beads array (BD Biosciences) for cytokine detection.

Statistical analysis

Statistical analyses were performed using Student’s t-test for comparison of sample means between two groups. For comparisons of more than two groups, one-way analysis of variance with Bonferroni post hoc test was used. The Pearson product-moment correlation coefficient was used to examine the relationship between two continuous variables. Multiple regression analysis was also performed in some situations. P-values <0.05 were considered to be statistically significant. All values are reported as the mean (s.d.).

Results

Serum sICOS levels

Serum samples were obtained from 68 Japanese patients with SSC and 20 healthy age- and sex-matched volunteers. We evaluated the association between sICOS levels and clinical disease features (Table 1). Twenty healthy age- and sex-matched volunteers served as normal controls [16 women and 4 men, average age 47.8 (16.1) years]. Serum sICOS levels were significantly higher in all patients with SSC or in each SSC subset than in healthy controls (P <0.01; Fig. 1A). Patients with early dcSSc had serum sICOS levels that were significantly higher than those in patients with lcSSc (P <0.05).
TABLE 1 The profile and clinical features of SSc patients

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Number (% of patients, unless otherwise indicated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female:male</td>
<td>53 (77.9); 15 (22.1)</td>
</tr>
<tr>
<td>lcSSc</td>
<td>34 (50.0)</td>
</tr>
<tr>
<td>dcSSc</td>
<td>34 (50.0)</td>
</tr>
<tr>
<td>Early dcSSc</td>
<td>21 (30.9)</td>
</tr>
<tr>
<td>Intermediate/late dcSSc</td>
<td>13 (19.0)</td>
</tr>
<tr>
<td>Age, mean (s.d.), years</td>
<td>51.3 (11.7)</td>
</tr>
<tr>
<td>Disease duration, mean (s.d.), years</td>
<td>4.3 (5.3)</td>
</tr>
<tr>
<td>Modified Rodnan total skin thickness score, mean (s.d.), points</td>
<td>12.3 (10.6)</td>
</tr>
<tr>
<td>ILD</td>
<td>38 (55.9)</td>
</tr>
</tbody>
</table>

| Pulmonary arterial hypertension            | 4 (5.9)                                             |
| Oesophagus                                 | 32 (47.0)                                           |
| Heart                                      | 1 (1.5)                                             |
| Kidney                                     | 1 (1.5)                                             |
| Joint                                      | 11 (16.2)                                           |
| Muscle                                     | 4 (5.9)                                             |
| ACAs                                       | 22 (32.4)                                           |
| Anti-topo I Ab                             | 22 (32.4)                                           |
| Anti-RNA polymerases                       | 1 (1.5)                                             |
| Anti-U1 RNP Ab                             | 1 (1.5)                                             |
| Anti-U3 RNP Ab                             | 1 (1.5)                                             |
| Elevated CRP (≥0.05 mg/dl)                 | 6 (8.8)                                             |
| Elevated ESR (>20 mm/h)                    | 28 (41.2)                                           |
| %VC, mean (s.d.)                           | 100.1 (18.6)                                        |
| %DLco mean (s.d.)                          | 65.3 (15.5)                                         |
| CS therapy                                 | 28 (41.2)                                           |
| CYC therapy                                | 2 (2.9)                                             |

D\text{LCO}: diffusion lung capacity for carbon monoxide; %VC: percentage vital capacity.

Patients with SSc with interstitial lung disease (ILD) had sICOS levels higher than those without ILD, although this increase was not statistically significant. Patients with elevated CRP exhibited significantly higher sICOS levels than those in patients with normal CRP levels ($P < 0.05$). Patients without ACAs showed significantly higher sICOS levels than patients with the Abs ($P = 0.05$), although the existence of anti-topo I Ab did not significantly affect the sICOS levels. Other clinical or laboratory factors did not significantly affect sICOS levels. We also performed multiple regressions using a stepwise method that specified the $\alpha$-level for either adding or removing a regression of 0.15. Among various clinical or laboratory parameters described above, the existence of ACA ($P = 0.01$) and elevated CRP ($P = 0.04$) was determined as predicting factors. As a result, the multiple regression equation predicting sICOS is $1011.8 + ACA (\cdot -1 \rightarrow +98.9, \cdot +1 \rightarrow -98.9) +$ elevated CRP ($\cdot -1 \rightarrow -204.9, \cdot +1 \rightarrow +204.9$) [$R^2$ (determination coefficient) $= 0.39$, $P < 0.01$, RMSE (root mean square error) = 270.0].

The levels of sICOS could be non-specifically elevated as negative feedback of T-cell activation like sIL-2R, a representative serum marker of T-cell activation. Therefore, serum levels of sIL-2R were also measured in the same serum samples for comparison (Fig. 1B). Although serum sIL-2R levels were elevated in patients with SSc compared with healthy controls, the difference was not statistically significant. Among each disease subset, early dcSSc or lcSSc showed increased sIL-2R levels compared with normal controls, but the differences were not significant. The sIL-2R levels were not significantly associated with any clinical or laboratory features. Moreover, the association between sICOS levels and sIL-2R levels was not significant in patients with SSc ($r=0.18$, $P = 0.14$). Therefore serum levels of sICOS were more closely associated with clinical features of SSc compared with serum levels of sIL-2R. Thus sICOS was significantly increased in patients with SSc, especially early dcSSc, and was inversely associated with the existence of ACAs.

ICOS expression on peripheral blood mononuclear cell in patients with SSc

Expression levels of ICOS and ICOSL by peripheral blood mononuclear cells (PBMCs) were examined by flow cytometry in more than six patients selected at random from each SSc subgroup (early dcSSc, intermediate/late dcSSc, lcSSc) (Table 2 and Fig. 2). Since the expression levels of this surface marker exhibited slight variability between experiments even in the same healthy controls, results are reported as the percentage of each surface marker in patients vs. that of the same healthy control ($n=3$) in each assay. Bulk CD4$^+$ T cells from patients with early dcSSc exhibited 37% higher mean ICOS expression levels than those from normal controls. ICOS expression on memory (CD45RO$^+$) CD4$^+$ T cells was significantly elevated (42%) in early dcSSc patients compared with normal controls. Mean ICOS expression on bulk CD8$^+$ T cells was significantly increased by 18% in patients with early dcSSc compared with controls. ICOS expression on memory (CD45RO$^+$) CD8$^+$ T cells was significantly elevated at 24% in early dcSSc patients compared with normal controls.

Tregs are commonly identified by CD25 (IL-2R$\alpha$) surface expression and/or intracellular expression of the FoxP3 transcription factor. Tregs are also characterized by low CD127 (IL-7R$\alpha$) expression [28], although CD25$^+$FoxP3$^+$ T cells and CD25$^+$CD127$^-$ T cells may represent different Treg populations [29, 30]. Mean ICOS expression on Tregs, defined as either CD4$^+$CD25$^+$FoxP3$^+$ or CD4$^+$CD25$^+$C127$^-$, was increased by 101% and 54%, respectively, in patients with early dcSSc compared with controls. The ICOS expression levels of each T cell subset from lcSSc were not significantly different from normal controls.

Expression levels of T-cell activation markers were also assessed and the association with ICOS expression was evaluated (Table 2). In general, expression levels of CD25, CD69 and HLA-DR on CD4$^+$ or CD8$^+$ T cells were significantly elevated in each SSc subset (early dcSSc, intermediate/late dcSSc and lcSSc) compared with normal controls. In patients with SSc, ICOS expression levels would be of interest.
Serum sICOS and sIL-2R levels were also compared with respect to each clinical factor. Levels of sICOS and sIL-2R were determined by sandwich ELISA. Bars show the group means. *P < 0.05 and **P < 0.01. EdcSSc: early dcSSc; I/ LdcSSc: intermediate and late dcSSc.

were significantly associated with that of HLA-DR on CD4+ T cells and CD8+ T cells (r = 0.49, P = 0.008 and r = 0.72, P = 0.008, respectively). However, there were no significant associations between ICOS expression levels and expression levels of CD25 or CD69 on CD4+ and CD8+ T cells in patients with SSc (data not shown). These findings indicate that increased ICOS expression on T cells may just be reflecting T-cell activation, but are more specifically increased in patients with early dcSSc compared with expression levels of other T-cell activation...
markers. Thus expression levels of ICOS were significantly increased on peripheral blood memory T cells and Tregs in patients with early dcSSc.

**ICOSL expression on antigen-presenting cells in SSc samples**

We next examined ICOSL expression on B cells and macrophages from SSc patient peripheral blood samples (Table 2 and Fig. 2). The mean ICOSL expression on CD19+ B cells was not significantly different between each SSc subset and normal controls. We then examined ICOSL expression on CD14+ macrophages and found this to be comparable between SSc patients and normal controls. Overall the ICOSL levels on B cells or macrophages were not significantly different between each SSc subset.

**Immunohistochemical examination of ICOS- and ICOSL-expressing cells**

Biopsy samples were taken from the lesional skin (dorsal aspect of the mid-forearm) of eight females in each SSc subgroup. In the affected skin tissue from patients with early dcSSc, a considerable number of ICOS-expressed mononuclear cells were found around the small vessels (Fig. 3A and C). ICOS+ cells were less commonly found in affected skin tissue from patients with intermediate/late dcSSc and lcSSc. ICOS+ cells were sparse by immunohistochemical staining of normal control skin tissue. Although the frequency of ICOS-positive cells among mononuclear cells was higher in patients with early dcSSc [75 (10%)] compared with that in normal controls and patients with late dcSSc or lcSSc, the difference was not statistically significant (Fig. 3B). Most CD3+ T cells were considered as expressing ICOS by immunohistochemical staining of serial sections (data not shown).

Although there were some variations between patients, moderate numbers of ICOSL-expressed mononuclear cells were detected around the small vessels of affected skin from patients with early dcSSc (Fig. 3A and C). ICOSL+ cells were modestly found in affected skin tissue from patients with intermediate/late dcSSc and lcSSc. ICOSL+ cells were only sparsely detected by immunohistochemical staining of normal control skin tissue. Although the frequency of ICOSL-expressed cells among mononuclear cells was higher in early dcSSc patients [18 (6%)] than in normal controls and patients with late dcSSc or lcSSc, the difference was not statistically significant (Fig. 3B). Most of the ICOSL+ cells were considered as monocytes/macrophages morphologically and by immunostaining with anti-CD68 Ab in serial sections (data not shown). CD20+ B cells were very few in the skin tissues from patients with SSc and normal controls (data not shown). Thus infiltration of ICOS+ cells and ICOSL+ cells was increased in the affected skin from patients with early dcSSc.

**ICOS and ICOSL mRNA expression in the skin**

Next we examined the expression of ICOS and ICOSL mRNA in the lesional skin in more than eight early dcSSc patients and normal control skin by real-time PCR (Fig. 3D). ICOS mRNA levels in patients with early dcSSc were significantly higher in the lesional skin than those in healthy control subjects (P < 0.05). Additionally, ICOSL mRNA also showed a significant elevation in the skin of early dcSSc patients compared with healthy subjects (P < 0.05). Thus mRNA expression levels of ICOS and ICOSL were augmented significantly in the skin of patients with early dcSSc, although the elevation was not so dramatic despite the marked increase of ICOS- or ICOSL-expressed mononuclear cells in the lesional skin by immunohistochemical staining.

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**Table 2** Expression levels of ICOS and ICOSL on each leucocyte subset in SSc subgroups

<table>
<thead>
<tr>
<th>Leucocyte subset</th>
<th>Early dcSSc (n = 8)</th>
<th>Intermediate/late dcSSc (n = 10)</th>
<th>lcSSc (n = 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICOS on CD4+ cells</td>
<td>137 (15)**</td>
<td>102 (11)</td>
<td>101 (23)</td>
</tr>
<tr>
<td>ICOS on CD4+CD45RO+ cells</td>
<td>142 (18)**</td>
<td>121 (18)*</td>
<td>94 (17)</td>
</tr>
<tr>
<td>ICOS on CD4+CD25+FoxP3+ cells</td>
<td>201 (46)**</td>
<td>113 (51)</td>
<td>106 (13)</td>
</tr>
<tr>
<td>ICOS on CD4+CD25+CD127+ cells</td>
<td>154 (23)**</td>
<td>144 (40)*</td>
<td>112 (64)</td>
</tr>
<tr>
<td>ICOS on CD8+ cells</td>
<td>118 (19)*</td>
<td>125 (23)*</td>
<td>95 (15)</td>
</tr>
<tr>
<td>ICOS on CD8+CD45RO+ cells</td>
<td>124 (17)*</td>
<td>136 (28)*</td>
<td>103 (12)</td>
</tr>
<tr>
<td>ICOSL on CD19+ cells</td>
<td>117 (29)</td>
<td>99 (24)</td>
<td>94 (24)</td>
</tr>
<tr>
<td>ICOSL on CD14+ cells</td>
<td>120 (37)</td>
<td>98 (12)</td>
<td>94 (20)</td>
</tr>
<tr>
<td>CD25 on CD4+ cells</td>
<td>157 (28)**</td>
<td>147 (17)**</td>
<td>128 (9)*</td>
</tr>
<tr>
<td>CD69 on CD4+ cells</td>
<td>127 (15)*</td>
<td>122 (11)*</td>
<td>129 (16)*</td>
</tr>
<tr>
<td>HLA-DR on CD4+ cells</td>
<td>174 (43)**</td>
<td>161 (15)**</td>
<td>177 (19)**</td>
</tr>
<tr>
<td>CD25 on CD8+ cells</td>
<td>108 (5)</td>
<td>98 (7)</td>
<td>106 (6)</td>
</tr>
<tr>
<td>CD69 on CD8+ cells</td>
<td>149 (18)*</td>
<td>114 (12)</td>
<td>145 (19)*</td>
</tr>
<tr>
<td>HLA-DR on CD8+ cells</td>
<td>127 (18)*</td>
<td>154 (14)*</td>
<td>128 (32)</td>
</tr>
</tbody>
</table>

Expression levels of ICOS and ICOSL were assessed by flow cytometry. Results [mean (s.d.)] are reported as the percentage of each surface marker in patients vs that of the same healthy control (n = 3) in each assay. *P < 0.05 vs normal controls, **P < 0.01 vs normal controls.
ICOS costimulation on T cells

As expression levels of ICOS were up-regulated in early dcSSc patients, we examined the effects of ex vivo ICOS ligation on T cells from early dcSSc patients. Purified CD4+ T cells from the peripheral blood of early dcSSc patients and age- and sex-matched healthy controls (n = 5) were costimulated with either anti-ICOS Ab or anti-CD28 Ab. The supernatants were used for cytometric beads array evaluation of cytokine production (Fig. 4). CD28 costimulation significantly increased the production of IL-2, IL-4 and IL-17A in SSc patients but not in normal controls. However, CD28 or ICOS costimulation did not significantly affect the production of TNF-α, IL-6 and IL-10. Thus ICOS costimulation induced production of pro-inflammatory (IFN-γ and IL-17A) and pro-fibrogenic cytokines (IL-4) from CD4+ T cells from early dcSSc patients.

Discussion

In the present study, the expression levels of ICOS on peripheral blood T cells were found to be significantly elevated in patients with early dcSSc. Additionally, mRNA expression levels of ICOS and ICOSL were augmented in the affected skin of early dcSSc patients.
Ex vivo ICOS costimulation of CD4⁺ T cells from SSc patients led to specific induction of pro-inflammatory and pro-fibrogenic cytokines. We found that serum sICOS levels were significantly increased in patients with SSc, and were inversely associated with the existence of ACAs and positively associated with elevated CRP. These findings suggest that augmented ICOS–ICOSL signalling contributes to the development of SSc via cytokine production from T cells, especially in the early progressive stage of disease. Furthermore, sICOS levels may be a useful biomarker for evaluating the disease activity and/or severity of SSc.

The current study demonstrates that expression of ICOS is significantly increased on memory T cells from SSc patients. ICOS-expressing T cells and ICOS mRNA were both significantly increased in the lesional skin from SSc patients. There are several reports regarding ICOS expression in other inflammatory diseases. One previous study demonstrated that ICOS expression is increased on peripheral CD4⁺ as well as CD8⁺ T cells in patients with SLE [20, 21]. Regarding ICOS expression on specific T-cell subsets, the finding of ICOS overexpression on CD4⁺ and CD8⁺ memory T cells in patients with active SLE, but not inactive SLE, is similar to our findings in SSc patients [31]. ICOS overexpression on peripheral CD4⁺ and CD8⁺ T cells has been shown to contribute to dysregulated T-cell proliferation, T-cell activation and pathogenic Ab production in SLE in vitro [21]. Another report demonstrated that ICOS expression is elevated on CD4⁺ T cells and contributes to the production of IFN-γ, IL-17 and TNF-α from PMBCs of Behçet’s disease patients with uveitis [32]. In addition, expression of ICOS on T cells was increased in SF of RA patients [33]. These findings highlight the importance of the ICOS signalling pathway in a variety of autoimmune or inflammatory diseases.

By contrast, a down-regulation of ICOSL has been reported on peripheral blood memory B cells in patients with SLE [20]. In vitro experiments indicate that ICOSL down-regulation on B cells is a signature of recent interaction with ICOS-expressing T cells [20]. In our study, ICOSL expression levels on peripheral blood B cells or macrophages from SSc patients were not significantly different from those of normal controls. We previously reported that ICOS-deficient mice show markedly increased ICOSL expression on splenic macrophages and B cells, whereas ICOSL-deficient mice exhibit striking increases in ICOS expression on splenic T cells [22]. Therefore it is possible that increased ICOS expression on T cells in SSc patients...
ICOS expression in scleroderma

Fig. 4 Cytokine production from CD4+ T cells by ICOS costimulation.

Peripheral CD4+ T cells from early dcSSc (EdcSSc) patients and normal controls (CTL) were stimulated with pre-coated anti-CD3 Ab plus anti-CD28 Ab, anti-ICOS Ab or control IgG for 48 h. Supernatants were used for cytometric bead array. *P < 0.05 and **P < 0.01 vs anti-CD3 Ab costimulation.

ICOS is expressed at low levels on resting naïve T cells and is rapidly up-regulated after TCR ligation and CD28 costimulation [9]. ICOS expression can be regulated by signalling molecules activated downstream of TCR engagement and CD28 costimulation, including the Src kinase Fyn and the MAP kinase ERK at the transcription level [34, 35]. ICOS expression can be also regulated by the RING-type ubiquitin ligase family member Roquin at the post-transcriptional level [36, 37]. It is also possible that ICOS expression is differentially regulated in each T-cell lineage [38]. Increased ICOS expression may just be reflecting the activation of T cells. Nonetheless, levels of ICOS expression on T cells were more specifically increased in early dcSSc compared with other activation markers. Therefore increased ICOS expression levels may be contributing to the development of SSc, especially in patients with early dcSSc.

ICOS expression was also increased in Tregs in patients with SSc. This is not surprising since ICOS is expressed on all T cells after activation [39]; thus enhanced ICOS on memory T cells and Tregs probably reflects cellular activation status, indicating that these T cells are continuing to receive antigen receptor and CD28 stimulation in vivo. Recent studies have demonstrated that Tregs could also be working as effector cells via production of IL-17A in response to IL-1β and IL-6 [40-42]. It has been reported that the CD4+ICOS+FoxP3+ subset of Tregs can be distinguished from other Tregs by the production of effector cytokines in mice [43, 44]. Therefore increased ICOS expression on Tregs in early dcSSc may reflect precursor inflammatory T cells. Under certain conditions they may lose their suppressor functions and convert to effector T cells, as suggested in the case of both active SLE and a murine melanoma model [44, 45]. Thus the net effect of ICOS-ICOSL signalling in host immune responses is probably dependent on the environment, which can lead to seemingly controversial results depending on the disease or disease models being examined [42, 44, 46]. In this study, in vitro ICOS costimulation induced the production of IFN-γ, IL-4 and IL-17A from peripheral blood T cells. IL-4 is a representative pro-fibrogenic cytokine, and IFN-γ and IL-17A are pro-inflammatory cytokines, although IFN-γ has anti-pro-fibrogenic effects. Recent studies suggest critical roles of IL-17A in fibrogenic diseases, including SSc [47-49]. Therefore augmented ICOS signalling may be contributing to the development of fibrosis via cytokine production in patients with SSc.

To the best of our knowledge, this study is the first to evaluate sICOS levels in human or animal serum samples. We found that sICOS levels were markedly increased in patients with early dcSSc. While SSC patients with ACA generally show mild symptoms except for pulmonary arterial hypertension, sICOS levels were significantly increased in SSC patients without ACA and SSC patients with elevated CRP. It has been reported that serum levels of sIL-2R reflect the T-cell activation and disease activity or severity in various inflammatory diseases including SSC [50]. However, serum levels of sICOS were more closely associated with clinical features of SSC compared with
serum levels of sIL-2R. The mechanism of shedding and the roles of sICOS remain unknown. Nonetheless, our
findings indicate that sICOS levels may be useful as a biomarker of disease activity and severity in SSc patients.

**Rheumatology key messages**

- ICOS expression on peripheral T cells and the lesional skin is augmented in patients with early dcSSC.
- Soluble ICOS levels may be useful as a serum marker of disease activity and severity in patients with SSc.

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