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CD40/CD40 Ligand Interactions Are Critical for Elicitation of Autoimmune-Mediated Fibrosis in the Lung

Jie Zhang-Hoover,* Annika Sutton, † and Joan Stein-Streilein2**

Pulmonary interstitial fibrosis (PIF), associated with persistent inflammation and increased collagen deposition in the interstitium, is often considered an autoimmune disease. Hapten immune PIF (HIPIF), a model for PIF, is elicited in the lung by a single intratracheal (i.t.) challenge in mice sensitized with hapten (2,4,6-trinitrobenzene sulfonic acid, TNBS). In this study, we characterized the role of CD40/CD40 ligand (CD40L) interactions in the elicitation of secondary cell-mediated immune responses that lead to development of fibrosis in the lung using an adoptive transfer model of HIPIF. The expression of CD40 was detected on bronchoalveolar lavage (BAL) cells 1–3 days after i.t. challenge with hapten in the HIPIF lung, but not lungs from the control mice. The CD40bright BAL cells morphologically resembled infiltrating monocytes. Furthermore, blocking CD40/CD40L interactions with blocking Ab decreased BAL production of Th1 mediators (IL-12 and TNF-α). Moreover, either blocking CD40/CD40L interactions with the Ab or using IL-12 knockout recipient mice prevented the increased collagen deposition (accumulation of hydroxyproline) in the lungs during HIPIF induction. We conclude that second signals (CD40/CD40L interactions) are required for elicitation of secondary immune responses that lead to PIF in vivo. The results support the notion that CD40/CD40L interactions are involved in the pathogenesis of an ongoing autoimmune disease. The Journal of Immunology, 2001, 166: 3556–3563.

CD40 is a coreceptor expressed on APCs that binds with CD40 ligand (CD40L)3 expressed on activated T cells during a primary immune response (1–3). CD40/CD40L interactions play a pivotal role in the initiation of most T cell-dependent immune responses (2–4). Although originally defined on B cells, it is now known that CD40 is expressed on both hematopoietic (B cells, activated macrophages, and dendritic cells) and nonhemopoietic cells (epithelial cells, fibroblast, and endothelial cells) (3, 5–7). Maturation of dendritic cells into fully competent APCs involves the up-regulation of CD40 on their surface (2, 3).

Although CD8+ T cells, eosinophils, mast cells, basophils, and NK cells are able to express the molecule, CD40L is expressed primarily by activated CD4+ T cells (2, 3). When CD40L ligates on dendritic cells or macrophages, B7 coreceptor molecules are up-regulated and inflammatory cytokines (IL-12, TNF-α, and IL-1) are produced (3, 8–11). Moreover, CD40/CD40L interactions are crucial in priming, expansion, and maturation of CD4+ T cells (2, 9, 12, 13) and are critical for the initiation and maintenance of macrophage/dendritic cell-mediated inflammation (7, 14–16). Blocking CD40/CD40L interactions reduces allograft rejection and prolongs graft survival (3, 17). It is well established that CD40/CD40L interactions are absolutely required for initiation of primary immune responses and accompanying inflammatory responses.

In contrast to what is known about CD40 and primary immune responses there are only a few studies evaluating the role of CD40 binding during secondary immune responses in vivo. Recently, Gerritte and colleagues showed that in vivo administration of anti-CD40L Ab inhibited the elicitation of experimental allergic encephalitis (EAE) in mice (16). Also, anti-CD40L immunotherapy reduced the severity of ongoing lupus nephritis (18). However, CD40 was not important for the elicitation of secondary immune responses observed by others. CD40/CD40L interactions were not critical for clearance of tumors after challenge in a vaccinated animal (2) nor modulated the outcome of colitis after induction by hapten 2,4,6-trinitrobenzene sulfonic acid (TNBS) (19).

The role of CD40 in pulmonary fibrosis was recently addressed by Phipps and colleagues when they showed that the blockade of CD40/CD40L interactions prevented fibrosis in lung injury models mediated by oxygen or radiation (nonimmune inflammation) (20, 21). Treatment of the mice with CD40L blocking Ab (MR1) before the oxygen or radiation exposure and even 48 h after oxygen exposure reduced the production of the proinflammatory enzyme cyclooxygenase-2 and blunted the injury-induced inflammation and lung fibrosis (20, 21). However, no studies have reported a role for CD40/CD40L interactions in secondary immune responses that lead to immune-mediated fibrosis.

Haptens include known protein-reactive chemicals, metals, salts, and drugs that are considered major environmental noxae targeting the immune system of vertebrates (22). Haptens are non-immunogenic but, because of their chemical reactivity, covalently bind to proteins and lipids as carriers and stimulate immune responses (22–24). Like viruses, haptens alter self-Ags, and part of the ensuing immune response includes an autoimmune component (22). Previously we reported that pulmonary interstitial fibrosis (PIF) was elicited in the lungs of hapten-sensitized mice after a local pulmonary challenge of the immunizing hapten (25). The intratracheally (i.t.) administered hapten induced a local recall cell-mediated immune response against self-Ag modified by hapten.
in the lung in particular. The experimental model for pulmonary fibrosis is called hapten immune PIF (HIPIF) and resembles human idiopathic PIF, not only because of its abnormal regulation of inflammation in the lung and unresolved fibrosis, but also because it shares an autoimmune etiology (29, 30).

The elicitation of HIPIF is Ag specific and dependent on the genetic susceptibility of mouse strain to express a contact hypersensitivity response toward TNBS (25, 26, 28). HIPIF does not occur in mice that are made tolerant to the Ag and can be adoptively transferred with sensitized cells to naive mice before i.t. challenge with TNBS (25, 31). Using a modified HIPIF model called adoptively transferred HIPIF (ADT-HIPIF) where we adoptively transferred sensitized cells into naive recipient mice, we showed that sensitized CD4+ and CD8+ cells from donor mice and alveolar macrophages from recipient mice were absolutely required for the elicitation of HIPIF (28, 32). These studies also showed that alveolar macrophages were necessary for the effective recruitment of inflammatory cells (IL-12R+ cells, activated T cells, and monocytes/immature dendritic cells) into the lung.

We reasoned that if HIPIF were to occur there must be a change in the normally suppressive phenotype of the alveolar macrophage to allow the recruitment of the inflammatory cells from the periphery and, perhaps, even promote the chronic secondary immune responses in the lung. Indeed data presented show that bronchoalveolar lavage (BAL) cells (including alveolar macrophages and infiltrating monocytes) produced inflammatory cytokines and expressed CD40 coreceptors on their surface. Importantly, CD40 was needed for a secondary immune response and fibrosis to occur because blocking the CD40/CD40L interactions reduced the amount of hydroxyproline (fibrosis) deposited in the lungs of sensitized and challenged mice. These results support the concept that CD40/CD40L interactions are required for secondary immune responses in vivo in general and for a pathogenic immune response in the lung in particular.

Materials and Methods

Animals

Female BALB/c ByJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the Schepens Eye Research Institute Vivarium until they reached the desired weight (20–24 g) for the experiments. The IL-12 p35 p40 knockout (α) mouse (BALB/c background) breeding pair was a gift from Dr. Keith Bishop (University of Michigan, Ann Arbor, MI), and mice used in the studies were bred in the Schepens Eye Research Institute Vivarium. All animals were treated humanely in accordance with National Institutes of Health guidelines and the approval of the Schepens Animal Care and Use Committee.

Reagents

TNBS, rat IgG, crystal violet, alkaline phosphatase-conjugated ExtrAvidin, and substrate p-nitrophenyl phosphate were purchased from Sigma (St. Louis, MO). Anti-CD40L Ab (MR1) was purchased from Bio Express (West Lebanon, NH). Normal Armenian hamster serum was purchased from Cayten (West Roxbury, MA), and the hamster IgG was purified from the serum using protein A-agarose (Life Technologies, Grand Island, NY) column. Biotin- and PE-conjugated anti-CD40 Abs (3/23), purified anti-IL-12 (p70) Ab (9A5), anti-IL-12 (p40/p70) Ab (C15.6), biotin-conjugated anti-IL-12 (p40/p70) Ab (C17.8), Fc blocker (2.4G2), and rat IgG2a (R35-95) were purchased from BD Pharmingen (San Diego, CA). A TNFα-ELISA kit (DuoSet) was purchased from Genzyme (Cambridge, MA). PermeaFix reagent was purchased from Ortho Diagnostic Systems (Raritan, NJ). R-PE-conjugated streptavidin was purchased from Jackson ImmunoResearch (West Grove, PA). Recombinant mouse (rm) IL-12 (p70) was purchased from R&D Systems (Minneapolis, MN).

Animal model

HIPIF. Mice were sensitized on the abdomens with a water-soluble form of the hapten TNBS (3% in PBS, 100 μl/mouse) at day 0. Five days after sensitization, the mice were inoculated i.t. with 50 μl of 1% TNBS (28).

ADT-HIPIF. Donor mice were skin sensitized with 100 μl of 3% TNBS. Five to seven days after skin sensitization, spleen and draining lymph nodes (axillary, inguinal, and brachial) were harvested and dissociated into a single cell suspension before determining the cell viability by the trypan blue exclusion method. Spleen and draining lymph node cells were mixed, counted, and used as donor cells for adoptive transfer. The spleen and lymph node cells from naive mice were similarly collected and adoptively transferred as control. Recipient mice were irradiated (200 rad, Mark 1 irradiator; J.L. Shepherd and Associates, Glendale, CA) 24 h before donor cells (3 × 107/mouse) were transferred through tail vein inoculation. One day after the adoptive transfer, recipient mice were challenged i.t. with 50 μl of 1% TNBS in PBS.

CD40L neutralizing Ab (MR1) and rmIL-12 (p70) treatment

Recipient mice were given Ab (MR1) or normal Armenian hamster IgG (200 μg/mouse, i.p.) 1 day before i.t. challenge. The recipient mice received additional Ab (100 μg/mouse, intranasally) at 4 h, 1, 3, and 7 days after i.t. challenge. rmIL-12 (p70) (100 or 500 ng) was inoculated i.p. per mouse on the day of i.t. challenge and was repeated 3 days after i.t. challenge. Each group for each dose contained five mice.

Flow cytometry analyses and sorting

BAL cells were collected by washing the lung with a total of 10 ml of PBS per lung (1 ml per wash). Cells in staining buffer (PBS, 1% BSA, 0.1% sodium azide) were incubated with blocking reagent (Fc blocker, 2 μg/10^6 cells, rat IgG 20 μg/10^6 cells), and then biotin-conjugated anti-CD40 or rat IgG2a (1 μg/10^6 cell) followed by PE-conjugated streptavidin. All cells were incubated on ice for 20 min and washed twice before the next reagent was added. To quench macrophage autofluorescence, cells were permeabilized and fixed with PermeaFix reagent to allow intracellular access of crystal violet (33) before the samples were analyzed by flow cytometry (EPICS XL; Beckman Coulter, Miami, FL).

For CD40+ cell sorting, the lavage cells were stained with PE-conjugated anti-CD40 Ab or control rat IgG2a (1 μg/10^6 cells) for 40 min on ice. CD40+ cells were sorted using flow cytometry (EPICS ELITE; Beckman Coulter, Miami, FL).

FIGURE 1. Flow cytometric analysis of CD40 expression on BAL cells from HIPIF mice. The histograms show the CD40 staining of BAL cells compared with their isotype control. BAL cells were collected 2 days after i.t. challenge and stained with biotin-conjugated anti-CD40 Ab followed by PE-conjugated streptavidin. Biotin-conjugated rat IgG2a was used as isotype control. Cells in a were harvested from the HIPIF mice. Cells in b were collected from i.t. challenge-only mice. Cells in c were obtained from naive mice. In each histogram the abscissa represents PE fluorescence intensity on the cells, and the ordinate represents relative cell number. The percentage of CD40+ cells is indicated in histogram a. The experiment was repeated four times.
and after CD40

... of a stained cytospin of BAL cells before

... shown. The percentage of cells in the gate is indicated in each histogram.

to the slides by centrifugation force and then subjected to Wright Giemsa staining (HEMA 3 Stain set; Fisher Scientific, Pittsburgh, PA), and then analyzed by light microscopy (oil, 400x) of a stained cytospin of BAL cells before and after CD40+ cell sorting to show cell morphology. The cells were fixed to the slides by centrifugation force and then subjected to Wright Giemsa staining. M, Monocyte; L, lymphocyte; AM, alveolar macrophage; N, neutrophil.

Coulter). Cytospin preparations (Cytospin 2; Shandon Southern Products, Astmoor, U.K.) were prepared from the sorted cells (5 x 10^6 cells/slide), followed by staining with Wright Giemsa staining (HEMA 3 Stain set; Fisher Scientific, Pittsburgh, PA), and then analyzed by light microscopy (oil, x40 and x60) (Nikon ECLIPSE E-800; Nikon, Melville, NY). Alveolar macrophages were identified by their large size (with a large cytoplasmic region and a single round nucleus); monocytes showed a kidney-shaped nucleus with light blue granules in the cytoplasam; lymphocytes had a spherical nucleus that was dark blue and surrounded by a small cytoplasmic region; neutrophils had a nucleus consisting of two to five lobes linked by fine threads of chromatin; and eosinophils contained a bilobed nucleus with pink granules in the cytoplasm.

**Cytokine ELISA**

The lung lavage was collected from each individual mouse as the return volume on 1 ml of PBS inserted i.t. The lavage wash was centrifuged at 200 x g for 10 min, and the supernatant was used for the ELISA. TNF-α ELISA was performed using the TNF-α ELISA kit according to the manufacturer’s instruction. IL-12 (p40/p70) ELISA was performed by using the capture Ab (C15.6, 2 μg/ml) and the detecting Ab (C17.8, 1 μg/ml) followed by alkaline phosphatase-conjugated ExtrAvidin (1:10,000 dilution) and substrate p-nitrophenyl phosphate. We used anti IL-12 mAbs, 9A5 (2 μg/ml) for the capture Ab and C17.8 (1 μg/ml) for the detecting Ab in an IL-12p70 ELISA.

**Hydroxyproline assay**

Changes in collagen deposition in the lung were measured by a colorimetric hydroxyproline assay (34). In brief, lungs recovered from the experimental mice were minced and hydrolyzed in 6 N HCl (2 ml/lung) for 16 h at 110°C. The samples were filtered through Whatman no. 1 filter paper, diluted with H₂O, neutralized with 10 N NaOH, and assessed spectrophotometrically. The amount of hydroxyproline in the lungs was calculated according to the standard curve generated using a serial dilution of trans-4-hydroxy-L-proline (Sigma).

**RT-PCR**

Total RNA was extracted from cells by Trizol reagent (Life Technologies, Rockville, MD). RNA purified from 3 x 10^6 BAL cells was dissolved in 30 μl diethyl procarbonate-treated H₂O. One microliter of the RNA sample was used for the one-step RT-PCR amplification using the Access RT-PCR system (Promega, Madison, WI) and Gene Amp PCR System 9600 (Perkin-Elmer, Norwalk, CT). The primer pairs for IL-12 p35 (sense: ggtcactagagactctccc, anti-sense: gigaagcgaggtcagacgcttc), IL-12 p40 (sense: cgtgctcatggctggtgcaaag, anti-sense: gaacacatgcccacttgctg) (35), TNF-α (sense: atgagcagaaagctagcagcttc, anti-sense: ccaagtagacgcgccggtc) (36), and β-actin (sense: gggcgccgtctggaccaaa, anti-sense: ctcttggtgtaacagcagcgttc) (37) were generated by Oligos Etc. (Wilsonville, OR). The RT reaction was one cycle of 48°C for 45 min followed by 94°C for 2 min. The PCR amplification was 40 cycles of 94°C for 30 s, 60°C for 1 min, and 68°C for 2 min followed by one cycle of 68°C for 7 min. The PCR products were separated on a 1% agarose gel and visualized using GelStar nucleic acid gel stain (FMC BioProducts, Rockville, MD) and UV illumination. The density of the bands on the gel was measured using Gel Doc 2000 (Bio-Rad, Hercules, CA).

**FIGURE 2.** Cell sorting and Wright Giemsa staining of CD40+ BAL cells from HIPIF mice. a, Sorting of CD40+ BAL cells using flow cytometry. BAL cells were collected from the HIPIF mice 1 day after i.t. challenge and stained with PE-conjugated anti-CD40 Ab. The gates used to sort the CD40+ BAL cells were based on PE vs Autofluorescence (525 nm) as shown. The percentage of cells in the gate is indicated in each histogram. b, Photomicrograph (oil, 400x) of a stained cytospin of BAL cells before and after CD40+ cell sorting to show cell morphology. The cells were fixed to the slides by centrifugation force and then subjected to Wright Giemsa staining. M, Monocyte; L, lymphocyte; AM, alveolar macrophage; N, neutrophil.

**FIGURE 3.** RT-PCR analyses and densitometry measurement of mRNA level of IL-12 p35 and p40 in BAL cells from HIPIF mice. a, Level of IL-12 p40 mRNA in BAL cells that were collected 2 days after i.t. challenge. b, Level of IL-12 p35 mRNA in BAL cells that were collected 2 and 5 days (D2, D5) after i.t. challenge. Lane A, RNA sample from the HIPIF mice. Lane B, RNA sample from the i.t. challenge-only mice. Lane C, RNA sample from naive mice. Lane M, m.w. marker. The PCR product was separated on a 1% agarose gel and visualized by using GelStar nucleic acid gel stain and UV illumination as shown in the pictures. The table on the right side of b shows the ratio of relative density of p35 band in lane A compared with relative density of p35 band in lane B from three independent experiments. For experiment 1, the RNA was pooled from three mice, and for experiments 2 and 3, the RNA represented two individual mice. The density of the bands was measured using Gel Doc 2000. Both bands in the D5 lane were considered as p35 product and summed for the density. The relative density of p35 bands was normalized to β actin.
ANOVA and post hoc tests were used to evaluate the difference among experimental groups. Values of \( p \leq 0.05 \) were considered significant. In some cases, individual \( p \) values are given.

**Results**

**BAL cells from HIPIF mice express CD40**

The elicitation of immune responses in sensitized mice is thought to involve different and perhaps fewer signals than needed for the induction of a primary immune response (2, 19). Because there is a controversy as to the absolute requirement for CD40 during elicitation of secondary immune responses, we began by measuring the expression of CD40 in the autoimmune fibrosis model, HIPIF.

In contrast to unsensitized mice that receive a single inoculation of hapten in their lungs, HIPIF mice that are sensitized before the i.t. challenge with hapten develop a lasting fibrosis (28). Moreover, adherent cells from the lungs of HIPIF mice are immune supporting, whereas the adherent cells from the lungs of challenge-only mice are immune suppressive (32). Thus, we reasoned that the functional phenotype of the BAL-adherent cells would differ in HIPIF mice. As a marker for activated and effective accessory cells, we assessed the expression of CD40 on BAL cells from the various experimental groups with flow cytometry. BAL cells were obtained from the experimental mice 2 days post i.t. challenge with hapten and stained with anti-CD40 Ab. The BAL cells from naive mice (C group) that were 90–95% alveolar macrophages (data not shown) expressed low level (mean fluorescence intensity of CD40 Ab vs isotype Ab staining was 0.876 vs 0.623, respectively) of CD40 on their surface. The BAL cells from sensitized and challenged mice (A group) contained a subpopulation of cells (8%) expressing high level (mean fluorescence intensity of CD40 Ab vs isotype Ab staining was 12.9 vs 0.886, respectively) of CD40 on their surface (Fig. 1). Challenge-only mice (B group) had no CD40 on their surface. The expression of CD40 on BAL cells was also increased 1 and 3 days after i.t. challenge in HIPIF mice compared with control (data not shown).

**The BAL cells with high CD40 expression are infiltrating monocytes/dendritic cells**

Because the BAL cells from sensitized or naive mice that were i.t. challenged contained both alveolar macrophages and infiltrating monocytes, both of which have the potential for expressing CD40 (2, 3), we identified the CD40 bright cells in HIPIF mice by their morphology. The CD40 bright cells from BAL cells collected 1 day post i.t. challenge were enriched by FACS sorting, stained with Wright Giemsa, and examined by light microscopy (Fig. 2). About 80% of the sorted cells as analyzed by flow cytometry were CD40 bright, and the same percentage of the sorted cells exhibited morphology consistent with their being monocytes. The rest of the cells were anti-CD40L Ab; B, i.t. challenge-only mice; C, naive mice. The treatment of each group is indicated under abscissa. In a, the HIPIF protocol was performed, and in b and c, the ADT-HIPIF protocol was performed. Sensitized or naive cells (30 × 10^6/mouse) were adoptively transferred (ADT) to syngeneic naive mice 1 day before i.t. challenge; Ab treatment is described in detail in Materials and Methods; αCD40L, anti-CD40L Ab (MR1); hIgG, normal Armenian hamster IgG. One day after i.t. challenge, lungs were washed using 1 ml of PBS, and supernatant from the recovered lavage was tested for IL-12 and TNF-α ELISA. Five mice were used in each group. The data is presented as mean ± SEM. An asterisk (*) indicates a statistical significant difference between the two groups indicated. IL-12 p70 ELISA sensitivity level was 5 pg/ml. Group B, B’, and C, IL-12 p70 was below detection level.
HIPIF mice treated with anti-CD40L Ab. The relative density of the bands was normalized to actin. The PCR product was separated on a 1% agarose gel and visualized by bands measured using Gel Doc 2000.

The effect of Ab blocking of CD40/CD40L interactions on cytokine production

The consequence of CD40/CD40L interactions in elicitation of hapten responses in the lungs of sensitized mice was studied first indirectly by monitoring inflammatory cytokine production. The level of IL-12 mRNA in lavage cells was measured by RT-PCR analysis in experimental and control mice 2 days after i.t. challenge (Fig. 3). Evaluation of IL-12 mRNA showed no differences in the p40 mRNA level in BAL cells collected from the various experimental groups of mice, but p35 mRNA level was consistently higher in BAL cells from HIPIF mice as compared with those from control mice at both 2 and 5 days after i.t. challenge. Reports show that the expression of IL-12 p35 is both transcriptionally and translationally regulated because multiple isoforms of p35 are expressed in different types of cells and stages of activation (38, 39). Therefore, we interpreted the double band seen in day 5 as two isoforms of p35. We included both bands in the densitometry analyses of the p35 RT-PCR product. During the elicitation of IL-12 protein, monocytes and T cells are recruited into the lung (32). Three days after i.t. challenge with TNBS, the HIPIF mice have significantly higher numbers of monocytes (9.58 ± 1.11 x 10^6 vs 5.28 ± 0.97 x 10^6) and T cells (5.54 ± 0.52 x 10^6 vs 2.09 ± 0.06 x 10^6) in BAL compared with the mice i.t. challenged-only (32). In addition, the cells in the HIPIF BAL are more activated (increased CD40 expression, IL-12 receptor expression, and TNF-α production) than the BAL cells from the control mice (32) (Fig. 1). Thus, the elevated level of IL-12 p35 mRNA in HIPIF BAL samples could be a result of increased numbers of infiltrating monocytes and enhanced activation of both monocytes and alveolar macrophages.

Active IL-12 protein is a heterodimer of p35 and p40. In the HIPIF lung p35 is inducible, whereas p40 is constitutively expressed. To show that the mRNA was indeed translated into a protein we measured IL-12 protein in the return from the first milliliter of lung wash in an IL-12 (p40/p70) ELISA and an IL-12 (p70) ELISA (Fig. 4, a and b). The results indicated that the HIPIF mice produced a higher level of IL-12 compared with the i.t. challenge-only or naive mice. Because HIPIF development is dependent on a Th1 immune inflammatory response (25, 26, 28, 32), the increase in IL-12 protein is biologically significant.

A number of publications show a direct relationship between CD40/CD40L interactions and IL-12 production (1–3, 10, 11). To confirm that IL-12 production in HIPIF mice was dependent on ligation of CD40, recipient mice were treated with CD40L-specific blocking Ab (MR1) on the day of transfer of hapten-sensitized cells (1 day before i.t. challenge with hapten), and IL-12 production was evaluated. BAL cells were harvested 1 day after i.t. challenge from several groups of HIPIF mice that were treated with different doses of MR1 (0, 50, 100, and 200 μg/mouse, i.p.). The mRNA level of IL-12 p35 and p40 in the BAL cells was analyzed by RT-PCR (Fig. 5). The BAL cells from HIPIF mice treated with 100 and 200 μg of anti-CD40L Ab had reduced IL-12 p35 mRNA levels (Fig. 5), as well as reduced production of IL-12 (p70) protein (Fig. 4a). The p35 mRNA levels on BAL cells were reduced by 37% at 2 days and 42% at 3 days after i.t. challenge from anti-CD40L (200 μg)-treated HIPIF mice (data not shown).

As expected, CD40L blocking Ab also interfered with TNF-α mRNA and protein production because there was a dose-dependent decrease of TNF-α mRNA in BAL cells and a reduction of TNF-α protein in BAL fluid from the mice treated with 200 μg CD40L Ab (Figs. 4c and 5). TNF-α mRNA levels were also reduced in BAL cells from anti-CD40L (200 μg)-treated HIPIF mice that were collected 2 and 3 days (53 and 51% reduction, respectively) after i.t. challenge (data not shown). These results support the postulate that CD40/CD40L interactions contribute to the effector phase of the hapten immune response in the lung by enhancing the production of proinflammatory cytokines IL-12 and TNF-α.

IL-12 is associated with the accumulation of hydroxyproline in HIPIF mice

To analyze the role of IL-12 in HIPIF, IL-12<sup>–/–</sup> BALB/c mice were used as recipient mice in ADT-HIPIF. TNBS-sensitized spleen and lymph node cells were collected from wild-type (WT) mice that were sensitized on their abdomen 5 days earlier and transferred to either WT or IL-12<sup>–/–</sup> mice.Recipient mice were challenged with TNBS (i.t.) 1 day later. The accumulation of hydroxyproline was measured in the lungs of experimental mice 14 days after i.t. challenge (Fig. 6). The IL-12<sup>–/–</sup> recipient mice had significantly less <i>p</i> ≤ 0.05 hydroxyproline deposition in the lung compared with the WT recipient mice. Thus, IL-12 not only contributes to the process that eventuates into fibrosis, but also must be produced by...
mice was 200.5 and 205.1.

The baseline hydroxyproline for C and C9, HIPIF mice with IL-120 mice as recipient mice; C, recipient mice; A, HIPIF mice were adoptively transferred (ADT) 1 day before i.t. challenge. The change (Δ) in hydroxyproline was calculated as experimental hydroxyproline - baseline hydroxyproline. The baseline hydroxyproline for C and C9 group mice was 200.5 and 205.1 μg/lung, respectively. Five mice were used in each experimental group. The data is presented as mean ± SEM. An asterisk (*) indicates a statistical significant difference between the two groups indicated.

Effect of CD40L blocking Ab on hydroxyproline accumulation in the HIPIF lung

Thus far, data show that CD40/CD40L interactions promote the production of proinflammatory and fibrogenic cytokines that lead to the development of pulmonary fibrosis. To evaluate the role of CD40/CD40L in pulmonary fibrosis, recipient mice (ADT-HIPIF model) were treated with CD40L blocking Ab, and hydroxyproline accumulation in the lung was measured. Compared with the HIPIF mice treated with hamster IgG (control Ab), the HIPIF mice treated with anti-CD40L Ab developed less accumulation of hydroxyproline (Fig. 7). Therefore, both the production of inflammatory cytokines and hydroxyproline accumulation were dependent on CD40/CD40L interactions. When HIPIF mice that were treated with CD40L Ab were given rmIL-12 (p70), the accumulation of hydroxyproline in the lung was restored (Fig. 8). These results indicate that IL-12 from CD40/CD40L interaction is biologically significant and is critical for the fibrogenic process in the HIPIF lung.

Discussion

HIPIF is mediated by an immune response against hapten-modified self-Ag and thus resembles an autoimmune process (26–28). Studies in several autoimmune disease models (EAE, lupus nephritis) show that CD40/CD40L interactions are important in both the priming and the effector phase of the disease process (2, 3).

Blocking the CD40/CD40L interactions using anti-CD40L Ab prevents the development of the disease and dramatically suppresses clinical signs even when treatment starts after onset of disease in both EAE and lupus nephritis models (16, 18). However, in TNBS-induced colitis, a model for Th1-mediated and autoimmune inflammatory bowel disease, blocking CD40/CD40L interactions prevents the induction phase of the disease but has no effect on the disease after the onset (19). Although HIPIF and TNBS-induced colitis are elicited by the same hapten TNBS and mediated by Th1 type immune responses, their requirement for the cosignaling molecule CD40 is different. This may suggest a role of local environment in regulating immune response and disease process. The intestine is home to a large variety of indigenous bacteria (intestinal microflora). Normally, hosts exhibit tolerance toward the indigenous intestinal flora; however, in the inflammatory bowel disease process, the tolerance is broken and the hyperresponsiveness toward intestinal flora is crucial in the pathogenesis of the disease (40). Therefore, the immune response toward the bacteria in the intestine may act as an adjuvant to bypass the total dependence on CD40 cosignaling in the ongoing colitis. In contrast, the lung, although not sterile, is not populated with an abundance of natural flora and therefore lacks the adjuvant effect. In the environment of the lung, resting alveolar macrophages suppress dendritic cell Ag-presenting function, and TGFβ from epithelial cells can further down-regulate inflammatory responses (41–43). This suppressive environment may raise the threshold for elicitation of an immune response in the lung. Our data support this notion because CD40/CD40L interactions are critical for elicitation of a secondary immune response in the lung.
signals activate the resting alveolar macrophages and further en-
thelial cells, and, more importantly, infiltrating monocytes, and these T cells interacts with CD40 on alveolar macrophages, epithe-
tory monocytes and presensitized activated T cells. The CD40L on the local TNBS challenge induces the infiltration of proinflamma-
tory cells. The administration of Ab and rmIL-12 is described in Materials and Methods. The change (Δ) in hydroxyproline was calculated as experimental hydroxyproline baseline hydroxyproline. The baseline hydroxyproline collected from naive mice was 215.8 μg/lung. Five mice were used in each experimental group. The data are presented as mean ± SEM. An asterisk (*) indicates a statistically significant difference between the two groups indicated.

It is known that multiple cell types within the lung express CD40 on their surface (3, 20, 44, 45). We and others showed that alveolar macrophages express low levels of CD40. During the elic-
itiation of IFN-γ, infiltrating monocytes express high levels of CD40 and can be distinguished from the CD40dim populations as CD40bright. Many studies report that signals derived from CD40/ CD40L interactions increase the production of proinflammatory cytokines (IL-12, TNF-α) and expression of B7 costimulatory molecules on macrophages, monocytes, and dendritic cells (3, 8–11). Activation of fibroblast and vascular endothelial cells through CD40 molecules stimulates their expression of adhesion molecules CD54 (ICAM-1) and CD106 (VCAM-1), and chemo-
tactic factors (3). Wiley and colleagues showed that exogenous CD40L administered into the lung induces recruitment of poly-
morphonuclear leukocytes and accumulation of macrophages with up-regulated Ia expression (44) presumably by binding to CD40 on lung epithelial cells and macrophages. Recent data from our labor-
atory showed that alveolar macrophage-derived TNF-α was impor-
tant for the recruitment of monocytes and the development of fibrosis in HIPIF (32). Therefore, we propose that in HIPIF mice the local TNBS challenge induces the infiltration of proinflamma-
tory monocytes and presensitized activated T cells. The CD40L on these T cells interacts with CD40 on alveolar macrophages, epithelial cells, and, more importantly, infiltrating monocytes, and perpetuates the production of TNF-α, IL-12, and GM-CSF. These signals activate the resting alveolar macrophages and further en-
hance the inflammatory cascade and recruitment of immune in-
flammatory cells by promoting chemokine production and adhe-
sion molecule up-regulation on endothelial cells and epithelial cells. The chronic immune inflammation in the lung eventually leads to matrix destruction and collagen deposition.

Haptenes are nonimmunogenic small reactive chemicals that con-
jugate to proteins and lipids (carrier molecules) to become immu-
ogenic (22–24). Haptenes are commonly used as tools to dissect Th1 and inflammatory responses in the skin and colon (19, 22, 23, 40, 46, 47). Here we show that pulmonary challenge with haptens in the lungs of sensitized mice leads to an abnormally regulated destructive immune response eventuating in fibrosis. Small reactive chemicals with haptenic properties are ubiquitous in our do-
mestic and industrial environment and therefore potentially are part of the unknown etiology of human idiopathic pulmonary fibrosis.

PIF in humans manifests as persistent inflammation and en-
hanced collagen deposition in the lung. Many types of abnormal immune regulation are described in the fibrotic lungs, which in-
clude the existence of anti-small nuclear protein Ab and immune complex deposition in the lung, infiltration of neutrophils, mono-
cytes, and activated T cells (29, 30). Although the insults that cause a large percentage of PIF remain unknown, the disease pro-
cess is considered to have an underlying autoimmune mechanism. Therapeutically, blocking CD40/CD40L interactions may be a benefit for fibrosis patients who exhibit an associated expression of CD40L on lymphocytes. Reagents that block CD40/CD40L inter-
actions have been tested extensively in allotransplantation models. With the success of humanized CD40L blocking Ab (hu5C8) in preventing acute rejection in allotransplantation in primates (17), it is possible that innovative treatments may be forthcoming for the treatment of PIF patients.

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