Keynote Lecture in the 13th Japanese Society of Immunotoxicology (JSIT 2006)

− Pathophysiological Development and Immunotoxicology: what we have found from the researches related to the silica and silicate such as asbestos −

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THE REPORT FROM THE 13TH ANNUAL MEETING OF THE JAPANESE SOCIETY OF IMMUNOTOXICOLOGY (JSIT 2006)

On September 14 and 15, 2006, the 13th annual meeting of the Japanese Society of Immunotoxicology (JSIT) was held in Kurashiki Geibun-kan, in Kurashiki city, Okayama Prefecture, Japan[1] (Fig. 1).

The JSIT started its activity in 1993 to explore the mechanisms of immunotoxicity caused by newly developed drugs, environmental substances and to develop strategies against impairment from immunotoxicity. The members are mainly scientists in research institutes of pharmacotherapeutic companies, national and independent administrative agencies, and departments of medical and pharmacological universities having an interest in these subjects.

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Fig. 1. Photographs from the 13th Annual Meeting of the JSIT. (A) Official poster, (B) Main Hall, (C) Poster Sessions, (D) Prof. Newman presenting his lecture, (E) Prof. Regal making a brief speech at the party, (F) Prof. Ueki's complimentary speech, (G) Prof. Otsuki singing his original "Song for the 13th JSIT" with accompaniment on the violins by Dr. and Mrs. Okamoto, (H) ~ (K) With extreme appreciation to all the staff members from the president

The 13th annual meeting was held with the sub-theme "Pathophysiological Development and Immunotoxicology".

During the two-day meeting, one keynote speech, three special lectures and two invited talks by specialists, one symposium, one workshop, two luncheon seminars and 29 general presentations were presented.

The symposium was entitled "Fibrous- and Nano-Materials and Immunotoxicological Researches" with four speakers organized by Dr. Seishiro Hirano (Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Japan) and Dr. Yasumitsu Nishimura (Department of Hygiene, Kawasaki Medical School, Kurashiki, Japan). Prof. Yasuo Morimoto (Department of Occupational Pneumology, Institute of Industrial Ecological Science, University of Occupational and Environmental Health, Kitakyushu, Japan) talked about "Factors in Hazard Evaluation of Fibrous Materials". Dr. Yasumitsu Nishimura focused on "Functional Alteration of Natural Killer Cells Exposed to Asbestos over s Long Period"; Dr. Akiko Furuyama (Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba, Japan) talked about "Transport of Nanoparticles through Aleveolo-Capillary Barrier"; and Prof. Masahiro Murakami (Faculty of Pharmacy, Osaka Ohtani University, Tondabayashi, Japan) made a presentation about "Nanoparticles as Drug Delivery System: Issues and Perspectives".

The workshop organized by Dr. Jun-ichi Sawada (Division of Biochemistry and Immunochemistry, The National Institute of Health Science, Tokyo, Japan) and Dr. Naohisa Tsutsui (Toxicology Laboratories, Pharmaceuticals Research Department, Mistubishi Pharma Corporation, Chiba, Japan) was entitled "A Guideline to Immunotoxicity Studies of Human Pharmaceutics". Five speakers presented their data and explained the guideline. Dr. Jun-ichi Sawada briefly introduced "ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) S8 Immunotoxicity Guideline"; Dr. Toshio Imai (Division of Pathology, National Institute of Health Science, Tokyo, Japan) and his colleague talked about "Pathology of Lymph Nodes and Peyer's Patches"; Dr. Shigeru Hisada (Safety Research Department, ASKA Pharmaceutical Co. Ltd., Kawasaki, Japan) focused his talk on "Interpretation of Stress-Related Changes"; Dr. Osamu Fueki (Office of New Drug I, Pharmaceuticals and Medical Devices Agency, Tokyo, Japan) and Dr. Tsutsui explained their "Q & A" format lecture about "Explanation of ICH S8 guideline". In addition, the invited speaker for this workshop, Dr. André H Penninks (TNO Quality of Life Product Manager, Experimental Immunology, AJ Zeist, The Netherlands) gave his lecture as in the name of the IILP group investigators and his talk was entitled "The Immunotoxicology Inter-Laboratory Project (IILP): The Validation of a T cell-Dependent Antibody Response (TDAR) using Keyhole Limpet Hemocyanin (KLH) in Rats".

The two luncheon seminars were "Immunotoxicology Assessment" by Dr. Lawrence Jacob (Molecular Biology and Immunology, Central Laboratory, Charles River Laboratories Preclinical Services, Edinburgh, Scotland, UK) and "*In Vitro and In Vivo* Predeiction of Monoclonal Antibody Cytokine Release Syndrome in Man" by Dr. Mark Wing (Huntington Life Science Ltd., UK).

The first special lecture was presented by Prof. Lee Newman (University of Colorado at Denver and Health Science Center, Denver, Colorado, USA). His talk was entitled "Immunotoxicology of Berylium Lung Disease" and the lecture was extremely exciting showing molecular approaches of the interaction between beryllium and immunocompetent cells such as T cells and antigen-presenting cells.

Prof. Jean Regal (Biochemistry and Molecular Biology, University of Minnesota Medical School,

Duluth, Minnesota, USA) presented her talk entitled "Models and Mechanisms of Chemical Respiratory Allergy: not all allergens are equal" as the second special lecture. She was invited to this meeting by as part of the scientific collaboration between the Immunotoxicology Specialty Section of the Society of Toxicology (SOT) in the USA and JSIT. Her lecture was also very interesting with a molecular approach for seeking and ranking genes related to the occurrence of respiratory allergy.

Two invited lectures were presented. The first was by Dr. Hoyroyuki Takano (Head, Environmental Health Sciences Division, National Institute for Environmental Studies, Tsukuba, Japan) and he talked about "Inflammation and Environmental Factors". The second was given by Prof. Kou Sakabe (Department of Public Health and Molecular Toxicology, Kitasato University School of Pharmaceutical Sciences, Environmental Medical Center, The Kitasato Institute, Tokyo, Japan) who focused on "Sick House Syndrome Research 2006". Both lectures were quite educational and all the audience had learned about recent advances in researches of these themes.

I, Prof. Takemi Otsuki, presented the Keynote Speech as the first program of the two-day meeting, and I talked about "Pathophysiological Development and Immunotoxicology: what we have found from researches related to silica and silicate such as asbestos". Here we describe our present summary of the immunological effects of silica and asbestos.

The JSIT are welcomed to our society by the many members of the Japanese Society of Hygiene who are interested in the immunological effects of environmental substances such as air pollutants, foods, drugs and others. The 14th annual meeting of the JSIT will be held on September 20-21, 2007, in Kobe. If you are interested in JIST, please feel free to contact me by e-mail (takemi@med.kawasaki-m.ac.jp), and I will be very happy to explain the JSIT.

KEYNOTE LECTURE

INTRODUCTION

Patients with silicosis who have been exposed to natural crystalline silica (SiO_2) are known to not only have pulmonary disorders but also immunological complications such as rheumatic arthritis (known as Caplan syndrome), systemic sclerosis (SSc), and systemic lupus erythematoses (SLE)[2) ~7)]. In addition, there are other epidemiological findings which indicate that exposure to silica-related compounds affect autoimmunity. Patients who received plastic surgery with implants containing silicone ($[SiO_2\text{-}O\text{-}]_n$) also show frequent complications of autoimmune disorders[8) ~11)]. This accumulation of findings clearly indicates that crystalline silica causes dysregulation and/or disturbance of the human immune system, particularly autoimmunity. In addition, asbestos, which is categorized as a silicate (mineralogical complexes containing metals, such as iron and magnesium, including chrysotile, crocidolite, and amosite), is known to cause malignant tumors[12) ~15)]. Some occurrence of these malignancies may be considered the result of a decline in tumor immunity due to exposure to asbestos of immuno-competent cells.

Silica and silicates may disturb immune functions such as autoimmunity and tumor immunity. Although silica-induced disorders of autoimmunity have been explained as adjuvant-type effects of silica, more precise analyses are needed and should reflect the recent progress in immunomolecular findings.

In this article, a brief summary of our investigations related to the immunological effects of silica/asbestos is presented. Details of each subject can be read in the references cited.

IMMUNOLOGICAL EFFECTS OF CHRYSOTILE, ASBESTOS

Asbestos (e.g. chrysotile, crocidolite, and amosite) is known to cause malignant lung cancer or mesothelioma[12) ~15)]. The International Agency for Research on Cancer (IARC) categorizes both asbestos and crystalline silica as group I carcinogens. According to the IARC classification, asbestos affects alveolar epithelial and mesothelial cells. There have been many studies concerning asbestos-induced apoptosis in these cells[16) ~24)]. Under experimental conditions, these cells undergo apoptosis upon relatively high-level, short-term exposure to asbestos as a result of the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) with activation of the mitochondrial apoptotic pathway. Furthermore, several non-small-cell lung cancer cell lines contain constitutively active signal transducer and activator of transcription 3 (STAT3)[25),26)]. It is also known that the inhibition of tumor-derived interleukin (IL)-10 and IL-10 receptor (IL-10R) interaction by an autocrine/paracrine loop results in a decrease of the constitutively active STAT3 and subsequent inhibition of Bcl-2 transcription and expression[27),28)]. Thus, it has been considered that during low-level, long-term exposure to asbestos, alveolar epithelial and mesothelial cells escape from the apoptotic pathway due to genetic changes and undergo malignant transformation.

We have also found that asbestos polyclonally activated CD4+ T cells and caused activation-induced cell death[29),30)]. In addition, peripheral blood mononuclear cells (PBMCs) from healthy donors (HD) exposed to asbestos in culture underwent apoptosis; however, many patients with asbestosis have had chronic occupational or other recurrent exposure to silicates. Therefore, there seems to be a need to develop an *in vitro* experimental model of chronic exposure to analyze the immunobiological effects of silicates during long-term exposure.

For this purpose, we employed a human T-cell leukemia virus type-1 (HTLV-1)- immortalized human polyclonal T cell line, MT-2, for the development of an *in vitro* model. Upon short-term, high-level exposure to chrysotile, MT-2 cells underwent apoptosis with the production of ROS via activation of the mitochondrial apoptotic pathway with the phosphorylation of p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal kinase (JNK) signaling molecules, shifting to a Bax-dominant Bax/Bcl-2 balance, release of cytochrome-c from mitochondria into cytosol, and activation of caspases 9 and 3 as shown on the left in Figure 2 and as previously reported[31)].

Next, we established a chrysotile-B (CB)-induced apoptosis-resistant subline of MT-2 (MT-2Rst), and characterized the cell biological differences between the original MT-2 cell line (MT-2Org) and MT-2Rst. The MT-2Rst cells were characterized by (i) enhanced expression of *bcl-2*, regaining of apoptosis sensitivity by the reduction of *bcl-2* by siRNA, (ii) excess IL-10 secretion and expression, and (iii) activation of STAT3 inhibited by 4-amino-5-(4- chlorophenyl)-7-(t-butyl) pyrazolol [3,4-d] pyrimidine (PP2), a specific inhibitor of Src family kinases. These results suggested that contact between cells and asbestos may affect the human immune system and trigger a cascade of biological events such as the activation of Src family kinases, enhancement of IL-10 expression, STAT3 activation and Bcl-2 overexpression as shown on the right in Figure 2 and as previously reported[32)]. This speculation was partially confirmed by the detection of elevated *bcl-2* expression levels in CD4+ peripheral blood T cells from patients with malignant mesothelioma compared with those from patients with asbestosis or HD[32)].

In addition, using the MT-2Rst cell line, the expression of the T cell receptor Vβ (TcRVβ) as described above, if asbestos possesses the super-antigenic potential to T cells, a certain number of various TcRVβ may be overexpressed without evidence of clonal expansion, as known when T cells are exposed to super-antigen

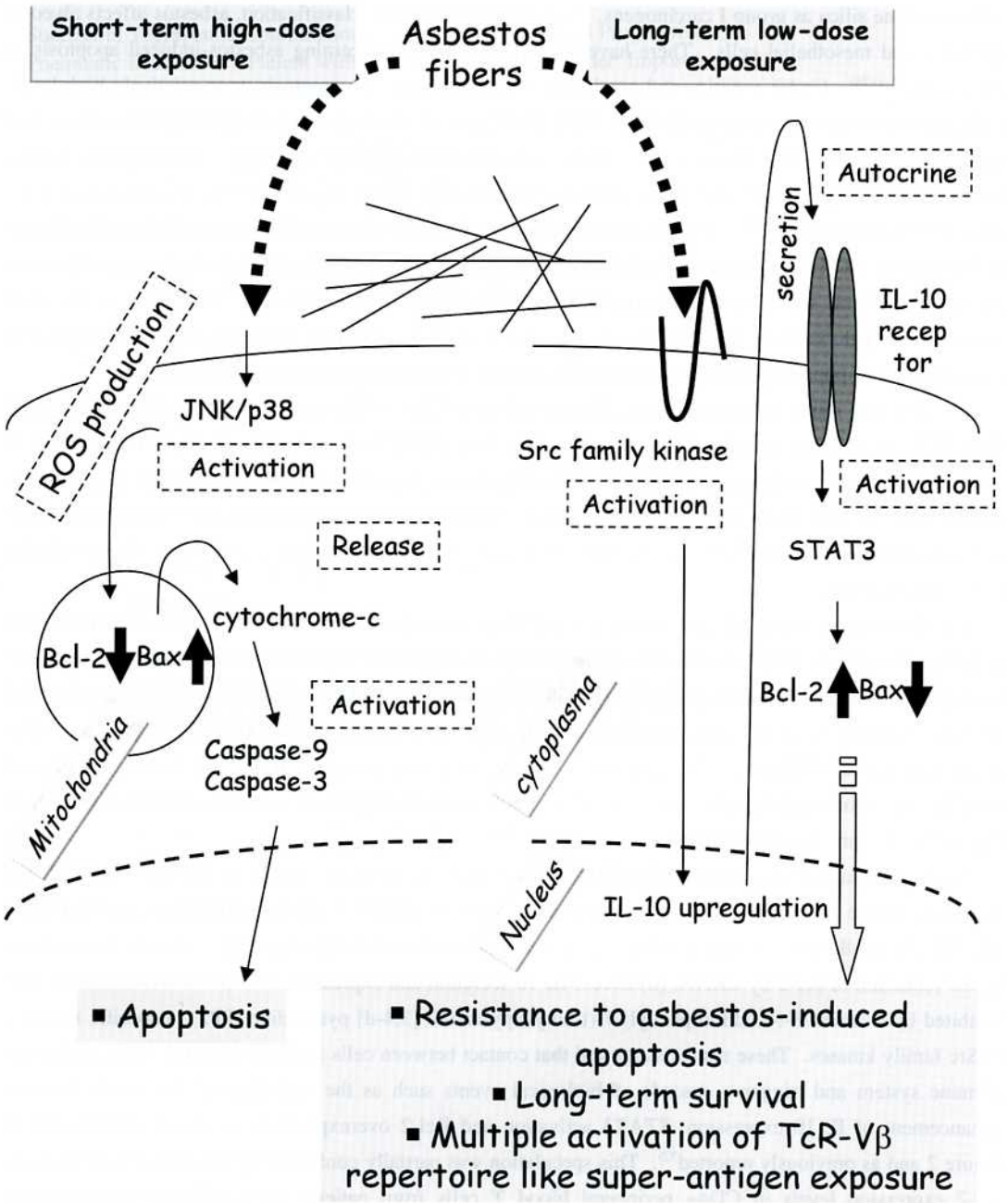

Fig. 2. Experimental findings of immunological effects of chrysotile, a form of asbestos, induced by short-term and high-dose exposure (left panel) or long-term and low-dose exposure using MT-2, an HTLV-1 immortalized human polyclonal T cell line.

such as staphylococcal enterotoxin B (SEB). In addition, 23 kinds of TcRVβ expression were examined in CD3+ peripheral blood T cells. As a result, MT-2Rst cells showed excess expression of various TcRVβ[33]. Although TcRVβ-overpresenting cells in MT-2Org were characterized as undergoing apoptosis due to first contact with crysotile, these cells in MT-2Rst had not significantly changed when they again contacted CB. These findings may be interpretered that the excess expression of various TcRVβ may be the remsult of contact between cells and CB, an asbestos fiber, during the acquisition of resistance to CB-induced apoptosis caused by long-term and low-dose exposure to CB. To support this interpretation, patients with asbestos-related diseases (ARD), such as asbestosis and malignant mesothelioma, were compared with silicosis patients (SIL) as a disease control and with HD. ARD patients showed a restricted overpresentation of TcRVβ without clonal expansion, whereas SIL revealed significant overpresentation of TcRVβ 7.2. These experimental and clinical analyses indicate the super-antigenic and dysregulation of the autoimmunity-inducing effects of asbestos and silica, respectively[33].

There are still many subjects to examine to obtain knowledge concerning the immunological effects of asbestos, particularly from the viewpoint of tumor immunity. Natural killer (NK) cells may be affected by exposure to asbestos, regulatory T cells (Treg), which regulate the auto-reaction, including tumor immunity, may change their function by exposure to asbestos. Immuno-competent cells may modify their characteristics not only with asbestos fibers *in vivo*, but also with malignant tumor cells such as mesothelioma cells. Thus, future investigations should be carried out and the discovery of biological tools to improve the prognosis of patients with asbestos-related malignancies in anticipated.

ALTERATIONS OF FAS-RELATED MOLECULES AND CD4+25+ REGULATORY T CELL FRACTION IN SILICOSIS PATIENTS

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 the Fas ligand. The signal-transducing death domain located in the intracellular domain of Fas then recruits FADD and pro-caspase 8 to form the active death-inducing signaling complex (DISC). 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[34) ~38].

The most typical alternatively spliced variant of the wild-type *fas* gene transcript is known as soluble *fas*. Since this variant transcript lacks 63 bp of the transmembrane domain, its product (soluble Fas) can be secreted from cells to suppress membrane Fas-mediated apoptosis by blocking the binding between membrane Fas and Fas ligand in the extracellular region[39),40)]. If there is a high level of soluble Fas 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 soluble Fas in patients with autoimmune diseases[41) ~44)]; therefore, we have compared the cellular and molecular changes of the levels of Fas and Fas-related molecules between SIL and HD:

I) Serum level of soluble Fas was higher in SIL than HD[45)].

II) Serum level of the soluble Fas ligand did not differ between SIL and HD[46)]. Although the Fas ligand is usually localized at the cell membrane on NK cells, activated T cells, and cytotoxic T cells, it is sometimes cleaved by matrix-metalloproteinase-like enzymes and secreted into extracellular spaces[47),48)].

III) Although the percentage of Fas-positive lymphocytes (membrane Fas expression) did not differ

between SIL and HD, the mean fluorescent intensity (MFI) of membrane Fas was lower in SIL than HD. In addition, the lower-membrane Fas expresser among lymphocytes was identified as the lower-*fas* message expresser[45),49)].

IV) Relative gene expression ratio of wild-type and soluble *fas* and various genes related to Fas-mediated apoptosis, such as the *decoy receptor 3 (dcr3)* gene, the apoptosis-accelerating genes *caspase-8, -3*, and *-9* and *cpan (cad)*, and the intracellular apoptosis-inhibitory genes *xiap, survivin, dff45 (icad), toso, i-flice*, and *s entrin*, in PBMCs was analyzed[50) ~53)].

DcR3 was initially discovered as a protein secreted from lung and colon cancer cells that prevents the Fas ligand from targeting them, and is also expressed on cytotoxic T cells and natural killer cells[54),55)]. Thus, DcR3 functions similarly to soluble Fas to inhibit membrane Fas-mediated apoptosis.

Then the findings were (i) soluble *fas* mRNA is dominantly expressed in PBMCs from SIL, but not in HD50), (ii) *dcr3* gene expression was higher in PBMCs from SIL than HD[52)] and this may induce the inhibition of Fas and Fas ligand binding similar to the higher presence of the soluble Fas molecule and (iii) the gene expression of intracellular inhibitors of Fas-mediated apoptosis such as *i-flice, sentrin, survivin*, and *icad* were lower in SIL than HD[52),53)].

V) Detection of alternatively spliced variants of *fas* and mutational screening of *fas* and *fas ligand* genes was performed[56)]. Although meaningful mutations in *fas* and *fas ligand* coding sequences were not detected, many alternative spliced variants were found and interpretation from the amino-acid translation from detected variants showed all of these as well as the typical soluble *fas* possessing the binding site of Fas ligand, but lacking the transmembrane domain and death domain. These findings indicate that all these variants may inhibit the binding between membrane Fas and Fas ligand similar to soluble Fas and DcR3 molecules[56)].

VI) Antibodies against Fas[57)] and caspase-8[58),59)] were found frequently in the serum of SIL. In addition, investigation of the function of the detected anti-Fas autoantibody showed autoantibody induces the Fas-mediated apoptosis of membrane Fas-expressing cells[57)].

VII) *In vitro* exposure to silica on T cells derived from HD causes the slow but precise activation of these cells when observed by CD69, a typical early marker of T cell activation, expression[60)].

VIII) The percentage of the peripheral blood CD4+25+ fraction, which includes CD4+25+FoxP3+ Treg suppressing excess auto-reaction in the scarce self-recognizing T cell fraction in peripheral blood, was slightly lower in SIL when compared with the age-predicted value calculated from the analysis of HD. In addition, the function of this fraction in SIL was lsee than that of HD when the function was examined by allo-reactive mixed lymphocyte reaction (MLR)[61)].

From these findings, a hypothesis for activated autoimmunity in SIL has been proposed as shown in Figure 3 and partially reported previously[49),62),63)]. The findings of the levels of factors in extracellular spaces, such as soluble Fas, DcR3, and the products from various alternatively spliced *fas* variants indicate that apoptosis mediated by membrane Fas seems to interfere with these molecules and is reduced. However, since there was a reduced expression of intracellular molecules for anti-Fas-mediated apoptosis such as *i-flice, sentrin* and *survivin* gene products in SIL compared with HD, it seemed likely that Fas-mediated apoptosis would be enhanced in the lymphocytes derived from SIL. In addition, the anti-Fas autoantibody found in serum from silicosis patients may contribute to the enhanced apoptosis of lymphocytes, because of the Fas-stimulating function of this antibody. Relative to HD, in which the apoptosis of lymphocytes is assumed to be neither enhanced nor reduced, it seemed that the two fractions of lymphocytes would respectively show

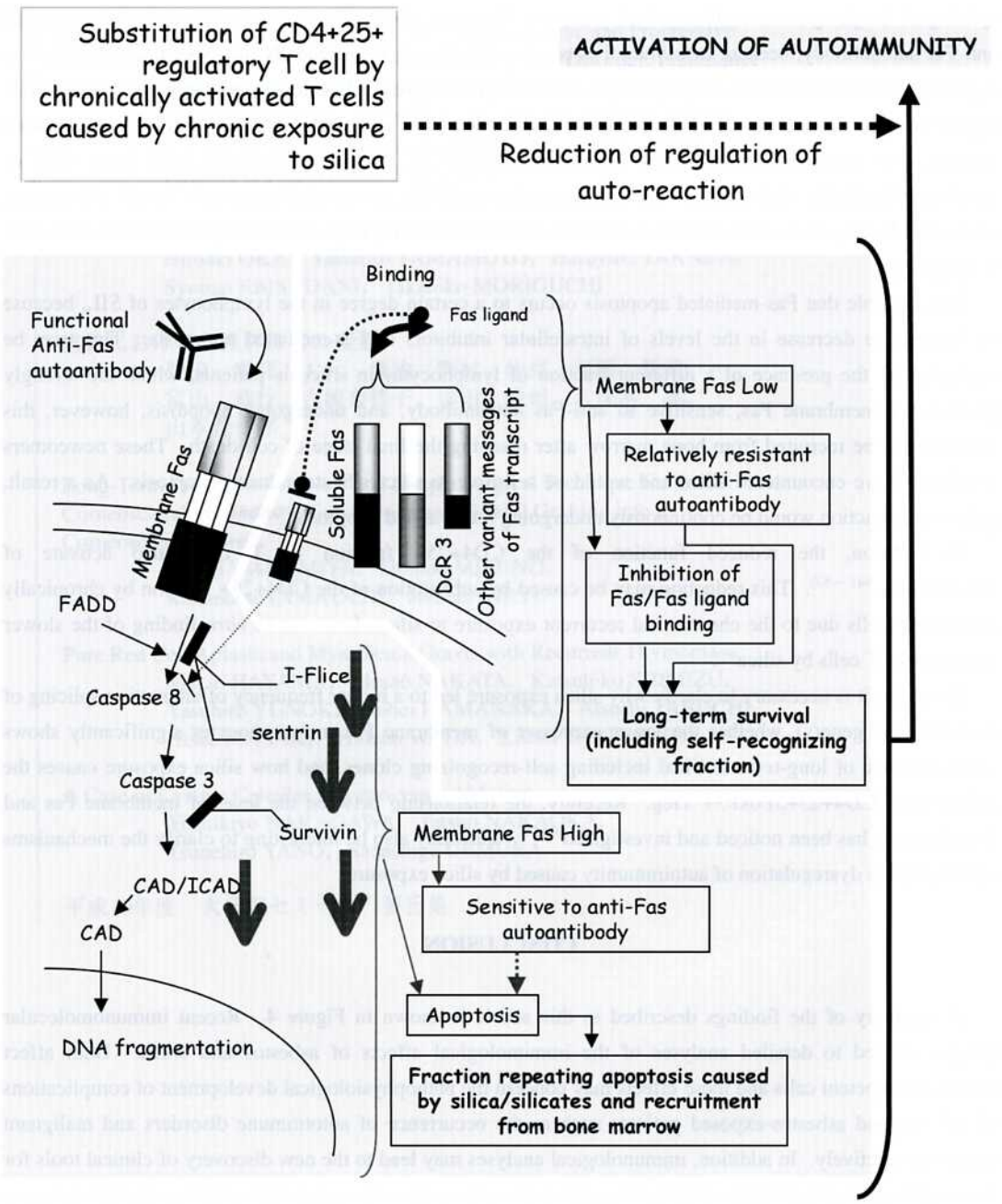

Fig. 3. Schematic presentation of the activation mechanisms of autoimmunity found in silicosis patients focusing on the alterations of Fas and Fas-related molecules, and reduced function of the CD4+25+ T cell fraction.

enhanced and reduced Fas-mediated apoptosis in the SIL.

Thus, there are two populations of CD4+ lymphocytes, a higher expresser of membrane Fas and a lower expresser of Fas, in SIL. The lower expressers may be developed by excess transcription of alternative splicing of the *fas* gene and other variant messages; therefore, these cells may be resistant to the functional anti-Fas autoantibody, because membrane Fas is relatively scarce. Consequently, it is speculated that there is a particular fraction of CD4+ T lymphocytes in SIL which expresses low levels of membrane Fas, secretes higher levels of soluble Fas, DcR3, and spliced variants, and is resistant to anti-Fas autoantibody-induced apoptosis, as shown in Figure 3 and previous reports[49),62),63)]. Since the patients with lower MFI of membrane Fas had a higher titer of anti-nuclear antigens (ANA), as reported previously[49)], self-recognizing clones in SIL may be included in this fraction, because these clones may survive longer and show resistance to apoptosis.

It is possible that Fas-mediated apoptosis occurs to a certain degree in the lymphocytes of SIL, because of the relative decrease in the levels of intracellular inhibitors of Fas-mediated apoptosis. This may be explained by the presence of a different fraction of lymphocytes in silicosis patients, which are strongly positive for membrane Fas, sensitive to anti-Fas autoantibody, and undergoing apoptosis; however, this fraction may be recruited from bone marrow after reaching the final stage of cell death. These newcomers would not have encountered silica and would be sensitive to silica/silicate-induced apoptosis. As a result, cells in this fraction would be continuously undergoing renewal and apoptosis[49),62),63)].

In addition, the reduced function of the CD4+25+ fraction of T cells also activate of autoimmunity[64) ~67)]. This reduction may be caused by substitution of the CD4+25+ fraction by chronically activated T cells due to the chronic and recurrent exposure to silica, from our *in vitro* finding of the slower activation of T cells by silica[60)].

However, it is necessary to clarify why silica exposure led to a higher frequency of alternative splicing of *fas* (or other) gene(s), whether the lower expresser of membrane Fas in lymphocytes significantly shows some evidence of long-term survival including self-recognizing clones, and how silica exposure causes the reduction of CD4+25+5FoxP3+ Treg. Recently, the relationship between the level of membrane Fas and Treg function has been noticed and investigated[68),69)]. This may also be interesting to clarify the mechanisms underlying the dysregulation of autoimmunity caused by silica exposure.

CONCLUSION

A summary of the findings described in this article is shown in Figure 4. Recent immunomolecular progression led to detailed analyses of the immunological effects of asbestos and silica. Both affect immuno-competent cells and these effects may concern the pathophysiological development of complications of silicosis and asbestos-exposed patients such as the occurrence of autoimmune disorders and malignant tumors, respectively. In addition, immunological analyses may lead to the new discovery of clinical tools for the modification of pathophysiological aspects of diseases such as the regulation of autoimmunity or tumor immunity using cell-mediated therapies, various cytokines and molecular targeting therapies. As asbestos-related malignancies are increasing and have been a medical and social problem since summer in 2005 in Japan, focused efforts should be performed to cure these diseases to remoce the nation-wide anxiety about these malignancies.

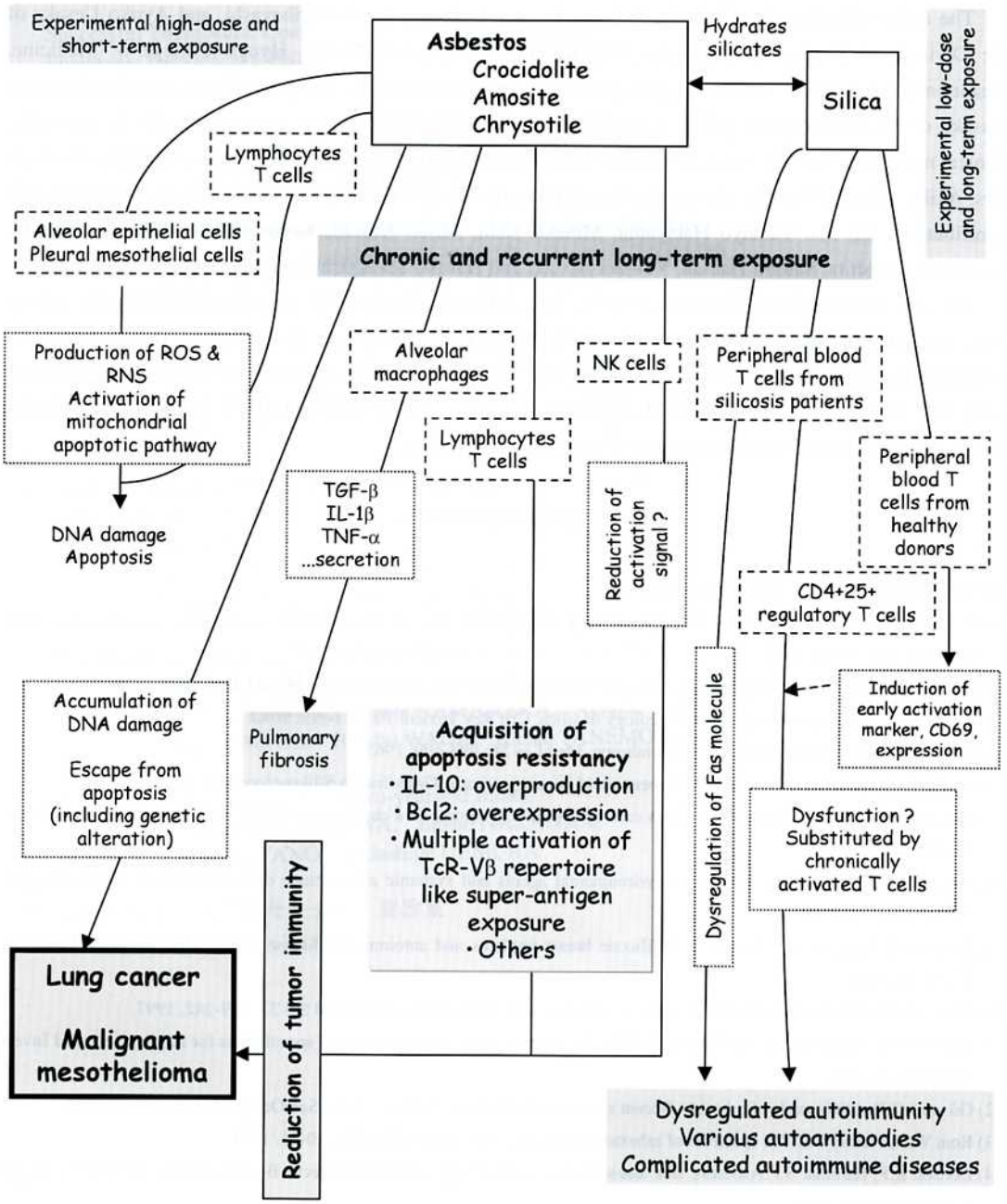

Fig. 4. Summary of immunological effects of silica/asbestos.

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