Induction of IL-17 production from human peripheral blood CD4+ cells by asbestos exposure

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Abstract. We have previously reported that chronic, recurrent and low-dose exposure to asbestos fibers causes a reduction in antitumor immunity. Investigation of natural killer (NK) cells using an in vitro cell line model and comprising in vitro activation using freshly isolated NK cells co-cultured with chrysotile fibers, as well as NK cells derived from asbestos-exposed patients with pleural plaque (PP) or malignant mesothelioma (MM), revealed decreased expression of NK cell activating receptors such as NKG2D, 2B4 and perforin, as well as suppressed proliferation of CTLs. Additionally, analysis of T helper cells showed that surface CXCR3, chemokine receptor, and the productive potential of interferon (IFN)γ were reduced following asbestos exposure in an in vitro cell line model and in peripheral CD4+ cells of asbestos-exposed patients. Moreover, experiments revealed that asbestos exposure enhanced regulatory T cell (Treg) function. This study also focused on CXCR3 expression and the Th-17 cell fraction. Following activation with T-cell receptor and co-culture with various concentrations of chrysotile fibers using freshly isolated CD4+ surface CXCR3 positive and negative fractions, the intracellular expression of CXCR3, IFNγ and IL-17 remained unchanged when co-cultured with chrysotile. However, subsequent re-stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin resulted in enhanced IL-17 production from human peripheral blood CD4+ cells by asbestos exposure. Asbestos and silica are the most well-known causative, environmental and occupational substances that induce pneumoconiosis (1-3). The initial event following entry of these substances into the human body involves the action of immune competent cells such as alveolar macrophages to treat these foreign materials by activating the NOD-like receptor family, pyrin domain containing 3 (NLRP3: NALP3) inflammasome to produce interleukin (IL)-1β and attract fibroblasts (4-6).

Since the immunological effects of asbestos fibers have not been thoroughly investigated, we decided to study the various effects of asbestos on human immune competent cells. Investigation of natural killer (NK) cells using in vitro chronic exposure in an NK cell line and freshly isolated NK cells derived from healthy volunteers (HV) showed a reduction in NK cell activating receptors such as NKG2D and 2B4, as well as intracellular granzyme B, and decreased phosphorylation of ERK (7,8). In addition to these findings, studies of peripheral blood NK cells from asbestos-exposed patients with pleural plaque (PP) or malignant mesothelioma (MM) showed decreased expression of NKP46 activating receptor (7-9). Furthermore, the differentiation and proliferation of CD8+ cytotoxic T lymphocytes (CTLs) were also impaired by...
asbestos exposure. The cytotoxicity for allogeneic targets decreased in peripheral blood mononuclear cells (PBMCs) exposed to chrysotile B (CB) asbestos, but not in cells exposed to crocidolite (CR) asbestos, when compared with PBMCs without exposure during a mixed lymphocyte reaction (MLR) (10). Exposure to CB during the MLR resulted in suppression of an increase in granulocyte B+ cells and interferon (IFN)γ positive cells. CB exposure also resulted in suppression of increases in CD45RO+ effector/memory cells and CD25+-activated cells in CD8+ lymphocytes, and a decrease in CD45RA+ cells. Furthermore, CB exposure suppressed the proliferation of CD8+ lymphocytes without yielding an increase in Annexin V+ apoptotic cells in CD8+ lymphocytes. Moreover, the production of IL-10, IFNγ and tumor necrosis factor (TNF)-α, but not IL-2, decreased in the presence of CB (10-13).

Our research also established a model involving chronic and continuous exposure to asbestos for the investigation of CD4+ T helper (Th) cells. Transient and relatively high-dose exposure to asbestos caused apoptosis in a human T-cell leukemia virus type (HTLV)-1 immortalized human polyclonal T-cell line, MT-2, by the production of reactive oxygen species (ROS). Activation of the pro-apoptotic MAK transduction signaling pathway such as p38 and JUN, and activation of the mitochondrial apoptotic pathway during continuous and relatively low-dose (in which the occurrence of apoptosis was <50%) exposure for more than one year resulted in MT-2 being more resistant to asbestos-induced apoptosis (14). Several independent sublines of MT-2 continuously exposed to asbestos showed upregulation of IL-10 and apoptosis (14). Several independent sublines of MT-2 continuously exposed to asbestos caused apoptosis in a human T-cell leukemia virus type (HTLV)-1 immortalized human polyclonal T-cell line, MT-2, by the production of reactive oxygen species (ROS). Activation of the pro-apoptotic MAK transduction signaling pathway such as p38 and JUN, and activation of the mitochondrial apoptotic pathway during continuous and relatively low-dose (in which the occurrence of apoptosis was <50%) exposure for more than one year resulted in MT-2 being more resistant to asbestos-induced apoptosis (14). Several independent sublines of MT-2 continuously exposed to asbestos showed upregulation of IL-10 and apoptosis (14). These results were also observed in CD4+ cells derived from patients with PP or MM (17,18). These findings were thought to be very important in assessing antitumor immunity in Th cells since CXCR3 expressing Th cells recruit IFNγ-producing antitumor Th into the area surrounding the tumor. However, the activity of both processes might be reduced in asbestos-exposed patients. Taken together, it might be of value to determine whether asbestos can induce inhibition of the cellular features of Th17 cells. The issue therefore is to determine the manner by which asbestos affects Th17 cells. In an effort to address this matter, freshly isolated human peripheral CD4+ cells were activated in vitro with chrysotile asbestos, since the use of chrysotile in industrial and commercial products is higher than that of amphibole asbestos, and the immunological effects were found to be similar for both forms of asbestos as we previously reported (32). We then investigated IL-17 expression and production in relation to CXCR3 expression. Since a reduction in CXCR3 expression was observed following asbestos exposure, the cellular roles of IL-17 production and expression in CXCR3 positive and negative cells were examined.

Materials and methods

Peripheral blood cell preparation and intracellular staining. Peripheral blood T cells were prepared from three HV. Peripheral blood was drawn from the vein with the aid of heparin. PBMCs were isolated using the Ficoll-Hypaque method. Cells were then stained with anti-CD4 monoclonal antibody (mAb) and anti-CXCR3 mAb. Both antibodies were purchased from BD Biosciences, Minneapolis, MN, USA. PBMCs stained by both antibodies were divided into two fractions comprising CD4+ and surface CXCR3 negative (CD4+CXCR3-) or positive (CD4+CXCR3+) cells (Fig. 1A). Cells in two fractions (CD4+CXCR3- or CD4+CXCR3+, 1x106 cells) were stimulated with 1 µg/ml of plate-coated anti-CD3 mAb and 1 µg/ml of soluble anti-CD28 mAb with 10 µg/ml of IL-2 for four weeks (Fig. 1B). These cultures received 0, 10, 25 or 50 µg/ml of chrysotile asbestos.

The chrysotile asbestos was removed following cultivation using the Ficoll-Hypaque method and intracellular expression of CXCR3, IFNγ and IL-17 was analyzed using mAbs for these molecules (BD Biosciences). Intracellular staining was performed using a Cytotix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer’s instructions. Cells were stained with individual mAbs for 30 min and then subjected to FACSCalibur flow cytometry.

Additionally, cells cultured with various concentrations of chrysotile were re-activated using phorbol 12-myristate 13-acetate (PMA) and ionomycin (IM) for 4 h. Human PBMCs were obtained for this study from H.V. who provided written informed consent according to guidelines established
Figure 1. (A) Representative flow cytometry analysis of peripheral blood mononuclear cells (PBMCs) (area P1). Cells were stained with anti-CD4 and anti-CXCR3 monoclonal antibodies, and CD4+ and surface CXCR3 negative (sCXCR3-) (area P3) or positive (sCXCR3+) (area P2) fractions were sorted by flow cytometry and then utilized for subsequent experiments. Cells in area P2 were not used for this analysis. (B) CD4+sCXCR3- and CD4+sCXCR3+ fractions were activated by anti-CD3 and anti-CD28 monoclonal antibodies with IL-2. In addition to this activation, cells were co-cultured with various concentrations of chrysotile asbestos fibers (0, 10, 25 or 50 µg/ml). These concentrations were compatible with our previous experiments which showed that transient and relatively high doses of chrysotile caused apoptosis in the human T-cell line. Following four weeks of cultivation and subsequent removal of chrysotile fibers, the intracellular expression of CXCR3, IFNγ and IL-17 was examined in cells from both fractions by flow cytometry. Additionally, cells from both fractions following activation and co-cultivation with chrysotile were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin (IM) for 4 h. Culture supernatants were then used for the measurement of IFNγ and IL-17. Cells were also analyzed for T-bet, IFNγ, RORγt and IL-17 mRNA expression.
Results

Intracellular CXCR3 expression following cultivation with chrysotile. CD4+ sCXCR3− and CD4+ sCXCR3+ cells were cultured in the presence of various concentrations of chrysotile (0, 10, 25 or 50 µg/ml) for four weeks with T-cell receptor (TCR) stimulation and IL-2. Following this activation period, intracellular CXCR3 expression was analyzed by flow cytometry (Fig. 2). The intracellular expression of CXCR3 was significantly higher in CD4+ sCXCR3+ cells compared with CD4+ sCXCR3− cells in both R1 (totally positive cell fraction) and R2 (highly positive fraction) regions. The results showed that although intracellular CXCR3 expression remained unaltered in cells derived from CD4+ sCXCR3− fractions, the presence of chrysotile tended to reduce the highly positive intracellular expression of CXCR3 in cells that were initially CD4+ sCXCR3−.

Intracellular IFNγ expression following cultivation with chrysotile. Levels of IFNγ were examined in CD4+ sCXCR3− and CD4+ sCXCR3+ fractions derived from cells cultured with various concentrations of chrysotile (Fig. 3). The intracellular expression of IFNγ was significantly higher in CD4+ sCXCR3+ cells compared with CD4+ sCXCR3− cells in both R1 (totally positive cell fraction) and R2 (highly positive fraction) regions. The results revealed that the initial CD4+ sCXCR3− cells tended to display reduced IFNγ expression when cells were co-cultured with relatively low doses of chrysotile, although exposure to chrysotile did not alter intracellular IFNγ expression in any of the three fractions.

Intracellular IL-17 expression following cultivation with chrysotile. Intracellular IL-17 expression was analyzed in CD4+ sCXCR3− and CD4+ sCXCR3+ fractions derived from cells following activation and cultivation with various concentrations of chrysotile (Fig. 4). The intracellular expression of IL-17 was significantly higher in CD4+ sCXCR3− cells compared with CD4+ sCXCR3+ cells in both R1 (totally positive cell fraction) and R2 (highly positive fraction) regions. The results showed that CD4+ fractions tended to display increased intracellular IL-17 expression, although there were no significant differences in intracellular IL-17 expression in any of the fractions.

IFNγ and IL-17 production following stimulation of cells cultured with chrysotile. Since analysis of the intracellular expression of IFNγ and IL-17 in cell fractions initially divided as CD4+ sCXCR3− and CD4+ sCXCR3+ following four weeks of cultivation with chrysotile asbestos did not show any marked alterations, these cells were re-stimulated by PMA and ionomycin for 4 h as shown in Fig. 1B. The concentration of IFNγ and IL-17 in culture supernatants was then measured.

As shown in Fig. 5, high levels of both IFNγ and IL-17 were found in CD4+ sCXCR3− fractions. In contrast, IFNγ production remained unaltered in cells cultured with chrysotile (Fig. 5A). However, IL-17 production was enhanced in cells
Figure 3. Cells from CD4+ sCXCR3- and CD4+sCXCR3+ fractions following activation and co-culture with chrysotile were analyzed for intracellular expression of IFNγ. Region 1 (R1) shows the total intracellular expression and region 2 (R2) indicates a highly positive fraction. (A) Actual intracellular IFNγ staining results of both CD4+sCXCR3 negative or positive fractions cultured in the absence or presence of chrysotile; (B) shows changes in the percentage of intracellular IFNγ positive cells. In both R1 and R2 regions, intracellular CXCR3 positive cells were higher in the CD4+sCXCR3 positive fraction compared with the CD4+sCXCR3 negative fraction, whereas there was no statistically significant difference between cells cultured in the absence or presence of chrysotile (three different concentrations). *Significant differences of <0.05. 'n.s.' Statistically not significant.

Figure 4. Cells from CD4+ sCXCR3- and CD4+sCXCR3+ fractions following activation and co-culture with chrysotile were analyzed for intracellular expression of IL-17. Region 1 (R1) shows total intracellular expression and region 2 (R2) indicates a highly positive fraction. (A) Actual intracellular IL-17 staining results of both CD4+sCXCR3 negative or positive fractions cultured in the absence or presence of chrysotile; (B) Changes in the percentage of intracellular IL-17 positive cells. In both R1 and R2 regions, intracellular CXCR3 positive cells were higher in the CD4+sCXCR3 positive fraction compared with the CD4+sCXCR3 negative fraction, whereas there was no statistically significant difference between cells cultured in the absence or presence of chrysotile (three different concentrations). *Significant differences of <0.05. 'n.s.' Statistically not significant.
cultured with chrysotile in a dose-dependent manner (Fig. 5B), although large variations were found.

**IFNγ, T-bet, RORγT and IL-17 mRNA expression.** Although IFNγ production remained unaltered in CD4+sCXCR3− and CD4+sCXCR3+ cells cultured with various concentrations of chrysotile asbestos, the production of IL-17 increased as shown in Fig. 6. Consequently, the expression of IFNγ, T-bet, RORγT and IL-17 mRNA was analyzed.

The results showed that cultivation with chrysotile did not modify the expression of T-bet, whereas IFNγ expression was reduced in cells initially categorized as CD4+sCXCR3−. These results indicated that although cultivation with chrysotile did not appear to alter the Th1 population, Th1 function was reduced and depended in particular on the initial expression of CXCR3.

The Th17 status in these three fractions derived from cells treated with PMA and ionomycin and followed by T-cell receptor stimulation with IL-2 and cultivation with various concentrations of chrysotile asbestos was then examined. IL-17 mRNA expression was enhanced in both CD4+sCXCR3− and CD4+sCXCR3+ fractions, although the expression of RORγT increased slightly in cells co-cultured with chrysotile.

**Discussion**

The causative mechanisms of asbestos-induced cancer are thought to include 1) DNA damage due to ROS production by iron found in asbestos fibers, 2) direct chromosomal/DNA damage by physical attack of cells near the inhaled asbestos fibers, and 3) adsorption of various carcinogenic substances around the fiber itself (33-36). The effect of these processes results in the generation of mesothelioma cells containing various genetic changes such as deletion of p16/p15 cyclin-dependent kinase-inhibitors (CDK-Is), deletion of NF2/merlin, and alteration (deletions and mutations) of BRCA1-associated protein 1/ubiquitin carboxyl-terminal hydrolase (BAP1) (37-39). Mesothelioma is thought to result after a 30 to 40-year latency period following initial exposure to asbestos. During these long latency periods, and under conditions leading to the occurrence of other malignancies such as cancers of the larynx, gastrointestinal tract and bladder (22,23), it is thought that individuals exposed to asbestos and possessing asbestos fibers in their body might have reduced antitumor immunity due to asbestos exposure and recurrent and chronic encounters between intra-body fibers and immune competent cells. It is on this basis that we have been investigating the immunological effects of asbestos on immune competent cells, particularly in regard to antitumor immunity (19-21,40).

Investigation of Th cells showed a reduction in CXCR3 surface expression and impaired IFNγ production capacity (17,18). The T-cell line model utilized in vitro stimulated freshly isolated human peripheral CD4+ cells and CD4+ cells derived from PP and MM (17,18). Since CXCR3 is thought to operate with IFNγ producing cells and functions to attack tumor cells, these findings also suggest that individuals exposed to asbestos possess reduced antitumor immunity (40).

We then investigated Th17 cells since they were considered to be important in the development of dysregulation of autoimmune and cancer (24-27). Some human cancers have been shown to possess increased levels of Th17 cells in the tumor, such as melanoma, ovarian and prostate cancers (24-27). Furthermore, the polarization of Th17 cells was considered...
to be opposite to that of CD4⁺CD25⁻ and Forkhead box protein P3 (FoxP3) positive regulatory T cells (Treg) (41,42). Since enhancement of the volume or function of Treg may cause a reduction in antitumor immunity that inhibits tumor-recognizing T cells, the peripheral balance and polarizing conditions defined by cytokine conditions among IL-6 and TGFβ and between Treg and Th17 (24-27) may be altered, and an increase in one population may cause a decrease in the other population. Therefore, an increase in number of cells or enhancement of Th17 function may reflect augmentation of antitumor immunity (24-27).

A consideration of the overall results and our previous data suggests that asbestos exposure reduces CXCR3 expression and decreases IFNγ production capacity in Th1 cells, and increases IL-17 production capacity in CD4⁺ and surface CXCR3⁺ fractions. This production capacity appears to be strongly related to the CXCR3 positive cell fraction (Fig. 5), and reduction in the surface CXCR3 positive fraction during asbestos exposure was associated with a reduction in IL-17 production capacity in peripheral blood derived from asbestos-exposed patients. These findings also indicated that the immunological effects of asbestos lead to a reduction in antitumor immunity, and that these mechanisms may play a role in the subsequent occurrence of mesothelioma and other cancers in asbestos-exposed individuals.

The status of Treg in asbestos-exposed patients and the effect of asbestos on Treg require investigation. Recently, we indicated that asbestos enhances Treg function through a cell-cell contact pathway, and increases production of typical soluble factors such as IL-10 and TGFβ. These investigations also showed a reduction in antitumor immunity in asbestos-exposed patients. However, it remains unclear whether chronic, recurrent and continuous exposure of T cells to asbestos influences the polarization of Th subpopulations such as Th1, Treg and Th17.

Additionally, studies of Treg and Th17 should determine whether there is an increase in the number and function of tumors surrounding these subpopulations. However, such investigations must collect all of the immune competent cells from rejected tumor specimens. These issues need to be examined in future studies in an effort to address unresolved questions such as the alteration of antitumor immunity in mesothelioma patients. However, our focus is to consider the gradual decrease in antitumor immunity in asbestos-exposed patients following initial exposure to asbestos. Therefore, it would be better to analyze a population that has a significant exposure history to asbestos, but which does not show any significant physical alterations or the presence of certain cancers or PP.

The overall results suggest that Treg and Th17 conditions should be analyzed with respect to antitumor immunity not only in PP and MM patients, but also in individuals who have a significant history of asbestos exposure without any changes in health and body-related issues.

An experimental model should be explored in order to confirm the reduction in antitumor immunity. For example, as previously reported, continuous exposure of the human NK cell line YT-A1 to chrysotile asbestos for more than five months resulted in reduced antitumor killing activity against the human erythroleukemia cell line K562, a commonly used target cell line to assay NK cell activity and compared with the original YT-A1 cell line, which has never been exposed to asbestos fibers (7-9). Regarding our previous reports showing
that asbestos fibers enhance Treg function (43) and increase its volume by acceleration of the cell cycle (44), the construction of a cell co-culture model using human cell lines such as a Treg cell model or responder T cell model with mesothelioma (or other cancer) model might be difficult. As we previously reported, subjecting a human Treg model comprising the MT-2 cell line to continuous exposure to asbestos resulted in increased production of IL-10 and TGFβ (43). The effects of soluble factors such as these cytokines can be assayed using a transwell culture model. However, these soluble factors act to reduce antitumor immunity by inhibiting the attack of tumor cells by T cells. Thus, the model should comprise two cell types such as tumor cells and tumor-attacking responder T cells. Of course, it would be possible to match the HLA of some mesothelioma cell line with HV who could be used to provide PBMCs, including tumor attacking T cells. Thereafter, PBMCs derived from HLA-matched HV can be activated in vitro in the absence or presence of asbestos fibers. The tumor killing activity may then be examined in vitro with or without cell culture supernatants derived from a human Treg cell line model cultured in the absence or continuous presence of asbestos fibers. Animal models transplanting human mesothelioma cells can also be employed with the induction of immune cells in the absence or continuous presence of asbestos. Furthermore, since antibodies are produced against tumor cells, human myeloma cell lines can be employed and we have successfully established many of these cell lines (45-47). These experiments should be performed in an effort to confirm our hypothesis that asbestos exposure causes a reduction in antitumor immunity.

In conclusion, we found that in vitro asbestos exposure of freshly isolated human T-cells induces IL-17 production. Further investigations regarding Th17 status in asbestos-exposed patients with PP or MM, and in individuals who do not exhibit any abnormalities, should be conducted to confirm the status of antitumor immunity in these asbestos-exposed individuals. Following confirmation of these hypotheses, certain kinds of food compositions or physiologically active substances derived from plants or other materials should be tested for their ability to neutralize the altered antitumor immunity in individuals exposed to asbestos in an effort to thwart the development of tumors.

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