IL-17 stimulates MMP-1 expression in primary human cardiac fibroblasts via p38 MAPK- and ERK1/2-dependent C/EBP-β, NF-κB, and AP-1 activation

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Cortez DM, Feldman MD, Mummidi S, Valente AJ, Steffensen B, Vincenti M, Barnes JL, Chandrasekar B. IL-17 stimulates MMP-1 expression in primary human cardiac fibroblasts via p38 MAPK- and ERK1/2-dependent C/EBP-β, NF-κB, and AP-1 activation. Am J Physiol Heart Circ Physiol 293: H3356–H3365, 2007. First published October 5, 2007; doi:10.1152/ajpheart.00928.2007.—Matrix metalloproteinases (MMPs) degrade collagen and mediate tissue remodeling. The novel cytokine IL-17 is expressed during various inflammatory conditions and modulates MMP expression. We investigated the effect of IL-17 on MMP-1 expression in primary human cardiac fibroblasts (HCF) and delineated the signaling pathways involved. HCF were treated with recombinant human IL-17. MMP-1 expression was analyzed by Northern blotting, RT-quantitative PCR, Western blotting, and ELISA; transcriptional induction and transcription factor binding by EMSA, ELISA, and reporter assay; and p38 MAPK and ERK1/2 activation by protein kinase assays and Western blotting. Signal transduction pathways were investigated using pharmacological inhibitors, small interfering RNA (siRNA), and adenoviral dominant-negative expression vectors. IL-17 stimulated MMP-1 gene transcription, net mRNA levels, protein, and promoter-reporter activity in HCF. This response was blocked by IL-17 receptor-Fc chimera and IL-17 receptor antibodies, but not by IL-6, TNF-α, or IL-1β antibodies. IL-17-stimulated type I collagenase activity was inhibited by the MMP inhibitor GM-6001 and by siRNA-mediated MMP-1 knockdown. IL-17 stimulated activator protein-1 [AP-1 (c-Fos, c-Jun, and Fra-1)], NF-κB (p50 and p65), and CCAAT enhancer-binding protein (C/EBP)-β DNA binding and reporter gene activities, effects attenuated by antisense oligonucleotides, siRNA-mediated knockdown, or expression of dominant-negative signaling proteins. Inhibition of AP-1, NF-κB, or C/EBP-β activity attenuated IL-17-stimulated MMP-1 expression. IL-17 induced p38 MAPK and ERK1/2 activation, and inhibition by SB-203580 and PD-98059 blunted IL-17-mediated transcription factor activation and MMP-1 expression. Our data indicate that IL-17 induces MMP-1 in human cardiac fibroblasts directly via p38 MAPK and ERK-dependent AP-1, NF-κB, and C/EBP-β activation and suggest that IL-17 may play a critical role in myocardial remodeling.

cytokines; interleukins; matrix metalloproteinases; fibrosis

EXTRACELLULAR MATRIX (ECM) turnover in the normal heart is a tightly regulated process. The alteration in the delicate balance between matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs) during myocardial injury and inflammation results in enhanced ECM degradation and remodeling (29, 30). MMPs belong to a family of related, but structurally distinct, zinc-dependent proteases that degrade various ECM proteins, including collagens, gelatins, fibronectin, and laminins (14). MMP-1 (EC 3.4.24.7), or collagenase type I, is the first identified metalloproteinase that degrades interstitial collagens (collagens I, II, III, and VII) in the myocardium (14).

Sustained production of inflammatory cytokines plays a central role in the initiation and progression of left ventricular hypertrophy to failure (11). Various cytokines have been shown to regulate MMP-1 expression at transcriptional and posttranscriptional levels (5, 23). IL-17, a recently discovered family of proinflammatory cytokines secreted mainly by a subset of T (Th17) cells, consists of six ligands (IL-17A, B, C, D, E, and F) that signal through five receptors (IL-17RA, B, C, D, and E) (4). IL-17 family members show little to no homology with other ILs and, therefore, constitute a family of their own (4). Enhanced expression of IL-17 has been reported in various models of inflammation, including rheumatoid arthritis, periodontitis, asthma, and organ rejection (4), and a causal role for IL-17 has been demonstrated in experimental autoimmune myocarditis (21, 28). However, a role for IL-17 in myocardial ischemic injury, hypertrophy, and remodeling has not been described. Since remodeling is characterized by hypertrophy and fibrosis and since fibroblasts play a critical role in fibrosis through expression of MMPs, we investigated whether IL-17 regulates MMP-1 expression in primary human cardiac fibroblasts (HCF).

MATERIALS AND METHODS

Materials. Recombinant human IL-6 (catalog no. 206-IL-010) and IL-17 (catalog no. 317-IL-050), neutralizing antibodies against IL-6, IL-1β, and TNF-α, and normal goat IgG (Ab 108-C) were purchased from R & D Systems (Minneapolis, MN). We previously reported the effectiveness of the anti-cytokine neutralizing antibodies (6, 19). Anti-p38, phosphorylated p38 [PhosphoPlus p38 MAP kinase (Thr180/Tyr182) antibody kit], ERK1/2 (catalog no. 9102), phosphorylated ERK1/2 (catalog no. 9101S), and anti-phosphorylated CCAAT enhancer-binding protein (C/EBP)-β (catalog no. 3084S) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Cycloheximide [InSolution cycloheximide (CHX)], a protein synthesis inhibitor (10 μg/ml in DMSO); SB-203580, a p38 MAPK inhibitor (1 μM in DMSO for 30 min); PD-98059, an ERK1/2 inhibitor (10 μM in DMSO for 1 h); and DMSO were purchased from EMD Biosciences (San Diego, CA). GM-6001, a nonspecific hydroxamic acid-based MMP inhibitor with potent inhibitory activity against collagenase, gelatinases, and stromelysin (15) (10 μM in DMSO for 15 min), was purchased from Upstate/Chemicon (Temecula, CA). Actinomy-

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cin D (ActD), an RNA synthesis inhibitor (2.5 μg/ml in DMSO); α-tubulin polyclonal antibodies; and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

**Cell culture.** HCF (catalog no. 6300, SclentCell Research Laboratories, San Diego, CA) were characterized by an immunofluorescent method using antibody to fibronectin (manufacturer’s technical data sheet). HCF were grown in fibroblast medium (FM) supplied by the manufacturer and supplemented with 2% FBS, fibroblast growth supplement, and antibiotics (complete medium). At 70% confluency, the complete medium was replaced with FM containing 0.5% BSA. After overnight incubation (quiescent cells), IL-17 was added, and the cells were cultured for the indicated time periods. At the end of the experimental period, culture supernatants were collected and snap frozen. Cells were harvested, snap frozen, and stored at -80°C.

Since IL-17 is a proinflammatory cytokine and induces the expression of other cytokines (27) that are known to stimulate MMP-1 expression (5, 23), we determined whether IL-17-mediated MMP-1 expression is dependent on IL-1β, IL-6, or TNF-α. HCF were incubated with IL-1β, IL-6, or TNF-α neutralizing antibodies (10 μg/ml for 1 h; R&D Systems) before addition of IL-17. Normal goat/mouse IgG served as a control.

**Adenoviral vectors and RNA interference.** Recombinant, replication-deficient adenoviral vectors encoding green fluorescent protein (Ad-CMV-GFP), dominant-negative (dn) IKK-β, dnIκB-α, and dnIκB-β (S32A/S36A) have been previously described (18). Ad-CMV-dnc-Jun was purchased from Vector Biolabs. Cells were infected at ambient temperature with adenoviruses in PBS at a multiplicity of infection (MOI) of 100. After 1 h, the PBS solution containing adenovirus was replaced with FM containing 0.5% BSA. Cells were washed out 48 h later, and knockdown of proteins was confirmed by Western blotting. C/EBP-α-flanking region of the MMP1 promoter-reporter assays. A 4,386-bp fragment (5'-ctc gag TCA GTG CAA GGT AAG TGA -3' [antisense]) served as an internal control. Northern blot analysis was carried out as previously described (6). MMP-1 cDNA (GenBank accession no. NM_002421.2) was amplified from reverse-transcribed HCF RNA by RT-PCR using the sense primer 5'-ATT ATT CTA TGT ATA TCG GGG CTT TTA G-3' and the antisense primer 5'-ATG TCC TTG GGG TAT CCT TG-3'. Expression of 28S rRNA was used as an internal control.

MMP-1 mRNA expression was also analyzed by real-time quantitative PCR (5'-CAT TGA TGG CAT CCA AGC C-3' (sense) and 5'-GGG TCT ACA GGG GGT CTT ATG A-3' (antisense)) using QuantiTect SYBR-Green Probe RT-PCR kit (Qiagen). Each sample was assayed in triplicate. For relative quantification, the cycle threshold (Ct) method (ratio = 2(ΔΔCt)) was used, with GAPDH as a control. For copy number determination, a calibration curve was obtained using serial dilutions of a linearized GAPDH cdNA with the GAPDH primer pair 5'-GAA GAT GGT GAT GGG ATT TC-3' (forward) and 5'-GAA GAT GGT GAT GGG ATT TC-3' (reverse).

**MMP-1 levels.** MMP-1 levels in culture supernatants were analyzed using an ELISA kit according to the manufacturer’s instructions (Amersham Biosciences).

**Western blot analysis.** ECM proteins (MMP-1,-2,-3,-8,-9,-10, and -13 and TIMP-1,-2, and -4) in the culture supernatants were analyzed by reporter assays using adenoviral vectors and RNA interference. Western blot analysis. ECM proteins (MMP-1,-2,-3,-8,-9,-10, and -13 and TIMP-1,-2, and -4) in the culture supernatants were analyzed by reporter assays using adenoviral vectors and RNA interference.

**EMSA, ELISA, and reporter assays.** NF-κB and AP-1 protein-DNA complex formation was assessed by EMSA (6, 18, 19) using HCF nuclear extracts and double-stranded consensus DNA for C/EBP (5'-TGG AGA TTA GCG AAT CTG CA-3'), NF-κB (5'-AGT TGA GGG GAC TTT CCC AGG C-3'), AP-1 (5'-GCC TGG ATG ATC CAC CCG GAA-3'), NF-κB (5'-GCC GAC TAG TCT CTG CA-3'), and AP-1 (5'-GCC TGG ATG ATC CAC CCG GAA-3'). Western blot analysis. ECM proteins (MMP-1,-2,-3,-8,-9,-10, and -13 and TIMP-1,-2, and -4) in the culture supernatants were analyzed by reporter assays using adenoviral vectors and RNA interference. Western blot analysis. ECM proteins (MMP-1,-2,-3,-8,-9,-10, and -13 and TIMP-1,-2, and -4) in the culture supernatants were analyzed by reporter assays using adenoviral vectors and RNA interference.
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RESULTS

IL-17 stimulates MMP-1 mRNA expression in human cardiac fibroblasts. Since the proinflammatory cytokine IL-17 signals via IL-17RA, we first used RT-PCR to determine whether HCF express IL-17RA. Our results demonstrated that HCF express IL-17RA under basal conditions (data not shown). We next investigated whether IL-17 can induce MMP-1 mRNA expression in HCF. HCF were treated with IL-17 (0–100 μg/ml) for 12 h and analyzed by Northern blotting and densitometry. HCF express MMP-1 mRNA at low levels under basal conditions (Fig. 1). These results demonstrate that 1) HCF express IL-17RA, 2) IL-17 is a potent inducer of MMP-1 expression, and 3) IL-17 induces MMP-1 expression in a time- and dose-dependent manner (Fig. 1).

IL-17 stimulates MMP-1 protein expression. We next investigated whether IL-17 also stimulates MMP-1 protein expression. Western blot analysis of whole cell homogenates using antibodies that recognize latent and active forms of MMP-1 revealed that, under basal conditions, HCF expressed latent and active forms of MMP-1 (Fig. 2A), whereas treatment with IL-17 resulted in a modest increase in the latent form but a
significant increase in the active form (Fig. 2A). Furthermore, IL-17 increased MMP-1 secretion from the HCF (Fig. 2B). These results demonstrate that IL-17 (1) stimulates MMP-1 protein expression, with a modest increase in the latent form and a significant increase in the active form, and (2) stimulates MMP-1 secretion (Fig. 2).

**IL-17 regulates MMP-1 expression at the transcriptional level.** We next determined whether IL-17-mediated MMP-1 expression is regulated at the transcriptional and/or translational level. HCF were treated with CHX or ActD for 1 h, IL-17 (10 ng/ml for 12 h) was added, and RNA was isolated and analyzed for MMP-1 expression by RT-qPCR. IL-17-mediated MMP-1 expression was significantly inhibited by the RNA synthesis inhibitor ActD, but not by the protein synthesis inhibitor CHX (Fig. 3A). Further studies indicated that the rate of degradation of MMP-1 mRNA was similar in ActD-treated and untreated controls (data not shown), indicating that mRNA stabilization did not contribute significantly to the induction of MMP-1 by IL-17. We next determined whether IL-17 induces MMP-1 promoter-reporter gene activity. Consistent with our earlier observations, IL-17 stimulated the MMP-1 promoter-reporter (pGL3-4334) activity, and incubation with IL-17 or IL-17R neutralizing antibodies blocked this effect (Fig. 3B). It has been reported previously that several IL-17 responsive genes contain NF-κB, AP-1, and C/EBP binding elements in their **cis-regulatory regions** (27). Since these elements also play a role in cytokine- and growth factor-induced MMP-1 expression, we determined whether these elements mediate IL-17-stimulated MMP-1 transcription. Transfection of the deletion constructs lacking C/EBP (pGL3-2685), C/EBP and NF-κB (pGL3-1524), or all three binding sites (pGL3-62) and treatment with IL-17 showed that IL-17-induced MMP-1 promoter-reporter activity was significantly attenuated by the deletion of C/EBP or NF-κB, but this inhibition was significantly more pronounced when all three sites were deleted (Fig. 3C). These results demonstrate that 1) IL-17 does not affect MMP-1 mRNA stability, 2) IL-17 regulates MMP-1 expression at the transcriptional level, and 3) IL-17-mediated MMP-1 transcription is dependent on C/EBP, NF-κB, and AP-1 (Fig. 3).

**IL-17 activates NF-κB, AP-1, and C/EBP.** Since we demonstrated that IL-17-mediated MMP-1 transcription is dependent on NF-κB, AP-1, and C/EBP (Fig. 3), we next investigated whether IL-17 induces activation of these TFs. TF activations were analyzed by EMSA and reporter assays, and their subunit composition was determined by ELISA. IL-17 induced NF-κB DNA binding activity, which was blunted by pretreatment with IL-17 or IL-17R neutralizing antibodies (Fig. 4A). Complementing these EMSA results, IL-17 also induced NF-κB-dependent reporter gene activity (Fig. 4B). ELISA of nuclear protein extracts revealed that p65 and p50 contribute to IL-17-mediated NF-κB activation (Fig. 4C). Similarly, IL-17 induced AP-1 DNA binding (Fig. 4D) and reporter gene activities (Fig. 4E), and c-Fos, FosB, c-Jun, Fra-1, and JunD contributed to its activation (Fig. 4F). IL-17 induced C/EBP DNA binding (Fig. 4G) and reporter gene activities (Fig. 4H), and C/EBP-β, but not C/EBP-α, contributed to its activation (Fig. 4I). These results demonstrate that IL-17 potently induces NF-κB (p65

**Fig. 2.** IL-17 induces MMP-1 protein expression. **A:** Western blot analysis of MMP-1 protein levels in whole cell homogenates from quiescent HCF treated with IL-17 (10 ng/ml) for 24 h. α-Tubulin served as internal control. **B:** ELISA quantification of MMP-1 levels in culture supernatants from quiescent HCF treated with IL-17 and/or cyclohexamide (CHX, 10 μg/ml) for 24 h. *P < 0.001 vs. untreated (ANOVA).

**Fig. 3.** IL-17 stimulates MMP-1 expression at transcriptional and posttranscriptional levels. **A:** quantification by RT-qPCR of MMP-1 expression in quiescent HCF treated with IL-17 and/or cyclohexamide (CHX, 10 μg/ml in DMSO) or actinomycin D (ActD, 10 μg/ml in DMSO) for 12 h. IL-17-mediated MMP-1 expression is regulated at transcriptional and posttranscriptional levels. **B:** IL-17 stimulation of MMP-1 promoter-reporter activity in quiescent HCF transfected with the full-length MMP-1 promoter-reporter vector (pGL3-4334; 3 μg) and 24 h later treated with IL-17 (10 ng/ml) for 12 h. Specificity of IL-17 was verified by 1 h of preincubation of cells with IL-17 or IL-17R neutralizing antibodies (10 μg/ml). pGL3-basic vector (3 μg) served as control. Cells were infected with adenovirus expressing β-galactosidase [Ad-β-Gal, 50 multiplicity of infection (MOI)]. Firefly luciferase and β-Gal activities were analyzed, and results are presented as fold increase relative to untreated and represent ratio of firefly luciferase to β-Gal activity. *P < 0.001 vs. untreated. †P < 0.05 vs. IL-17. **C:** deletion of CCAAT enhancer-binding protein (C/EBP), NF-κB, or activator protein (AP)-1 binding sites blunts IL-17-mediated MMP-1 promoter-reporter activity in quiescent HCF transfected with the full-length vector (pGL3-4334) or deletion constructs lacking C/EBP (pGL3-2685), NF-κB (pMMP1–1524), or all 3 binding sites (pGL3-62). Ad-β-Gal served as control. *P < 0.01 vs. untreated. †P < 0.05 vs. pGL3-4334. §P < 0.01 vs. pGL3-4334.
and p50), AP-1 (c-Fos, FosB, c-Jun, Fra-1, and JunD), and C/EBP-β activation in HCF (Fig. 4).

Targeting TF activation blunts IL-17-mediated MMP-1 expression. We have demonstrated that IL-17 induces NF-κB, AP-1, and C/EBP activation (Fig. 4). We have also shown that deletion of the NF-κB, AP-1, or C/EBP binding site blunts IL-17-mediated MMP-1 transcription (Fig. 3). We next investigated whether targeting NF-κB, AP-1, or C/EBP-β activation will inhibit IL-17-mediated MMP-1 expression. Activation of NF-κB, AP-1, and C/EBP-β was targeted by adenoviral transduction with dominant-negative expression vectors, phospho-rothioated antisense oligonucleotides (ODN), or RNA interference. IL-17-induced MMP-1 promoter-dependent reporter gene activity was significantly attenuated by adenoviral transduction with dnIKK-β, dnp65, or dnp65 (S32A/S36A) (Fig. 5A). Similarly, c-Fos, c-Jun antisense ODN, or Ad.dnc-Jun significantly attenuated IL-17-dependent MMP-1 promoter reporter activity (Fig. 5B), as did siRNA-mediated C/EBP-β knockdown (Fig. 5C). Furthermore, targeting NF-κB, AP-1, or C/EBP-β attenuated IL-17-mediated MMP-1 mRNA expression (Fig. 5D). These results demonstrate that activation of NF-κB, AP-1, or C/EBP-β is a significant mechanism in IL-17-mediated MMP-1 transcription and mRNA expression (Fig. 5).

IL-17 induces p38 MAPK and ERK1/2 activation. MAPKs are important mediators of a variety of physiological and pathological cellular processes, including cell death, cell survival, proliferation, and migration (20). Since IL-17 induced MMP-1 transcription and mRNA expression via NF-κB, AP-1, and C/EBP-β activation (Fig. 5) and since these TFs serve as nuclear effectors of MAPKs, we investigated whether IL-17 induces MAPK activation in HCF. Quiescent HCF were treated with IL-17 for 30 min, and cleared cell lysates were then analyzed for MAPK activation by Western blotting using activation-specific antibodies. Kinase activity was determined by immune complex kinase assays. IL-17 induced p38 MAPK

Fig. 4. IL-17 stimulates AP-1, NF-κB, and C/EBP activation. A: IL-17 stimulation of NF-κB DNA binding activity in quiescent HCF treated with IL-17 (10 ng/ml) for 2 h. Nuclear protein was extracted and analyzed by EMSA using labeled double-stranded consensus NF-κB oligodeoxynucleotides (ODNs). Specificity of IL-17 was verified by preincubation of HCF with IL-17 or IL-17R antibodies. B: IL-17 stimulation of NF-κB-dependent reporter gene activity in quiescent HCF transduced with Ad-NF-κB-Luc (50 MOI). Ad-MCS-Luc (50 MOI) served as control; Ad-β-Gal (50 MOI) served as internal control. After 24 h, cells were treated with IL-17 (10 ng/ml), and firefly luciferase and β-Gal activities were determined. C: contribution of p50 and p65 to IL-17-mediated NF-κB activation. Nuclear extracts from quiescent HCF were treated with IL-17 as described in A and analyzed by ELISA for p50 and p65. OD, optical density. D–I: IL-17-mediated AP-1 and C/EBP activation determined by EMSA (AP-1 (D) and C/EBP (G)), reporter (AP-1 (E) and C/EBP (H)) assays, and ELISA (AP-1 (F) and C/EBP (I)). Arrows in A, D, and G indicate transcription factor-specific DNA-protein complexes. *P < 0.001 vs. respective untreated (B, E, and H); P < 0.01 vs. untreated (C, F, and I).
phosphorylation (Fig. 6A) and activity (Fig. 6B), which were blocked by the inhibitor SB-203580. Similarly, IL-17 induced ERK1/2 phosphorylation (Fig. 6C) and activity (Fig. 6D), which were blunted by PD-98059. These results demonstrate that IL-17 potently induces p38 MAPK and ERK1/2 activation in HCF (Fig. 6).

**IL-17 induces TF activation and MMP-1 mRNA expression via p38 MAPK and ERK1/2.** Our results show that IL-17 potently induces NF-κB, AP-1, and C/EBP-β activation in HCF (Fig. 4). IL-17 also induced p38 MAPK and ERK1/2 activation (Fig. 6). Therefore, we next investigated whether IL-17 induces TF activation via p38 MAPK and ERK1/2. Quiescent HCF were treated with SB-203580 or PD-98059 and then with IL-17. TF activation was analyzed after 2 h by ELISA of nuclear protein extracts. MMP-1 mRNA expression was analyzed after 12 h by RT-qPCR using total RNA. IL-17 stimulated nuclear translocation of NF-κB p65, an effect significantly attenuated by SB-203580 and PD-98059 (Fig. 7A). Similarly, SB-203580 and PD-98059 attenuated IL-17-mediated AP-1 (c-Fos; Fig. 7B) and C/EBP (C/EBP-β; Fig. 7C) activation. Furthermore, SB-203580 and PD-98059 attenuated IL-17-mediated MMP-1 mRNA expression (Fig. 7D). These results demonstrate that IL-17 induces TF activation and MMP-1 mRNA expression via p38 MAPK and ERK1/2 activation (Fig. 7).

**Inhibition of MMP-1 expression blocks IL-17-mediated HCF migration.** Fibroblast migration and proliferation are critical processes in wound healing and scar formation following ischemia, infarction, and inflammation (12). Since MMPs degrade ECM and facilitate cell migration, we investigated whether IL-17 induces HCF migration in an MMP-1-dependent manner. IL-17 does indeed stimulate HCF migration, and pretreatment with the broad-spectrum MMP inhibitor GM-6001 or siRNA-mediated MMP-1 knockdown attenuated IL-17-dependent HCF migration (Fig. 8A); knockdown of MMP-1 was confirmed by Western blotting (Fig. 8B). The effect of GM-6001 appeared to be more pronounced than was MMP-1 knockdown. However, neither control siRNA (Fig. 8A) nor GFP siRNA (data not shown) modulated IL-17-mediated HCF migration. These results demonstrate that IL-17 induces HCF migration.
migration, at least in part, in an MMP-1-dependent manner (Fig. 8).

**IL-17 induces MMP-2, -3, -9, and -13 and TIMP-1 expression in HCF.** In addition to MMP-1, MMP-2, -9, and -13 also play critical roles in ECM degradation and myocardial remodeling. These MMPs also contain similar TF binding sites in their promoters and, therefore, may be transcriptionally regulated by IL-17. The proteolytic activity of MMPs is tightly regulated by various physiological inhibitors termed TIMPs. Since TIMPs such as TIMP-1 contain similar cis elements, including AP-1 and NF-κB, in their promoter regions, we hypothesized that IL-17 may regulate their expression as well. Therefore, we used an antibody array to investigate the expression of various MMPs and TIMPs (Fig. 9A). Confirming our earlier results obtained using ELISA (Fig. 2B), results in Fig. 9B show that IL-17 potently induces MMP-1 expression. In addition, IL-17 also induced the expression of MMP-2, -3, -9, and -13 (Fig. 9B). However, IL-17 failed to significantly affect MMP-8 and -10 expression. Similarly, IL-17 induced TIMP-1, but failed to modulate TIMP-2 and -4, expression (Fig. 9B). These experiments were performed three times, and the results are summarized in Fig. 9C. Together, these results indicate that
IL-17 induces the expression of various MMPs and TIMP-1, which play a role in myocardial remodeling (Fig. 9).

DISCUSSION

The results from this study show that the novel cytokine IL-17 is a potent inducer of MMP-1 expression in primary HCF and stimulates MMP-1 expression independently of IL-1β, IL-6, and TNF-α. IL-17 regulates MMP-1 expression at the transcriptional level and is dependent on AP-1, NF-κB, and C/EBP-β activation. More importantly, IL-17 induces HCF migration in an MMP-1-dependent manner. Since MMPs degrade ECM and facilitate migration, our results suggest that IL-17 may be potentially important in myocardial injury, remodeling, and failure.

IL-17s constitute a newly discovered and unique family of cytokines that show no structural homology to other ILs (4). IL-17 is expressed mainly by a subset of CD4+ T cells, i.e., Th17 cells. Many cell types express IL-17Rs and are, therefore, targets of IL-17. Increased IL-17 levels have been detected in various models of inflammation, including rheumatoid arthritis and periodontitis (1, 4). However, few studies have reported the role of IL-17 in myocardial inflammation and injury. Sonderegger et al. (28) demonstrated that administration of IL-23 neutralizing antibodies attenuated experimental autoimmune myocarditis (EAM). Since IL-23 stimulates a pathogenic IL-17-producing T cell population, these authors hypothesized that targeting IL-17 would reduce EAM. They targeted IL-17 expression by an active vaccination approach that breaks B cell tolerance and found that neutralization of IL-17 effectively reduced heart autoantibody responses and myocardial inflammation (28). Recently, Rangachari et al. (21) showed that mice lacking T-bet, a T-box TF essential for Th1 lineage differentiation, develop severe EAM. Using T-bet−/− IL-12Rβ1−/− and T-bet−/− IL-12p35−/− mice and antibody depletion experiments, these authors reported that IL-23 and IL-17 are critical for EAM pathogenesis.

EAM serves as an animal model for postinfectious myocarditis and cardiomyopathy. Dilated cardiomyopathy is characterized by increased MMP-1 expression (13). It is therefore plausible that IL-17 may play a causal role in dilated cardiomyopathy via enhanced expression of various MMPs, including MMP-1. Although the above-mentioned studies demonstrate a role for IL-17 in myocarditis and cardiomyopathy, Li et al. (16) showed that local expression of soluble IL-17R-immunoglobulin chimera (sIL-17R-Ig) prolongs graft survival in rat cardiac allografts by suppressing cytokine responses and leukocyte infiltration. Together, these studies suggest that IL-17 may be a therapeutic target to reduce cardiac inflammation and injury.

Although the above-mentioned studies show that neutralization of IL-17 blunts myocardial inflammation mainly by regulating Th1 cell responses and attenuating inflammatory and immune cell infiltration (16, 21, 28), it is not known whether IL-17 affects myocardial biology directly. It is also not known whether myocardial constituent cells express IL-17 and whether IL-17 affects myocardial cells differentially.

Fig. 9. IL-18 stimulates secretion of MMPs and tissue inhibitors of MMPs (TIMPs). A: antibody array that detects various MMPs and TIMPs simultaneously was used to assess whether IL-17 induces other MMPs and TIMPs. POS, positive control; NEG, negative control. B: IL-17 stimulates secretion of MMPs and TIMPs. Quiescent HCF were stimulated with saline or IL-17 (10 ng/ml) for 24 h, and culture supernatants were collected and analyzed for extracellular matrix proteins. C: quantitation of signals in B by image analysis. Intensity of signals was normalized to saline-treated control samples, and results are expressed as fold increases. *P < 0.05; **P < 0.001 vs. respective saline (by ANOVA).
multiplexed immunoassays, we observed for the first time that human cardiac fibroblasts secrete various proinflammatory cytokines, including low levels (4.1 pg/ml) of IL-17 at basal conditions (unpublished observations). Since no cells of a non-HCF phenotype contaminate these cultures, our results suggest that cells other than Th17 lineage may secrete IL-17. Thus fibroblast-secreted IL-17 may affect fibroblasts and other myocardial cells via autocrine and paracrine mechanisms.

In the present study, we show that IL-17 induces MMP-1 expression in cardiac fibroblasts, and these stimulatory effects are independent of other proinflammatory cytokines. IL-17 induced MMP-1 expression via enhanced transcription, rather than MMP-1 mRNA stability. Furthermore, IL-17 stimulated MMP-1 transcription via NF-κB, AP-1, and C/EBP-β activation. It has been previously demonstrated that cytokines induce MMP-1 expression via c-Jun induction (22). Although we have not investigated whether IL-17 induces c-Jun expression, our studies show that IL-17-mediated AP-1 DNA binding involves various subunits including c-Jun, and treatment with c-Jun antisense ODN and adenoviral transduction of dnc-Jun attenuate IL-17-mediated MMP-1 transcription. Our results also show that IL-17-mediated AP-1 activation includes Fra-1. Since Fra-1 confers invasiveness and motility in various cancer cell lines (2), it is plausible that Fra-1 may function in a similar fashion in mediating IL-17-dependent fibroblast migration.

In addition to AP-1, our results indicate that IL-17-mediated MMP-1 expression is dependent on NF-κB and C/EBP-β activation. In support of our studies, Raymond et al. (23) recently demonstrated that NF-κB and C/EBP-β cooperatively induce IL-1β-mediated MMP-1 transcription in chondrocytes. They further demonstrated that IL-1β induces phosphorylation of C/EBP-β on threonine 235, enhancing C/EBP-β transactivation potential. In that study, IL-1β induced C/EBP-β phosphorylation via ERK activation (23). Our results show that IL-17 induces MMP-1 expression via p38 MAPK and ERK activation. Together, these studies demonstrate that p38 MAPK- and ERK-dependent coordinated activation of NF-κB, AP-1, and C/EBP-β plays a role in IL-17-mediated MMP-1 induction in cardiac fibroblasts. Since MMPs such as MMP-3 and -9 are also responsive to AP-1 and NF-κB activation (6, 26, 33), IL-17 may induce their expression and, thus, play a critical role ECM regulation and myocardial remodeling.

In fact, our results demonstrate that, in addition to MMP-1, IL-17 induces the expression of other members of the MMP family, such as MMP-2, -3, -9, and -13, which play critical roles in myocardial remodeling. Similar to MMP-1, these MMPs are also regulated predominantly at the transcriptional level. On the basis of the composition of cis-regulatory regions in their promoters, MMPs are arbitrarily grouped into three categories (32). Group 1 consists of MMP-1, -3, -7, -9, -10, -12, and -13, which contain a TATA box and an AP-1 binding site proximal to the transcriptional start site. MMP-9 also contains an NF-κB site at the distal region, and its expression is regulated at the transcriptional levels via AP-1 and NF-κB. Group 2 consists of MMP-8 and -11, which contain a TATA box but lack a proximal AP-1 site. Group 3 consists of MMP-2 and -14, which lack the TATA box and the proximal AP-1 site. Because of these variations, it is possible that these MMPs are regulated differently. Our results show that IL-17 potently induces the expression of various group 1 MMPs. However, lack of significant induction of MMP-10 expression suggests that its induction is not solely dependent on AP-1 activation. The MMP-8 promoter, which contains a TATA box but lacks a proximal AP-1 site, failed to respond to IL-17. In contrast to its effects on MMP-1 induction, IL-17 failed to induce MMP-8 expression, despite three potential C/EBP binding sites at -70, -112, and -164 in its cis-regulatory region. IL-17 also induces the expression of MMP-2 in HCF. MMP-2 contains neither a TATA box nor the proximal AP-1 site. However, MMP-2 contains two AP-2 binding sites at -61 and -1649. It is possible that IL-17 may induce MMP-2 via AP-2 activation. However, Bergman et al. (3) demonstrated that the MMP-2 promoter contains an AP-1 binding site at -1670 that binds FosB and JunB, suggesting that further critical analysis of TF binding sites is necessary.

Transcription is a complex process and is regulated at multiple stages. It is possible that IL-17 may regulate MMP expression at transcriptional and posttranscriptional levels. Despite few CpG islands in the promoter regions of MMPs, epigenetic mechanisms have recently been shown to significantly affect MMP expression (31). For example, increased promoter methylation was shown to suppress MMP-9 transcription (9). Therefore, it is possible that IL-17 may regulate expression of MMPs via genetic and epigenetic mechanisms. It is also possible that IL-17-induced MMP induction is mediated by an intermediary, inasmuch as IL-17 is a potent inducer of various proinflammatory cytokines that are known to induce MMP expression. However, in the present study, we have shown that neutralization of IL-1β, IL-6, and TNF-α fails to inhibit IL-17-mediated MMP-1 induction.

MMPs are synthesized as proenzymes and, upon secretion, bind to various ECM components (31, 32). These stored proforms are immediately available and become activated during inflammation and injury. In addition to ECM degradation, MMPs also release ECM-bound growth factors and other biological molecules. For example, TGF-β, a growth factor readily expressed after myocardial ischemic injury (7), is secreted as a latent and inactive form due to an intact prodomain, the latency-associated peptide (17). MMP-1 degrades latency-associated peptide and releases mature TGF-β, and mature TGF-β downregulates MMP-1 expression. This dynamic coregulation and downregulation of MMP-1 expression may result in reduced tissue injury. In fact, active MMP-1 has been shown to induce cardiomyocyte death (8), and these cytotoxic effects are blunted by exogenous addition of mature TGF-β. However, studies are in progress to determine whether IL-17 coregulates MMP-1 and TGF-β expression.

Our studies have important clinical implications. 1) We have established for the first time that IL-17, a novel proinflammatory cytokine, induces primary cardiac fibroblast migration in an MMP-1-dependent manner. Since fibroblast migration and proliferation are two critical steps in cardiac fibrosis, our results indicate that IL-17 may play a role in myocardial remodeling. 2) IL-17 induced NF-κB, AP-1, and C/EBP-β activation. Therefore, IL-17 may upregulate NF-κB, AP-1, and C/EBP-β-responsive proinflammatory cytokines, chemokines, adhesion molecules, and MMPs in fibroblasts and other myocardial constituent cells. IL-17 may synergize with these mediators and induce myocardial inflammation and injury. 3) Neutrophils, at least initially, play a role in myocardial ischemic injury. IL-17, a potent inducer of neutrophil chemoattractants (24), may amplify the inflammatory cascade during...
ischemic injury via recruitment of neutrophils to the site of injury/inflammation. Therefore, targeting IL-17 expression may reduce fibrosis and remodeling following myocardial inflammation and injury.

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REFERENCES


