Tumor Necrosis Factor and Transforming Growth Factor β Regulate Clock Genes by Controlling the Expression of the Cold Inducible RNA-binding Protein (CIRBP)*‡

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Background: CIRBP facilitates expression of clock genes by stabilizing their transcripts.

Results: Down-regulation of expression of clock genes by TNF and TGFβ is mediated by inhibition of CIRBP production.

Conclusion: TNF and TGFβ impair the expression of Cirbp and thereby influence circadian gene expression.

Significance: A novel regulatory pathway that links immune activation with circadian gene expression is identified.

The circadian clock drives the rhythmic expression of a broad array of genes that orchestrate metabolism, sleep wake behavior, and the immune response. Clock genes are transcriptional regulators engaged in the generation of circadian rhythms. The cold inducible RNA-binding protein (CIRBP) guarantees high amplitude expression of clock. The cytokines TNF and TGFβ impair the expression of clock genes, namely the period genes and the proline- and acidic amino acid-rich basic leucine zipper (PAR-bZip) clock-controlled genes. Here, we show that TNF and TGFβ impair the expression of Cirbp in fibroblasts and neuronal cells. IL-1β, IL-6, IFNα, and IFNγ do not exert such effects. Depletion of Cirbp is found to increase the susceptibility of cells to the TNF-mediated inhibition of high amplitude expression of clock genes and modulates the TNF-induced cytokine response. Our findings reveal a new mechanism of cytokine-regulated expression of clock genes.

Clock genes mediate circadian rhythmicity and thereby control mammalian metabolism and sleep-wake behavior (1, 2). The heterodimerized transcription factor CLOCK-BMAL1 (brain and muscle ARNT-like protein) activates transcription by binding to E-box motives of period (Per), cryptochrome (Cry), and the following members of the PAR-bZip family of transcription factor genes: the D-site albumin promoter-binding protein (Dhp), tyrosph morph embryonic factor (Tef), and hepatic leukemia factor (Hlf) (3, 4). PER and CRY proteins inhibit the function of CLOCK-BMAL1 complexes, thereby inhibiting their own gene expression. This feedback-loop mechanism generates circadian oscillations of Per (Per1, Per2, and Per3) and Cry (Cry1 and Cry2) expression. The same positive and negative regulatory components also govern the rhythmic expression of the nuclear orphan receptor Rev-Erbα, which in turn represses the transcription of Bmal1 through direct binding to a REV-ERBα response element in the Bmal1 promoter. Thereby, REV-ERBα interconnects the cyclic expression of positive- and negative-loop members. These pathways drive the 24-h rhythms in physiology, behavior, and the immune response to microbes. The cross-talk between the clock system and the immune system has been addressed in several recent studies. The extent of secretion of TNF and IL-6 by LPS-stimulated macrophages follows a circadian rhythm (5). The macrophage response to LPS is decreased in mice with an inactivation of the Clock gene (6). Compared with bone marrow-derived macrophages obtained from wild type (WT) mice, the expression of cytokines including IL-1β, IL-6, TNF, IFNα, and IFNγ was significantly lower in clock mutant mice. Inactivation of the clock genes Cry1 and Cry2 leads to increased production of TNF, IL-1β, and IL-6 in experimental arthritis (7). Per2 gene knock-out mice show a defective natural killer cell function with decreased IFNγ and IL-1β serum concentrations following LPS challenge (8). A nonfunctional Per2 gene also affects TLR9 expression and thereby the vaccine response when using TLR9 ligands as adjuvant (9). Taken collectively, the data show that the circadian clock controls immune responsiveness.

A compelling recent study shed light on post-transcriptional regulation required for circadian orchestration of the clock network. Loss-of-function experiments indicate that the cold-inducible RNA-binding protein (CIRBP) is required for high amplitude circadian gene expression (10). The RNA-binding protein CIRBP belongs to the highly conserved glycine-rich RNA-binding protein family and is thought to modulate gene expression by binding to transcripts in the 5′-untranslated region (UTR) or 3′-UTR of mRNA and thereby affect
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| Table 1 |
| --- |
| **Sequences for custom-made Cirbp TaqMan assays** |
| Primer forward 5’—3’: CAGATCTTCGAAAGTGGTGTT | Primer reverse 5’—3’: CGCGCGTGCGAACCCCTGAT |
| Probe: GGCCTGGGTGTTGCACCCCTTT |

Treatment of Cells with siRNA against Cirbp—NIH-3T3 cells were transfected with DharmaFECT 1 transfection reagent (Dharmacon) and 25 nm siRNA (Dharmacon) against Cirbp. Gapdh and no target siRNA were used as positive and negative controls, respectively. After transfection cells were incubated for 36 h for RNA analysis and 48 h for protein analysis.

RNA Isolation and Gene Expression Analysis—Whole cell RNA from cultured cells was extracted using NucleoSpin-RNA II kit (Macherey-Nagal). Subsequently, RNA was reverse-transcribed using random hexamers (Fermentas) and M-MuLV reverse transcriptase (Roche Applied Science). One µg of total RNA was amplified in an ABI 7900 HT detection system (Applied Biosystems) using the TaqMan Universal PCR Master Mix (Applied Biosystems). For sequences for custom made Cirbp TaqMan assays, see Table 1. The relative levels of each RNA were calculated by the 2-ΔΔCT method; eEF1a1 and GAPDH were used as housekeeping genes. Each CT value used for these calculations is the mean of triplicates of the same reaction. Relative RNA levels are expressed as ×-fold variations compared with Zeitgeber time = 0 (time course experiments) or as percentages of the average control groups. RNA stability measurements were performed by using 5 µg/ml the transcription blocker actinomycin D (ActD) (Sigma). The compound was added to the cultures 2 h after starting cytokine treatment (see above). Cells were harvested at the indicated time points, and the amount of extracted RNA was analyzed with qPCR after reverse transcription.

Cytokine and Chemokine Array—Expression of cytokine and chemokine genes was tested by using a StellARray assay (Bar Har Biotechnology) for the expression of immunology-related genes in mice. The array was performed accordingly to the manufacturer’s instructions.

Analysis of CIRP by Western Blotting—Cells were lysed with the immunoprecipitation lysis buffer (Pierce) as described in the protocol. Whole protein extracts (40 µg) in LDS sample buffer (Invitrogen) and DT T were applied on a NuPAGE 12% Bis-Tris gel (Invitrogen). The proteins were separated at constant 150 V in a MES SDS running buffer (Invitrogen). Subsequently blotting on a PVDF membrane was performed in a full wet tank blot. Membranes were incubated with a CIRBP rabbit polyclonal antibody recognizing the C terminus of mouse CIRBP (27). As secondary antibody an HRP-conjugated goat anti-rabbit (ab79051; Abcam) was used and incubated for 1 h. As a loading control an antibody to the mouse nuclear matrix protein p84 was used (1 h, ab487). The secondary antibody used with the anti-p84 was a goat to mouse HRP (1 h, ab97023). For densitometric measurements the Western blots were analyzed with the ImageJ software. The relative values were normalized to the loading controls.

Cirbp Complementation and Overexpression—Cirbp−/− MEFs (24) were grown as described above and transiently transfected with a Cirbp-overexpressing plasmid. Cells (8 × 10⁵

EXPERIMENTAL PROCEDURES

Treatment of Cells with Cytokines and Blockers—NIH-3T3 fibroblasts, HT22 neuronal cells, C57BL/6 WT mouse embryonic fibroblasts (MEFs) and Cirbp−/− MEFs were grown in DMEM high glucose (4.5 g/liter) medium supplemented with 10% FCS and penicillin/streptomycin (1 ×). Cells were kept at 37 °C and 5% CO₂. For RT-qPCR analysis, cells were seed in triplicate in 12-well plates in a density of 1 × 10⁵/well. For Western blotting, cells were seed in T25 flasks in a density of 1 × 10⁶/flask. Two days after seeding, cells were synchronized by serum deprivation (1% FCS) for 1 h and then treated with murine cytokines. TNF, TGFβ, IL-1β, IL-6, and IFNγ were obtained from Peprotech and IFNα from Miltenyi Biotech.

the rate of translation initiation and stability of the transcript (10–12). Cirbp is constitutively expressed mainly in testis, lung, heart, kidney, hippocampus, and cerebral cortex of the adult rat (13). The expression of Cirbp mRNA and protein is up-regulated by cellular stress, including mild cold stress (32 °C) and hypoxic conditions (14). In hypoxia, CIRBP is released from lysosomes of macrophages and is found in the serum of patients with shock due to hemorrhages or trauma (15). Surprisingly, CIRBP is found to bind to the LPS receptor TLR4 and thereby activates the release of proinflammatory cytokines including TNF and IL-6. These data point to a pivotal role of CIRBP in the innate immune response.

CIRBP may serve as a chaperone that assists in the folding/unfolding, assembly/disassembly, and transport of various proteins (11, 16). High amplitude expression of transcripts associated with circadian clock function has been found to depend on CIRBP. Among the transcripts interacting with CIRBP are the mRNAs encoding Sirtuin-1 (SIRT1), PER2, PER3, and DBP, which were decreased in Cirbp-depleted cells (10, 12). The phenotype caused by Cirbp deficiency resembles most closely that observed in cells depleted of Clock. In both Cirbp- and Clock-depleted cells, amounts of Bmal1 mRNA were increased, whereas Per3, Per2, and Dbp mRNA were decreased (10). This phenotype is also reminiscent of the pattern of clock gene expression described in TNF- and TGFβ-treated cells. These cytokines have been shown recently to lead to inhibition of expression of Per1, Per2, and Per3 and of the PAR-bZip clock-controlled genes Dpb, Tef, and Hlf and to up-regulation of Bmal1 (17–19). Dysregulation of clock gene expression by cytokines has been described in fibroblasts, human pancreas cancer cells, and in leukocytes in vitro, and in the liver of mice treated with TNF or LPS (20–23).

The molecular mechanisms, which lead to cytokine-induced dysregulation of expression of clock genes, are not yet clear. Using reporter genes, TNF is found to inhibit CLOCK-BMAL1-induced activation of E-box regulatory elements in clock gene promoters (17). Because the RNA-binding protein CIRBP was previously described to be required for high amplitude circadian gene expression, we explored the hypothesis that cytokines may interfere with Cirbp expression. Our data show that TNF and TGFβ inhibit the production of Cirbp and thereby enhance the suppressive effect of the cytokines on clock genes. Moreover, down-regulation of Cirbp expression is found to have an impact on basal and TNF-stimulated cytokine expression.

**TREATMENT OF CELLS WITH SI-RNA AGAINST CIRBP**—NIH-3T3 cells were transfected with DharmaFECT 1 transfection reagent (Dharmacon) and 25 nm siRNA (Dharmacon) against Cirbp. Gapdh and no target siRNA were used as positive and negative controls, respectively. After transfection cells were incubated for 36 h for RNA analysis and 48 h for protein analysis.

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**CYTOKINE AND CHEMOKINE ARRAY**—Expression of cytokine and chemokine genes was tested by using a StellARray assay (Bar Har Biotechnology) for the expression of immunology-related genes in mice. The array was performed accordingly to the manufacturer’s instructions.

**ANALYSIS OF CIRP BY WESTERN BLOTTING**—Cells were lysed with the immunoprecipitation lysis buffer (Pierce) as described in the protocol. Whole protein extracts (40 µg) in LDS sample buffer (Invitrogen) and DT were applied on a NuPAGE 12% Bis-Tris gel (Invitrogen). The proteins were separated at constant 150 V in a MES SDS running buffer (Invitrogen). Subsequently blotting on a PVDF membrane was performed in a full wet tank blot. Membranes were incubated with a CIRBP rabbit polyclonal antibody recognizing the C terminus of mouse CIRBP (27). As secondary antibody an HRP-conjugated goat anti-rabbit (ab79051; Abcam) was used and incubated for 1 h. As a loading control an antibody to the mouse nuclear matrix protein p84 was used (1 h, ab487). The secondary antibody used with the anti-p84 was a goat to mouse HRP (1 h, ab97023). For densitometric measurements the Western blots were analyzed with the ImageJ software. The relative values were normalized to the loading controls.

**CIRBP COMPLEMENTATION AND OVEREXPRESSION**—Cirbp−/− MEFs (24) were grown as described above and transiently transfected with a Cirbp-overexpressing plasmid. Cells (8 × 10⁵
per T-25 flask) were seeded and transfected with 8.8 μg of a pCMV6::Cirbp expression vector (Origene) and FuGENE HD transfection reagent (Promega). 48 h later cells were treated with TNF (10 ng/ml) or left untreated as control. Using the same procedure, WT MEFs were transfected with the pCMV6::Cirbp plasmid to generate a stable Cirbp-overexpressing cell line.

Statistics—Graphs are plotted as a mean of triplicates ± S.E. Student’s t test was performed with PRISM software.

RESULTS

TNF and TGFβ Inhibit the Expression of Cirbp—The effect of cytokines on Cirbp expression was assessed in NIH-3T3 fibroblasts and HT22 neuronal cells. The individual cytokines were tested in concentrations, which have been described to mediate maximal bioactivity in their standard assays including studies on clock gene expression (17). In NIH-3T3 cells TNF decreases Cirbp mRNA expression by 50% (Fig. 1A). A pronounced reduction of Cirbp mRNA was also observed with TGFβ and to a lesser extent with IL-1β (inhibition 22%). Neither IL-6 nor IFNγ affected Cirbp mRNA expression. The inhibitory effect of IFNα was significant, the extent of inhibition, however, being only 15%. Down-regulation of Cirbp mRNA was also seen when treating HT22 hippocampal neurons with TNF and TGFβ, maximal inhibition being 37 and 22% respectively (Fig. 1B). In HT22 cells IL-1β, IL-6, IFNα, and IFNγ failed to alter Cirbp expression.

To assess the relationship between the expression of Cirbp and clock genes, we analyzed the expression level of Dpb mRNA. Confirming recent studies TNF and TGFβ inhibit Dpb mRNA expression in NIH-3T3 cells (Fig. 1C). Although the effect of IL-1β was much less pronounced, no effects on Dpb expression were seen when treating the cells with IL-6, IFNα, or IFNγ. When treating NIH-3T3 cells with both TNF and TGFβ, the effect of low dose TNF on Dpb mRNA expression was increased significantly by TGFβ. When testing HT22 cells, Dpb mRNA expression was found to be profoundly inhibited by TNF and TGFβ (Fig. 1D). Whereas Cirbp mRNA expression in neuronal cells treated with IL-1β, IL-6, IFNα, and IFNγ was not altered, the cytokines decreased Dpb mRNA expression, the percentages of inhibition being 25, 24, 37, and 32%, respectively (Fig. 1D). Taken collectively, the most efficient interference with the expression of both Cirbp and Dpb mRNA in NIH-3T3 as well as in HT22 cells is seen with TNF and TGFβ. However, the concomitant treatment of the cells with both cytokines did not show additive effects of TGFβ on the TNF-induced inhibition of Cirbp expression (Fig. 2).

Because Cirbp appears to be regulated at two levels, the first at the transcriptional level and the second at the translational...

FIGURE 1. TNF and TGFβ inhibit the expression of Cirbp (A and B) and of Dpb (C and D) mRNA in NIH-3T3 (A and C) and HT22 cells (B and D) exposed for 4 h to cytokines (gray bars) (untreated control, white bars). Data of RT-qPCR assays of Cirbp and Dpb expression show the mean ± S.E. (error bars) of triplicates from one representative experiment of three; independent t test, *, p < 0.05; **, p < 0.01; ***, p < 0.001.
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was significantly lower, the extent of decreased expression being comparable at all time points tested. At the protein level TNF inhibited CIRBP in both HT22 and NIH-3T3 cells treated for 8 h and 24 h with the cytokine (Fig. 4B). The effect of TGFB was transient. The cytokine failed to inhibit CIRBP expression in 3T3 cells exposed for time periods, which exceed 4 h. In HT22 cells TGFB was found to have effects at 8 h, but not at 24 h. Taken collectively, the most uniform effects were observed with TNF which inhibited the expression of CIRBP in both fibroblasts and neuronal cells over a prolonged time period.

Mort et al. (10) observed rhythmic Cirbp expression in NIH-3T3 cells only upon exposure to temperature rhythms. In the aforementioned study synchronization of the cultured cells was performed by serum shock rather than serum deprivation, the method being used here. Indeed, when adapting our method and adding 50% horse serum Cirbp mRNA did not show a rhythmic expression. However, TNF also decreased Cirbp expression in this culture condition.

CIRBP belongs to the family of RNA-binding proteins and thereby may modulate mRNA stability. We examined whether treatment of cells with TNF alters the decay of Dbp mRNA after adding the transcription blocker ActD. A 2-h pretreatment with TNF before adding ActD did not alter the decline of Dbp mRNA over time compared with untreated cells (Fig. 4C). Moreover, the decline of Dbp mRNA was not different in Cirbp−/− MEFs compared with WT MEFs. These data do not support the hypothesis that diminution of Cirbp by TNF destabilizes Dbp mRNA and thereby prevents from its high amplitude expression. When investigating the effect of TNF on the stability of Cirbp mRNA following ActD treatment, the cytokine was not found to influence the decline of the Cirbp mRNA (Fig. 4D).

The Cytokine-induced Inhibition of the Production of CIRBP Correlates with Their Effects on Clock Gene Expression—A relationship of the effects of TNF on the expression of Cirbp and Dbp is supported when analyzing the kinetics of the expression of the respective genes. Whereas in NIH-3T3 cells Cirbp mRNA and Dbp mRNA were not altered by treatment of the cells with TNF for 30 min or 60 min, a robust inhibition of the expression of both genes was seen at 120 min and 240 min, respectively (Fig. 5, A and B).

Cirbp Silencing with siRNA Enhances TNF- and TGFB-induced Suppression of Dbp mRNA—Because CIRBP enables high amplitude expression of clock genes including Dbp (10) and because, as shown here, TNF and TGFB suppress both Cirbp and Dbp expression, we determined whether the effect of TNF and TGFB on Dbp is mediated through CIRBP. This hypothesis was tested by suppressing Cirbp expression by siRNA. NIH-3T3 cells were transfected for 36 h with siRNA against Cirbp and siNoTarget control. Thereafter, cells were treated with TNF, which was added in a high or low dose concentration. TNF added in a high dose (10 ng/ml) reduced both Cirbp and Dhb mRNA (Fig. 6, A and B). The expression of Cirbp and of Dbp was less affected when treating MEFs with a low TNF dose. However, the depletion of Cirbp by siRNA made the cells become significantly more responsive to the effect of low dose TNF to inhibit Dbp expression (Fig. 6B). Whereas in the
absence of Cirbp depletion the TNF-mediated inhibition of Dbp expression was 35%, the respective value in cells treated with siRNA against Cirbp was 69% (p < 0.01). Treatment of Cirbp-depleted and nondepleted cells with the high dose of TNF was followed by an inhibition of Dbp expression of 91 and 89%, respectively. Analogous experiments with TGFβ also show that the inhibitory effect of TGFβ on Dbp mRNA expression was more pronounced in cells treated with siRNA against Cirbp compared with control cells (37% versus 46%). However, the difference did not reach statistical significance (Fig. 6, C and D). Western blot analysis confirmed the knockdown of Cirbp by siRNA, the effect being enhanced by high dose TNF (Fig. 7).

**Cells with an Inactivation of the Cirbp Gene Respond to TNF with Increased Inhibition of Clock Genes**—Because we found that siRNA against Cirbp sensitizes the cells to the effect of TNF to suppress Dbp mRNA, we analyzed the expression of clock genes in (MEFs), which were established from mice with an inactivation of the Cirbp gene (Cirbp−/− MEFs) (27). Compared with WT MEFs, the effect of TNF to lower the expression of clock genes was found to be more pronounced in Cirbp−/− MEFs (Fig. 8). Upon treatment with TNF the expression of Per3 and the PAR-bZip clock-controlled genes Dbp, Hlf, and Tef was significantly lower in Cirbp−/− MEFs than in TNF-treated control MEFs.

**Cytokine Effects on the Expression of Dbp in Cirbp-overexpressing Cells**—To disclose Cirbp-independent effects in selected clones in Cirbp−/− MEFs, the cells were transfected with a pCMV6::Cirbp expression vector. Overexpression of Cirbp in Cirbp−/− MEFs was found to render the cells less responsive to the inhibitory effect of TNF on Dbp expression (Fig. 8). Next we studied the effect of overexpression of Cirbp in WT MEFs. Whereas WT MEFs treated with TNF (Fig. 9, A and
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FIGURE 6. The expression of Cirbp and Dbp mRNA was tested in Cirbp-depleted cells that were treated with TNF (A and B) and TGFβ (C and D). The data show the effect of cytokines and of siRNA against Cirbp and Dbp that were added to the cultures alone or in combination. Error bars, S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 7. Control Western blot to show the effective repression of CIRBP by down-regulating Cirbp with siRNA. p84 was used as a loading control; the numbers show the ratio from the protein of interest to the loading control.

B) or TGFβ (Fig. 9, C and D) showed a reduction of the expression of Cirbp and Dbp of approximately 50% compared with the untreated cells, overexpression of Cirbp led not only to a higher expression of Dbp but also made the cells unresponsive to the effect of the two cytokines to inhibit the expression of Dbp (Fig. 9, A–D).

Cirbp-depleted Cells Show an Augmented Cytokine Response to TNF Treatment—Next, we addressed the influence of TNF-induced inhibition of Cirbp production on genes that are not part of the molecular clock. Because some cytokines including TNF, IL-1β, CCL2, and CXCL12 as well as cytokine receptors, namely CXCR4 and CX3CR1, have been reported to display circadian rhythms (28), we investigated the influence of Cirbp depletion on the TNF-induced modulation of cytokine and cytokine receptor genes. Using real-time PCR we analyzed the expression of 94 genes in WT and Cirbp−/− MEFS. We found the TNF response in Cirbp−/− MEFS to be augmented in 73 (77%) of the 94 genes tested (supplemental Table 1). A reduced response was seen in 14 (15%) of the 94 genes (supplemental Table 2). The depletion of Cirbp was found to alter the expression of 5 of the 6 genes which have been described to display a rhythmic expression (see above), Compared with WT MEFS, Cirbp−/− MEFS treated with TNF showed an increase of Cxcl12 (49.18-fold), Cxcr4 (11.88-fold), Cx3cr1 (7.41-fold), and Il-1β (2.48-fold) (Fig. 10). Whereas Ccl2 expression was not different, the induction of Tnf by TNF treatment was inhibited (4.11-fold). These data indicate that Cirbp is involved in the modulation of TNF-induced cytokine/cytokine receptor expression.

DISCUSSION

The data presented here show for the first time that TNF and TGFβ modulate clock gene expression by impairing the production of CIRBP. This conclusion is supported by the finding that overexpression of Cirbp protects cells from the inhibitory effects of TNF and TGFβ on the expression of clock genes. This contrasts the findings in Cirbp-depleted cells which show an increased susceptibility to the inhibitory effect of TNF on the expression of Per3 and PAR-bZip clock genes including Dbp, Hlf, and Tef. Because CIRBP is an RNA-binding protein it may play multiple roles in gene expression by regulating the processing and fate of RNA transcripts. The stability of CIRBP target mRNAs has been shown to be increased in testis (29). CIRBP represses the usage of proximal polyadenylation sites by binding to the common 3′-UTRs (12). It has been proposed that down-regulation of CIRBP leads to the preferred use of the proximal polyadenylation sites and shortening of 3′-UTR (12). This effect would increase mRNA stability through reduction of microRNA-mediated repression (30). When assessing the effect of Cirbp depletion we find the decline of Dbp mRNA over time not to be different in Cirbp−/− MEFS or TNF-treated NIH-3T3 cells compared with respective controls. Thus, the effect of TNF to impair CIRBP production and thereby to modulate clock gene expression may involve other mechanisms.

FIGURE 8. MEFS from WT mice (open bars) were compared with Cirbp−/− MEFS (gray bars) for their responsiveness to TNF. Data show the TNF-induced expression of the indicated genes as percentage from their expression in untreated MEFS. In a separate experiment the extent of TNF-induced inhibition of Dbp was assessed in Cirbp−/− MEFS which were complemented after transfection with pCMV6:Cirbp (C) and in mock transfected Cirbp−/− MEFS (−). Error bars, S.E. **, p < 0.01; ***, p < 0.001.
CIRBP has been shown to bind to Clock transcripts and to increase their cytoplasmic accumulation (10). The ectopic expression of Clock mRNA regulates high amplitude expression of genes, which show circadian oscillations including clock genes (10). Besides its effect on Cirbp expression, TNF has been shown to interfere with E-box-mediated transcription induced by CLOCK-BMAL1 (17). Transient transfections of NIH-3T3 cells with luciferase reporter genes and the native 3- or 1.7-kb promoter sequences of mouse Per1 and Per3, respectively, showed the promoter activity to be suppressed by TNF. Moreover, TNF inhibited the expression of E-box reporter constructs of the Dbp gene that was co-transfected with plasmids expressing CLOCK and BMAL1 proteins; the expression of mutated E-box reporter constructs was not altered by TNF (17). Studies using fibroblasts with a deletion of either Per1 and Per2 or Cry1 and Cry2 ruled out the possibility that TNF activates the repressor loop mediated by PER-CRY complexes (31).

TNF and TGFβ effects on fibroblasts are in the center of cytokine-mediated pathologies in various immune-mediated diseases, including rheumatoid arthritis, inflammatory bowel diseases, and pulmonary fibrosis. By their effects on Cirbp expression TNF and TGFβ may not only regulate expression of clock genes, but may also modulate immune-mediated effector functions. Cirbp protects MEFs from TNF-induced apoptosis and neurons from H2O2-mediated cell damage (32, 33). By lowering Cirbp expression, TNF may counteract Cirbp-mediated antiapoptotic effects. As outlined above, the expression of cytokines is influenced by the circadian clock (28). When using cells deficient in Cirbp we found the TNF response to differ significantly. 73 (77%) of 94 cytokine/cytokine receptor genes showed an increased expression. Thus, Cirbp not only plays a role in the TNF-induced dysregulated expression of clock genes, but may also influence the cytokine response in innate immunity and in immune-mediated diseases.

The effect of TNF and TGFβ on CIRBP production and clock gene expression may play a pivotal role in the 24 h-based oscillations of physiological systems. Numerous studies show the circadian clock to participate in the regulation of metabolism (34, 35). Oscillation of clocks in hepatocytes, which are entrained by feeding cycles, modulates gluconeogenesis through interference with glucagon and inhibition of cyclic AMP signaling (36). As outlined in the Introduction, glucose homeostasis is also influenced by clock genes through their action on insulin synthesis and sensitivity (37–39). By modulating the expression of Nocturnin, clock genes regulate lipid absorption in the small intestine and adipogenesis (40). Clock
gene expression has been shown to be coupled to the sleep-wake distribution (2). Several examples point to the existence of a cross-talk between cytokines and metabolism (41), TNF influences metabolic processes including glucose homeostasis, lipid metabolism, and xenobiotic metabolism (42, 43). Furthermore, TNF leads to changes of sleep-wake behavior and body temperature (44, 45). TGFβ modulates key aspects of metabolic processes such as hepatic phospholipid and bile homeostasis. Both the organic solute transporter OSTα and CYP7A1, the rate-limiting enzyme of bile acid synthesis, are regulated by TGFβ (46). Moreover, TGFβ is involved in energy metabolism and energy-sensing pathways (47, 48). The cytokine is induced by an increase of blood lactose and stimulates production of reactive oxygen intermediates by reducing complex IV and mitochondrial respiration. TGFβ enhances fatty acid oxidation and lipoprotein lipase (49).

As outlined above, the circadian clock guides the daily oscillations of metabolism, sleep-wake behavior, endocrine pathways, and of the immune response. TNF and TGFβ influence these physiological systems and interfere with the expression of the components of the hierarchical, multilayered regulatory network of the clock network (17–19). The data presented here propose a new molecular circuit, which couples cytokine production with low levels of expression of clock genes. The linker is provided by CIRBP, which is required for efficient expression of Clock mRNA but down-regulated by TNF and TGFβ.

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