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,1 Kazutoshi Shibuya,5,6 Kenzo Hiroshima,7 James West,8 and Koichiro Tatsumi1

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

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

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...
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 Med-Systems, 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 H₂O, 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% H₂O₂ 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 immered in diaminobenzidine 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(+) Fas Ab(+); F: CD40 Ab(+) IFN-γ(−) Fas Ab(+) Fas Ab(+); G: CD40 Ab(−) IFN-γ(+) Fas Ab(+) Fas Ab(+); H: CD40 Ab(+) IFN-γ(+) Fas Ab(+) 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 diaminobenzidine 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 (HPAECiP) were purchased from Lonza (Walkersville, MD) and ScienCell Research Laboratories (Carlsbad, CA), respectively. HPAECiP 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%), hydrocortisone (0.04%), human epidermal growth factor (0.1%), ascorbic acid (0.1%), gentamicin/amphotericin-B (GA-1000, 0.1%), ascorbic acid (0.1%), and heparin (0.1%). HPAECiP 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%), apo-transferrin (10 μg/ml), ascorbic acid (100 μg/ml), gentamycin/amphotericin-B (GA-1000, 0.1%), ascorbic acid (0.1%), hydrocortisone (1 μg/ml), and heparin (0.4%). HPAECiP were incubated with phycoerythrin-conjugated anti-human CD40 mAb (R&D Systems) and FITC-conjugated anti-human Fas Ab (eBioscience) at 0.2, 2.0, and 20.0 μg/ml and/or recombinant human IFN-γ (1,000 U/ml) were added to HPMVEC and HPAECiP for 24 h before analysis.

Surface expression of CD40 and Fas on HPMVEC and HPAECiP. HPMVEC and HPAECiP were incubated with phycoerythrin-conjugated mouse anti-human CD40 mAb (R&D Systems) and 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 HPAECiP 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 HPAECiP 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.

Secrecion of CCR5 ligands into culture supernatants. Culture medium was removed from 60% confluent HPMVEC and HPAECiP, 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 (FEV1)/forced vital capacity (FVC) ratio < 0.70 with %FEV1 < 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.

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.
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 χ² 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.
involves 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.

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 serine 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. 4A). 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. 4B). 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 up-regulated 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. 6A). 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. 6B). 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. 6C). 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. 6D).

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. 2I). 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. 7D).

Plasma sCD40L was increased in patients with COPD. Circulating levels of soluble CD40 ligand (sCD40L) are up-regulated 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 %FEV1 and FEV1/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 %FEV1 (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. 2I). 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 in 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. 2I) although
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 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. 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 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.

Table 1. Participant characteristics

<table>
<thead>
<tr>
<th>Healthy Smoker</th>
<th>COPD (n = 69)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>72.4 ± 2.2</td>
<td>73.3 ± 0.7</td>
</tr>
<tr>
<td>Male/Female</td>
<td>19/0</td>
<td>62/7</td>
</tr>
<tr>
<td>BMI</td>
<td>23.2 ± 0.6</td>
<td>19.9 ± 0.3</td>
</tr>
<tr>
<td>FEV₁/FVC, %</td>
<td>79.4 ± 0.3</td>
<td>45.3 ± 1.1</td>
</tr>
<tr>
<td>%FEV₁, %</td>
<td>104.7 ± 4.1</td>
<td>44.5 ± 1.8</td>
</tr>
<tr>
<td>Pack/Year</td>
<td>27.9 ± 4.9</td>
<td>34.6 ± 2.1</td>
</tr>
<tr>
<td>Plasma sCD40L, ng/ml</td>
<td>0.8 ± 0.2</td>
<td>2.1 ± 0.2</td>
</tr>
</tbody>
</table>
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/Th1 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.

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

REFERENCES

1. Afford SC, Randhawa S, Eliopoulos AG, Hubscher SG, Young LS, Adams DH. CD40 activation induces apoptosis in cultured human hepa-


11. Donjoerovic D, Scott DW. Activation-induced cell death in B lympho-


