IL-17A/F Modulates Fibrocyte Functions in Cooperation with CD40-Mediated Signaling.

Original article

Title: IL-17A/F modulates fibrocyte functions in cooperation with CD40-mediated signaling.

Running title: Effects of IL-17A/F on fibrocytes

Authors: Hisako Hayashi, Akiko Kawakita, Shintaro Okazaki, Motoko Yasutomi, Hiroki Mirai, Yusei Ohshima

Affiliations: Department of Pediatrics, Faculty of Medical Sciences, University of Fukui, Japan

Corresponding author

Yusei Ohshima, M.D., Ph.D.

Department of Pediatrics, Faculty of Medical Sciences, University of Fukui,

23-3 Shimoaizuki, Matsuoka, Yoshida-gun, Fukui, 910-1193, JAPAN

Tel: +81-776-61-3111

FAX: +81-776-61-8129

e-mail: yohshima@u-fukui.ac.jp

Abstract

T helper 17 (Th17) cells that produce Interleukin (IL)-17A and IL-17F have been found to participate in the development of bronchial asthma and bleomycin-induced pulmonary fibrosis. However, whether they play a causative role in the airway remodeling observed in these respiratory diseases remains unclear. Because fibrocytes are involved in tissue repair and fibrosis, and are presumably precursors of lung fibroblasts and myofibroblasts, we examined the effects of IL-17A/F on fibrocyte functions. Both IL-17A and IL-17F enhanced fibrocytes' α-smooth muscle actin (SMA) expression. Priming fibrocytes with IL-17A enhanced their CD40-mediated IL-6 production, whereas IL-17F-priming increased the CD40-mediated mRNA expression of collagen I, vascular endothelial growth factor (VEGF), and angiogenin. $CD4^+$ T cells co-cultured with fibrocytes produced IL-17A, which was inhibited by blocking CD40 and CD40 ligand interactions. These findings suggest that cooperative interactions between fibrocytes and Th17 cells play an important role via CD40- and IL-17A/F-mediated signaling for collagen and proangiogenic factor production, which may lead to the extracellular matrix deposition and neo-vascularization seen in airway remodeling.

Keywords: IL-17A; IL-17F; fibrocytes; airway remodeling; CD40

1. Introduction

Fibrocytes are unique bone marrow-derived mesenchymal progenitor cells that are characterized by expression of several markers of both hematopoietic cells (CD34, CD45, and LSP-1) and stromal cells (collagen I and proly-4-hydroxylase). Fibrocytes are presumably derived from a subpopulation of $CD14^+$ peripheral blood monocytes [1-5]. Fibrocytes are involved in chronic inflammation, tissue repair and fibrosis, and might be a significant source of lung fibroblasts and myofibroblasts in response to lung injury and in remodeling of the airway wall [6-12].

Fibrocytes participate in tissue remodeling by producing extracellular matrix proteins such as collagen, and by secreting matrix metalloproteinases. Moreover, fibrocytes are an important source of inflammatory cytokines, chemokines and proangiogenic factors, such as vascular endothelial growth factor (VEGF) [13]. Fibrocytes express major histocompatibility complex class II and have shown antigen-presenting capabilities both *in vitro* and *in vivo* [2,3]. Recent data obtained from clinical setting suggest that the enumerating circulating fibrocytes may be a biomarker for disease progression in chronic lung diseases including asthma and pulmonary fibrosis [14,15]. A greater understanding of the immunologic mediators that influence fibrocyte biology may suggest new opportunities for therapeutic manipulation of these cells in the process of fibrogenesis and neo-vascularization.

Interleukin (IL)-17A is the signature cytokine produced by the T helper 17 (Th17) cell subset. IL-17A expression has been implicated both in the pathogenesis of various autoimmune diseases and in some fibrotic disorders and the airway remodeling that occurs in chronic asthma [16-18]. IL-17 levels are increased in lung tissues, BAL fluids, sputum, and peripheral blood from patients with allergic asthma [19,20]. In the sputum of asthmatic patients, the increased levels of IL-17 mRNA expression were correlated with the number of neutrophils [21]. The percentages of Th17 cells and the level of IL-17 in airway and plasma tend to increase with disease severity in asthmatic patients

Although IL-17 supposedly contributes to airway remodeling by contributing to the accumulation of neutrophils or the release of matrix metalloproteinases and cytokines including IL-6, TNF-α, IL-11, HB-EGF by resident cells [17,22-24], much less is known about Th17 cells' ability to exacerbate airway remodeling and the mechanisms that may underlie this activity. Therefore, in this study we examined the effects of IL-17 on the proliferation and function of fibrocytes, and the mechanisms underlying Th17-mediated remodeling. Because it is difficult to obtain a sufficient number of fibrocytes with a high purity from pulmonary tissues, we took advantage of $CD14^+$-monocyte-derived fibrocytes to analyze the function of fibrocytes *in vivo*. We show that the cooperation between fibrocytes and Th17 cells may possibly play an important role in airway remodeling by CD40/CD40 ligand interactions.

2. Materials and methods

2.1. Cell culture

Peripheral blood was obtained from healthy volunteers with informed consent. The study was approved by the ethics committees of University of Fukui. 15 x 10^6 PBMCs isolated by Ficoll-Paque Plus (GE Healthcare Biosciences, Piscataway, NJ) were suspended in 2 ml of RPMI1640 + 10% FBS + penicillin, and streptomycin (Invitrogen Co. Carlsbad, CA) and cultured in the wells of fibronectin-coated 6-well plates. After 48 h, non-adherent cells were removed and medium was changed periodically to develop fibrocytes, as previously described [10]. Non-adherent cells were washed out over time with changes of medium. 10 ng/ml of platelet derived growth factor-BB (PDGF-BB), VEGF, basic fibrocyte growth factor (bFGF), or Interleukin 1β (IL-1β) (PeproTec Inc. Rocky Hill, NJ) was added on day 7. After 2 weeks of culture, fibrocytes were harvested and then washed twice. 1.2 x 10^5 cells and 0.6 x 10^5 cells were cultured in FCS-free RPIM1640 in fibronectin-coated 12 and 24-well plates, respectively. Medium was replaced the next day with fresh FCS-free RPIM1640. The cells were primed with 10 ng/ml of IL-17A/E (PeproTec Inc.) for 1 hour and then were stimulated with 0.2 μg/ml of soluble CD40 ligand (sCD40L; Alexis Biochemicals, San Diego, CA) in the presence or absence of dexamethasone (Sigma-Aldrich). After 48 h of stimulation, cell proliferation was assessed by adding a colorimetric assay reagent of Cell Counting Kit-8 (Dojindo, Kumamoto,

Japan). The plates were incubated for 4 h at 37°C, and 450 nm UV absorbance of each sample was measured in a microplate reader. Assay was done in triplicate wells.

2.2. RNA isolation and RT-PCR

After 24 h culture with stimulation, total RNA were isolated using an RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan). First-strand cDNA was synthesized from 1 μg of total RNA using the Superscript First Strand System (Invitrogen Co.). mRNA levels were quantified by real-time quantitative PCR using Taqman Pre-Developed Assay Reagents (Applied Biosystems Japan Ltd., Tokyo, Japan) according to the manufacturer's instructions. For each sample, the difference in threshold cycle number between the target gene and β-actin was used to determine the standardized expression level (ΔCt). Subtracting the mean ΔCt of the control non-stimulated samples from the mean ΔCt of stimulated samples yielded the ΔΔCt value that was used to calculate relative expression levels in the stimulated samples according to the formula $2^{-\Delta\Delta Ct}$. The error was estimated by evaluating the $2^{-\Delta\Delta Ct}$ term using ΔΔCt ± SEM [25].

2.3. Flow cytometric analysis

Cells were pretreated with BD Cytofix/Cytoperm (BD Biosciences, San Diego, CA) and then incubated with anti-α-SMA mAb (Sigma-Aldrich Co.), and anti-collagen I mAb (Millipore

Billerrica, MA), or isotype control IgG, along with human IgG in BD Permwash (BD Biosciences). After washing, FITC-conjugated $F(ab')_2$ of goat anti-rabbit IgG (Rockland, Gilbertsville, PA) were added. Cells were analyzed on a FACSCalibur and CellQuest Pro (BD Biosciences), and specific mean fluorescence intensities (ΔMFI) were determined by subtracting the isotype-matched control antibody-fluorescence. Because the specific binding of anti-α-SMA mAb and anti-collagen I mAb to cells was not detectable without permeablization steps, the ΔMFI represents their intracellular expression levels.

2.4. T lymphocyte activation by fibrocytes

$CD4^+$ T cells were isolated from PBMC by using a MACS $CD4^+$ T cell isolation Kit, according to the supplier's recommended protocol (Miltenyi Biotec, Bergisch Gladbach, Germany). A total of 0.3×10^5 fibrocytes were co-cultured with 1×10^6 $CD4^+$T cells in the presence or absence of 1 μg/ml of anti-CD40 mAb or isotype-control IgG in the wells of fibronectin-coated 48-well plates. After 48 h, supernatants were collected.

2.5. Cytokine measurements

IL-6, IL-10, IFN-γ, and TGF-β1 concentrations were determined by ELISA kits (R&D Systems Inc., Minneapolis, MN). IL-17 and VEGF levels were determined with ELISA kits

(Invitrogen Co.).

2.6. Statistical analysis

Comparisons of 2 groups used unpaired Student's *t*-tests, unless an F-test showed that the variances were significantly different. When variances were significantly different, Welch's test was used. A *p* value less than 0.05 denoted a statistically significant difference.

3. Results

3.1. Phenotypes of fibrocytes generated from PBMCs

Because only small numbers of fibroblast-like adherent cells developed from PBMCs using serum-based or serum-free culture conditions, we tested a variety of growth factors, which could be produced by fibrocyte in a autocrine fashion [2], including PDGF-BB, VEGF, bFGF, and IL-1β, to maximize the fibrocyte yield and purity of adherent cells after 2 weeks of cultures. PDGF-BB enhanced the development of $CD14^-$ fibroblast-like cells with a highest purity and quantity (Fig. 1A and B). Thus for the following experiments, PBMC were cultured in the presence of PDGF-BB for the last 7 days. The induced adherent cells expressed α-SMA, calponin, collagen type I, and trace amounts of CD34, consisting with the typical phenotype of *in vitro* cultured fibrocytes [3]. Although they also expressed MHC class II, CD86 and CD40, they did not expressed other cell lineage markers (i.e. dendritic cells; CD1 and CD83, monocytes; CD14, endothelial cells; Willbrand factor, T cells; CD3 and B cells; CD19) (Fig 1C).

3.2. Large amounts of IL-17A/F enhance fibrocyte proliferation.

It has been shown that Th2 cytokines, IL-4 and IL-13 augmented fibrocyte differentiation from $CD14^+$ precursors whereas the Th1cytokine interferon-γ inhibits differentiation [26], and that activated T-cell subsets enhanced fibrocyte outgrowth depended on cell-contacts with

$CD14^+$ precursors [1]. Consistent with the previous reports, 10 ng/ml of IL-13 increased the proliferation of fibrocytes, whereas IFN-γ decreased it (Fig 2A). Lower concentrations of IL-17A and IL-17F did not show any significant effects on the fibrocyte proliferation, while higher concentrations of IL-17A and IL-17F (50 ng/ml) enhanced fibrocyte proliferation (Fig 2B). Stimulation with a soluble form of CD40 ligand (sCD40L), which is expressed on activated T cells, augmented the proliferation of both IL-17A/F-treated and non-treated fibrocytes.

3.3. IL-17A/F enhanced α-SMA and collagen expression.

Both IL-17A and IL-17F slightly, but consistently increased the constitutive expression of α-SMA, indicating that they directly induced maturation of fibrocytes. Stimulation with sCD40L also enhanced α-SMA expression irrespective of IL-17A/F-priming (Fig. 3A). Neither IL-17A nor IL-17F alone increased collagen I production in terms of the mRNA and protein levels, but both enhanced intracellular collagen I and mRNA expression in response to sCD40L stimulation (Fig. 3B and 3C).

3. 4. IL-17A and IL-17F differentially regulate cytokine production by fibrocytes.

The fibrocytes constitutively produced IL-6, IL-10 and TGF-β1 (Fig 4A - 4C). Production of

IL-6 and IL-10 was enhanced by stimulation with sCD40L, whereas TGF-β1 production was inhibited. Neither IL-17A nor IL-17F alone increased IL-6 production. Of note, priming with IL-17A but not IL-17F increased sCD40L-mediated IL-6 production. In addition, both IL-17A and IL-17F decreased IL-10 and TGF-β1 production in a dose-dependent manner, irrespective sCD40L-stimulation. Neither IL-17A nor IL-17F alone showed any effects on the constitutive mRNA expressions of VEGF and angiogenin (Fig. 4D and 4F). Priming with IL-17F, but not IL-17A significantly enhanced the mRNA expressions of VEGF and angiogenin in conjunction with sCD40L. Although IL-17A and IL-17F are known to bind to the same receptor, they might have distinct roles in priming fibrocytes.

3.5. Interactions between fibrocytes and $CD4^+$ T cells results in IL-17 production.

Because of their expression of MHC class II molecules and CD86, fibrocyte can presumably stimulate effector $CD4^+$ T cells as antigen presenting cells. Thus, we asked whether fibrocytes interacted with $CD4^+$ T cells. As shown in Fig. 5, when cultured alone, both fibrocytes and $CD4^+$ T cells produced little, if any IL-17 and IFN-γ. However, when $CD4^+$ T cells and fibrocytes were co-cultured, these cytokines were produced. IL-17 production, but not IFN-γ production by $CD4^+$ T cells co-cultured with fibrocytes was inhibited by anti-CD40 blocking antibody, indicating that CD40/CD40L interactions were involved in IL-17 production rather

than IFN-γ production.

3.6. Dexamethasone suppressed a-smooth muscle actin expression and cytokine production by fibrocytes.

The possible therapeutic effects of glucocorticoid on airway remodeling remain controversial. In our system, dexamethasone inhibited IL-17A/F-induced up-regulation of α-smooth muscle actin expression, irrespective of stimulation with sCD40L (Fig. 6A). Dexamethasone suppressed the IL-6 production that was enhanced by sCD40L and the constitutive and inducible IL-10 production in a dose dependent manner (Fig. 6B, 6C). Constitutive TGF-β1 productions was also suppressed by dexamethasone, but with a lesser extent (Fig. 6D)

4. Discussion

In the present study, we have demonstrated that IL-17A and IL-17F can modulate fibrocyte functions to induce pro-angiogenic cytokine and collagen production in response to CD40 signaling and allows these cells to differentiate into a myofibroblastic phenotype, suggesting proangiogenic and profibrogenic roles of IL-17A/F in tissue remodeling. IL-17A and IL-17F share the high amino acid sequence homology and bind to the same two receptors, such as IL-17RA and IL17RC. IL-17A has a higher binding affinity for IL-17RA, whereas IL-17F has a higher affinity for IL-17RC. Thus, they perform overlapping functions, but they also have distinct functions [27]. Consistent with the notion, IL-17A and IL-17F differentially primed fibrocytes in terms of CD40-mediated IL-6 and VEGF production and the mRNA expression of angiogenin.

TGF-β1 is known to be a pleiotropic growth factor that directly induces a variety of responses associated with lung fibrosis and airway remodeling [28]. Wilson *et al.* reported that IL-17A, production of which is dependent on TGF-β1, was essential for the development of bleomycin-induced pulmonary fibrosis and speculated that the profibrogenic activity of TGF-β1 may be partially attributed to the induction of IL-17A [29]. Because IL-17A/F inhibited TGF-β1 production by fibrocytes, the profibrogenic effects of IL-17A/F, such as α-smooth muscle expression and collagen production could not be attributed to the endogenous TGF-β1

production.

Several cytokines, including IL-6 have been implicated in the differentiation, expansion and/or survival of Th17 cells [30]. Recently, it has been shown that a Th17 response was much more dependent on CD40-mediated T cell-APC interactions than was a Th1 response [31]. Since fibrocytes are known to function as an antigen-presenting cell [32], crosstalk between T cells and fibrocytes through CD40L expression and IL-6 production, respectively, might augment a Th17 response rather than a Th1 response.

Although whether PDGF directly induced the differentiation of monocytes into fibrocytes or prevented fibrocyte lineage-committed cells from undergoing apoptosis was not determined, culturing PBMC in the presence of PDGF-BB yields a high quantity and purity of fibrocytes. Airway smooth muscle cell-derived PDGF has been shown to promote fibrocyte migration to smooth muscle bundles *in vitro* [33]. PDGF might play an important role in the accumulation of fibrocytes in the airway smooth muscle compartment in asthma.

Asthmatic subjects exhibit increased numbers of VEGF mRNA-expressing cells in the bronchial mucosa, which are correlated with the degree of vascularity. $CD34^+$ cells were one of the major sources of VEGF and angiogenin within the bronchial mucosa of asthmatics [34]. Although IL-17F alone cannot directly stimulate the growth of vascular endothelial cells [35], IL-17F and CD40L expressed by activated Th17 cells might participate in neovascularization in

asthmatic airway by enhancing VEGF and angiogenin expression by $CD34^+$ fibrocytes.

Accumulating evidence suggests that aberrant IL-17 production is a key determinant of severe forms of asthma [36]. A recent study indicated that airway hyper-reactive responses induced by transferred Th17 cells are steroid-resistant [37]. In patients with chronic kidney diseases, it has been shown that the number of interstitial fibrocytes was significantly decreased by glucocorticoid therapy [38]. Given that cytokine production and α-SMA expression by fibrocytes were susceptible to the inhibitory effects of glucocorticoids, fibrocyte-mediated airway remodeling might be alleviated by steroid-treatment, irrespective of the involvement of IL-17A/F.

In conclusion, cooperativity between fibrocytes and Th17 cells may play an important role via CD40- and IL-17A/F-mediated signaling for collagen and proangiogenic factor production, which may lead to extracellular matrix deposition, basement membrane thickening, and the neo-vascularization observed in airway remodeling. These fibrocyte-mediated responses in airway remodeling might be ameliorated by glucocorticoid treatment.

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Figure legends

Figure 1 Phenotypes of fibrocytes generated from PBMCs

Fibrocytes were generated from $1X10^7$ PBMCs in the presence of the indicated growth factors. (A) The numbers of fibrocytes (hatched bars) and $CD14^+$ macrophages (open bars) are shown after two weeks of culture. The data are expressed as means ± SEM (n = 4). (B) Morphology and (C) lineage marker expression of the fibrocytes generated in the presence of PDGF-BB were analyzed by phase-contrast microscopy and flow cytometric, respectively. Cells were stained with the indicated lineage marker-specific Ab (thick lines) or isotype control Ab (thin lines).

Figure 2 Effects of IL-17A/F and CD40-stimulation on fibrocyte proliferation

Fibrocytes were cultured (A) in the presence of 10 ng/ml of the indicated cytokines or (B) in the presence of various concentration of IL-17A (closed symbols) or IL-17F (open symbols) with (circles) or without (squares) sCD40L. After 2 days of culture, relative cell proliferation was determined by a colorimetric assay. The data are expressed as means ± SEM (n = 4). $*p < 0.05$

Figure 3 Effects of IL-17A/F on α-smooth muscle actin and collagen expression

Fibroblasts were primed with IL17A/F and then stimulated with (hatched bars) or without (open

bars) sCD40L. (A) Intracellular α-smooth muscle actin expression and (B) collagen I expression were quantified by flow cytometry after 2 days of culture. (C) Collagen I mRNA expression was determined by real-time PCR. The indicated relative expression levels and the errors were calculated with the formula $2^{-\Delta\Delta Ct}$. Results are means ± SEM (n = 4). *$p < 0.05$

Figure 4 Effects of IL-17A/F on constitutive and CD40-mediated cytokine production by fibrocytes

Fibrocytes were primed with IL17A (closed symbols) or IL-17F (open symbols) and then stimulated with (circles) or without (squares) sCD40L. After 2 days of culture, (A) IL-6, (B) IL-10, (C) TGF-β1, and (D) VEGF concentrations in the supernatants were determined by ELISA. (E) VEGF and (F) angiogenin mRNA expression by fibrocytes stimulated with (hatched bars) or without (open bars) sCD40L was determined by real-time PCR. The indicated relative expression levels and the errors were calculated with the formula $2^{-\Delta\Delta Ct}$. Results are means ± SEM (n = 4). *$p < 0.05$, **$p < 0.01$

Figure 5 IL-17 and IFN-γ production by $CD4^+$ T cells co-cultured with fibrocytes

$CD4^+$ T cells were co-cultured with fibrocytes in the presence or absence of anti-CD40 blocking Ab or control mouse IgG1. IL-17 and IFN-γ concentrations in supernatants were determined by

ELISA. Results are means ± SEM (n = 4). *$p < 0.05$, nd; not detected

Figure 6 Dexamethasone inhibited α-smooth muscle actin and cytokine production by fibrocytes.

Fibrocytes primed with IL-17A (closed symbols) or IL-17F (open symbols) were stimulated with (circles) or without (squares) sCD40L in the presence of 1μM or the indicated concentrations of dexamethasone. After 2 days of culture, (A) α-smooth muscle actin expression was determined by flow cytometry and (B) IL-6, (C) IL-10, and (D) TGF-β1 concentrations in supernatants were determined by ELISA. Results are means ± SEM (n = 4). *$p < 0.05$, **$p < 0.01$