IL-17A stimulates granulocyte colony-stimulating factor production via ERK1/2 but not p38 or JNK in human renal proximal tubular epithelial cells

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The IL-17 family consists of six members, including IL-17A, IL-17B, IL-17C, IL-17D, IL-17E, and IL-17F. IL-17A, a prototype of the IL-17 family, is a cytokine that is mainly produced by activated CD4+ and CD8+ T cells (34). Recent studies have demonstrated that IL-17A induces cytokine and growth factor production in various cell types, including synovial fibroblasts, colonic myofibroblasts, bronchial and renal epithelial cells, airway smooth muscle cells, and vascular endothelial cells (22). Therefore, increasing attention has been paid to the role of IL-17A in the pathogenesis of various inflammatory diseases, such as rheumatoid arthritis, inflammatory bowel disease, inflammatory skin disorders, allograft rejection, airway inflammation, and multiple sclerosis (22).

It has been reported that IL-17A in renal tubular epithelial cells (RTECs) is associated with the pathogenesis of renal allograft rejection (20, 32, 33), unilateral urethral obstruction (3), and kidney ischemia-reperfusion injury (IRI) (19). A recent report showed an additional role of IL-17A as a mediator of innate immunity; in the case of kidney IRI, IL-17A can be produced by neutrophils other than TH17 cells (the subset of T helper cells that produces IL-17), and this IL-17A positively regulates neutrophil transmigration to the injured kidney (19). It has been reported that IL-17A promotes granulopoiesis that is largely related to the induction of granulocyte colony-stimulating factor (G-CSF) and stem cell factor (26, 27). Although previous reports demonstrated that IL-17A increases IL-6, IL-8, and MCP-1 expression in RTECs in vitro (32, 33), the production of G-CSF, a critical granulopoietic growth factor, by IL-17A in RTECs has not been reported to date.

IL-17F, a protein containing 153 amino acids, is approximately 50% homologous with IL-17A, which is the highest homology of all members of the IL-17 family (10, 17). The genes encoding IL-17A and IL-17F lie next to each other on chromosome 6, which likely arises from a gene duplication event (10). There is considerable overlap in the biological functions of these cytokines (7, 24, 29). We were the first to report that IL-17F stimulates mouse mesangial cells to induce chemokine production (12). The involvement of IL-17F in the inflammation of RTECs has yet to be defined.

In the present study, we investigated the role of IL-17A and IL-17F in the production of G-CSF, and their synergistic activity with other proinflammatory cytokines, such as TNF-α and IL-1β in cultured human renal proximal tubular epithelial cells (HRPTECs), as well as the underlying signaling pathways. The MAPK family, including ERK1/2, p38 MAPK, and JNK, has been shown to play a significant role in the mediation of signals triggered by cytokines, growth factors, and environmental stress, and is known to be involved in various cellular functions (2, 15, 25, 30). Here, we demonstrated for the first time that IL-17A, but not IL-17F, induces G-CSF via the activation of ERK1/2, but not of p38 MAPK or JNK, as well as mRNA transcription and protein translation, and synergizes with TNF-α and IL-1β.

MATERIALS AND METHODS

Reagents. Recombinant human IL-17A, IL-17F, TNF-α, and IL-1β were obtained from R&D Systems (Minneapolis, MN). Actinomycin D and cycloheximide were obtained from Sigma (St. Louis, MO). The inhibitor of MEK1/2 (U0126) was purchased from Cell Signaling Technology (Beverly, MA); the inhibitor of JNK (SP600125) was
Human proximal tubular cell culture. Human proximal tubular cells (HK-2 cells) that exhibit biochemical and morphological features of normal proximal tubular cells in culture were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were grown in DMEM containing 5% FBS, a 1% streptomycin-penicillin mixture, 44 mM NaHCO₃, and 14 mM HEPES in an atmosphere of 5% CO₂ and 95% air at 37°C in a humidified incubator. Experiments were performed with cells up to the fifth passage, as it has been shown that there are no phenotypic changes up to this passage number. To assess cell viability after treatment with MAPK inhibitors, 50 ml of the cell culture supernatant were taken, and lactate dehydrogenase (LDH) levels were measured using CytoTox 96 (Promega, Madison, WI) in accordance with the manufacturer’s instructions.

Quantification of human G-CSF. G-CSF protein levels present in harvested cell culture supernatants were determined using a commercially available ELISA kit (R&D Systems) according to the manufacturer’s instructions.

RESULTS

IL-17A induces G-CSF protein production in cultured HRPTECs. HRPTECs were exposed to increasing concentrations (0, 10, and 100 ng/ml) of IL-17A for 24 h, or to 100 ng/ml IL-17A for 2, 6, and 24 h, and the G-CSF protein levels in the supernatant fractions were measured. IL-17A significantly induced G-CSF production in HRPTECs in a dose-dependent manner (Fig. 1A). In addition, we also observed a significant time-dependent increase in secreted G-CSF following IL-17A stimulation at doses of both 10 and 100 ng/ml (Fig. 1A).

IL-17A-induced G-CSF production is dependent on mRNA transcription and protein translation in cultured HRPTECs. To determine whether the IL-17A-induction of secreted G-CSF is dependent on the translation of IL-17A-induced G-CSF mRNA, we performed Western blot analysis. HRPTECs were incubated with medium alone or medium containing IL-17A for 2, 6, or 24 h, and the G-CSF protein levels in the supernatant fractions were measured. IL-17A significantly induced G-CSF protein production in HRPTECs in a dose-dependent manner (Fig. 1A).

Flow cytometric assay. Cells were incubated for 30 min in the dark on ice with the antibodies PE-conjugated mouse anti-human IL-17RA (eBioscience, San Diego, CA), PE-conjugated mouse anti-human IL-17RC (R&D Systems), or isotype controls (BD Biosciences, Franklin Lakes, NJ). The cells were then washed and analyzed using flow cytometry (BD FACSCalibur; BD Biosciences). The results were analyzed using Cell Quest Software (BD Biosciences).

Statistical analysis. Data are presented as means ± SE. Student’s t-test was performed to determine significant differences, and values of P < 0.05 were considered statistically significant.
centrations (1, 10, and 100 ng/ml) of IL-17A (Fig. 2). mRNA expression following stimulation with increasing concentration reached a maximum level at 6 h following stimulation. In HRPTECs, the time course of G-CSF mRNA expression in HRPT-ECs stimulated with 100 ng/ml IL-17A or medium alone was evaluated using Q-PCR (Fig. 2A). G-CSF mRNA expression reached a maximum level at 6 h following stimulation. In addition, we also observed a significant increase in G-CSF mRNA expression following stimulation with increasing concentrations (1, 10, and 100 ng/ml) of IL-17A or medium for 6 h, and G-CSF mRNA expression levels were analyzed using Q-PCR. The horizontal dotted lines show expression levels in the media control. The values were normalized to GAPDH and then expressed as the G-CSF/GAPDH mRNA ratio. The presented results are representative of 2 independent experiments. Values are means ± SE (n = 4).

dependent on mRNA transcription or protein translation, we pretreated HRPTECs with cycloheximide (10 μg/ml), a protein translation inhibitor, or actinomycin D (1 μg/ml), an mRNA synthesis inhibitor, 1 h before stimulation with IL-17A. G-CSF concentration in the media was analyzed after 24 h of stimulation. Both cycloheximide and actinomycin D completely abrogated IL-17A induction of secreted G-CSF (Fig. 1B). These findings indicate that the induction of G-CSF by IL-17A depends on both de novo mRNA and protein synthesis.

IL-17A induces G-CSF mRNA expression in cultured HRPTECs. The time course of G-CSF mRNA expression in HRPTECs stimulated with 100 ng/ml IL-17A or medium alone was evaluated using Q-PCR (Fig. 2A). G-CSF mRNA expression reached a maximum level at 6 h following stimulation. In addition, we also observed a significant increase in G-CSF mRNA expression following stimulation with increasing concentrations (1, 10, and 100 ng/ml) of IL-17A (Fig. 2B).

IL-17A synergizes with TNF-α and IL-1β for G-CSF protein production by cultured HRPTECs. We next investigated whether IL-17A interacted with the proinflammatory cytokines TNF-α and IL-1β during G-CSF production in HRPTECs (Fig. 3). Stimulation of HRPTECs with 1 ng/ml TNF-α did not enhance G-CSF protein production. Stimulation with a combination of IL-17A and TNF-α resulted in a synergistic enhancement of secreted G-CSF. There was a 12.9-fold increase in G-CSF production following treatment with IL-17A (100 ng/ml) combined with TNF-α over that following IL-17A (100 ng/ml) treatment alone. Similar results were obtained when HRPTECs were stimulated with IL-17A combined with IL-1β. There was a 6.23-fold increase in G-CSF production following treatment with IL-17A (100 ng/ml) combined with IL-1β over that following IL-17A (100 ng/ml) treatment alone.

IL-17A induces activation of ERK1/2 but not of p38 or JNK in cultured HRPTECs. To determine the signaling pathways involved in IL-17A stimulation of G-CSF production in HRPTECs, untreated or IL-17A-stimulated cells were lysed, and Western blot analysis was performed using antibodies against members of the MAPK family, including p38 MAPK, ERK1/2, and JNK. Western blot analysis demonstrated that phosphorylation of ERK1/2 was initiated at 5 min, reached a maximum level at ~10–20 min, and returned to baseline levels at 180 min after IL-17A-stimulation of HRPTECs (Fig. 4A). No activation of p38 MAPK or JNK was seen at any time point, even after 180-min stimulation of the cells with IL-17A (data not shown). In addition, preincubation of the cells with a MEK1/2 inhibitor, U0126 (10 μM), completely abrogated IL-17A induction of secreted G-CSF (Fig. 4A). G-CSF mRNA expression levels were analyzed using Q-PCR. The horizontal dotted lines show expression levels in the media control. The values were normalized to GAPDH and then expressed as the G-CSF/GAPDH mRNA ratio. The presented results are representative of 3 independent experiments. Values are means ± SE (n = 4).

G-CSF production following treatment with IL-17A (100 ng/ml) combined with IL-1β over that following IL-17A (100 ng/ml) treatment alone.

IL-17A and IL-1β synergistically enhanced G-CSF production in HRPTECs. Synergistic effects of IL-17A on TNF-α- and IL-1β-induced G-CSF production in HRPTECs are shown. The cells were pretreated with TNF-α, IL-1β, or DMSO vehicle control for 1 h, followed by stimulation with medium or IL-17A (10, 100, and 200 ng/ml) for 24 h. The G-CSF protein levels in the supernatant were measured by ELISA. The presented results are representative of 3 independent experiments. Values are means ± SE (n = 4). *P < 0.05 compared with cultures without the addition of IL-17A.
Effects of IL-17F on G-CSF protein production in cultured HRPTECs. In contrast to IL-17A, IL-17F did not induce G-CSF in HRPTECs and no significant synergistic effects were observed when IL-17F was combined with TNF-α or IL-1β (Fig. 6A). Western blot analysis indicated that neither ERK1/2 (Fig. 6B), p38 MAPK, nor JNK (data not shown) phosphorylation was observed in IL-17F-stimulated HRPTECs.

IL-17A and IL-17F induce G-CSF protein production in cultured primary human renal cortical epithelial cells. IL-17A, but not IL-17F, significantly induced G-CSF production in cultured HRPTECs. To determine whether this differential effect was a cell-specific phenomenon, we further analyzed the effect of these cytokines on G-CSF production in primary human renal cortical epithelial cells. Primary human renal cortical epithelial cells were exposed to 100 ng/ml IL-17A or IL-17F for 6 or 24 h, and the G-CSF protein levels in the supernatant fractions were measured. IL-17A significantly induced G-CSF production in primary human proximal tubular cells (6 h: 86.92 ± 3.55 pg/ml; 24 h: 142.02 ± 3.93 pg/ml). Unexpectedly, we also observed a significant increase in G-CSF production following IL-17F stimulation (6 h: 33.35 ± 0.77 pg/ml; 24 h: 60.64 ± 5.88 pg/ml).

Expression of IL-17RA and IL17RC on HRPTECs and on primary human renal cortical epithelial cells. To investigate the reason IL-17F was unable to trigger G-CSF production by HRPTECs, we examined the expression of extracellular domains of IL-17RA and IL17RC on HRPTECs and on primary human renal cortical epithelial cells by using flow cytometry (Fig. 7). High IL-17RA expression was detected on both HRPTECs and primary human renal cortical epithelial cells. Unexpectedly, the expression of IL-17RC was higher on HRPTECs than on primary human renal cortical epithelial cells.

DISCUSSION

In the present study, we found that stimulation of cultured HRPTECs with IL-17A resulted in significant increases in the levels of secreted G-CSF protein and in G-CSF mRNA expression. These effects appeared to be mediated mainly by ERK1/2 signaling pathways. Induction of G-CSF in response to IL-17A occurred in both a dose- and time-dependent manner, and was dependent on mRNA transcription and protein translation. In addition, IL-17A synergized with TNF-α or IL-1β for induction of G-CSF production.
In the present study, we showed that IL-17A synergizes with the proinflammatory cytokines TNF-α or IL-1β, which are mainly derived from monocytes/macrophages, for G-CSF production in HRPTECs. It has been reported that TNF-α and IL-1β can be produced by IL-17A in human macrophages (14). Our results suggest the possibility that IL-17A and TNF-α/IL-1β might cooperate strongly to promote G-CSF production from cultured HRPTECs. Although the precise mechanisms underlying the synergy of IL-17A and TNF-α/IL-1β in G-CSF induction have not been fully defined, several reports have suggested that IL-17A enhances chemokine gene expression via mRNA stabilization or synergistic induction of transcription factors such as C/EBPβ. Consistent with our previous study of cultured mesangial cells (12), we found that IL-17A did not have an obvious effect on the stability of G-CSF mRNA induced by TNF-α or IL-1β in tubular epithelial cells (data not shown).

To investigate the cellular mechanisms underlying IL-17A activation in HRPTECs, we investigated the p38 MAPK, ERK1/2, and JNK pathways. These MAPK pathways are known to play a central role in a large variety of cellular activities ranging from cell survival and proliferation to the expression of proinflammatory cytokines. In the present study, we found an increase in the phosphorylation of ERK1/2 in response to IL-17A stimulation in cultured HRPTECs. Activation of the ERK1/2 but not the p38 MAPK or JNK pathways by IL-17A is in agreement with a previous study that investigated human bronchial epithelial cells (16). To investigate the precise roles of individual MAPKs in G-CSF release in IL-17A-stimulated cultured HRPTECs, we tested the effect of a series of well-characterized pharmacological inhibitors of the MAPK signaling pathways, including the p38 MAPK inhibitor SB203580, the MEK1/2 inhibitor U0126, and the JNK inhibitor SP600125. We found that the level of secreted G-CSF protein induced by IL-17A was significantly suppressed by U0126, but not by SB203580 or SP600125, indicating that the ERK1/2 pathway is the important MAPK pathway for upregulation of G-CSF expression. It was noted, however, that induction of G-CSF was not completely inhibited by the MEK1/2 inhibitor U0126, suggesting that additional signaling pathways are involved in G-CSF release by IL-17A in HRPTECs.

Interestingly, secreted G-CSF protein was not detected following IL-17F stimulation of HRPTEC culture. This result was in contrast to the observed dramatic upregulation of G-CSF production by IL-17F alone in bronchial epithelial cells (21), or in combination with other proinflammatory cytokines in human lung microvascular endothelial cells (23). Thus IL-17F, which shares many biological profiles with IL-17A, differs from IL-17A with regard to G-CSF production in HRPTECs. In

Mobilization of neutrophils to sites of tissue injury under the influence of chemokines is a hallmark of inflammation and tissue damage. The extent of neutrophil infiltration into the kidney reflects the magnitude of kidney injury. A recent report by Li et al. (19) showed that IL-17A could be produced by neutrophils other than Th17 cells in kidney IRI and that, in the case of kidney IRI, it positively regulated neutrophil transmigration to the injured kidney. Our in vitro observations extend their in vivo evidence of IL-17A-dependent neutrophil transmigration in renal tubular injury. Although it has been reported that IL-17A can potentially stimulate granulopoiesis via the induction of endogenous G-CSF (1, 4, 13, 26–28), no previous study has evaluated the potential effect of IL-17A on granulopoietic growth factor induction in renal cells. Our data are the first to suggest that the IL-17A-dependent regulation of G-CSF production could be involved in neutrophil transmigration in renal tubular injury.

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humans, IL-17RA binds with an extremely low affinity to IL-17F, whereas IL-17RC binds with higher affinity to IL-17F than to IL-17A (18). Therefore, cells with high IL-17RC expression could be highly responsive to IL-17F, whereas those with low IL-17RC expression and high IL-17RA expression might respond better to IL-17A (5). In contrast, a number of studies have suggested that binding to the IL-17RC receptor might not be essential for the activity of IL-17A and IL-17F (11, 31, 35). Indeed, we found that secreted G-CSF protein was not detected following IL-17F stimulation of HRPTEC culture that significantly expressed IL-17RC on the cell surface, whereas significant G-CSF production was detected in IL-17F-stimulated primary human cortical epithelial cells that do not express IL-17RC. In addition, IL-17RC splice variants have been identified (6, 18), and these IL-17RC splice variants do exhibit ligand preference as certain forms bind preferentially to IL-17A or IL-17F (5). The expression of specific IL-17RC splice variants might be important for understanding why IL-17F stimulation of HRPTECs does not induce upregulation of G-CSF production. It has been reported that the binding of IL-17A or IL-17F to their receptors results in the formation of an IL-17RA-IL-17RC complex, and that the SEFIR domains of both IL-17RA and IL-17RC are required to activate NF-κB, MAPKs, and C/EBP pathways in response to IL-17A or IL17F (8, 9). We speculate that a lack of interaction between IL-17F and IL-17RC results in a lack of activation of ERK1/2 or other MAPKs following IL-17F stimulation in the present study.

In conclusion, we demonstrated that IL-17A is able to induce G-CSF production via ERK1/2 signaling pathways, as well as modulate mRNA transcription and protein translation, and that IL-17A synergizes with TNF-α and IL-1β for stimulation of G-CSF production in cultured HRPTECs. These data suggest that IL-17A may play an important role in neutrophil transmigration and activation via stimulation of G-CSF production in tubular injury.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS

Author contributions: Y.H., M.I., Y.K., K.M., and Y.W. performed experiments; M.I., M.K., N.H., F.K., and T.A. approved final version of manuscript. M.I. drafted manuscript; M.I. edited and revised manuscript; M.I., T.S., M.K., N.H., and F.K. interpreted results of experiments; M.I. wrote the paper.

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