

Interleukin-10 Inhibits Lipopolysaccharide-induced Tumor Necrosis Factor- α Translation through a SHIP1-dependent Pathway^{*S}

Received for publication, February 19, 2012, and in revised form, August 10, 2012. Published, JBC Papers in Press, September 6, 2012, DOI 10.1074/jbc.M112.348599

Catherine S. Chan^{‡S¶}, Andrew Ming-Lum^{‡S¶}, Gary B. Golds^{‡S¶}, Shaina J. Lee^{‡S¶}, Raymond J. Anderson^{||}, and Alice L.-F. Mui^{‡S¶¶1}

From the [‡]Immunity and Infection Research Centre, Vancouver Coastal Health Research Institute, Vancouver, British Columbia V6H 3Z6, the Departments of ^SSurgery and [¶]Biochemistry and Molecular Biology, University of British Columbia, Vancouver, British Columbia V6T 1Z3, and the ^{||}Departments of Chemistry and Oceanography, University of British Columbia, Vancouver, British Columbia V6T 1Z1, Canada

Background: IL-10 inhibits TNF α expression in the anti-inflammatory response.

Results: IL-10 activates SHIP1 phosphatase to inhibit TNF α translation through polysome disassembly and Mnk1 activation.

Conclusion: SHIP1 is involved in the regulatory mechanism of IL-10 for controlling TNF α translation.

Significance: This suggests a STAT3-independent pathway for IL-10 in controlling inflammation through SHIP1.

Production of the proinflammatory cytokine TNF α by activated macrophages is an important component of host defense. However, TNF α production must be tightly controlled to avoid pathological consequences. The anti-inflammatory cytokine IL-10 inhibits TNF α mRNA expression through activation of the STAT3 transcription factor pathway and subsequent expression of STAT3-dependent gene products. We hypothesized that IL-10 must also have more rapid mechanisms of action and show that IL-10 rapidly shifts existing TNF α mRNA from polyribosome-associated polysomes to monosomes. This translation suppression requires the presence of SHIP1 (SH2 domain-containing inositol 5'-phosphatase 1) and involves inhibition of Mnk1 (MAPK signal-integrating kinase 1). Furthermore, activating SHIP1 using a small-molecule agonist mimics the inhibitory effect of IL-10 on Mnk1 phosphorylation and TNF α translation. Our data support the existence of an alternative STAT3-independent pathway through SHIP1 for IL-10 to regulate TNF α translation during the anti-inflammatory response.

The host defense against bacteria and other pathogens includes production of inflammatory cytokines such as TNF α by macrophages. Bacterial cell wall products such as LPS bind to receptors on the macrophage and initiate a signal transduction cascade, resulting in TNF α transcription (1). For translation to occur, p38 MAPK must be activated to relieve translational silencing that is mediated, in part, by proteins interacting with

AU-rich elements (AREs)² in the 3'-UTR of TNF α mRNA (2–6). One p38 MAPK substrate involved in regulating TNF α translation is Mnk1 (MAPK signal-integrating kinase 1) (7). Mnk1 can be activated by p38 MAPK or ERK1/2 and phosphorylates a number of ARE-binding proteins (5, 8) as well as eIF4E, a component of the translation initiation complex (9, 10).

Although TNF α is beneficial for potentiating the inflammatory response, the magnitude and duration of its production must be controlled and appropriately terminated to prevent tissue damage. The anti-inflammatory cytokine IL-10 is a key moderator of inflammation, and loss of normal levels of IL-10 or its receptor results in inflammatory disorders in both mouse and man (11–14). Studies on the mechanism by which IL-10 receptor signaling inhibits TNF α expression have concentrated on activation of the STAT3 transcription factor and induction of STAT3-regulated genes (15–19). The gene products implicated in inhibiting TNF α expression include Bcl-3, an inhibitor of TNF α transcription (20); tristetraprolin, which destabilizes TNF α mRNA (21); and ETV3 and SBNO2, which modify the chromatin structure of the TNF α gene (22). However, we reasoned that IL-10 must also have a more immediate mechanism of action because physiologically in a whole organism, IL-10 is not encountered by macrophages until they have already become activated. In fact, an activated macrophage itself is a major source of IL-10 through autocrine production, which intrinsically delays the exposure of the activated macrophage to IL-10. We thus investigated and found that IL-10 also has immediate effects on TNF α translation, which do not require *de novo* transcription of STAT3-dependent genes.

This STAT3-independent pathway involves SHIP1 (SH2 domain-containing inositol 5'-phosphatase 1). SHIP1 has already been implicated in negative regulation of immune and inflammatory cell activation by antagonizing the PI3K pathway (23, 24). We now show that IL-10 inhibits TNF α translation through SHIP1-dependent inhibition of Mnk1. This inhibitory

^{*} This work was supported by Research Grant MOP-84539 from the Canadian Institutes of Health Research (to A. L.-F. M.), and Canadian Cancer Society Research Institute Grant 017289 (to R. J. A.). A. M.-L. held CIHR Doctoral Research, and Michael Smith Foundation for Health Research (MSFHR) awards. The CIHR Transplantation Training Program provided graduate scholarships (to A. M. L., G. B. G., and C. S. C.).

^S This article contains supplemental "Experimental Procedures" and Figs. 1–3.

¹ To whom correspondence should be addressed: Jack Bell Research Center, 2660 Oak St., Vancouver, BC V6H 3Z6, Canada. Tel.: 604-875-4111 (ext. 62242); Fax: 604-875-4497; E-mail: alice.mui@ubc.ca.

² The abbreviations used are: ARE, AU-rich element; hnRNP1, heterogeneous nuclear ribonucleoprotein A1.

effect appears to be modulated by phosphorylation of one of two of its upstream kinases, p38 MAPK. Consistent with this finding, activation of SHIP1 using a small-molecule SHIP1 agonist mimicked the effect of IL-10 by inhibiting Mnk1 activation and TNF α translation.

EXPERIMENTAL PROCEDURES

Cells and Reagents—RAW 264.7 cells were obtained from American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 9% (v/v) Fetal calf serum (Thermo Fisher Scientific). Primary cells (peritoneal macrophages) from 6–20-week-old male and female BALB/c wild-type (SHIP1^{+/+}) or SHIP1^{-/-} mice (Dr. Gerald Krystal, BC Cancer Research Centre, Vancouver, British Columbia, Canada) were isolated by peritoneal lavage with 3–5 ml of sterile PBS (Thermo Fisher Scientific). Cells were collected and transferred to Iscove's modified Dulbecco's medium (Thermo Fisher Scientific) supplemented with 10% (v/v) fetal calf serum, 10 μ M β -mercaptoethanol, 150 μ M monothiolglycolate, and 1 mM L-glutamine. All cells were cultured at 37 °C and at 5% CO₂ and 95% humidity.

Antibodies against phospho-Mnk1 (Thr-197/Thr-202), phospho-ERK1/2 (p44/p42 MAPK; Thr-202/Tyr-204), phospho-p38 (Thr-180/Tyr-182), phospho-eIF4E (Ser-209), PI3K p85, and Akt (pan) were purchased from Cell Signaling Technologies. Anti-SHIP1 (P1C1) antibody was purchased from Santa Cruz Biotechnology. CGP57380 (Calbiochem) was dissolved in dimethyl sulfoxide, and AQX-MN100 (Aquinox Pharmaceuticals, Vancouver, British Columbia, Canada) was dissolved in ethanol. Recombinant mouse IL-10 was expressed and purified in-house.

Immunoblot Analysis—Peritoneal macrophages were plated at a density of 3×10^5 cells/well on a 24-well tissue culture plate and allowed to adhere for 1 h, followed by replacement with fresh medium for 3 h prior to stimulation with 1 ng/ml LPS (*Escherichia coli* serotype 0111:B4; Sigma) with and without 100 ng/ml IL-10 for 1 h. Cells were rinsed with cold PBS and lysed with 250 μ l of hot 2 \times Laemmli solubilization buffer. Proteins were separated by 10% SDS-PAGE, followed by electroblotting onto PVDF membrane. Blots were developed with the appropriate Alexa Fluor[®] 660 or 800-conjugated anti-IgG antibodies (Invitrogen) and imaged using a LI-COR Odyssey imager.

SHIP1 and Scrambled Knockdowns—siRNA oligonucleotides targeting SHIP1 or a nonspecific scrambled sequence were designed using BLOCK-iTTM siRNA online design software (Invitrogen) and cloned into a microRNA expression vector (pTRIPZ, Thermo Fisher Scientific) under the control of a doxycycline/tetracycline-inducible promoter. Lentivirus produced from these vectors was used to transduce RAW 264.7 cells in the presence of 8 μ g/ml protamine sulfate and selected for resistance in 3 μ g/ml puromycin. Further selection was provided by induction of GFP expression by 2 μ g/ml doxycycline (Sigma) and sorting for GFP fluorescence.

TNF α mRNA Fractionation and Quantification—siRNA lentivirus-transduced cells were treated with 2 μ g/ml doxycycline 48 h prior to stimulation, when the cells were replated at a density of 8×10^6 cells in a 10-cm tissue culture plate at \sim 32 h. Parental RAW 264.7 cells and peritoneal macrophages were plated

as described above but without doxycycline treatment. For Mnk1 inhibitor treatment, cells were replaced with fresh medium containing vehicle (dimethyl sulfoxide) or 75 μ M CGP57380 30 min prior to stimulation. Cells were stimulated with 1 ng/ml LPS for 45 min, followed by the addition of 100 ng/ml IL-10 or 10 μ M AQX-MN100 for 15 min where indicated.

Cells were rinsed with 5 ml of cold PBS and then lifted in 500 μ l of buffer containing 10 mM Tris-HCl (pH 7.4), 10 mM potassium chloride, 10 mM magnesium chloride, 20 mM dithiothreitol, 150 μ g/ml cycloheximide, 0.5% Nonidet P-40, and 500 units of Protector RNase (Roche Diagnostics) with a cell scraper and lysed for 30 min at 4 °C. The soluble lysates were collected by centrifugation at $12,000 \times g$ for 10 min, and sucrose was added to 500 μ l of the lysates to 10% (w/v). A 125- μ l aliquot was set aside to extract the total unfractionated RNA, and 450 μ l was layered on top of a stepwise sucrose gradient containing 450 μ l of 30% (w/v), 250 μ l of 50% (w/v), and 200 μ l of 70% (w/v) sucrose. All sucrose solutions were generated in the above lysis buffer lacking Nonidet P-40. Samples were separated by ultracentrifugation at $152,000 \times g$ for 35 min, followed by collection of 125- μ l fractions. Each fraction was supplemented with 150 ng of total human mRNA and 30 μ g of GlycoBlue (Invitrogen) prior to extraction of RNA with TRIzol reagent (Invitrogen) following the manufacturer's protocol and then treated with DNase (Roche Diagnostics). cDNA was generated using a RevertAid H minus first strand cDNA synthesis kit (Roche Diagnostics) and analyzed by SYBR Green-based real-time quantitative PCR using mouse TNF α and human GAPDH gene-specific primers. The recovery of mouse TNF α mRNA in each fraction was normalized by comparison with the level of human GAPDH present in each fraction. The amount of mouse TNF α mRNA in each fraction is plotted as a percentage of the sum of mouse TNF α mRNAs in all fractions. The amount of mouse TNF α mRNA in the starting unfractionated sample was normalized to endogenous mouse GAPDH levels.

TNF α Production Measurements—Parental RAW 264.7 cells were seeded at a density of 2×10^5 cells in 24-well tissue culture plates overnight and then pre-activated with 1 ng/ml LPS for 45 min. The cells were rinsed with 1 ml of fresh medium and replaced with 1 ng/ml LPS with 0–100 ng/ml IL-10 for 15 min prior to collecting the media supernatant for ELISA analysis with a OptEIATM mouse TNF ELISA kit (BD Biosciences).

RESULTS

IL-10 Regulates TNF α Translation through SHIP1—In a previous study, Kontoyiannis *et al.* (25) reported that the co-addition of IL-10 and LPS reduced the amount of TNF α produced, at least in part through inhibiting TNF α protein translation. Because certain populations of macrophages would be activated by LPS prior to exposure to IL-10 in many physiological settings, we examined whether IL-10 could inhibit TNF α translation if added to LPS-pre-activated macrophages. RAW 264.7 cells were stimulated for 45 min with LPS and then treated for 15 min with IL-10. RNA was isolated under non-denaturing conditions as described by Kontoyiannis *et al.* (25) and fractionated over a sucrose gradient to separate the heavier polyribosome-associated (polysome) mRNA from the lighter monoribosome-associated (monosome) mRNA. The relative amounts

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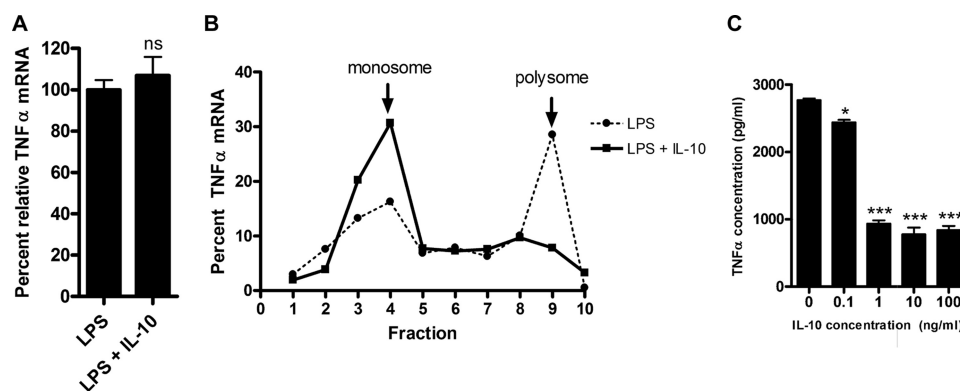


FIGURE 1. IL-10 inhibits TNF α synthesis through polysome disassembly in pre-activated cells. *A*, relative total TNF α mRNA from RAW 264.7 cells pre-activated with 1 ng/ml LPS for 45 min and then treated with 100 ng/ml IL-10 for an additional 15 min. Relative TNF α mRNA was normalized against mouse GAPDH mRNA and then expressed as a percentage against cells that were not treated. Statistical significance between treatments was calculated by Student's unpaired two-tailed *t* test with 95% confidence. *ns*, not significant ($p = 0.3077$). *B*, TNF α mRNA distribution in total mRNA from *A* fractionated over a sucrose density gradient. Fraction 10 indicates the bottom of the gradient. mRNA levels were measured by real-time quantitative PCR in each fraction and expressed as a percentage of the sum of the TNF α mRNA signal from all 10 fractions. Results are representative of two independent experiments. *C*, TNF α protein production in cell supernatants was measured by ELISA from pre-activated cells from *A* except that cells were treated with a range of IL-10 concentrations. Results are representative of three experiments. Statistical significance between treatments was calculated by one-way analysis of variance with 95% confidence. *, $p < 0.05$; ***, $p < 0.001$.

of TNF α mRNA associated with polysomes and monosomes were quantified to determine the amount of rapidly translated TNF α . As shown in Fig. 1*A*, the addition of IL-10 for 15 min did not change the total amount of TNF α mRNA present, but did shift the TNF α mRNA population completely from polysomes to monosomes (Fig. 1*B*). This decrease in polysome-associated TNF α mRNA is reflected in decreased amounts of TNF α protein detected in culture supernatants of LPS-pre-activated cells treated with 1–100 ng/ml IL-10 (Fig. 1*C*).

The IL-10 receptor signaling pathway is well known for activation of the STAT3 transcription factor (15–19). However, in other studies, we found that IL-10 also activates the SHIP1 inositol 5'-phosphatase.³ The action of the SHIP1 pathway is predicted to mediate the immediate responses to IL-10 because, unlike a STAT3 transcription factor-dependent pathway, it does not depend on *de novo* gene transcription (23, 24). We thus examined the ability of IL-10 to shift TNF α mRNA to monosomes in RAW 264.7 cells in which SHIP1 expression had been reduced by siRNA knockdown. siRNA sequences were cloned into the pTRIPZ vector, which expresses siRNA sequences in the context of a microRNA element and in a doxycycline-inducible manner. Fig. 2*A* shows SHIP1 expression in cells transduced with SHIP1 or scrambled siRNA of a nonspecific sequence and treated with or without doxycycline for 48 h. Densitometry of the SHIP1 bands suggested that <15% of SHIP1 expression remained when cells were treated with doxycycline.

SHIP1 or scrambled siRNA cells were stimulated for 45 min with 1 ng/ml LPS and then treated with 100 ng/ml IL-10 for 15 min, and monosome/polysome-associated TNF α mRNA was analyzed as described above. As shown in Fig. 2*B*, IL-10 was unable to shift TNF α mRNA to the monosomes in SHIP1 knockdown cells. However, IL-10 shifted TNF α mRNA to the monosomes as efficiently in the scrambled cells as it did in the parental RAW 264.7 cells (Fig. 1). These studies were

repeated in primary peritoneal macrophages freshly isolated from wild-type (SHIP1^{+/+}) or SHIP1^{-/-} mice with similar results (Fig. 2*C*).

IL-10 Inhibits TNF α Translation through SHIP1-mediated Inhibition of Mnk1—Several groups have shown that LPS induction of TNF α translation in macrophages involves Mnk1 (26–28). We thus tested whether IL-10 inhibits Mnk1 phosphorylation and whether this requires SHIP1. Peritoneal macrophages from wild-type (SHIP1^{+/+}) and SHIP1^{-/-} mice were stimulated with 1 ng/ml LPS with or without 100 ng/ml IL-10 and subjected to immunoblot analyses with antibody specific to phosphorylated Mnk1. As shown in Fig. 3*A*, IL-10 inhibited Mnk1 phosphorylation in SHIP1^{+/+} cells, but not in SHIP1^{-/-} cells. Fig. 3*B* summarizes the densitometry of three to five independent experiments. We also examined the phosphorylation status of the p38 MAPK and ERK1/2 kinases because of their reported role as upstream activators of Mnk1 (29). We found that IL-10 also reduced the LPS-induced phosphorylation of p38 MAPK only in SHIP1^{+/+} cells (Fig. 3, *A* and *B*). ERK1/2 phosphorylation did not appear to be significantly inhibited by IL-10 regardless of the presence of SHIP1 (Fig. 3, *A* and *B*). Together, these results suggest that IL-10 regulates Mnk1 through SHIP1 by inhibiting the p38 MAPK pathway.

Finally, we confirmed Mnk1 regulation of TNF α mRNA polysome assembly using the Mnk1 inhibitor CGP57380, which has been previously shown to decrease TNF α induced upon LPS stimulation (26). RAW 264.7 cells were pretreated with 75 μ M CGP57380 or dimethyl sulfoxide vehicle for 30 min prior to stimulation with 1 ng/ml LPS for 45 min. mRNAs were then isolated and fractionated as described for Fig. 1. As shown in Fig. 3*C*, CGP57380 effectively abolished association of TNF α mRNA with polysomes.

SHIP1 Activation Is Sufficient to Inhibit Mnk1 Phosphorylation and TNF α Translation—Our data suggest that IL-10 inhibits TNF α mRNA association with polysomes via activation of SHIP1. SHIP1 is an allosterically regulated enzyme, and we have previously described a small-molecule agonist of

³ A. Ming-Lum, E. So, S. T. Cheung, C. S. Chan, E. MacCarrell, T. Chang, A. Ghanipour, P. Qasimi, L. M. Sly, S. W. Chung, R. J. Anderson, C. J. Ong, G. Krystal, and A. L.-F. Mui, unpublished data.

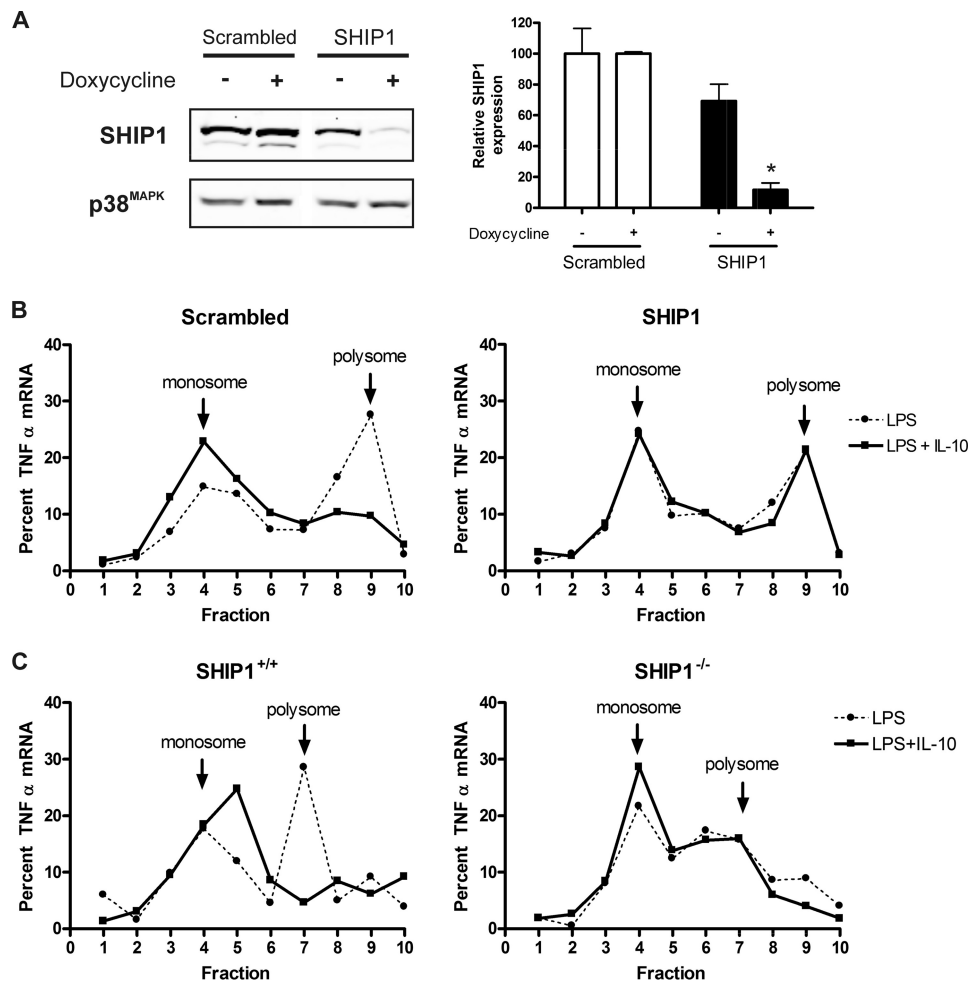


FIGURE 2. IL-10 regulates TNF α translation through SHIP1 in pre-activated cells. *A*, siRNA-inducible knockdown of SHIP1. Stably transduced cells carrying siRNA constructs of SHIP1 or a scrambled sequence under a doxycycline-inducible promoter, and the level of SHIP1 expression was assessed by immunoblotting and densitometric analysis after 48 h of induction. Protein levels were normalized against p38 MAPK, and relative SHIP1 levels were plotted as a percentage of SHIP1 from the scrambled cells. Statistical significance between treatments was calculated by Student's unpaired two-tailed *t* test with 95% confidence. *, *p* < 0.05. *B*, gradient fractionation of total mRNA from scrambled or SHIP1 siRNA knockdown pre-activated cells as described in the legend to Fig. 1. Results are representative of two experiments. *C*, gradient fractionation of total mRNA from wild-type (SHIP1^{+/+}) or SHIP1 knock-out (SHIP1^{-/-}) pre-activated peritoneal macrophages as described in the legend to Fig. 1. Results are representative of two experiments.

SHIP1 called AQX-MN100, which binds to the allosteric activation site of SHIP1 (30) and stimulates SHIP1 phosphatase activity. We thus used AQX-MN100 to determine whether SHIP1 activation alone would be sufficient to inhibit LPS-induced phosphorylation of Mnk1 and shift TNF α mRNA from polysomes to monosomes.

SHIP1^{+/+} and SHIP1^{-/-} peritoneal macrophages were treated with LPS in the absence or presence of IL-10 or AQX-MN100, and cell lysates were analyzed for Mnk1 phosphorylation as described above. As shown in Fig. 4A, both IL-10 and AQX-MN100 reduced LPS-induced Mnk1 phosphorylation in SHIP1^{+/+} cells. In fact, the inhibitory effect of AQX-MN100 was ~30% stronger than that of IL-10 (Fig. 4B). However, neither IL-10 nor AQX-MN100 was able to inhibit Mnk1 phosphorylation in SHIP1^{-/-} cells. This suggests that SHIP1 activation is required and is sufficient to inhibit Mnk1 phosphorylation. We then examined the effect of AQX-MN100 treatment on TNF α mRNA polysome assembly. Cells were treated with 1 ng/ml LPS for 45 min prior to the addition of 10 μ M AQX-MN100 or ethanol vehicle as a control for 15 min. mRNA was isolated and

subjected to sucrose density fractionation as described above. Fig. 4C shows that AQX-MN100 treatment shifted the TNF α mRNA from polysomes to monosomes, as observed with IL-10 treatment (Fig. 1).

DISCUSSION

IL-10 inhibition of TNF α production occurs at the level of transcription, mRNA stability, and translation (17, 21, 25). Studies on the mechanism by which IL-10 exerts its actions suggest that IL-10 activation of the STAT3 transcription factor and induction of STAT3-responsive gene products are sufficient for the anti-inflammatory response of IL-10 (15–19). However, we reasoned that, in a physiological setting, IL-10 target cells are usually already activated prior to exposure to IL-10, so it must also have more immediate mechanisms of action. We surveyed the occurrence of known IL-10 regulatory effects on TNF α mRNA expression when IL-10 is added after the cells are activated. As described previously by Denys *et al.* (31), IL-10 has only marginal effects on TNF α mRNA transcription if added to cells after LPS addition. However, we did find

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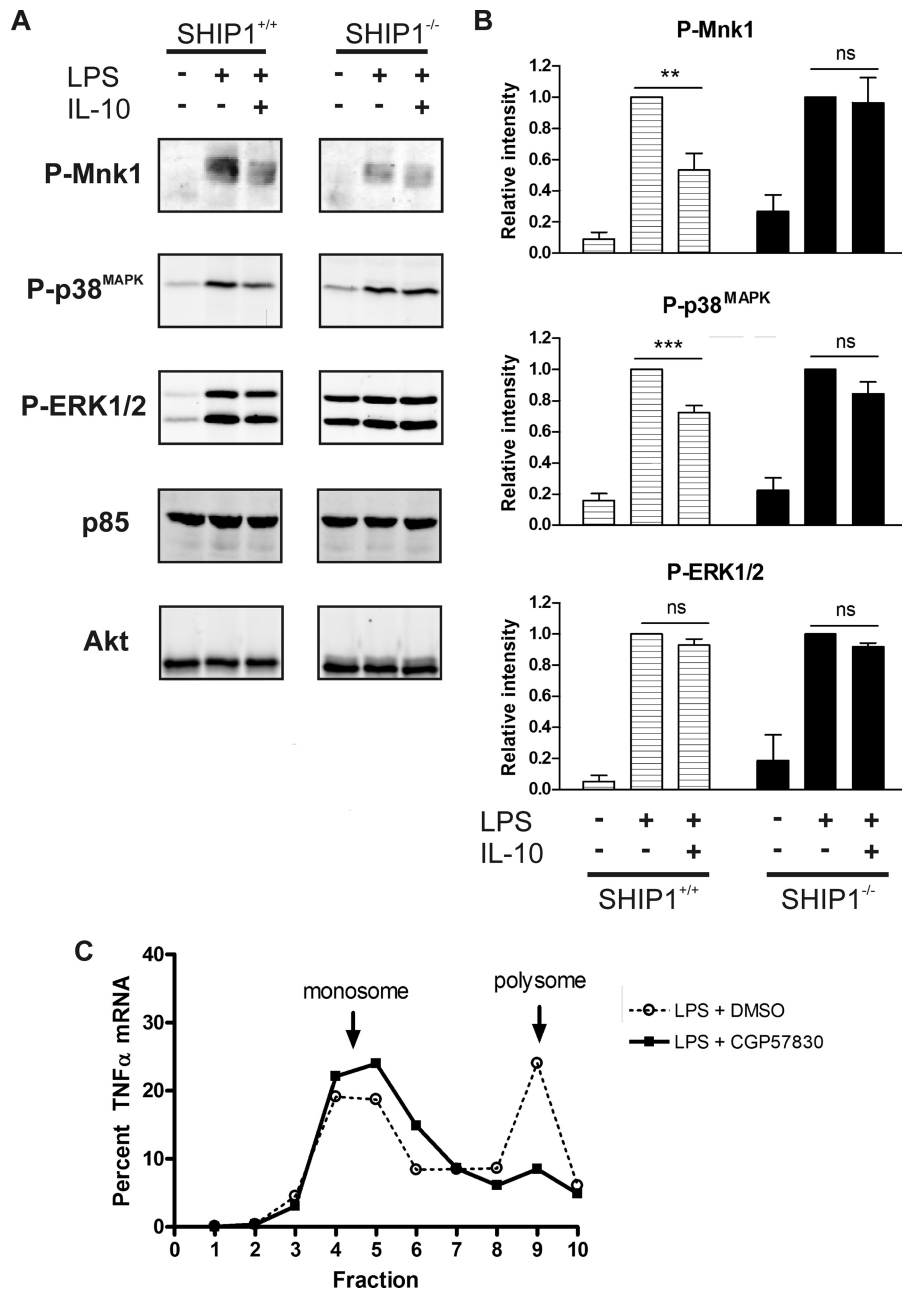


FIGURE 3. IL-10 inhibits TNF α translation through SHIP1 via Mnk1. *A*, immunoblot analysis of cell lysates from peritoneal macrophages isolated from wild-type (SHIP1^{+/+}) or SHIP1 knock-out (SHIP1^{-/-}) mice stimulated with 1 ng/ml LPS in the absence or presence of 100 ng/ml IL-10 for 1 h. Results are representative of three to five experiments. *B*, densitometric analyses were done by normalizing the intensity to Akt or PI3K p85 protein levels. The average relative intensities from four experiments were then plotted relative to the LPS-only fractions. Statistical significance between LPS \pm IL-10 samples was calculated by Student's paired two-tailed *t* test with 95% confidence. **, $p < 0.01$; ***, $p < 0.001$; ns, not significant ($p = 0.8280$ (phospho-Mnk1), $p = 0.1059$ (phospho-p38), and $p = 0.4069$ (phospho-ERK1/2) for SHIP1^{+/+} mice and $p = 0.1722$ (phospho-ERK1/2) for SHIP1^{-/-} mice). *C*, gradient fractionation of total mRNA from RAW 264.7 cells pretreated with vehicle (dimethyl sulfoxide (DMSO)) or 75 μ M Mnk1 inhibitor (CGP57830) as described in the legend to Fig. 1, except without IL-10 treatment.

that IL-10 could efficiently shift the population of TNF α mRNA from polysomes to monosomes even when IL-10 was added to cells already activated by LPS.

Kontoyiannis *et al.* (25) were the first to show that when IL-10 is added simultaneously with LPS to macrophage cells, the proportion of TNF α mRNA in polysomes is significantly reduced compared with cells treated with LPS alone. Their data also suggest that IL-10 inhibition of p38 MAPK is responsible for the decreased polysome association, a conclusion consistent with the known requirement for p38 MAPK in TNF α transla-

tion (32). Subsequently, Mnk1 was discovered to be a p38 MAPK-activated kinase required for TNF α translation in T lymphocytes (5), prompting us to examine the effect of IL-10 on Mnk1. Our data show that IL-10 inhibits the activation of Mnk1 through SHIP1. Of the two known upstream activators of Mnk1, p38 MAPK and ERK1/2, our data suggest that IL-10 inhibition of Mnk1 occurs through SHIP1-dependent inhibition of p38 MAPK activation. The outcome of the SHIP1-dependent inhibition of p38 MAPK and Mnk1 kinases is an immediate decrease in translation of existing TNF α mRNA.

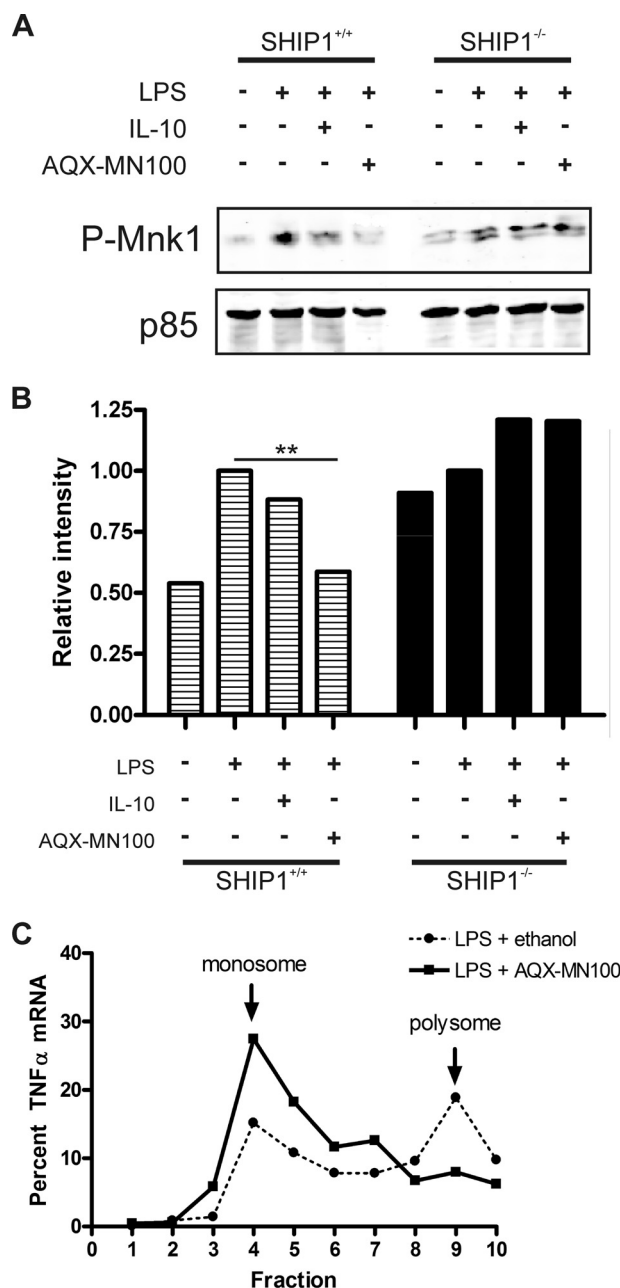


FIGURE 4. Small-molecule agonist-activated SHIP1 inhibits TNF α translation through Mnk1. *A*, immunoblot analysis of peritoneal macrophage cell lysates isolated from wild-type (SHIP1^{+/+}) or SHIP1 knock-out (SHIP1^{-/-}) mice stimulated with 10 ng/ml LPS in the absence or presence of 100 ng/ml IL-10 or 10 μ M AQX-MN100 for 1 h. Results are representative of two experiments. *B*, densitometric analysis of phospho-Mnk1 levels in *A* normalized to the levels of PI3K p85 protein and plotted relative to the LPS-only samples. Statistical significance between treatments was calculated by one-way analysis of variance with 95% confidence. **, $p < 0.01$. *C*, gradient fractionation of total mRNA from pre-activated RAW 264.7 cells as described in the legend to Fig. 1, except with 10 μ M AQX-MN100 treatment instead of IL-10 treatment.

It is important to identify the Mnk1 substrate whose activity is attenuated by IL-10 treatment. The RNA-binding proteins eIF4E and heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) have been described as being Mnk1 substrates in macrophage or T cells and have been described to have a role in regulating TNF α translation (5, 26). The translation initiation factor eIF4E binds to the 5'-cap structure of mRNAs and the

scaffold protein eIF4G to form a translation preinitiation complex along with the small ribosomal subunit (33). Although we and others (26, 27, 34) found that LPS induces Mnk1-dependent phosphorylation of eIF4E at Ser-209, we did not observe IL-10 inhibition of eIF4E phosphorylation (supplemental Fig. 1). However, the role of Ser-209 phosphorylation in eIF4E function is not clear (8, 35). It has been suggested that the phosphorylation of eIF4E is required for oncogenic transformation in cancer cells rather than regulating translation in normal cells, where it appears to be dispensable during normal development (36–38). eIF4E activity is also regulated through binding to the 4E-BP repressor protein to form an inactive complex (39). Phosphorylation of 4E-BP1 by Akt kinase causes release of eIF4E (40). LPS also activates the Akt pathway in macrophages (41), and although we observed increased 4E-BP1 phosphorylation upon LPS stimulation, no changes were observed with IL-10 treatment (supplemental Fig. 2), suggesting that IL-10 does not regulate eIF4E through its binding proteins. The hnRNPA1 protein binds to the TNF α mRNA ARE and has been shown to undergo phosphorylation in response to T cell receptor stimulation in the Jurkat T cell line (5). Phosphorylation reduces the affinity of binding to TNF α mRNA, but like eIF4E, the role of hnRNPA1 in translation is also still not clear (8). However, we did not observe changes in the amount of hnRNPA1 protein binding to the TNF ARE in an RNA pull-down assay of lysates from macrophages treated with LPS in the presence or absence of IL-10 (supplemental Fig. 3). Thus, we do not have evidence that hnRNPA1 is a substrate of Mnk1 in macrophages. Future studies will be directed toward identifying the Mnk1 substrate that mediates LPS-stimulated TNF α mRNA translation and whose action is inhibited by IL-10 signaling. Other potential Mnk1 substrates include ARE-binding proteins that are implicated in regulating translation. These include TIA-1 (42), TIAR (43), CUGBP2 (44), FXR1P (45), and tristetraprolin (46).

In conclusion, we have described a new mechanism by which IL-10 inhibits TNF α expression. This SHIP1-dependent inhibition of Mnk1 and TNF α translation occurs very rapidly and may represent the first response to IL-10. On the other hand, IL-10-induced, STAT3-regulated gene products mediate the subsequent long-term inhibitory effect of IL-10 on target cells. The implication that SHIP1 is involved in regulating the translation of proinflammatory genes such as TNF α also provides a new target for treating inflammatory diseases and disorders.

Acknowledgments—We thank Eva So and Sylvia Cheung for critical reading of the manuscript.

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