The Biosynthesis Characteristics of TTP and TNF Can Be Regulated through a Posttranscriptional Molecular Loop\(^*\)\(^{S1}\)

Received for publication, July 29, 2010, and in revised form, October 1, 2010. Published, JBC Papers in Press, November 18, 2010, DOI 10.1074/jbc.M110.168757

Naveed Aslam\(^{1}\) and Irum Zaheer\(^{5}\)

From the \(^{4}\)Department of Neurobiology and Anatomy, University of Texas Medical School, Houston, Texas 77030 and the \(^{5}\)Department of Internal Medicine, Methodist Hospital, Houston, Texas 77030

---

The abundant expression of tumor necrosis factor (TNF) is a hallmark of chronic inflammation of the gastrointestinal tract. Prolonged inflammation can lead to inflammatory bowel disease. TNF biosynthesis is regulated both at transcription and posttranscriptional levels. However, the stimulation-induced increase in translation rate is much larger. This might indicate the possibility of a posttranscriptional regulatory mechanism. How, during basal conditions, is the free concentration of TNF tightly regulated at low levels? The stability and translational efficiency of TNF transcript are regulated by an AU-rich element (ARE) in the 3′-UTR of messenger RNA. A transacting protein, TTP, binds to ARE and enhances the mRNA turnover. Here, we examine a proposal that TNF homeostasis is regulated by a TTP-TNF interaction loop at the posttranscriptional level. We propose a computational framework of this regulatory loop by modeling the role of AREs in mediating the messenger RNA stability and translation. This posttranscriptional regulatory loop between TTP and TNF is composed of two feedback loops (i.e. positive and negative). The mutual interaction of these feedback loops regulates the biosynthesis response of TNF during basal and inflammatory conditions. Here, we also propose an explanation for why the p38 inhibitors become insensitive for TTP knock-out mice.

---

Tumor necrosis factor (TNF) is a proinflammatory cytokine (1). Its impaired biosynthesis is linked with different disease states, such as rheumatoid arthritis, inflammatory bowel disease, and acute respiratory distress syndrome (1–3). TNF is active both in soluble and trans-membrane forms and is involved in a variety of cellular signaling cascades, including growth, activation, and apoptosis. The macrophage cells mediate a central role in regulating the inflammatory activities during these disease states. These cells generate proinflammatory cytokines (e.g. TNF) during the inflammatory stimulation. The LPS-induced TNF biosynthesis by macrophages is regulated at both transcriptional and posttranscriptional levels (4–7).

Previous observations indicate that during resting conditions, the TNF transcript is highly labile and is prone to degradation. However, an inflammatory stimulation enhances the stability of TNF transcript, which in turn increases the translation of TNF transcript and thus its free concentration (8, 9). Experimental observations also demonstrate that the stability of TNF messenger RNA is modulated through a functional coding sequence located in the 3′-untranslated region (3′-UTR) of its transcript (1, 8, 10). This regulatory machine consists of A- and U-rich elements with a characteristic AUUUA pentanucleotide. These sequences are known as AU-rich elements (AREs).\(^2\) In addition to TNF, the AREs are involved in modulating the stability of several different classes of transcripts (e.g. c-fos, c-myc, c-jun, cox-2, Mmp-13, cyclins, IL-1β, IL-3, and many others) (10–16). The AU-rich elements are classified into different classes, based on their ability to induce the rapid degradation of a transcript (17). The control of mRNA stability through an ARE-mediated mechanism can be linked with different pathological conditions (8, 14, 18–28).

Partially, the destabilizing characteristics of these elements are regulated through their interaction with AU-binding proteins (AUBPs) (14, 29). An inflammatory stimulation could mediate the posttranslational modifications of AUBPs, such as phosphorylation. This in turn might alter the binding characteristics of AUBPs with AREs and thus probably enhance the stability of an otherwise liable transcript. Therefore, in the absence of stimulation, AREs act as the determinant of transcript instability. Experimental observations also indicate that during basal conditions, the AREs act to repress the TNF translation (8, 30). In contrast, during inflammation, these elements mediate the posttranscriptional accumulation of TNF transcript (8) and thus positively regulate its translation rate. The role of AREs in regulating the stability of TNF message is also established through developing an ARE mutant mouse (8). These results show that deletion of ARE from 3′-UTR increases the stability of TNF message and relieves the permanent translation silencing. Because this leads to more translatable mRNA, the TNF biosynthesis response to an inflammatory stimulation will be enhanced, and also the duration for which TNF concentration is non-negligible is prolonged. This essentially could lead to a loss of anti-inflammatory translational control and can be related with diseases like chronic inflammatory arthritis, inflammatory bowel disease, etc.

Tristetraprolin (TTP) is an ARE-binding protein (14). TTP belongs to a zinc finger family of proteins with a Cys-Cys-
Regulation of Biosynthesis Characteristics of TTP and TNF

Cys-His (CCCH) signature. This protein is implicated as a key player in regulating the stability and translation of TNF messenger RNA (8, 26). Previous experimental work demonstrates that TTP protein regulates the expression of TNF message through modulating the stability of its transcript. The TTP binds with ARE of TNF messenger RNA and inhibits the TNF biosynthesis by destabilizing its RNA without imposing translational control (8, 26). TTP knock-out seems to be normal at birth but soon develops arthritis and inflammatory disorders. This is probably due to overexpression of TNF protein, which could be directly related with increased mRNA stability in TTP knock-out mice (8).

Additional experiments indicate that in response to an LPS signal, the transcription and translation rates of TNF are up-regulated. However, the transcription rates only increase 4–5-fold, whereas the translation rates are enhanced almost 80–150-fold (8). This difference indicates a posttranscriptional regulation. Despite observations demonstrating the role of TTP in mediating the stability of TNF transcript and additional observations indicating the role of TNF protein in modulating the expression of TTP transcript, the exact molecular mechanism of this regulatory process at the posttranscriptional level remains elusive. In this paper, we examine a proposal that the homeostasis in TNF biosynthesis can be maintained by a TTP-TNF interaction loop at the posttranscriptional level. Here, we develop a computational framework of this regulatory loop by modeling the role of AREs in mediating the transcript stability and translation. The proposed posttranscriptional regulatory loop between TTP and TNF is composed of two feedback loops (i.e., positive and negative feedback loops). The mutual interaction of these feedback loops regulates the biosynthesis response of TNF during basal and inflammatory conditions.

Our proposed molecular model of the TTP-TNF interaction loop is based on the following experimental observations (8, 26, 31–36). 1) TTP and TNF interact through a net negative feedback loop, which is also responsible for maintaining the TNF homeostasis. 2) TNF stimulates the expression of its own transcript (26). 3) TNF also modulates the expression of TTP transcript (26). 4) TTP binds with its mRNA through an ARE element and mediates the degradation of its own transcript. 5) An inflammatory signal mediates the phosphorylation of TTP probably through the p38 pathway. 6) Non-phosphorylated TTP is an active molecule and has a fast turnover rate. These observations suggest that a complex regulatory mechanism might be modulating the homeostasis in TNF biosynthesis. Here, through these observations, we put together a molecular model of a posttranscriptional regulatory loop. The proposed model might improve the understanding of the molecular mechanism involved in regulating the homeostasis in TNF biosynthesis. Specifically, we explore the following three questions. 1) During the resting conditions, what could be the molecular mechanism through which TTP regulates the stability of TNF messenger RNA? 2) What happens to this regulatory loop during inflammation? 3) What is the mechanism through which AREs regulate the stability and translation of TNF transcript? Our results imply that regulation of TNF biosynthesis is due to a balance between the negative and positive feedback loop. We also show that a net negative feedback between TTP and TNF molecules could be implicated in maintaining the homeostasis in TNF biosynthesis during resting phase. Through our approach, we also explain how an inflammatory signal disrupts TNF homeostasis. Our results show that during inflammation, the negative feedback effect of TTP on TNF biosynthesis is reduced, leading to transient increase in TNF biosynthesis. These results are consistent with various experimental observations (8, 26, 31–36).

**Materials and Methods**

**Biochemical Reactions**—The biochemical interactions for the TTP-TNF molecular loop (Fig. 1) are based on standard Michaelis-Menten type kinetics. The following set of reactions is used to describe the molecular interactions of this loop. The dynamic variables used are X to represent TTP and Y to represent TNF; a subscript P represents phosphorylation, and subscript m represents mRNA. The phosphatase P is approximated as a fixed parameter. The parameter L denotes the LPS, which represents the inflammatory stimulus. Here T represents the concentration of translation machinery.

\[
\begin{align*}
X_m + X &\xRightarrow{k_1} C_1 \xrightarrow{k_{11}} X + \text{Degraded TTP mRNA} \quad \text{(Eq. 1)} \\
Y_m + X &\xRightarrow{k_3} C_2 \xrightarrow{k_{33}} X + \text{Degraded TNF mRNA} \quad \text{(Eq. 2)} \\
C_2 + Y &\xRightarrow{k_5} C_4 \xrightarrow{k_{55}} Y_m + Y + X \quad \text{(Eq. 3)} \\
X + L &\xRightarrow{k_7} C_4 \xrightarrow{k_{77}} X_p + L \quad \text{(Eq. 4)} \\
C_1 + Y &\xRightarrow{k_9} C_5 \xrightarrow{k_{99}} X_m + Y + X \quad \text{(Eq. 5)} \\
X_p + P &\xRightarrow{k_{111}} X + P \quad \text{(Eq. 6)} \\
X_m + L &\xRightarrow{k_{75}} C_{45} \quad \text{(Eq. 7)} \\
C_{45} + T &\xRightarrow{k_{85}} X + L + T + X_m \quad \text{(Eq. 8)} \\
Y_m &\xRightarrow{k_{12}} Y'_m(\text{repressed state of TNF mRNA}) \quad \text{(Eq. 9)}
\end{align*}
\]
Regulation of Biosynthesis Characteristics of TTP and TNF

The binding of TTP (X) with its own message (Xm) and with the transcript of TNF (Ym) promotes the ARE-mediated degradation of these two messages through biochemical complexes C1 and C2. These ARE-mediated degradation events are described through Equations 1 and 2. The TNF protein also acts on the biochemical complexes C1 and C2 and enhances the expression of TTP and TNF transcript. These events are described by Equations 3 and 5. The inflammatory stimulus LPS acts on TTP and phosphorylates it through p38

$$Y_m + L \rightarrow Y_m$$  \hspace{1cm} (Eq. 10)

$$Y_m + L \rightarrow C_6$$  \hspace{1cm} (Eq. 11)

$$C_6 + T \rightarrow Y + L + T + Y_m$$  \hspace{1cm} (Eq. 12)

$$\text{miR16} + Xm \rightleftharpoons C_7$$  \hspace{1cm} (Eq. 13)

$$C_7 + X \rightleftharpoons C_8 \rightleftharpoons X + \text{miR16} + (\text{Degraded TNF mRNA})$$  \hspace{1cm} (Eq. 14)

The inflammatory stimulus LPS acts on TTP and phosphorylates it through p38

Events are described by Equations 3 and 5. The inflammatory stimulus LPS acts on TTP and phosphorylates it through p38

The LPS-mediated phosphorylation of TTP also enhances the stability of TTP protein. Phosphatase ‘P’ mediates the dephosphorylation of TTP through a biochemical reaction as described by Equation 6. The LPS stimulation also regulates the translation of TNF through its transcript Xm. The translation event of TTP through LPS is described by two reactions (Equations 7 and 8). Equation 7 describes the activation of TTP transcript through LPS, and Equation 8 describes the recruitment of translation machinery, which leads to the translation of TTP protein (in Equation 8, “S” in Kst represents a supplementary process to Equation 4). The ARE also mediates the inactivation of TNF transcript as described through Equation 9. This repression is relieved through an LPS-mediated event as described by Equation 10. The active form of TNF transcript Ym undergoes translation in an LPS-mediated step as shown by Equations 11 and 12. The stability of TNF mRNA can also be regulated through microRNA. The TTP protein indirectly assists the miR16 in targeting the ARE-mediated degradation of TNF transcript. Here, this role of TTP is described by Equation 13. This reaction event only loosely describes the possibility of indirect TTP and miR16 interaction. Equation 14 describes the role of microRNA in degrading the TNF mRNA through an ARE-mediated complex.

Temporal Dynamics—We integrated these differential equations resulting from Equations 1–14 through nonlinear solvers (using Math-Works MATLAB). For the TTP and TNF dynamic coefficients, some values are estimated from experiments. We then scaled unknown rate constants to obtain dynamics that are comparable with experimental results. All of the molecular concentrations in the model are expressed as ng/ml except if otherwise stated, and times are represented as h. The initial concentrations of all of the molecular species are set at 0.00001 ng/ml.

RESULTS

Posttranscriptional Regulatory Loop between TTP and TNF—The molecular model described in this paper is based on a posttranscriptional regulatory loop between TTP and TNF. This inhibition-activation model (Fig. 1) is composed of two molecular components (TTP and TNF). This regulatory loop explains how, at the posttranscriptional level, the stability and translation of TNF and TTP transcripts might be regulated. This model examines the hypothesis that TNF biosynthesis is regulated through a balance between positive and negative feedback regulatory loops within the TTP-TNF molecular pair. In this model, during resting conditions, the TTP protein mediates the destabilization of its own messenger RNA and TNF messenger RNA through an ARE-mediated process. Thus, under resting conditions, a negative feedback loop between TTP and TNF molecules tightly regulates the TNF expression. An inflammatory signal transiently introduces a positive feedback effect into this system by reducing the activity of TTP protein (through phosphorylation) and promoting the expression of TNF transcript. Thus, in this model, the inflammatory stimulation enhances the stability of TNF transcript and relieves the ARE-mediated translation block of TNF transcript, which in turn enhances the biosynthesis rate of cytokine.

The detailed molecular structure of this posttranscriptional loop is described in Fig. 2. This molecular wiring diagram indicates that the TTP mRNA is either free (translatable) or in the form of a biochemical complex with TTP protein (i.e. C1 (TTP-TTP mRNA)). Similarly, the TNF mRNA is either free (translatable) or in the form of a biochemical complex with TTP (i.e. C3 (TTP-TNF mRNA)). The TTP protein regulates...
the stability of TNF messenger RNA and also the stability of its own transcript. The transcript stability is regulated through two biochemical complexes (C1 and C2, respectively). ARE-mediated degradation of these transcripts is taking place through complexes C1 and C2 and is modeled here as an irreversible kinetic process (36). Here, the TTP protein can be in non-phosphorylated and phosphorylated forms. The phosphorylation of TTP is regulated through an LPS-mediated inflammatory signal. The non-phosphorylated form of TTP is active but is unstable and has very fast degradation, whereas the phosphorylated form of TTP is inactive but stable. The phosphorylated form also undergoes dephosphorylation through a PP2A-mediated reaction event. Here, the TTP protein plays a role of AUBP. It binds with ARE located in the 3'-UTR of TTP and TNF transcript. TTP thus acts as a key regulator of transcript stability. The model proposed in this work is based on experimental observations in RAW 264.7 cells (34). This posttranscriptional molecular loop is described by set of biochemical reactions (Equations 1–14).

The Dynamic Characteristics of TTP and TNF Biosynthesis—We implemented the transcription of both TTP and TNF through a single kinetic constant. As the simulations are initialized, the transcription rate rapidly increases to a certain value and is maintained at that level for 2 h, followed by a rapid shut-off of transcription although the LPS stimulation is still present (34). The same transcription protocol is used for both stimulated and non-stimulated conditions. The temporal dynamics depicted in Fig. 3 clearly show two phases in the
Regulation of Biosynthesis Characteristics of TTP and TNF

biosynthesis characteristics of TTP and TNF. Both of the molecules have a slow induction (3–4 h) phase followed by an extended (22–23 h) resolution phase in their biosynthesis profile. These simulations represent the condition of wild type RAW 264.7 cells (34). The solid lines represent the non-stimulation (resting) condition, whereas the dotted lines represent the stimulation (inflammatory condition) with LPS (1.0 μg/ml) (Fig. 3). Here, the non-phosphorylated form of TTP is unstable and degrades at a much faster rate than the phosphorylated form of TTP. The turnover time of the non-phosphorylated form of TTP is set between 30 and 45 min, whereas the turnover time of phosphorylated form of TTP is set between 4 and 8 h (31). The basal concentration of all the species is set at very small value of 0.00001 ng/ml. The free concentration of TNF protein during resting conditions is almost negligible (Fig. 3a, solid line). However, as the system is stimulated through LPS, the free concentration of TNF rapidly increases to a maximum value of 2.1 ng/ml in almost 3 h (Fig. 3a, dashed line). The induction phase is followed by a slow resolution during which the increase in TNF concentration is again reduced to basal levels. The free concentration of TTP during non-stimulated conditions is almost negligible (Fig. 3a, solid line). Here, during the LPS stimulation, the free concentration of total TTP is (Fig. 3b, dashed line), rapidly induced to 22 ng/ml in almost 3 h. The total TTP is distributed in the non-phosphorylated and phosphorylated forms (supplemental Fig. S1). The non-phosphorylated form of TTP has a fast induction and rapidly reaches to its maximum level of 3.1 ng/ml (supplemental Fig. S1a), followed by a rapid return to its basal concentration. In contrast, the concentration of phosphorylated form of TTP increases in a relatively slower manner (supplemental Fig. S1b; induction of 3–4 h to its maximum concentration of 19.0 ng/ml) and followed by slow clearance. Interestingly, the temporal characteristics of the phosphorylated form and those of total TTP (Fig. 3b, dashed line) are almost identical, thus raising the possibility that new synthesis and phosphorylation of TTP are taking place almost in parallel. These results show that both TNF and TTP are rapidly induced through an inflammatory stimulation and are cleared through a rather slow degradation mechanism (Fig. 3, a and b, dotted lines). The temporal dynamics of TTP biosynthesis in these computational experiments are consistent with previous experimental observations in RAW 264.7 cells (34).

One critical parameter that could influence the biosynthesis of TTP and TNF is the strength of LPS stimulation (supplemental Fig. S2). The AU-rich element has a dual role in these simulations. It regulates the stability of TNF transcript through TTP, and additionally it relieves the inactive state of TNF transcript through the LPS-stimulated state (see “Materials and Methods”). During resting phase, ARE does not allow the net accumulation of TNF transcript; therefore, the TNF biosynthesis rates are very small. However, through LPS stimulation, it mediates the accumulation of cytokine transcript, thus increasing its biosynthesis rates and consequently its concentration in response to an inflammatory signal. An additional posttranscriptional mechanism to regulate the biosynthesis of TNF is mediated through microRNA (35). The TTP is again indirectly involved with microRNA miR16 to assist the ARE-mediated degradation. The effect of miR16 on TNF biosynthesis is dependent on its concentration and has saturable characteristics (supplemental Fig. S4).

Inhibition of the p38 Pathway and Its Effect on Biosynthesis of TTP and TNF—The previous experimental observations suggest a possible link between p38 and TTP in the regulation of TNF mRNA stability and thus its secretion (31, 32, 34). These results indicate that TNF secretion is highly sensitive to p38 pathway inhibitor. The results from RAW 264.7 cells also show that p38 MAPK inhibitor destabilized the endogenous TTP protein, reducing its half-life (31). Additional observations in similar systems indicate that the phosphorylation of TTP reduces its activity but increases its stability (31). Using simulations of the molecular network of this posttranscriptional loop, we set out to test if this model can account for the experiments that block the p38 MAPK pathway and its effect on TNF biosynthesis. We implemented the blocking of the p38 MAPK pathway through inhibiting the rate parameter k_8 (Equation 4). Here, the p38 MAPK pathway inhibitor specifically blocks the phosphorylation of TTP. We first implemented the simulations for the wild type RAW 264.7 cell system (Fig. 4). Here, the solid lines represent the non-stimulated condition, whereas the dashed lines represent the stimulation with an LPS concentration of 1.0 μg/ml. We carried out the blocking of the p38 MAPK pathway in a dose-dependent manner. The concentration of free TNF at no blocking and at 40, 75, 90, 95, 99, and 100% blocking conditions of the p38 pathway is depicted by Fig. 4a. These results show that the TNF biosynthesis is sensitive to a p38 pathway inhibitor. However, completely blocking this pathway does not completely abolish the TNF biosynthesis. The dose-response curve (supplemental Fig. S3) shows that the TNF concentration is sensitive to the p38 inhibitor. Blocking the p38 pathway will reduce the LPS sensitivity to induce the biosynthesis of TNF. The maximum amount of TNF generated and the duration of its persistence (Fig. 4a) is dependent on the extent of blocking. The dose-response curve (supplemental Fig. S3) shows that the percentage of maximum TNF levels sharply drops between 40 and 75% blocking of the p38 pathway. The blocking results from our simulations are consistent with previous experimental observations obtained through p38 inhibitors SB203580 and SB202190 (31, 33). Similarly, the concentration of total TTP is also affected by the p38 pathway inhibitor (Fig. 4b). The maximum concentration of total TTP generated and the duration of its persistence (Fig. 4b) are dependent on the extent of p38 pathway blocking levels. These simulations are carried out at no blocking and at 40, 75, 90, 95, 99, and 100% blocking of the p38 pathway (Fig. 4b). These results show that the maximum concentration of TTP and duration of its persistence decrease as the blocking of the p38 pathway is increased, and reversal in TTP concentration is much faster at higher blocking levels of the p38 pathway.

The TTP Knock-out and TNF Biosynthesis Characteristics—The deficiency of TTP in intact animals is linked to excessive biosynthesis of TNF. The TNF mRNA is a direct target of TTP, and its stability is enhanced in the absence of TTP. This increase in the stability of TNF transcript is related to enhanced biosynthesis response for TTP knock-out (TTP-KO).
Regulation of Biosynthesis Characteristics of TTP and TNF

animals and is probably the main cause of chronic inflammatory syndromes like erosive arthritis, autoimmunity, and myeloid hyperplasia (33). Through our simulation framework, we also implemented conditions mimicking the effect of TTP knock-out. The knock-out results show that the concentration of TNF in the non-stimulated condition is almost negligible (Fig. 5a, dashed line) in comparison with simulations representing the wild type RAW 264.7 cells (Fig. 5a, solid line). This increase in TNF biosynthesis is due to increase in the stability of TNF mRNA in TTP-KO simulations. Our simulation results also show that the free concentration of TTP in stimulated TTP-KO is almost negligible and is comparable with its concentration in non-stimulated wild type RAW 264.7 cells. We also carried out the blocking of the p38 path-

FIGURE 4. The simulations mimicking the inhibition of p38 pathway and its effect on LPS-induced biosynthesis of TTP and TNF in wild type RAW 264.7 cells. The solid line represents the non-stimulated condition, whereas the dotted line represents stimulation with an LPS concentration of 1.0 μg/ml. The blocking of the p38 pathway is accomplished by manipulating the parameter k8. a, concentration of free TNF under resting and LPS-stimulated conditions at different p38 pathway blocking levels. Simulations are carried out at no blocking and at 40, 75, 90, 95, 99, and 100% blocking of the p38 pathway. This result shows that TNF biosynthesis is sensitive to the p38 pathway. However, completely blocking this pathway does not completely abolish the TNF induction. b, concentration of total TTP under basal and LPS-stimulated conditions at different p38 pathway blocking levels. Simulations are carried out at no blocking and at 40, 75, 90, 95, 99, and 100% blocking of the p38 pathway. This result shows that the maximum concentration of TTP decreases as the blocking of the p38 pathway is increased, and the reversal in TTP concentration is much faster at higher blocking levels of the p38 pathway.

FIGURE 5. The biosynthesis characteristics of TNF and TTP. These simulations mimic the TTP knock-out in RAW 264.7 cells. The solid line represents the non-stimulated condition, whereas the dotted line represents stimulation with an LPS concentration of 1.0 μg/ml for wild type RAW 264.7, and the black dotted line represents simulation with an LPS concentration of 1.0 μg/ml for TTP knock-out in RAW 264.7 cells. a, concentration of free TNF for simulations mimicking the TTP knock-out and wild type RAW 264.7 cells. The TNF concentration rapidly increases to 3.3 ng/ml during the first 2 h for TTP knock-out conditions in RAW 264.7 cells. The maximum TNF level is almost 60% higher for TTP knock-out when compared with simulations representing the wild type cells. b, concentration of free TTP for simulations mimicking the TTP knock-out and wild type RAW 264.7 cells. This result indicates that for TTP knock-out, there is no increase in the total TTP concentration.
way in TTP-KO (Fig. 6). Interestingly, our results demonstrate that p38 inhibitor has no effect on TNF biosynthesis for TTP-KO. This result of complete insensitivity of p38 inhibitor on TNF secretions in TTP-KO simulation is consistent with previous experimental observations (33).

**The Simultaneous Inhibition of the p38 and Proteasome Pathway**—Our simulation results and previous experimental observations indicate that blocking the p38 pathway reduces the total amount of TTP. Once the p38 pathway is inhibited, it leads to rapid dephosphorylation followed by degradation of TTP through 20 S/26 S proteasomes. An interesting experimental observation in RAW 264.7 cells suggests that proteasome inhibitor (MG-132) can reverse the effect of p38 pathway inhibitor (31). These experimental results show that the application of MG-132 caused a dose-dependent reversal of the destabilizing effect of SB202190 on the TTP concentration (31). Through our molecular model, we also set out to account for this experimental observation. We started our simulations by first 100% blocking the p38 pathway with no proteasome inhibition. In subsequent simulations, while keeping the p38 pathway blocking levels at 100%, we started to block the proteasome pathway in a dose-dependent manner. Our results (Fig. 7) indicate that even with the simulation framework, the proteasome pathway blocking reversed the destabilizing effect of p38 pathway inhibitor. Interestingly, this reversal is only observed for biosynthesis characteristics of TTP (Fig. 7b), not for TNF (Fig. 7a). Here, the blue solid line represents the non-stimulated condition, whereas the red dotted line represents the LPS-stimulated condition but no blocking (Fig. 7). The green dotted line represents the 100% p38 pathway blocking. The solid black lines denote the 100% p38 pathway blocking and different levels of proteasome blocking (Fig. 7). Our results (Fig. 7a) show that at 100% p38 blocking, as the proteasome blocking levels are increased, the TNF concentration start to decrease. In contrast, the concentration of TTP increases with proteasome blocking (Fig. 7b). At almost 85% proteasome blocking, the destabilizing effect of the p38 pathway on TTP biosynthesis characteristics is completely reversed (Fig. 7b). Our simulation results are quantitatively able to reproduce the experimental observation.

**DISCUSSION**

This study proposes a molecular model for regulating the biosynthesis characteristics of a cytokine through a posttranscriptional molecular loop. Here, we test a hypothesis that the biosynthesis characteristics of an inflammatory cytokine might be regulated at the posttranscriptional level through a regulatory loop. On the basis of previous experimental observations (8, 26, 31–36), we have selected the TNF-TTP molecular pair as a specific example to examine this hypothesis. The proposed regulatory model is based on observations that the biosynthesis characteristics of a cytokine can be regulated through an ARE-mediated control mechanism of transcript stability and translation. The ARE sequence is located in the 3'-UTR of a cytokine mRNA. Usually, the stability characteristics of an ARE bearing cytokine message are regulated through posttranslational modifications of its AUBP. In this molecular loop, the AUBP for TNF transcript is TTP, and LPS-induced phosphorylation of TTP enhances its stability while reducing its binding with TNF transcript. The increased stability of TNF transcript leads to its net accumulation and thus enhances its biosynthesis response. Here, ARE also mediates the permanent translational silencing of TNF transcript during resting conditions. However, through LPS-mediated stimulation, ARE acts to relieve this repression.

The TTP-TNF molecular model consists of two inner loops. One of them is based on positive whereas the other inner loop is based on negative feedback.
The non-stimulated condition, whereas the stimulated condition again shows the maximum value of around 22 ng/ml. These simulation results are consistent with previous experimental observations from RAW 264.7 cells (34).

During the resting conditions, a net negative feedback signal is much stronger and overrides any positive feedback thus tightly regulating the concentration of TNF at low basal levels. This persistent negative feedback signal is responsible for TNF homeostasis during normal cellular function. However, an inflammatory signal enhances the expression of TNF transcript and also mediates the phosphorylation of TTP, thus reducing its activity and binding with TNF transcript. Therefore, during the early phase of inflammation, the negative effect is much stronger, thus increasing the biosynthesis rate of TNF during the induction phase. After the initial induction phase, the negative feedback effects are starting to increase mainly due to TNF-mediated expression of TTP and slow dephosphorylation of its inactive form. According to the molecular circuit of this proposed model, a net negative feedback loop eventually will check any transient increase of TNF concentration and will bring it back to basal levels, thus restoring the TNF homeostasis even when the inflammation signal is still present. However, this corrective action might take hours to reduce the TNF concentration to basal levels. Our simulation results clearly show an induction and a slow resolution phase in TNF biosynthesis characteristics. These results indicate the possibility of a positive feedback during induction and a slow compensatory negative effect during the resolution phase.

One of the key posttranslational regulatory mechanisms involves the phosphorylation of newly synthesized proteins. The phosphorylation paradigm of most proteins usually involves a rapid process. Typically, a newly synthesized protein undergoes phosphorylation on a fast time scale through an active kinase. TTP, however, provides an interesting example where the protein synthesis and phosphorylation are probably taking place on parallel time scales. Previous experimental observations from RAW 264.7 cells indicate that the new synthesis of TTP is accompanied by gradual phosphorylation of TTP (34). The posttranscriptional regulatory model of this work provides the similar TTP biosynthesis and phosphorylation characteristics. Our results indicate that the total concentration of TTP gradually increases (3–4 h) to a maximum level. An analysis of the dynamics of non-phosphorylated and phosphorylated forms of TTP (supplemental Fig. S1) clearly shows that the non-phosphorylated form is rapidly induced (less than 1 h) to its maximum concentration of 3 ng/ml, whereas the phosphorylated form gradually increases to its maximum concentration of 19 ng/ml (in almost 4 h). Also, as we notice the dynamics of total TTP (Fig. 3b), the concentration of total TTP gradually increases (within 4 h) to its maximum value of around 22 ng/ml. These simulation results are in a way consistent with previous experimental observations in RAW 264.7 cells (34).
One working assumption of this model is the regulatory role of TTP phosphorylation. The naive newly synthesized form of TTP has high activity but is unstable in character, whereas the phosphorylated form is inactive but is more stable (31). Thus, although the phosphorylation of TTP improves the stability, it comes at the expense of activity. Previous experiments show that during LPS-stimulated conditions, the p38 MAPK and ERK pathways synergistically regulate the stability and subcellular distribution of TTP protein because inhibiting both of these pathways results in rapid dephosphorylation and destabilization of TTP protein (31). The experimental observations also indicate that phosphorylation events at two or more sites are probably involved in regulating the stability of TTP. However, in our molecular model, we only consider the single phosphorylation event that is induced by an LPS-activated p38 pathway signal. Our model does not account for multisite phosphorylations of TTP. We have also made no attempt to rigorously model the different signaling pathways involved in the phosphorylation of TTP. In our model, a kinetic parameter is used to represent the lumped effect of different upstream pathways involved in the LPS-mediated phosphorylation of TTP. The phosphorylated form of TTP has strong affinity for 14–3–3 proteins (34). These interactions are thus probably regulating both the turnover and dephosphorylation of TTP. How exactly 14–3–3 proteins regulate the characteristics of TTP remains unclear. Along with the stability, the 14–3–3 protein also tightly regulates the localization of its target proteins. From previous experimental observations, it is speculated that 14–3–3 protein mediates the accumulation of phosphorylated TTP within a nuclear compartment during inflammation. However, the regulatory loop model described in this work is only a single-compartment model. Here, we have not incorporated the interaction of the phosphorylated form of TTP with 14–3–3 proteins and their subsequent effect on migration of TTP from the cytoplasmic to the nuclear compartment.

This study also shows that the p38 pathway inhibitor blocks the TNF secretion in a dose-dependent manner. Blocking the p38 pathways also reduces the total concentration of TTP again in a dose-dependent manner. In our simulations, the p38 pathway is involved in stabilization of TTP protein through phosphorylation. The non-phosphorylated form of TTP is very active but unstable, whereas the phosphorylated form is stable but inactive. Only the active form of TTP is involved in ARE-mediated degradation of TNF transcript. The p38 pathway inhibitor acts through blocking the phosphorylation of TTP. Thus, during the inhibition, a more active form of TTP is available, which directs the TNF transcript toward degradation and hence reduces the biosynthesis of TNF. Our results also indicate that even 100% blocking of the p38 pathway did not completely abolish the TNF biosynthesis. This is due to the fact that other LPS-mediated pathways are also involved in regulating the TNF biosynthesis, and these pathways are not affected by p38 inhibitor. Interestingly, for simulations representing the TTP knock-out case, the p38 pathway inhibitor is completely ineffective. The TTP knock-out results show higher levels of TNF secretions indicating that TNF remains completely insensitive to p38 pathway inhibition. This probably occurs because in TTP knock-out the TTP is not available to mediate the degradation of TNF transcript; therefore, even for a high blocking level of the p38 pathway, there is no effect on TNF biosynthesis.

Recent experimental results also indicate that in RAW 264.7 cells, the inhibition of the p38 MAPK pathway leads to a rapid dephosphorylation of LPS-induced TTP. The phosphorylated form of TTP is highly unstable and probably degrades through the proteasome pathway (20 S/26 S) (31). These results also indicate that a proteasome inhibitor, such as MG132, protects the phosphorylated form of TTP from degradation (31). Interestingly, these results depict that the proteasome inhibitor reversed the effect of a p38 MAPK pathway inhibitor. The MG132 reversed the effect of SB202190 (p38 MAPK pathway inhibitor) in a dose-dependent manner, with almost 10 μM MG132 completely reversing the effect of p38 MAPK inhibitor in RAW 264.7 cells (31). Surprisingly, with our minimal model, we are able to predict this behavior. Our results indicate that around 85% blocking of the proteasome pathway balanced the destabilizing effect of p38 MAPK pathway inhibitor. However, this reversal was only observed in the biosynthesis characteristics of TTP, not for TNF. Interestingly, in our hands, the proteasome inhibitor was not able to recover the biosynthesis characteristics of TNF.

In this paper, we do not rigorously model the transcription of TTP and TNF. Here, the transcription is incorporated into the model through a single kinetic constant. As simulations are initiated, the transcription rate increases to a certain level and is maintained at that level for 2 h before it drops to basal levels. This pulse type of transcription protocol is adapted because some previous experimental results show a rapid and dramatic induction of TTP transcription, followed by a similar rapid shut-off almost after 2 h in RAW 264.7 cells (34). Therefore, we created a simplistic representation of such a behavior in our simulation framework without going into the molecular details of transcription pathways.

REFERENCES

Regulation of Biosynthesis Characteristics of TTP and TNF
