Peripheral regulatory T cells from silicosis patients are susceptible to CD95-mediated apoptosis

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ABSTRACT Given that silicosis patients (SILs) suffer not only from pulmonary fibrosis but also from complications associated with autoimmune diseases such as rheumatoid arthritis and systemic sclerosis, silica may have the effect of disturbing self-tolerance. One advancement in the field of immunoregulation concerned discovery of the regulatory T (Treg) cell, characterized as CD4+CD25+ and FoxP3+. Treg cells regulate the activation of responder T cells, and a reduction in the size (absolute and/or relative number) and function of Treg cells leads to excessive activation of responder T cells against various antigens including self-antigens. This mechanism may be associated with the occurrence of autoimmune diseases. Our previous investigations showing reduced Treg cell function in the CD4+CD25+ fraction of SILs indicated alterations of Treg cell size or function. Here, we examined the expression of pd-1, a T cell activation marker, in CD4+CD25- and CD4+CD25+ fractions, expression of surface CD95 known as Fas, an apoptosis inducing receptor, in Treg cells, and susceptibility against apoptosis inducing anti-CD95 antibody in CD4+CD25- and CD4+CD25+ cells. We observed chronic activation of responder T and Treg cells in SILs, higher expression of CD95 and higher susceptibility to the CD95 stimulating antibody in Treg cells from SILs. Taken together, the reduction of Treg cell function and size caused by excessive loss of Treg cells and substitution by chronically activated responder T cells caused by silica exposure may be important in facilitating the disturbance of autoimmunity in SILs.

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Key words：**Silicosis, Regulatory T cells, Activated T cells**

INTRODUCTION

Silicosis is defined by chronic and progressive pulmonary fibrosis caused by occupational inhalation of small silica particles. Silicosis patients (SILs) suffer from respiratory dysfunction and pulmonary complications such as tuberculosis, chronic bronchitis and emphysema[1–3]. Additionally, SILs often have complications associated with autoimmune diseases such as rheumatoid arthritis [4–6] (known as Caplan syndrome) and systemic sclerosis[7–9]. The effects of silica on autoimmunity have also been recognized following the observation that patients who receive plastic surgery with implants containing silicone ([SiO2-O-]n) show

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frequent complications involving autoimmune disorders[10,11]. These findings clearly indicate that crystalline silica causes dysregulation and/or disturbance of the human immune system.

One recent immunological finding relates to the discovery of CD4+CD25^{bright} and transcription factor forkhead box P3 (*foxp3*) gene expressing regulatory T (Treg) cells, and that Treg cells contribute to maintaining self-tolerance by down-regulating immune responses to self and non-self antigens (Ags) in an Ag-non-specific manner, presumably at the T cell activation stage; elimination/reduction of CD4+CD25^{bright} T cells relieves this general suppression, thereby not only enhancing immune responses to non-self Ags, but also eliciting autoimmune responses to certain self-Ags. Genetic anomalies of *foxp3* cause autoimmune and inflammatory disease in rodents and humans by affecting the development and function of CD4+CD25bright Treg cells[12-14].

It is known that Treg cells suppress the activation of responder T cells against foreign and self antigens. Thus, deficiencies in Treg cell function or decreases in the proportion/size of Treg cells have been observed to influence the pathogenesis of collagen or autoimmune diseases such as multiple sclerosis[15], rheumatoid arthritis[16], systemic lupus erythematosus[17], and pemphigus vulgaris[18].

Our group has investigated alterations of the immune system in SILs, with a focus on CD95 (also known as Fas), an apoptotic cell death inducing receptor[19-21]. Our findings showed that SILs exhibited elevated serum soluble Fas levels[22], increased relative expression of soluble *fas* and *dcr3* genes in peripheral blood mononuclear cells[23,24], high levels of other variant messages of *fas* transcripts[25], relatively decreased expression of genes encoding several physiological inhibitors (such as surviving and toso)[26], and a predominance of lower-membrane Fas expression in lymphocytes, which predominantly transcribe soluble fas, compared with soluble Fas transcript in healthy donors (HDs)[21]. The CD95 receptor has been implicated in controlling T cell expansion by triggering T cell-autonomous apoptosis. CD95-dependent elimination is a major regulatory mechanism in autoimmune responses and acts in concert with the CD95-mediated regulation of chronically activated autoimmune T cells. Thus, various alterations of CD95-related molecules may be a manifestation of impaired autoimmunity in SILs.

Additionally, we previously reported on the reduced function of the CD4+CD25+ regulatory T cell fraction in SILs[27] and the *in vitro* activation of responder T cells by silica particles[28].

In this study, given our previous findings which showed several alterations in lymphocytes or T cells from SILs, we attempt to confirm that silica exposure is the environmental factor which leads to the dysregulation of autoimmunity[19-21].

MATERIALS AND METHODS

Isolation of human peripheral blood mononuclear cells (PBMCs) and patients studied

PBMCs were isolated from heparinized peripheral blood of healthy donors (HDs: n=4 age 35 ± 11) and SILs(n=8 age 73 ± 6.8) using a Ficoll-Hypaque density gradient. Specimens were taken from HDs and SILs from whom informed consent had been obtained. The Institutional Ethics Committee of Kawasaki Medical School, Kusaka Hospital and Urakami Iin had approved the project. All SILs were brickyard workers in Bizen City, Okayama Prefecture, Japan, and were followed at Kusaka Hospital and Urakami Iin, and the substances which these SILs had been handled were estimated to include as high as 40 to 60% of free silica. The subjects were diagnosed with pneumoconiosis according to the ILO 2000 guideline[29]. They showed no clinical symptoms of autoimmune diseases

including sclerotic skin, Raynaud's phenomenon, facial erythema or arthralgia.

Cell staining and flow cytometry

PBMCs were stained with anti-CD4 PE or anti-CD4 FITC or anti-CD4 PE-Cy7, anti-CD25 FITC or anti-CD25-allophycocyanin, anti-CD95-allophycocyanin (BD Pharmingen). For the intracellular staining of Foxp3, surface staining was first performed; cells were fixed and permeabilized, and then incubated with anti-FoxP3-PE (eBioscience) using an anti-human regulatory T cell staining kit (eBioscience) according to the manufacturer's instructions. Cells were sorted using a FACSaria™ flow cytometer (BD Bioscience) and analyzed with FACS-Diva software (BD Bioscience). The $CD25^{bright}$ gate was adjusted to less than 1~3% in CD4+ T cells and the CD25+ gate was greater than the CD25 bright zone in several HDs, and previously reported[27].

Gene expression in responder T (Tres) cells and Treg fractions from HDs and SILs.

RNA extraction, cDNA synthesis and real-time reverse transcription-polymerase chain reaction (RT-PCR) with SYBR-G using Tres and Treg cells from HDs and SILs were performed as previously reported[21]. The relative expression levels of pd-1 and gapdh were examined with a fluorescence thermocycler (Mx3000P® QPCR System, Stratagene Corporation, La Jolla, CA) using the following primer sets:

pd-1: (forward: GTGTCACACAACTGCCCAAC, reverse: CTGCCCTTCTCTCTGTCACC, 162 bp),
Gapdh: (forward: GAGTCAACCGATTTGGTCGT, reverse: TTGATTTTGGAGGGATCTCG, 238 bp).

The relative expression of the pd-1 gene was calculated as follows:

A: number of PCR cycles required to reach a certain level of fluorescence for the gapdh product.
B: number of PCR cycles required to reach the same level of fluorescence for the pd-1 gene derived from the same samples.

The relative expression of the pd-1 gene is given by 1/2[B-A] with the gapdh expression being 1.0. Real-time RT-PCR products were assessed by standard agarose gel electrophoresis and staining with ethidium bromide.

Cell culture and apoptosis assay

Apoptosis was analyzed by staining cells with FITC-annexin V and propidium iodide (PI) (Roche, Basel, Switzerland) according to the manufacturer's instructions. Finally, the percentages of annexin V-positive PI-negative cells and annexin V-positive and PI-positive cells were measured using a FACSCalibur™ (Becton Dickinson) flow cytometer. Specific cell death was calculated as follows; (percent of experimental cell death - percent of spontaneous cell death)/ (100-percent of spontaneous cell death) × 100.

Statistical analysis

Differences in pd-1 mRNA expression, the mean fluorescence intensity (MFI) of surface CD95, and the percentage of apoptosis induced by apoptosis-inducing anti-CD95 Ab (CH11 clone) were examined using the nonparametric Mann-Whitney U test. Statistical analysis was performed using StatView 5.0J software (SAS Institute, Cary, NC).

RESULTS

Peripheral CD4+25+ and CD4+25- fractions from SILs are chronically activated.

It was recently revealed that $CD4+CD25^{bright}$ Foxp3+ T cells did not exhibit significant levels of PD-1[30], which is known as the inhibitory receptor molecule of programmed cell death and is upregulated on activated Tres cells. Therefore, the PD-1 molecule/gene could be utilized to discriminate CD4+CD25+ resting T cells from activated T cells. We measured the expression levels

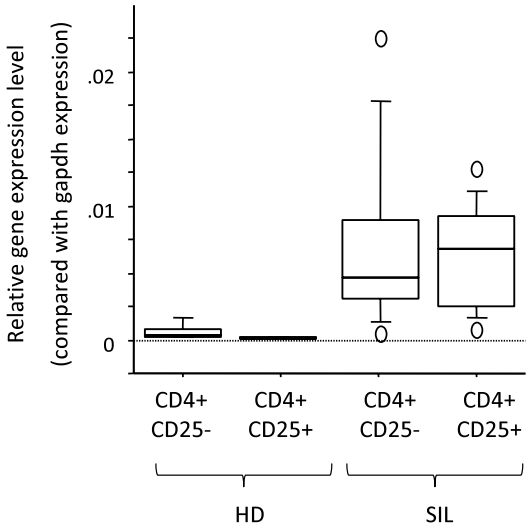

Fig. 1. The relative expression levels of pd-1 in CD4+CD25- and CD4+CD25+ fractions from HDs (healthy donors) and SILs (silicosis patients). The pd-1 gene, a marker gene for activated T cells, was overexpressed in the CD4+CD25+ fraction of SILs but not in HDs. Both CD4+CD25- and CD4+CD25+ fractions from SILs showed higher expression of pd-1 compared those from HDs.

of *pd-1* using real-time PCR. *pd-1* gene expression levels in CD4+CD25+ and CD4+CD25- fractions were very low in HDs and high SILs, as shown in Fig. 1. These results supported our previous experimental finding which suggested that silica can stimulate Tres cells, and that activated T cells present in the CD4+CD25+ fraction from SILs, in addition to the CD4+CD25+ fraction, may include activated Tres and activated Treg cells.

CD95 is highly expressed in Treg cells from SILs but not from HDs

The surface expression of CD95 in Treg cells has recently been investigated, and these cells seemed to show higher expression of CD95 and a tendency to progress towards apoptosis[31–33]. CD4+FoxP3+ T cells represent only a minor population (less than 5%) of peripheral blood CD4+ T cells (Fig. 2). Statistically, the MFI of Fas molecules in

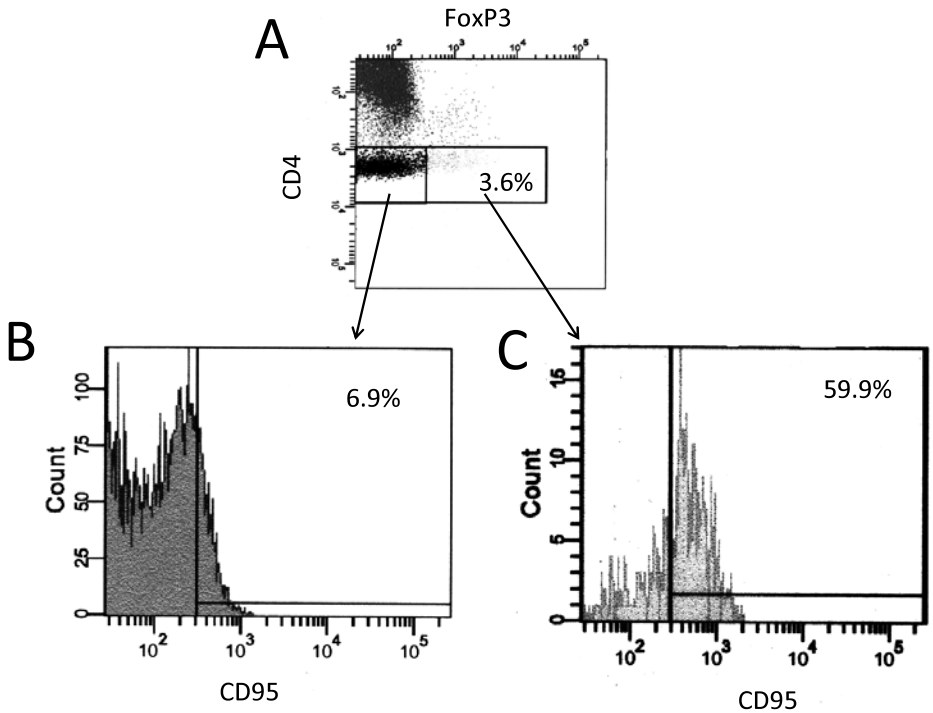

Fig. 2. Expression levels of CD95 in CD4+FoxP3+ and CD4+FoxP3- T cells. Representative CD4+FoxP3+ T cells in PBMCs from HDs revealed a CD4+ fraction of 3.6% (panel A). Representative surface CD95 expression in peripheral CD4+FoxP3+ cells from HDs (panel B) and CD4+FoxP3- cells from HDs (panel C). The CD95 expression is higher in CD4+FoxP3+ cells compared with CD4+25- cells.

CD4+FoxP3+ cells was significantly up-regulated in SILs compared with that in HDs (Fig. 3). These data indicated that Treg cells in SILs are activated by chronic low-dose and long-term exposure to silica, since Fas is known as one of the activated markers of Treg cells.

CD4+CD25+ Treg cells from SILs were more sensitive to anti-CD95 Ab-induced apoptosis compared with cells from HDs.

In an effort to determine whether freshly isolated Treg cells from SILs are sensitive to CD95-mediated apoptosis, FACSAria-sorted Treg and Tres cells were cultured with anti-CD95 Ab (anti-APO-1) for six and twelve hours. As shown in Fig. 4, T cells from CD4+CD25+ fractions derived from both HDs and SILs showed a time-dependent

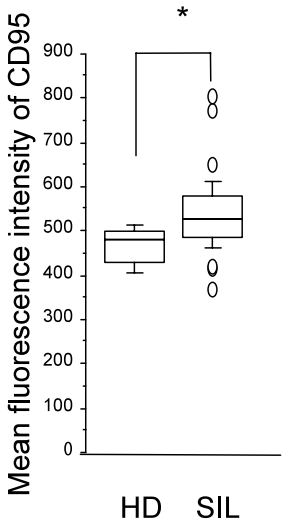

Fig. 3. Comparison of the MFI of Fas on CD4+FoxP3- or CD4+FoxP3+ cells from fourteen HDs and twenty-one SILs. Significant differences are shown with asterisks (*p < 0.05).

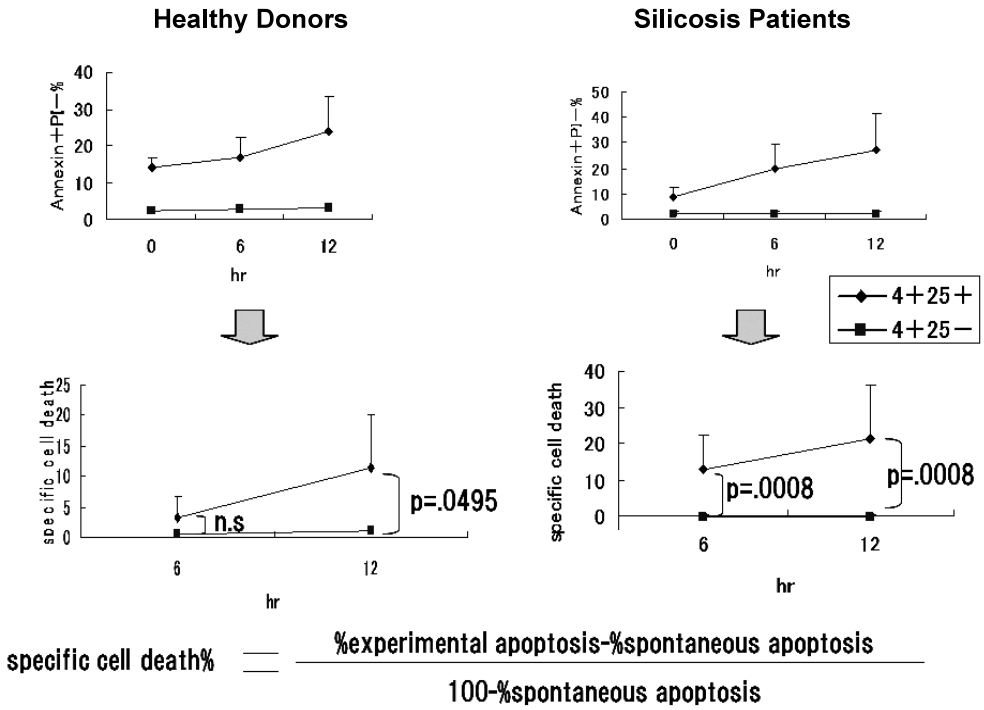

Fig. 4. In vitro silica-induced specific cell death in Tres and Treg from HDs or SILs. CD4+CD25- and CD4+CD25+ cells from SILs (n=8) and HDs (n=4) were cultured with apoptosis-inducing anti-CD95 Ab for six and twelve hours. Apoptotic cells were defined as Annexin V+ and PI-. Upper panels show actual changes in apoptotic cells of the CD4+CD25- (square) and CD4+CD25+ (diamond) fractions from HDs (left) and SILs (right). Lower panels show specific cell death calculated using the formula shown in the figure. There is a significant increase in specific apoptosis in CD4+CD25+ cells when cells from SILs were cultured for six and twelve hours, and in cells from HDs cultured for twelve hours.

increase in apoptotic cells defined by annexin+ and PI-, although CD4+CD25- cells from both HDs and SILs did not show any increase in apoptotic cells.

The specific cell death (%) was defined as shown in Fig. 4. This relates only to the percentage of cell death caused by anti-CD95 Ab. The appearance of apoptotic cells gradually increased in CD4+CD25+ cells from both HDs and SILs, and at twelve hours incubation the specific cell death in CD4+CD25+ cells from HDs and SILs were significantly higher compared with CD4+CD25- cells. However, at six hours incubation only CD4+CD25+ cells from SILs showed a significant increase in the number of apoptotic cells. This may suggest that CD4+CD25+ cells from SILs were susceptible to CD95-mediated apoptosis.

The specific cell death of CD4+CD25+ cells from both HDs and SILs with apoptosis-inducing anti-CD95 Ab at six hours incubation is shown in Fig. 5. Unlike the case with HDs, a significant increase in apoptosis of CD4+CD25+ cells from SILs was clearly evident. This indicates that CD4+CD25+ cells from SILs were susceptible to CD95-mediated apoptosis.

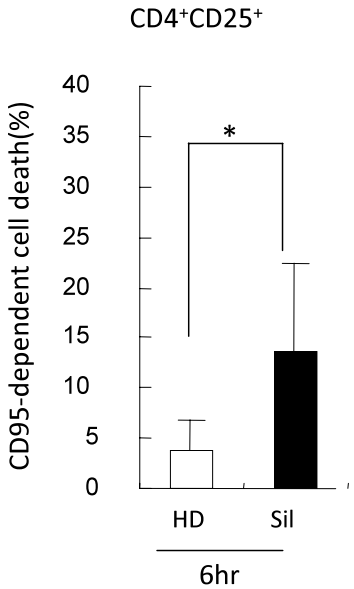

Fig. 5. The comparison of silica-induced specific cell death of Treg derived from HDs or SILs. Statistically, the specific apoptosis defined in Fig. 4 in CD4+CD25+ cells from SILs cultured with anti-CD95 Ab for six hours was higher than that in cells from HDs.

DISCUSSION

Our previous data showed that Treg cell function of the CD4+CD25+ fraction from SILs was reduced, whereas the size of CD4+CD25+ cells did not differ markedly from that of HDs[27]. Additionally, we showed that silica could activate peripheral blood T lymphocytes *in vitro* as monitored by CD69, an early activation marker of T cells[28]. Thus, our previous finding concerning the reduction of Treg cell function in SILs involved contamination by chronically activated Tres cells in the CD4+CD25+ fraction, since it is well known that activated T cells express CD25 on their surface. However, if contamination by activated Tres cells in the CD4+CD25+ fraction had only occurred, the size of the CD4+CD25+ fraction should have increased, and since activated Treg cells were present in this fraction, normal Treg cells should also have been present. Consequently, the total Treg function of the CD4+CD25+ fraction in SILs may not be altered.

Thus, a loss of true Treg cells from the CD4+CD25+ fraction in SILs is required for there to be no change in the size of CD4+CD25+ cells and reduction of Treg cells in SILs. The investigation then shifted focus to the CD95, since we previously demonstrated various alterations in CD95 and a CD95-related molecule in SILs[19-26].

The surface expression of CD95 in Treg cells has recently been investigated, and these cells seemed to show higher expression of CD95 and a tendency to progress towards apoptosis[31-33]. Similarly, the results of this study also showed that CD95 was more highly expressed in Treg cells (CD4+CD25+FoxP3+ cells) from SILs and HDs compared with that expression observed in Tres cells. Additionally, CD95 expression in Treg cells from SILs was significantly higher compared with

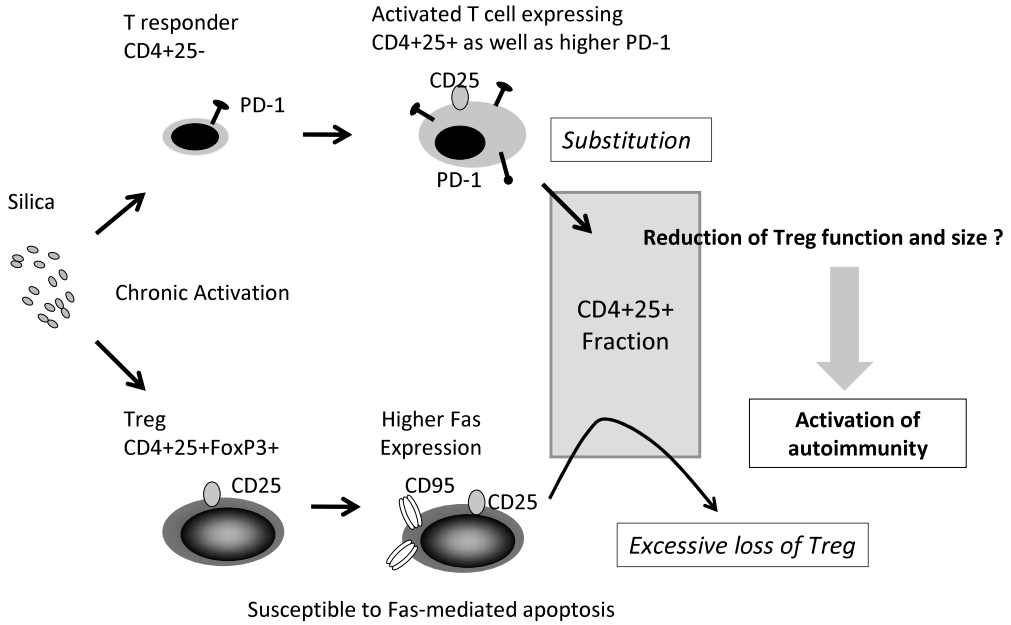

Fig. 6. Schematic presentation of the mechanisms involved that lead to a reduction of Treg function and size in SILs. Chronic exposure to silica causes activation of Tres and Treg cells. Activated Tres cells express CD25 and are present in the CD4+CD25+ fraction, where Treg cells are also present if there is no chronic stimulation such as silica exposure. Activated Treg cells express CD95 and then become susceptible to CD95-mediated apoptosis, leading to their absence from the CD4+CD25+ fraction. Taken together, Treg cell function and size in the CD4+CD25+ fraction from SILs are reduced, and may cause impairment of autoimmunity.

that of HDs. This may be the result of chronic activation of Treg cells by silica exposure.

The susceptibility of Treg cells from SILs was also upregulated compared than that from HDs, particularly in the early phase of cultivation with apoptosis-inducing anti-CD95 Ab *in vitro*. Thus, this may represent one mechanism which leads to early loss of Treg cells from the CD4+CD25+ fraction in SILs.

The effects of chronic silica exposure are outlined in Fig. 6. Chronic silica exposure induces chronic activation of both Tres and Treg cells. Activated Tres cells begin to express CD25 and are then present in the CD4+CD25+ fraction, which predominantly comprises Treg cells without any stimulation. Additionally, activated Treg cells then begin to express CD95 cell death receptor, progress towards apoptosis, and are then absent from the CD4+CD25+ fraction. Thus, as reported previously, the CD4+CD25+ fraction in SILs showed no marked changes in size, but possessed reduced function.

Further investigations are required to determine whether the number of true Treg cells (CD4+CD25+FoxP3+) are reduced in the CD4+CD25+ fraction of SILs (previously we only examined the number of CD4+CD25+ cells), whether CD95-neutralizing Ab can rescue CD95-mediated apoptosis in Treg cells from HDs and SILs, and the outcome of re-stimulating Treg cells from SILs with silica *in vitro*.

In conclusion, it is clear that occupational exposure can influence the development and course of autoimmune disease. Silica may act as an adjuvant, enhance the immune response, modify apoptosis to self-reactivity, or induce regulatory mechanisms. These mechanisms may be relevant with respect to the onset of autoimmune and collagen diseases.

Therefore, further investigations concerning the immunological effects of silica are necessary in an effort to delineate the pathophysiology of many autoimmune diseases.

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