ORIGINAL ARTICLE: CLINICAL RESEARCH

SARCOIDOSIS VASCULITIS AND DIFFUSE LUNG DISEASES 2012; 29; 19-25

HISTORICAL CHANGES IN EPIDEMIOLOGY OF DIFFUSE PANBRONCHIOLITIS

C. Kono[1,3], T. Yamaguchi[1], Y. Yamada[1,3], H. Uchiyama[2], M. Kono[3], M. Takeuchi[4], Y. Sugiyama[5], A. Azuma[6], S. Kudoh[6], T. Sakurai[3], K. Tatsumi[3]

[1] Department of Respiratory Medicine, Japan Railway Tokyo General Hospital; [2] JR East Health Promotion Center, East Japan Railway Company; [3] Department of Respirology, Graduate School of Medicine, Chiba University; [4] Division of Biostatistics, Graduate School of Medicine, Kitasato University; [5] Division of Pulmonary Medicine, Jichi Medical University; [6] Division of Pulmonary Medicine, Nippon Medical School, Japan

ABSTRACT. *Background and objective:* Japanese pulmonologists, experienced in treating patients with diffuse panbronchiolitis (DPB) prior to the 1980s, have uniformly observed that new incidences of DPB are now a rare event in Japan. However, there is no epidemiological data to support this observation. We examined epidemiological trends of the number of patients with DPB in a large company. *Design:* The computerized health records of JR East Company employees were used to identify patients with DPB and then these were followed up using the assessments of these patients in JR Tokyo General Hospital and two other JR hospitals. The whole study period was 27 years (1976-2003), although detailed analyses were carried out for three specific periods; the first was 1976-1980, the second was 1989-1993, and the third was 1999-2003. *Results:* In the first period, 11 DPB cases (four incidence, and seven prevalence) were detected among a total of 355,572 workers. In the second period, three DPB cases (one incidence, and two prevalence) were identified from a total of 180,359 workers. In the third period, no case was found in a total of 144,485 workers. *Conclusion:* This epidemiological trend suggests that both the incidence and prevalence of DPB may have decreased. *(Sarcoidosis Vasc Diffuse Lung Dis 2012; 29: 19-25)*

KEY WORDS: diffuse panbronchiolitis, epidemiology, incidence, prevalence, BMI

INTRODUCTION

In 1969, Yamanaka et al. reported diffuse panbronchiolitis (DPB) for the first time as a disease characterized by numerous micronodular pulmonary lesions composed of chronic inflammatory cells infiltrating the walls of the respiratory bronchioles (1). DPB has since come to be internationally accepted as a disease entity.[2] At the time of its discovery, DPB had a poor prognosis because of recurrent respiratory infections leading to respiratory failure. Since 1985, when long-term, low-dose erythromycin (EM) therapy was introduced, the prognosis of DPB has markedly improved (2, 3). In addition, most Japanese pulmonologists with experience in treating patients with DPB prior to the 1980s have anecdotally noted a decrease in the appearance of DPB, al-

Received: 20 March 2010
Accepted after Revision: 12 May 2011
Correspondence: Takayuki Sakurai
Department of Respirology, Graduate School of Medicine, Chiba University
1-8-1, Inohana, Chuo-ku, Chiba, 260-8670, Japan
Fax +81 43 226 2176
E-mail: taksak@tiara.ocn.ne.jp

C. Kono: was the main author of this manuscript and responsible for data collection, and analysis.
T. Yamaguchi, Y. Yamada, H. Uchiyama, H. Amano, M. Kono, M. Takeuchi, Y. Sugiyama, A. Azuma, S. Kudoh, K. Tatsumi: all carried out data collection and analysis.
T. Sakurai: is the corresponding author of this manuscript, and carried out data analysis.

though no epidemiological survey has yet been done. Therefore, we examined the incidence of the number of patients with DPB over the past 30 years in a relatively large Japanese company.

Methods

The clinical outpatients records from Japan Railway (JR) Tokyo General Hospital along with Sapporo, and Sendai JR hospitals were analyzed. In addition, computerized health records from 1972 of the male population of JR East Company employees were evaluated (most employees were men). These employees had undergone an annual health check which included height, weight, chest X-ray, audiometry, visual testing, and a medical interview by public health nurses of the JR East Health Promotion Center. Compliance with the annual check-ups remained, for a long period of time, as high as 99%. Any employees with long-term nasal and respiratory symptoms, and/or X-ray abnormalities on the annual health check-ups were sent to JR Hospitals for further examination. JR East health records were available from 1972 to assess health findings from the time of initial employment. The study covered 27 years (1976-2003), although detailed analyses were limited to the following three periods: the first was 1976-1980 and with a total of 355,574 examinees, the second was 1989-1993 with 180,359 subjects, and the third period was 1999-2003 with 144,485 examinees. All employees had undergone health check at employment, and passed it except for extremely sick, visual impairment and difficulty in hearing. There is no difference in the economical or familial background among the study population.

Study profiles

In the first and second periods, a DPB diagnosis was based on the initial clinical criteria established by Homma and Yamanaka in 1969 (1). In the third period, the 1998 revised criteria developed by the Ministry of Health and Welfare of Japan (Table 1) were adopted. The main difference between the two sets of criteria is that, in the revised set, chest CT scans are included for radiographical evaluation to detect centrilobular granular shadows. The revised criteria may exhibit greater sensitivity for detecting patients with DPB than the initial criteria. We reapplied the revised criteria to the cases of DPB diagnosed in the first and second periods. As a result, all DPB diagnoses based on the initial clinical criteria were found to meet the requisite for diagnosis with the revised criteria.

Table 1. Diagnostic criteria proposed in 1998 according to the Ministry of Health and Welfare of Japan

Indispensable signs
1. Symptoms: chronic cough, sputum, and dyspnea on exertion.
2. Past history or coexistence of chronic sinusitis
3. Chest radiographic findings: bilateral diffuse small nodular shadows on a plain chest
4. X-ray film or diffuse centrilobular nodular shadows on chest CT images.

Reference signs
1. Physical signs: coarse crackles, sometimes with rhonchi, wheezes or squawk, on auscultation of the chest.
2. Pulmonary function tests and blood gas analysis: FEV_1 < 70% and PaO_2 < 80 mmHg.
3. Elevated titers of cold hemagglutinin

- *Definite cases* should fulfill three indispensable criteria with at least two of the three reference criteria.
- *Probably definite cases* should fulfill three indispensable criteria.
- *Possible cases* should fulfill 1 and 2 of indispensable criteria.

Statistical analysis

We determined the incidence and prevalence of DPB in three 5-year periods (1976-1980, 1989-1993, and 1999-2003), and calculated the age-adjusted incidence rate with the direct method using a standard model population in 1985. Body characteristics were calculated as mean = SD for continuous variables, and two-sample t tests were performed to analyze differences between DPB patients and controls. Analysis of variance was performed to compare the time-dependent changes of prevalence rate in DPB. This was followed by McNemar test (with Yates' revised) and a post hoc Bonferroni's multiple-comparison test. P values less than 0.05 were considered to be statistically significant.

This study was partly supported by a grant to the Diffuse Lung Disease Research Group and Respiratory Failure Research Group from the Ministry of Health, Labour and Welfare, Japan.

Results

Incidence and prevalence rates

The clinical course of detected DPB cases are shown in Fig. 1. According to medical interviews, most patients with DPB had a long period of respiratory prodromal symptoms, including nasal discharge or obstruction indicating chronic sinusitis. The longest such period was 29 years, in case #12, followed by 25 years in case #2, 8~14 years in six cases, 1~3 years in four cases, and no such period in case #9. Cases #5 and #13 were excluded in part from the statistical analysis because data before the first period were missed.

The incidence and prevalence of DPB each year during the study period are shown in Table 2. The numbers of prevalence cases are affected by a number of factors including, retirement, death, moving to other hospitals, and missing. In the first period, 11 cases (incidence of four, and prevalence of seven) were observed among 355,572 workers. In the second period, three cases (incidence of one, and prevalence of two) were observed among 180,359 workers. In the third period, no case of DPB was detected among 144,485 workers. Overall, the incidence was three cases in 1976, one case in 1977, and one in 1990. The incidence was zero after 1977 except for one case in 1990.

Incidence and prevalence rates for DPB are shown in Table 3. The incidence rate was 1.12 per 100,000 in the first period, 0.55 in the second period, and 0 in the third period. The age-adjusted incidence rate was 0.88 per 100,000 in the first period, 0.28 in the second period, and 0 in the third period. The prevalence rate was 13.78 per 100,000 in the first period, 6.63 in the second period, and 0 in the third period (this was despite DPB being more widely detected by chest CT scan in the third period). Considering the progress of radiographical evaluations, these findings suggest that the incidence of DPB has decreased with time since 1976 in the JR East Compa-

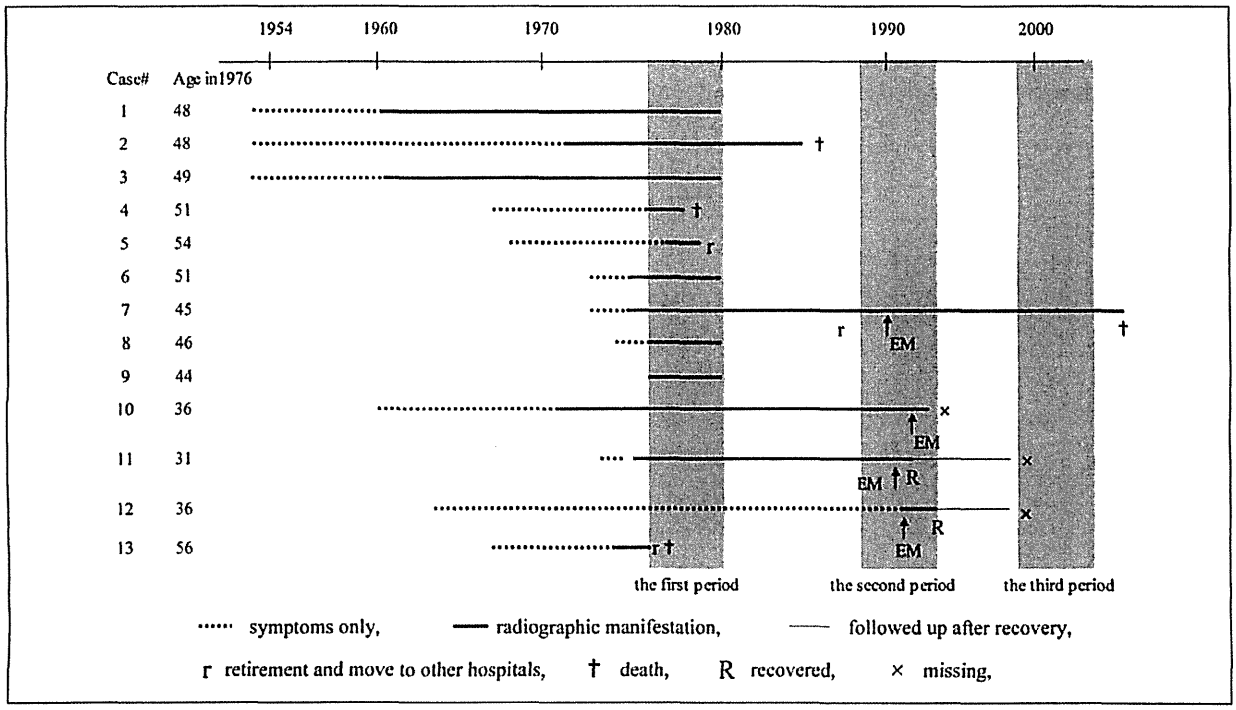


Fig. 1. Clinical course of detected diffuse panbronchiolitis (DPB) cases. This figure shows the progress of all the DPB cases. We could not follow all of their courses completely, because most cases moved to another hospital, or were missing after retirement, and some data between 1981 and 1988 were not available. Only case #2 and #7 could be followed completely. Case #13 was not counted in the first period because of his retirement. Case#10 and #11 discontinued their attendance at our hospital. Case #12 moved to another hospital due to a transfer

Table 2. The number of incidence and prevalence cases in each year

	The first period					The second period					The third period				
	1976	1977	1978	1979	1980	1989	1990	1991	1992	1993	1999	2000	2001	2002	2003
Incidence cases	3	1	0	0	0	0	1	0	0	0	0	0	0	0	0
Prevalence cases	10	11	10	9	9	2	3	3	3	1	0	0	0	0	0

Table 3. DPB crude incidence rates and annual average prevalence rates

	Incidence rate (10^{-5})	Prevalence rate (10^{-5})
The first period (1976~1980)	1.12	13.78
The second period (1989~1993)	0.55	6.63
The third period (1999~2003)	0	0

ny. Time dependent changes of prevalence rates in DPB, was followed by McNemar test (with Yates' revised) and a post hoc Bonferroni's multiple-comparison test, which were p=0.045 and p=0.039 (period I/II), p=0.133 and p=0.125 (period II/III).

Patient physique before onset

The height and body mass index (BMI) of DPB patients was examined before onset, and the BMI of DPB patients was compared with age- and job-matched controls. We hypothesized that a small physique was associated with the onset of DPB. According to medical interviews, three DPB patients had only slight prodromal symptoms that passed the employment health check-up as having no abnormal medical conditions. Height and weight data in 1954 were obtained from the JR East Health Promotion Center. Ten patients were compared with 30 controls in the first period. For these ten cases (cases #1-9, and #13), 30 age- and job-matched controls were chosen at random from male employees in 1976. Height, weight, and BMI are presented in Fig. 2. The mean height of DPB patients was 158.6 ± 4.7 cm, while that of the 1976 controls was 161.5 ± 6.9 cm, in 1976 (p>0.05). There was no change between 1954 and 1976. In 1954, the mean BMI of the patients tended to be lower (19.5) than that of the controls (20.7), although it was not significant in statistical analysis (p>0.05).

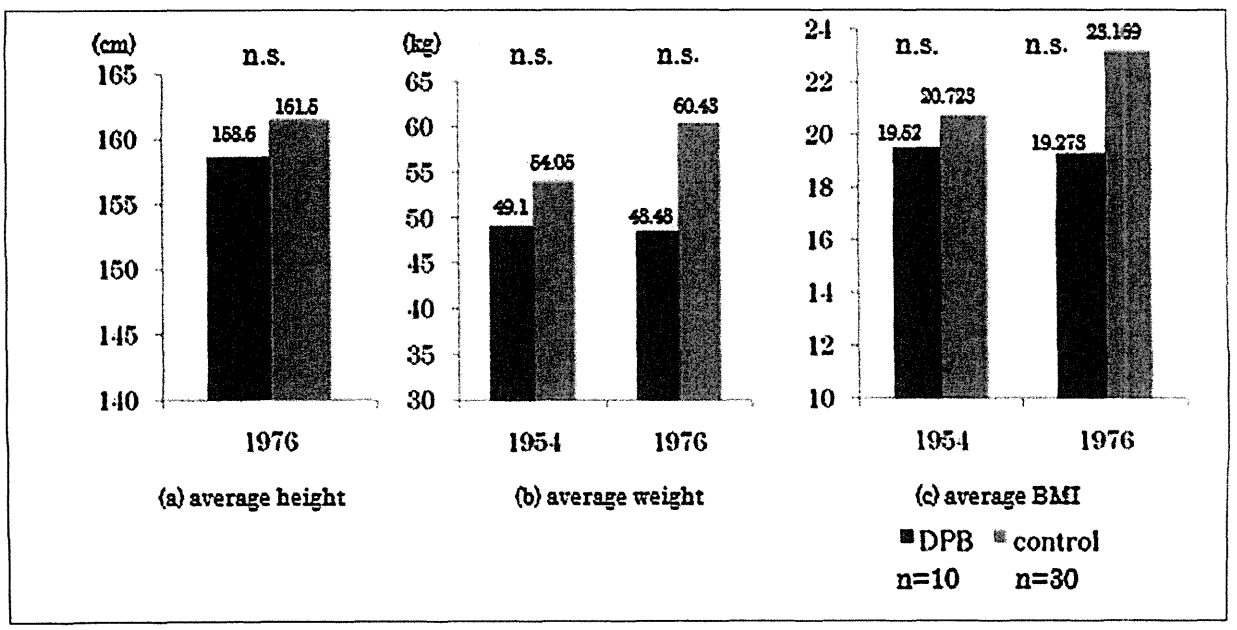


Fig. 2. Body characteristics in diffuse panbronchiolitis (DPB) patients. The average height, weight, and BMI of DPB patients tended to be lower than the controls in 1976, affected by DPB incidence or prodrome. The average BMI of DPB patients tended to be lower than controls, as of 1954; about 20 years before onset of the disease. n.s. ; not significant

Discussion

Very few epidemiological studies of DPB have been available, other than that by Saitoh et al., who performed case-control studies on the occurrence of DPB in 1982 (4). The findings of the present study indicate that the incidence and prevalence rates of DPB have decreased in the JR East Company.

In the small number of incident cases in the present study, it would be difficult to consider that the described clinical pattern and its trend are representative of the overall situation in Japan. However, the study population included around sixty-eight thousands individuals without unusual medical conditions at the time of their employment. Therefore, our findings may contribute to determine the prevalence of DPB in our country, and may also be invaluable in revealing the time when prodromic symptoms and X-ray abnormalities first appear. It is unclear in daily clinical practice when DPB patients first develop abnormal chest X-ray findings.

Homma collected more than 1,000 cases of probable DPB, and 82 histologically confirmed cases, through a nation-wide survey from 1978-1980. They noted that secondary ectasia of proximal bronchioli probably occurred in the advanced stage of DPB (5). We also observed these findings in case #7, in which typical centrilobular granular shadows were initially noted, with progression to diffuse, ring-shaped shadows indicative of diffuse bronchiectasis (Fig. 3). Secondary ectasia of proximal bronchioli seemed to occur in the advanced stage of DPB. Cases with diffuse bronchiectasis similar to case #7 are thought to progress from DPB.

Although the etiology of DPB remains unknown, the participation of genetic factors was sug-

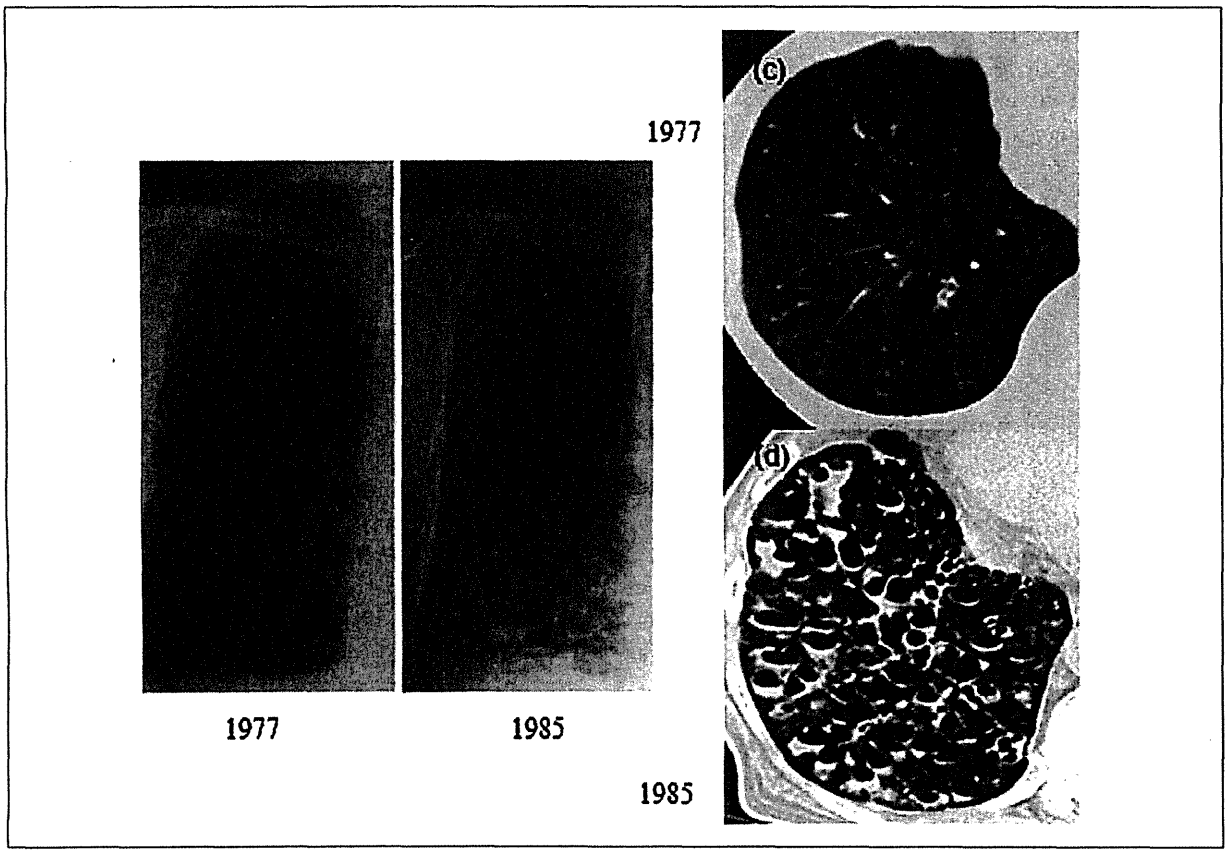


Fig. 3. Chest X-ray and CT findings in Case #7 (a) Chest X-ray and (c) CT in 1977 showing diffuse centrilobular nodules. (b) Chest X-ray and (d) CT in 1985 progressing to diffuse bronchiectasis

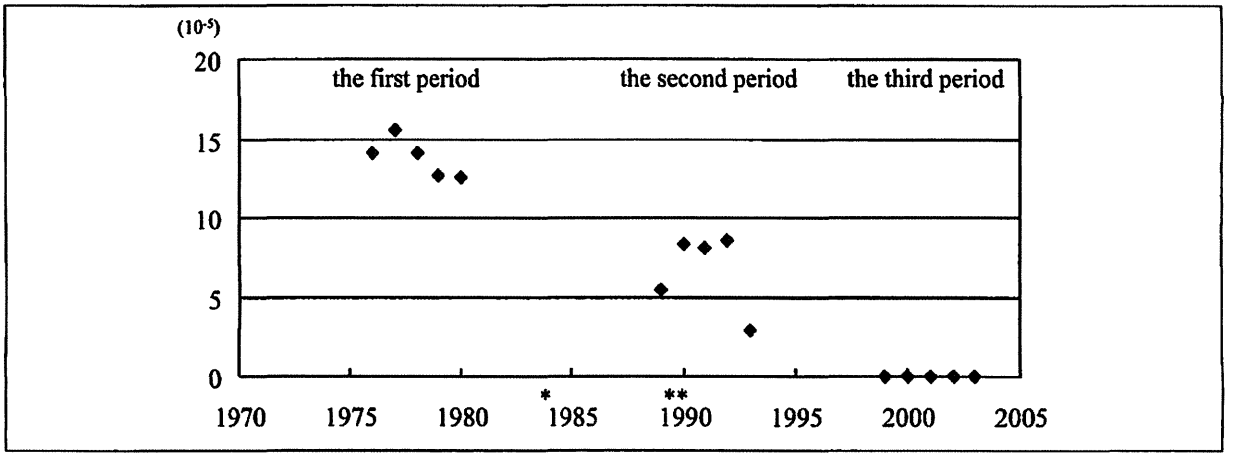


Fig. 4. Time-dependent changes of prevalence rates in diffuse panbronchiolitis (DPB). Macrolide therapy was introduced for DPB between the first and second period, and was introduced for chronic sinusitis in the second period. *1984, first report of macrolide therapy for DPB. **1990, first report of macrolide therapy for chronic sinusitis

gested by Keicho and coworkers. They found that the major disease-susceptibility gene for DPB exists between the two HLA loci on chromosome 62 (6). However, not all DPB patients have genetic disorders and not all individuals with genetic disorders have DPB, suggesting that non-genetic factors may also play a role in the aetiology of this condition.

Differences in diet and environment may contribute to the development of DPB. It could also be that small physique is associated with DPB. In comparison of BMI between DPB patients and controls in 1954, we found that the mean BMI of DPB patients from 1954 tended to be lower that that of the controls. In present study, it was not significant in statistical analysis between the BMI of the patients and that of the controls. We estimate that few number of the patients would lead it. According to medical interviews from ten DPB patients, seven had no prodromal symptoms and three had only slight prodromal symptoms that passed the employment health check-up as a normal medical condition. This suggests that DPB patients had smaller physiques compared with controls 20 years prior to the onset of DPB.

Although adult height is genetically determined, it may be influenced by diet in childhood and adolescence (7). Adult height has been used as a proxy indicator of nutrition early in life in relation to subsequent risk of incidence and mortality from chronic respiratory disease. Although it is possible that a gene involved in the onset of DPB is linked to another gene involved in height determination, we found no evidence to support this hypothesis. According to Japanese Health and Welfare statistics, mean height for men in their 40's was 162.9 cm in the first period, 166.4 cm in the second period, and 168.8 cm in the third period (this increase being the result of improved nutrition in Japan). Although the reason for the relationship between DPB and malnutrition is unclear, chronic periodontal disease due to malnutrition may result in the release of cytokines such as IL-1 and TNF-α, which may stimulate respiratory epithelial cells (8). It may thus be that the release of cytokines due to malnutrition causes chronic respiratory diseases such as DPB. Furthermore, the bronchial mucosa become fragile as a result of malnutrition in childhood, since the growth of bronchi continues until about eight years of age. We speculate that improved nutrition suppresses the onset of DPB. A further epidemiological study of the prevalence of chronic sinusitis or prodromal symptom of DPB is required.

Since the efficacy of long-term, low-dose EM therapy in patients with DPB was reported in 1984 (2, 3), the prognosis of DPB has dramatically improved. In the 1970s, prior to the introduction of EM therapy, the overall 5-year survival rate was 62.9%. Between 1980 and 1984, the survival rate was still limited to 72.4%, but after 1985, when EM therapy was introduced, the 5-year survival rate improved significantly to 91.4% (4). New applications of 14-membered ring macrolides, including EM,

clarithromycin and roxithromycin were reported, and these agents proved to be effective, not only for DPB, but also for various chronic respiratory disease such as bronchiectasis and chronic sinusitis.

Macrolides therapy were effective in many DPB patients except for secondary diffuse bronchiectasis, advanced stage in DPB. They were introduced to DPB therapy between the first and second period of our study; and for the treatment of chronic sinusitis in the second period. It appears that patients with a mild degree of DPB improved between the first and second periods. Also since chronic sinusitis prior to the development of DPB could be treated between the second and third periods, the incidence rate of DPB decreased (Fig. 4). In addition, most DPB cases complicated with chronic sinusitis were treated with macrolides, and were thus dramatically improved by this treatment. Thus, we anticipate that treatment for chronic sinusitis may be a preventive treatment for lower respiratory tract inflammation and may explain the decreased incidence of DPB after the introduction of macrolide therapy.

Our findings indicate that incidence and prevalence rates of DPB have decreased over time in a large Japanese working population. Small physique may possibly be associated with the development of DPB, and long-term, low-dose EM therapy, at an early stage, could decrease the prevalence rate of DPB.

Acknowledgements

We would like to give heartful thanks to the late Dr Yutaka Hosoda, JR East Health Promotion Center, whose enormous support and insightful comments were invaluable during the course of our study.

References

1. Yamanaka A, Sasaki S, Tamura S, Saito K. Problems in chronic obstructive bronchial diseases, with special reference to diffuse panbronchiolitis. Naika 1969; 23: 442-51 [in Japanese].
2. Azuma A and Kudoh S. Diffuse panbronchiolitis in East Asia. Respirology 2006; 11: 249-61.
3. Kudoh S, Azuma A, Yamamoto M, Izumi T, Ando M. Improvement of survival in patients with diffuse panbronchiolitis treated with low-dose erythromycin. Am J Respir Crit Care Med 1998; 157: 1829-32.
4. Saitoh M, Hosoda Y, Odaka M. The onset of DPB: a case-control study. The 1982 Report of the Ministry of Health and Welfare, Intractable disease epidemiological studies. 1982; 225-42 [in Japanese].
5. Homma H, Yamanaka A, Tanimoto S, et al. Diffuse panbronchiolitis, a disease of the transitional zone of the lung. Chest 1983; 83: 63-9.
6. Keicho N, Ohashi J, Tamiya G, et al. Fine localization of a major disease-susceptibility locus for diffuse panbronchiolitis. Am J Hum Genet 2000; 66: 501-7.
7. La Vecchia C, Decarli A, Negri E, Ferraroni M, Pagano R. Height and the prevalence of chronic disease. Rev. Epidemiol. Sante Publique 1992; 40: 6-14.
8. Scannapieco FA, Ho AW. Potential associations between chronic respiratory disease and periodontal disease: analysis of National Health and Nutrition Examination Survey III. J Periodontol 2001; 72: 50-6.

CD40 amplifies Fas-mediated apoptosis: a mechanism contributing to emphysema

Ayako Shigeta, Yuji Tada, Ji-Yang Wang, Shunsuke Ishizaki, Junichi Tsuyusaki, Keita Yamauchi, Yasunori Kasahara, Ken Iesato, Nobuhiro Tanabe, Yuichi Takiguchi, Akemi Sakamoto, Takeshi Tokuhisa, Kazutoshi Shibuya, Kenzo Hiroshima, James West and Koichiro Tatsumi

Am J Physiol Lung Cell Mol Physiol 303:L141-L151, 2012. First published 18 May 2012;
doi: 10.1152/ajplung.00337.2011

American Journal of Physiology - Lung Cellular and Molecular Physiology publishes original research covering the broad scope of molecular, cellular, and integrative aspects of normal and abnormal function of cells and components of the respiratory system. It is published 24 times a year (twice monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991. Copyright © 2012 the American Physiological Society. ESSN: 1522-1504. Visit our website at http://www.the-aps.org/.

Am J Physiol Lung Cell Mol Physiol 303: L141–L151, 2012.
First published May 18, 2012; doi:10.1152/ajplung.00337.2011.

CD40 amplifies Fas-mediated apoptosis: a mechanism contributing to emphysema

Ayako Shigeta,[1] Yuji Tada,[1] Ji-Yang Wang,[2] Shunsuke Ishizaki,[1] Junichi Tsuyusaki,[1] Keita Yamauchi,[1] Yasunori Kasahara,[1] Ken Iesato,[1] Nobuhiro Tanabe,[1] Yuichi Takiguchi,[3] Akemi Sakamoto,[4] Takeshi Tokuhisa,[4] Kazutoshi Shibuya,[5,6] Kenzo Hiroshima,[7] James West,[8] and Koichiro Tatsumi[1]

Departments of [1]Respirology, [3]Medical Oncology, and [4]Developmental Genetics, Graduate School of Medicine, Chiba University, Chiba, Japan; [2]Laboratory for Immune Diversity, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Kanagawa, Japan; [5]Department of Pathology, School of Medicine, Toho University, Tokyo, Japan; [6]Department of Dermatology, Peking University First Hospital, Beijing, China; [7]Department of Pathology, Tokyo Women's Medical University Yachiyo Medical Center, Chiba, Japan; [8]Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University, Nashville, Tennessee

Submitted 14 October 2011; accepted in final form 15 May 2012

Shigeta A, Tada Y, Wang J-Y, Ishizaki S, Tsuyusaki J, Yamauchi K, Kasahara Y, Iesato K, Tanabe N, Takiguchi Y, Sakamoto A, Tokuhisa T, Shibuya K, Hiroshima K, West J, Tatsumi K. CD40 amplifies Fas-mediated apoptosis: a mechanism contributing to emphysema. *Am J Physiol Lung Cell Mol Physiol* 303: L141–L151, 2012. First published May 18, 2012; doi:10.1152/ajplung.00337.2011.—Excessive apoptosis and prolonged inflammation of alveolar cells are associated with the pathogenesis of pulmonary emphysema. We aimed to determine whether CD40 affects alveolar epithelial cells and endothelial cells, with regard to evoking apoptosis and inflammation. Mice were repeatedly treated with agonistic-anti CD40 antibody (Ab), with or without agonistic-anti Fas Ab, and evaluated for apoptosis and inflammation in lungs. Human pulmonary microvascular endothelial cells and alveolar epithelial cells were treated with agonistic anti-CD40 Ab and/or anti-Fas Ab to see their direct effect on apoptosis and secretion of proinflammatory molecules in vitro. Furthermore, plasma soluble CD40 ligand (sCD40L) level was evaluated in patients with chronic obstructive pulmonary disease (COPD). In mice, inhaling agonistic anti-CD40 Ab induced moderate alveolar enlargement. CD40 stimulation, in combination with anti-Fas Ab, induced significant emphysematous changes and increased alveolar cell apoptosis. CD40 stimulation also enhanced IFN-γ-mediated emphysematous changes, not via apoptosis induction, but via inflammation with lymphocyte accumulation. In vitro, Fas-mediated apoptosis was enhanced by CD40 stimulation and IFN-γ in endothelial cells and by CD40 stimulation in epithelial cells. CD40 stimulation induced secretion of CCR5 ligands in endothelial cells, enhanced with IFN-γ. Plasma sCD40L levels were significantly increased in patients with COPD, inversely correlating to the percentage of forced expiratory volume in 1 s and positively correlating to low attenuation area score by CT scan, regardless of smoking history. Collectively CD40 plays a contributing role in the development of pulmonary emphysema by sensitizing Fas-mediated apoptosis in alveolar cells and increasing the secretion of proinflammatory chemokines.

alveolus; inflammation; IFN-γ; chemokine regulated on activation normal T cells expressed and secreted (RANTES)

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is characterized by the progressive development of airflow limitation that is not fully reversible. COPD is a leading global cause of morbidity and mortality, and the number of patients with COPD is expected to increase as the world population continues to age. The pathogenesis of COPD is complex, and several mechanisms are involved, including chronic airway inflammation, protease/antiprotease imbalance, cell senescence, and deficiency of growth and/or angiogenic factors (3, 6, 8). Several animal models of COPD have been established, including chronic inhalation of lipopolysaccharide (5), repeated exposure to ozone (35), interferon (IFN)-γ transgenic mice (39), and the vascular endothelial growth factor (VEGF) signaling blockade model (22). In each of these models excessive apoptosis of alveolar component cells is closely associated with the pathogenesis of COPD, especially of pulmonary emphysema.

CD40 is a member of the tumor necrosis factor receptor superfamily and binds to CD40 ligand (CD40L). The CD40/CD40L plays a central role in the activation of adaptive immunity (18) but is also involved in the induction of apoptosis in overactivated cells (14, 38). Whether CD40 stimulation induces or protects from apoptosis depends on the cell type. Thus CD40 stimulation protects airway epithelial cells from oxidant-induced apoptosis (27); conversely, it enhances Fas-mediated apoptosis in hepatocytes and salivary gland epithelial cells (1, 31). Recent studies demonstrated that the CD40/CD40L system is upregulated in response to cigarette smoking (19), viral infection (36), pulmonary hypertension (12), and hypoxia (7), all of which are associated with COPD. Analysis of CD40L in patients with COPD in the Framingham study showed that high expression was a significant risk factor but only in the context of high levels of smoking (37). Therefore, it has been suggested that CD40L is a context-specific risk factor for COPD, but the effect of CD40 on apoptosis of alveolar component cells, such as alveolar epithelial cells and pulmonary arterial endothelial cells, as well as on inducing sustained inflammation, has not been determined. The aim of this study is to assess whether CD40/CD40L plays a contributing role in COPD, either alone or in combination with other factors.

MATERIALS AND METHODS

Animal treatment protocols. Male C57BL/6J mice (8 wk old) were purchased from CLEA (Tokyo, Japan). All mice were housed in specific pathogen-free animal facility with free access to food and water. In the first set of experiments, mice were divided into eight groups ($n = 5$): *1*) control (isotype control antibody), *2*) anti-CD40

Address for reprint requests and other correspondence: Y. Tada, Dept. of Respirology, Graduate School of Medicine, Chiba Univ., Chiba, Japan, 1-8-1 Inohana Chuo-ku, Chiba 260-8670, Japan (e-mail: ytada25@yahoo.co.jp).

antibody (Ab), *3*) IFN-γ, *4*) anti-CD40 Ab + IFN-γ, *5*) anti-Fas Ab, *6*) anti-Fas Ab + anti-CD40 Ab, *7*) anti-Fas Ab + IFN-γ, and *8*) anti-Fas Ab + anti-CD40 Ab + IFN-γ. Agonistic anti-mouse monoclonal antibody to CD40 (FGK45) (Alexis Biochemicals, San Diego, CA) (30 μg) and/or recombinant mouse IFN-γ (Bender MedSystems, Vienna, Austria) (100 ng) and/or anti-mouse monoclonal antibody to Fas (Jo2) (BD Biosciences, San Jose, CA) (0.1 μg/g) were dissolved in a total volume of 60 μl of PBS and injected intratracheally by MicroSprayer (Penn-Century, Philadelphia, PA) every 3 days for a total of eight times. An initial dose-ranging study was also performed to determine the minimum effective dose for each compound. For example, CD40 Ab (30 μg) and IFN-γ (100 ng) were used because these doses increased the cellularity in mice lung in the preliminary study. Furthermore, anti-Fas Ab was used at 0.1 μg/g because this dose did not induce acute lung injury seen in 2-μg/g inhalation in our study and previous report (29). Isotype-matched antibodies were used as controls, including purified NA/LE Rat IgG2a κ isotype control (BD Biosciences) (30 μg) and purified NA/LE Hamster IgG2 λ isotype control (BD Biosciences) (0.1 μg/g). All animal protocols were approved by the Committee on Animal Welfare of Chiba University.

Expression of CD40 and Fas in vivo. After treatment, mice were killed by intraperitoneal injection of pentobarbital sodium (100 mg/kg) and phenotyped on *day 27*. The left lung was filled with 0.5% low melting agarose at a constant pressure of 25 cm H_2O, allowing homogenous expansion of the lung parenchyma. The lungs were fixed in 10% formalin for 48 h, embedded in paraffin, and sectioned sagittally (2 μm) for histological analysis. Immunohistochemistry was performed using the following primary antibodies; anti-CD40 antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA) and anti-CD95 antibody (1:100; Abcam, Cambridge, UK). A total of 200 cells per mouse ($n = 5$) randomly selected by independent two pathologists and CD40 or Fas-positive cells were counted under a light microscope.

Evaluation of alveolar enlargement. Sections (2 μm) were stained with hematoxylin and eosin, and then the evaluation of mean linear intercept (MLI) was performed as described (9). In brief, 5 fields of 500 × 100 μm grid per mouse were selected randomly, following that the total length of each line divided by the number of alveolar intercepts gave the average distance between alveolate surfaces. All samples were assessed by light microscopy (Nikon ECLIPSE E400).

Evaluation of apoptosis in vivo. Evaluation of apoptotic cells in mouse lung were determined by terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) and immunohistochemical staining of activated caspase-3.

TUNEL staining was performed using an ApopTag Plus Peroxidase In Situ Apoptosis Detection Kit (Millipore, Billerica, MA), according to the manufacturer's instructions. Briefly, after deparaffinization and rehydration, sections were digested with proteinase K at a concentration of 20 μg/ml for 15 min. Endogenous peroxidase activity was quenched with 3% H_2O_2 for 5 min. The slides were incubated in a humid atmosphere at 37°C for 60 min with terminal deoxynucleotidyl transferase (TdT) buffer containing TdT and digoxigenin-dNTP. The slides were then washed with PBS and incubated with anti-digoxigenin-peroxidase for 30 min. After being rinsed with PBS, the slides were immersed in diaminobenzidene solution. The slides were counterstained for 10 min with 0.5% methyl green.

Caspase-3 staining was performed using cleaved caspase-3 antibody (1:100; Cell Signaling, Beverly, MA) as a primary antibody. After deparaffinization and rehydration, sections were heated at 120°C in autoclaved sterilizer for 10 min and naturally cooled for 30 min. They were then exposed to 1% hydrogen peroxide/methanol for 30 min to block endogenous peroxidase activity and rinsed in TBS. Next they were treated with 8% skimmed milk for 30 min. Cleaved caspase-3 antibody in TBS was applied to the sections in a moisture chamber at 4°C overnight. They were then sequentially treated with biotinylated secondary antibody and peroxidase-labeled streptavidin.

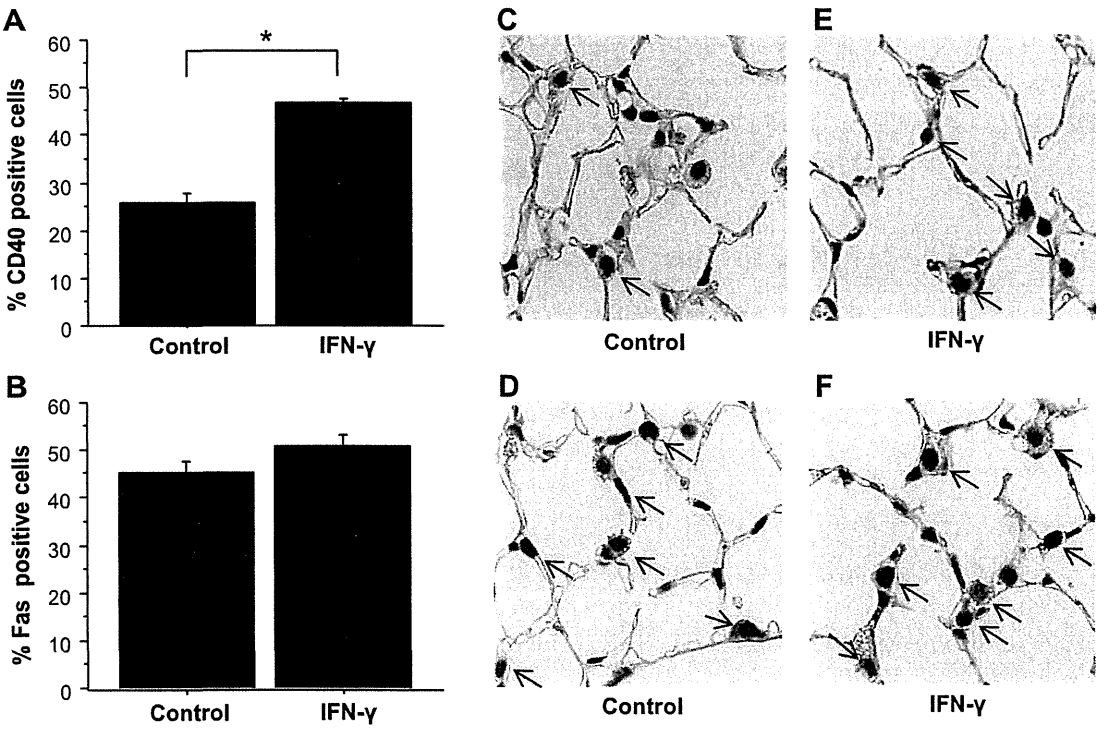


Fig. 1. CD40 (*A*) and CD95 (Fas, *B*) expression in mouse lung alveoli; arrows point to positive cells. Expression of CD40 and Fas in mouse lung alveoli after intratracheal injection with isotype control (*C* and *D*) or IFN-γ (*E* and *F*). IFN-γ stimulation significantly induced enhanced CD40 expression (*A*: isotype control 26.0 ± 1.8%, IFN-γ 46.8 ± 1.0%, $P < 0.0001$; $n = 5$). *$P < 0.05$.

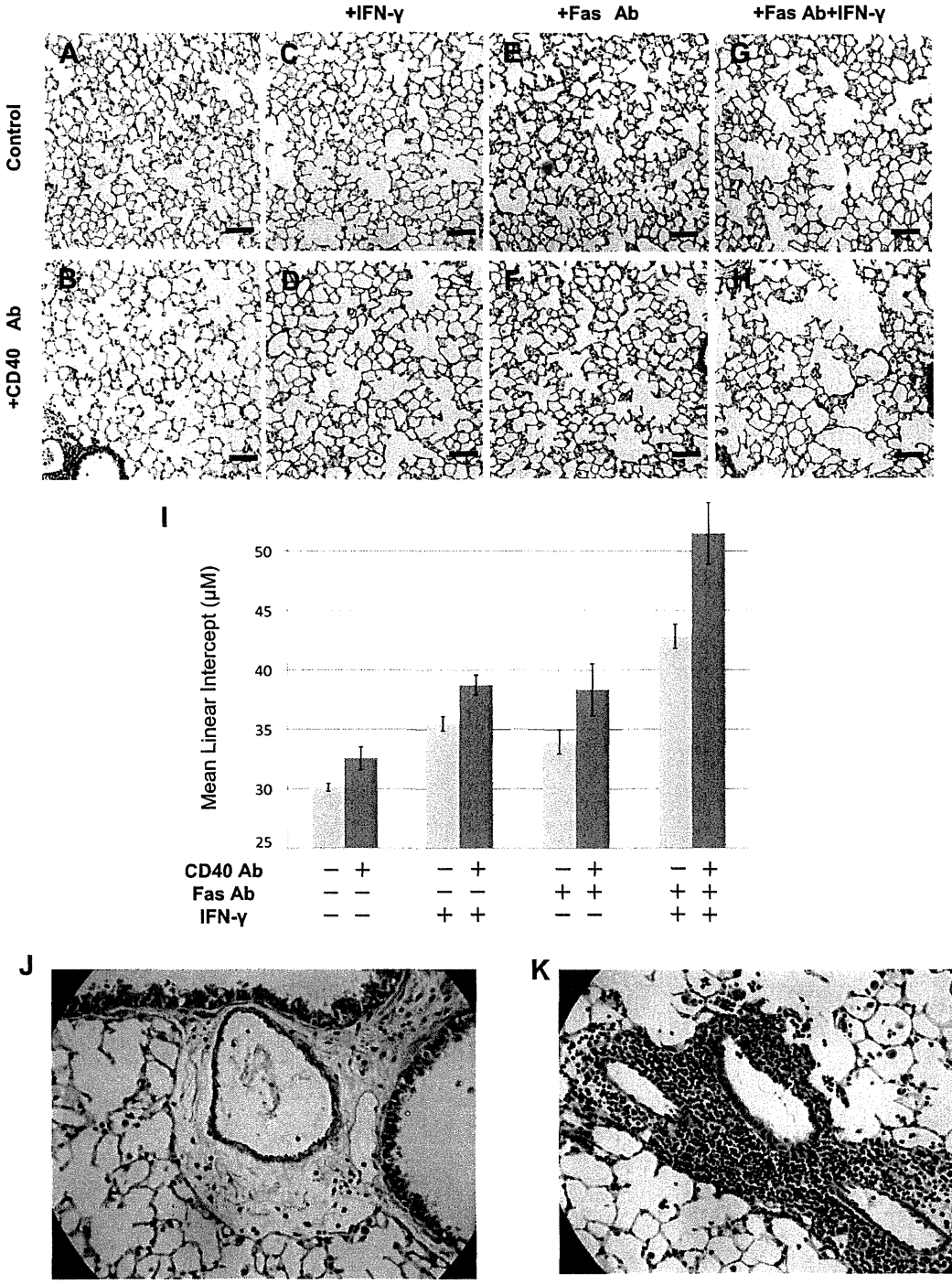


Fig. 2. Repeated inhalation of act-CD40 mAb (CD40 Ab), IFN-γ, and act-Fas mAb (Fas Ab) induced alveolar wall destruction and emphysematous changes in mice. *A–H*: representative hematoxylin and eosin-stained lung tissue sections from 5 mice (×100, scale bar = 50 μm). *A*: CD40 Ab(−) IFN-γ(−) Fas Ab(−); *B*: CD40 Ab(+) IFN-γ(−) Fas Ab(−); *C*: CD40 Ab(−) IFN-γ(+) Fas Ab(−); *D*: CD40 Ab(+) IFN-γ(+) Fas Ab(−); *E*: CD40 Ab(−) IFN-γ(−) Fas Ab(+); *F*: CD40 Ab(+) IFN-γ(−) Fas Ab(+); *G*: CD40 Ab(−) IFN-γ(+) Fas Ab(+); *H*: CD40 Ab(+) IFN-γ(+) Fas Ab(+). *I*: quantification of mean linear intercept (MLI) in mice. Data are means ± SE from 5 experiments. The effects of IFN-γ, CD40 Ab, and Fas Ab as individual factors were significant at $P < 0.0001$ by multiple-factor ANOVA. By Fisher's least-significant-difference (LSD) post hoc analysis, CD40 had a significant effect ($P < 0.05$) in increasing MLI in combination with either Fas Ab or the combination of Fas Ab and IFN-γ, but not when used alone. Mononuclear cell accumulation at perivascular site in mouse lungs after repeated inhalation of CD40 Ab (*K*), compared with isotype control (*J*).

Finally, they were visualized by diaminobenzidene reaction and counterstained with hematoxylin.

A total of 200 cells per mouse (n = 5) randomly selected by two independent pathologists was examined under a light microscope, and TUNEL- and caspase-3-positive cells were counted.

Evaluation of inflammation in lungs by bronchoalveolar lavage. Bronchoalveolar lavage (BAL) of mice was performed as describe elsewhere (40). In brief, the trachea was exposed and lavaged three times with 1 ml of PBS. The BAL fluid (BALF) was centrifuged at 300 *g* for 5 min at 4°C. Total cell counts and the differential cell counts were determined using hemocytometer by counting 200 cells per mouse stained with Diff-Quick (Sysmex, Kobe, Japan). Resulting supernatants were stored at −80°C and used for measurement of CCR5 ligands [macrophage inflammatory protein (MIP)-1α, MIP-1β, and chemokine regulated on activation normal T cells expressed and secreted (RANTES)] by commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instruction.

Cell culture. Human pulmonary microvascular endothelial cells (HPMVEC) and human pulmonary alveolar epithelial cells (HPAEpiC) were purchased from Lonza (Walkersville, MD) and ScienCell Research Laboratories (Carlsbad, CA), respectively. HPAEpiC was composed of 95% of alveolar type I cell and 5% of alveolar type II cell. HPMVEC were placed in BD BioCoat well plates coated with type I collagen (Becton Dickinson, Franklin Lakes, NJ) and maintained in EGM-2 BulletKit medium (Lonza), consisting of basal medium (CCMD-130) with the following supplements: fetal bovine serum (2.0%), hydrocortisone (0.04%), human epidermal growth factor (0.1%), VEGF (0.1%), human fibroblast growth factor-basic (with heparin, 0.4%), long recombinant 3-insulin-like growth factor-1 (0.1%), gentamicin/amphotericin-B (GA-1000, 0.1%), ascorbic acid (0.1%), and heparin (0.1%). HPAEpiC were placed in BioCoat poly-D-lysine-coated well plates (Becton Dickinson) and maintained in Alveolar Epithelial Cell Medium (ScienCell Research), consisting of basal medium with the following supplements: fetal bovine serum (10 μg/ml), apo-transferrin (10 μg/ml), insulin (5 μg/ml), epidermal growth factor (10 ng/ml), fibroblast growth factor-2 (2 ng/ml), epinephrine (500 ng/ml), hydrocortisone (1 μg/ml), retinoic acid (10^{-7} M), penicillin G sodium salt (100 U/ml), and streptomycin (100 μg/ml). These cells were incubated at 37°C in 5% CO_2 overnight and grown to ~60–80% confluence before being used for further analyses. Cells at *passages 4–6* were used for all experiments.

Surface expression of CD40 and Fas on HPMVEC and HPAEpiC. HPMVEC and HPAEpiC were incubated with phycoerythrin-conjugated anti-human CD40 mAb (R&D Systems) and FITC-conjugated mouse anti-human CD95 mAb (R&D Systems). FITC-conjugated mouse anti-human IgG (R&D Systems) was used as control antibody. Surface expression of CD40 and Fas (CD95) was assessed on a BD FACS Calibur flow cytometer using CELL Quest software (Becton Dickinson). In some experiments, recombinant human IFN-γ (BD Biosciences) (1,000 U/ml) was added to the cultures of HPMVEC and HPAEpiC for 24 h before analysis.

Evaluation of apoptosis in vitro. Functional anti-human CD40 Ab (eBioscience, San Diego, CA) at 0.2, 2.0, and 20.0 μg/ml and/or anti-human Fas Ab (eBioscience) at 2.0 μg/ml were added to HPMVEC and HPAEpiC cultures. After a 12-h incubation, cells were stained with FITC-conjugated Annexin V (Invitrogen, Carlsbad, CA) and examined by flow cytometry. In some experiments, recombinant human IFN-γ (1,000 U/ml) was added, together with anti-human CD40 Ab and/or anti-human Fas Ab.

Secretion of CCR5 ligands into culture supernatants. Culture medium was removed from 60% confluent HPMVEC and HPAEpiC, and cells were washed three times with PBS. Cells were incubated with serum-free medium and functional anti-human CD40 Ab (eBioscience) (2 μg/ml) for 12 h. In some experiments, recombinant human IFN-γ (1,000 U/ml) was added to the cultures. The concentrations of CCR5 ligands, i.e., MIP-1α, MIP- 1β, and RANTES, were determined using a Bio-Plex suspension array system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions.

Patients. The patient group consisted of 69 subjects with smoking-related COPD, who were recruited from the respiratory outpatient clinic at Chiba University Hospital. On the basis of past history, physical examination, and spirometric data, COPD was diagnosed according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines (30), that is, forced expiratory volume in 1 s (FEV_1)/forced vital capacity (FVC) ratio < 0.70 with $\%FEV_1$ < 80%. Participants matched for age, sex, and pack/year smoking history without impaired respiratory function on spirometry were enrolled as healthy smokers (n = 19). All the subjects are not current smokers, and patients with COPD had been clinically stable for more than 2 mo, without any symptoms of acute exacerbation. Subjects with liver disease, renal disease, cancer, autoimmune disease, and infection were excluded from the study.

All studies were approved by the Institutional Review Board of Chiba University Graduate School of Medicine, in the name of "No. 2217 Evaluation of QOL and prognosis in COPD. Written informed consent was obtained from all participants in this study.

CT scan. Evaluation for the presence of emphysema was done using a high-resolution computed tomography (HRCT) at full inspiration (Aquilion 64 scanner; TOSHIBA Medical Systems, Tokyo, Japan). HRCT images were photographed with a window setting appropriate for the lungs [window level −700 to −900 Hounsfield units (HU) and width 800 to 1,000 HU]. The presence of emphysema on HRCT was defined as well-demarcated areas of decreased attenuation compared with a contiguous normal lung and marginated by either a very thin wall (>1 mm), no wall at all, and/or multiple bullae

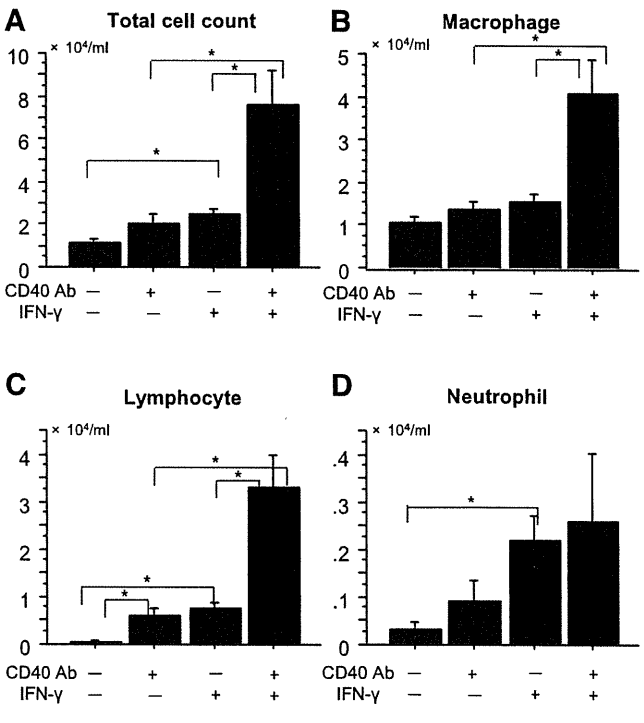


Fig. 3. *A–D*: total and differential cell counts in bronchoalveolar lavage fluid (BALF) after intratracheal injection. Sole CD40 activation significantly increased the number of lymphocytes compared with control (P = 0.0093; n = 4) but further enhanced when combined with IFN-γ. *P < 0.05.

(<1 cm) with an upper lung field predominance. Emphysema was scored visually in bilateral upper, middle, and lower lung fields according to the method used by Goddard et al. (17, 32). Briefly, the score in each field was calculated for the dimensions according to the ratio of low-attenuation area (LAA) as follows: score 0, LAA < 5%; score 1, 5% ≤ LAA < 25%; score 2, 25% ≤ LAA ≤ 50%; score 3, 50% ≤ LAA ≤ 75%; score 4, 75% ≤ LAA. The severity of emphysema was graded in accordance with the sum of scores for six dimensions (minimum score 0 to maximum score 24). CT images were analyzed independently by two pulmonologists with no information of the patients' clinical status.

Measurement of plasma sCD40L. All samples were collected at the time of diagnosis, namely before any intervention for COPD was initiated. Peripheral venous blood samples were collected by single puncture with 18-gauge needle by physicians. All samples were centrifuged at 3,000 *g* for 10 min and stored at −80°C until analysis. Soluble CD40L in plasma were determined using commercial ELISA kit (R&D Systems) according to the manufacturer's instruction.

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed using Stat View 5.0 (SAS Institute, Cary, NC). Group comparisons were made by using χ^2 test and unpaired Student's *t*-test. Pearson's correlation coefficients and multiple-regression analysis were used to identify the variables that influenced plasma sCD40L level. Multiple-factor analysis of variance was used to assess the independent and synergistic effects of treatment with combinations of CD40 Ab, Fas Ab, and IFN-γ. In these tests, rather than compare any two specific conditions, the statistical test deter-

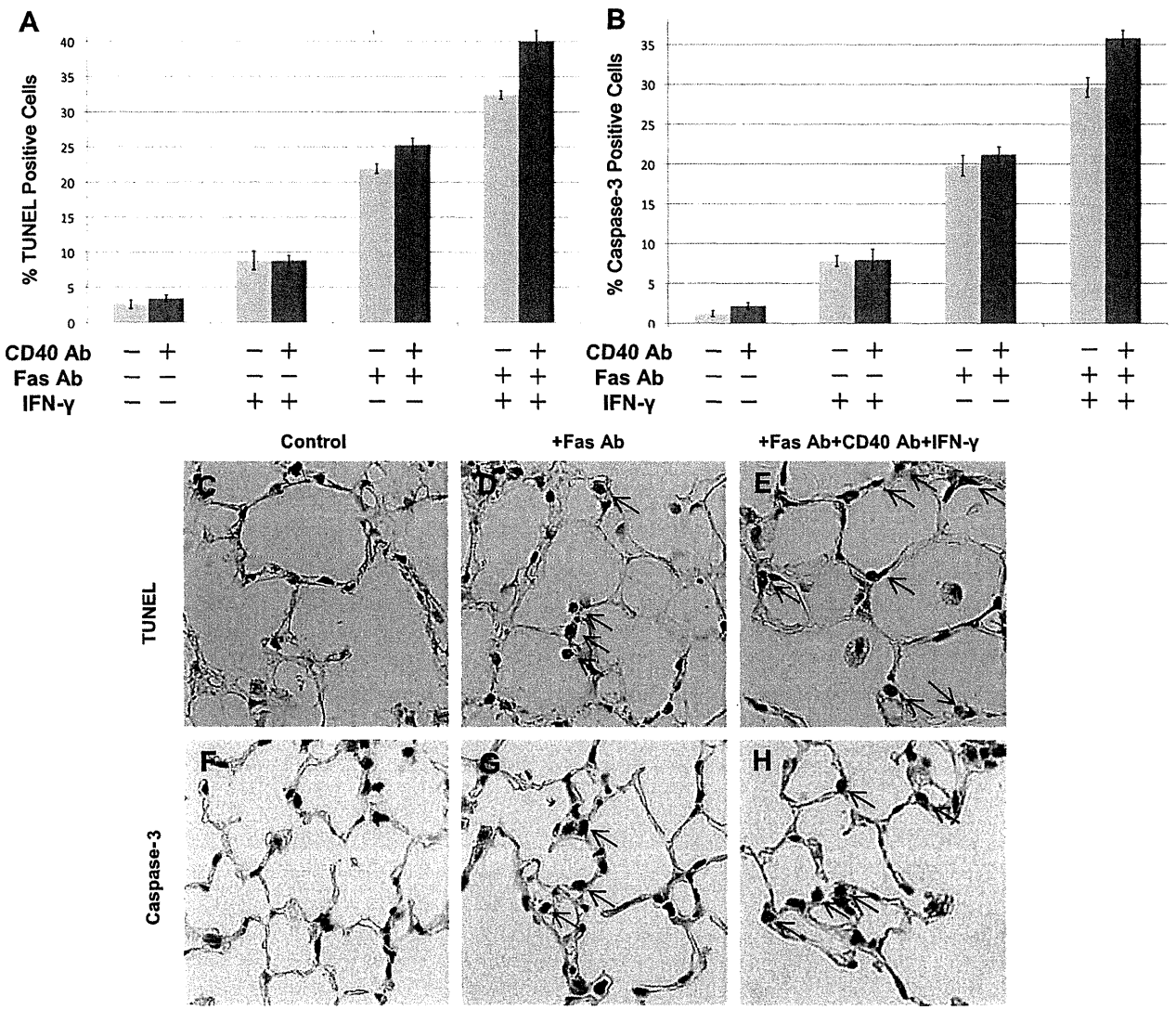


Fig. 4. Quantification of terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)- (*A*) and Caspase-3-positive (*B*) cells in mouse lung. Data are means ± SE from 5 experiments. Multiple-factor ANOVA was used to test the effects of IFN-γ, CD40 Ab, and Fas Ab. *C–H*: localization of TUNEL- and Caspase-3-positive cells (white-encircled) in lung tissue sections from 5 mice. By multiple-factor ANOVA, the effects on the TUNEL$^+$ staining of all 3 singly were significant at $P < 0.0001$, and there was synergy between CD40 Ab and Fas Ab ($P = 0.0004$) and also between IFN-γ and Fas Ab ($P < 0.0001$). By Fisher's LSD post hoc analysis, CD40 had a significant effect ($P < 0.05$) in increasing TUNEL staining in combination with either Fas Ab or the combination of Fas Ab and IFN-γ, but not when used alone. By multiple-factor ANOVA, effects of IFN-γ, CD40 Ab, or Fas Ab on the Caspase-3 staining were significant at $P < 0.0001$, $P = 0.003$, and $P < 0.0001$, respectively. Interactions between Fas Ab and both CD40 Ab and IFN-γ were significant at $P = 0.03$ and $P < 0.0001$ respectively. By Fisher's LSD post hoc analysis, CD40 significantly ($P < 0.05$) increased Caspase-3 staining in combination with Fas Ab and IFN-γ, but not under other conditions.

mines whether each factor has significant effect across conditions and then determines whether those effects are altered in an additive or synergistic way when combined with other factors. It is these statistical values that are usually reported, rather than comparisons of any two conditions; where post hoc tests were performed, they were done by Fisher's least-significant difference and reported as such. The statistical significance of dose-response curves was determined by the correlation z-test using log-transformed doses, i.e., apoptosis rate plotted against log (dose).

RESULTS

CD40 activation induced emphysematous changes in mice. We first determined whether CD40 activation, alone or in combination with other factors, was capable of inducing an emphysematous phenotype in mice. IFN-γ and Fas activation were chosen as the two cofactors, IFN-γ because it is well known to be involved in the pathogenesis of emphysema (3, 6, 8) and produces an accepted mouse model of emphysema (39), and Fas because synergy between CD40/CD40L and Fas has been demonstrated previously (1, 38) and Fas-driven apoptosis may be mechanistically relevant. Mice received activating antibodies to CD40 (CD40 Ab), IFN-γ, and activating antibodies to Fas (Fas Ab), individually or in combination, by inhalation every 3 days for 24 days. Inhalation of activating Fas Ab has been reported to cause acute lung injury associated with the infiltration of massive leukocyte (23, 29). To see the chronic effects for alveolar destruction, we started Fas Ab instillation from a smaller dose and gradually escalated to determine the final dose.

Before performing inhalation experiments, we first confirmed constitutive expression of CD40 and Fas in alveoli. Both CD40 and Fas were expressed by mouse lung alveoli, and IFN-γ stimulation enhanced the expression of CD40 (Fig. 1). In assessment of emphysematous change, IFN-γ and Fas-activation each independently increased MLI. Although CD40 activation alone did not show a strong effect on MLI, it increased MLI induced by Fas Ab, IFN-γ, or Fas Ab + IFN-γ (Fig. 2, *A–I*). These data indicate that CD40 activation is capable of enhancing alveolar wall destruction by Fas and by IFN-γ, resulting in emphysematous changes in mice lung.

In addition to the presence of dilation of alveoli and an enlargement of respiratory bronchiole alveolar duct complex, a decrease in the numbers of alveoli recognized as a closed curve means an enlargement of continuous terminal airway with disruption of septa, which has been known as the essential of remodeling in case of emphysema. Of interest, mononuclear cell accumulation, predominantly lymphocytes, at the perivascular site was observed only in the CD40-activation group (Fig. 2, *J* and *K*). This implies that CD40 stimulation elicits lymphocyte activation or induction of chemotactic molecule secretion from local tissues. In vivo, CD40 activation, as well as IFN-γ, significantly increased inflammatory cell accumulation in lung, especially lymphocytes, in BALF (Fig. 3). CD40 activation dramatically enhanced the accumulation of inflammatory cell when combined with IFN-γ.

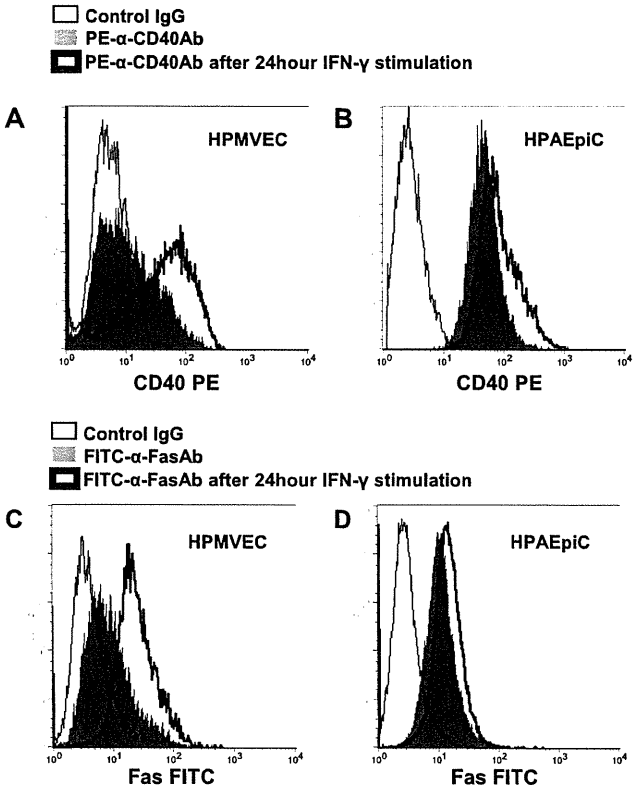


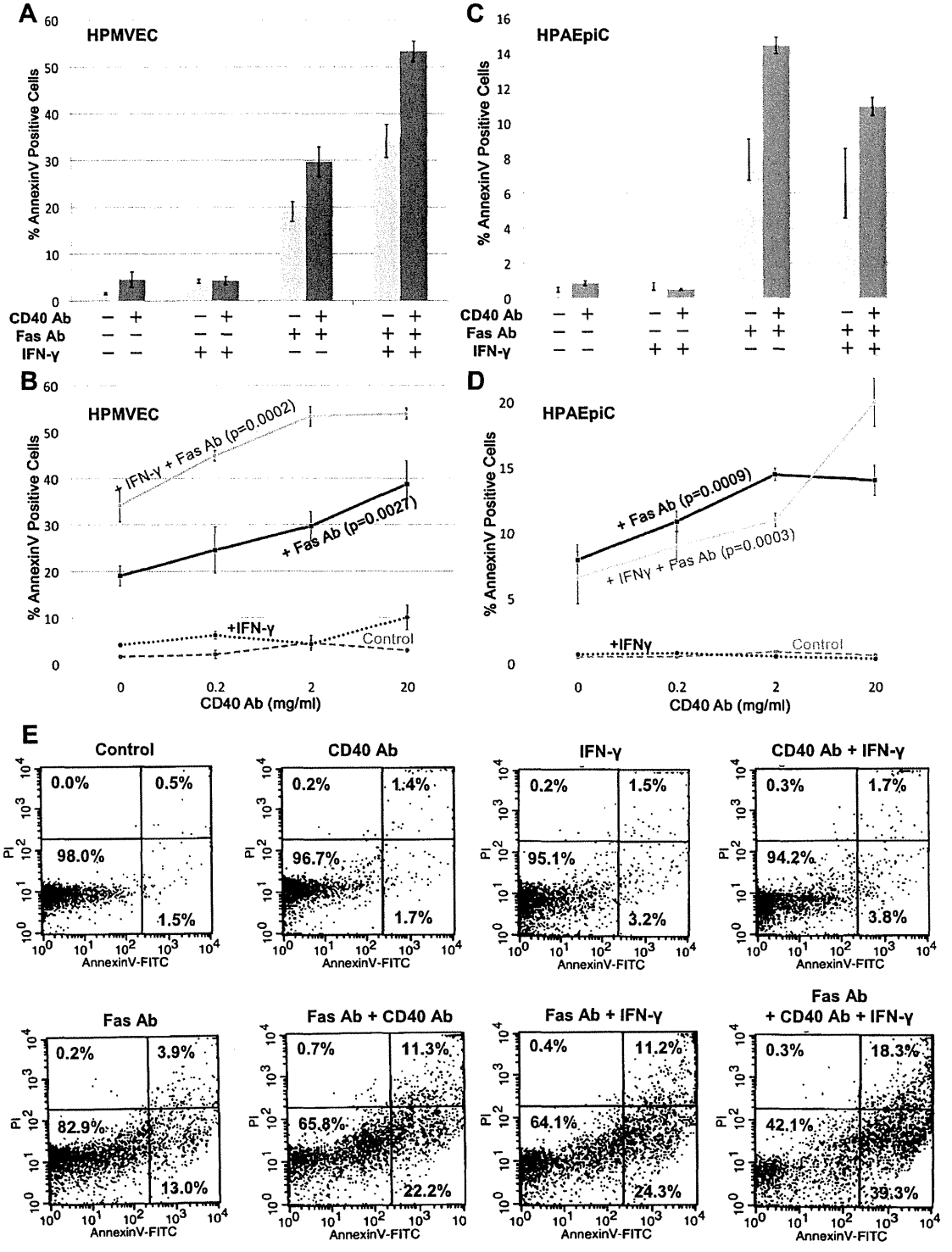
Fig. 5. *A* and *B*: expression of CD40 in human pulmonary microvascular endothelial cells (HPMVEC) (*A*) and human pulmonary alveolar epithelial cells (HPAEpiC) (*B*). *C* and *D*: expression of Fas in HPMVEC (*C*) and HPAEpiC (*D*). Data for control IgG, in the absence of IFN-γ, and in the presence of IFN-γ are shown as white histogram with thin line, gray shaded histogram, and white histogram with thick line, respectively. PE, phycoerythrin.

CD40 activation increased apoptotic cells in alveoli. Apoptosis is thought to play a major role in emphysema (2) and has been shown to drive emphysematous changes in mouse models (5, 22). To determine whether the increase in MLI in mice treated with activating antibody to CD40 correlated with increased apoptosis, TUNEL and caspase-3 staining were performed on lung sections, with positive cells counted as a proportion of the total cells. In TUNEL staining, CD40 activation, IFN-γ, and Fas activation each independently increased

Fig. 6. HPMVEC (*A* and *B*) and HPAEpiC (*C* and *D*) apoptosis following CD40, IFN-γ, and Fas stimulation. Data are means ± SE from 3 experiments. Multiple-factor ANOVA was used in *A* and *C* to test the effects of IFN-γ, CD40 Ab, and Fas Ab. The correlation z-test was used in *B* and *D* to test the dose response for increasing concentrations of activating mAb to CD40 in combination with Fas Ab and/or IFN-γ. *A*: effects of all 3 singly were significant at $P < 0.0001$; there was synergy between Fas Ab and CD40 Ab ($P = 0.0004$) and between Fas Ab and IFN-γ ($P < 0.0001$). *B*: there was no effect of CD40 alone; however, increasing CD40 activation, in combination with Fas Ab and Fas Ab + IFN-γ, increased the percentage of apoptotic cells. *C*: both CD40 Ab and Fas Ab increased apoptosis, and this was significant at $P < 0.0003$. IFN-γ did not significantly affect apoptosis in this model, either as a single factor or in combination with other factors. The combined effects of Fas Ab and CD40 Ab were synergistic ($P = 0.0004$). *D*: there was no effect of CD40 alone; however, in combination with Fas Ab, increasing CD40 activation significantly increased the percentage of apoptotic cells (P values indicated). By multiple-factor ANOVA, IFN-γ had no effect on apoptosis in this model. *E*: representative flow cytometry plots for Annexin V and phosphatidyl inositol in HPMVEC.

the numbers of apoptotic nuclei, and there was a mild synergy between CD40 and Fas, as well as between IFN-γ and Fas (Fig. 4*A*). In caspase-3 staining, CD40 activation slightly increased the number of caspase-3-positive cells, whereas IFN-γ and Fas activation significantly increased caspase-3-positive cells. Moreover, in accordance with the TUNEL results, there was a mild synergy between CD40 and Fas (Fig. 4*B*). These results indicate that CD40 activation slightly increased cell death in lung although its major effect in apoptosis was to enhance the death signal by Fas.

CD40 and Fas were constitutively expressed on HPMVEC and HPAEpiC. We next determined whether CD40 directly affects alveolar endothelial and epithelial cells, alone or in



combination with cofactors in vitro. Previous results on CD40 were based on experiments using human umbilical vein endothelial cells, and little is known about CD40 in lung-derived cells. Flow cytometry was therefore performed to confirm the cell surface expression of CD40 and Fas in HPMVEC and in HPAEpiC. Both CD40 (Fig. 5, *A* and *B*) and Fas (Fig. 5, *C* and *D*) were constitutively expressed in both cell types, although at a lower level in endothelium. CD40 and Fas were both upregulated by incubation with IFN-γ in both cell types, although to a greater extent in endothelium than in epithelium. Enhancement of Fas expression by CD40 activation, which occurs in B cells (38), was not observed in either cell type (data not shown).

Sensitization of Fas-mediated cell death by CD40 and/or IFN-γ stimulation. After confirming the expression of CD40 and Fas on both cell types, we determined whether CD40 would increase apoptosis in cultured cells; if this were so, it implies that the effect is on the cells themselves, rather than being mediated through bystander inflammation. HPMVEC or HPAEpiC were incubated for 12 h in medium containing activating anti-CD40 Ab, activating anti-Fas Ab, IFN-γ, or combinations of these, and apoptotic cells were evaluated by flow cytometry.

In endothelial cells (HPMVEC), the effects of both CD40 Ab and IFN-γ, as individual factors, on Annexin V-positive cell counts were weak. Fas Ab induced cell death in ~20% of cells. However, both CD40 Ab and IFN-γ enhanced Fas-mediated cell death ($P = 0.0004$ and $P < 0.0001$ by multiple-factor ANOVA, respectively, Fig. 6*A*). The enhanced effect of CD40 Ab in combination with Fas Ab and Fas Ab + IFN-γ was further assessed in a dose-response study (Fig. 6*B*). CD40 activation, either alone or in combination with IFN-γ, had no significant effect on cell death. However, increased CD40 activation, in combination with either Fas Ab or Fas Ab + IFN-γ, resulted in significantly increased cell death, suggesting that cell death is primarily triggered by Fas ligation.

In epithelial cells (HPAEpiC), the induction of cell death was generally less pronounced than in HPMVEC. In HPAEpiC, IFN-γ had no significant effect on cell death, either alone or in combination with Fas Ab or CD40 Ab. CD40 activation had little effect alone but significantly enhanced Fas-mediated cell death (Fig. 6*C*). CD40 activation also showed a strong and significant dose-response effect in the amplification of Fas-mediated cell death but had no effect alone or in combination with IFN-γ (Fig. 6*D*).

Taken together, these data indicate that CD40 potentiates Fas-mediated cell death in both endothelial and epithelial cells but that the effect of IFN-γ is specific to endothelial cells. It is plausible that the effect of IFN-γ on cell death is entirely the result of its effects on CD40 and Fas, which it induces to a much greater extent in endothelial cells than in epithelial cells (Fig. 5, *A–D*).

Induction of CCR5 ligands by CD40 stimulation. The findings thus far indicate that CD40 amplified Fas-mediated apoptosis in mice lungs (Fig. 4) and in pulmonary endothelial and epithelial cells (Fig. 6) but showed little effect on apoptosis, either alone or in combination with IFN-γ (Figs. 4 and 6). When combined with IFN-γ, however, CD40 did drive the loss of alveoli in mice (Fig. 2*I*). This suggests that, although amplification of Fas-mediated apoptosis is one effect of CD40 in emphysema, other independent effects may also exist.

In other systems, CD40 has been shown to regulate proinflammatory molecules (13, 26, 36) involved in sustained inflammation, resulting in mild alveolar loss in the lung. Among them, we noticed the important role of CCR5 ligands such as MIP-1α, MIP-1β, and RANTES because these molecules may be involved in IFN-γ-induced inflammation and remodeling in the pathogenesis of pulmonary emphysema. CCR5 ligands play a role in the progression of emphysema by an IFN-γ-dependent mechanism that involves the regulation of cell death, as well as caspase, matrix metalloproteinase, and antiprotease activities (3, 24). Thus their concentrations were measured in culture supernatants from HPMVEC and HPAEpiC treated by activating CD40 Ab and/or IFN-γ. In HPMVEC, both CD40 Ab and IFN-γ, as independent factors, significantly induced RANTES and MIP-1β secretion in culture supernatant (Fig. 7). Synergy between CD40 Ab and IFN-γ was observed for secretion of RANTES. In HPAEpiC, the levels of CCR5 ligands were below the threshold of detection (data not shown). RANTES is a potent chemoattractant for monocyte and T cell, and its expression is often upregulated, especially in acute exacerbations of COPD (8, 24). We measured RANTES in BALF from treated mice and confirmed that RANTES was also increased in vivo by sole CD40 stimulation or in combination with IFN-γ (Fig. 7*D*).

Plasma sCD40L was increased in patients with COPD. Circulating levels of soluble CD40 ligand (sCD40L) are upregulated in systemic vascular diseases, such as ischemic heart disease, stroke, pulmonary hypertension, and even in cigarette smokers (12, 16, 19, 20). Furthermore, we found that CD40 stimulation induces emphysematous change in mouse lung. Therefore, we lastly wanted to determine whether or not sCD40L was upregulated in patients with COPD. The COPD group showed significant decreases in $\%FEV_1$ and FEV_1/FVC as expected. Body mass index was also decreased in the COPD group (Table 1). Plasma sCD40L levels were significantly higher in the COPD group compared with the healthy smoker group (COPD 2.1 ± 0.2 ng/ml, healthy smoker 0.8 ± 0.2 ng/ml: $P = 0.008$) (Fig. 8). Notably, plasma sCD40L level in patients was oppositely correlated to $\%FEV_1$ ($R = 0.68$, $P < 0.0001$) and positively correlated to LAA score ($R = 0.54$, $P < 0.0001$) regardless of pack/year smoking (Fig. 9, *A* and *B*, Table 2). These findings may indicate that high concentration of plasma sCD40L is associated with impaired lung function, as well as alveolar structural destruction, regardless of smoking history.

DISCUSSION

In the present study, we showed in mice that CD40 agonists, both alone and in concert with Fas agonists and IFN-γ, drive the enlargement of airspaces (Fig. 2*I*). In concert with the mouse data, patients with COPD have elevated plasma levels of sCD40L, which was correlated to impaired lung function and alveolar destruction (Fig. 9). A likely mechanism is the amplification of Fas-mediated apoptosis by CD40, as demonstrated both in mice (Fig. 4) and in human pulmonary endothelial and epithelial cells (Fig. 6). IFN-γ enhanced the airspace enlargement caused by CD40 agonist (Fig. 2*I*) although

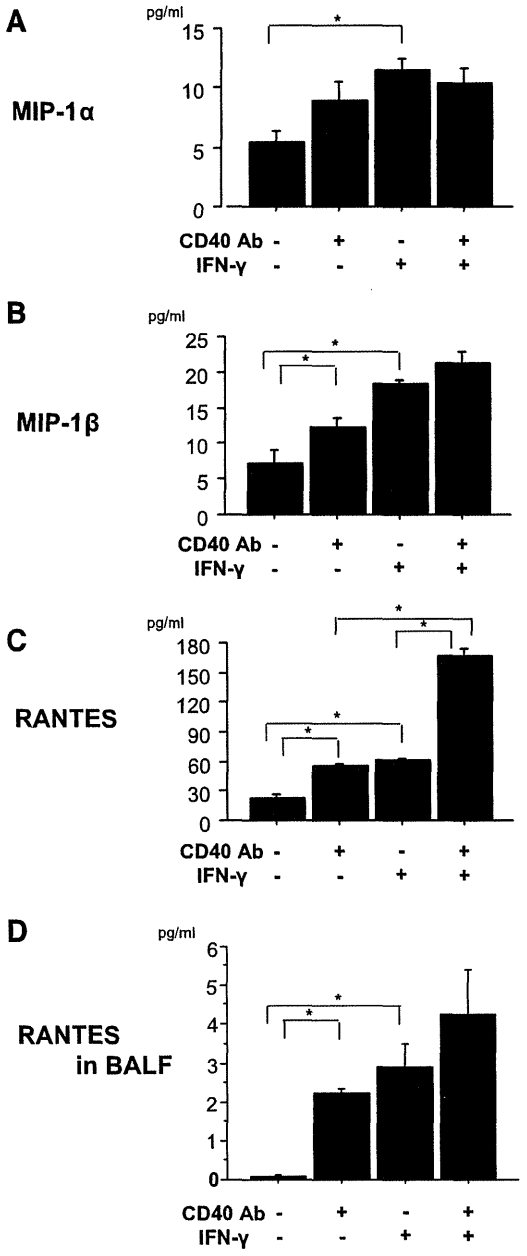


Fig. 7. *A–C*: CCR5 ligand levels in cell supernatants as assessed using the Bioplex system (n = 5), after a 12-h incubation with CD40 agonist and/or IFN-γ in HPMVEC. *D*: chemokine regulated on activation normal T cells expressed and secreted (RANTES) level in BALF from mice with treatments (n = 4). *P < 0.05. MIP, macrophage inflammatory protein.

synergy between CD40 and IFN-γ in apoptotic induction was not obvious (Figs. 4 and 6). It might indicate that collaboration between CD40 and IFN-γ in mediating emphysema probably does not occur through enhanced apoptosis but rather through enhanced inflammation (Fig. 7).

Fas-mediated apoptosis of alveolar cells has previously been reported only in acute lung injury and pulmonary fibrosis (23, 29). However, in the context of emphysema, endothelial and epithelial cell apoptosis in COPD were thought to be driven by

Table 1. *Participant characteristics*

	Healthy Smoker (n = 19)	COPD (n = 69)	P Value
Age, yr	72.4 ± 2.2	73.3 ± 0.7	n.s.
Male/Female , n	19/0	62/7	n.s.
BMI	23.2 ± 0.6	19.9 ± 0.3	< 0.0001
FEV_1/FVC, %	79.4 ± 0.3	45.3 ± 1.1	< 0.0001
$\%FEV_1$, %	104.7 ± 4.1	44.5 ± 1.8	< 0.0001
Pack/Year	27.9 ± 4.9	34.6 ± 2.1	n.s.
Plasma sCD40L, ng/ml	0.8 ± 0.2	2.1 ± 0.2	0.008

Applicable values are means ± SE. COPD, chronic obstructive pulmonary disorder; BMI, body mass index; n.s., not significant; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity.

the release of perforin and granzyme from $CD8^+$T cells (3, 8). The interaction of Fas/FasL system in the development of COPD has long been discussed, only in the context of circulating FasL levels in the patient's serum (34, 41), and it remains controversial. However, because $CD8^+$ T cells are a major source of functional FasL, it is quite possible that these cells also mediate alveolar or endothelial apoptosis through the Fas/FasL pathway, in addition to perforin and granzyme release, thus reconciling these two mechanisms. Sensitization of Fas by CD40 stimulation has only been reported in activated B cells and a few other cell types (1, 31, 38). Although it is not surprising that the same mechanism appears to function in pulmonary cells, this finding is relevant and of potential importance in the pathophysiology of emphysema. The role of IFN-γ in emphysema has been studied in a mouse transgenic overexpression model. In that model, the pathogenesis of emphysema was attributed to overactivation of matrix metalloproteinase-12 although alveolar apoptosis was not confirmed (39). This is consistent with the present data, which showed that IFN-γ did not induce apoptosis on its own, either in epithelial or endothelial cells. The present findings suggest a potential mechanism for the effect of IFN-γ in emphysema, namely the induction of CD40 and Fas. However, the major mechanism for the effect of IFN-γ and the interaction between CD40 and IFN-γ appeared to be through the induction of

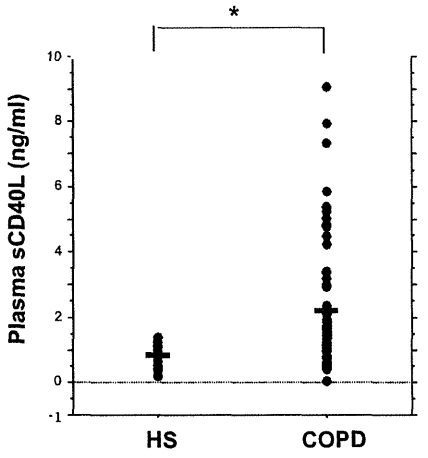


Fig. 8. Plasma sCD40L level in patients with chronic obstructive pulmonary disease (COPD) (n = 69) was increased compared with that of in healthy smoker (HS; n = 19). COPD 2.1 ± 0.2 ng/ml, HS 0.8 ± 0.2 ng/ml, P = 0.008. Values are means ± SE.

CCR5 ligands, such as MIP-1β and RANTES. In the present study, the increase in RANTES attributable to CD40 activation was comparable to that stimulated by high concentrations of IFN-γ, and this was further increased by the combination of CD40 with IFN-γ. Thus, in concert with IFN-γ, CD40 plays a role in sustaining inflammation by increasing the secretion of RANTES.

One of the limitations of this study was that we have not investigated the recruitment of professional antigen-presenting cells, such as dendritic cells and macrophages, although these cells have been suggested to play important roles in emphysema (3, 6). Surface expression of CD40 on pulmonary dendritic cells has been reported to be increased by smoke inhalation in mice (11). However, Matute et al. (4, 25) reported that, in acute lung injury, Fas directly affects alveolar cells, not via myeloid cells such as alveolar macrophages. Indeed, we did not detect increased numbers of apoptotic immune cells in lungs of our mouse models. CD40 signaling is an effective driver of antigen-presenting cells for maturation and producing Th1/Tc1 cytokines as IFN-γ (15, 33); thus we speculate that the effects on immune cell by these molecules did not alter the present results. Increased lymphocyte recruitment was observed in the CD40 agonist model systems and was consistent with the findings of increased chemokine secretion in vitro and in vivo. This observation suggested that increased recruitment of inflammatory cells may be one of the mechanisms through which CD40 facilitates IFN-γ-induced emphysematous change.

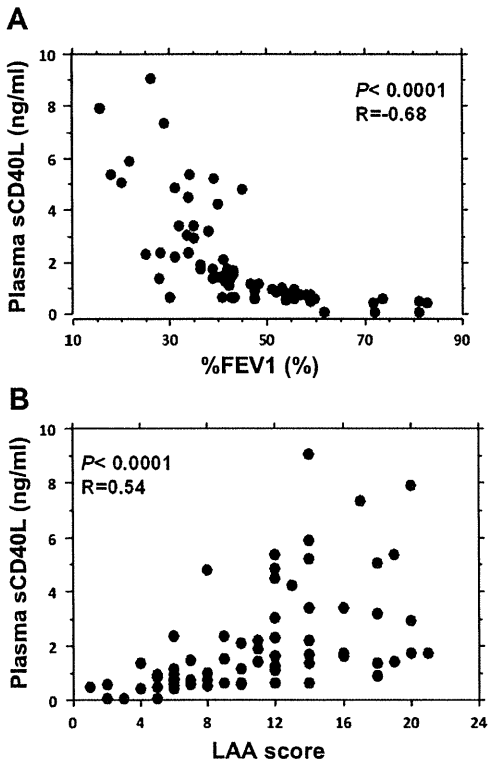


Fig. 9. Correlations between the percentage of forced expiratory volume in 1 s (%FEV_1) or low-attenuation area (LAA) score, and plasma sCD40L level. Plasma sCD40L level in patients was oppositely correlated to %FEV_1 (R = 0.68, $P < 0.0001$, *A*) and positively correlated to LAA score (R = 0.54, $P <$ 0.0001, *B*).

Table 2. *Relationship between sCD40L and emphysema-associated factors*

Variable	SRC	*P* value
%FEV_1	−0.656	<0.0001
Pack/Year	0.14	0.12
LAA score	0.51	<0.0001
Pack/Year	0.11	0.32

Multiple-regression analysis to find which is more associated, pack/year or % FEV_1 and low-attenuation area (LAA), with plasma sCD40L level in patients with COPD (n = 69). SRC, standardized regression coefficients.

We analyzed only type-1-dominant alveolar cell (HPAEpiC: composed of 95% of alveolar type I cell) in the present study. However, alveolar type II cells are considered to be local progenitor cells that have the ability to compensate for the loss of type I cells and are resistant to apoptotic signals (28). Because growing evidence suggests that impaired repair of alveoli is a critical mechanism in emphysema (10, 21), it will be interesting to analyze the response of both type I and type II alveolar cells.

Taking all the findings together, we conclude that CD40 enhances Fas-mediated apoptosis in alveolar cells and secretion of proinflammatory chemokines, both of which are associated with the development of pulmonary emphysema.

This study adds insight to reconsider the role of TNF-family molecules in the pathogenesis of the disease. Blocking or normalization of the CD40 signaling pathway might be an alternative therapeutic strategy for the treatment of pulmonary emphysema.

ACKNOWLEDGMENTS

The authors thank M. Shinozaki (Department of Pathology, Toho University), N. Tsuchiya, C. Matsumoto and M. Fukutake (Tsumura & Co.) for their excellent technical assistance, and Dr. Stephen I. Rennard (Pulmonary and Critical Care Medicine, Nebraska Medical Center) for insightful comments and suggestions throughout the study.

GRANTS

This research was partially supported by the Ministry of Education, Science, Sports and Culture, Grant-in-Aid for Scientific Research (C) (23591114), the Health Science Research Grants for Research on Emerging and Re-emerging, Infectious Diseases (H22-Shinko-Ippan-008 and H23-Shinko-Ippan-018) from the Ministry of Health, Labor and Welfare of Japan, a grant from the Strategic Basis on Research Grounds for Non-governmental Schools at Heisei 20th from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant from the Strategic Research Foundation Grant-aided Project for Private schools at Heisei 23rd from Ministry of Education, Culture, Sports, Science and Technology of Japan (2011–2015).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: A. Shigeta, Y. Tada, and K.T. conception and design of research; A. Shigeta, S.I., J.T., K.Y., A. Sakamoto, K.S., and K.H. performed experiments; A. Shigeta and J.W. analyzed data; A. Shigeta, Y. Tada, J.-Y.W., A. Sakamoto, K.S., K.H., and J.W. interpreted results of experiments; A. Shigeta and J.W. prepared figures; A. Shigeta and Y. Tada drafted manuscript; A. Shigeta, Y. Tada, J.-Y.W., S.I., J.T., K.Y., Y.K., K.I., N.T., Y. Takiguchi, A. Sakamoto, T.T., K.S., K.H., J.W., and K.T. approved final version of manuscript; Y. Tada, J.-Y.W., Y.K., K.I., N.T., Y. Takiguchi, A. Sakamoto, T.T., K.S., K.H., J.W., and K.T. edited and revised manuscript.

REFERENCES

1. **Afford SC, Randhawa S, Eliopoulos AG, Hubscher SG, Young LS, Adams DH.** CD40 activation induces apoptosis in cultured human hepatocytes via induction of cell surface fas ligand expression and amplifies fas-mediated hepatocyte death during allograft rejection. *J Exp Med* 189: 441–446, 1999.
2. **Aoshiba K, Yokohori N, Nagai A.** Alveolar wall apoptosis causes lung destruction and emphysematous changes. *Am J Respir Cell Mol Biol* 28: 555–562, 2003.
3. **Barnes PJ.** Immunology of asthma and chronic obstructive pulmonary disease. *Nat Rev Immunol* 8: 183–192, 2008.
4. **Bem RA, Farnand AW, Wong V, Koski A, Rosenfeld ME, van Rooijen N, Frevert CW, Martin TR, Matute-Bello G.** Depletion of resident alveolar macrophages does not prevent Fas-mediated lung injury in mice. *Am J Physiol Lung Cell Mol Physiol* 295: L314–L325, 2008.
5. **Brass DM, Hollingsworth JW, Cinque M, Li Z, Potts E, Toloza E, Foster WM, Schwartz DA.** Chronic LPS inhalation causes emphysema-like changes in mouse lung that are associated with apoptosis. *Am J Respir Cell Mol Biol* 39: 584–590, 2008.
6. **Brusselle GG, Joos GF, Bracke KR.** New insights into the immunology of chronic obstructive pulmonary disease. *Lancet* 378: 1015–1026, 2011.
7. **Chakrabarti S, Rizvi M, Pathak D, Kirber MT, Freedman JE.** Hypoxia influences CD40-CD40L mediated inflammation in endothelial and monocytic cells. *Immunol Lett* 122: 170–184, 2009.
8. **Chung KF, Adcock IM.** Multifaceted mechanisms in COPD: inflammation, immunity, and tissue repair and destruction. *Eur Respir J* 31: 1334–1356, 2008.
9. **Churg A, Wang RD, Tai H, Wang X, Xie C, Wright JL.** Tumor necrosis factor-alpha drives 70% of cigarette smoke-induced emphysema in the mouse. *Am J Respir Crit Care Med* 170: 492–498, 2004.
10. **Crosby LM, Waters CM.** Epithelial repair mechanisms in the lung. *Am J Physiol Lung Cell Mol Physiol* 298: L715–L731, 2010.
11. **D'Hulst AI, Vermaelen KY, Brusselle GG, Joos GF, Pauwels RA.** Time course of cigarette smoke-induced pulmonary inflammation in mice. *Eur Respir J* 26: 204–213, 2005.
12. **Damas JK, Otterdal K, Yndestad A, Aass H, Solum NO, Froland SS, Simonsen S, Aukrust P, Andreassen AK.** Soluble CD40 ligand in pulmonary arterial hypertension: possible pathogenic role of the interaction between platelets and endothelial cells. *Circulation* 110: 999–1005, 2004.
13. **Dechanet J, Grosset C, Taupin JL, Merville P, Banchereau J, Ripoche J, Moreau JF.** CD40 ligand stimulates proinflammatory cytokine production by human endothelial cells. *J Immunol* 159: 5640–5647, 1997.
14. **Donjerkovic D, Scott DW.** Activation-induced cell death in B lymphocytes. *Cell Res* 10: 179–192, 2000.
15. **Freeman CM, Martinez FJ, Han MK, Ames TM, Chensue SW, Todt JC, Arenberg DA, Meldrum CA, Getty C, McCloskey L, Curtis JL.** Lung dendritic cell expression of maturation molecules increases with worsening chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 180: 1179–1188, 2009.
16. **Garlichs CD, Kozina S, Fateh-Moghadam S, Handschu R, Tomandl B, Stumpf C, Eskafi S, Raaz D, Schmeisser A, Yilmaz A, Ludwig J, Neundorfer B, Daniel WG.** Upregulation of CD40-CD40 ligand (CD154) in patients with acute cerebral ischemia. *Stroke* 34: 1412–1418, 2003.
17. **Goddard PR, Nicholson EM, Laszlo G, Watt I.** Computed tomography in pulmonary emphysema. *Clin Radiol* 33: 379–387, 1982.
18. **Grewal IS, Flavell RA.** The role of CD40 ligand in costimulation and T-cell activation. *Immunol Rev* 153: 85–106, 1996.
19. **Harding SA, Sarma J, Josephs DH, Cruden NL, Din JN, Twomey PJ, Fox KA, Newby DE.** Upregulation of the CD40/CD40 ligand dyad and platelet-monocyte aggregation in cigarette smokers. *Circulation* 109: 1926–1929, 2004.
20. **Heeschen C, Dimmeler S, Hamm CW, van den Brand MJ, Boersma E, Zeiher AM, Simoons ML.** Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 348: 1104–1111, 2003.
21. **Huh JW, Kim SY, Lee JH, Lee JS, Van Ta Q, Kim M, Oh YM, Lee YS, Lee SD.** Bone marrow cells repair cigarette smoke-induced emphysema in rats. *Am J Physiol Lung Cell Mol Physiol* 301: L255–L266, 2011.
22. **Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF.** Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 106: 1311–1319, 2000.
23. **Kuwano K, Hagimoto N, Kawasaki M, Yatomi T, Nakamura N, Nagata S, Suda T, Kunitake R, Maeyama T, Miyazaki H, Hara N.** Essential roles of the Fas-Fas ligand pathway in the development of pulmonary fibrosis. *J Clin Invest* 104: 13–19, 1999.
24. **Ma B, Kang MJ, Lee CG, Chapoval S, Liu W, Chen Q, Coyle AJ, Lora JM, Picarella D, Homer RJ, Elias JA.** Role of CCR5 in IFN-gamma-induced and cigarette smoke-induced emphysema. *J Clin Invest* 115: 3460–3472, 2005.
25. **Matute-Bello G, Lee JS, Liles WC, Frevert CW, Mongovin S, Wong V, Ballman K, Sutlief S, Martin TR.** Fas-mediated acute lung injury requires fas expression on nonmyeloid cells of the lung. *J Immunol* 175: 4069–4075, 2005.
26. **Melter M, Reinders ME, Sho M, Pal S, Geehan C, Denton MD, Mukhopadhyay D, Briscoe DM.** Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis in vivo. *Blood* 96: 3801–3808, 2000.
27. **Merendino AM, Bucchieri F, Gagliardo R, Daryadel A, Pompeo F, Chiappara G, Santagata R, Bellia V, David S, Farina F, Davies DE, Simon HU, Vignola AM.** CD40 ligation protects bronchial epithelium against oxidant-induced caspase-independent cell death. *Am J Respir Cell Mol Biol* 35: 155–164, 2006.
28. **Mura M, Binnie M, Han B, Li C, Andrade CF, Shiozaki A, Zhang Y, Ferrara N, Hwang D, Waddell TK, Keshavjee S, Liu M.** Functions of type II pneumocyte-derived vascular endothelial growth factor in alveolar structure, acute inflammation, and vascular permeability. *Am J Pathol* 176: 1725–1734, 2010.
29. **Neff TA, Guo RF, Neff SB, Sarma JV, Speyer CL, Gao H, Bernacki KD, Huber-Lang M, McGuire S, Hoesel LM, Riedemann NC, Beck-Schimmer B, Zetoune FS, Ward PA.** Relationship of acute lung inflammatory injury to Fas/FasL system. *Am J Pathol* 166: 685–694, 2005.
30. **Pauwels RA, Buist AS, Calverley PM, Jenkins CR, Hurd SS.** Global strategy for the diagnosis, management, and prevention of chronic obstructive pulmonary disease. NHLBI/WHO Global Initiative for Chronic Obstructive Lung Disease (GOLD) Workshop summary. *Am J Respir Crit Care Med* 163: 1256–1276, 2001.
31. **Ping L, Ogawa N, Sugai S.** Novel role of CD40 in Fas-dependent apoptosis of cultured salivary epithelial cells from patients with Sjogren's syndrome. *Arthritis Rheum* 52: 573–581, 2005.
32. **Sakao S, Tatsumi K, Igari H, Watanabe R, Shino Y, Shirasawa H, Kuriyama T.** Association of tumor necrosis factor-alpha gene promoter polymorphism with low attenuation areas on high-resolution CT in patients with COPD. *Chest* 122: 416–420, 2002.
33. **Tada Y, O-Wang J, Yu L, Shimozato O, Wang YQ, Takiguchi Y, Tatsumi K, Kuriyama T, Takenaga K, Sakiyama S, Tagawa M.** T-cell-dependent antitumor effects produced by CD40 ligand expressed on mouse lung carcinoma cells are linked with the maturation of dendritic cells and secretion of a variety of cytokines. *Cancer Gene Ther* 10: 451–456, 2003.
34. **Takabatake N, Nakamura H, Inoue S, Terashita K, Yuki H, Kato S, Yasumura S, Tomoike H.** Circulating levels of soluble Fas ligand and soluble Fas in patients with chronic obstructive pulmonary disease. *Respir Med* 94: 1215–1220, 2000.
35. **Triantaphyllopoulos K, Hussain F, Pinart M, Zhang M, Li F, Adcock I, Kirkham P, Zhu J, Chung KF.** A model of chronic inflammation and pulmonary emphysema after multiple ozone exposures in mice. *Am J Physiol Lung Cell Mol Physiol* 300: L691–L700, 2011.
36. **Tripp RA, Jones L, Anderson LJ, Brown MP.** CD40 ligand (CD154) enhances the Th1 and antibody responses to respiratory syncytial virus in the BALB/c mouse. *J Immunol* 164: 5913–5921, 2000.
37. **Walter RE, Wilk JB, Larson MG, Vasan RS, Keaney JF Jr, Lipinska I, O'Connor GT, Benjamin EJ.** Systemic inflammation and COPD: the Framingham Heart Study. *Chest* 133: 19–25, 2008.
38. **Wang J, Watanabe T.** Expression and function of Fas during differentiation and activation of B cells. *Int Rev Immunol* 18: 367–379, 1999.
39. **Wang Z, Zheng T, Zhu Z, Homer RJ, Riese RJ, Chapman HA Jr, Shapiro SD, Elias JA.** Interferon gamma induction of pulmonary emphysema in the adult murine lung. *J Exp Med* 192: 1587–1600, 2000.
40. **Yamauchi K, Kasuya Y, Kuroda F, Tanaka K, Tsuyusaki J, Ishizaki S, Matsunaga H, Iwamura C, Nakayama T, Tatsumi K.** Attenuation of lung inflammation and fibrosis in CD69-deficient mice after intratracheal bleomycin. *Respir Res* 12: 131, 2011.
41. **Yasuda N, Gotoh K, Minatoguchi S, Asano K, Nishigaki K, Nomura M, Ohno A, Watanabe M, Sano H, Kumada H, Sawa T, Fujiwara H.** An increase of soluble Fas, an inhibitor of apoptosis, associated with progression of COPD. *Respir Med* 92: 993–999, 1998.

Clinical and Vaccine Immunology

Immunogenicity of a Monovalent Pandemic Influenza A H1N1 Virus Vaccine with or without Prior Seasonal Influenza Vaccine Administration

Hidetoshi Igari, Akira Watanabe, Shunsuke Segawa, Akiko Suzuki, Mariko Watanabe, Takayuki Sakurai, Masaharu Watanabe, Koichiro Tatsumi, Mikio Nakayama, Kazuo Suzuki and Takeyuki Sato

Clin. Vaccine Immunol. **2012, 19(10):1690. DOI: 10.1128/CVI.00077-12.**
Published Ahead of Print 1 August 2012.