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Immunological Effects of Silica and Related Dysregulation of Autoimmunity

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1. Introduction

Silicosis is known as environmental and occupational pulmonary fibrosis and the most typical form of pneumoconiosis results from long-term exposure (ten years or more) to relatively low concentrations of silica dust and usually appears ten to thirty years after the first exposure (Hoffman & Wanderer, 2010; Madl, 2008; Rimal, 2005). Patients with this type of silicosis, especially in the early stages, may not have obvious signs or symptoms of disease, but abnormalities may be detected by x-ray. Chronic cough and exertional dyspnea are common clinical findings. Radiographically, chronic simple silicosis reveals a profusion of small (less than 10 mm in diameter) opacities, typically rounded, and predominating in the upper lung zones. Patients with silicosis are particularly susceptible to tuberculosis infection—known as silicotuberculosis (Brown, 2009). It is thought that silica damages pulmonary macrophages, inhibiting their ability to kill mycobacteria. Pulmonary complications of silicosis also include chronic bronchitis and airflow limitation, non-tuberculous *Mycobacterium* infection, fungal lung infection, compensatory emphysema, and pneumothorax (Cohe & Velho, 2002; Rees & Murray, 2007). Lung cancer is also considered to be associated with silicosis and the International Agency for Research on Cancer (IARC) categorized crystalline silica as a causative of lung cancer (Cocco, 2007; IARC, 1997; Pelucchi, 2006). In addition, it is well known that silicosis patients (SILs) often experience complications due to autoimmune diseases (Shanklin & Smalley, 1998; Steenland & Goldsmith, 1995; Uber & McReynolds, 1982) such as rheumatoid arthritis (known as Caplan syndrome) (Caplan, 1959, 1962), systemic lupus erythematosus (SLE) (Bartsch, 1980; Yamazaki 2007), systemic scleroderma (SSc) (Barnadas, 1986; Cowie, 1987; Hausteine, 1990; Hausteine & Anderegg, 1998; Sluis-Cremer, 1985) and anti-neutrophil cytoplasmic autoantibody (ANCA)-related vasculitis/nephritis (Bartůňková, 2006; Mulloy, 2003; Tervaert, 1998).

Silica-induced dysregulation of autoimmunity has been thought to be caused by the adjuvant effect of silica (Cooper, 2008; Davis, 2001; Parks, 1999). Although this represents

one mechanism by which silica might be involved in the development of autoimmune diseases, silica can influence circulating immunocompeting cells and dysregulate the T responder (Tresp) survival and activation status, since several different autoimmune diseases may be associated with silica dust exposure as mentioned above. In addition, silica may affect the regulatory T cell (Treg, CD4+25+FoxP3+), since Treg has been considered the most important subpopulation of T cells for the control of Tresp activation by the recognition of foreign and/or auto-antigens (Baecher-Allan, 2004; Bluestone & Tang, 2005; Schwartz, 2005). If the function or number of Treg is reduced, continuous stimulation of Tresp is thought to be maintained.

Furthermore, recent findings regarding the NOD-like receptor family, pryin domain containing 3 (NLRP3, Nalp3)-inflammasome, have contributed substantially to our understanding of the sequential cellular events occurring when silica is inhaled into the pulmonary region and alveolar macrophages try to treat silica particles as a foreign substance (Cassel, 2008; Dostert, 2008; Hormung, 2008).

At first, initial recognition of silica occurs by cell membrane receptors such as the macrophage receptor with collagenous structure (MARCO), scavenger receptor (SR)-AI and SR-AII (Brown, 2007; Hamilton, 2006; Thakur, 2009). The next stage involves capture of silica by macrophages and entrapment within lysosomes and their activation of the nucleotide-binding domain and leucine-rich repeat containing proteins, the NLRP3 inflammasome, to cleave pro-caspase 1 to an active form (Cassel, 2008; Dostert, 2008; Hormung, 2008). Thereafter, cleavage of pro-interleukin (IL)-1 β occurs to an active form for release to form fibrotic nodules and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the macrophages yielded (37-40). As a consequence, the induction of cellular and tissue damages occur due to the production of ROS and RNS and the apoptosis of alveolar macrophages. Various cytokines/chemokines such as IL-1 β , tumor necrosis factor (TNF)- α , macrophage inflammatory protein (MIP)-1/2, monocyte-chemoattractant protein-1 (MCP-1) and IL-8 are produced that cause chronic inflammation and proliferation of collagenic fibers (Barrett, 1999; Hamilton, 2008; Hubbard, 2001; Porter, 2002). Silica particles are released from alveolar macrophages and the similar cellular reactions described above by newly-recognizing nearby macrophages will be repeated. Finally, silica particles are transferred to regional lymph nodes. As these cellular and molecular reactions are continuously repeated, pulmonary fibrosis will gradually and progressively appear.

Even though details of these initial biological sequential reactions are recognized, it is still unclear how silica causes dysregulation of autoimmunity. From this viewpoint, we have been investigating the following perspectives:

1. Alteration of Fas and related molecules to affect long-term survival of lymphocytes.
2. Chronic activation of Tresp exposed to silica particles.
3. Alteration of Treg function and/or numbers exposed to silica particles.

In this chapter, we describe and summarize our experimental findings regarding the above three viewpoints, and insights concerning silica-induced dysregulation of autoimmunity will be discussed. Investigation using patient materials such as serum and lymphocytes were approved by the Institutional Ethics Committee of Kawasaki Medical School, Kusaka Hospital or Hinase-Urakami lin. The specimens were only obtained from patients who gave documented informed consent. All of the patients were Japanese brickyard workers in Bizen City (Okayama prefecture, Japan), and were monitored at either Kusaka Hospital or the Hinase-Urakami Clinic. The silica in materials handled by these workers (e.g., dirt, sand, mud, concrete), and thus presenting the potential risk of being inhaled by these individuals

in their work environment, was estimated to reach levels as high as 40–60% (by mass). The subjects were diagnosed with pneumoconiosis according to the ILO 2000 Guideline (ILO, 2004). These patients displayed neither clinical symptoms related to autoimmune diseases (e.g., sclerotic skin, Raynaud's phenomenon, facial erythema, or arthralgia) nor any cancers.

2. Alteration of Fas/CD95 and its related molecules in SILs

The discovery of Fas has led to a remarkable improvement in our understanding of apoptosis and its signal transduction (Matiba, 1997; Nagata, 1996; Nagata & Golstein, 1995). Abnormal regulation of apoptosis, particularly in relation to the Fas/Fas ligand (FasL) pathway, has been thought to play a role in the pathogenesis of autoimmune diseases (Eguchi, 2001; Rudin, 1996; Yonehara, 2002). Mutations of the *fas* gene and the *fas ligand* gene which lead to defects in apoptosis have been found in autoimmune strains of mice (*lpr* mice and *gld* mice, respectively) and human autoimmune lymphoproliferative syndrome (ALPS) in childhood (Nagata, 1998; Nagata & Suda, 1995; Mountz & Edwards, 1992; Steinberg, 1994). Fas/CD95, which is mainly expressed on the cell membrane of lymphocytes, usually exists as membrane-type Fas and forms a Fas-trimer after binding with FasL (Matiba, 1997; Nagata, 1996; Nagata & Golstein, 1995). The signal-transducing death domain located in the intracellular domain of Fas then recruits Fas-associated protein with Death Domain (FADD) and pro-caspase 8 to form the active death-inducing signaling complex (DISC) (Curtin & Cotter, 2003; Yu & Shi, 2008). Thereafter, activated caspase-8 triggers a caspase-cascade involving the activation of CAD/CPAN/DFF40 by removing its inhibitor, ICAD/DFF45, DNA fragmentation, and finally apoptotic cell death (Sabol, 1998; Sakahira, 1998).

The most typical alternatively spliced variant of the wild-type *fas* gene transcript is known as soluble Fas (sFas). Since this variant transcript lacks 63 bp of the transmembrane domain, its product (sFas) can be secreted from cells to suppress membrane Fas-mediated apoptosis by blocking the binding between membrane Fas and the FasL in the extracellular region (Matiba, 1997; Nagata, 1996; Nagata & Golstein, 1995). If there is a high level of sFas in the extracellular regions, lymphocytes in these regions may avoid apoptosis and survive longer. Actually, there have been several studies showing elevated serum levels of sFas in patients with autoimmune diseases (Cheng, 1994; Knipping, 1995; Tokano, 1996).

The following findings were obtained from our series of analyses of specimens from SILs. The detection of autoantibody to Fas and caspase-8, as well as topoisomerase I and desmoglein (Takata-Tomokuni, 2005; A. Ueki, 2001a, 2002; H. Ueki, 2001). Anti-Fas autoantibody detected in SILs was functionally active and caused Fas-mediated apoptosis (Takata-Tomokuni, 2005). The level of serum sFas was higher in SILs than healthy volunteers (HVs), although the level of serum soluble FasL did not differ between SILs and HVs (Tomokuni, 1997, 1999). The mean fluorescent intensity (MFI) of membrane Fas was lower with lymphocytes from SILs than those from HVs, although total numbers of Fas-positive lymphocytes (membrane Fas expression) did not differ between the two populations (Otsuki, 2005). The weaker membrane Fas expressers (among lymphocytes) were identified to be weaker *fas* message expressers (Otsuki, 2005). The gene expression levels of extracellular inhibitor competing membrane Fas-FasL binding such as sFas, decoy receptor 3 (DCR3), and other alternatively spliced variants of the *fas* gene were higher in peripheral blood mononuclear cells (PBMC) from SILs than HVs (Otsuki, 2000a, 2000b). The intracellular apoptosis-inhibitory genes including *i-flice*, *sentrin*, *survivine* and *icad* showed a lower expression in PBMC from SILs than HDs (Guo, 2001; Otsuki, 2000c).

Although significant mutations of *fas* and *fas ligand* genes were not detected, these results indicated that two populations of lymphocytes may exist in the peripheral blood of SILs. As shown on the right side of Fig. 1, one population is a weaker membrane Fas expresser and these cells may have developed out of an excessive transcription of the alternatively spliced *fas* gene and other variant messages. Therefore, these cells may be resistant to the functional anti-*fas* autoantibody, secrete higher levels of sFas, DCR3 and spliced variants, and are resistant to Fas-mediated apoptosis (Murakami, 2007, Otsuki, 2005, 2007). As reported previously (Otsuki, 2005), patients with a weaker MFI of membrane Fas often have a higher titer of anti-nuclear antibodies (ANA), and self-recognizing clones in silicosis may be included in the fraction because these clones may survive longer and show resistance to apoptosis.

The other population, shown on the left side of Fig. 1, represents stronger membrane Fas expressers that may be sensitive to Fas-mediated apoptosis including cell death caused by anti-Fas autoantibody, show a reduced expression of intracellular inhibitor genes of Fas-mediated apoptosis, and undergo apoptosis. These cells may be recruited from bone marrow after reaching the final stage of cell death. This recruited fraction would not have encountered silica and would be sensitive to Fas-mediated apoptosis. As a result, cells in this fraction would be continuously undergoing renewal and then apoptosis (Murakami, 2007, Otsuki, 2005, 2007).

The overall findings support the supposition that the long-term surviving subpopulation of T cells may include self-recognizing clones. However, these results provide no evidence that the lymphocytes in SILs are activated continuously. Thus, an investigation that incorporates experimental and patient-oriented studies is required to observe the chronic activation of Tresp by silica.

3. Chronic activation of Tresp by exposure to silica

To investigate the hypothesis that silica chronically activates Tresp, we first examined the *in vitro* activation of Tresp by exposure to silica (Wu, 2005). Freshly isolated PBMCs from HVs were cultured with or without phytohaemagglutinin (PHA), Min-U-silica (25 or 50 µg/ml) or chrysotile A (an asbestos, 50 µg/ml) for ten days. The expression of CD69 was used as the marker for early activation of T cells. Results showed that only silica can upregulate CD69 expression in T cells slowly and gradually in a dose-dependent manner in regard to cell surface expression (as shown in Fig. 2-A) and the message level (Wu, 2005). Although the data is not shown here, it was evident that PHA can stimulate T cells and that CD69 expression was observed at day 1 as the peak and then gradually reduced until day 5 (Wu, 2005). Additionally, chrysotile A was not able to induce CD69 expression (Wu, 2005). In this study, the necessity of the existence of phagocytosed cells in contact with lymphocytes was also found, and soluble factors secreted from phagocytosed cells contributed to approximately half of the induced CD69 expression in T cells (Wu, 2005). These results indicated the importance of the NLRP3 inflammasome in these experimental situations. Moreover, if Tresp in SILs encounter silica at the pulmonary circulation and also regional lymph nodes where silica is accumulated after it is handled by alveolar macrophages, they can be exposed to silica chronically and recurrently. In view of this consideration, the activation of Tresp in circulating peripheral blood Tresp and collateral evidence of Tresp activation were then investigated.

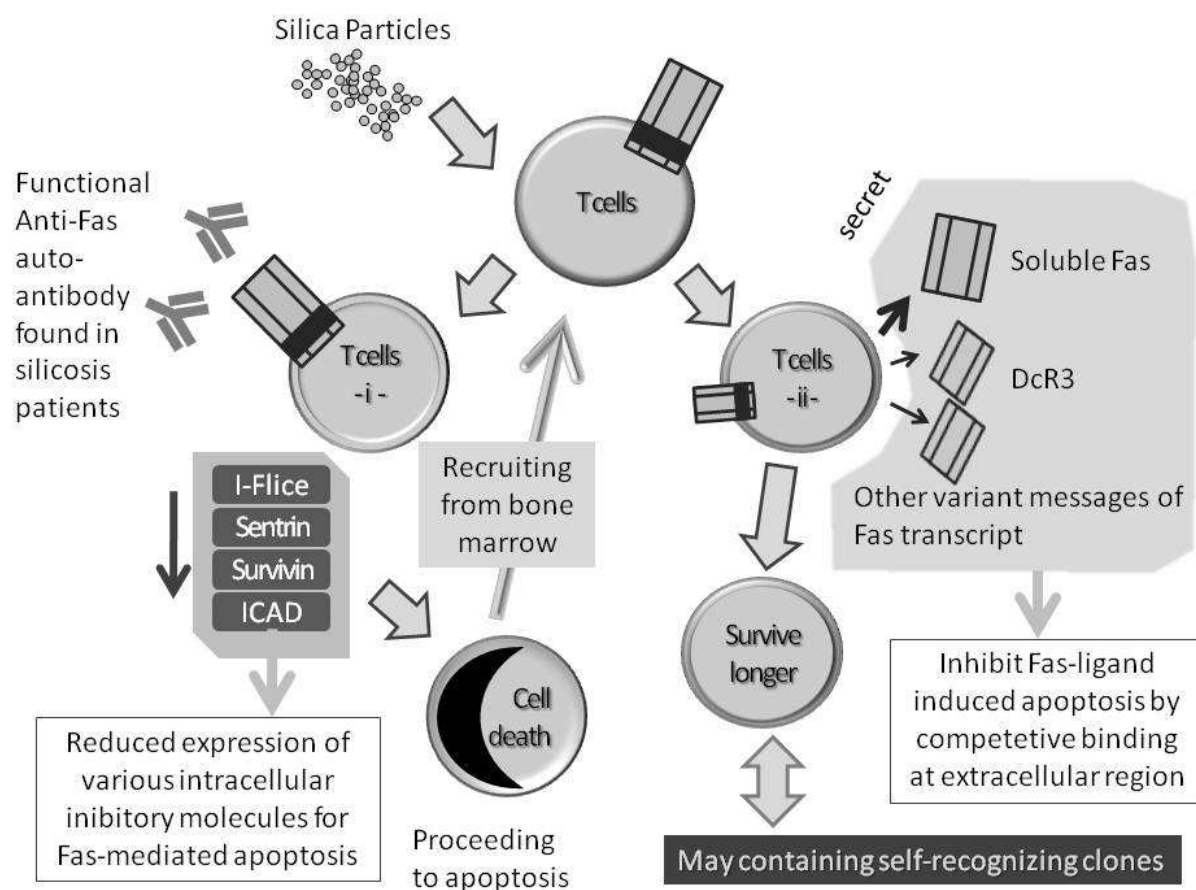


Fig. 1. Schematic model of the dysregulation of Fas and Fas-related molecules found in patients with silicosis. Two groups (temporarily designated T cell - i - and - ii -) may exist among lymphocytes from these patients: a population repeatedly undergoing apoptosis caused by silica and recruited from bone marrow, and another population surviving in the long term by avoiding apoptosis due to self-producing inhibitory molecules such as soluble Fas that may include self-recognizing clones.

With the recent recognition of Treg, most of the peripheral CD4+25+ T cells, particularly the higher expresser of CD25, are considered as Treg (Baecher-Allan, 2004; Bluestone & Tang, 2005; Schwartz, 2005). However, activated Tresp also express CD25 on their surface. Although Treg is defined in regard to the nuclear forkhead box P3 (FoxP3) gene as the master gene of Treg to manifest Treg function in order to inhibit the Tresp activation response against auto, foreign, cancerous and transplanted antigens (Baecher-Allan, 2004; Bluestone & Tang, 2005; Schwartz, 2005), observation of FoxP3 expression by flow cytometry requires the permeabilization of cell surface and nuclear membranes. This procedure is not suitable for subsequent biological examinations using sorted cells. Thus, in the following experiments, CD4+25+ cells were sorted to examine gene expression and the inhibitory function of the fraction.

As the marker for activation, we again used CD69 as an early activation marker and programmed cell death-1 (PD-1) genes (Saresella, 2008; Wang, 2009). Peripheral blood CD4+25- and CD4+25+ cells derived from HVs or SILs were collected by flow cytometry and relative gene expressions of CD69 and PD-1 were analyzed by real-time RT-PCR in

comparison to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression (Hayashi, 2010). As shown in Fig. 2-B, the CD4+25- fraction from both HVs and SILs revealed a higher expression of CD69 than CD25+ cells. In addition, CD69 expression in the CD25- fraction of SILs was significantly higher than that of HVs. Furthermore, as shown in Fig. 2-C, the expression of PD-1 was higher in the CD25- and CD25+ fractions of SILs than HVs. These findings supported the view that Tresp in SILs were chronically and recurrently activated and possessed long-term survival. Since CD69 expression was limited in the early stage of T cell activation, it is significant that the CD25+ fraction from both populations showed lower expression. However, both the CD25- and CD25+ fractions showed a higher expression of

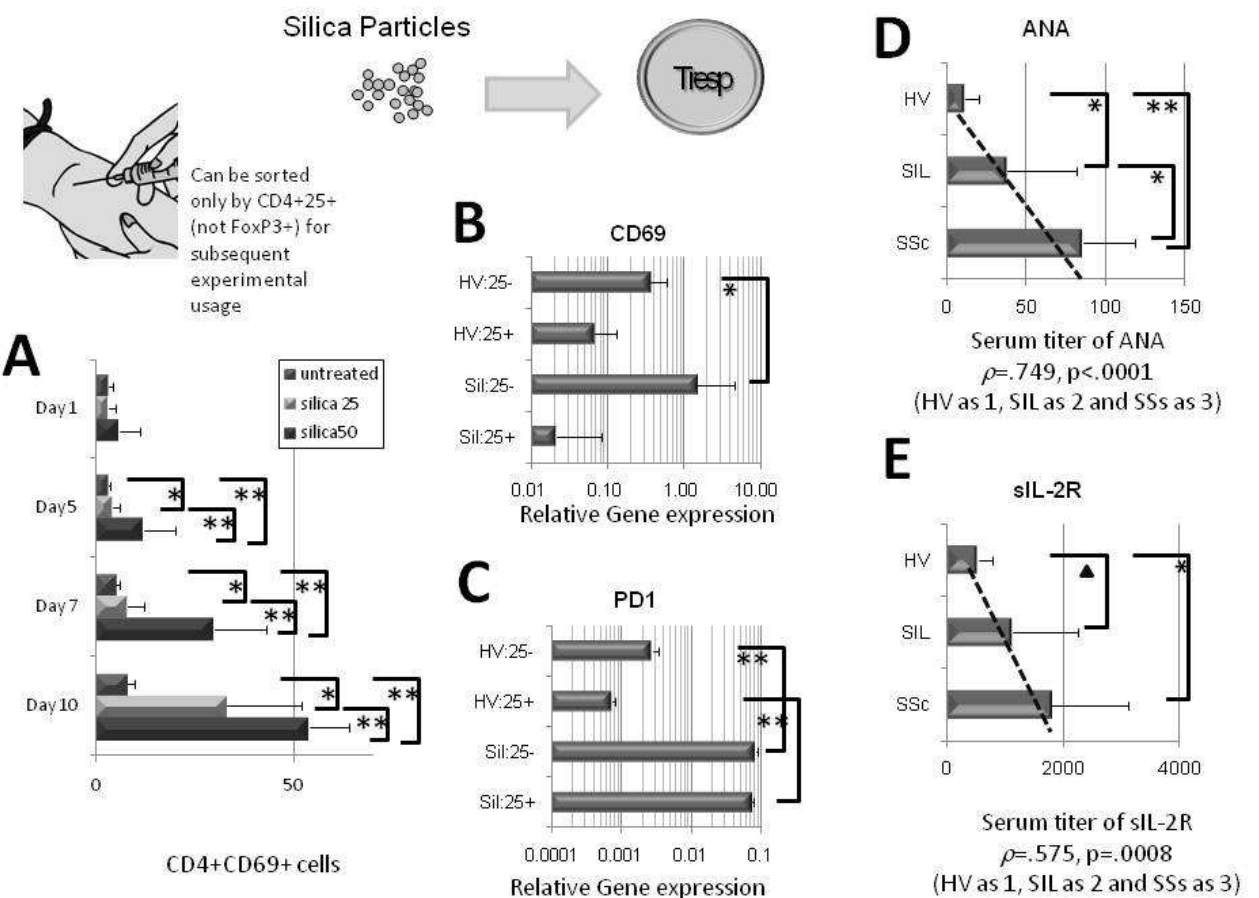


Fig. 2. Various examinations to recognize the effects of silica exposure on responder T cells (Tresp). * : $p<0.05$, ** <0.01 and \blacktriangle : $0.05<p<0.1$. [A] Peripheral blood mononuclear cells from healthy volunteers (HVs) were incubated with or without silica particles (25 or 50 $\mu\text{g}/\text{ml}$) for ten days. CD69 expression in CD4+ cells was analyzed by flow cytometry. [B] and [C] Peripheral blood CD4+25- and CD4+25+ cell fractions derived from HVs and silicosis patients (SILs) were sorted by flow cytometry, extracted total RNAs from individual fractions, and synthesized cDNA. Real-time RT-PCR analyses were employed to compare the gene expression of CD69 and PD-1, respectively. [D] and [E] Serum levels of the ANA titer and soluble IL-2 receptor (sIL-2R), respectively, were measured by ELISA methods and compared among HVs, SILs and patients with systemic sclerosis (SSc). In addition, after a numbered disease status set to 1 for HVs, 2 for SILs and 3 for SSc, correlations between disease status number and titers of ANA or sIL-2R were analyzed.

PD-1 (Hayashi, 2010). This may suggest that silica can activate both Tresp and Treg, and that the CD25⁺ fraction in SILs may include chronically activated Tresp in which surface CD25 expression occurred continuously due to recurrent stimulation by silica (Hayashi, 2010).

To investigate another marker of Tresp activation, we measured the serum soluble IL-2 receptor (sIL-2R) in SILs and compared results with those obtained from HVs and patients with SSc, since sIL-2R is known to arise in the serum of apparently healthy individuals who subclinically possess neoplastic (i.e., certain lymphoid malignancies such as T cell leukemia and early cell leukemia), autoimmune or inflammatory diseases (Carlson, 1992; Nelson & Willerford, 1998; Pizzolo, 1991; Rubin & Nelson, 1990; Zerler, 1991). The high-affinity IL-2R is a multichain receptor which possesses at least three IL-2 binding chains: IL-2R α /CD25 (55 kDa), IL-2R β /CD122 (75 kDa) and IL-2R γ /CD132 (64 kDa). sIL-2R is the naturally occurring soluble form of IL-2R α . For this analysis, the serum titer of anti-nuclear antigens (ANA) was measured in HVs, SILs and SSc using the Enzyme-Linked ImmunoSorbent Assay (ELISA)-based MESACUP ANA TEST (MBL Co. Ltd., Nagoya, Japan), which includes several recombinant proteins such as RNP, SS-A/Ro, SS-B/La, Scl-70, Jo-1 and Ribosomal P *in vitro* transcribed U1 RA and CENP-B protein, and purified antigen (Sm, SS-A/Ro, Scl-70m Histone and DNA) (Hayashi, 2009). As shown in Fig. 2-D, the ANA titer in SSc was the highest among the three groups, and significantly higher than that of HVs or SILs, whereas the ANA titer in SILs was also significantly higher than that of HVs. In addition, if disease status was numbered and set to 1 for HV, 2 for SIL and 3 for SSc, a significant positive correlation was obtained between the serum titer of ANA and disease status. Even our patients did not manifest any clinical symptoms for autoimmune diseases, and SILs subclinically tended to present a dysregulation of autoimmunity. Following these findings, serum sIL-2R was also analyzed in a manner similar to that used for the serum ANA titer. As shown in Fig. 2-E, SSc patients showed significantly higher serum sIL-2R than HVs or SILs, and the level shown by SILs tended to be higher than that shown by HVs. In addition, a significant positive correlation was detected between serum sIL-2R and disease status. These results suggest that sIL-2R may be used to detect immunological alteration in SILs, and that Tresp in SILs is activated chronically to an unknown higher level of sIL-2R (Hayashi, 2009).

4. Chronic activation of Treg by exposure to silica

As we have investigated Tresp activation in SILs as described above, the next point of interest was the function and activation of Treg. It has been revealed that CD4⁺CD25⁺ Treg contribute to maintaining self-tolerance by down-regulating the immune response to self and non-self antigens in an antigen-non-specific manner, presumably at the T cell activation stage (Baecher-Allan, 2004; Bluestone & Tang, 2005; Schwartz, 2005). Elimination and/or reduction of CD4⁺CD25⁺ T cells relieves this general suppression, thereby enhancing immune responses to non-self Ags and eliciting autoimmune responses to certain self-antigens. Recent studies have shown that CD4⁺CD25⁺ Treg specifically express transcription factor Foxp3 (Baecher-Allan, 2004; Bluestone & Tang, 2005; Schwartz, 2005). Genetic anomalies in Foxp3 cause autoimmune and inflammatory diseases in rodents and humans by affecting the development and function of CD4⁺CD25⁺ Treg (Baecher-Allan, 2004; Bluestone & Tang, 2005; Schwartz, 2005). Clinically, a deficiency in Treg function or decrease in the proportion of Treg has been shown to influence the pathogenesis of collagen or autoimmune diseases such as multiple sclerosis (O'Connor & Anderton, 2008), rheumatoid arthritis (Toh &

Miossec, 2007), systemic lupus erythematosus (Mudd, 2006), and pemphigus vulgaris (Yokoyama & Amagai, 2010). These findings at the cellular and molecular levels provide firm evidence that CD4+25+Foxp3+ Treg cells are an indispensable cellular constituent of the normal immune system, and that these cells play crucial roles in establishing and maintaining immunologic self-tolerance and immune homeostasis.

As mentioned above, CD25 molecules are also expressed on non-Treg subsets such as antigen-activated responder/effector T cells. Therefore, Foxp3 has been utilized as a useful marker to identify CD25+ regulatory T cells from CD25+ activated Tresp, although several distinguishable markers such as CD127 and PD-1 have been utilized to distinguish Treg from activated CD4+CD25+ Tresp (Liu, 2006; Hartigan-O'Connor, 2007; Saresella, 2008; Wang, 2009).

As described above, since the peripheral blood CD4+25+ fraction showed higher PD-1 expression (Fig. 2-C) and several findings demonstrate the chronic and recurrent activation of Tresp in SILs, the peripheral CD4+25+ fraction in SILs may be contaminated by these activated Tresp expressing CD25 on the base of Treg (Hayashi, 2010).

Thus, we first analyzed the function of the Treg fraction (actually, the peripheral CD4+25+ fraction sorted by flow cytometry in which Treg is mainly included and there is no availability of FoxP3-sorted cells for biological use as mentioned above) (Wu, 2006). As shown in Fig. 3-A, the inhibitory function of the CD4+25+ sorted fraction from SILs was lower than that of HVs when this fraction was added to the mixed lymphocyte culture (MLR) (Tresp was stimulated by irradiated allo-PBMCs) with the ratio 1:1/4 or 1:1/2, and tended to be lower when added with the ratio 1:1/8 or 1:1. There may be a reduced number of true Treg in the CD4+25+ fraction from SILs or an impaired function of true Treg (Wu, 2006). Taken together with the results of chronic and recurrent activation of Tresp in SILs (Hayashi, 2010; Wu, 2005), these findings support the possibility that the CD4+25+ fraction in SILs may include activated Tresp due to silica exposure. To examine this possibility, Treg-specific gene expression such as FoxP3 and cytotoxic T-lymphocyte antigen 4 (CTLA-4) was analyzed in CD4+25- and CD4+25+ fractions derived from HVs and SILs. As shown in Fig. 3-B and 3-C, the CD4+25+ fraction from SILs lost the dominant expression levels of both genes. These results suggested the CD4+25+ fraction in SILs was contaminated with chronically activated Tresp by exposure to silica (Hayashi, 2010; Wu, 2006). As expected and shown in Fig. 3-D, the percentage of the CD4+25+ fraction in peripheral lymphocytes was significantly smaller in HVs than SILs. Although the CD4+FoxP3+ fraction did not differ between HVs and SILs, the CD25+FoxP3- population was higher in SILs than HVs (Hayashi, 2010; Wu, 2006). These analyses indicated that the CD4+25+ fractions in SILs were contaminated by chronically activated Tresp due to exposure to silica (Hayashi, 2010; Wu, 2006). Although this may explain the results of reduced inhibitory function of the CD4+25+ fraction from SILs, there may be another possibility regarding the number of true Treg in SILs. Even the percentage of the CD4+FoxP3+ fraction did not differ between SILs and HVs, and a certain loss of Treg may occur, otherwise the reduced inhibitory function may not be fully explained.

We again take an interest with the Fas/CD95 molecule. As Tresp upregulated its CD25 expression due to chronic exposure to silica, Treg may have excess expression of Fas/CD95 because it has been shown that Treg expresses Fas/CD95 and is more sensitive to Fas-mediated apoptosis than Tresp (Fritzsching, 2005, 2006). To investigate this possibility, peripheral blood mononuclear cells from HVs and SILs were stained with CD4, CD25,

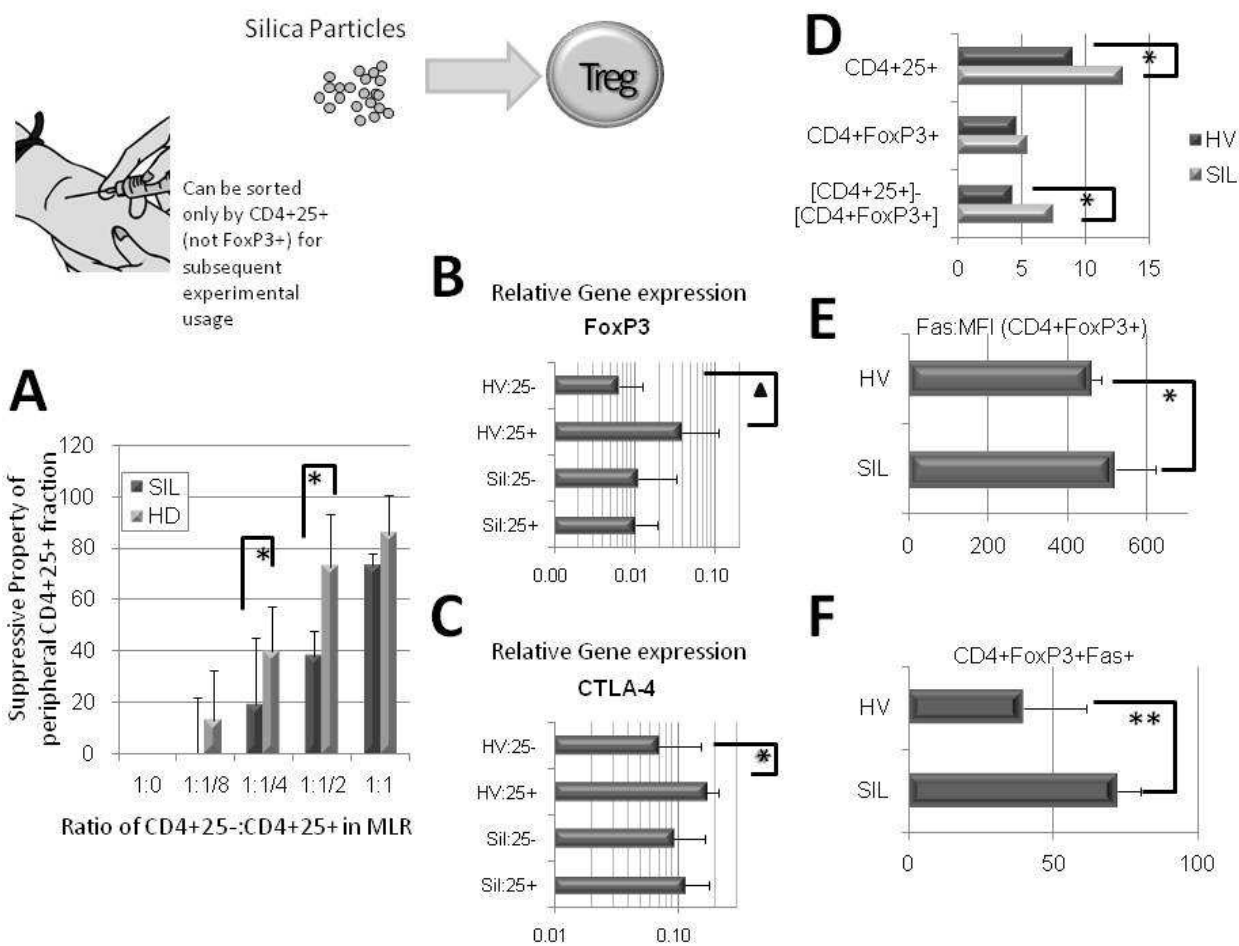


Fig. 3. Various examinations to recognize the effects of silica exposure on regulatory T cells (Treg). * : $p < 0.05$, * * < 0.01 and \blacktriangle : $0.05 < p < 0.1$. [A] The CD4+25- and CD4+25+ fractions from healthy volunteers (HVs) and silicosis patients (SILs) were collected by flow cytometry. CD4+25- cells with or without various ratios of CD4+25+ cells, such as 1:0, 1:1/8, 1:1/4, 1:1/2 and 1:1, were applied to a mixed lymphocyte reaction (MLR). Allogenic irradiated peripheral blood mononuclear cells were used as a stimulator. Graphs express suppressive properties of added CD4+25+ fractions. The degree to which the added CD4+25+ fraction reduced Cd4+25-DNA synthesis was measured by the ^3H -thymidine incorporation assay. [B] and [C] Peripheral blood CD4+25- and CD4+25+ cell fractions derived from HVs and SILs were sorted by flow cytometry, extracted total RNAs from individual fractions, and synthesized cDNA. Real-time RT-PCR analyses were employed to compare the gene expression of FoxP3 and CTLA-4, respectively. [D] Peripheral blood CD4+25+ and CD4+FoxP2+ populations were compared between HVs and SILs. [E] and [F] Peripheral blood CD4+FoxP3+ cells derived from HVs and SILs were compared in regard to CD95/Fas expression by means of fluorescent intensity and positive cell percentage, respectively.

CD95/Fas and FoxP3, and CD95/Fas expression (MFI) and positive cell frequency were analyzed in the CD4+FoxP3+ cell fraction. As shown in Fig. 3-E (MFI) and 3-F (positive cell frequency), Treg from SILs showed significantly higher expression levels of CD95/Fas than those from HVs. In addition, CD4+25+ cells from SILs were significantly more sensitive against Fas-mediated apoptosis inducing monoclonal antibody (CH-11) than those from HVs (data not shown), and proceeded faster to apoptosis as previously reported (Hayashi,

2010). All of these findings indicate that Treg may lose its true Treg ability due to chronic activation of Treg by recurrent exposure to silica mediated by excess expression of Fas/CD95 on the Treg cell surface.

5. Silica-induced dysfunction of the Treg fraction in SILs

Our results and those of our previous findings suggest that silica can reconstitute the peripheral CD4+CD25+ fraction to facilitate a decline in the number and function of Treg by the activation of both Tresp and Treg cells (Hayashi, 2010; Maeda, 2010), as outlined in Fig. 4.

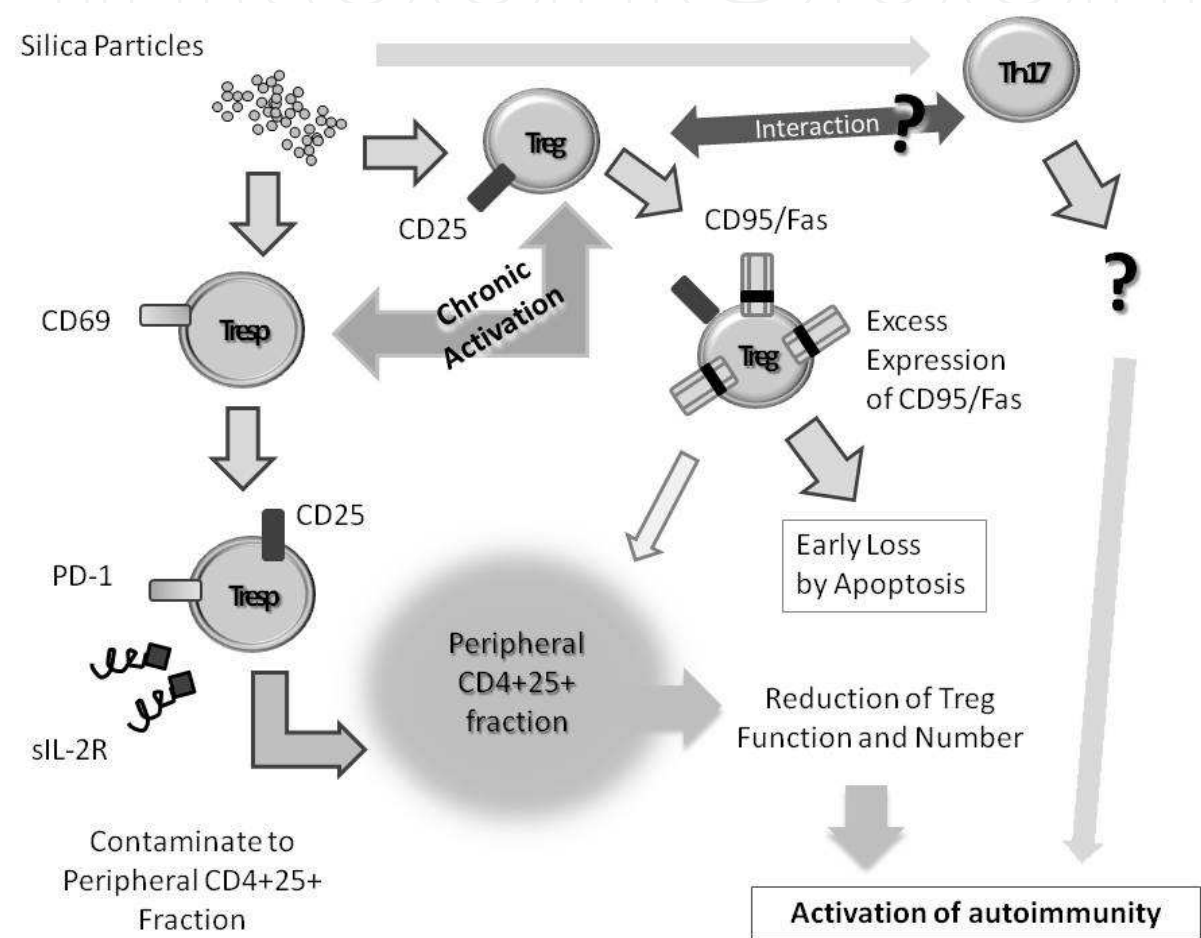


Fig. 4. Schematic representation of the immunological effects of silica exposure on alteration of autoimmunity. Silica chronically activates CD4+FoxP3 T cells (Treg), resulting in the induction of higher Fas expression. This up-regulated Fas marks Treg for Fas-mediated apoptosis. However, silica induces the change of CD4+FoxP3- T cells (Tresp) to CD4+25+FoxP3- activated Tresp. This population contaminates the peripheral CD4+25+ fraction in which Treg should be located. This imbalance between a decreased Treg and increased activated Tresp results in a dysfunction of the so-called CD4+25+ Treg fraction, which may trigger the occurrence of autoimmune diseases such as SSc. However, the roles and alterations of Th17 in silica-exposed patients are unknown and should be clarified through further research in order to obtain a better understanding of the immunological effects of silica on the human immune system.

Many issues remain to be resolved, such as delineating the complications of SSc in SILs (Barnadas, 1986; Cowie, 1987; Haustein, 1990; Haustein & Anderegg, 1998; Sluis-Cremer, 1985), or those complications associated with malignant tumors such as mesothelioma and lung cancer in patients exposed to the mineral silicate asbestos (Greillier, 2008; Toyokuni, 2009; Miura, 2008). Regarding the relationship between tumor immunity and Treg function, it may be that Treg enhances cell numbers or function to reduce tumor immunity (Chattopadhyay, 2005; Danese & Rutella, 2007; Kretschmer, 2006). If this is the case, future investigations will need to determine whether silica and asbestos possess opposite effects on Treg. Furthermore, specific parameters will need to be examined such as the degree of silica exposure, the progression of respiratory diseases (Otsuki, 1999), and identification of a possible individual factor such as the HLA type (A. Ueki, 2001b) that leads to the development of autoimmune complications in SILs.

In addition, the recent discovery of T helper type 17 cells (Th17) has contributed to the recognition of the occurrence of autoimmunity (Afzali, 2007; Awasthi & Kuchroo, 2009; Harrington, 2006; Jin, 2008; Louten, 2009; Stockinger, 2007). Research on the biology of Th17 cells suggests a critical role for Th17 in the development of inflammatory and autoimmune diseases. Furthermore, Th17 has been shown to interact with Treg cells (Afzali, 2007; Awasthi & Kuchroo, 2009; Harrington, 2006; Jin, 2008; Louten, 2009; Stockinger, 2007). TGF- β not only regulates the generation of Foxp3⁺ Treg cells, but together with IL-6 initiates Th17 differentiation. A reciprocal relationship between Th17 and Treg development has been proposed, since the generation of Foxp3⁺ Treg cells and Th17 cells both require TGF- β signaling. If the frequencies of Th17 and Treg were regulated by each other, silica-induced early loss of Treg may have an inverse effect by increasing the Th17 population, and represents another way to induce dysregulation of autoimmunity in SILs. Although we have just begun to investigate the status of Th17 in SILs, this is another important and critical issue to be resolved for a better understanding of environmental disturbance of autoimmunity such as that involving silica-induced autoimmune diseases (Shanklin & Smalley, 1998; Steenland & Goldsmith, 1995; Uber & McReynolds, 1982).

In the future, a comprehensive understanding of the immunological effects of silica may lead to the discovery of preventive and therapeutic molecular targets for autoimmune diseases, and will help to clarify the pathophysiological mechanisms involved in the development of dysregulation of autoimmunity.

6. Acknowledgments

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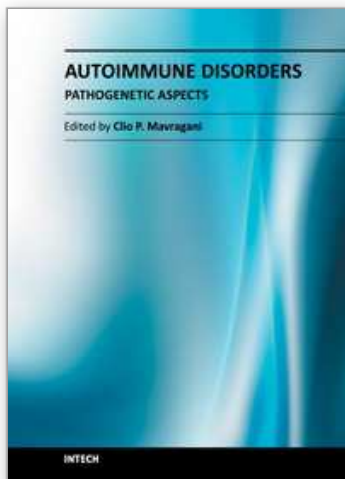
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