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IL-17 Boosts Proinflammatory Outcome of Antiviral Response in Human Cells

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Excessive inflammation during bacterial and viral infections is destructive to the host and involves elevated production of proinflammatory cytokines. It is especially deleterious in organs with space constraints such as lung and the CNS. Indeed, a number of viruses that infect lungs, such as avian influenza virus, SARS-associated coronavirus, and respiratory syncytial virus, elicit a very high level of proinflammatory cytokines; however, it is unclear what triggers their production. In this study, we show that IL-17 commonly produced during viral infection specifically augments a proinflammatory response by directly synergizing with antiviral signaling. Costimulation of primary human fibroblasts with IL-17 greatly enhanced respiratory syncytial virus-induced or synthetic dsRNA-based viral mimic polyinosinic:polycytidylic acid-induced expression of proinflammatory genes without affecting expression of IFN-β–stimulated or IFN-stimulated genes. Knockdown of expression of known mediators of the antiviral signaling pathway revealed that the IL-17–poly(I:C) synergy depends on the presence of the transcriptional factors RelA and IFN regulatory factor 3 and IκB kinases. Moreover, this synergy was blocked by an IκB kinase inhibitor, BAY 11-7082. These findings shed light on the molecular mechanisms behind IL-17–dependent immunopathology observed in viral infections. The Journal of Immunology, 2011, 187: 000–000.

Interleukin-17 is a cytokine mainly produced by Th17 and γδ T cells that is best known for its protective properties during bacterial infections and involvement in autoimmune disorders. IL-17 exacerbates inflammatory responses during viral infection (1–3), and influenza virus-infected IL-17RA−/− mice show decreased production of proinflammatory cytokines and neutrophil infiltration (1). Furthermore, IL-17 blocking Abs reduce, whereas recombinant IL-17 enhances, lung pathology in Thelier’s murine encephalomyelitis virus-infected mice (4). An age-dependent increase in IL-17 levels exacerbates inflammation and infection-associated mortality in HSV-2- and murine cytomegalovirus-infected mice (3). In addition, exogenous IL-17 enhances IL-8 production in rhinovirus-infected epithelial cells (5). Also, elevated Th17 numbers correlate with hepatitis B virus and SIV infection progression (6, 7). IL-17 is largely produced by γδ T cells, neutrophils, NKT cells, and Th17 cells (8). IL-17 is also produced by a recently described CD8 T cell population, Tc17 cells, which are important for protection against influenza in mice (9).

IL-17 stimulates epithelial, stromal, and endothelial cells (expressing IL-17 receptor) to induce inflammatory mediators attracting immune cell types to the site of IL-17 action (10–12). IL-17 stimulates epithelial, stromal, and endothelial cells (expressing IL-17 receptor) to induce inflammatory mediators attracting immune cell types to the site of IL-17 action (10–12). IL-17 binds to a heterodimeric IL-17RA/RC receptor to initiate a signaling cascade resulting in AP-1 and NF-κB activation (13, 14). In addition, IL-17 stimulation results in mRNA stabilization of cytokines induced by TNF-α (15, 16).

Despite the crucial role of IL-17 in modulating immune pathophysiology of viral infection, the molecular mechanisms of this regulation remain largely unknown. Viral detection by pattern recognition receptors like TLR3 or RIG-I/MDA-5 helicases elicits a strong immune response in addition to the immunopathology induced by viral replication (17). As an example of this, intranasal application of polyinosinic:polycytidylic acid [poly(I:C)], a synthetic dsRNA viral mimic, causes severe inflammation in mice (18). Notably, a recent study has shown that IL-17 is an essential factor in poly(I:C)-induced lung inflammation (19).

In this study, we hypothesized that IL-17 can directly synergize with viral products in superinducing the proinflammatory response. Using primary human skin fibroblasts, we show that IL-17 boosts poly(I:C)- and respiratory syncytial virus (RSV)-induced synthesis of proinflammatory cytokines without affecting the production of antiviral molecules. In addition, our inhibition experiments using small interfering RNA (siRNA) and chemical inhibitors demonstrate a critical role of the transcription factors RelA and IFN regulatory factor 3 (IRF3) and IκB kinases (IKKs) in mediating IL-17–poly(I:C) synergistic induction of proinflammatory genes.

Materials and Methods

Reagents and cells

Primary human skin fibroblasts (HSFs; purchased from American Type Culture Collection) were cultured in DMEM (PAA) supplemented with 10% FBS (Life Technologies) and 1% penicillin/streptomycin (PAA) at 37˚C in 5% CO2 and 95% humidity. Cells were seeded at a density of 2 × 103 cells/ml (for 12-well plates) or 5 × 103 (for 6-well plates) and cultured...
overnight for use in experiments the following day. BAY 11-7082 was from Sigma. After the initial titration experiments, cells were stimulated with 25 ng/ml human IL-17A (Peprotech) and/or 2.5 μg/ml poly(I:C) (InvivoGen).

**Viral infection**

RSV (A2 strain) was grown in HEP-2 cells. All work was performed according to institutional and U.K. Home Office guidelines. HSFs were plated a day before infection at 2×10^3 cells/well (in 12-well plates). Prior to infection, cells were washed twice with PBS, and a serum-free DMEM was added. RSV was then added to cells at multiplicity of infection (MOI) of 2 for 1 h at 37°C. After that, the medium was changed to DMEM with serum and antibiotics, and cells were incubated for 1–3 d at 37°C in 5% CO₂ before lysis for RNA or protein extraction.

**Abs**

The anti–IL-6, anti–IL-8, and anti–IFN-γ–induced protein 10 (IP-10) Abs used for ELISA were from BD Pharmingen. Rabbit anti-ISG15 was from Cell Signaling; and monoclonal anti–β-actin was from Sigma. The monoclonal anti-IKKε, anti–TBK1, and polyclonal anti–IKKe Abs were from Imgenex; rabbit anti–RelA, anti–IRF3, and anti–IKKe were from Santa Cruz Biotechnology. Mouse Act1 Ab was purchased from ebioscience. Goat anti-RSV Ab was from AbD Serotec.

**RNA interference**

Human p65 (RelA), IRF3, and IKKε were targeted using On-target Plus siRNA pools (Dharmacon); a nontargeting pool, siC, was used as a control siRNA (Dharmacon). siRNAs for IKKβ, TBK1/IKKδ, and IKKe are described in Ref. 20. HSFs plated a day before transfection at 70–90% confluency were transfected with siRNA in a serum-free medium, OptiMEM (Invitrogen), using lipid-based transfection reagent Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. The transfection medium was replaced with DMEM containing 10% serum and antibiotics 4 h posttransfection. Twenty-four hours posttransfection, cells were trypsinized and split 1:2 into 12-well plates and incubated for a further 24 h before stimulation with IL-17 and or poly(I:C) and subsequent analysis.

**RNA extraction, cDNA synthesis, and quantitative PCR**

Total RNA was extracted from cells using Qiagen RNeasy Mini Kit (Qiaogen). Cells were lysed in RLT buffer (Qiaegen), and cDNA synthesis was performed using extracted RNA, oligo(dT) primer, and Superscript III reverse transcriptase (Invitrogen). The cDNA was PCR-amplified using EfficienSee Fast quantitative PCR master mix plus dTTP (Eurogentec). The Taqman gene expression assays for human RPLPO (housekeeper control), Act1, IL-6, IL-8, CXCL1, NFKBIZ, CCL8, IFN-β, IP-10, ISG15, OA2S, CXCL11, and TNF were acquired from Applied Biosystems.

**ELISA**

Levels of IL-6, IL-8, and IP-10 in culture supernatants were determined by ELISA (BD Pharmingen), according to the manufacturer’s instructions. Absorbance was measured at 450 nm using a spectrophotometric plate reader (Labsystems Multiscan Biochromic). The data were analyzed using Ascent software (Thermo Labsystems).

**Western blot**

Cells were lysed in sample buffer (2×, 0.05% bromophenol blue, 20% glycerol, 4% SDS, 0.25 M TrisHCl pH 6.8, and 200 mM DTT), the proteins in the lysates were separated by SDS-PAGE using NuPAGE Novex 4–12% Bis-Tris Gels (Invitrogen), immunoblotted, and the protein bands were visualized using Amersham ECL detection system (GE Healthcare).

**Statistical analysis**

Data are presented as means with error bars of the SEM or representative data are shown. Statistical significance was calculated for experiments, where n = 3, using the paired Student t test.

**Results**

**IL-17 specifically enhances the proinflammatory response in cells infected with RSV**

To investigate the impact of IL-17 on gene expression during viral infection, we used primary HSFs that do not produce TNF in response to infection with RSV or stimulation with poly(I:C), thus circumventing previously described IL-17–TNF synergism in cytokine induction (15, 16). Viral envelope proteins of RSV were detectable already at 24 h and peaked at 48 h after infection of HSFs (Fig. 1A). IL-17 did not appear to affect synthesis of RSV proteins (Fig. 1A). mRNA expression of proinflammatory mediators, such as IL-6, IL-8, CCL8, CXCL1, and NFKBIZ, was significantly increased in virus-infected but not in control resting cells or cells stimulated with IL-17 alone (Fig. 1B and Supplemental Fig. 1A). Moreover, addition of IL-17 to RSV-infected cells resulted in a significant increase of IL-6 and IL-8 protein secretion (Fig. 1C) by these cells. Notably, expression of antiviral genes, such as IFN-β, ISG15, IP-10, MX1, and OAS2, were not affected by the presence of IL-17 at either the mRNA level (Fig. 1D and Supplemental Fig. 1B) or the protein level (Fig. 1A and Supplemental Fig. 1C).

Thus, IL-17 stimulation results in an enhanced proinflammatory response in RSV-infected cells without affecting the production of antiviral proteins. To validate further the specificity of IL-17 signaling in HSFs, we knocked down expression of the IL-17 receptor adapter Act1/TRAF3IP2 using specific siRNA (Supplemental Data 1). As shown in Supplemental Fig. 2, IL-17 stimulation resulted in a significant increase of IL-6 and IL-8 protein secretion by these cells. Notably, expression of antiviral genes, such as IFN-β, ISG15, IP-10, MX1, and OAS2, were not affected by the presence of IL-17 at either the mRNA level (Fig. 1D and Supplemental Fig. 1B) or the protein level (Fig. 1A and Supplemental Fig. 1C).
As expected, IL-17–induced but not TNF-induced gene expression was inhibited by Act1 depletion. Therefore, we confirmed that the recombinant IL-17 we used in this study signals specifically via IL-17R–Act1 axis in HSFs.

RSV activates cytosolic RIG-I/MDA-5–dependent pathway in HSFs

Viruses trigger antiviral signaling when their dsRNA products are recognized by TLR3 in endosomes (17, 21) or by RLH family helicases, RIG-I and MDA-5, in the cytosol (22). We observed that in HSFs, RSV is mostly recognized via the cytosolic RLH-dependent pathway, as siRNA-mediated knockdown of the RLH adapter MAVS, but not the TLR3 adapter TIR-domain–containing adapter-inducing IFN-β (TRIF), inhibited RSV-induced IFN-β expression (Fig. 2A). In addition, knockdown of MAVS suppressed TRIF mRNA induction by RSV (Fig. 2A). Therefore, IL-17 acts in synergy with RIG-I–dependent pathway during RSV infection in HSFs.

IL-17 synergizes with both TLR3-mediated and RIG-I/MDA-5–mediated antiviral signaling pathways

To examine whether IL-17 can also synergize with antiviral signaling triggered by the TLR3 pathway, we used a synthetic compound, poly(I:C), which mimics dsRNA derived from virus genomic RNA or replicative intermediates. Poly(I:C) is known to signal via the endosomal pathway, which we confirmed in this study by blocking IFN-β expression in HSFs with siRNA-mediated knockdown of TRIF (Fig. 2B). In addition, poly(I:C)–but not RSV-induced gene expression was blocked in cells treated with chloroquine, which prevents endosomal acidification (23) and TRIF signaling (Supplemental Fig. 3A). We therefore stimulated HSFs with poly(I:C) and IL-17 separately or in combination for 3, 8, and 24 h and measured inducible gene expression. The combined poly(I:C)/IL-17 treatment of HSFs resulted in a strong upregulation of the proinflammatory genes at the protein (Fig. 3A) and mRNA (Fig. 3B and Supplemental Fig. 3B) levels. Once again, addition of IL-17 had no extra effect on the protein and mRNA expression levels of antiviral genes (Fig. 3C and Supplemental Fig. 3C). Therefore, IL-17 stimulation selectively changes the outcome of poly(I:C) signaling in HSFs by greatly enhancing proinflammatory gene expression without affecting induction of antiviral genes.

Collectively, these data suggest that IL-17 stimulation acts in synergy with both virus-activated endosomal TLR3 and cytosolic RIG-I and MDA-5 to trigger enhanced proinflammatory response but has no role in further induction of antiviral genes. TNF was not involved in the observed synergy between IL-17 and RSV or poly(I:C), as this cytokine is not produced at the protein level in stimulated HSFs (data not shown).
**FIGURE 4.** Poly(I:C)/IL-17 synergistic induction of proinflammatory cytokines depends on IKKs. A and B, HSFs were transfected with siRNA (100 nM each) against IKKα, IKKβ, TBK1, and IKKe, or with nontargeting siRNA. Cells were incubated with siRNA for 24 h and split 1:2 24 h before lysing cells (A) or before 24 h stimulation with IL-17 (25 ng/ml) or poly(I:C) (2.5 μg/ml), or both or left nonstimulated. A, The effect of gene knockdown on IKK protein expression was determined by Western blotting with corresponding Abs. β-Actin was used as a protein loading control. B, The effect of gene knockdown on IL-6 and IL-8 secretion was measured by ELISA in the culture supernatants. Data are shown as mean ± SD of a representative of three independent experiments. C, HSFs were preincubated with 0, 1, or 5 μM BAY 11-7082 for 1 h and then stimulated for 24 h with IL-17 (25 ng/ml) or poly(I:C) (2.5 μg/ml), or both or left nonstimulated. IL-6 and IL-8 protein secretion was measured by ELISA. Data are shown as mean ± SEM of three independent experiments. *p < 0.02 (one-tailed paired t test). N.S., nonstimulated; siC, nontargeting siRNA.

**IL-17 enhances proinflammatory outcome of the antiviral response via IKKs**

IL-17 binding to its receptor initiates a signaling cascade resulting in activation of the canonical IKK complex (24, 25), containing IKKα and IKKβ catalytic subunits, which phosphorylates IkBα, targeting it for ubiquitin-dependent proteasomal degradation, and as a result releasing NF-κB to the nucleus (26). TLR3 and RIG-I/MDA-5 signaling pathways trigger the noncanonical IKKs, such as TBK1 and IKKe, which phosphorylate IRF3, resulting in its dimerization and nuclear translocation (20, 27). We therefore hypothesized that IKKs are likely to be involved in the observed synergy between IL-17 and poly(I:C). siRNA-mediated knockdown of canonical and noncanonical IKK gene expression in HSFs (Fig. 4A) revealed that poly(I:C)/IL-17–induced IL-6 and IL-8 production was significantly reduced in cells with downregulated IKKs (Fig. 4B).

In addition, mRNA expression of IL-6 and IL-8 was abrogated by depletion of either IKK (Supplemental Fig. 4A). To further validate these results, we incubated cells with a previously characterized IKK inhibitor, BAY 11-7082 (28). Preincubation of HSFs with increasing concentrations of BAY 11-7082 suppressed IL-6 and IL-8 production in response to poly(I:C)/IL-17 (Fig. 4C). mRNA expression of IL-6 and IL-8 was also significantly reduced (Supplemental Fig. 4B).

Moreover, siRNA-mediated knockdown of two downstream targets of the IKK complexes, NF-κB and RelA and IRF3 (Fig. 5A), in HSFs also effectively reduced the poly(I:C)/IL-17–induced mRNA expression of IL-6 and IL-8 (Fig. 5C).

Taken together, these results demonstrate that synergistic upregulation of proinflammatory gene expression in HSFs by IL-17 and poly(I:C) is mediated by IKKs and also depends on the IRF3 and RelA transcription factors.

**Discussion**

IL-17 is linked to increased immunopathology in several viral infections (1–3), but the molecular mechanisms behind its role remain largely undefined. In this study, we demonstrate that IL-17 specifically boosts proinflammatory, but not antiviral, gene expression in human cells infected with RSV or stimulated with the viral mimic poly(I:C). Moreover, we investigate the molecular mechanisms behind the synergistic effect of IL-17 and viral signaling, which at the transcriptional level involves IKKs and transcription factors IRF3 and RelA. It would therefore be interesting to assess whether the IKK inhibitors, which we show interfere with...
IL-17/poly(I:C) synergistic induction of proinflammatory genes in vitro, could be used to block IL-17-dependent responses in vivo. IL-17 is known to trigger TNF-α-induced cytokine mRNA stabilization (15). However, in our system, poly(I:C)/IL-17 synergy is independent of TNF-α, as TNF protein is not detectable in HSFs infected with RSV or stimulated with poly(I:C) even in the presence of IL-17 (data not shown). Significantly, IL-17 causes pathogenic effects to an equal degree in wild-type and of IL-17 (data not shown). Significantly, IL-17 causes pathogenic unique among the TLRs in its inability to ensure mRNA stabili-

References


Figure S1. IL-17 enhances pro-inflammatory but not antiviral gene expression in RSV-infected cells. (A-C) HSFs were infected with RSV (MOI 2) or stimulated with 25ng/ml of human IL-17, or both for 48 hours. mRNA expression was analysed by RT-PCR (A,B); protein secretion in the supernatants of cultured cells - by ELISA (C). Data are shown as fold induction over non-stimulated (N.S.) cells normalised to the value of expression in RSV/IL-17 stimulated cells which were given the value of 100% and are mean ± S.E.M of three independent experiments: ** p<0.005; * p<0.05 (one-tailed paired t-test).
Figure S2. Specificity of IL-17 induced gene expression in HSFs. HSFs were transfected with siRNA (100 nM each) against Act1 or with non-targeting siRNA (siC). Cells were incubated with siRNA for 24 hours and split 1:2 24 hours before 4 hour stimulation with IL-17 (25 ng/ml) or TNF-α (10 ng/ml) or left non-stimulated (N.S.). The knockdown efficiency was verified by Western blotting using monoclonal Act1 antibody. β-actin was used as a protein loading control. The effect of Act1 knockdown on mRNA induction was determined by RT-PCR. Data are shown as means +/- SD of a representative experiment. Gene expression is presented as fold induction over N.S. siC cells normalized to the value of the house-keeper gene expression (RPLPO).
Figure S3. IL-17 superinduces pro-inflammatory but not antiviral gene expression triggered by a viral mimic poly(I:C) in HSFs. (A) HSFs were either infected with RSV at MOI 2 (A) or stimulated with 2.5 μg/ml poly(I:C) in the presence or absence of 10 μM chloroquine (CQ) for 24 hours. mRNA induction was measured by RT-PCR. Data are shown as mean ± SD of a representative of three independent experiments. (B,C) HSFs were stimulated for 3, 8 and 24hrs with IL-17 (25 ng/ml) or poly(I:C) (2.5 μg/ml), or both or left non-stimulated (N.S.). The induction of mRNA expression of pro-inflammatory (B) and antiviral (C) genes was measured by RT-PCR. Data are shown as fold induction over non-stimulated (N.S.) cells normalised to the value of expression in poly(I:C)/IL-17 stimulated cells which were given the value of 100% and are mean ± S.E.M of three independent experiments: * p<0.05, ** p<0.01, *** p<0.001 (one-tailed paired t-test).
Figure S4. IL-17/poly(I:C) induced activation of pro-inflammatory cytokine expression is dependent on IKK kinases. (A) HSFs were transfected with siRNA (100 nM each) against IKKα, IKKβ, TBK1, IKKe or with non-targeting siRNA (siC). Cells were incubated with siRNA for 24 hours and split 1:2 24 hours before stimulation with IL-17 (25 ng/ml) or poly(I:C) (2.5 μg/ml) or both or left non-stimulated (N.S.). (B) HSFs were pre-incubated with 0, 1 or 5 μM of BAY 11-7082 for 1 hour and then stimulated for 24 hours with IL-17 (25 ng/ml) or poly(I:C) (2.5 μg/ml) or both or left non-stimulated (N.S.). IL-6 and IL-8 mRNA induction was determined by RT-PCR. Data are shown as fold induction over N.S. siC cells normalised to the value of expression in poly(I:C)/IL-17 stimulated siC cells which were given the value of 100% and are mean ± S.D of a representative out of three independent experiments (A) or mean ± S.E.M of three independent experiments: ** p<0.01, *** p<0.001 (one-tailed paired t-test) (B, D).