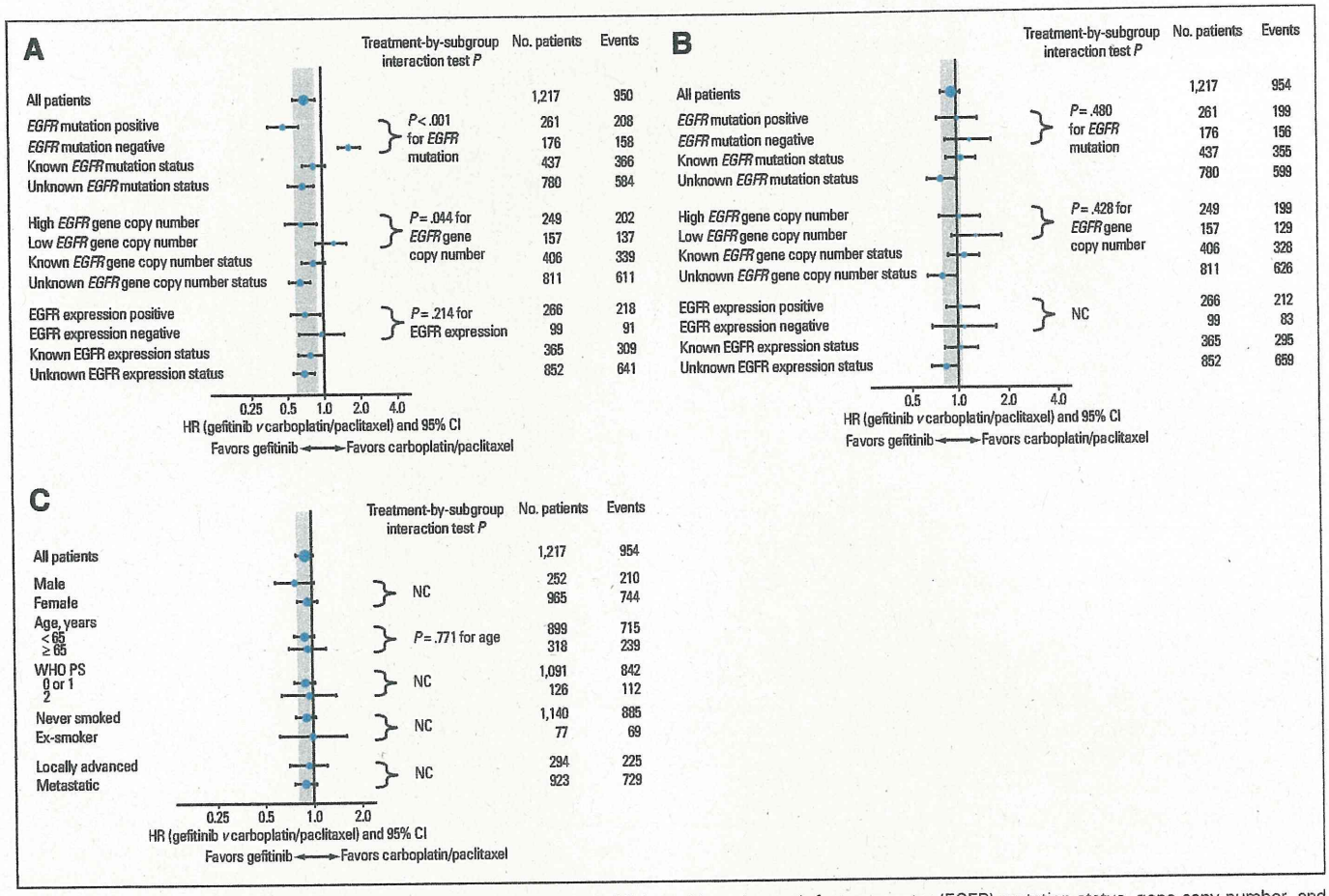

Fig 3. Forest plot of progression-free survival (PFS) and overall survival (OS) by epidermal growth factor receptor (EGFR) mutation status, gene copy number, and protein expression status (intent-to-treat population). (A) PFS by biomarker status. (B) OS by biomarker status. (C) OS by clinical subgroup. Hazard ratio < 1 implies a lower risk of progression or death for patients treated with gefitinib. The size of the point estimate reflects the number of events in the subgroup, with a larger circle indicating more events. Cox analysis with covariates (performance status [PS], 0 to 1 or 2; smoking history, never-smoker, light ex-smoker; and sex). OS by biomarker status; no formal adjustment for multiple testing was made, therefore, statistical significance at the traditional 5% level (95% CI < 1) cannot be claimed. Protocoled interaction tests were calculated only for OS and clinical subgroups if there was a significant interaction test for PFS. NC, not calculated.

EGFR gene copy number, and 365 (30.0%) for EGFR protein expression (Fig 1); the percentage of patients with a positive EGFR biomarker status was 59.7% (261 of 437), 61.3% (249 of 406), and 72.9% (266 of 365), respectively. A summary of EGFR biomarker status is presented in the Data Supplement.

The demographics, baseline characteristics, and efficacy results of patients with evaluable samples for assessment of *EGFR* mutation status, gene copy number, and protein expression were generally comparable with the ITT population (Table 2). There was a high degree of overlap between patients who were positive for all three biomarkers; 190 patients (78.5%) with high *EGFR* gene copy number also harbored an *EGFR* mutation; 132 patients were positive for all three biomarkers.

EGFR *Mutation Status*

Demographic and baseline characteristics by *EGFR* mutation status are shown in the Data Supplement. PFS results by *EGFR* mutation status have been previously published[19] (Fig 3A).

There was no differential treatment effect for OS by *EGFR* mutation (treatment by *EGFR* mutation interaction test $P = .480$). There was no significant difference in OS for gefitinib versus carboplatin/paclitaxel in the subgroups of patients with *EGFR* mutation–positive tumors (104 and 95 events, respectively; HR, 1.00; 95% CI, 0.76 to 1.33; $P = .990$; median OS, 21.6 *v* 21.9 months); *EGFR* mutation–negative tumors (82 and 74 events, respectively; HR, 1.18; 95% CI, 0.86 to 1.63; $P = .309$; median OS, 11.2 *v* 12.7 months), or mutation status unknown tumors (298 and 301 events, respectively; HR, 0.82; 95% CI, 0.70 to 0.96; $P = .015$; Figs 2B, 2C, 2D, and 3B). Postdiscontinuation treatments by *EGFR* mutation status are listed in Table 1.

EGFR *Gene Copy Number*

EGFR gene copy number was a predictive biomarker for the effect of gefitinib compared with carboplatin/paclitaxel on PFS (treatment by *EGFR* gene copy number interaction test $P = .044$; Fig 3A). In patients with high *EGFR* gene copy number (fluorescent in situ hybridization scores 5 and 6; n = 249), PFS was significantly longer with gefitinib versus carboplatin/paclitaxel (HR, 0.66; 95% CI, 0.50 to 0.88; $P = .005$). ORR also significantly favored gefitinib in these patients (58.9% *v* 44.8% for gefitinib *v* carboplatin/paclitaxel, respectively; odds ratio [OR], 1.79; 95% CI, 1.08 to 2.96; $P = .024$). Conversely, in

Table 2. Demographics, Baseline Characteristics, and Analysis Outcomes for Patients with Evaluable Tissue Samples for Each Biomarker Compared With the ITT Population

Variable	Evaluable for *EGFR* Mutation Status* (n = 437)					Evaluable for *EGFR* Gene Copy Number Status* (n = 406)					Evaluable for EGFR Protein Expression Status* (n = 365)					ITT Population (n = 1,217)				
	No.	%	HR	OR	95% CI	No.	%	HR	OR	95% CI	No.	%	HR	OR	95% CI	No.	%	HR	OR	95% CI
Demographic characteristic																				
Female	335	76.7				313	77.1				285	78.1				965	79.3			
Age < 65 years	326	74.6				303	74.6				262	71.8				899	73.9			
WHO PS 0 or 1	402	92.0				375	92.4				334	91.5				1,091	89.6			
Never-smoker	405	92.7				375	92.4				334	91.5				1,140	93.7			
Locally advanced	83	19.0				77	19.0				67	18.4				295	24.2			
Efficacy																				
PFS			0.85		0.69 to 1.06			0.83		0.66 to 1.03			0.79		0.62 to 0.99			0.74		0.65 to 0.85
ORR				1.21	0.83 to 1.78				1.31	0.88 to 1.95				1.43	0.94 to 2.18				1.59	1.25 to 2.01
OS			1.05		0.85 to 1.29			1.10		0.89 to 1.37			1.04		0.82 to 1.30			0.90		0.79 to 1.02

NOTE. Hazard ratio (HR) < 1 implies a lower risk of progression or death on gefitinib; odds ratio (OR) > 1 implies a greater chance of response on gefitinib.
Abbreviations: ITT, intent to treat; EGFR, epidermal growth factor receptor; PS, performance status; PFS, progression-free survival; ORR, objective response rate; OS, overall survival.
*Irrespective of whether positive or negative for each biomarker.

patients with low *EGFR* gene copy number (n = 157), PFS was numerically longer (HR, 1.24; 95% CI, 0.87 to 1.76; $P = .237$) and ORR was numerically higher (26.3% *v* 22.2%; OR, 0.80; 95% CI, 0.38 to 1.68; $P = .558$) with carboplatin/paclitaxel versus gefitinib.

A total of 190 patients (78%) with high *EGFR* gene copy number also harbored *EGFR* mutations. Of the 153 patients with low *EGFR* gene copy number, only 51 (33%) were also *EGFR* mutation positive. Post hoc analyses found that PFS was significantly shorter with gefitinib versus carboplatin/paclitaxel in patients with high *EGFR* gene copy number in the absence of a coexisting *EGFR* mutation (n = 55; HR, 3.85; 95% CI, 2.09 to 7.09), although patients with *EGFR* mutation achieved significantly longer PFS with gefitinib versus carboplatin/paclitaxel irrespective of whether they had high (HR, 0.48; 95% CI, 0.34 to 0.67; n = 190) or low (HR, 0.51; 95% CI, 0.25 to 1.04; n = 51) *EGFR* gene copy number (Figs 4A to 4D).

There was no differential treatment effect for OS by *EGFR* gene copy number (treatment by *EGFR* gene copy number interaction test $P = .428$). There was no significant difference in OS for gefitinib versus carboplatin/paclitaxel in patients with high *EGFR* gene copy number (104 and 95 events, respectively; HR, 1.03; 95% CI, 0.78 to 1.37; $P = .816$) or low *EGFR* gene copy number (67 and 62 events, respectively; HR, 1.30; 95% CI, 0.92 to 1.85; $P = .137$; Fig 3B).

EGFR Protein Expression

There was no differential treatment effect for PFS by EGFR protein expression (treatment by EGFR protein expression status interaction test $P = .214$; Fig 3A). PFS was significantly longer for gefitinib versus carboplatin/paclitaxel in patients with EGFR protein expression–positive tumors (HR, 0.73; 95% CI, 0.55 to 0.96; $P = .024$; n = 266). There was no significant difference in PFS between treatments in patients with EGFR protein expression–negative tumors (HR, 0.97; 95% CI, 0.64 to 1.48; $P = .893$; n = 99).

ORRs were similar between the gefitinib and carboplatin/paclitaxel groups for patients with either EGFR protein expression–positive (51.5% *v* 41.8%; OR, 1.49; 95% CI, 0.92 to 2.42; $P = .109$) or EGFR protein expression–negative (34.0% *v* 26.1%; OR, 1.44; 95% CI, 0.60 to 3.47; $P = .415$) tumors.

There was no significant difference in OS for gefitinib versus carboplatin/paclitaxel in patients with EGFR protein expression–positive (107 and 105 events, respectively; HR, 1.05; 95% CI, 0.80 to 1.37; $P = .731$) or EGFR protein expression–negative (46 and 37 events, respectively; HR, 1.09; 95% CI, 0.70 to 1.70; $P = .692$) tumors.

Activating EGFR *Mutation Type*

Of the 261 patients with *EGFR* mutation–positive tumors, 53.6% (n = 140) had tumors with exon 19 deletions, and 42.5% (n = 111) had exon 21 L858R mutations (Data Supplement); demography was generally similar between these groups (Data Supplement).

In post hoc analyses, PFS was significantly longer for gefitinib versus carboplatin/paclitaxel in both the exon 19 deletions (HR, 0.38; 95% CI, 0.26 to 0.56) and the exon 21 L858R mutation (HR, 0.55; 95% CI, 0.35 to 0.87; Figs 5A and 5B) subgroups. Within-treatment analysis indicated no significant difference in PFS with gefitinib in the exon 19 deletions versus exon 21 L858R mutation subgroup (HR, 0.78; 95% CI, 0.51 to 1.19). ORR was significantly higher with gefitinib (84.8%) versus carboplatin/paclitaxel (43.2%; OR, 7.23; 95% CI, 3.19 to 16.37) in the exon 19 deletions subgroup and higher (but not statistically significant) in the L858R subgroup (60.9% *v* 53.2%; OR, 1.41; 95% CI, 0.65 to 3.05).

DISCUSSION

Gefitinib showed similar OS to doublet chemotherapy with no significant difference in the overall population or in patients with *EGFR* mutation–positive or *EGFR* mutation–negative status. The significant treatment-related differences for PFS and ORR according to *EGFR* mutation status were not observed for OS. Although there may be other contributing factors, the subsequent treatments that patients received are likely to have confounded the true effect of the initial, randomized first-line treatment on OS. Of the *EGFR* mutation–positive subgroup randomly assigned to carboplatin/paclitaxel, 64.3% received EGFR TKIs postdiscontinuation. Fewer patients with unknown mutation status randomly assigned to carboplatin/paclitaxel received EGFR TKIs (47.5%) compared with patients with *EGFR* mutation–positive status (64.3%), which may potentially contribute to the numerical trend in favor of gefitinib in this subgroup; statistical significance at the traditional 5% level ($P < .05$) cannot be claimed because no adjustment was made for multiple testing. The First-SIGNAL study had a study design similar to that of IPASS[23] and

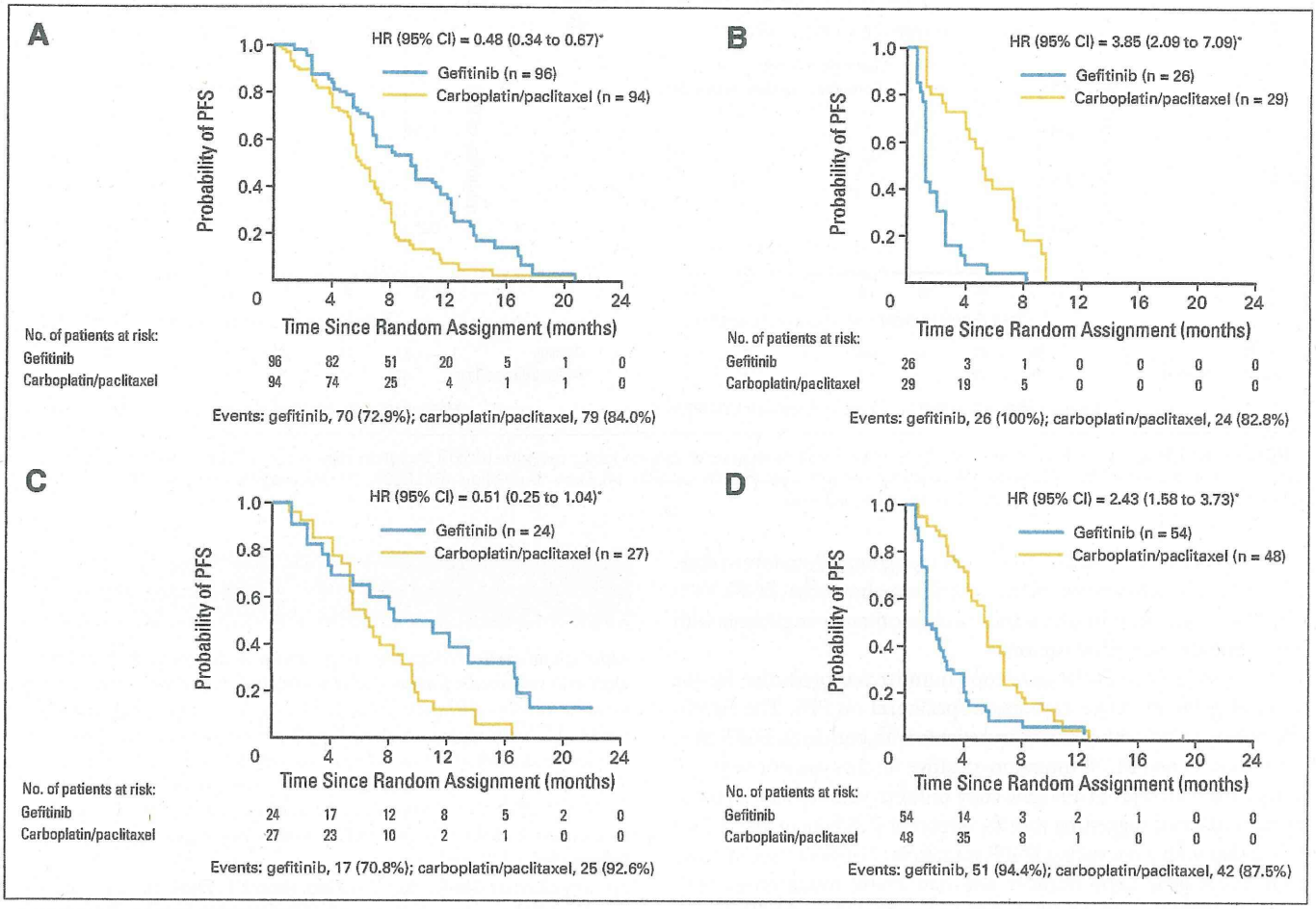

Fig 4. Kaplan-Meier curves for progression-free survival (PFS) by epidermal growth factor receptor (*EGFR*) mutation status and *EGFR* gene copy number. Hazard ratio (HR) < 1 implies a lower risk of progression/death for patients treated with gefitinib. (A) High *EGFR* gene copy number *EGFR* mutation–positive. (B) High *EGFR* gene copy number *EGFR* mutation–negative. (C) Low *EGFR* gene copy number *EGFR* mutation–positive. (D) Low *EGFR* gene copy number *EGFR* mutation–negative. (*) Cox analysis with covariates (performance status [0-1, 2], smoking history [never, light ex-smoker], and sex).

reported no significant difference in OS (primary end point) between gefitinib versus gemcitabine/cisplatin (overall population, 182 events; 59% maturity; mutation-positive HR, 0.82; 95% CI, 0.35 to 1.92; $P = .648$; median survival, 30.6 *v* 26.5 months, respectively). The randomized Japanese NEJ002 study also reported that OS did not differ significantly between gefitinib and carboplatin/paclitaxel in patients selected by *EGFR* mutation status (median survival, 30.5 *v* 23.6 months, respectively; $P = .31$), likely explained by treatment crossover.[24]

Although collection of tumor material was not mandatory or feasible in all patients, IPASS has the largest group of patients with *EGFR* mutation–positive tumors studied in a randomized controlled trial in NSCLC and has confirmed *EGFR* mutation to be the strongest predictive biomarker for the effect of gefitinib with a statistically significant interaction test for PFS. Patients with mutation-negative tumors have a poorer outcome in terms of PFS and ORR with gefitinib compared with carboplatin/paclitaxel, indicating that in the first-line setting, gefitinib should not be used in preference to doublet chemotherapy in patients with a negative mutation status.

Our findings were broadly consistent with those of previous first-line, single-arm studies of gefitinib in patients with *EGFR* mutation–positive tumors.[25-32] Recently, outcomes similar to those of IPASS among patients with *EGFR* mutation–positive tumors have been reported in two randomized phase III studies[24,33] comparing first-line gefitinib with doublet chemotherapy, with PFS as the primary end point. The NEJ002 study prospectively randomly assigned 230 patients with *EGFR* mutation–positive tumors to gefitinib or carboplatin/paclitaxel. PFS favored gefitinib over carboplatin/paclitaxel (PFS HR, 0.30; 95% CI, 0.22 to 0.41; $P < .001$; median PFS, 10.8 *v* 5.4 months; tumor response rate, 73.7% *v* 30.7%, respectively; $P < .001$).[24] The similarly designed West Japan Thoracic Oncology Group 3405 (WJTOG3405) study reported increased PFS with gefitinib over cisplatin/docetaxel in 172 patients with *EGFR* mutation–positive tumors (PFS HR, 0.49; 95% CI, 0.34 to 0.70; $P < .001$; median PFS, 9.2 *v* 6.3 months; 295 events; 95% maturity).[33] Tumor response rates (n = 117) were 62.1% and 32.2%. In the First-SIGNAL study, PFS (secondary end point) increased with gefitinib compared with gemcitabine/cisplatin in 42 patients with *EGFR* mutation–positive tumors (PFS HR, 0.61; 95% CI, 0.31 to 1.22; $P = .084$; median PFS, 8.4 *v* 6.7 months).[23] The OPTIMAL study compared erlotinib with gemcitabine/cisplatin in 154 patients with *EGFR* mutation–positive tumors and also reported a significant difference in PFS (HR, 0.16; 95% CI, 0.10 to 0.26; $P = .001$).[34] The similarly designed European Tarceva

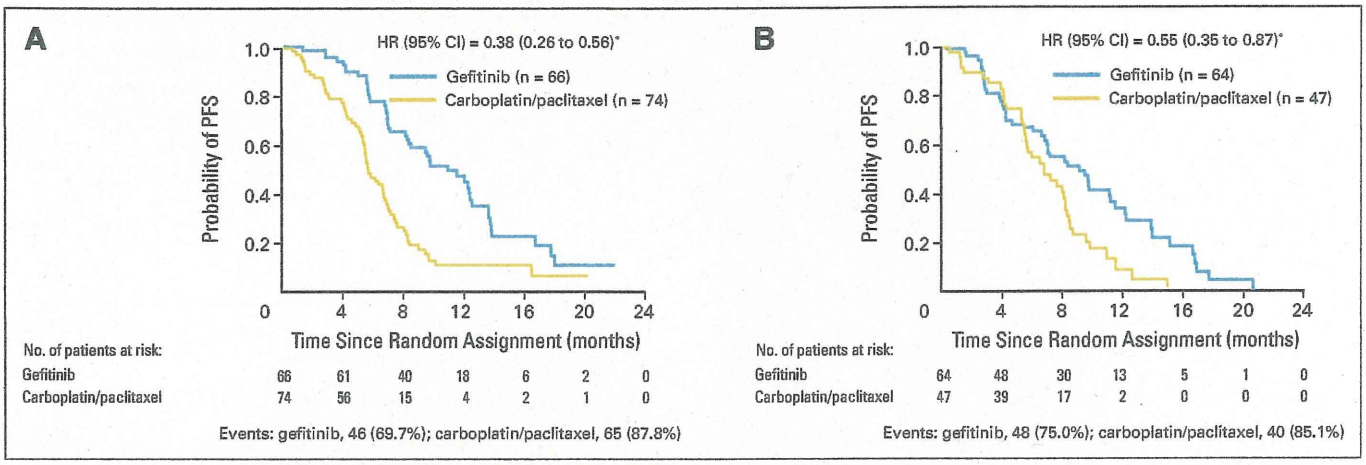

Fig 5. Kaplan-Meier curves for progression-free survival (PFS) by epidermal growth factor receptor (*EGFR*) mutation type (intent-to-treat population). Hazard ratio (HR) < 1 implies a lower risk of progression/death for patients treated with gefitinib. (A) Exon 19 deletion. (B) L858R. (*) Cox analysis with covariates (performance status [0-1, 2], smoking history [never, light ex-smoker], and sex).

versus Chemotherapy (EURTAC) study is ongoing. Therefore to date, including IPASS, five randomized studies have shown that EGFR TKIs offer significant benefits over standard chemotherapy in patients with *EGFR* mutation–positive tumors.

In IPASS, high *EGFR* gene copy number was predictive for the effect of gefitinib versus carboplatin/paclitaxel on PFS. The significantly longer PFS with gefitinib in patients with both high *EGFR* gene copy number and *EGFR* mutation–positive tumors was not observed in patients with high *EGFR* gene copy number without an accompanying mutation, suggesting that the apparent PFS benefit was driven by overlap with a coexisting *EGFR* mutation (77.6% of patients with high *EGFR* gene copy number also had *EGFR* mutation–positive tumors). Patients with *EGFR* mutation–positive tumors without accompanying high *EGFR* gene copy number showed longer PFS with gefitinib than with carboplatin/paclitaxel, suggesting that *EGFR* mutations determine the treatment outcomes independent of the status of *EGFR* gene copy number.

Post hoc analyses of PFS by *EGFR* mutation type showed that PFS was significantly longer for gefitinib than for carboplatin/paclitaxel in both the exon 19 deletions and exon 21 L858R subgroups, with a slightly greater advantage in the exon 19 deletions subgroup. First-line, single-arm studies[35,36] have reported an increased response to EGFR TKIs in patients with exon 19 deletions *v* exon 21 L858R mutation. However, IPASS (HR, 0.78; 95% CI, 0.51 to 1.19), WJTOG3405 (HR, 1.13; 95% CI, 0.63 to 2.03; $P = .681$), and NEJ002 (11.5 *v* 10.8 months; $P = .90$) randomized phase III studies and the prospective phase II iTARGET study ($P = .600$) showed no significant difference in PFS for gefitinib between the exon 19 deletions and exon 21 L858R mutation subgroups.[24,25,33]

In summary, *EGFR* mutation was the strongest predictive biomarker for benefit of gefitinib over carboplatin/paclitaxel on PFS and ORR. Post hoc analyses suggested that the predictive value of *EGFR* gene copy number for PFS benefit with gefitinib was driven by the overlap of high *EGFR* gene copy number with a positive *EGFR* mutation status. Treatment-related differences for PFS seen in patients with a positive *EGFR* mutation status were not apparent for OS. The OS results were likely confounded by the high proportion of patients receiving different types of subsequent therapies and, in particular, crossing over to the alternative treatment.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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Review Article

Asbestos Induces Reduction of Tumor Immunity

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Asbestos-related cancers such as malignant mesothelioma and lung cancer are an important issue in the world. There are many conflicts concerning economical considerations and medical evidence for these cancers and much confusion regarding details of the pathological mechanisms of asbestos-induced cancers. For example, there is uncertainty concerning the degree of danger of the iron-absent chrysotile compared with iron-containing crocidolite and amosite. However, regarding bad prognosis of mesothelioma, medical approaches to ensure the recognition of the biological effects of asbestos and the pathological mechanisms of asbestos-induced carcinogenesis, as well as clinical trials to detect the early stage of mesothelioma, should result in better preventions and the cure of these malignancies. We have been investigating the immunological effects of asbestos in relation to the reduction of tumor immunity. In this paper, cellular and molecular approaches to clarify the immunological effects of asbestos are described, and all the findings indicate that the reduction of tumor immunity is caused by asbestos exposure and involvement in asbestos-induced cancers. These investigations may not only allow the clear recognition of the biological effects of asbestos, but also present a novel procedure for early detection of previous asbestos exposure and the presence of mesothelioma as well as the chemoprevention of asbestos-related cancers.

1. Introduction

The fact that inhaled asbestos causes malignant mesothelioma and lung cancer is an enormous medical and social problem. Canada's decision to expand asbestos mining and export to developing countries in which asbestos has not been banned is unsettling [1]. People are sometimes influenced by economical forces even though they know many people have suffered from malignant diseases caused by these minerals, and their decisions appear to be made for financial reasons.

In Japan, the asbestos issue erupted in the summer of 2005 [2–4]. Residents were suddenly informed that asbestos, which was used in large amounts from the early 1950s up to the early 1990s in Japan with a maximum usage of approximately 352,000 tons in 1974, caused malignant mesothelioma (MM). Residents that lived near the asbestos handling manufacturer Kubota Corporation, in Amazasaki City, Hyogo Prefecture, developed MM. They had never worked in the asbestos-handling manufacture industry. In addition, medical information regarding MM induced anxiety in the Japanese people, since the prognosis is very poor, and there is no certain way to detect the cancer in the very early stage of the disease. Furthermore, people could not remember being exposed to asbestos 30 to 40 years ago. To reduce the anxieties of the Japanese people, epidemiological analyses regarding the Amagasaki area proceeded, and clinical and basic research was conducted on the biological effects of asbestos and early detection of mesothelioma. It is in this context that the authors

became involved in the project "Comprehensive Approach on Asbestos-Related Diseases", supported by the "Special Coordination Funds for Promoting Science and Technology" (Dr. Takemi Otsuki, Department of Hygiene, Kawasaki Medical School, Kurashiki, Japan) from 2006 to 2010. In this project, a case and clinical specimen registration system was established. A feasibility clinical trial was established and involved a combined trimodality therapy using anticancer chemotherapy with cisplatin and pemetrexed, following by extrapleural pneumonectomy and postoperative radiation therapy for early-stage mesothelioma patients [5, 6]. Furthermore, early detection procedures were developed using serum or pleural effusions to measure soluble mesothelin-related peptide (SMRP) and other markers such as osteopontin, vascular endothelial growth factor (VEGF) and angiopoietin-1 [7–9], as well as procedures for detection of circulating mesothelioma cells and circulating epithelial cells using peripheral blood [10, 11].

For the basic research, the project "Comprehensive Approach on Asbestos-Related Diseases" included three subgroups: (1) analyses of cellular and molecular characteristics using mesothelioma cell lines, (2) investigation of asbestos-induced carcinogenesis using an animal model, and (3) study of the immunological effects of silica/asbestos.

The first subgroup explored novel tumor suppressor gene(s) in mesothelioma cells and found that the serine/threonine-protein kinase (LATS2) gene is inactivated in approximately one-third of mesothelioma cell lines and is a candidate for a novel tumor suppressor in MM [12]. In addition, they found the possibility that the Yes-associated protein (YAP) involved the NF2/Merlin-hippo signaling pathway and that LATS2 may constitutively dephosphorylate and act as an oncogene to bind with the TEAD transcription factor to enhance the cell cycle and resistance to apoptosis [13]. In addition, mesothelioma-specific epigenetic profiles were identified for differential diagnosis with lung adenomatous cancers [14].

The second subgroup confirmed the importance of iron in asbestos-induced carcinogenesis. Findings showed that not only iron-containing crocidolite and amosite, but also chrysotile asbestos caused mesothelioma when these materials were injected into the peritoneal region of a rat. Even individual rats having mesothelioma caused by the injection of iron-absent chrysotile showed numerous depositions of iron in the spleen, liver, and kidney. In addition, adding nitrilotriacetate (NTA) to chrysotile-injected rats induced the acceleration of mesothelioma formation, suggesting the critical participation of iron for asbestos-induced carcinogenesis even for chrysotile. Although the detailed mechanisms of this phenomenon are now being explored, the binding ability of chrysotile to hemoglobin and other proteins and the induced hemolysis is a concern [15–18]. Moreover, the importance of a homozygous deletion of CDKN2A/2B was found in rat mesothelioma with the suggestion that this deletion seems to be fundamental for the development of mesothelioma, since these genes are also known to be homozygously deleted in human mesothelioma [19].

We have performed the third subtheme concerning the "immunological effects of silica/asbestos". In this paper, we introduce our findings and considerations regarding involvement of reduced tumor immunity caused by asbestos exposure to immunocompetent cells as the basic condition in asbestos-exposed people who may develop MM.

2. Immunological Effects of Asbestos

Asbestos comprises a set of six naturally occurring silicate minerals (chrysotile as Serpentine and crocidolite, amosite, actinolite, anthophyllite, and tremolite as Amphibole) exploited commercially for their desirable physical properties. They all have in common their asbestiform structure, possessing long (having more than 1 : 3 aspect ratio, usually approximately 1 : 20) and thin fibrous crystals [20, 21]. Silica (SiO_2) certainly affects the human immune system, because people exposed to silica not only suffer from respiratory disorders known as silicosis, but also experience complications with autoimmune disorders such as rheumatoid arthritis (known as Caplan's syndrome), systemic sclerosis, systemic lupus erythematosus, and antineutrophil cytoplasmic antibody- (ANCA-) related vasculitis/nephritis [22–27]. We have, therefore, been exploring the mechanisms involved in silica-induced dysregulation of autoimmunity using case peripheral blood specimens. We had found that there is dysregulated expression of the CD95/Fas molecule, which is very important for the survival of self-recognizing T cell clones. Additionally, analyses of Fas and Fas-related molecules in silicosis patients suggested that there are two populations of T cells: one is the long-term surviving populations probably including self-recognizing clones, and the other is a population repeating apoptosis caused by silica and recruiting from the bone marrow [28, 29]. In addition, our recent studies regarding CD4+25+ and forkhead box P3 (FoxP3)+ regulatory T cells (Treg) suggested that (1) silica activates both responder T cells (Tresp) and Treg, (2) Tresp chronically-activated by silica becomes CD4+25+ (and programmed cell death-1 (PD-1) + as an activated cell marker) expressers, (3) Treg activated by silica express higher CD95/Fas and are sensitive to Fas-mediated apoptosis, and (4) after the ongoing progression of these events, the composition of the peripheral CD4+25+ fraction in silicosis patients changes to reflect a loss of Treg and a gain of activated Tresp, and this reduction of Treg function results in activation of autoimmunity in silicosis patients [30–32].

Since silica influences the human immune system, its mineral silicate, an asbestos, may also have an effect. As we considered silica's immunological effects from the complications of silicosis and autoimmune diseases, the most important complication of asbestos-exposed people is the occurrence of malignant disease such as MM and lung cancer. In addition, some epidemiological studies suggested a relationship between asbestos exposure and other cancers of the gastrointestinal tract, larynx, kidney, liver, pancreas, ovary, and hematopoietic systems [33–35]. Thus, if asbestos affects the immune system, a reduction of tumor immunity may result and then make people exposed to asbestos

sensitive to the development of malignancies. Of course, asbestos itself possesses carcinogenic activities. As shown in Figure 1, asbestos fibers having iron (or even chrysotile as mentioned above) produce reactive oxygen/nitrogen species (ROS/RNS) causing DNA damage to nearby cells, and fibers are sometimes directly inserted into the cells and injure chromosomes, while retained fibers may adsorb other carcinogens on their surface (known as an asbestos body) [15, 16, 18, 36, 37]. As a result, specific DNA alterations may result, such as inactivation (mostly homozygous deletion) of $p16^{INK4a}/p14^{ARF}$, *NF2/Merlin*, and *LATS2*, and the activation of *YAP* as mentioned above [12, 13]. However, it is difficult to explain why the development of mesothelioma requires 30 to 40 years, and how asbestos-exposed people possess sensitive features for other cancers.

We have been considering that asbestos may affect immunocompetent cells such as CD4+ Tresp, Treg, Th17 T cells, CD8+ cytotoxic T cells (CTL), monocyte-macrophage, natural killer (NK) cells, natural killer T (NKT) cells, and dendritic cells (DC). Firstly, to observe the effects of low-dose and continuous exposure to asbestos (we initially chose chrysotile because this is the most frequently used asbestos in Japan, and our investigations suggested it was not carcinogenic), we employed a human adult leukemia/lymphoma virus-1 (HTLV-1) immortalized polyclonal T cell line, MT-2 [38, 39]. In the next part of this paper, analysis of asbestos exposure to the MT-2 cell line is documented.

3. Transient and Continuous Exposure to Asbestos on a Human T Cell Line

Initially, the cellular alteration of MT-2 cells exposed to transient and high-dose chrysotile was observed to compare various published investigations showing the ability of asbestos exposure to induce ROS production and mitochondrial-pathway-dependent apoptosis in normal alveolar epithelial cells and mesothelial cells, which are the target cells of asbestos-induced carcinogenesis. As shown on the left side of Figure 2, transient and relatively high-dose exposure (25–50 μg/mL, not likely to comprise adhesive cells such as alveolar epithelial or mesothelial cells, since we are using suspended cells, and thus, μg/mL was used instead of μg/cm^2) caused production of ROS as measured by production of O_2^- using flow cytometry, phosphorylation of proapoptotic molecules in the mitogen-activated protein kinase (MAPK) pathway such as p38 and c-Jun N-terminal kinase (JNK), release of cytochrome-c from mitochondria to the cytosol, BAX overexpression, cleavage of caspase-9 and -3, and thereafter the appearance of apoptosis [40]. These findings resembled the effects of asbestos on alveolar epithelia and mesothelial cells [41–44].

We then conducted a trial to establish a low-dose and continuous exposure cell line model by adding 5 or 10 μg/mL of chrysotile (doses which cause apoptosis in less than half of cells exposed transiently) to the MT-2 cell culture. After more than eight months exposure with monthly monitoring for the occurrence of apoptosis, and when these cells were re-exposed to fibers one week after being released from continuously exposed chrysotile, an MT-2 subline which showed resistance to chrysotile-induced apoptosis had been established. As shown on the right side of Figure 2, the continuously exposed subline of MT-2 showed activation of Src-family kinase, increased expression and production of interleukin (IL)-10, phosphorylation of signal transducer and activator of transcription 3 (STAT3) with overexpression of BCL-2 (located downstream of STAT3) [45, 46]. In addition, transforming growth factor (TGF)-β was upregulated [47, 48]. Actually, we had established three independent continuously exposed sublines to chrysotile B and three other sublines exposed to chrysotile A. The altered gene expression of these six continuously exposed sublines in comparison with the original MT-2 cell line was very similar, and the cellular and molecular characteristics of these cell lines in regard to tumor immunity with the *ex vivo* chrysotile exposure model using freshly isolated peripheral blood CD4+ T cells derived from healthy donors was investigated and confirmed using peripheral blood specimens derived from asbestos-exposed patients such as patients with pleural plaque (PP) or MM.

Chemokine receptor, CXC chemokine receptor (CXCR)3, expression and relation with interferon (IFN)-γ.

Using the above-mentioned MT-2 original cell line and the continuously exposed chrysotile sublines, molecules related to tumor immunity were investigated. For example, CXCR3 expression was a focus of investigations, since CXCR3 downregulation in sublines was detected in comparison with the original line using cDNA microarray analysis. It is known that CXCR3 expression and IFN-γ production are induced by T-cell activation and lead to the enhancement of antitumor immune function [49, 50].

From findings using the MT-2 cell line model, as shown in Figure 3(a), all six continuously exposed sublines showed reduced CXCR3 expression on their surface and mRNA expression levels with reduced production and expression of IFN-γ. Production of the Th1-type CXCR3 ligand CXCL10/IP10 was also significantly reduced in all six continuously exposed sublines when compared with the original line. In addition, another Th1-type chemokine, CCL4/MIP-1β mRNA, was also expressed at low levels in all six sublines compared with the MT-2 original line as previously reported. However, CCR5, the Th1-type receptor for CCL4/MIP-1β, was not reduced significantly through mRNA expression in MT-2Rsts cells. These results indicated that continuous exposure of MT-2 original cells to asbestos altered the expression of Th1-related chemokines (CXCL10/IP10 and CCL4/MIP-1β) and chemokine receptors (CXCR3) [51].

Thereafter, we tried to determine whether freshly isolated human peripheral CD4+ T cells show a similar alteration *ex vivo* when proliferation is maintained by IL-2-containing medium in the presence of chrysotile as shown in Figure 3(b). After 40 days of coculture supplemented with IL-2 in the presence or absence of chrysotile, cell surface CXCR3 expression decreased in a dose-dependent manner. Thus, we examined cell surface expression of CXCR3 and CCR5 in CD4+ T cells derived from six healthy donors, since both receptors are preferentially expressed in Th1/effector T cells. The expression of CXCR3 was significantly reduced

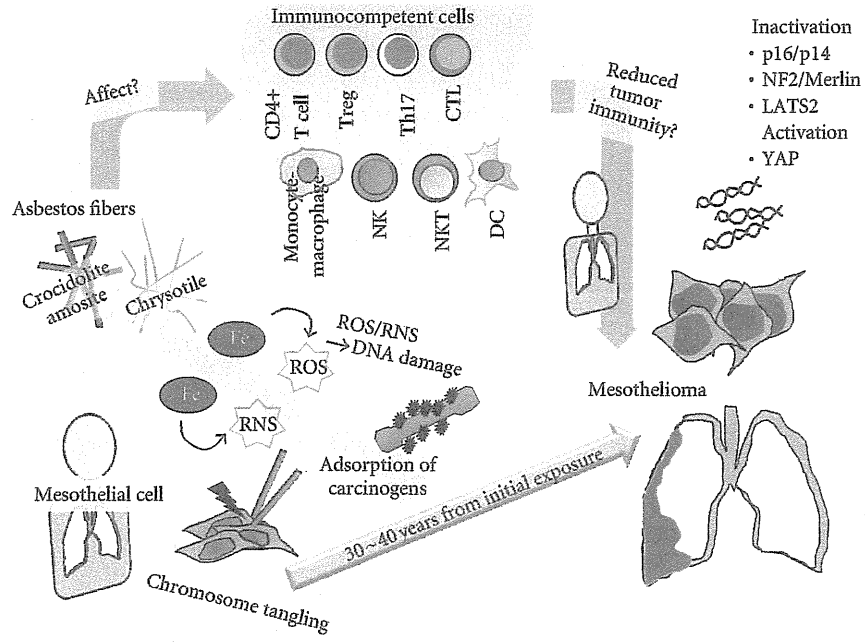

FIGURE 1: Schematic model showing mechanisms of asbestos-induced carcinogenesis and genomic/epigenetic changes found in mesothelioma cells and the relationship of the immunological effects of asbestos in regard to reduced tumor immunity.

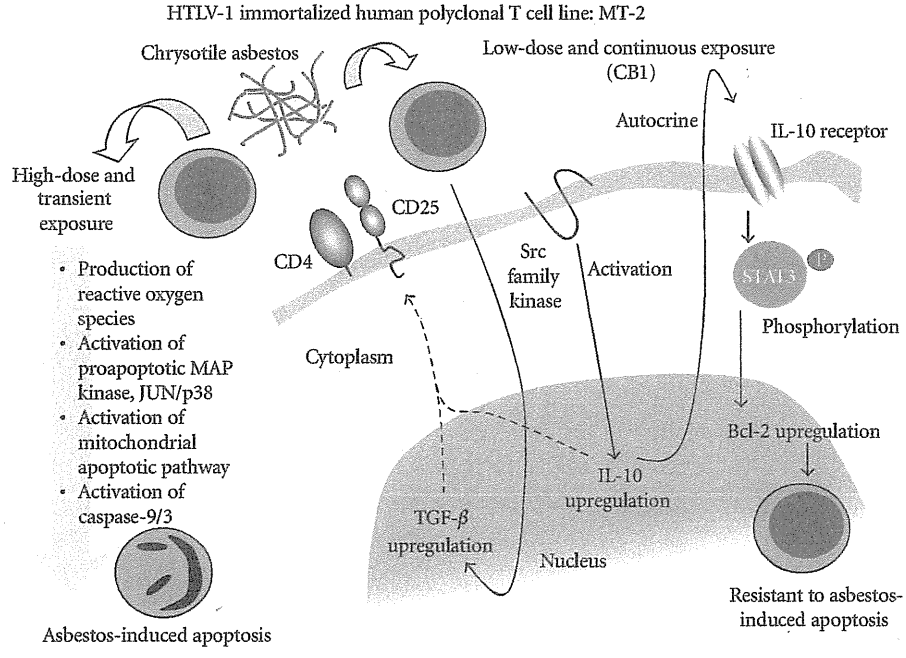

FIGURE 2: Summarized findings of cellular and molecular events caused by high-dose and transient exposure (left side) and lowdose and continuous exposure (CB1: one of the sublines established) (right side) to chrysotile asbestos using an HTLV-1 immortalized human polyclonal T cell line, MT-2.

following exposure to 10 μg/mL of chrysotile for 28 days although this difference seemed to depend on one case in which the expression decreased remarkably. Even if the culture conditions for the CD4+ T cells was limited to a period of around four weeks, four of the six healthy donors showed a decrease of CXCR3 expression to various degrees, and it might be concluded that asbestos exposure potentiates reduction of CXCR3 expression in CD4+ T cells. On the other hand, the expression of CCR5 varied among all healthy donors, and there were no significant changes after

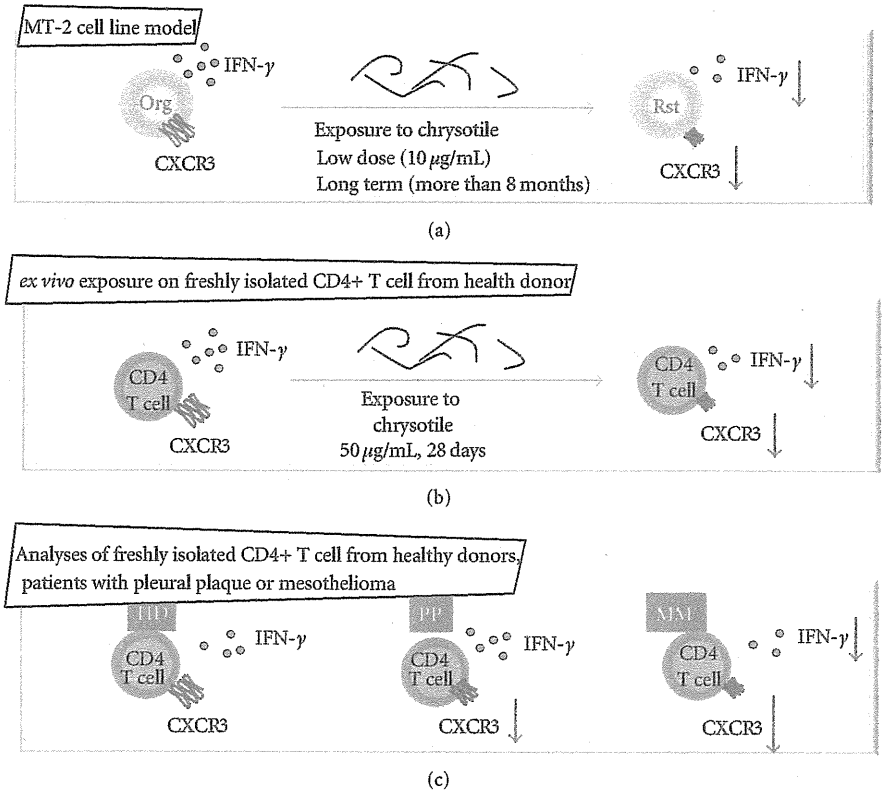

FIGURE 3: Schematic representation of asbestos-induced reduction of expression of a chemokine receptor, CXCR3, and expression and production of IFN-γ using the MT-2 cell line model (Org; MT-2 original cell line, and Rst: sublines exposed continuously to a low-dose of chrysotile), an *ex vivo* exposure model using freshly isolated CD4+ T cells from healthy donors (HD), as well as analyses of freshly isolated CD4+ T cells from healthy donors and patients with pleural plaque (PP) and malignant mesothelioma (MM).

seven and 28 days of coculture with chrysotile, as shown previously by the cell line model. These results indicated that CXCR3 expression might be specifically reduced by asbestos exposure. In addition, these experiments revealed decreased IFN-γ expression and production when CD4+ T cells from healthy donors were cultured with chrysotile for 28 days [52].

Finally, analyses of changes in surface CXCR3 expression on freshly isolated CD4+ T cells from asbestos-exposed patients such as PP or MM were compared with those from health donors. In addition, IFN-γ expression of CD4+ T cells from these patients and healthy donors was measured with stimulation using anti-CD3/CD28 antibodies with IL-2. As summarized in Figure 3(c), CXCR3 expression was reduced in CD4+ T cells from asbestos patients. A comparison of PP and MM patients showed that the expression level of CXCR3 on CD4+ T cells from MM was decreased although the difference was not statistically significant. Moreover, IFN-γ expression was only reduced in stimulated CD4+ T cells from MM patients, not in those from PP patients [52].

With the findings that CD4+CXCR3+ T cells in lymphocytes from MMs showed a tendency for an inverse correlation with CXCR3's ligand, CXCL10/IP10 in plasma, our results indicate a reduction of tumor immune function in asbestos-exposed patients and suggest that CXCR3, IFN-γ, and CXCL10/IP10 may be candidates to detect and monitor disease status.

4. Alteration of NK Cells and Others

As shown in Figure 4, the effects of asbestos on other immunocompetent cells such as Treg, CD8+ CTL, and NK cells were investigated. As mentioned above with the MT-2 cell line model, sublines continuously exposed to chrysotile showed overproduction of IL-10 and TGF-β. It is well known that these cytokines are a typical soluble factor produced from Treg to function with a suppressive effect on activated responder T cells. On the other hand, it is also reported that MT-2 cells possess a Treg function, since cells express CD4 and CD25 with nuclear expression of FoxP3. Taken together, continuous exposure to chrysotile produces a stronger Treg function, at least with the capacity to produce soluble functional factors (i.e., IL-10 and TGF-β) [47, 48]. At present, we have been studying alteration of Treg function using the MT-2 cell line model, and preliminary findings indicate asbestos may enhance Treg function.

In regard to tumor immunity, CD8+ CTL and NK cells are very important players, since they directly kill tumor cells even when individually restricted with major histocompatibility complexes. Investigations have just started with

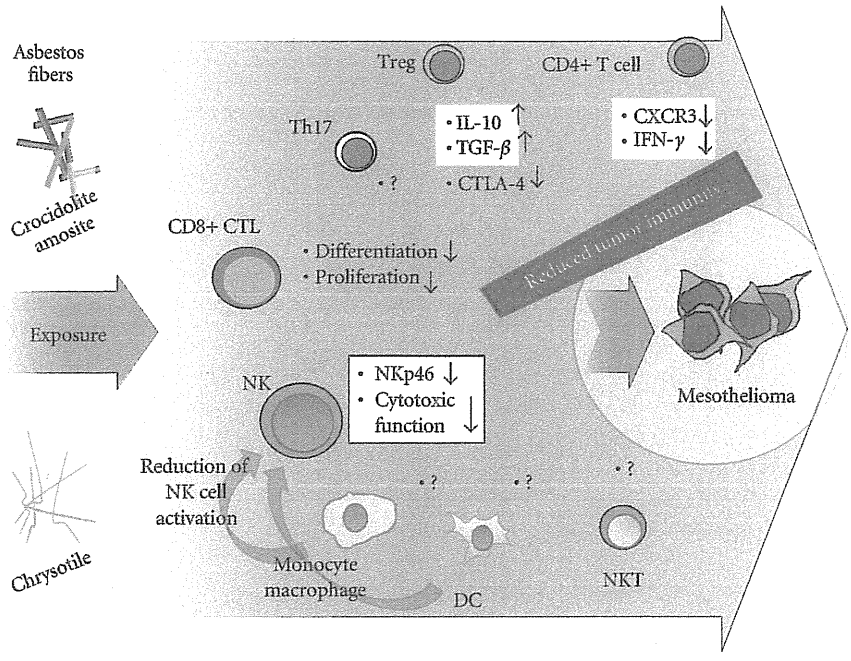

FIGURE 4: Schematic representation of findings showing asbestos-induced reduction of tumor immunity on CD4+ T cells, CD4+25+FoxP3+ regulatory T cells (Treg), T helper (Th)17, CD8+ cytotoxic T cells (CTL), natural killer (NK) cells, monocyte-macrophage, dendritic cells (DC), and natural killer T cells (NKT).

CD8+ CTL, but in *ex vivo* experimental conditions designed to produce CD8+ CTL proliferation and differentiation using freshly isolated peripheral blood mononuclear cells from healthy donors, the addition of asbestos seems to result in reduced proliferation and differentiation of CTL. Although detailed analyses concerning the roles of cytokines surrounding CTL differentiation are being performed, our ongoing studies suggest that asbestos reduces CTL activities.

Regarding NK cells, cellular and molecular analyses have been conducted using a human NK cell line, YT-A1, exposed continuously to asbestos in an *ex vivo* exposure model using freshly isolated NK cells from health donors, as well as asbestos-exposed patients such as PP and MM.

Focusing on the NK cell-activating receptors, including NKG2D (also known as KLRK1 (killer cell lectin-like receptor subfamily K, member 1), klr and CD314, binding to a diverse family of ligands that include MHC class I chain-related A and B proteins and UL-16 binding proteins, where ligand-receptor interactions can result in the activation of NK and T cells), 2B4 (also known as NAIL; Nmrk; NKR2B4; SLAMF4 and CD244, mediate nonmajor histocompatibility complex (MHC) restricted killing), and NKp46 (also known as NCR1 (natural cytotoxicity triggering receptor 1), LY94 and CD335, constituting a natural cytotoxic receptor family with NKp44 and NKp30, and being important in killing tumor cells and dendritic cells), the YT-A1 human NK cell line exposed continuously to chrysotile asbestos reveal-ed reduced expression of NKG2D and 2B4 [53]. The reduced phosphorylation of extracellular signal-regulated kinase (ERK) and subsequent reduction of degranulation of perforin and granzyme B resulting from reduced cytotoxicity were observed in this cell line model [54]. Similar to the cell line model, the *ex vivo* exposure model using freshly isolated NK cells from healthy donors revealed a reduction of NKp46 expression. Furthermore, freshly isolated NK cells from MM patients showed reduced killing function compared with those from healthy donors and revealed a lower expression of NKp46 [53]. Moreover, the expression level of NKp46, but not NKG2D or 2B4, and the cytotoxic activity of individual freshly isolated NK cells from health donors and MM patients clearly showed a reverse correlation, indicating that the target molecule of asbestos-induced dysfunction of NK cells is NKp46 [54]. Although further analyses are required regarding the interaction between asbestos-exposed NK cells and other immunocompetent cells such as dendritic cells, monocytes, and macrophages, molecular mechanisms to reduce NKp46 expression and other aspects need to be explored, and surface NKp46 expression levels may be the candidate to monitor the level of tumor immunity in asbestos-exposed patients [55].

Further investigations are needed to examine the effects of asbestos exposure on other types of immunocompetent cells such as Th17 dendritic cells, NKT, and the monocyte-macrophage lineage, and to investigate why asbestos seems to reduce tumor immunity in the total network of the immunological surveillance system.

In addition, although we have mainly analyzed the effects of chrysotile asbestos on the human immune system,

differences and similarities between the different types of fibers should also be investigated.

5. Conclusion

We have been investigating the effects of asbestos exposure on the human immune system in regard to tumor immunity and found that people exposed to asbestos possess reduced tumor immunity, making them sensitive to cancer development. Although these studies may contribute to the clear recognition of the biological effects of asbestos, the variety of alterations in immunocompetent cells may be the factor that allows detection of previous asbestos exposure and the occurrence of cancer in people that live or have lived near asbestos-handling manufacturers. Furthermore, to recover tumor immunity using physiologically active substances in foods or derived from plants may be an effective method for the chemical prevention of asbestos-induced cancers.

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Decreased CXCR3 Expression in CD4$^+$ T Cells Exposed to Asbestos or Derived from Asbestos-Exposed Patients

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Asbestos causes malignant tumors such as lung cancer and malignant mesothelioma (MM). To determine whether asbestos exposure causes reduction of antitumor immunity, we established an *in vitro* T-cell line model of low-dose and continuous exposure to asbestos using an human adult T-cell leukemia virus–1 immortalized human polyclonal T-cell line, MT-2, and revealed that MT-2 cells exposed continuously to asbestos showed resistance to asbestos-induced apoptosis. In addition, the cells presented reduction of surface CXCR3 chemokine receptor expression and IFN-γ production. In this study, to confirm that these findings are suitable for clinical translation, surface CXCR3 and IFN-γ expression were analyzed using freshly isolated human CD4$^+$ T cells derived from healthy donors and patients with pleural plaque (PP) or MM. The results revealed that CXCR3 and IFN-γ expression in the *ex vivo* model were reduced in some cases. Additionally, CXCR3 expression in CD4$^+$ T cells from PPs and MMs was significantly reduced compared with that from healthy donors, and CD4$^+$ T cells from patients with MMs exhibited a marked reduction in IFN-γ mRNA levels after stimulation *in vitro*. Moreover, CD4$^+$CXCR3$^+$ T cells in lymphocytes from MMs showed a tendency for an inverse correlation with its ligand CXCL10/IP10 in plasma. These findings show reduction of antitumor immune function in asbestos-exposed patients and indicate that CXCR3, IFN-γ, and CXCL10/IP10 may be candidates to detect and monitor disease status.

Keywords: asbestos; pleural plaque; malignant mesothelioma; CXCR3; IFN-γ

CLINICAL RELEVANCE

In this study, we show that chrysotile asbestos has the potential to reduce chemokine receptor CXCR3 expression in human peripheral CD4$^+$ T cells using an *ex vivo* model, and CXCR3 expression is decreased in peripheral CD4$^+$ T cells from patients with asbestos-related diseases, such as pleural plaque or malignant mesothelioma. Our findings suggest that antitumor immunity might not function normally in patients with asbestos-related disease because the low expression of CXCR3 induces depressed chemotaxis and indicate that CXCR3 may be effective tool for the prediction of impaired antitumor immune status in these patients.

The cellular and molecular biologic effects of asbestos fibers on alveolar epithelial cells and pleural mesothelial cells have been investigated using various animal models and culture cells (1–5). Studies of fibrogenesis have shown that the initial recognition of asbestos by alveolar macrophages (AMs) is important, and AM-releasing cytokines, such as TNF-α, TGF-β, and IL-1β and IL-8, play a critical role in activating mesenchymal cells to induce the proliferation of collagen fibers (5, 6). Moreover, recent advances resulting from cellular and molecular investigations regarding activation of the nucleotide oligomerization domain–like receptor family, the pryin domain–containing 3 (NLRP3)-inflammasome caused by asbestos, are leading to a better understanding of this recognition (7–9). Reactive oxygen species and reactive nitrogen species play important roles in carcinogenesis (1–6), in which substances that damage DNA are generated through the activity of iron contained in the asbestos. Alveolar and mesothelial cells exposed to asbestos then develop apoptosis with activation of a mitochondrial pathway and a B-cell lymphoma 2 (Bcl-2)-associated x protein (Bax) dominant balance of the Bax/Bcl-2 complex, cytosomal release of cytochrome-c from mitochondria, and activation of caspase 9 and 3 (1–6).

The above findings are based on studies using animal models and cell culture experiments in which exposure conditions were temporary and involved high doses. In human asbestos-related diseases, the latency is quite long and may range from 10 to 30 years for fibrogenic changes known as asbestosis and around 40 years for malignant mesothelioma (MM) (10–12).

To investigate the immunological effects of asbestos on human immunocompetent cells, we constructed an *in vitro* experimental T-cell line model of continuous asbestos exposure using a human adult T-cell leukemia virus type 1–immortalized human polyclonal T-cell line (MT-2) because this cell line is not tumor-cell derived and is reported to have a normal karyotype (13–16). In our experiments, chrysotile was used as the asbestos because it may be important to compare this substance with silica (SiO_2), which causes silicosis and alteration of autoimmunity (17, 18). In addition, the dose of chrysotile used in industries around the world is much higher than that of other fibers, and carcinogenicity is considered much lower for chrysotile (10–12).

As we reported recently, our experimental models, which use the MT-2 original cell line and six sublines exposed to chrysotile independently and continuously, revealed that all of the sublines exhibited reduction of surface expression of CXC chemokine receptor 3 (CXCR3) and production of IFN-γ (19). It is well known that IFN-γ is important in antitumor immunity

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and antiviral immunity (20–24). In addition, CXCR3 is thought to be involved in antitumor immune function. Some reports have investigated the state of chemokines surrounding mesothelioma tumor cells (25–28). It has been found that the ligand of CXCR3, C-X-C motif chemokine 10 (CXCL10)/IFN-γ–induced protein 10 kD (IP10), is present in pleural fluid collected from patients with MM and that human mesothelioma tumors are abundantly infiltrated with $CD4^+$ T cells (29). Furthermore, CXCL10/IP10 mRNA expression is significantly higher in MM compared with normal mesothelial cell lines and pleural mesothelium (30). CXCL10/IP10 inhibits tumor growth and metastasis through a decrease in tumor-associated angiogenesis and recruits Th1/effector T cells by binding the chemokine receptor CXCR3 (20, 31, 32). Therefore, it is important to regulate lymphocyte transport mediated by chemokine receptors to enhance antitumor immune responses because CXCR3-positive Th1/effector $CD4^+$ T cells are recruited to CXCL10/IP10-enriched tumor sites, where IFN-γ is secreted and subsequent suppression of tumor growth is induced (33).

We examined CXCR3 and IFN-γ expression in $CD4^+$ T cells co-cultured with chrysotile and showed the potential of chrysotile to inhibit CXCR3 and IFN-γ expression. In addition, we investigated CXCR3 and IFN-γ expression in $CD4^+$ T cells from healthy donors (HDs) and asbestos-exposed patients with pleural plaque (PP) or MM and found low-level expression of CXCR3 in $CD4^+$ T cells from patients with PPs and MMs and decreased mRNA levels of IFN-γ in cultured $CD4^+$ T cells from patients with MMs. These findings highlight parameters that are effective in predicting the risk of impaired antitumor immune status in patients with PP and MM.

MATERIALS AND METHODS

Ex Vivo Activation and Exposure to Asbestos on Human $CD4^+$ T Cells

After receiving informed consent, blood samples were obtained from six HDs (mean age, 34.8 ± 9.7 yr; range, 25–50 yr; three men and three women), and peripheral blood mononuclear cells (PBMCs) were isolated using the Ficoll-Paque method. $CD4^+$ T cells were isolated by positive selection using anti-CD4–coated beads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in accordance with the manufacturer's instructions. Freshly isolated $CD4^+$ T cells (2×10^5 cells/well) in 96-well, U-bottomed plates were stimulated with anti-CD3 (clone UCHT1) and anti-CD28 (clone CD28.2) antibodies (Beckman Coulter, Inc., Fullerton, CA) at 2 μg/ml and cultured in RPMI 1640 medium containing 10% FBS and recombinant IL-2 (rIL-2) (10 ng/ml) (PeproTech Inc., Rocky Hill, NJ). After 3 days, activated $CD4^+$ T cells were transferred to 24-well plates at 1×10^6 cells/well and expanded in medium supplemented with 10 ng/ml rIL-2 for 1 week and further cultured in the absence or presence of 2 to 10 μg/cm² of chrysotile-B. Chrysotile-B was kindly provided by the Department of Occupational Health, National Institute for Occupational Health, South Africa (34). Before analysis, cultured $CD4^+$ T cells were released from chrysotile-B by the Ficoll-Paque method and cultured for 3 days. For intracellular staining, cultured $CD4^+$ T cells (1×10^6 cells/ml) were stimulated with 5 ng/ml phorbol 12-myristate 13-acetate and 250 ng/ml ionomycin for 6 hours in the presence of monesin.

Flow Cytometry

Surface antigens were stained as reported previously (19) with the following antibodies: anti-CD4 (clone RPA-T4), CXCR3 (clone 1C6), and CC chemokine receptor 5 (CCR5) (clone 2D7) (BD Biosciences Pharmingen, San Diego, CA). Intracellular staining was performed using a Cytofix/Cytoperm kit (BD Biosciences) in accordance with the manufacturer's instructions. Cells were stained with anti–IFN-γ (clone 4S.B3) antibody (BD Biosciences Pharmingen) for 30 minutes at 4°C and analyzed on a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ).

Patients and Isolation of PBMCs

Blood from a different series of 19 HDs (mean age, 42.3 ± 12.8 yr; range, 29–70 yr; 9 men and 10 women), 13 patients with PP (mean age, 71.5 ± 5.3 yr; range, 58–79 yr; 11 men and 2 women), and eight patients with MM (mean age, 67.4 ± 8.5 yr; range, 59–79 yr; seven men and one woman) was collected in citrate phosphate dextrose. Plasma was collected from supernatants after centrifugation at 3,000 rpm for 5 minutes. PBMCs were isolated as described above and subjected to FACS analysis. For analysis of mRNA expression and cytokine production, $CD4^+$ T cells were isolated as described above. Freshly isolated $CD4^+$ T cells were cultured in RPMI 1640 medium containing 10% FBS at 2×10^4 cells/100 μl in 96-well, U-bottomed plates and stimulated with anti-CD3 and anti-CD28 antibodies at 2 μg/ml. After 5 days, the supernatants and cells were collected. Informed consent was obtained from all donors, and the project and procedures used were approved by the Institutional Ethics Committees of Kawasaki Medical School and Okayama Rosai Hospital.

RESULTS

Changes of CXCR3 Cell Surface Expression in $CD4^+$ T Cells Cultured with Chrysotile-B

As reported recently, our *in vitro* T-cell line model of low-level and continuous exposure to chrysotile asbestos showed reduction of Th1-type cell surface expression of CXCR3 (19). Therefore, we tried to determine whether freshly isolated human peripheral $CD4^+$ T cells show a similar alteration *ex vivo* when proliferation is maintained by IL-2–containing medium in the presence of chrysotile-B. After 40 days of co-culture, cell surface CXCR3 expression decreased in a dose-dependent manner (2–10 μg/cm²) (Figure 1A). Thus, we examined cell surface expression of CXCR3 and CCR5 in $CD4^+$ T cells derived from six HDs because both receptors are preferentially expressed in Th1/effector T cells. The expression of CXCR3 was significantly reduced after exposure to 10 μg/cm² of chrysotile for 28 days, although this difference seemed to depend on one case in which the expression decreased remarkably (Figure 2, *upper panel*). Even if the culture conditions for the $CD4^+$ T cells was limited to a period of around 4 weeks, four of six HDs showed a decrease of CXCR3 expression to various degrees, and it might be concluded that asbestos exposure potentiates reduction of CXCR3 expression in $CD4^+$ T cells. On the other hand, the expression of CCR5 varied among all HDs, and there were no significant changes after 7 and 28 days of co-culture with chrysotile-B (Figure 1B, *lower panel*), as shown previously by the cell line model (19). These results indicated that CXCR3 expression might be specifically reduced by asbestos exposure.

IFN-γ Expression under *Ex Vivo* Exposure Conditions

We also showed previously that IFN-γ production in the T-cell line model was decreased by chronic exposure to chrysotile (19). Thus, we examined intracellular expression of IFN-γ in $CD4^+$ T cells exposed to chrysotile-B (10 μg/cm²) from three healthy subjects under the same *ex vivo* exposure conditions used for the analysis of surface CXCR3 expression. After 28 days of co-culture, mRNA expression of IFN-γ was reduced in all three cases, although there were no significant differences ($P = 0.3226$) (Figure 2A). In addition, intracellular staining showed that IFN-γ–positive cells tended to be reduced in $CD4^+$ T cells from all three subjects when cultured with chrysotile-B ($P = 0.0511$) (Figure 2B).

These findings from the *ex vivo* study indicated that chronic exposure to asbestos reduced CXCR3 and IFN-γ expression in $CD4^+$ T cells, although the reductions were not statistically significant.

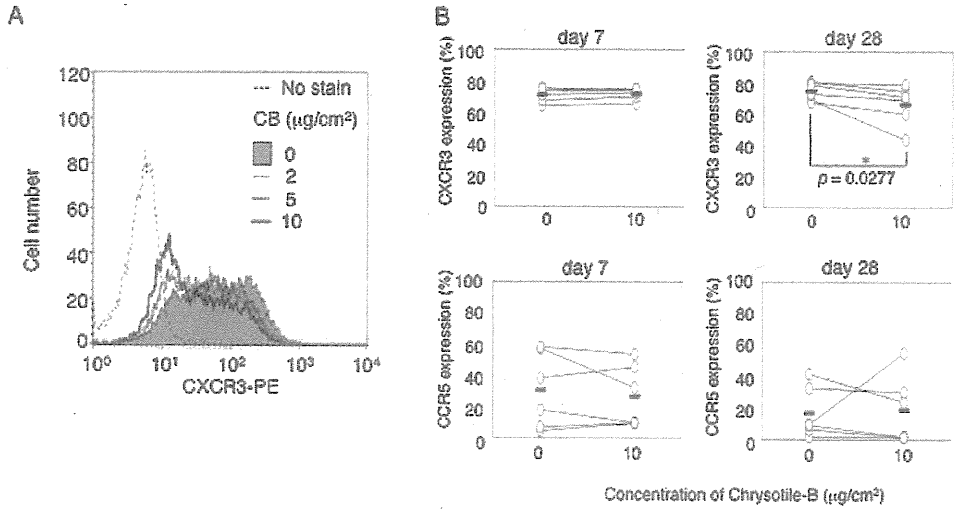

Figure 1. Chronic exposure to chrysotile reduces expression of CXCR3 in IL-2–cultured human CD4+ T cells. Peripheral CD4+ T cells from healthy donors were stimulated with anti-CD3/CD28 antibodies and maintained with IL-2–containing medium. Ten days later, exposure to chrysotile-B (CB) was initiated. (*A*) Histogram shows representative example of CXCR3 expression on CD4+ T cells after 40 days of exposure to various doses of CB. (*B*) Graphs show expression of CXCR3 (*upper panel*) and CCR5 (*lower panel*) after 7 and 28 days of culture in the absence or presence of 10 μg/cm² CB. Data are presented for six healthy subjects. The *P* value was obtained using the Wilcoxon signed-rank test. $^*P < 0.05$.

Expression of Cell Surface CXCR3 in Human CD4+ T Cells from Patients with Asbestos-Related Disease

To investigate whether peripheral CD4+ T cells in people exposed to asbestos also show reduction of CXCR3, we analyzed cell surface CXCR3 and CCR5 expression in peripheral CD4+ T cells from patients with asbestos-related PP and MM by flow cytometry (Figure 3A). Given that the population of lymphocytes in MMs was significantly lower than that in the other groups (Figure 3B, *left panel*), gated lymphocytes were analyzed for CXCR3 and CCR5 expression in CD4+ T cells. The percentage of CD4+ T cells in lymphocytes revealed no differences among the three groups (Figure 3B, *right panel*).

The percentage of CD4+CXCR3+ T cells in lymphocytes from PPs and MMs were significantly lower than that of HDs, and MMs showed the lowest percentage compared with the other groups (Figure 3C, *left panel*). In contrast, the percentage of CD4+CCR5+ T cells in lymphocytes showed no differences (Figure 3C, *right panel*). Furthermore, the expression of CXCR3 in CD4+ T cells from PPs and MMs was significantly lower than that of HDs (Figure 3D, *left panel*), although CCR5 expression in CD4+ T cells showed no differences (Figure 3D, *right panel*).

To confirm whether there is an aging effect concerning decreased expression of CXCR3, 19 HDs were divided into two groups: younger (mean age, 34.9 ± 5.8 yr; range, 25–45 yr; $n = 13$) and older (mean age, 58.3 ± 7.6 yr; range, 51–70 yr; $n = 6$). The difference between these groups regarding age was significant (Figure 4A). However, our findings indicated that there were no significant differences in the percentage of CD4+CXCR3+ T cells in lymphocytes (Figure 4B, *upper panel*) and CXCR3 expression in CD4+ T cells (Figure 4B, *lower panel*) between the two groups. Thus, these results confirmed that aging is not associated with a reduction of CXCR3 expression and that decreased expression of CXCR3 in peripheral CD4+ T cells occurs in people exposed to asbestos.

IFN-γ mRNA Levels in Stimulated Peripheral CD4+ T Cells from Patients with Asbestos-Related Diseases

Real-time RT-PCR was used to detect the mRNA levels of IFN-γ in stimulated peripheral CD4+ T cells from 13 HDs, 13

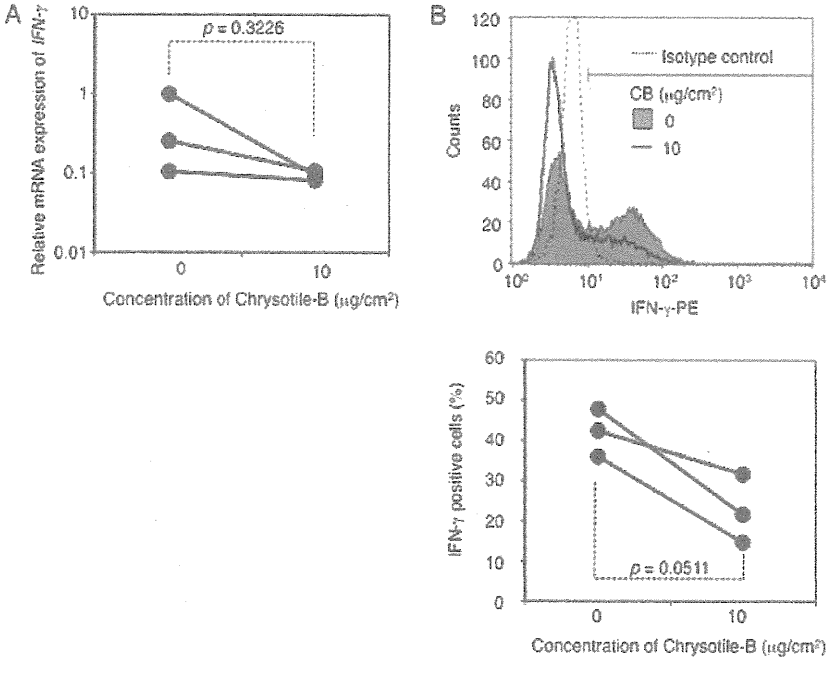

Figure 2. IFN-γ production in IL-2–cultured human CD4+ T cells from patients showing reduced CXCR3 expression by exposure to chrysotile. CD4+ T cells from three subjects were activated with anti-CD3/CD28 antibodies and cultured with IL-2–containing medium in the absence or presence of 10 μg/cm² CB for 30 days. (*A*) IFN-γ mRNA in subjects who showed decreased expression of surface CXCR3 in CD4+ T cells by CB exposure was measured by real-time RT-PCR. (*B*) Histogram shows representative staining for intracellular IFN-γ. Graph indicates the percentage of IFN-γ–positive cells. Data are shown for three subjects. The *P* value was obtained using the paired *t* test. $^*P < 0.05$.

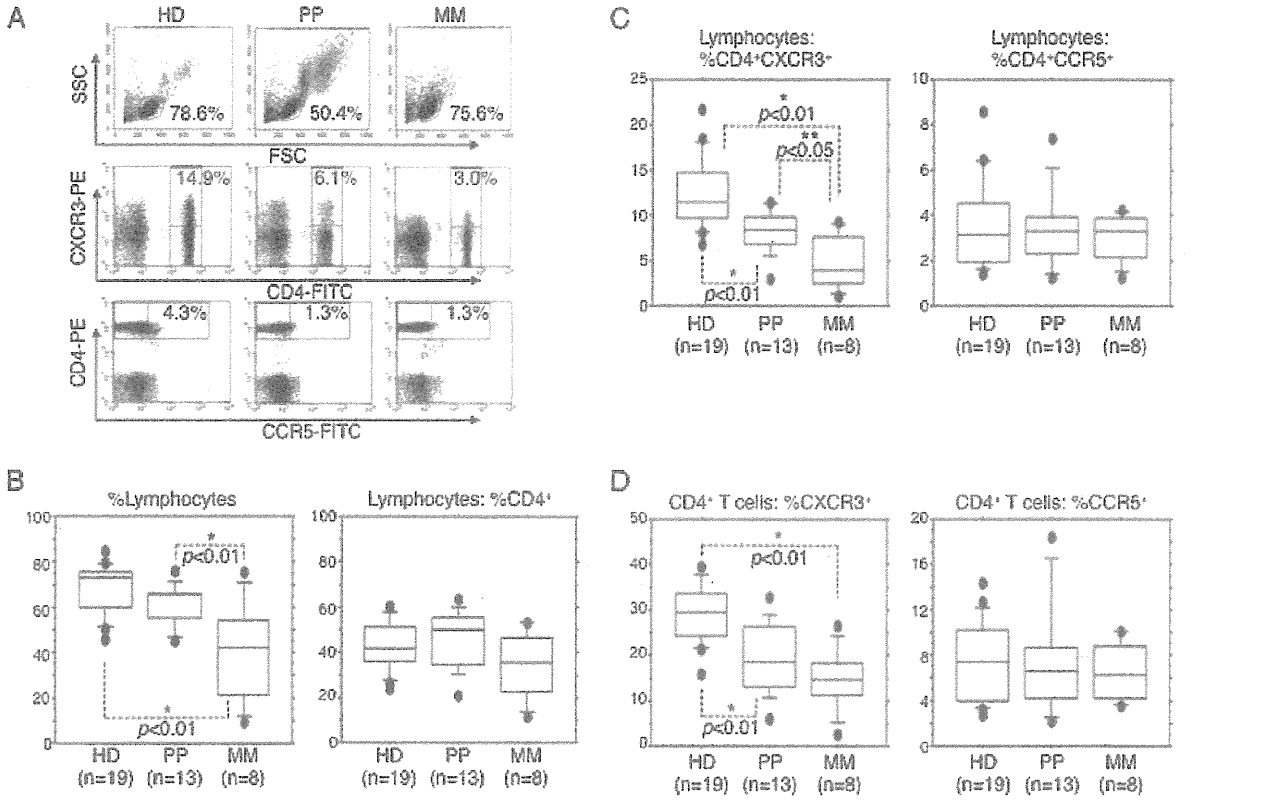

Figure 3. Peripheral $CD4^+$ T cells in patients with pleural plaque (PP) or malignant mesothelioma (MM) show decreased CXCR3 expression. (*A*) A forward/side scatter dot plot was used to gate lymphocytes, and CXCR3 and CCR5 expressions in peripheral $CD4^+$ T cells from HDs (n = 19), PPs (n = 13), and MMs (n = 8) were analyzed by FACS. Representative FACS profiles are indicated. Numbers in each dot plot indicate percentage of lymphocytes (*top*), $CD4^+CXCR3^+$ T cells (*middle*), and $CD4^+CCR5^+$ T cells (*bottom*). (*B*) The percentage of gated lymphocytes in total cells (*left*) and $CD4^+$ T cells in gated lymphocytes (*right*). (*C*) The percentage of $CD4^+CXCR3^+$ T cells (*left*) and $CD4^+CCR5^+$ T cells (*right*) in gated lymphocytes. (*D*) The percentage of CXCR3-positive (*left*) and CCR5-positive (*right*) cells in gated $CD4^+$ T cells. The box plot shows 10% (*lower whisker*), 25% (*lower hinge of box*), the median (*line in the box*), 75% (upper hinge of box), 90% (*upper whisker*), and the extreme points (*black dots*). The *P* value was obtained using the Student-Newman-Keuls test. $^*P < 0.01$; $^{**}P < 0.05$.

PPs, and 7 MMs. mRNA expression levels of IFN-γ relative to GAPDH were significantly decreased only in MMs compared with HDs and PPs (Figure 5A). Additionally, the Spearman's rank correlation test showed that there was no significant correlation between levels of CXCR3 expression in peripheral $CD4^+$ T cells and IFN-γ mRNA levels of stimulated $CD4^+$ T cells in all three groups (Figures 5B–5D), although HDs showed a tendency for a positive correlation (P = 0.1295) and PPs showed a tendency for a negative correlation (P = 0.0502). Unlike suppressed CXCR3 expression, these results suggested that decreased mRNA expression of IFN-γ was dependent on the occurrence of mesothelioma and was not necessarily associated with asbestos exposure.

IFN-γ and Production of other Cytokines in Human $CD4^+$ T Cells from Patients with Asbestos-Related Disease

The cytometric bead array assay was performed to measure the concentration of Th1/Th2/Th17 cytokine (IL-2, IL-4, IL-6, IL-10, TNF-α, IFN-γ, and IL-17A) in the supernatants from cultured peripheral $CD4^+$ T cells. There were no significant differences in IFN-γ production from cultured peripheral $CD4^+$ T cells (Figure 6), although there were statistically significant differences in IFN-γ mRNA expression (Figure 5A). Thus, the expected result for IFN-γ–producing activity was not obtained under these conditions. The results for other cytokines showed that the IL-6 concentration in PPs and MMs was significantly higher than that of HDs (Figure 6). Although the IL-4 concentration in MMs was significantly higher than that of HDs, the level might be too low for the cytokine to exert its physiological function (Figure 6).

Correlation between the Percentage of $CD4^+CXCR3^+$ T Cells in Lymphocytes and Levels of Plasma CXCL10/IP10

Because the ligand for CXCR3, CXCL10/IP10, is secreted not only from monocytes, endothelial cells, and fibroblasts (20, 31, 32) but also from mesothelial cells and transformed (mesothelioma) cells (30), the plasma levels of CXCL10/IP10 in HDs, PPs, and MMs were measured by CBA assay. The levels of plasma CXCL10/IP10 had no correlation with CXCR3 expression in $CD4^+$ T cells (data not shown). Therefore, the correlation between the percentage of $CD4^+CXCR3^+$ T cells in lymphocytes and plasma chemokine levels was determined using Spearman's rank correlation test. The plasma concentration of CXCL10/IP10 in PPs or MMs tended to be higher when compared with that of HDs, although there were no significant differences (Figure 7A). Therefore, there was no significant correlation between the percentage of $CD4^+CXCR3^+$ T cells and CXCL10/IP10 concentration in all three groups (Figures 7B–7D), although MMs showed a tendency for an inverse correlation (P = 0.081) in comparison with HDs (P = 0.927) and PPs (P = 0.578).

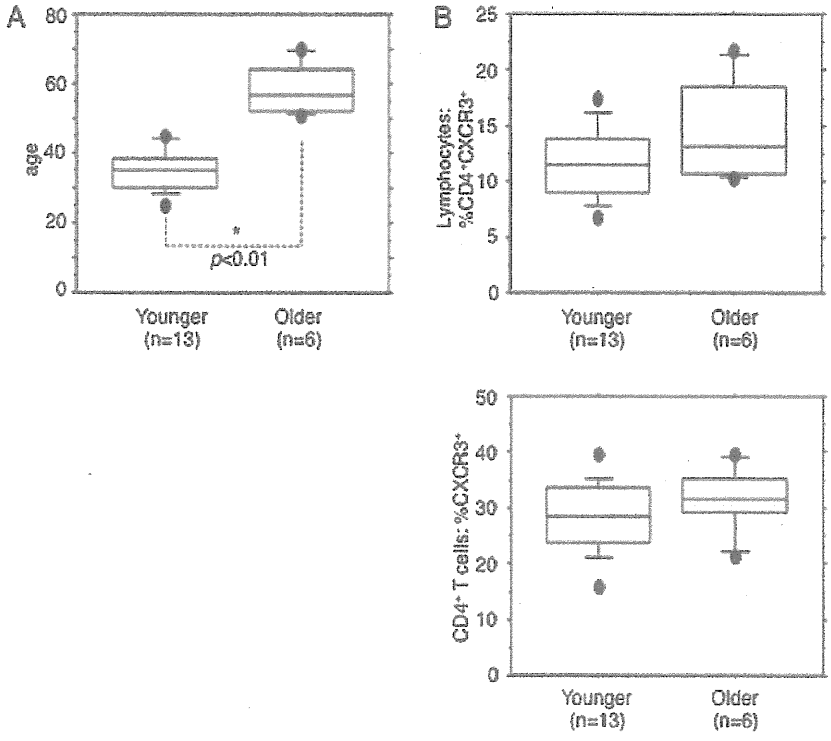

Figure 4. Age of subjects does not affect CXCR3 expression in CD4$^+$ T cells. (*A*) Nineteen HDs were divided into younger (< 50 yr old; n = 13) and older (⩾ 50 yr old; n = 6) groups. (*B*) The percentage of CD4$^+$CXCR3$^+$ T cells in gated lymphocytes (*top*) and CXCR3-positive cells in gated CD4$^+$ T cells (*bottom*). The box plot is presented as described in Figure 3. The P value was obtained using the Student-Newman-Keuls test. *$P < 0.01$.

DISCUSSION

There are few publications regarding the effects of asbestos on the general immune system, particularly antitumor immunity. However, the mechanisms by which local immune cells, such as alveolar macrophages, recognize asbestos fibers have been investigated, particularly the discovery of the NLRP3–inflammasome and its ability to recognize and process asbestos fibers and activate IL-1β and caspase 1 (7–9). Asbestos is a mineral silicate that contains magnesium, iron, and calcium. Patients exposed to silica particles may develop a disease known as silicosis, which is often complicated with autoimmune diseases such as rheumatoid arthritis (known as Caplan's syndrome), systemic sclerosis, and anti–neutrophil cytoplasmic autoantibody–related vasculitis/nephritis (35–37). However, the most important complication resulting in asbestos-exposed patients is the occurrence of cancer, such as lung cancer and MM (10–12). In particular, MM is known to be caused by low-level and long-term exposure to asbestos (10–12).

Therefore, asbestos fibers may affect the human immune system, particularly antitumor immunity. In fact, we have been reporting impaired NK cell function with reduced expression of

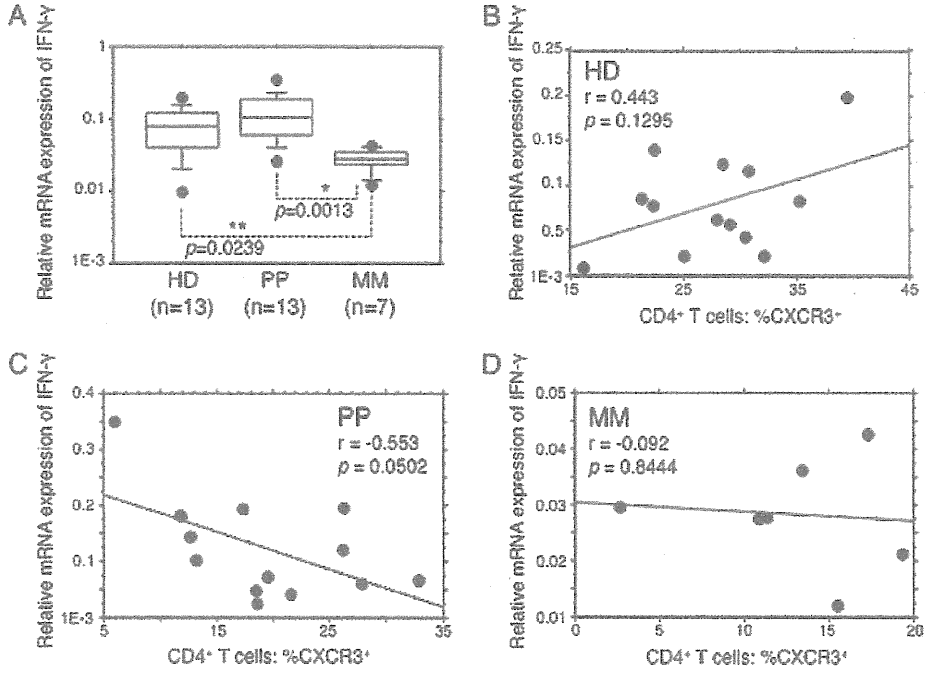

Figure 5. Peripheral CD4$^+$ T cells in MMs show reduced IFN-γ mRNA expression. Freshly isolated peripheral CD4$^+$ T cells from HDs (n = 13), PPs (n = 13), and MMs (n = 7) were stimulated with anti-CD3/CD28 antibodies for 5 days. (*A*) Total RNA was isolated from CD4$^+$ T cells stimulated with anti-CD3/CD28 antibodies for 5 days, and relative mRNA expression of IFN-γ was estimated by real-time RT-PCR. The box plot is presented as described in Figure 3. The P value was obtained using the Mann-Whitney U test. *$P < 0.01$; **$P < 0.05$. (*B*) Correlations between mRNA expression of IFN-γ and cell surface expression of CXCR3 in CD4$^+$ T cells in HDs (*B*), PPs (*C*), and MMs (*D*) were analyzed using Spearman's rank correlation test.

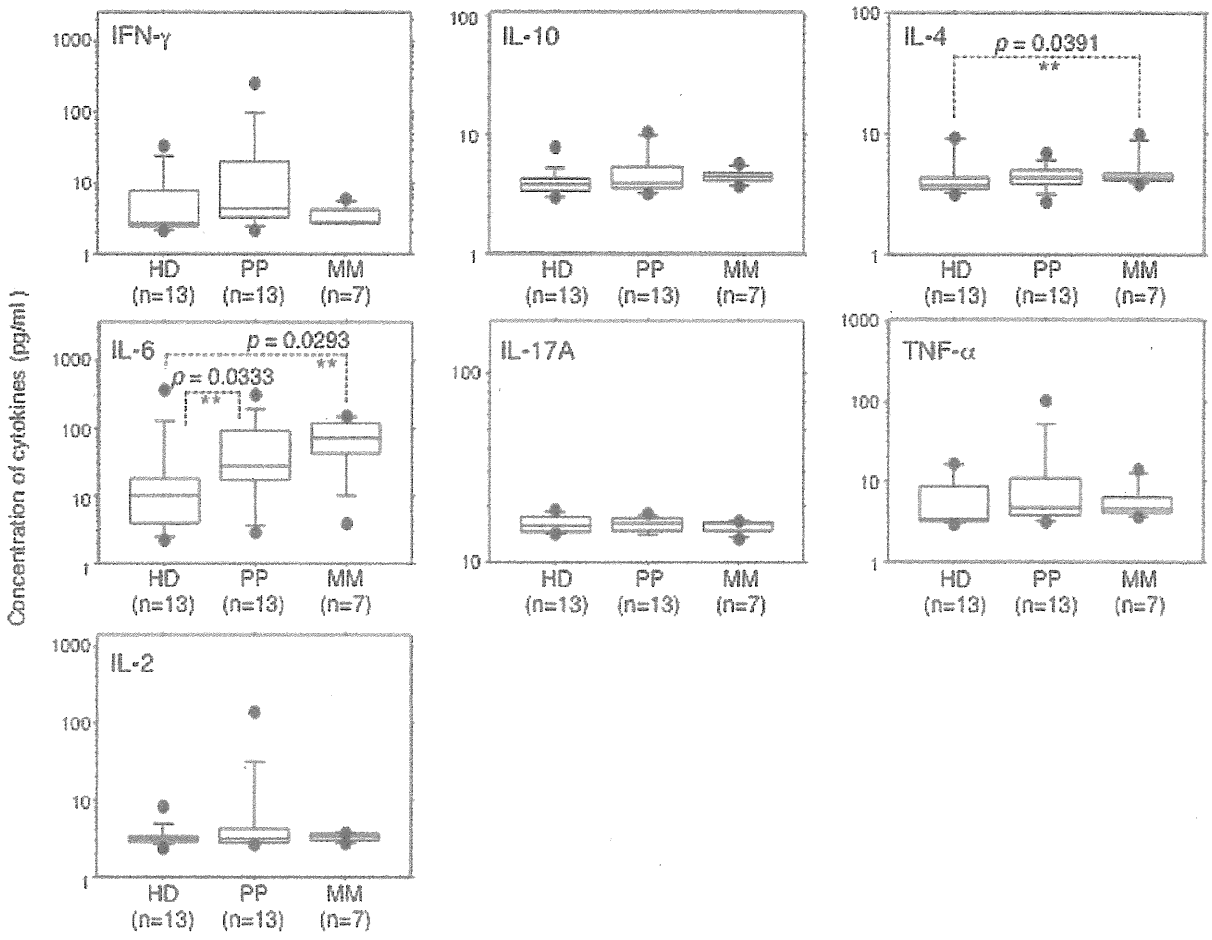

Figure 6. Peripheral CD4$^+$ T cells in patients with PP or MM show increased IL-6 production. Freshly isolated peripheral CD4$^+$ T cells from HDs (n = 13), PPs (n = 13), and MMs (n = 7) were stimulated with anti-CD3/CD28 antibodies for 5 days, and the concentration of various cytokines indicated in the graphs were measured using a CBA Human Th1/Th2/Th17 Cytokine Kit. The box plot is presented as described in Figure 3. The P value was obtained using the Mann-Whitney U test. **$P < 0.05$.

the NK-activating receptor NKp46 (38, 39). In addition, we attempted to establish an *in vitro* T-cell model of continuous and low-level exposure to asbestos and reported that the MT-2 subline exhibited resistance to asbestos-induced apoptosis with activating Src-family kinases, up-regulation of IL-10, phosphorylation of STAT3, and subsequent up-regulation of Bcl-2 (15, 16). Furthermore, six independent sublines of MT-2 original cells continuously exposed to chrysotile showed reduced Th1-related IFN-γ production, CXCL10/IP10 production, and cell surface expression of the CXCL10/IP10 receptor CXCR3 (19).

If these findings obtained using the *in vitro* cell line model are compatible with those pertaining to asbestos-exposed patients, such as those possessing PPs and MMs, these molecules may represent clinical markers for asbestos exposure, disease progression, and even therapeutic targets. Thus, we tried to confirm alteration of surface CXCR3 expression and IFN-γ production using freshly isolated human CD4$^+$ T cells derived from HDs and patients with PP and MM. To use the *ex vivo* model of low-dose and long-term exposure to chrysotile asbestos, these CD4$^+$ T cells should be cultured for several months. However, because IL-2–dependent proliferation of CD4$^+$ T cells is not permanent, our experiment was limited to culture for several weeks in the presence of IL-2 after stimulation of anti-CD3/CD28 antibodies. The results showed that four of six cases exhibited reduced CXCR3 expression within the limitation of the *ex vivo* model. Moreover, intracellular IFN-γ expression was clearly reduced in this *ex vivo* experimental situation, although the number of applied cases was small. These findings encouraged us to examine CXCR3 expression and IFN-γ production using the samples derived from asbestos-exposed patients, such as those possessing PPs and MMs.

Analysis of the case samples revealed that the expression of CXCR3 in CD4$^+$ T cells from PPs and MMs was decreased when compared with that of HDs, even though another Th1-type chemokine receptor, CCR5, did not differ between the three groups. Because CXCR3 is implicated in the migration of Th1/effector T cells, decreased CXCR3 expression in CD4$^+$ T cells by asbestos exposure may reduce normal Th1/effector T-cell recruitment to tumor sites, resulting in impairment of anti-tumor immune functions. Meanwhile, asbestos is accumulated in several lymph nodes (40–42). Thus, it seems that CXCR3 expression in CD4$^+$ T cells is inhibited by contact with asbestos in the lymph nodes of patients with PP and MM. In addition, CD4$^+$CXCR3$^+$ T cells in lymphocytes were significantly reduced in MMs rather than PPs because the population of lymphocytes was significantly decreased in MMs compared with PPs. This suggests that CD4$^+$CXCR3$^+$ T cells are gradually decreased and that they depend not only on asbestos exposure but also on disease status, such as the occurrence of mesothelioma.

IFN-γ mRNA levels in CD4$^+$ T cells derived from MMs stimulated *in vitro* were lower than those of HDs or PPs, whereas there were no significant differences in IFN-γ pro-