IL-17 stimulates inflammatory responses via NF-κB and MAP kinase pathways in human colonic myofibroblasts

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Hata, Kazunori, Akira Andoh, Mitsue Shimada, Sanae Fujino, Shigeki Bamba, Yoshio Araki, Takafumi Okuno, Yoshihide Fujiyama, and Tadao Bamba. IL-17 stimulates inflammatory responses via NF-κB and MAP kinase pathways in human colonic myofibroblasts. Am J Physiol Gastrointest Liver Physiol 282: G1035–G1044, 2002.—Colonic subepithelial myofibroblasts (SEMFs) may play a role in the modulation of mucosal inflammatory responses. We investigated the effects of interleukin (IL)-17 on IL-6 and chemokine IL-8 and monocyte chemotactant protein (MCP)-1 secretion in colonic SEMFs. Cytokine expression was determined by ELISA and Northern blotting. Nuclear factor kappa B (NF-κB) DNA-binding activity was evaluated by electrophoretic gel mobility shift assay (EMSA). The activation of mitogen-activated protein kinase (MAPK) was assessed by immunoblotting. IL-6, IL-8, and MCP-1 secretions were rapidly induced by IL-17. IL-17 induced NF-κB activation within 45 min after stimulation. A blockade of NF-κB activation markedly reduced these responses. MAPK inhibitors (SB-203580, PD-98059, and U-0126) significantly reduced the IL-17-induced IL-6 and chemokine secretion. The combination of either IL-17 + IL-1α or IL-17 + tumor necrosis factor (TNF)-α enhanced cytokine secretion; in particular, the effects of IL-17 + TNF-α on IL-6 secretion were much stronger than the other responses. This was dependent on the enhancement of IL-6 mRNA stability. In conclusion, human SEMFs secreted IL-6, IL-8, and MCP-1 in response to IL-17. These responses might play an important role in the pathogenesis of gut inflammation.

INFLAMMATORY BOWEL DISEASES (IBDs), such as ulcerative colitis and Crohn’s disease, are characterized by recurrent flare of inflammation on a background of chronic enterocolitis. The activation of T cells has been regarded as an important factor in the pathogenesis of IBD (8, 17, 25, 44).

Interleukin (IL)-17 is a newly identified T cell-specific cytokine (19, 51). Human IL-17 is a ~20-kDa glycoprotein of 155 amino acids, the sequence of which exhibits close homology to both cytotoxic T lymphocyte-associated antigen-8 and the open reading frame 13 of T-lymphotropic Herpesvirus saimiri. IL-17 secretion is restrictedly limited in activated CD4+ and CD8+ T lymphocytes, predominantly in the memory CD45RO+ cells (2, 26, 46). In particular, both the Th1 and Th2 subsets of CD4+ cells release IL-17. On the other hand, the IL-17 receptor is widely distributed on various cell types (50, 52), and there is increasing evidence that IL-17 is a potent mediator of the inflammatory responses in various tissues. For example, IL-17 induces several genes associated with inflammation, including IL-6, granulocyte colony stimulating factor, leukemia inhibitory factor, and intercellular adhesion molecule-1 (1, 6, 9, 10, 20, 26).

Subepithelial myofibroblasts (SEMFs) are present immediately subjacent to the basement membrane in the normal intestinal mucosa, juxtaposed against the bottom of the epithelial cells (30, 37, 38). These cells are specialized mesenchymal cells that exhibit the ultrastructural features of both fibroblasts and smooth muscle cells and can be characterized by positive immunoreactivity for both α-smooth muscle actin and vimentin (30, 37, 38, 43, 49). Previous studies have suggested that intestinal SEMFs may be classified as members of a family of functionally related cells, including hepatic Ito cells, glomerular mesangial cells, and orbital and synovial fibroblasts (49). The location of SEMFs below the basement membrane suggests that these cells may play a role in regulation of a number of epithelial cell functions, such as epithelial proliferation and differentiation, and/or extracellular matrix metabolism affecting the growth of the basement membrane. Recently, Mahida et al. (30) reported a method to isolate pure populations of SEMFs from the human colonic mucosa. These cells retain their representative and differentiated phenotypes, such as the positive expression of α-smooth muscle actin, vimentin, fibronectin, type IV collagen, and laminin (30). Recent studies using these cells have demonstrated that SEMFs can modulate the migration (restitution) of epithelial cells (33) and that they express cyclooxygenases (30). Another study (23) demonstrated that the proliferation of these cells is controlled by various growth factors. However, there is little information available on the immunologic functions of SEMFs.

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which may play an important role in the pathogenesis of IBD.

In this study, we investigated the potential role of IL-17 in the induction of inflammatory responses in SEMFs. In particular, we focused on the role of IL-17 in the induction of IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 secretion. The observations in this study indicate that T cells play an important role in the inflammatory responses of the intestine through the secretion of IL-17.

MATERIALS AND METHODS

Reagents. Recombinant human IL-1β, IL-17, and tumor necrosis factor (TNF-α) were obtained from R&D Systems (Minneapolis, MN). The inhibitor of p44/42 mitogen-activated protein kinases (MAPKs; PD-98059 and U-0126) (3, 18) and the inhibitor of p38 MAPK (SB-203580) (14) were purchased from Cell Signaling Technology (Beverly, MA). All other reagents used in this study were purchased from Sigma (St Louis, MO).

Culture of human colonic myofibroblasts. Primary cultures of SEMFs were generated according to the methods described by Mahida et al. (30). In brief, samples of the human adult colonic mucosa were obtained (with informed patient consent) from surgical specimens (>5 cm from the tumor margin) from patients undergoing a partial colectomy for carcinoma. The mucosa samples were completely denuded of epithelial cells by three 30-min incubations at 37°C in 1 mM EDTA (Sigma). The deepithelialized mucosal samples were subsequently cultured at 37°C in a 5% CO₂ atmosphere in DMEM (GIBCO, Grand Island, NY) containing 10% fetal bovine serum (GIBCO). The denuded tissues were maintained in culture for up to 6 wk, and established colonies of myofibroblasts were cultured in DMEM containing 10% fetal bovine serum. All culture media were supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. The studies were performed on passages 2–6 of myofibroblasts isolated from six resection specimens.

Immunohistochemistry. Mouse monoclonal antibodies against α-smooth muscle actin and vimentin were obtained from Sigma. SEMF cells were grown on coverslips and then fixed with 3% paraformaldehyde and 0.05% glutaraldehyde in phosphate buffer before immunoperoxidase staining using an enhanced chemiluminescence Western blotting system (Amersham). The detection was performed using an enhanced chemiluminescence Western blotting system (Amersham).

Measurement of radioactivity. Radioactivity of each band of Northern blotting was determined by the Instant Imager electronic autoradiography system (model 2024/47257; Packard, Meriden, CT). For comparison, each radioactivity was converted to relative radioactivity to the value of medium alone.

Statistical analysis. Data are expressed as means ± SD. Statistical significance of changes was determined by the Mann-Whitney U-test. Differences resulting in P values <0.05 were considered to be statistically significant.

RESULTS

SEMF cells. Morphological features of SEMF cells were compatible with the characteristic features of myofibroblasts reported in the colon and other tissues (30, 37, 38, 43, 49) (Fig. 1A). Immunohistochemical studies of SEMFs at passages 3–6 showed that the cells expressed α-smooth muscle actin (Fig. 1B) and vimentin (Fig. 1C). Cells were also positive for laminin, fibronectin, and type IV collagen (data not shown). Filamentous immunostaining was clearly observed for α-smooth muscle actin and vimentin. These findings are consistent with these cells being myofibroblasts, with phenotypic features similar to myofibroblasts derived from other tissues.
**Induction of IL-6, IL-8, and MCP-1 secretion by IL-17.** Human SEMFs were incubated for 24 h with increasing concentrations of IL-17. The amount of IL-6, IL-8, and MCP-1 secreted into the supernatants was determined by ELISA. As shown in Figs. 2A and 2B, the addition of IL-17 induced a dose-dependent and time-dependent increase in IL-6, IL-8, and MCP-1 secretion.

**Induction of IL-6, IL-8, and MCP-1 mRNA expression by IL-17.** Kinetics of the effects of IL-17 on IL-6, IL-8, and MCP-1 mRNA expression were evaluated in human SEMFs (Fig. 3, A and B). Cells were stimulated with IL-17 (500 ng/ml), and the abundance of IL-6, IL-8, and MCP-1 mRNA was determined by Northern blotting. IL-17 induced a rapid increase in the accumulation of IL-6 mRNA and reached a maximum at 1–3 h.
after stimulation. Thereafter, the induced IL-6 mRNA levels decreased gradually. Similarly, IL-17 induced an increase in the accumulation of IL-8 and MCP-1 mRNA, and these reached a maximum at 3 h. These levels also decreased gradually thereafter.

Modulation of transcription factor activation. It has been reported that activation of transcriptional factor NF-κB plays a central role in the induction of the IL-6, IL-8, and MCP-1 genes (29, 48). To elucidate the mechanisms underlying the response to IL-17, we evaluated the activation of the transcription factor NF-κB in human SEMFs. As demonstrated in Fig. 4A, stimulation with IL-17 (500 ng/ml) for 1.5 h induced an increase in NF-κB DNA-binding activity. Effects of IL-17 were rather weak compared with those induced by IL-1β and TNF-α. The specificity of this reaction was confirmed by the addition of cold oligo-DNA, which abolished the reactive band. The addition of antibodies directed against the 50,000 mol wt (p50) and a 65,000 mol wt (p65) subunits of NF-κB significantly suppressed the reactive band. These results indicate that IL-17 activates NF-κB in human SEMFs.

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mol wt (p65) subunits of NF-κB induced supershifts of the binding complexes, indicating that this binding complex was a heterodimer consisting of p50 and p65 subunits.

Kinetics of NF-κB DNA-binding activity induced by IL-17 was determined (Fig. 4B). IL-17 induced a rapid increase in NF-κB DNA-binding activity within 45 min (0.75 h), and then this was gradually decreased to basal level.

Effects of NF-κB inhibitors. To confirm the role of NF-κB activation, we assessed the actions of NF-κB inhibitors, such as the pyrrolidine derivative of dithiocarbamate (PDTC) (46) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) (41) on IL-6, IL-8, and MCP-1 mRNA expression. A role for oxygen radicals in mediating NF-κB activation has been postulated, and antioxidants such as PDTC have been shown to block NF-κB activation in several cell lines (46). TPCK blocks NF-κB activation by preventing the degradation of the predominant inhibitory molecule IκBα and inhibits the translocation of NF-κB into the nucleus (41). As shown in Fig. 5A, PDTC and TPCK completely blocked IL-17-induced NF-κB DNA-binding activity. The addition of PDTC blocked IL-17-induced IL-6, IL-8, and MCP-1 gene expression (Fig. 5B). TPCK potently attenuated IL-17-induced IL-6, IL-8, and MCP-1 mRNA expression in human SEMFs (Fig. 5B). These findings indicate that the activation of NF-κB may play a major role in the induction of IL-6, IL-8, and MCP-1 mRNA expression in these cells.

IL-17 induces the activation of MAPKs. In various cells, the MAPK family has been shown to play an important role in regulating gene expression in response to inflammatory mediators (12, 21, 22). However, it has not been fully studied whether MAPKs participate in IL-17 signaling. To assess whether similar responses are involved in our system, we evaluated the effects of IL-17 on MAPK phosphorylation in human SEMFs. As shown in Fig. 6A, IL-17 induced the

![Fig. 6. A: effects of mitogen-activated protein kinase (MAPK) inhibitors on IL-17-induced MAPK activation. Cells were pretreated with 20 μM MAPK inhibitors (SB-203580, PD-98059 or U-0126) for 15 min. Cells were stimulated with IL-17 (500 ng/ml) for 15 min and lysed for Western blotting. B: effects of MAPK inhibitors on IL-17-induced IL-6, IL-8, and MCP-1 secretion. Cells were incubated for 24 h with IL-17 (500 ng/ml) in the presence or absence of 20 μM MAPK inhibitors, and the concentrations of IL-6, IL-8, and MCP-1 in the supernatants were determined by ELISA. Values are expressed as means ± SD (n = 4). There were significant differences from the values in the absence of MAPK inhibitors (*P < 0.05).](http://ajpgi.physiology.org/)

![Fig. 7. Combined effects of IL-17 + TNF-α or IL-17 + IL-1β on IL-6, IL-8, and MCP-1 secretion. Cells were cultured for 24 h in the presence of various concentrations of the cytokines, and the concentration of IL-6, IL-8, and MCP-1 in the supernatants was determined by ELISA. Values are expressed as means ± SD (n = 4 experiments). Nos. in parentheses, ng/ml.](http://ajpgi.physiology.org/)
phosphorylation of p42/44 [extracellular signal-regulated kinases (ERK)] and p38 MAPKs as early as 15 min after stimulation. These results indicate that MAPK pathways are rapidly activated by IL-17 in human SEMFs. Phosphorylation of the MAPKs was actually blocked by MAPK inhibitors in these cells (Fig. 6A). The cells were pretreated for 15 min with the inhibitors of p42/44 MAPKs (PD-98059 and U-0126) and the inhibitor of p38 MAPK (SB-203580) and were then stimulated with cytokines for 15 min. PD-98059 and U-0126 inhibited p42/44 MAPK phosphorylation but did not affect p38 MAPK phosphorylation. SB-203580 blocked p38 MAPK phosphorylation but did not affect p42/44 MAPK phosphorylation.

Suppression of the IL-6, IL-8, and MCP-1 induction by MAPK inhibitors. To evaluate the effects of MAPKs on the induction of IL-6, IL-8, and MCP-1 secretion by IL-17 in human SEMFs, the effects of SB-203580, PD-98059, and U-0126 were examined. As shown in Fig. 6B, each inhibitor significantly reduced the IL-17-induced IL-6, IL-8, and MCP-1 secretion. These results indicate that p42/44 and p38 MAPKs play an important role in IL-17-induced IL-6, IL-8, and MCP-1 secretion.

Combination effects of IL-17 + TNF-α and/or IL-1β + IL-1β. Combined effects of IL-17 + TNF-α or IL-17 + IL-1β were evaluated. Cells were incubated with stimulators for 24 h, and IL-6, IL-8, and MCP-1 levels were then determined. As shown in Fig. 7A, IL-17 dose dependently enhanced both TNF-α- and IL-1β-induced IL-6 secretion. Enhancing effects of IL-17 on TNF-α-induced IL-6 secretion were much stronger than those on IL-1β-induced IL-6 secretion. IL-17 dose dependently enhanced both TNF-α- and IL-1β-induced IL-8 and MCP-1 secretion (Fig. 7B), but these were modest compared with the effect of IL-17 on TNF-α-induced IL-6 secretion.

Combined effects of IL-17 + IL-1β or IL-17 + TNF-α on IL-6, IL-8, and MCP-1 mRNA expression were investigated (Fig. 8, A and B). Cells were stimulated for 3 h, and then the IL-6, IL-8, and MCP-1 mRNA levels were determined by Northern blotting. These combinations increased the IL-6, IL-8, and MCP-1 mRNA levels compared with the effects of each individual cytokine. In particular, the combined effect of IL-17 + TNF-α on IL-6 mRNA expression was strong.

To define the mechanism involved in the strong induction of IL-6 mRNA by IL-17 + TNF-α, we investigated NF-κB activation. As shown in Fig. 8C, electrophoretic gel mobility shift assays demonstrated that the effects of IL-17 on both IL-1β- and TNF-α-induced NF-κB DNA-binding activities were modest, suggesting that the transcriptional mechanism might not play a major role in the strong induction of IL-6 by IL-17 + TNF-α.

IL-17 enhances TNF-α-induced IL-6 mRNA stability. To evaluate the posttranscriptional regulation of IL-6 mRNA, we looked at the effects of IL-17 on the TNF-α-induced IL-6 or IL-8 mRNA stability. Cells were stimulated with cytokines for 3 h, and then a chasing approach using the transcription inhibitor actinomycin D and Northern blot analysis were performed. As shown in Fig. 9A, the levels of TNF-α-induced IL-6 mRNA decreased rapidly, but IL-17 markedly enhanced its stabilities. Furthermore, this effect was completely attenuated by the addition of SB-203580, an inhibitor of p38 MAPK, although the addition of PD-98059, the inhibitor of p42/44 MAPKs, had no effect. These observations indicate that the potent enhancing effects of IL-17 on TNF-α-induced IL-6 mRNA expression are mainly mediated by the induction of IL-6 mRNA stabilization and that the activation of p38 MAPK plays an important role in this process. On the other hand, TNF-α-induced IL-8 mRNA was stable, and this was not modulated by the addition of IL-17 (Fig. 9B).
DISCUSSION

Chronic mucosal inflammation is characterized by inflammatory cell infiltration with epithelial cell proliferation and migration, accompanied by an increased turnover of extracellular matrix components in the subepithelial region (40, 47). In this process, SEMFs may play an important role, although the precise functions of these cells remain unclear due to a lack of suitable experimental models. For this purpose, isolated cells are a useful tool for the study of the cellular responses associated with mucosal inflammation. In this study, we used normal human colonic SEMFs isolated by the method reported by Mahida et al. (30).

IL-6 plays an important role in the development of the acute phase response in various tissues via its broad proinflammatory actions (36, 39). Evidence obtained in studies of experimental animals and supported by data from humans suggests that the excessive production of IL-6 is involved in the pathogenesis of IBD (3, 15). However, the local biosynthetic site for IL-6 in the intestine remains unclear.

Chemokines also have a broad range of actions on the recruitment and function of specific populations of leukocytes at the site of inflammation. These factors also play an important role in the initiation and maintenance of the host inflammatory response (7, 34). Chemokines are structurally divided into several groups based on the presence of an intervening amino acid between the first two cysteine residues (e.g., CXC, and CC chemokines) (47, 48). The CXC chemokines act as chemoattractants and activators of neutrophils (e.g., IL-8), whereas the CC chemokines function mainly as chemoattractants for monocytes, eosinophils, T cells, and basophils (e.g., MCP-1).

T cell activation has been reported to play an important role in the pathogenesis of IBD (8, 17, 25, 44). The infiltration of T cells is a characteristic feature of the chronic inflammation in IBD, and neutrophil infiltration becomes more prominent in accordance with the progression of disease activity. The IL-6, IL-8, and MCP-1 secretion by human SEMFs in response to IL-17 emphasizes the importance of T cell products in the induction of inflammation in the intestine. Furthermore, in human SEMFs, the combination of IL-17 with either IL-1β or TNF-α strongly enhances IL-6, IL-8, and MCP-1 secretion; the effect of IL-17 + TNF-α on IL-6 secretion was particularly strong. These responses were clearly observed, even at low concentrations. These results indicate that the cytokines produced by monocytes/macrophages (IL-1β and TNF-α) and T cells (IL-17) can cooperate in the induction of IL-6, IL-8, and MCP-1 secretion in human SEMFs at the low concentrations that are physiologically relevant.

Many cytokine-inducible responses are mediated by DNA-binding proteins such as NF-κB (28). The promoter regions of the human IL-6, IL-8, and MCP-1 genes have been cloned and have been shown to contain putative consensus binding motifs for NF-κB (29, 48). Our results demonstrated that the activation of NF-κB was necessary for IL-17-induced IL-6, IL-8, and MCP-1 gene expression in human SEMFs. Evidence supporting this conclusion may be summarized. First, IL-17 rapidly induced nuclear proteins that exhibited binding to an oligonucleotide containing a NF-κB consensus recognition motif. Binding specificity was confirmed by experiments in which binding was blocked by the addition of excess cold NF-κB oligonu-
compound SB-203580 is a specific inhibitor of p38 MAPK (14). SB-203580 caused a significant decrease in the IL-17-induced IL-6, IL-8, and MCP-1 secretion, indicating that p38 activation was involved. This observation is compatible with the recent report by Craig et al. (13) indicating that the stimulation of p38 MAPK by the MAPK kinase MKK6 activates NF-κB DNA-binding activity and induces IL-6 secretion. In addition, we addressed the role of ERK1/2 in our system. PD-98059 is a specific inhibitor of MAPK/ERK kinase (MEK1) (3), the kinase directly upstream to ERK1/2, and U-0126 is a specific inhibitor of MEK1 and MEK2 (18). U-0126 blocked the phosphorylation of ERK1/2 more potently than PD-98059. PD-98059 and U-0126 caused a significant inhibition of the IL-17-induced IL-6, IL-8, and MCP-1 secretion, thus we concluded that ERK1/2 MAPKs also participate in the IL-6, IL-8, and MCP-1 secretion induced by IL-17 in human SEMFs.

Molecular mechanisms involved in the strong induction of IL-6 secretion by the combination of IL-17 + TNF-α remains to be clarified. One possible approach may be to evaluate the changes in the NF-κB binding activity. However, IL-17 exerted modest effects on both IL-1β- and TNF-α-induced NF-κB DNA-binding activity, suggesting that transcriptional mechanisms did not play a role. We next evaluated the changes in mRNA stabilities. As shown in Fig. 9, TNF-α-induced IL-6 mRNA decreased rapidly, whereas TNF-α-induced IL-8 mRNA was stable. The addition of IL-17 markedly prolonged the half-life of TNF-α-induced IL-6 mRNA, indicating that the strong induction of IL-6 mRNA by IL-17 + TNF-α was mainly mediated by the enhancement of the stability of the IL-6 gene. As reported (27, 31, 35) in other genes, the activation of p38 MAPK was involved in the induction of IL-6 gene stabilization by IL-17 + TNF-α, because SB-203580 completely attenuated these responses. Because the effects of PD-98059 on IL-6 gene stability were negligible, ERK1/2 MAPKs did not play a role in the induction of IL-6 gene stabilization by IL-17 + TNF-α. It has been reported that MKK-6- or MKK-3-induced-p38 activation phosphorylates the kinase MAPK-activated protein kinase 2 (MAPKAPK2) (31). The mRNA stabilization by p38 activation is considered to be mediated by MAPKAPK2, although the relevant substrates of MAPKAPK2 have not fully been identified (31). The precise mechanisms and molecules participating in IL-17 + TNF-α-induced IL-6 gene stabilization should be determined in the future.

In conclusion, this study demonstrated for the first time that IL-17 induces IL-6, IL-8, and MCP-1 secretion in human colonic SEMFs. Although the importance of proinflammatory cytokines in the pathogenesis of IBD is becoming increasingly apparent, the precise mechanisms responsible for the cellular responses have not been fully identified. It is likely that many inflammatory genes are induced in SEMFs in the colonic mucosa. Further investigations using SEMFs will clarify the regulatory mechanisms involved in the pathogenesis of IBD.

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