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*J Immunol* 2009; 182:1660-1666; doi: 10.4049/jimmunol.182.3.1660
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IL-17 Signaling for mRNA Stabilization Does Not Require TNF Receptor-Associated Factor 6

Justin Hartupee, Cai Ni Liu, Michael Novotny, Dongxu Sun, Xiaoxia Li, and Thomas A. Hamilton

IL-17 alone is a relatively weak inducer of gene expression, but cooperates with other cytokines, including TNF-α, to generate a strong response in part via prolongation of mRNA t1/2. Because TNFR-associated factor 6 (TRAF6) has been reported to be essential for signaling by IL-17, we examined its involvement in IL-17-mediated mRNA stabilization. Although overexpression of TRAF6 in HeLa cells activates NF-κB, it does not stabilize transfected KC mRNA. Furthermore, a dominant-negative TRAF6 abrogates NF-κB activation, but does not block IL-17-induced chemokine mRNA stabilization. IL-17 can stabilize KC and MIP-2 mRNAs comparably in TNF-α-treated mouse embryo fibroblasts from TRAF6+/− and TRAF6−/− mice. TRAF6 is known to couple upstream signals with activation of p38 MAPK and mitogen activated protein kinase activated protein kinase 2, both of which have been shown to be important for Toll/IL-1R-mediated mRNA stabilization in various cell types. Inhibition of p38 MAPK, however, does not block IL-17-induced KC mRNA stabilization, and IL-17 can stabilize KC mRNA equally in mouse embryo fibroblasts from both wild-type and mitogen activated protein kinase activated protein kinase 2/3 doubly-deficient mice. Finally, IL-17 can stabilize KC and MIP-2 mRNAs comparably in TNF-α-treated mouse embryo fibroblasts from TRAF6+/− and TRAF6−/− mice. Collectively, these findings demonstrate the existence of a TRAF6/p38 MAPK-independent pathway that couples the IL-17R with enhanced mRNA stability. Because the most potent effects of IL-17 on gene expression are obtained in cooperation with other cytokines such as TNF-α, these findings suggest that this pathway is a major contributing mechanism for response to IL-17. The Journal of Immunology, 2009, 182: 1660–1666.

Interleukin-17 is recognized to play a critical role in several forms of inflammation associated with host defense or autoimmunity (1–3). In response to stimulation with IL-17, resident tissue cell populations produce a variety of proinflammatory gene products resulting in the influx of neutrophils (2–4). However, the molecular mechanisms by which IL-17 regulates target gene expression are poorly understood. It has been repeatedly observed that whereas IL-17 alone exerts only modest effect on gene expression, the combination of IL-17 with other inflammatory stimuli (particularly TNF-α) leads to a strong, synergistic response (4, 5). Because IL-17 will almost certainly be present in vivo in a microenvironment containing multiple cytokines, including TNF-α, the ability to amplify responses induced by other stimuli is likely to be physiologically important.

Although many genes induced during inflammation exhibit a significant increase in transcription, often dependent upon activation of the transcription factor NF-κB, the mature mRNAs frequently exhibit very short t1/2, but serve as a check to ensure that the protein is not produced inappropriately (6–10). These remarkably short t1/2 are often prolonged via signals from inflammatory stimuli enabling robust gene expression during the response (11–14). In many settings, this stimulus-induced stabilization requires the action of the p38 MAPK cascade, including its downstream kinases mitogen activated protein kinase activated protein kinase (MK)2 and 3 (11, 12, 15–17).

A number of reports have indicated that activation of NF-κB is a major mechanism by which IL-17 regulates target gene expression (18, 19). However, IL-17-mediated NF-κB activation is modest in comparison with that induced by agents such as TNF-α and IL-1 (20–22). Several laboratories have shown that IL-17 serves as a strong stimulus to induce stabilization of constitutively unstable mRNAs, and this may represent an important mechanism by which this cytokine regulates target gene expression (20, 23, 24). The ability of IL-17 to prolong the t1/2 of unstable mRNAs provides a mechanistic basis for the synergistic response induced by TNF-α and IL-17, because TNF-α serves as a strong stimulus for NF-κB-dependent transcription, but is unable to prolong the t1/2 of the mRNA (14, 20, 25).

Although the signal transduction pathway(s) initiated in response to IL-17 remains largely undefined, several reports implicate participation of adaptor proteins, including NF-κB activator 1 (Act1) and TNFR-associated factor (TRAF)6 (21, 22, 26). IL-17-induced activation of NF-κB and JNK and modulation of IL-6 expression were shown to be dependent on TRAF6 using TRAF6-deficient mouse embryo fibroblasts (MEFs) (26). Using mice deficient in Act1, this signaling adaptor was found to be required both for response to IL-17 alone and for the more robust responses seen with the combination of IL-17 and TNF-α (21, 22). We subsequently showed that Act1 was both necessary and sufficient for IL-17 to promote stabilization of chemokine mRNA (20).

3 Abbreviations used in this paper: MK, mitogen activated protein kinase activated protein kinase; Act1, NF-κB activator 1; ActD, actomyosin D; dn, dominant negative; dox, doxycycline; Lcn2, lipocalin 2; MEF, mouse embryo fibroblast; tet, tetracycline; TRAF6, TNFR-associated factor 6; UTR, untranslated region.
mRNA stabilization in IL-17-mediated amplification of TNF-α depend upon the action of p38 MAPK or downstream kinases mRNAs. In addition, IL-17-induced mRNA stabilization did not require the action of rDNA3 or expression vectors encoding FLAG-tagged Act1 or TRAF6 along with 2 μg of pTRE2 KCΔ3. After overnight culture, dox was added, and KC and GAPDH mRNA levels were determined, as described in A. C. The autoradiographs from B were quantified and presented graphically as percentage of remaining mRNA vs time. The time at the intercept at 50% remaining RNA was used to determine t1/2. D. HeLa tet-off cells were cotransfected with 4 μg of empty vector (pcDNA3) or expression vectors encoding FLAG-tagged Act1 or TRAF6 along with 2 μg of pTRE2 KCΔ3. After overnight culture, dox was added, and KC and GAPDH mRNA levels were determined, as described in A. C. The autoradiographs from B were quantified and presented graphically as percentage of remaining mRNA vs time. The time at the intercept at 50% remaining RNA was used to determine t1/2. E. HeLa tet-off cells were cotransfected with 4 μg of empty vector (pcDNA3) or expression vectors encoding FLAG-tagged Act1 or TRAF6 along with 2 μg of 5′×κB luciferase reporter and 0.25 μg of pTK renilla luciferase (C). Cell lysates were collected 24 h after transfection, and luciferase activities were determined. Values represent the mean of duplicate samples that have been normalized to Renilla. E. Expression levels of Act1 and TRAF6 were determined by Western blot using anti-FLAG Abs. The data are representative of at least three independent experiments.

In the present report, we have examined the requirement for TRAF6 in IL-17-induced mRNA stabilization. Surprisingly, using multiple experimental approaches, we find that TRAF6 is not required in signaling for stabilization of TNF-α-induced chemokine mRNAs. In addition, IL-17-induced mRNA stabilization did not depend upon the action of p38 MAPK or downstream kinases MK2 and MK3. These results further highlight the importance of mRNA stabilization in IL-17-mediated amplification of TNF-α-stimulated gene expression and identify a TRAF6/p38/MK2/MK3-independent pathway operating downstream of Act1. Because the potency of response to IL-17 is markedly greater when used in combination with TNF-α, this pathway may represent the predominant means through which IL-17 promotes inflammatory gene expression.

Materials and Methods
Reagents
G418, formamide, MOPS, salmon sperm DNA, and diethyl-pyrocarbonate were purchased from Sigma-Aldrich. DMEM, Dulbecco’s PBS, penicillin, and streptomycin were obtained from Cell Culture Services of the Lerner Research Institute. FBS was purchased from BioWhittaker. PolyFect Transfection Reagent was obtained from Qiagen, and Tri-Reagent was purchased from Molecular Research Center. FBS was purchased from BioWhittaker. PolyFect Transfection Reagent was obtained from Qiagen, and Tri-Reagent was purchased from Molecular Research Center. Human and mouse rL-17, rTNF-α, and rIL-1α were purchased from R&D Systems. SB203580 was purchased from Calbiochem. Nylon transfer membrane was purchased from Micron Separations. Luciferase assay buffer and passive lysis buffer were obtained from Promega. PerkinElmer Life Sciences was the source of [α-32P]dCTP and Western Lightning Chemiluminescence Reagent Plus. Protein assay reagents were purchased from Bio-Rad. Anti-FLAG M2 Ab was purchased from Sigma-Aldrich; Ab against GAPDH was purchased from Chemicon International; and anti-mouse IgG HRP-linked Ab was purchased from Amersham.

Preparation of peritoneal macrophages
C57BL/6 mice were purchased from Jackson ImmunoResearch Laboratories and were housed in microisolator cages with autoclaved food and bedding to minimize exposure to viral and microbial pathogens. All procedures were approved by the Institutional Animal Care and Use Committee. Thioglycollate-elicited peritoneal macrophages were prepared, as described previously (17), and cultured overnight in RPMI 1640 medium containing l-glutamine, penicillin, streptomycin, and 5% FBS before treatments, as described in the text.

Plasmids
Plasmids encoding KC cDNA under control of a tetracycline (tet)-regulated promoter (pTRE2 KCΔ3) and the NF-κB reporter 5×κB luciferase have been previously described, as have the plasmids and methods used to prepare radiolabeled CXCL1 (KC), GAPDH, (CXCL2) MIP-2, and IκBc cDNAs (20, 28–30). Expression plasmids encoding epitope (FLAG)-tagged Act1, TRAF6, and dominant-negative (dn) TRAF6 have been previously described (31, 32).

Cell culture and transfection
HeLa tet-off cells were purchased from BD Clontech; cultured in DMEM containing 10% FBS, penicillin, and streptomycin; and kept under selection with G418. For transfection, HeLa tet-off cells were plated in 100-mm dishes and allowed to grow for 24 h to 70% confluency. Transfection was performed using PolyFect Transfection Reagent, according to the manufacturer’s protocol (Qiagen). Wild-type, TRAF6−/−, and Act1−/− MEFs have been previously described and were cultured in DMEM containing 10% FBS, penicillin, and streptomycin (21, 33). MEFs from MK2/MK3 doubly-deficient mice were provided by M. Gaestel (Institute of Biochemistry, Medical School Hannover, Hannover, Germany) (17).

Northern blot, real-time PCR, Western blot, and luciferase assay
Total RNA was isolated using Tri-Reagent, according to the manufacturer’s protocol, and Northern hybridization was performed, as previously described (28). To calculate mRNA t1/2, the autoradiographs were quantified using the NIH Image software. mRNA levels for each gene of interest were normalized to GAPDH mRNA levels and plotted as log of the percentage of remaining mRNA vs time. The best fit to linear decay was determined, and the t1/2 was calculated from the intersection at the point corresponding to 50% residual RNA. Quantitative real-time PCR was performed, as previously described (21). The cDNAs were synthesized with random hexamers using Moloney murine leukemia virus reverse transcriptase (Promega) and real-time PCR conducted using SYBER Green PCR reagent. The data are representative of at least three independent experiments.
Luciferase assays were performed according to the manufacturer’s protocol (Promega). Western blots were conducted, as described previously (35).

Although KC mRNA rapidly decays following addition of dox, transcription will drive strong transcription of the KC transgene, enabling the analysis of mRNA decay in the absence of the requirement for a transcriptional stimulus (28, 37). HeLa cells stably expressing the tet-controlled trans activator are transiently transfected with reporter plasmid (pTRE2 KCΔ3) containing the KC 5′ untranslated region (UTR), coding region, and a portion of the 3′ UTR that confers both instability and stimulus-induced stabilization. In the absence of tet, or its analog doxycycline (dox), the tet repressor will drive strong transcription of the KC transgene, but following addition of dox to the culture medium transcription is abolished and the decay of the mRNA can be followed (Fig. 1A). Although KC mRNA rapidly decays following addition of dox, reflecting the constitutive instability of the mRNA, addition of IL-17 along with dox is able to prolong the t_1/2 of the message.

We have previously shown that overexpression of Act1, the adaptor linking directly to the IL-17R, results in enhanced stability of KC mRNA (20). This finding indicates that signals originating from this adaptor are sufficient to initiate the stabilization response. As a first test of the role of TRAF6 in mRNA stabilization, we determined whether overexpression of this molecule would also be sufficient to engage the mRNA stabilization process. Surprisingly, whereas overexpression of Act1 stimulates KC mRNA stabilization, overexpression of TRAF6 does not alter the t_1/2 of the message (Fig. 1B and C). Although TRAF6 overexpression did not affect KC mRNA stability, it promoted strong activation of NF-κB, as measured by a cotransfected luciferase reporter (Fig. 1D) demonstrating the functional activity of the overexpressed molecule. Western blot analysis indicated that each of the adaptor proteins demonstrating the functional activity of the overexpressed molecule.

**Results**

**Overexpression of Act1, but not TRAF6, mediates stabilization of KC mRNA**

Robust expression of KC mRNA from the endogenous gene requires both transcription via the activation of NF-κB and stabilization of the constitutively unstable mRNA (14, 36). We have used the tet-off system to control transcription of transgenic KC mRNA, enabling the analysis of mRNA decay in the absence of the requirement for a transcriptional stimulus (28, 37). HeLa cells stably expressing the tet-controlled trans activator are transiently transfected with reporter plasmid (pTRE2 KCΔ3) containing the KC 5′ untranslated region (UTR), coding region, and a portion of the 3′ UTR that confers both instability and stimulus-induced stabilization. In the absence of tet, or its analog doxycycline (dox), the tet trans activator will drive strong transcription of the KC transgene, but following addition of dox to the culture medium transcription is abolished and the decay of the mRNA can be followed (Fig. 1A). Although KC mRNA rapidly decays following addition of dox, reflecting the constitutive instability of the mRNA, addition of IL-17 along with dox is able to prolong the t_1/2 of the message.

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TRAF6 is not required for IL-17-induced mRNA stabilization

Two experimental strategies were used to determine whether TRAF6 was required for IL-17-induced mRNA stabilization. First, we tested the ability of a dn version of TRAF6 (31) to interfere

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**FIGURE 2.** The dnTRAF6 does not block IL-17-induced mRNA stabilization. A, HeLa tet-off cells were transfected with 2 μg of pTRE2 KCΔ3, 2 μg of 5×κB luciferase reporter, 1 μg of pcDNA3, and 1 μg of dnTRAF6 or empty vector (pcDNA3). One set of cultures was treated with dox alone or in combination with IL-17 (25 ng/ml) for the indicated times. Total RNA was collected, and KC and GAPDH mRNAs were determined by Northern hybridization. NT, Represents untreated cells and serves as the zero time point for all treatment conditions. B, The autoradiographs from four separate experiments similar to A were quantified, as described in the legend to Fig. 1, and t_1/2 for each condition were determined. The mean ± 1 SD for each condition was determined and presented. C, A second set of cultures transfected as in A was treated for 6 h with IL-1α (10 ng/ml) or IL-17 (100 ng/ml), or left untreated. Cell lysates were prepared, and luciferase activity was determined. Values represent the mean of duplicate samples.

**FIGURE 3.** TRAF6 is not required for IL-17-induced mRNA stabilization. A, Wild-type and TRAF6-deficient MEFs were treated for 1 h with TNF-α (10 ng/ml). Fresh medium was then added containing ActD (5 μg/ml) alone or with IL-17 (10 ng/ml). Total RNA was collected at the indicated times, and KC and GAPDH levels were determined by Northern hybridization. NT, Represents cells treated with TNF-α for 1 h and serves as the zero time point for all treatment conditions. B, The autoradiographs in A were quantified, as described in the legend to Fig. 1, and the percentage of remaining KC mRNA relative to GAPDH is shown for each experimental condition. C, Data from three independent experiments were analyzed for KC mRNA t_1/2, and the mean ± 1 SD is shown.
with IL-17-induced KC mRNA stabilization. TRAF6 has previously been linked to NF-κB activation in response to both IL-17 and IL-1α (26, 33). IL-17 treatment stabilized KC mRNA in HeLa tet-off cells transfected with pTRE2 KC/Δ3 in the presence of either empty vector or an expression vector encoding dnTRAF6 (Fig. 2A). A similar result was obtained in four separate experiments, which collectively show that IL-17 prolongs the t1/2 of KC mRNA by a mean value of more than 2-fold in the presence or absence of dnTRAF6 (Fig. 2B). Under the same conditions, even high doses of IL-17 (100 ng/ml) serve as a very weak stimulus for NF-κB activation compared with lower doses of IL-1α (10 ng/ml) (Fig. 2C). Consistent with prior reports, however, NF-κB activation following IL-17 or IL-1α treatment was diminished in cells expressing the dnTRAF6.

As a second test for the requirement of TRAF6, we compared the ability of IL-17 to induce mRNA stabilization in wild-type and TRAF6-deficient MEFs. We have previously shown that KC mRNA transcribed in response to TNF-α is highly unstable and decays rapidly following the addition of actinomycin D (ActD), whereas the addition of IL-17 along with ActD is able to prolong the t1/2 of the TNF-α-induced KC mRNA (20). The ability of IL-17 treatment to prolong the t1/2 of KC mRNA remains intact in the TRAF6-deficient cells (Fig. 3, A and B). Similar results were obtained in two additional experiments, as demonstrated by comparison of mean t1/2 values in the two different cell populations either with or without IL-17 treatment (Fig. 3C). It is noteworthy that KC mRNA instability was reduced in the TRAF6−/− cells even in the absence of IL-17, but treatment with IL-17 resulted in a quantitatively comparable increase in t1/2 in both wild-type and TRAF6-deficient cells. These results support the conclusion that TRAF6 is not required for IL-17-induced chemokine mRNA stabilization.

**IL-17-induced mRNA stabilization does not require p38 or MK2/MK3**

p38 MAPK and its downstream kinase MK2 have been shown to play a critical role in stimulus-induced stabilization of proinflammatory mRNAs in a variety of settings (11, 12, 15–17), including IL-17. TRAF6 is known to link to p38 activation, and thus, the finding that TRAF6 was not required for IL-17-induced mRNA stabilization raised questions regarding the role of the p38 MAPK cascade in the IL-17 response. As a first test to assess the role of p38, we determined the effect of the p38 MAPK inhibitor SB203580 on IL-17-induced KC mRNA stabilization. The presence of the inhibitor did not affect the ability of IL-17 to stabilize KC mRNA in HeLa tet-off cells (Fig. 4A). In contrast, as we have previously reported (38, 39), the ability of LPS to stabilize KC mRNA in mouse macrophages is highly sensitive to the inhibitory effects of SB203580 (Fig. 4B).

To further assess the role of the p38/MK2 pathway in IL-17-induced stabilization, we tested the ability of IL-17 to prolong KC mRNA t1/2 in MEFs from MK2/MK3-deficient mice. Wild-type and MK2/MK3-deficient MEFs were stimulated with TNF-α alone or with IL-17 for 1 h, followed by the addition of ActD. The TNF-α-induced KC mRNA was unstable in both cell populations, although the magnitude of response to TNF-α was markedly reduced in MK2/3−/− cells (Fig. 4C). However, in both cell populations, the t1/2 of KC message induced by the combination of TNF-α and IL-17 was significantly and comparably prolonged in comparison with that seen in cells stimulated by TNF-α alone (Fig. 4C). Comparable results were obtained in three similar experiments, as demonstrated by comparison of the mean t1/2 values (Fig. 4C). These findings provide further support for the conclusion that IL-17-induced mRNA stabilization does not require the p38 MAPK pathway, including MK2 and MK3.

**TRAF6 is not required for IL-17-mediated amplification of TNF-α-induced gene expression**

Prior reports demonstrate that the modest ability of IL-17 alone to induce activation of NF-κB and the transcription of select target genes is dependent upon TRAF6 (22, 26). The present study, however, shows that IL-17-induced stabilization of chemokine mRNA
transcribed in response to TNF-α is TRAF6-independent. The requirement for TRAF6 in NF-κB activation, but not mRNA stabilization, enables a comparison of the relative contribution of the two mechanisms by examining a broader spectrum of IL-17-induced genes in TRAF6-deficient cells. Using quantitative real-time PCR, we determined the expression of a set of five genes that have been previously identified as sensitive to IL-17-mediated amplification based upon oligonucleotide microarray analysis of gene expression in fibroblasts stimulated with TNF-α alone or with IL-17 (5, 20). Wild-type and TRAF6-deficient MEFs were stimulated for 3 or 9 h with TNF-α alone or in combination with IL-17. For comparison, the same treatments were also performed on Act1−/− and Act1−/− MEFs because IL-17-induced gene expression has previously been reported to be fully dependent on Act1 (21). In four of the five genes studied, the response to IL-17 in the TRAF6-deficient MEFs was comparable to that seen in wild-type cells (Fig. 5). A single dramatic exception was observed for behavior of the gene encoding cxcl5. cxcl5 mRNA levels, however, although modestly induced in response to TNF-α alone in wild-type cells, were below the level of detection in all samples from TNF-α-treated TRAF6-deficient cells. Hence, there appears to be a deficiency in TNF-α-stimulated cxcl5 gene transcription in the TRAF6-deficient cell population rather than a requirement for TRAF6 in IL-17-mediated enhancement of cxcl5 expression. Although IL-6 mRNA levels are reproducibly higher in the TRAF6−/− cells than in wild-type cells, it is clear that in both cell populations IL-17 is able to amplify the TNF-α-induced response. These results are in dramatic contrast to the behavior of the same genes in Act1-deficient MEFs, in which the response to IL-17 is completely lost. The magnitude of responses in the different MEF cell populations (wild type, TRAF6−/−, Act1−/−, or Act1−/−) shows variability, but the effects of IL-17 are qualitatively similar for the TRAF6 pair and qualitatively different for the Act1 pair.

To further assess the IL-17-mediated amplification of TNF-α-stimulated gene expression in the presence or absence of TRAF6, we compared the t1/2 of two additional mRNAs (MIP-2 and IκB). As was seen for KC, the t1/2 in TNF-α-stimulated cells are short
and markedly prolonged for both mRNAs in the presence of IL-17 (Fig. 6). As seen with KC mRNA, IL-17 stabilizes the MIP-2 message effectively even when added in the presence of ActD in both cell populations. For IκBζ mRNA, we compared decay following stimulation with TNF-α either alone or in combination with IL-17 because starting mRNA levels were significantly higher. Although the levels of IκBζ mRNA in cells treated with TNF-α alone are low and our measure of t1/2 may be an underestimate, the results demonstrate substantive stabilization of the IκBζ mRNA in the presence of IL-17, and this response is comparable in both cell populations.

Discussion
IL-17-induced inflammatory gene expression has been linked with both transcriptional and posttranscriptional mechanisms, although the signaling pathways that couple IL-17R engagement with such responses remain poorly understood. In the present study, we undertook to determine whether signaling events linked with the activation of NF-κB via TRAF6 were also requisite for the prolongation of specific mRNA t1/2. The data presented establish that the ability of IL-17 to stabilize a selection of TNF-α-induced mRNAs does not require the participation of TRAF6. This conclusion is based upon three complementary experimental observations. First, although overexpression of TRAF6 can stimulate the activation of NF-κB, it cannot stimulate mRNA stabilization. Second, a dn version of TRAF6 can effectively block activation of NF-κB, but does not block mRNA stabilization in response to stimulation with IL-17. Finally, IL-17 remains fully capable to stabilize TNF-α-induced KC, MIP-2, and IκBζ mRNAs in TRAF6-deficient cells. Hence, the current findings identify a pathway distinct from NF-κB activation that leads from the IL-17R to enhanced stability of specific mRNAs.

TRAF6 has been shown to be involved in TLR-mediated activation of MAPKs, including p38 through the action of TAK1 (33, 40, 41). p38 MAPK and its downstream targets MK2 and MK3 have well-established roles in stimulus-induced mRNA stabilization and have been reported to participate in such responses to IL-17 (11, 12, 15–17, 23, 42). The observation that TRAF6, however, was not necessary for the stabilization of KC and MIP-2 mRNAs in response to IL-17 in the present studies raised the possibility that this response did not involve the action of p38. More direct tests of this hypothesis using a pharmacologic inhibitor of p38 or cells genetically deficient in MK2 and MK3 further support the conclusion that IL-17-induced stabilization does not require the p38/MK2 pathway. Thus, although KC mRNA stabilization in response to TLR ligands requires p38 in mouse macrophages (38, 39), the response to IL-17 in the nonmyeloid cell populations studied in this work is both p38 and MK2/3 independent. The differences between the current findings and prior work both in TLR- and IL-17-induced mRNA stabilization are likely to reflect distinct mechanisms that may function differentially depending upon the cell type. In this regard, we have recently reported that IL-1α-induced stabilization of KC and MIP-2 mRNA is also independent of both TRAF6 and the p38/MK2 pathway in these same cell populations (35). Collectively, these findings suggest a common pathway downstream of Act1 and IL-1R-associated kinase 1 that mediates mRNA stabilization in nonmyeloid cells in response to IL-17 and IL-1α, respectively.

The TRAF6/p38 pathway is believed to promote the stabilization of AU-rich mRNAs by targeting tristetraprolin, an RNA-binding protein that promotes rapid decay of mRNAs containing multiple copies of the pentameric sequence AUUUA (43–45). Indeed, KC mRNA decay (and LPS-mediated stabilization) in peritoneal macrophages depends upon this mechanism (38). It is noteworthy, however, that IL-17- and IL-1α-mediated stabilization of KC in nonmyeloid cells does not require tristetraprolin and depends upon a tristetraprolin-insensitive sequence in the 3′ UTR that contains no AUUUA motifs (S. Datta, M. Novotny, P. Pavicic, C. Zhao, T. Herjan, J. Hartupee, and T. Hamilton, manuscript in preparation). This different sequence requirement provides an additional criterion distinguishing this IL-17 signaling pathway.

The variable dependence of responses to IL-17 on TRAF6 appears to relate directly to the mechanism involved; activation of NF-κB requires TRAF6, whereas stabilization of mRNA does not. Thus, the assessment of several well-recognized IL-17-inducible mRNAs for dependence on TRAF6 provides some measure of the relative contribution of the two mechanistic pathways. It should be noted, however, that our study is limited to examining the response to IL-17 in the context of costimulation with TNF-α. Because TNF-α is a potent transcriptional activator, but a poor stimulus for mRNA stabilization, this feature of our experimental design focuses attention on the mRNA stabilization pathway by overriding the requirement for transcriptional stimulation. The results demonstrate that many of the IL-17-mediated responses are TRAF6 independent and are, therefore, likely to have some dependence upon the mRNA stabilization pathway. It is, however, worth considering whether transcriptional versus posttranscriptional mechanisms are involved in controlling the expression of each of these mRNAs. Of the five TRAF6-independent responses demonstrated in Figs. 5 and 6, three clearly appear to involve mRNA stabilization, as follows: KC (CXCL1), MIP-2, and IκBζ (20, 24). IL-6 is known to be unstable and sensitive to mRNA stabilization (16), but the IL-17 response may also use an indirect transcriptional mechanism via the induction of C/EBPβ and C/EBPδ (46, 47). Lipocalin 2 (Lcn2) has been shown to be inducible by the combination of TNF-α and IL-17 (5) and involves transcription, but not mRNA stabilization (48). Interestingly, several recent reports demonstrate convincingly that Lcn2 expression is dependent upon both NF-κB and the expression of IκBζ (49, 50). This latter finding, along with prior work (24) and that reported in this study, suggests that transcriptional induction of the lcn2 gene by IL-17 represents a downstream consequence of the stabilization of IκBζ. Thus, it appears that multiple mechanisms are contributing to the pattern of enhanced gene expression in IL-17-stimulated cells through both direct and indirect means.

Previous reports showing that the response to IL-17 is dependent upon TRAF6 were focused on the action of IL-17 alone (18, 26), whereas those reported in this study reflect the IL-17-mediated amplification of response to TNF-α. It is noteworthy that the former responses are, in fact, relatively modest in magnitude, whereas the combination of TNF-α and IL-17 often induces robust increases in gene expression that are much greater than the response to either cytokine alone (see Fig. 5) (5, 20, 21). It seems unlikely that IL-17 will be encountered alone at inflammatory sites in vivo, and hence, the cooperative effects seen with TNF-α are more likely representative of physiologic responses. The finding that most of the cooperative responses that we assessed were TRAF6-independent suggests that the mRNA stabilization pathway is at least one major mechanism by which IL-17 signals the amplification of inflammatory gene expression. Because this mechanism appears to function in a cell type-dependent fashion, there may be circumstances in vivo in which TRAF6-dependent, noncooperative IL-17-mediated responses occur, and this remains to be fully explored.

Disclosures
The authors have no financial conflict of interest.


