Ingredients such as trehalose and hesperidin taken as supplements or foods reverse alterations in human T cells, reducing asbestos exposure-induced antitumor immunity

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Abstract. Exposure of human immune cells to asbestos causes a reduction in antitumor immunity. The present study aimed to investigate the recovery of reduced antitumor immunity by several ingredients taken as supplements or foods, including trehalose (Treh) and glycosylated hesperidin (gHesp). Peripheral blood $CD4^+$ cells were stimulated with IL-2, anti-CD3 and anti-CD28 antibodies for 3 days, followed by further stimulation with IL-2 for 7 days. Subsequently, cells were stimulated with IL-2 for an additional 28 days. During the 28 days, cells were cultured in the absence or presence of 50 μg/ml chrysotile asbestos fibers. In addition, cells were treated with 10 mM Treh or 10 μM gHesp. Following culture for 28 days, reverse transcription-quantitative PCR was performed to assess the expression levels of transcription factors, cytokines and specific genes, including matrix metalloproteinase-7 (MMP-7), nicotinamide nucleotide transhydrogenase (NNT) and C-X-C motif chemokine receptor 3, in unstimulated cells (fresh) and cells stimulated with PMA and ionomycin (stimuli). The results demonstrated that compared with the control group, chrysotile-exposure induced alterations in MMP-7, NNT and IL-17A expression levels were not observed in the 'Treh' and 'gHesp' groups in stimulated cells. The results suggested that Treh and gHesp may reverse asbestos exposure-induced reduced antitumor immunity in T helper cells. However, further investigation is required to confirm the efficacy of future trials involving the use of these compounds with high-risk human populations exposed to asbestos, such as workers involved in asbestos-handling activities.

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Introduction

Asbestos exposure causes pneumoconiosis and other benign diseases, including pleural plaque (PP), diffuse pleural thickening, benign asbestos pleural effusion and round atelectasis (1-4). Additionally, asbestos induces malignant diseases, such as lung cancer and malignant mesothelioma (MM) (1-4). Despite advances in therapeutic strategies, MM remains one of the worst known malignancies to date, with a 5-year survival rate <20% (5-7). As with other malignant diseases, anti-programmed cell death 1, anti-programmed death-ligand 1 and anti-cytotoxic T-lymphocyte-associated protein 4 monoclonal antibodies have been used for the treatment of patients with MM as monotherapy and in combination with other therapies (8-11). However, recent clinical trials investigating these immune checkpoint inhibitors in MM have not been sufficient to determine whether they are significantly effective.

The biological effects of asbestos fibers impact not only lung epithelial cells and pleural mesothelial cells, but also various immune cells (12,13). The effects of asbestos exposure on natural killer (NK) cells (14,15), cytotoxic T lymphocytes (CTLs) (16), effector T helper cells (Th) (16,17) and regulatory T cells (Treg) (16,18) have been investigated, and have indicated a reduction in antitumor immunity.

Regarding Treg cells, our previous studies employed a cell line model using MT-2 cells (19,20), a human T cell leukemia/lymphoma virus type 1 immortalized polyclonal human T cell line (21,22) (Fig. 1A). The MT-2 cell line

possesses Treg-like inhibitory functions (23,24). Continuous low-dose (5-10 μg/ml) exposure of MT-2 sublines to chrysotile or crocidolite asbestos fibers induced resistance to apoptosis (20), whereas transient high-dose (50-100 μg/ml) exposure was more likely to induce cell apoptosis via production of reactive oxygen species (ROS) and activation of the mitochondrial apoptotic signaling pathway (19). The continuous exposure of MT-2 sublines indicated enhanced Treg function via cell-cell contact and excess production of soluble factors, including IL-10 and TGF-β (18). Furthermore, nicotinamide nucleotide transhydrogenase (NNT) was overexpressed in MT-2 sublines (25), which rescued ROS-induced cellular damage due to iron-containing asbestos fibers. Matrix metalloproteinase-7 (MMP-7) expression levels were also upregulated in MT-2 sublines (12).

Moreover, investigation of the effects of exposure of Th cells to asbestos fibers identified a reduction in C-X-C motif chemokine receptor 3 (CXCR3) expression (17), an important molecule located at tumor sites that recruits T cells to attack the tumor (Fig. 1B). Additionally, $CD4^+$ Th cells transiently stimulated by phorbol 12-myristate 13-acetate (PMA) and ionomycin (IM) after relative long-term exposure to asbestos during stimulation with IL-2 and subsequent stimulation using anti-CD3 and CD28 antibodies displayed reduced intracellular expression of IFN-γ (18,26). Although CXCR3 expression on the Th cell surface was reduced by asbestos exposure, IL-17 expression was increased in $CXCR3^+$ Th cells after *in vitro* stimulation (27).

As for patients exposed to asbestos with PP and MM, $CD4^+$ Th cells derived from these patients displayed decreased expression levels of CXCR3 [healthy volunteers (HV) > PP > MM] (26), and IL-10 and TGF-β levels in the plasma were increased (20,28) (Fig. 1C).

Taken together, the aforementioned studies indicated a reduction in Th, Treg, CTL and NK cell antitumor immunity following asbestos exposure (12,13), which might account for the decreased effectiveness of immune checkpoint inhibitors against MM. In addition, reductions in antitumor immunity may provide an explanation for the rapid progression of MM and other asbestos-induced cancer after a long-term latency period of 30-50 years following asbestos exposure (1-11).

Therefore, if certain compounds taken as supplements or foods could reverse asbestos exposure-induced reductions in antitumor immunity, then past and current workers at asbestos-handling factories, demolition contractors and others involved in general asbestos-handling activities may benefit (29,30). Trehalose (Treh) (31,32) and hesperidin (Hesp) (33,34) were selected as potential candidate compounds for investigation in the present study.

Treh is a disaccharide composed of two glucose molecules bound by an α, α-1,1 linkage (35). Some bacteria, fungi, plants and invertebrate animals synthesize Treh as a source of energy to survive freezing and water shortages (30,31,35). Hayashibara Co., Ltd. succeeded in mass-producing Treh from starch using malto-oligosyltrehalose synthase and malto-oligosyltrehalosetrehalohydorolase (35). Treh is currently used in food, cosmetics and pharmaceutical ingredients (35). In the medical field, Treh has been reported to display neuroprotective activity (36), thus may be used for the treatment of Parkinson's disease (37), and is known to improve glucose tolerance (38). Hesp is a flavanone glycoside found in citrus fruit in the aglycone form (33,34). Hesp has been reported to serve a role in protecting plants from external toxins, but also displays antioxidant properties (39). Pharmacological effects have been investigated in terms of inflammation, hypertension, dyslipidemia, allergy, anxiety and cancer prevention (40,41).

Hayashibara Co. Ltd. has also succeeded in producing glycosyl hesperidin (gHesp) (42,43), a proprietary glycosylation technology that increases the water solubility of hesperidin, a polyphenol, by up to ~100,000 times. Furthermore, gHesp displayed improved absorption into the body compared with Hesp.

Therefore, if Treh and Hesp can modify asbestos-induced cellular and molecular alterations in T cells, they may serve as candidates for reversing asbestos exposure-induced reductions in antitumor immunity.

Materials and methods

$CD4^+$ T cells from HV. Freshly isolated peripheral $CD4^+$ Th cells derived from a HV were used in the present study. The HV was a 64-year-old Japanese male who was recruited from Kawasaki Medical School (Kurashiki, Japan) in March 2020. Blood was collected three times between April 2020 and May 2020. Venous blood was collected from the HV four times. To collect blood, ~10 ml of venous blood was drawn from the median cubical vein with the aid of heparin (Fig. 2). Mononuclear cells were isolated using the Ficoll-Hypaque method (density gradient centrifugation: 1,700 x g for 30 min at room temperature). Subsequently, cells were stained with anti-CD4 microbeads (cat. no. 130-045-101; Miltenyi Biotec GmbH) and $CD4^+$ cells were collected by positive selection using MS autoMACS® Columns (Miltenyi Biotec GmbH). $CD4^+$ cells were seeded ($2x10^5$ cells/well) into a 96-well U-bottomed plate. Subsequently, cells were stimulated with 10 ng/ml IL-2 (cat. no. 200-02; PeproTech, Inc.), 2 μg/ml anti-CD3 monoclonal antibody (cat. no. IM1304; Beckman Coulter, Inc.) and 2 μg/ml anti-CD28 monoclonal antibody (cat. no. IM1376; Beckman Coulter, Inc.). After three days, proliferating cells from two wells were collected and re-seeded into one well of a 24 flat-bottomed plate containing 10 ng/ml IL-2. All experiments were performed at 37˚C.

After seven days, cells were collected and re-seeded ($1x10^6$ cells/well) into a 24-well flat-bottomed plate containing 10 ng/ml IL-2, 10 mM Treh (Hayashibara Co., Ltd.) or 10 μM gHesp (Hayashibara Co., Ltd.) in the absence or presence of 50 μg/ml chrysotile asbestos (Japan Association for the Study of Fiber Materials). Following continuous culture for 28 days, the culture medium was changed and supplemental substances were replaced every 3-4 days.

After 28 days, half of the cells from each group were harvested as fresh cells for RNA extraction. The remaining half of the cells from each group were re-stimulated with 5 ng/ml PMA (cat. no. P1585; Sigma-Aldrich; Merck KGaA) and 250 ng/ml IM (cat. no. 19657; Sigma-Aldrich; Merck KGaA) for 6 h. Subsequently, cells were harvested as stimuli cells for RNA extraction. Stimulation times using PMA and IM were selected according to a previous study (31).

Ethical approval. The Ethics Committee of the Kawasaki Medical School and Kawasaki Medical School Hospital

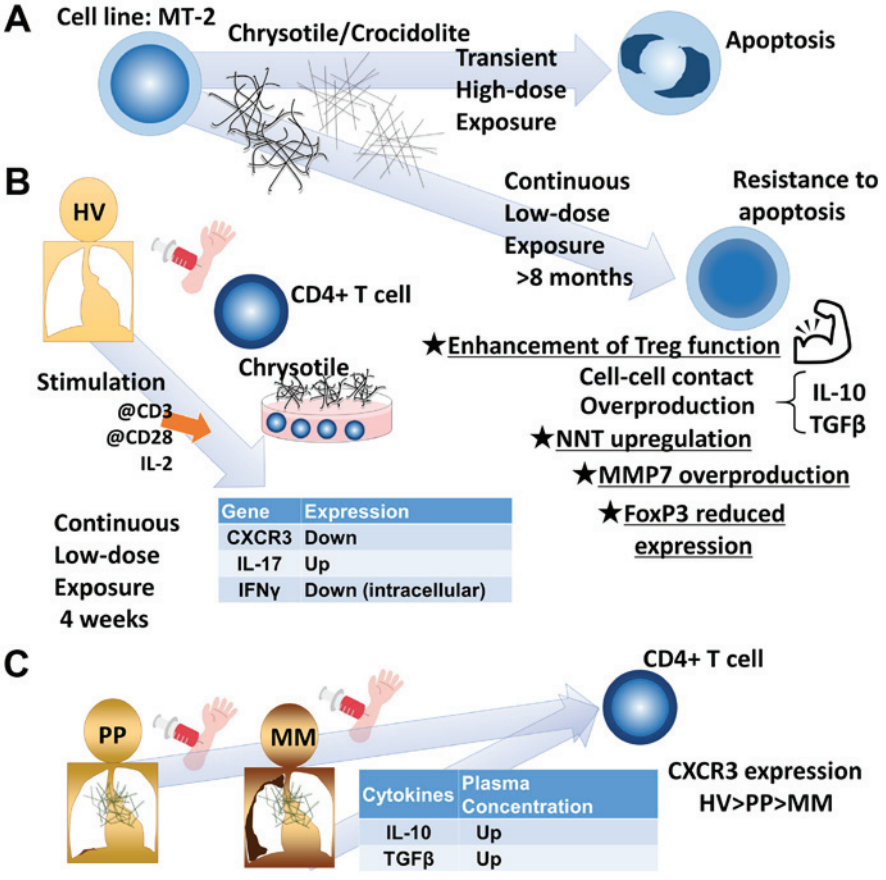

Figure 1. Schematic presentation of reduction in antitumor immunity of Th cells. (A) In a cell line model using the human T cell leukemia/lymphoma virus type 1 immortalized polyclonal T cell line (MT-2) transient (1-3 days) and relatively high-dose (50-100 μg/ml) exposure resulted in apoptosis. However, long-term (>8 months), continuous and relatively low-dose (5-10 μg/ml) exposure resulted in sublines that were resistant to asbestos-induced apoptosis. The sublines also displayed enhanced Treg function with cell-cell contact, as well as overproduction of soluble factors (IL-10 and TGF-β). Furthermore, the expression levels of MMP-7 and NNT were markedly increased. Overall, Treg function was upregulated, although expression of FoxP3 was reduced. (B) An *ex vivo* culture model using chrysotile asbestos with freshly isolated peripheral blood $CD4^+$ T cells from HVs displayed reduced expression and production of cell surface CXCR3 and intracellular IFN-γ. However, IL-17 expression and production was enhanced in this culture model. (C) Finally, freshly isolated $CD4^+$ T cells and plasma from patients exposed to asbestos with PP or MM displayed higher plasma levels of IL-10 and TGF-β compared with HVs, which was similar to the MT-2 cell line. Additionally, expression of cell surface CXCR3 on $CD4^+$ Th cells gradually decreased from HV to PP and MM. Th, T helper; MMP-7, matrix metalloproteinase-7; NNT, nicotinamide nucleotide transhydrogenase; FoxP3, forkhead box P3; HV, healthy volunteer; CXCR3, C-X-C motif chemokine receptor 3; PP, pleural plaque; MM, malignant mesothelioma.

(approval no. 883) approved the present study. Specimens were only obtained from HV who provided written informed consent.

RNA extraction and reverse transcription-quantitative PCR (qPCR). Total RNA was extracted from harvested cells (fresh and stimuli) using an RNase Plus Mini Kit (cat. no. 74104; Qiagen GmbH). Total RNA was reverse transcribed into cDNA using the PrimeScript II 1st Strand synthesis Kit (cat. no. 6210A; Takara Bio, Inc.) according to the manufacturer's protocol. Subsequently, qPCR was performed using the SYBR-Green method (Takara Bio, Inc.) and the Mx3000P qPCR System (Agilent Technologies, Inc.) as previously described (25-27). The sequences of the primers used for qPCR are listed in Table I. The expression levels of transcription factors, forkhead box P3 (FoxP3), T-box transcription factor TBX21 (Tbet), GATA binding protein 3 (GATA3) and retinoic acid receptor-related orphan receptor C (RORC), cytokines, including IL-4, IL-10, IL-17A, IFN-γ and TGF-β, and molecules and genes that have been modified by asbestos long-term exposure, including MMP-7, NNT and CXCR3, as determined in our previous studies (16-20,25-27). The following thermocycling conditions were used for qPCR: Initial denaturation at 95˚C for 3 min; 40 cycles of denaturation at 95˚C for 5 sec and annealing/extension at 60˚C for 20 sec; and melting curve analysis at 95˚C for 1 min, 60˚C for 30 sec and 95˚C for 30 sec. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (44)

Statistical analysis. Statistical analyses were performed using SPSS software (version 21; IBM Corp) and Microsoft Excel 2016 (Microsoft Corporation). All the experiments were performed three times. RT-qPCR assays were performed in duplicate. Data are presented as the mean ± SD. mRNA expression levels are expressed on a Log10 scale. Differences regarding exposure to chrysotile were examined in 'no Treh, no gHesp', 'Treh' and 'gHesp' groups. Alterations in mRNA expression levels in individual genes were compared between 'no Treh, no gHesp' cells with or without chrysotile exposure, 'Treh' cells with or without chrysotile exposure and 'gHesp' cells with or without chrysotile exposure. Comparisons between two non-homoscedastic samples were analyzed using

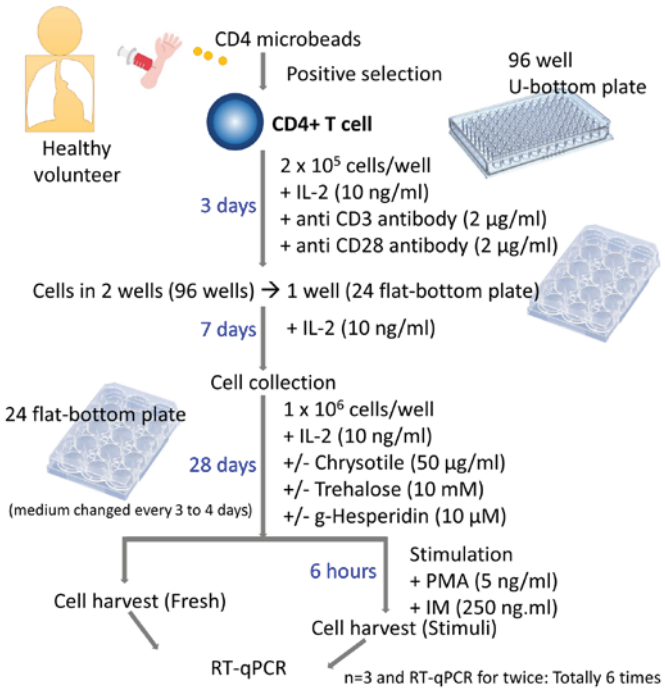

Figure 2. Schematic presentation of procedures conducted in the present study. PMA, phorbol 12-myristate 13-acetate; IM, ionomycin; RT-qPCR, reverse transcription-quantitative PCR.

the unpaired Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Comparison of gene expression in fresh cells. The expression patterns of genes in fresh cells are presented in Fig. 3. IL-17A expression levels in the fresh samples are not presented because IL-17A expression levels were too low.

Compared with the control groups, chrysotile exposure significantly decreased FoxP3 expression in 'no Treh, no gHesp' and 'Treh' cells, but slightly increased FoxP3 expression in 'gHesp' cells. Chrysotile exposure-induced reductions in Tbet expression were not observed in the 'Treh' and 'gHesp' groups. Compared with the control groups, GATA3 expression was only significantly reduced by chrysotile exposure in the 'Treh' group. By contrast, compared with the control groups, the expression of RORC was significantly increased by chrysotile exposure in all three groups ('no Treh, no gHesp', 'Treh' and 'gHesp'). However, mRNA expression levels of IFN-γ in the Treh group were markedly lower in chrysotile-treated cells compared with control cells.

Model-specific genes MMP-7 and NNT were significantly upregulated in all three groups by chrysotile exposure compared with the control groups. By contrast, CXCR3 expression levels were not significantly different between the control and chrysotile-exposed groups in all three groups. The results suggested that neither Treh or gHesp altered asbestos exposure-induced alterations in CXCR3 gene expression.

Although IL-4 and TGF-β expression levels were significantly different between the control and chrysotile-exposed groups for all three groups ('no Treh, no gHesp', 'Treh' and 'gHesp'), the differences among the three groups were not compared in the present study. Compared with the control groups, chrysotile exposure significantly reduced IL-4 expression and significantly enhanced TGF-β expression in all three groups. By contrast, there were no significant alterations in IL-10 expression levels between the control and chrysotile exposure groups in the 'no Treh, no gHesp' and 'Treh' groups, although chrysotile exposure significantly reduced IL-10 expression in 'gHesp' cells compared with the control group. Similarly to Tbet (a key transcription factor for Th1 differentiation) (45), the expression levels of IFN-γ (a representative cytokine of Th1) were significantly decreased by chrysotile exposure in the 'no Treh, no gHesp' group compared with the control group, but this effect was not observed in the 'Treh' or 'gHesp' groups.

The aforementioned results were obtained using fresh cells, which were cultured with Treh or gHesp in the absence or presence of chrysotile asbestos for 28 days, representing

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'→3')
FoxP3	F: TCCCAGAGTTCCTCCACAAC
	R: AGAAGCAGCGGACACTCAAT
Tbet	F: CCGTGACTGCCTACCAGAAT
	R: TCATGCTGACTGCTCGAAAC
GATA3	F: CTCATTAAGCCCAAGCGAAG
	R: TTTTTCGGTTTCTGGTCTGG
RORC	F: AAATCTGTGGGGACAAGTGG
	R: TCCCTCTGCTTCTTGGACAT
CXCR3	F: ACACCTTCCTGCTCCACCTA
	R: GTTCAGGTAGCGGTCAAAGC
MMP-7	F: GAGTGCCAGATGTTGCAGAA
	R: AAATGCAGGGGGATCTCTTT
NNT	F: GGGTTCAGTGTCTGCTCTCT
	R: TAGCACCAGACGAGCCTATG
IFN-γ	F: TGACCAGAGCATCCAAAAGA
	R: CTCTTCGACCTCGAAACAGC
IL-4	F: ACTGCACAGCAGTTCCACAG
	R: CTCTGGTTGGCTTCCTTCAC
IL-10	F: AACCTGCCTAACATGCTTCG
	R: CATTCTTCACCTGCTCCACG
IL-17A	F: ACCAATCCCAAAAGGTCCTC
	R: CCCACGGACACCAGTATCTT
TGF-β	F: TTCAACACATCAGAGCTCCG
	R: ATAACCACTCTGGCGAGTCG
GAPDH	F: GAGTCAACGGATTTGGTCGT
	R: TTGATTTTGGAGGGATCTCG

FoxP3, forkhead box P3; Tbet, T-box transcription factor TBX21; GATA3, GATA binding protein 3; RORC, retinoic acid receptor-related orphan receptor C; CXCR3, C-X-C motif chemokine receptor 3; MMP-7, matrix metalloproteinase-7; NNT, nicotinamide nucleotide transhydrogenase; F, forward; R, reverse.

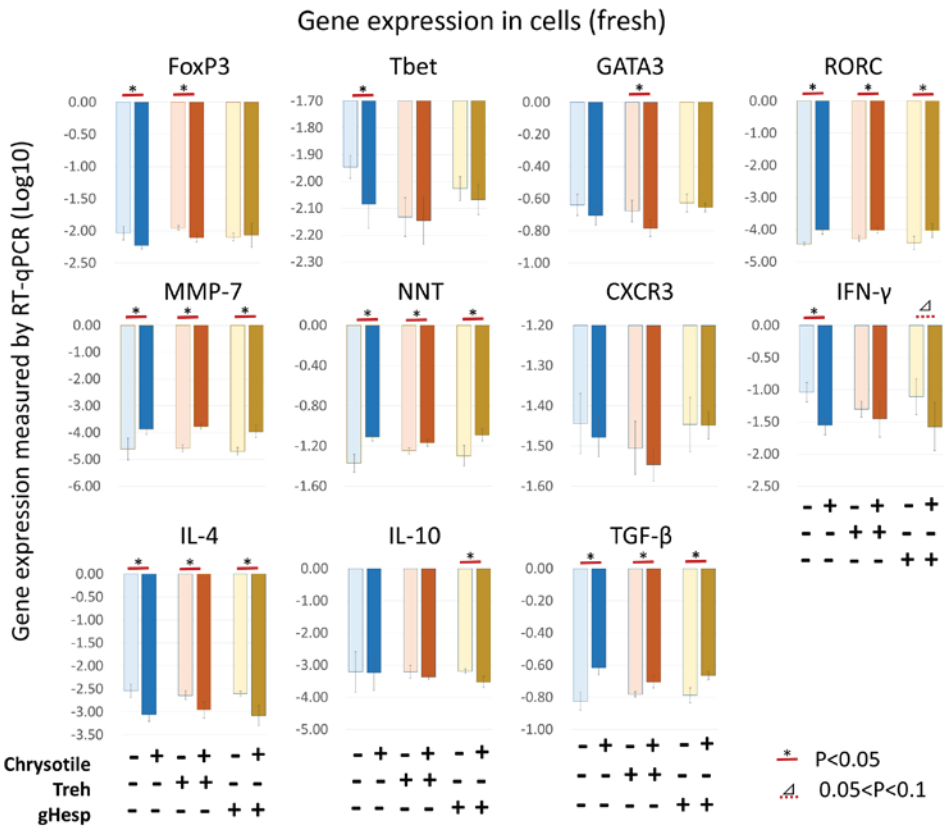

Figure 3. Gene expression in fresh cells. FoxP3, Tbet, GATA3, RORC, MMP-7, NNT, CXCR3, IFN-γ, IL-4, IL-10 and TGF-β mRNA expression levels were measured via RT-qPCR. FoxP3, forkhead box P3; Tbet, T-box transcription factor TBX21; GATA3, GATA binding protein 3; RORC, retinoic acid receptor-related orphan receptor C; CXCR3, C-X-C motif chemokine receptor 3; MMP-7, matrix metalloproteinase-7; NNT, nicotinamide nucleotide transhydrogenase; Treh, trehalose; gHesp, glycosylated hesperidin; RT-qPCR, reverse transcription-quantitative PCR.

relatively long-term exposure. However, Th cells may exert their functions when they are stimulated to proliferate by antigen exposure and other factors. Therefore, expression levels in stimuli cells stimulated by PMA and IM were subsequently assessed.

Comparison of gene expression in stimuli cells. The expression patterns of genes in stimuli cells are presented in Fig. 4.

As for transcription factors, Tbet and RORC expression levels were significantly upregulated by chrysotile exposure compared with the control group in the 'no Treh, no gHesp' and 'Treh' groups, which suggested that asbestos exposure prepared T cells to differentiate into not only Th17 cells, but also Th1 cells. However, based on the knowledge that Th1 differentiates together with tumor cells (46,47), the results suggested that these Th1 cells could become tumor-attacking T cells. However, GATA3 and FoxP3 expression levels were not significantly different between control and chrysotile-exposed groups in the three groups, 'no Treh, no gHesp', 'Treh' and 'gHesp'; however, an exception to this was that FoxP3 expression was significantly decreased by chrysotile exposure in the 'gHesp' group compared with the control group.

Subsequently, the expression levels of MMP-7, NNT and CXCR3 were examined. The results demonstrated that MMP-7 and NNT expression levels were significantly increased by chrysotile exposure compared with the control group in the 'no Treh, no gHesp' group, but this effect was not observed in the 'Treh' and 'gHesp' groups. Additionally, compared with the control group, NNT expression was notably reduced by chrysotile exposure in the 'gHesp' group.

Following stimulation with PMA and IM, the expression of IFN-γ was notably higher compared with fresh cells; the relative expression levels in fresh cells were $Log10^{-1}$-$Log10^{-1.5}$, whereas the relative expression levels in stimuli cells were >$Log10^{0.5}$. No significant differences between the control and chrysotile exposure groups were observed for other assessed cytokines, including IL-4, IL-10 and TGF-β. For example, IL-10 expression levels were not significantly different between the control and chrysotile exposure groups in the 'Treh' and 'gHesp' groups. With TGF-β, only the Treh group displayed notably downregulated expression levels in the chrysotile exposure group compared with the control group. However, similar tendencies were observed for IL-10 and TGF-β expression levels in all three groups, which made it difficult to interpret the subtle alterations in expression levels of these cytokines. On the other hand, the significantly enhanced expression of IL-17A induced by chrysotile exposure was negated by the addition of Treh or gHesp. However, compared with the control group, there was significant RORC upregulation following chrysotile exposure in the 'Treh' group and a similar but not significant trend was observed in the 'gHesp' group. Similar effects on RORC expression were observed in the 'no Treh, no gHesp group', as it has been previously reported that IL-17 production is induced by asbestos exposure in human peripheral blood $CD4^+$ cells (27), and the addition of Treh or gHesp counteracted this additive effect.

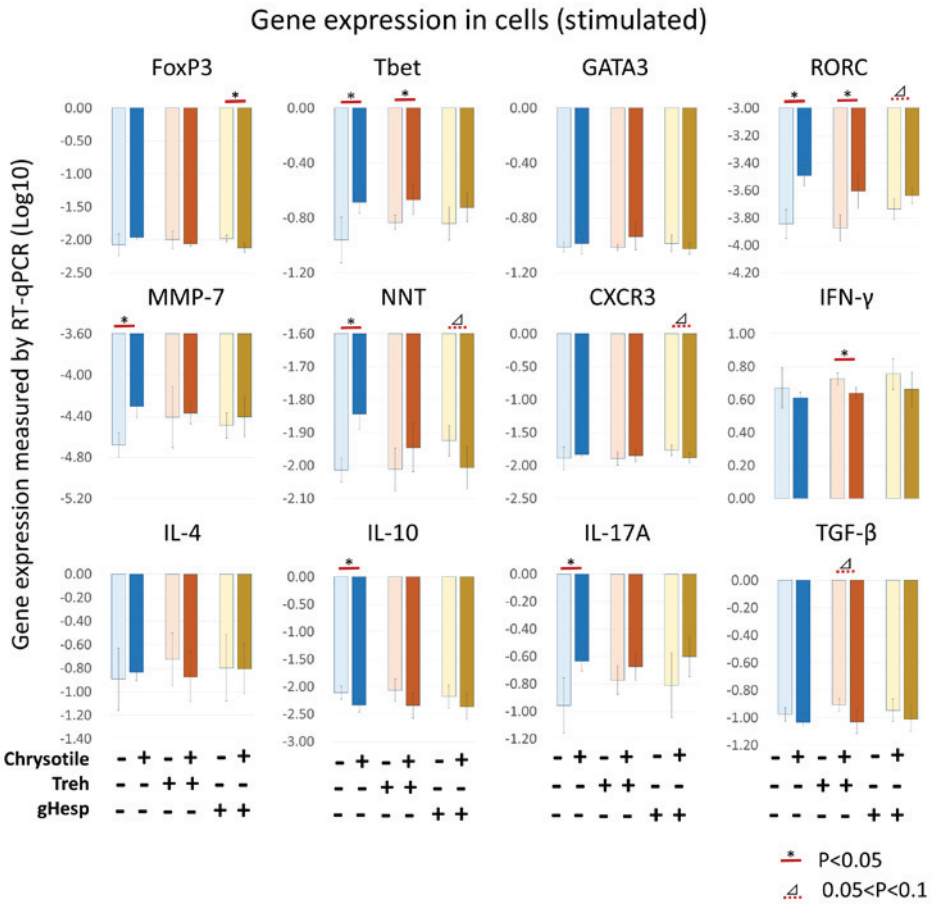

Figure 4. Gene expression in stimuli cells. FoxP3, Tbet, GATA3, RORC, MMP-7, NNT, CXCR3, IFN-γ, IL-4, IL-10, IL-17A and TGF-β mRNA expression levels were measured via RT-qPCR. FoxP3, forkhead box P3; Tbet, T-box transcription factor TBX21; GATA3, GATA binding protein 3; RORC, retinoic acid receptor-related orphan receptor C; CXCR3, C-X-C motif chemokine receptor 3; MMP-7, matrix metalloproteinase-7; NNT, nicotinamide nucleotide transhydrogenase; Treh, trehalose; gHesp, glycosylated hesperidin; RT-qPCR, reverse transcription-quantitative PCR.

Discussion

The latency period that exists prior to the occurrence of asbestos-related malignancies, including MM, following initial exposure to asbestos fibers is estimated to be 30-50 years (1-4). Our previous studies employed immune cell lineages, including NK cells, CTLs, Th1 cells and Treg cells, exposed to asbestos fibers or derived from patients exposed to asbestos, for example patients with PP and MM (14-18). The cell types could account for the relatively long latency period of asbestos-induced carcinogenesis. If so, restoration of reduced antitumor immunity to normal levels would be substantial. Immunosurveillance against initial cancer cells may prevent the progression of asbestos-induced cancer, which could potentially benefit past and current workers at asbestos-handling factories, demolition contractors and others involved in general asbestos-handling activities (29,30).

Therefore, identifying compounds suitable for this type of chemoprevention is important. In the present study, Treh and gHesp were selected as potential candidate compounds as both compounds have already been used as food ingredients or supplements (31-34), meaning both products are relatively safe to administer to high-risk populations (31-34).

The present study was designed as an initial trial to determine whether Treh or gHesp altered asbestos exposure-induced cellular and molecular alterations in $CD4^+$ Th cells in an *ex vivo* experiment. Byun *et al* (48) introduced Treh as an autophagy inducer in the context of generating potential therapeutic strategies for the treatment of cancer, infectious diseases and immune disorders. Other groups have referred to Treh as an autophagy inducer in terms of its neuroprotective activities (49). It has been reported that Treh-induced autophagy is not mediated by mTOR (50). However, with respect to immunity, studies have focused on how Treh modifies the immune status following viral infections, including human cytomegalovirus 9 (51) and human rhinovirus (52).

gHesp, a flavanone glycoside found in citrus fruit, has been reported to induce various pharmacological effects, such as reduction in cholesterol and blood pressure in rats (53,54). Additionally, the sedative effects of gHesp have been investigated and have been reported to be mediated by opioid receptors (55,56). With respect to immunity and inflammation, the antioxidant effects of citrus fruit suggest that gHesp may be effective against Coronavirus disease 2019 infection (57). Ding *et al* (58) reported that gHesp attenuated influenza A virus-induced lung injury in rats via its anti-inflammatory activities (49). Thus, gHesp might possess anti-inflammatory effects that are not observed with Treh. The antioxidant and anti-inflammatory properties of gHesp suggest that it may serve as a potential chemopreventive substance.

The aim of the present study differed slightly from a simple examination of the antioxidant and anti-inflammatory effects

of gHesp and autophagy-inducing effects of Treh, since the alterations that are observed in Th or Treg cells are caused by relatively long-term continuous exposure to asbestos. Therefore, it was difficult to gain a perspective on the results before executing the experiments. In our experimental model, which examined Treh and gHesp using fresh cells, the cells had already been stimulated and cultured in the absence or presence of chrysotile. Furthermore, there were similar findings to our previous investigations with respect to upregulation and enhancement of genes, including RORC, MMP-7, NNT and TGF-β (12,17,20,25-27,59). Moreover, chrysotile exposure-induced downregulation of FoxP3, IFN-γ, Tbet and IL-4 expression levels observed in the ' no Treh, no gHesp' group in the present study was comparable with our previous findings (27,4,60). Using an MT-2 cell line model, it was reported that exposure to asbestos enhanced Treg function but reduced FoxP3 gene expression (43,52). Furthermore, using freshly isolated T cells from HV displayed increased IL-17 production in an *ex vivo* culture model, similar to the present study (27). The aforementioned results indicated that asbestos exposure, at least in the isolated cell model, resulted in Th cells being driven toward Th17, and not Th1, Th2 or Treg subtypes. However, in the present study, comparisons of gene expression between the 'no Treh, no gHesp' and 'Treh' or 'gHesp' groups were not performed.

In the stimuli group, mRNA was extracted from cells after 28 days incubation with asbestos fibers and 6 h of stimulation with PMA or IM. Since T cells are typically exposed to specific antigens and need to receive signals to proliferate, unlike the fresh state, the stimuli state may resemble the biological state of T cells. In the stimuli set, significant differences between the chrysotile-exposed group and control group were not observed in the 'Treh' and 'gHesp' groups, but significant differences were observed in the 'no Treh, no gHesp' group, which suggested that Treh and gHesp inhibited chrysotile-induced alterations, indicating that gHesp inhibited chrysotile-induced alterations, such as the upregulation of MMP-7, NNT and IL-17A. The significant differences in RORC expression between the chrysotile-exposed group and control group were not observed in the 'gHesp' group, but significant differences were observed in the 'Treh' and 'no Treh, no gHesp' groups. The aforementioned results suggested that Treh and/or gHesp may restore alterations in Th cells caused by relatively long-term continuous exposure to chrysotile asbestos.

Although the experimental setup in the present study was very specific, the results provided valuable information regarding chemoprevention with respect to asbestos-induced antitumor immunity. Treh and gHesp can be ingested safely on a daily basis as supplements or in the form of food ingredients (30-42). Therefore, Treh and gHesp could be supplied to populations at high risk of asbestos exposure.

Future studies should examine different experimental settings to verify the results of the present study by using cell line models or freshly isolated T cells from patients with PP or MM who have been exposed to asbestos. There is difficulty associated with setting up animal models comprising continuous low-dose exposure of immune cells to asbestos. Therefore, the use of human cell models that include cell lines and cells from patients exposed to asbestos may prove useful in verifying the results of the present study. A variety of additional *in vitro* and *in vivo* investigations are required to verify the results and conclusions of the present study. Following further investigation, it is hoped that chemopreventive substances, such as Treh and gHesp, could be supplied to reduce antitumor immunity resulting from asbestos exposure.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

SL and SY drafted the manuscript. TA, TOh, TI, YN and TOt reviewed and edited the manuscript. TI, YN, NS and TOt provided supervision for individual experiments. SL, NKT, TI, YS, BS, NS, YN and SY confirmed the authenticity of the raw data. YN, NKT and TOh supervised the study design. SY, SL, TA, SE, SM, AY, AH, TOt and TOh made substantial contributions to the conception of the study. SL, TA, TOt and TOh designed the study. SY, SL, TA, NKT, TI, YS, BS, NS, YN and TOt acquired the data. SY, SL, TA, SE, SM, AY, AH, TOt and TOh analyzed the data. SY, SL, TA, SE, SM, AY, AH, TOh and TOt interpreted the data. SL, SY, BS and YS drafted the manuscript. SL, NKT, TI, NS and YN revised the manuscript critically for important intellectual content. SL, YN and TOt provided final approval of the version to be published. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The Ethics Committee of the Kawasaki Medical School and Kawasaki Medical School Hospital (approval no. 883) approved the present study. The healthy volunteer provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. Toshio Ariyasu, Shin Endo, Satomi Miyata, Akiko Yasuda, Akira Harashima and Tsunetaka Ohta are employees of Hayashibara Co., Ltd, Okayama, Japan, where treharose and hesperidin, which were used in the present study, were obtained.

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