

## CONCISE REPORT

# Early T cell activation in the skin from patients with systemic sclerosis

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**Objectives:** To determine if T cells in skin lesions in systemic sclerosis (SSc) express the early activation antigen CD69 that participates in cell-cell interactions.

**Methods:** Skin biopsy specimens from 17 patients with SSc were analysed by immunohistochemistry using the indirect peroxidase method and monoclonal antibodies for CD3 (T cell marker), CD69 (early T cell activation marker), and CD68 (macrophage marker).

**Results:** Mononuclear cells, containing mostly T cells and macrophages, were increased in SSc skin lesions and were present in perivascular areas. CD69 was expressed in these mononuclear cells. There was no correlation between the number of CD3+, CD69+, or CD68+ cells and the Rodnan skin score or disease duration.

**Conclusions:** The expression of early T cell activation antigen CD69 in skin lesions suggests that T cells may actively participate in cell-cell contact with fibroblasts to promote fibrosis.

Recent studies suggest that T cells may have an important role in the pathogenesis of systemic sclerosis (SSc).<sup>1</sup> In patients with SSc, T cells exhibit the intermediate activation markers interleukin 2 receptor (IL2R) and HLA-DR in peripheral blood<sup>2</sup> and skin lesions.<sup>3</sup> In peripheral blood there are increased levels of T cell cytokines, such as IL4<sup>4</sup> and IL17.<sup>6</sup> T cell derived cytokines can cause fibrosis in tissues and small blood vessels in SSc. For instance, IL4 is a profibrotic cytokine. It induces collagen production *in vitro* and *in vivo*, whereas disruption of the IL4 receptor gene in animal models of SSc prevents fibrosis (reviewed by Sakkas and Platsoucas<sup>1</sup>). T cells are also necessary for the production of antitopoisomerase autoantibodies in SSc.<sup>1</sup> Mononuclear cell infiltrates, consisting mainly of T cells and macrophages, appear in the skin early in the disease process.<sup>7–8</sup>

We have recently found that the skin-infiltrating T cells in patients with SSc exhibit oligoclonal expansion and that the same T cell clone persists over time in individual patients.<sup>9</sup> These findings suggest that T cells in skin lesions have been activated and expanded in response to an antigen. One antigen that is expressed on the cell surface early upon T cell activation is CD69.<sup>10</sup> Therefore, we investigated the expression of CD69 in skin biopsy specimens from patients with SSc.

## PATIENTS AND METHODS

### Patients

Seventeen patients with SSc (16 women, 1 man) with a mean age of 50.8 years (range 34–75) were included in the study. Patients were from central and northern Greece and attended the rheumatology outpatient clinic of the University Hospital of Larisa. They all fulfilled the classification criteria for SSc of the American College of Rheumatology. Sixteen patients had

diffuse SSc and one patient had limited disease. Disease duration varied from 12 to 228 months. The modified Rodnan skin score was assessed<sup>11</sup> as described; skin thickness was rated as 0 (normal), 1+ (mild), 2+ (moderate), and 3+ (severe). Normal skin biopsy specimens from seven adults were used as controls. Skin biopsy specimens were obtained from patients after approval by the ethical committee of the University Hospital of Larisa and written informed consent. Specimens were embedded in OCT and kept at –30°C.

### Monoclonal antibodies

The following monoclonal antibodies were used: anti-CD3 (dilution 1:100; mouse antihuman IgG1, clone UCHT1; R&D Systems, Minneapolis, MN, USA), anti-CD69 (dilution 1:50; mouse antihuman IgG1, clone FN50; DAKO, Glostrup, Denmark), and anti-CD68 (dilution 1:200; mouse antihuman IgG1, clone KP1; DAKO).

### Immunohistochemistry

Cryostat sections of skin biopsy specimens (6 µm) were air dried and fixed in cold (–20°C) acetone for 30 minutes, and then treated with cold methanol-H<sub>2</sub>O<sub>2</sub> to block endogenous peroxidase activity. Sections were stained with monoclonal antibodies using the avidin-biotin immunoperoxidase complex (ABC) method (Vectastain Elite ABC Kit) according to the supplier's instructions (Vector Laboratories Burlingame CA, USA). Briefly, serial sections were first incubated with diluted normal blocking serum for 20 minutes and then with the primary antibody for 1 hour. Then the specimens were incubated with diluted biotinylated secondary antimouse antibody for 30 minutes and subsequently with the ABC reagent for 30 minutes. Between steps, sections were washed in phosphate buffered saline. Finally, sections were developed with 3', 3'-diaminobenzidine as chromogen, lightly counterstained with Harris's haematoxylin, and examined under a light microscope. Positive cells were counted three times, and results were expressed as the average number of positive cells per high power field (×400).

### Statistical analysis

Statistical analysis was carried out using the prism software.

## RESULTS

All patients had antinuclear autoantibodies, and 11 patients had anti-DNA topoisomerase I autoantibodies. The mean modified Rodnan skin score was 28.6. (range 9–46.5). Inflammatory cells were detected in skin biopsy specimens of all patients. They were around small vessels and included CD3+, CD69+, and CD68+ cells (fig 1). Table 1 shows the degree of T cell and macrophage infiltration. The number of immunoreactive cells was increased in biopsy specimens from patients with SSc compared with controls. The mean

**Abbreviations:** IL, interleukin; IL2R, interleukin 2 receptor; SSc, systemic sclerosis

number of CD3+ cells per high power field in SSc skin lesions was 59.9 ( $v$  0.97 in controls, two tailed  $p=0.02$ ). Cells immunoreactive for CD69 were detected in all biopsy specimens. The mean number of CD69+ cells in SSc skin lesions was 18.9 ( $v$  0.03 in controls, two tailed  $p=0.006$ ), whereas the mean number of CD68+ cells in SSc lesions was 77.2 ( $v$  5.54 in controls, two tailed  $p<0.0001$ ) (table 1).

There was a small decrease in the number of CD3+ and CD69+ cells as the disease duration increased. In patients with disease duration of  $<1$  year, the mean number of CD3+ cells was 76.5, whereas the respective number was 20 in patients with disease duration of  $>5$  years. However, there was no significant correlation between CD3 immunoreactivity and disease duration (Pearson correlation,  $r=-0.36$ ,  $p=0.08$ ). Similarly, there was no correlation between the number of CD3+, CD69+, or CD68+ cells and the Rodnan skin score.

## DISCUSSION

In this study mononuclear cell infiltrates were found in perivascular areas of skin lesions from patients with SSc. These infiltrates contained mainly CD3+ T cells and CD68+ macrophages, and were immunoreactive for the early activation antigen CD69. In sequential skin biopsy sections stained for CD3 and CD69 it appeared that CD69 was expressed on T cells.

Evidence of T cell activation has been reported previously in SSc skin lesions. For example, products of activated T cells, such as soluble IL2R, were found in suction skin blisters from patients with SSc.<sup>3</sup> This study showed that T cells expressed CD69, one of the earliest known molecules expressed on the cell surface upon T cell activation.<sup>10</sup> Activated T cells interact with other cells through cellular contact and through soluble mediators, the cytokines. The CD69 molecule is involved in contact cell interactions of T cells with other cells such as monocytes and fibroblasts<sup>12</sup> and is expected to influence fibroblast function in this way.

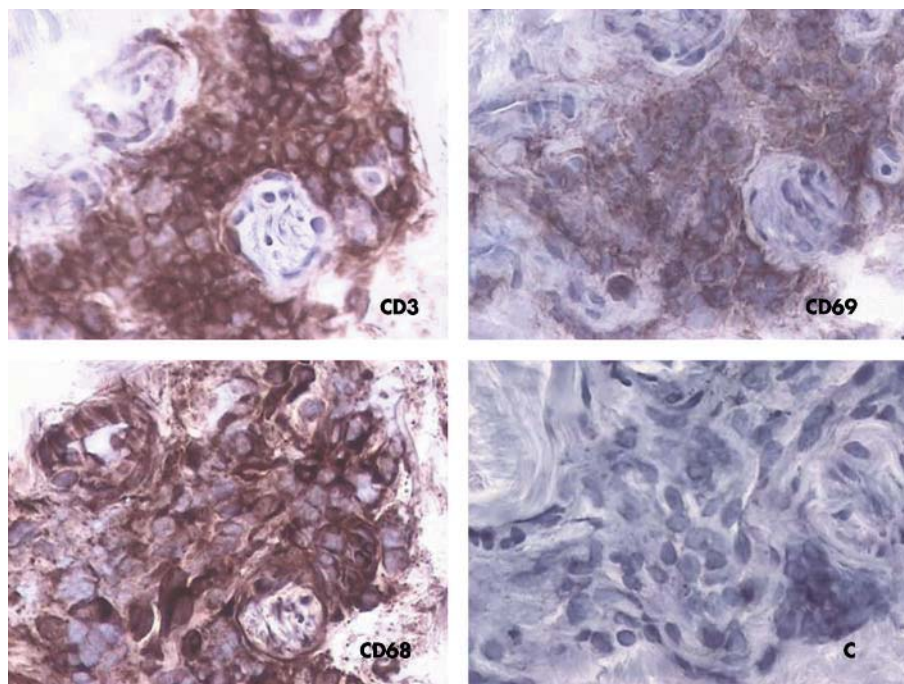
In a previous study we found that in SSc the skin-infiltrating T cells exhibit oligoclonal expansion.<sup>9</sup> The

**Table 1** Mononuclear cell infiltration of skin lesions from patients with SSc. Positive cells were counted three times and expressed as a mean number per high power field ( $\times 400$ )

Patients	Disease duration (months)	No of CD3+ cells/HPF	No of CD69+ cells/HPF	No of CD68+ cells/HPF
1	60	7	3	61
2	18	180	14	52
3	23	26	25	56
4	166	16	13	99
5	38	112	47	68
6	17	13	1	47
7	18	210	35	45
8	228	21	19	84
9	39	9	2	141
10	22	140	37	120
11	38	55	4	70
12	51	17	12	53
13	57	56	7	102
14	94	52	40	120
17	45	20	15	34
18	36	14	2	52
19	12	70	45	108
<b>Mean</b>		<b>59.9</b>	<b>18.9</b>	<b>77.2</b>

oligoclonal expansion of T cells indicates an antigen driven T cell activation. In contrast, T cells activated non-specifically by cytokines exhibit polyclonal expansion. Taken together, these findings suggest that T cell activation in skin lesions from patients with SSc may take place in situ early in the disease process and it may be this T cell activation that initiates a series of cell-cell interactions that lead to tissue fibrosis and microvascular injury.

The numbers of CD3+ and CD69+ cells in SSc skin lesions were found to decrease over time, though not significantly, and the numbers of T cells did not correlate with the Rodnan skin score. This is not surprising given the complex natural history of SSc. At the onset of the disease there is an



**Figure 1** Immunoperoxidase staining of a skin lesion from a patient with SSc. Sequential sections were stained with anti-CD3, anti-CD69, anti-CD68 monoclonal antibody or with IgG1 (control).

inflammatory phase—inflammation may appear before any histological evidence of fibrosis.<sup>8</sup> Fibrosis dominates the skin lesion in a subsequent phase of the disease and, finally, may regress.<sup>13</sup> In this initial inflammatory phase, T cells seem to have a central role. It is logical that in this inflammatory phase, treatments of patients with SSc should target T cells. Indeed, treatments directed against T cells in a few uncontrolled small studies support this concept. In these studies the proportion of patients whose skin improved was higher in patients with short disease duration<sup>14</sup> than in those with long disease duration.<sup>15</sup>

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