The neurosteroid pregnenolone promotes degradation of key proteins in the innate immune signaling to suppress inflammation

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Pregnenolone is a steroid hormone precursor that is synthesized in various steroidogenic tissues, in the brain, and in lymphocytes. In addition to serving as the precursor for other steroid hormones, pregnenolone exerts its own effect as an anti-inflammatory molecule to maintain immune homeostasis in various inflammatory conditions. Pregnenolone and its metabolic derivatives have been shown to have beneficial effects in the brain, including enhancing memory and learning, reversing depressive disorders, and modulating cognitive functions. A decreased level of pregnenolone has been observed in neuroinflammatory diseases, which emphasizes its role in neuroprotection and neuroregeneration. Although the anti-inflammatory property of pregnenolone was recognized several decades ago, its mechanism of action remains unknown. Here we report that pregnenolone promotes ubiquitination and degradation of the TLR2/4 adaptor protein TIRAP and TLR2 in macrophages and microglial cells. Pregnenolone and its metabolites suppressed the secretion of tumor necrosis factor α and interleukin-6 mediated through TLR2 and TLR4 signaling. Pregnenolone has been reported to induce activation of cytoplasmic linker protein 170, and this protein has recently been shown to promote targeted degradation of TIRAP. We observed enhanced degradation of TIRAP and TLR4 suppression by cytoplasmic linker protein 170 in the presence of pregnenolone. Our experimental data reveal novel nongenomic targets of pregnenolone and provide important leads to understand its role in restoring immune homeostasis in various inflammatory conditions.

Pregnenolone is synthesized from cholesterol in steroidogenic tissues such as the adrenal gland, gonads, and placenta by the mitochondrial enzyme CYP11A1 (1, 2). It is also synthesized as a neurosteroid in the central and peripheral nervous system, mainly in glial cells and neurons (3). In the brain, pregnenolone and its metabolic derivatives, such as pregnenolone sulfate, allopregnanolone, and dehydroepiandrosterone (DHEA), have been shown to enhance learning and memory, relieve depression, and modulate cognitive functions of the brain (4). Pregnenolone and its metabolites have a neuroprotective role in various neuroinflammatory diseases, including Alzheimer's disease (AD)[4] and multiple sclerosis (MS), and in neuropsychiatric disorders such as schizophrenia, depression, and autism (5–7). Pregnenolone is also capable of inhibiting cannabis intoxication by blocking cannabinoid receptor–induced intracellular signaling pathways (8). In addition to brain and classical steroidogenic tissues, lymphocytes have also been reported to synthesize pregnenolone, which induced immunosuppression (9).

Pregnenolone has previously been considered an inactive steroid without any biological function by itself other than serving as the precursor for other steroid hormones. However, its anti-inflammatory and anti-fatigability properties were already recognized in the 1930s (10, 11). Pregnenolone has been in use as an anti-inflammatory agent and as a steroid for well-being for several decades (12, 13). However, the mechanism by which pregnenolone exerts its anti-inflammatory property remains unknown. In general, steroids exhibit anti-inflammatory properties by binding to their intracellular receptors. Subsequently, these receptors translocate to the nucleus to negatively regulate genes associated with the secretion of inflammatory cytokines (14). Progesterone, which is the metabolic derivative of pregnenolone, has been reported to exert its effects through progesterone receptors (15, 16). However, a specific receptor for the genomic action of pregnenolone has so far not been identified. Studies have revealed some of the nongenomic targets of pregnenolone, such as microtubule-associated protein 2 (MAP-2) and cytoplasmic linker protein 170 (CLIP170) (17, 18). Pregnenolone interacts with MAP-2, which enhances microtubule

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[4] The abbreviations used are: AD, Alzheimer's disease; MS, multiple sclerosis; TLR, Toll-like receptor; LPS, lipopolysaccharide; Pam3CSK4, Pam3CysSerLys4; TNF, tumor necrosis factor; BMDM, bone marrow-derived macrophage; IL, interleukin; IFN, interferon; HEK, human embryonic kidney; HA, hemagglutinin; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; Aβ, β-amyloid; HRP, horseradish peroxidase; TIRAP, Toll/IL-1 receptor domain–containing adapter protein; esiRNA, endoribonuclease-prepared siRNA.

polymerization and facilitates neural growth and plasticity (17). CLIP170 is a microtubule plus end–tracking protein involved in various cellular processes, including regulation of microtubule dynamics, cell migration, mitosis, and intracellular transport. Pregnenolone has been reported to activate autoinhibited CLIP170, which promotes microtubule polymerization and cell migration in zebrafish embryos and mammalian cells (18).

Although limited experimental data are available regarding how pregnenolone modulates microtubule dynamics, the targets of its anti-inflammatory properties remain obscure. Toll-like receptors (TLRs) are crucial components of innate immunity, and their aberrant activation leads to various inflammatory disorders. TLR2 and TLR4, which detect a wide range of pathogen-associated molecular patters and endogenous ligands, play a crucial role in various inflammatory diseases. Here we report that pregnenolone promotes the degradation of key proteins in the TLR signaling pathway. Pregnenolone induced targeted ubiquitination and degradation of the TLR2/4 adaptor protein TIRAP and also of TLR2. TIRAP functions as the bridging adaptor that recruits MyD88 to TLR2/4 receptors. Pregnenolone-induced degradation of TIRAP and TLR2 resulted in attenuation of LPS or Pam3CysSerLys4 (Pam3CSK4) mediated secretion of TNFα in macrophages and microglial cells. We previously reported that CLIP170 induces ubiquitination and degradation of TIRAP (19). Pregnenolone induced activation of CLIP170, which enhanced its ubiquitin ligase–like property toward TIRAP and suppression of LPS-induced TLR4 signaling. Our findings provide novel insights into the mechanism by which pregnenolone suppresses activated macrophages to curb the aberrant inflammatory responses in various conditions.

Results

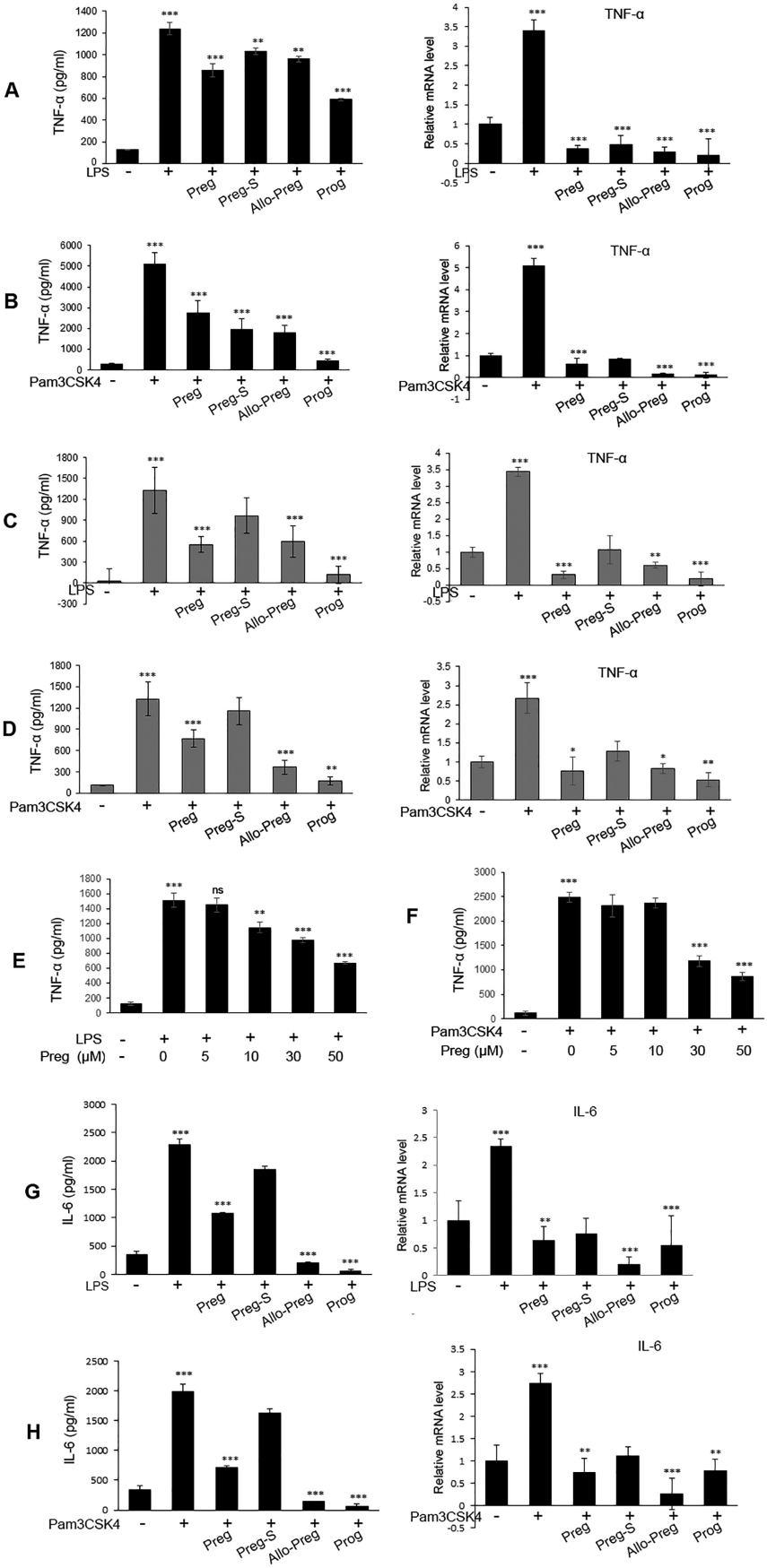
Pregnenolone suppresses LPS and Pam3CSK4-induced TNFα and IL-6 in macrophages and microglial cells

Pregnenolone is known to exhibit anti-inflammatory properties other than modulating microtubule dynamics. We investigated the effect of pregnenolone and its metabolic derivatives, such as pregnenolone sulfate, allopregnanolone, and progesterone, on secretion of TNFα by mouse and human macrophages and mouse microglial cells. RAW264.7 cells, bone marrow–derived macrophages (BMDMs), THP1 cells, or N9 microglial cells were treated with pregnenolone or its metabolites, followed by induction with LPS or Pam3CSK4 and quantification of TNFα by ELISA and quantitative PCR. Pregnenolone, allopregnanolone, and progesterone significantly suppressed LPS- or Pam3CSK4-induced TNFα in macrophages as well as in microglial cells (Fig. 1, *A–D*, and Fig. S1, *A* and *B*). Treatment of RAW264.7 macrophages with increasing concentrations of pregnenolone suppressed TNFα secretion in a dose-dependent manner (Fig. 1, *E* and *F*). Pregnenolone, allopregnanolone, and progesterone could also suppress IL-6 in LPS- or Pam3CSK4-induced RAW264.7 cells (Fig. 1, *G* and *H*). We did not observe suppression of LPS-induced IFN-β in RAW264.7 cells, which indicates that pregnenolone or it metabolites do not affect the MyD88-independent pathway of TLR4 signaling (Fig. S2*A*). Next we analyzed the expression levels of *TIRAP* and *MyD88* in RAW264.7 cells treated with pregnenolone or its metabolites. We observed down-regulation of *TIRAP* expression in cells treated with allopregnenolone or progesterone (Fig. S2*B*). Down-regulation of pro-inflammatory mediators by progesterone has been reported upon stimulation with LPS (16). Other compounds did not affect the expression of *TIRAP* or *MyD88* (Fig. S2, *B* and *C*). Taken together, our experimental data suggest that pregnenolone and its metabolites can efficiently suppress TLR2- and TLR4-mediated pro-inflammatory signaling.

Pregnenolone promotes enhanced degradation of TIRAP and TLR2

TIRAP serves as the bridging adaptor that recruits myD88 to membrane-bound TLR2 and TLR4 to initiate the signaling cascade (20). Targeted degradation of TIRAP is reported to occur in immune cells, which negatively regulates TLR2/4 signaling to maintain cellular homeostasis after their stimulation. The suppressor of cytokine signaling 1 protein has been reported to play a key role in this process; it induces targeted ubiquitination and proteasome-mediated degradation of TIRAP (21). Recently, our studies have revealed that the microtubule plus end–binding protein CLIP170 also promotes enhanced ubiquitination and degradation of TIRAP, which negatively regulates TLR2/4 signaling. CLIP170 has been shown to exist in a circular autoinhibited conformation to prevent its nonproductive interaction with other proteins (22). Pregnenolone has been reported to interact with CLIP170, which relieves its autoinhibition and promotes its activity. Therefore, we sought to examine whether treatment of cells with pregnenolone induces degradation of TIRAP. We overexpressed FLAG-TIRAP in HEK293T cells by transient transfection, followed by treatment of cells with various concentrations of pregnenolone, pregnenolone sulfate, allopregnenolone, progesterone, and the precursor of pregnenolone, cholesterol, for 1 h. Next, the cells were lysed and subjected to immunoblotting to detect the levels of FLAG-TIRAP. We observed enhanced degradation of FLAG-TIRAP with increasing concentrations of pregnenolone (Fig. 2*A*). Treatment with pregnenolone sulfate or allopregnenolone induced slight degradation, whereas progesterone or cholesterol did not affect the level of FLAG-TIRAP (Fig. 2, *A* and *B*). Next, we analyzed the degradation of TIRAP in the presence of pregnenolone *in vitro*. The lysates of HEK293T cells overexpressing FLAG-TIRAP were mixed with pregnenolone, followed by analysis of the levels of FLAG-TIRAP at various time points. We observed enhanced degradation of FLAG-TIRAP with increasing time in the presence of pregnenolone (Fig. 2*C*). Degradation of FLAG-TIRAP was not observed in cells co-treated with pregnenolone and the proteasome inhibitor MG132 (Fig. 2*D*). To examine whether pregnenolone-induced degradation is specific for TIRAP, we examined the degradation of MyD88, which serves as the sorting adaptor for TLR2/4 signaling. HEK293T cells overexpressing FLAG-MyD88 were treated with various concentrations of pregnenolone or its derivatives. We did not observe degradation of FLAG-MyD88 in the presence of pregnenolone or its metabolic derivatives (Fig. 2, *E* and *F*).

To further confirm the pregnenolone-induced degradation of TIRAP, we analyzed the endogenous level of TIRAP in


A
TNF-α (pg/ml)
LPS
Preg
Preg-S
Allo-Preg
Prog
TNF-α
Relative mRNA level
B
Pam3CSK4
C
D
E
ns
Preg (μM)
F
G
IL-6
H

RAW264.7, mouse primary peritoneal macrophages and microglial cells. Cells were treated with increasing concentrations of pregnenolone or pregnenolone sulfate, followed by detection of endogenous levels of TIRAP and MyD88 by immunoblotting. Pregnenolone promoted the degradation of endogenous TIRAP in both macrophages and microglial cells, whereas it did not affect the level of MyD88 (Fig. 2, *G* and *H*, and Fig. S3). As observed before, pregnenolone sulfate induced slight degradation of TIRAP (Fig. 2, *G* and *H*). The experimental data clearly indicate that pregnenolone promotes enhanced degradation of TIRAP in mammalian cells.

Pregnenolone attenuated LPS or Pam3CSK-induced signaling, which are mediated by TLR4 and TLR2 receptors, respectively. Next, we analyzed whether pregnenolone promotes degradation of TLRs. HEK293T cells overexpressing FLAG-tagged TLR2, TLR4, or TLR9 were treated with pregnenolone, followed by detection of FLAG-tagged proteins. Pregnenolone induced degradation of FLAG-TLR2, but it did not affect the level of FLAG-TLR4 or FLAG-TLR9 (Fig. 3*A*). To further examine the degradation of TLR2, HEK293T cells overexpressing FLAG-TLR2 were treated with pregnenolone or its metabolic derivatives, followed by detection of FLAG-tagged proteins by immunoblotting. We observed enhanced degradation of FLAG-TLR2 with pregnenolone, pregnenolone sulfate, and allopregnanolone, whereas progesterone or cholesterol did not affect the level of FLAG-TLR2 (Fig. 3, *B* and *C*). Further, we analyzed the degradation of FLAG-TLR2 *in vitro*, which indicated enhanced degradation of FLAG-TLR2 in pregnenolone-treated samples (Fig. 3*D*). Next, we examined the degradation of endogenous TLR2 in macrophages and microglial cells. RAW264.7 or N9 microglial cells were treated with increasing concentrations of pregnenolone, followed by detection of TLR2 by immunoblotting. Enhanced degradation of TLR2 was observed with pregnenolone, especially at the higher concentration (Fig. 3, *E* and *F*). These studies indicate that, in addition to TIRAP, pregnenolone also targets TLR2 for degradation.

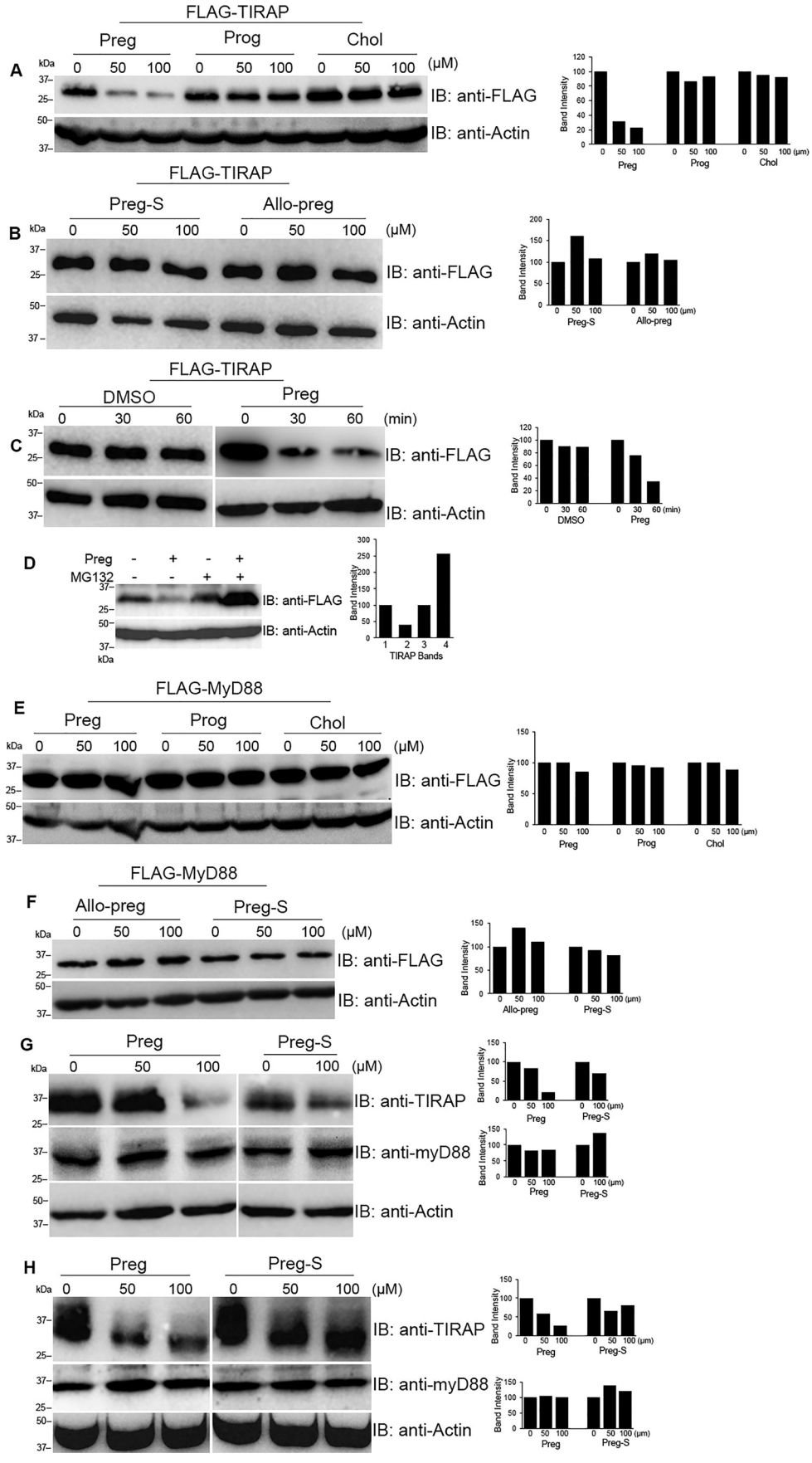
In eukaryotes, ubiquitination of substrate protein is a prerequisite for proteasome-mediated degradation. Therefore, we analyzed whether pregnenolone enhances the ubiquitination TIRAP or TLR2. HEK293T cells were co-transfected with FLAG-tagged TIRAP or TLR2 and HA-ubiquitin, followed by treatment of cells with pregnenolone. Next, FLAG-TIRAP or FLAG-TLR2 were immunoprecipitated, followed by immunoblotting with anti-HA antibody to detect the ubiquitin-conjugated substrates. We observed enhanced ubiquitination of FLAG-TIRAP and FLAG-TLR2 in the presence of pregnenolone (Fig. 3, *G* and *H*). The experimental data indicate that pregnenolone promotes ubiquitination of TIRAP and TLR2 to induce its degradation.

Pregnenolone enhances the effects of CLIP170

Pregnenolone has been shown to interact with the coiled-coil domain of CLIP170, which relieves its autoinhibition and enhances its interaction with microtubule resulting CLIP170-dependent microtubule polymerization and microtubule abundance in zebrafish embryos (18). We examined the pregnenolone-induced activation of CLIP170 by FRET analysis. HEK293T cells were transfected with plasmids harboring YFP-CLIP170-CFP, YFP-CLIP170-K1-CFP, or CFP-YFP tandem. A plasmid harboring YFP-CLIP170-K1-CFP served as the negative control, where a mutation in the first zinc knuckle of CLIP170 abolished the interaction between NH_2 and C termini, whereas CFP-YFP served as the positive control. Next, the cell lysates were incubated with pregnenolone or vehicle, followed by analysis of fluorescent spectra of the lysates. The samples were excited at 425 nm, where the CFP acted as a donor, and YFP served as an acceptor. The cell lysate that expressed CFP-YFP tandem exhibited marked acceptor emission after donor excitation because of FRET (Table S1). The cell extract that contained the YFP-CLIP170-CFP fusion exhibited a higher ratio of fluorescence at acceptor emission compared with YFP-CLIP-170-K1-CFP. The ratio of YFP to CFP fluorescence emission was decreased upon treatment of the YFP-CLIP170-CFP lysate with pregnenolone compared with the lysates treated with DMSO vehicle control (Table S1). The experimental data suggest that pregnenolone relieves the folded-back state of CLIP170 that separates the NH_2 and C termini, thereby decreasing the ratio of YFP to CFP fluorescence emission.

We previously reported that CLIP170 promotes enhanced ubiquitination and degradation of TIRAP, which attenuated TLR2/4-mediated signaling (19). Because pregnenolone induces activation of CLIP170, we sought to analyze whether pregnenolone enhances the TIRAP degradation and TLR suppression properties of CLIP170. RAW264.7 macrophages overexpressing FLAG-TIRAP and MYC-CLIP170 were treated with pregnenolone or DMSO followed by immunoblotting to detect the level of FLAG-TIRAP. The degradation of FLAG-TIRAP appeared to be enhanced further in the presence of pregnenolone in MYC-CLIP170- and FLAG-TIRAP– overexpressing cells (Fig. 4*A*). We observed that pregnenolone promoted enhanced ubiquitination of TIRAP (Fig. 3*G*). CLIP170 has been reported to exhibit a ubiquitin ligase–like property toward TIRAP (19). Because pregnenolone activates CLIP170, we wished to examine whether pregnenolone enhances the ubiquitin ligase property of CLIP170. HEK293T cells were co-transfected with FLAG-TIRAP, MYC-CLIP170, and HA-ubiquitin in various combinations, followed by treatment of cells with pregnenolone. Cells overexpressing CLIP170, which was treated with pregnenolone, exhibited more FLAG-TIRAP ubiquitination compared with DMSO-treated samples (Fig. 4*B*).

Figure 1. Pregnenolone and its metabolic derivatives attenuate LPS- or Pam3CSK4-induced secretion of TNFα. *A–D*, levels of secreted TNFα in RAW264.7 (*A* and *B*) or differentiated human THP1 cells (*C* and *D*) that were treated with pregnenolone or its metabolic derivatives (50 μM). Pregnenolone (*Preg*) or its metabolites significantly suppressed LPS-induced (*A* and *C*) or Pam3CSK4-induced (*B* and *D*) TNFα in mouse and human macrophages. *Preg-S*, pregnenolone sulfate; *Allo-Preg*, allopregnenolone; *Prog*, progesterone. *E* and *F*, levels of secreted TNFα in RAW264.7 cells that were treated with increasing concentrations of pregnenolone. Pregnenolone suppressed LPS-induced (*E*) or Pam3CSK4-induced (*F*) secretion of TNFα in a dose-dependent manner. *G* and *H*, suppression of LPS-induced (*G*) or Pam3CSK4-induced (*H*) IL-6 by pregnenolone or its metabolites in RAW264.7 cells. Data are presented as mean ± S.D. from three independent experiments. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; *ns*, not significant.


FLAG-TIRAP
Preg
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A
kDa
0 50 100 0 50 100 0 50 100 (μM)
37–
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IB: anti-FLAG
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Preg
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Preg-S
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B
IB: anti-FLAG
IB: anti-Actin
Preg-S
Allo-preg
FLAG-TIRAP
DMSO
Preg
0 30 60 0 30 60 (min)
C
IB: anti-FLAG
IB: anti-Actin
D
Preg - + - +
MG132 - - + +
IB: anti-FLAG
IB: anti-Actin
TIRAP Bands
FLAG-MyD88
E
IB: anti-FLAG
IB: anti-Actin
FLAG-MyD88
F
Allo-preg
Preg-S
IB: anti-FLAG
IB: anti-Actin
G
Preg
Preg-S
IB: anti-TIRAP
IB: anti-myD88
IB: anti-Actin
H
Preg
Preg-S
IB: anti-TIRAP
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This suggests that pregnenolone-induced activation of CLIP170 enhances its ubiquitin ligase property toward TIRAP.

Next, we analyzed CLIP170-induced suppression of TLR4 signaling in the presence of pregnenolone. RAW264.7 cells were transfected with the CLIP170 expression plasmid or CLIP170 endoribonuclease-prepared siRNA (esiRNA), followed by treatment with pregnenolone. Cells were then induced with LPS, and we quantified the secreted TNFα. An enhanced level of TNFα suppression was observed by CLIP170 in the presence of pregnenolone (Fig. 4*C*). The effect of pregnenolone was reduced in CLIP170-silenced macrophages, as an elevated level of TNFα was observed in CLIP170-silenced macrophages compared with the nontargeting control (Fig. 4*D*). The experimental data suggest that pregnenolone enhances the TLR4 suppression property of CLIP170 by promoting its activation.

Discussion

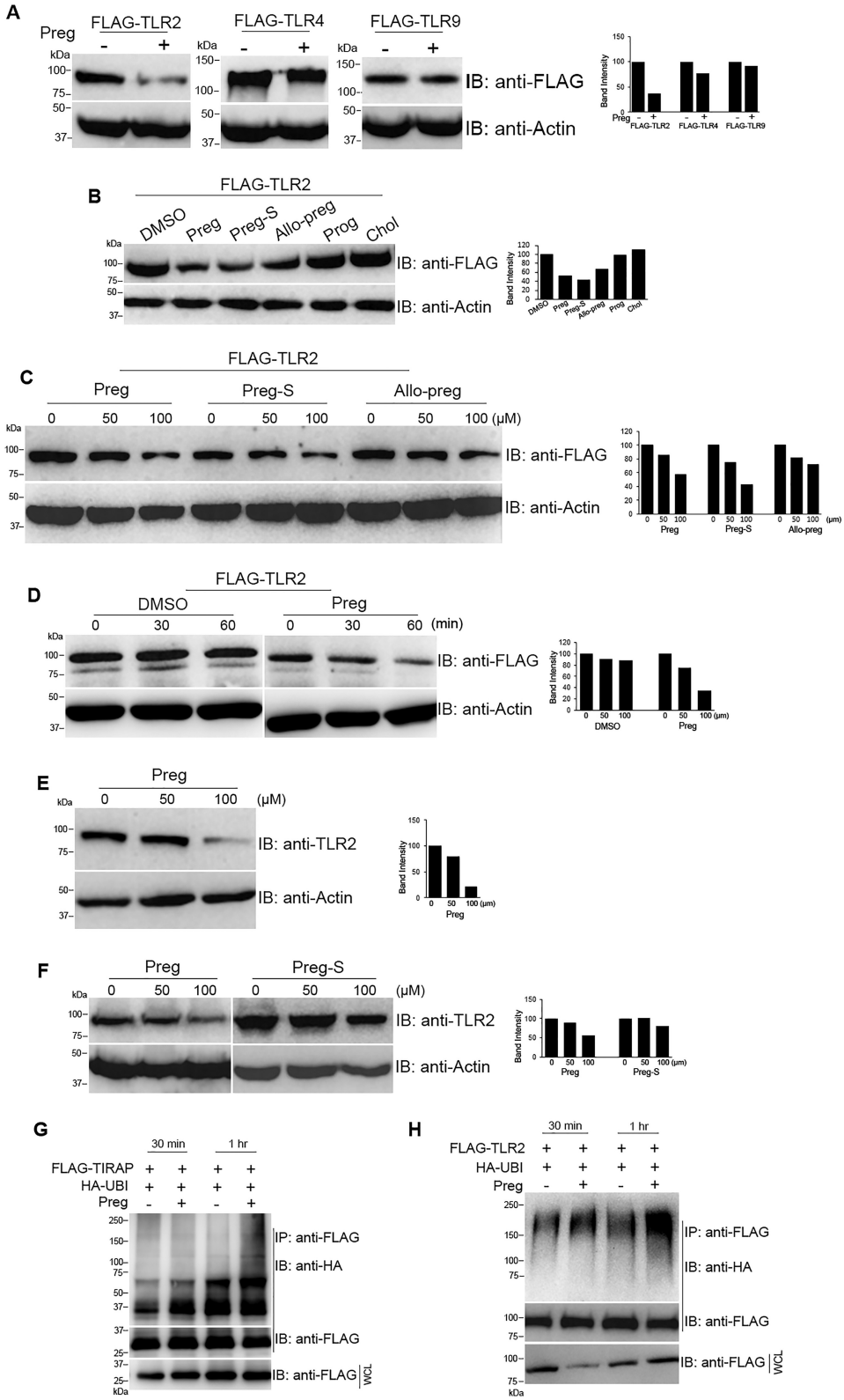
In addition to acting as the precursor of other hormones, pregnenolone has its own biological effects. Pregnenolone has been shown to exhibit anti-inflammatory properties, and its role in preventing neuroinflammation and enhancing neuroprotection has been well documented (4, 7). Metabolites of pregnenolone, such as pregnenolone sulfate and allopregnanolone, have also been reported to modulate neuroinflammation and promote neuroprotection. Preclinical studies have shown that pregnenolone exhibits antidepressant properties, improves memory and cognitive functions, controls pain and stress, and relieves symptoms of mood disorder. However, the molecular mechanism behind the anti-inflammatory and neuroprotective functions of pregnenolone remains largely unknown. Glucocorticoids exert their immunosuppressive effect by binding to the glucocorticoid receptor (23). However, pregnenolone is believed to use a glucocorticoid receptor–independent mechanism to suppress the inflammatory response, as mifepristone, which antagonizes the glucocorticoid receptor, did not affect the immunosuppressive property of pregnenolone (24). In contrast, mifepristone, which also blocks the progesterone receptor, has been shown to neutralize the anti-inflammatory property of progesterone in BV2 microglial cells (16).

CLIP170 has been identified as one of the subcellular targets of pregnenolone (18). CLIP170 exist in a circular autoinhibited state by intramolecular interaction between its N-terminal cytoskeleton-associated protein-glycine-rich (CAP-Gly) domain and the C-terminal zinc finger domain (22). This conformational state prevents nonproductive protein–protein interactions. Proteins that interact with CLIP170 induce an open extended conformation of CLIP170 that promotes its binding to microtubules. Pregnenolone has been reported to bind to CLIP170, which induces its open extended conformational change, resulting in its binding to microtubules followed by enhanced microtubule polymerization and cell migration (18). Our previous studies revealed that CLIP170 promotes enhanced degradation of the TLR2/4 adaptor protein TIRAP, which attenuates pro-inflammatory responses mediated by TLR2 and TLR4 (19). Pregnenolone is capable of suppressing pro-inflammatory cytokines induced by LPS and Pam3CSK4 in macrophages and microglial cells. The neuroprotective role of pregnenolone is attributed mainly to its anti-inflammatory property. Here we demonstrate that pregnenolone induces ubiquitination and subsequent degradation of TIRAP and TLR2, which lead to attenuation of TLR2- and TLR4-mediated secretion of TNFα and IL-6. Pregnenolone-induced activation of CLIP170 resulted in enhanced degradation of TIRAP and TLR4 suppression by CLIP170. Based on our findings, it can be envisaged that the neuroprotective role of pregnenolone may be attributed to its ability to eliminate key proteins in the TLR signaling pathway through CLIP170. CLIP170 has been reported to have a role in neuronal development, and a defect in *CLIP170* has been reported in autosomal recessive intellectual disability (25).

TLR2 and TLR4, which detect various microbial and endogenous ligands, play a major role in the inflammation of the central nervous system. Microglial cells, which are residential macrophages in the brain, express TLR2 and TLR4, and they respond to various pathogen-associated molecular patters and endogenous ligands, leading to their polarization to either the M1 or M2 phenotype. The M1 phenotype is destructive, which is characterized by the secretion of various pro-inflammatory cytokines, induction of apoptosis of neuronal cells, and suppression of neurogenesis (26). M2-polarized microglial cells are anti-inflammatory, and they exert various neuroprotective responses, including scavenging of tissue debris and tissue repair and remodeling (27). TLR4-mediated activation of microglial cells is neurotoxic and contributes to the pathophysiology of many neurodegenerative diseases, including AD,

Figure 2. Pregnenolone induces degradation of TIRAP. *A* and *B*, immunoblotting of HEK293T cells transfected with FLAG-TIRAP and treated with the indicated concentrations of pregnenolone (*Preg*) or its metabolic derivatives or its precursor, cholesterol (*Chol*). Pregnenolone induced degradation of FLAG-TIRAP, whereas progesterone (*Prog*) or cholesterol did not affect the level of FLAG-TIRAP in the treated cells. A slight degradation of FLAG-TIRAP was detected in cells treated with pregnenolone sulfate or allopregnenolone at higher concentration. *IB*, immunoblot; Allo-Preg, allopregnanolone; *Preg-S*, pregnenolone sulfate. *C*, degradation of FLAG-TIRAP *in vitro*. HEK293T cells transfected with FLAG-TIRAP were lysed, and the lysates were treated with pregnenolone or DMSO for the indicated time, followed by immunoblotting and detection of FLAG-TIRAP using anti-FLAG antibody. Pregnenolone induced degradation of FLAG-TIRAP with increasing time. The FLAG-TIRAP level was not affected in DMSO-treated cells. *D*, pregnenolone does not induce degradation of TIRAP in the presence of MG132. HEK293T cells were transfected with FLAG-TIRAP, followed by co-treatment with pregnenolone/DMSO/MG132, as indicated. FLAG-TIRAP was detected by immunoblotting as described before. The increased intensity of the FLAG-TIRAP band in the presence of MG132 and pregnenolone could be due to enhanced ubiquitination of TIRAP. *E* and *F*, pregnenolone did not induce degradation of MyD88. Shown is immunoblotting of HEK293T cells transfected with FLAG-MyD88 and treated with pregnenolone, its metabolic derivatives, or cholesterol. Degradation of FLAG-MyD88 was not detected in cells treated with pregnenolone or its metabolic derivatives. *G* and *H*, pregnenolone induces degradation of endogenous TIRAP in macrophages and microglial cells. Shown is immunoblotting of RAW264.7 (*G*) or N9 microglial cells (*H*) treated with pregnenolone or pregnenolone sulfate. Pregnenolone induced degradation of endogenous TIRAP in both macrophages and microglial cells, whereas it did not affect the level of MyD88. As observed before, a slight degradation of TIRAP was observed in the presence of pregnenolone sulfate. The immunoblots in *C*, *D*, and *H* are representative of two independent experiments. The other immunoblots are representative of three independent experiments. Actin served as the loading control. The *right panels* of the immunoblots show densitometry analysis of the represented immunoblots, where the test protein bands were normalized to actin.

which is caused by deposition of β-amyloid peptides (Aβ) and subsequent damage to neurons (28–30). Deficiency of TLR4 strongly inhibits Aβ-induced microglial activation, resulting in decreased levels of pro-inflammatory cytokines (31). TIRAP is required for the MyD88-dependent pathway of TLR4 signaling, and it positively regulates M1 polarization of microglial cells that are treated with LPS/IFN-γ (32). Silencing of TIRAP in microglial cells attenuates expression of M1-related pro-inflammatory cytokines. Our experimental data suggest that enhanced ubiquitination and degradation of TIRAP by preg-



nenolone can suppress TLR4-induced inflammatory responses in microglial cells.

Compared with other TLRs, TLR2 is ubiquitously expressed in various cell types, and it can detect a wide range of microbial components and endogenous ligands, leading to secretion of pro-inflammatory cytokines and chemokines (33, 34). Studies have shown that TLR2 can detect Aβ peptides, which triggers M1 polarization of microglial cells, and the resulting neurotoxicity contributes to the pathogenesis of AD (35). Deficiency of TLR2 results in alternative M2-type activation of microglial cells, which suppresses inflammation and improves neuroprotection by phagocytosis of Aβ aggregates (29). Stimulation of TLR2 on CD4+ T cells has been shown to induce its differentiation to TH17 cells, which secrete various pro-inflammatory cytokines, which contributes to the pathogenesis of MS (34). In agreement with this, C57BL6 mice deficient in TLR2 are protected from induction of experimental autoimmune encephalomyelitis (36). Also, up-regulation of TLR2 has been reported in MS brain lesions (37). Given that pregnenolone induces degradation of TLR2 and its adaptor molecule TIRAP, it could prevent destructive M1-type activation of microglial cells, which can promote neuroprotection and neuroregeneration. Pregnenolone has been reported to have a protective role in AD, and a lower level of pregnenolone sulfate has been reported in AD patients (5).

Recent studies have revealed pregnenolone as a lymphosteroid that regulates the action of lymphoid tissues (9). Pregnenolone has been reported to be actively synthesized in T helper 2 cells, which suppresses T helper cell proliferation and B cell class switching to restore immune homeostasis in a helminth infection model (9). Up-regulation of Cyp11A, which synthesizes pregnenolone from cholesterol, has been reported in IL-4 – differentiated CD4+ and CD8+ T cells. Cyp11A1 promotes conversion of IFN-γ–producing CD8+ T cells to IL-13–producing cells in allergic lung disease (38). However, the mechanism by which pregnenolone or its metabolites regulate the polarization of CD8+ T cells remains obscure.

In summary, we identified new nongenomic targets of pregnenolone that may contribute to its anti-inflammatory and neuroprotective properties. We assume that pregnenolone induces targeted ubiquitination and degradation of TLR2 and TIRAP through the activation CLIP170. Pregnenolone and its metabolic derivatives have many beneficial roles in neurodegenerative diseases and psychiatric disorders. Our findings provide crucial leads for understanding the underlying mechanism of pregnenolone-mediated neuroprotection and immunosuppression, which may ultimately help to develop novel treatment strategies for various immune and inflammatory disorders.

Experimental procedures

Cell culture and transfection

Human embryonic kidney (HEK) 293T (ATCC) or RAW264.7 macrophages (ATCC) were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), 1× penicillin–streptomycin solution (Gibco), and 100 μg/ml normocin (Invivogen). RPMI 1640 medium (Sigma) supplemented with 10% fetal bovine serum and 1× penicillin–streptomycin solution (Gibco) was used to culture THP1 (ATCC) and N9 microglial cells (a gift from Dr. Anirban Basu, National Brain Research Centre, India). Cells were grown in a 37 °C humidified atmosphere of 5% CO_2. All transfections were performed using TurboFect (Thermo Fisher Scientific) or FuGENE HD (Promega) according to the manufacturer's instructions.

To isolate BMDMs from mice, bones were collected from the hind legs of 4- to 6-week-old C57BL6 mice, followed by isolation of bone marrow cells using a standard procedure (39). For differentiation, cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1× penicillin–streptomycin solution, and 25 ng/ml mouse colony-stimulating factor (R&D Systems). Peritoneal macrophages were isolated from the peritoneal cavity of 6-week-old C57BL6 mice using a standard procedure (40).

Stimulation of macrophages and quantification of cytokines

RAW264, BMDM, THP1, and N9 microglial cells (0.05×10^6) were seeded in 48- or 24-well plates and allowed to adhere overnight. Next, the cells were treated with various concentrations of pregnenolone, its metabolic derivatives, or DMSO alone for 1 h, followed by treatment with LPS (100 ng/ml) or Pam3CSK4 (100 ng/ml). Subsequently, the cells or culture supernatants were harvested 1 h post-induction for TNFα and IFN-β, 3 h post-induction for IL-6 in RAW264.7 and BMDM cells, 2 h post-induction for TNFα in THP1 cells, or after overnight induction in the case of N9 microglial cells. The cytokines were quantified by quantitative PCR as well as ELISA using the Quantikine ELISA kit (R&D Systems) according to the manufacturer's protocols. All assays were performed independently at least three times. Data were analyzed using Sigma Plot software, and statistical significance was determined using a *t* test.

Figure 3. Pregnenolone promotes degradation of TLR2. *A–C*, immunoblotting (*IB*) of HEK293T cells transfected with FLAG-tagged TLR2, TLR4, or TLR9 and treated with pregnenolone (*Preg*), its metabolites, or cholesterol (*Chol*). Pregnenolone promoted the degradation of FLAG-TLR2, whereas it did not affect the level of FLAG-TLR4 or FLAG-TLR9 (*A*). Enhanced degradation of FLAG-TLR2 was detected in the presence of pregnenolone, pregnenolone sulfate (*Preg-S*), and allopregnenolone (*Allo-preg*) (*B* and *C*). Progesterone or cholesterol did not induce degradation of FLAG-TLR2 (*B*). *D*, *in vitro* degradation of FLAG-TLR2 in the presence of pregnenolone. HEK293T cells overexpressing FLAG-TLR2 were lysed, and the lysates were treated with pregnenolone or DMSO for the indicated times. Pregnenolone induced degradation of FLAG-TLR2 with increasing time. *E* and *F*, pregnenolone induces degradation of endogenous TLR2 in macrophages. Shown is immunoblotting of RAW264.7 (*E*) or N9 microglial cells (*F*) treated with pregnenolone or pregnenolone sulfate. Pregnenolone induced degradation of endogenous TLR2 in macrophages and microglial cells. A slight degradation of TLR2 by pregnenolone sulfate was detected in microglial cells (*F*). *G* and *H*, pregnenolone induces ubiquitination of TIRAP and TLR2. The immunoblot shows immunoprecipitated FLAG-TIRAP (*G*) and FLAG-TLR2 (*H*) from HEK293T cells that were co-transfected with FLAG-tagged TIRAP or TLR2 and HA-ubiquitin (*HA-UBI*) and treated with pregnenolone. Cells treated with pregnenolone displayed enhanced ubiquitination of FLAG-TIRAP or FLAG-TLR2. All immunoblots are representative of two independent experiments. Actin was used as the loading control. The *right panels* of the immunoblots show densitometry analysis of the represented immunoblots, where the TLR2 bands were normalized to actin.

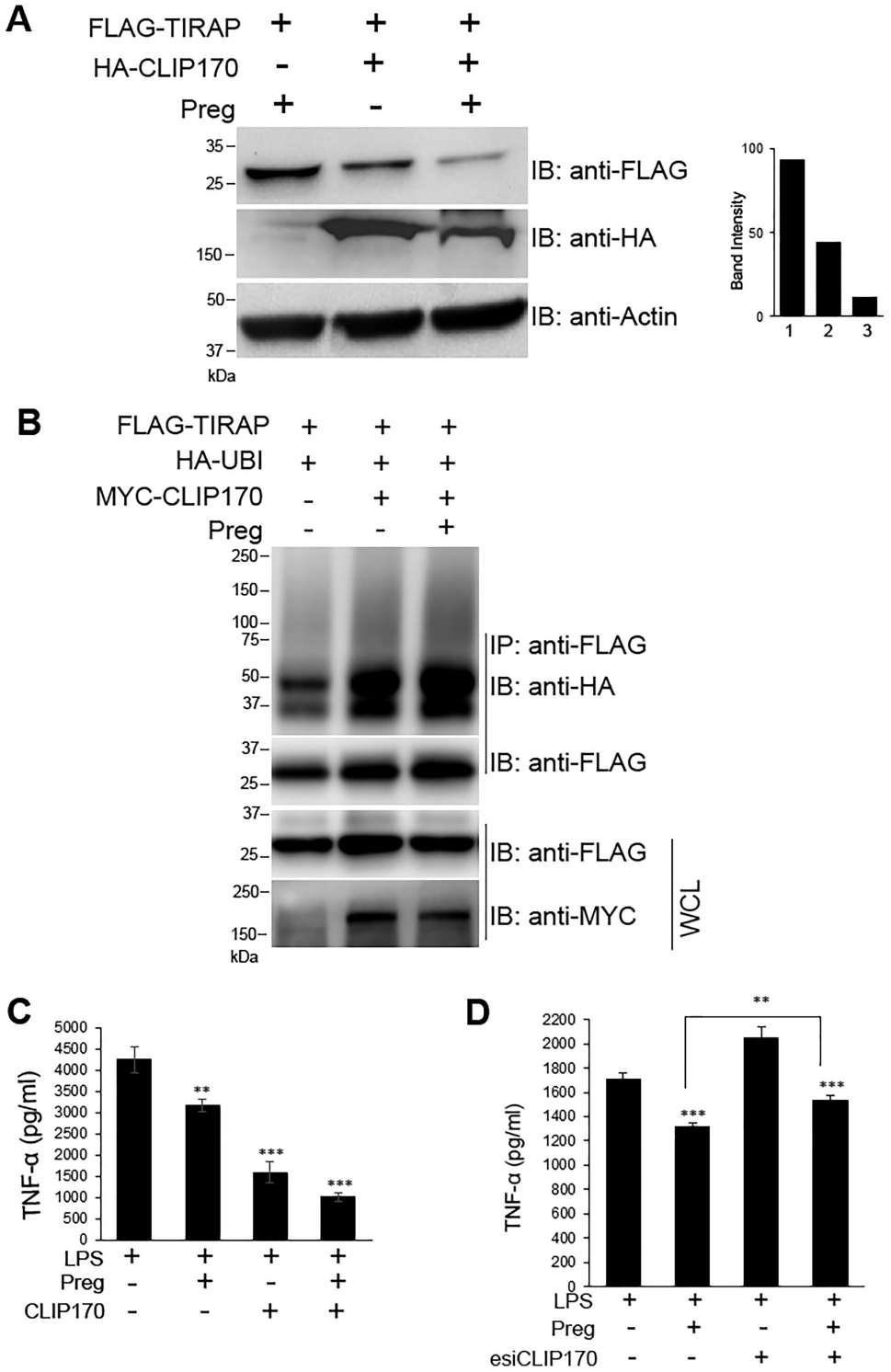


Figure 4. Pregnenolone promotes enhanced degradation of TIRAP in the presence of CLIP170. *A*, immunoblotting (*IB*) of RAW264.7 cells co-transfected with FLAG-TIRAP and MYC-CLIP170 and treated with pregnenolone (*Preg*). Enhanced degradation of FLAG-TIRAP was observed in the presence of CLIP170 and pregnenolone. *B*, pregnenolone enhances ubiquitination of TIRAP in the presence of CLIP170. The immunoblot shows immunoprecipitated FLAG-TIRAP from HEK293T cells co-transfected with FLAG-TIRAP, MYC-CLIP170, and HA-ubiquitin (*HA-UBI*). CLIP170-transfected cells exhibited enhanced ubiquitination of FLAG-TIRAP in the presence of pregnenolone. Immunoblots (*A* and *B*) are representative of two independent experiments. *C* and *D*, pregnenolone synergizes the TLR4 suppression property of CLIP170. Shown are the levels of secreted TNFα in RAW264.7 cells transfected with the CLIP170 expression plasmid or CLIP170 esiRNA, followed by treatment with pregnenolone. Enhanced suppression of TNFα was detected in CLIP170-overexpressing cells that were treated with pregnenolone (*C*). CLIP170-silenced cells exhibited slightly elevated levels of TNFα compared with cells transfected with control siRNA (*D*). Data are presented as mean $\pm$ S.D. from three independent experiments. **, $p < 0.01$; ***, $p < 0.001$.

Protein degradation experiments

To analyze the degradation of overexpressed proteins, HEK293T cells (0.5×10^6) were seeded into 12-well plates and transfected with 500 ng of FLAG-tagged TIRAP/myD88 (a gift from Dr. Douglas Golenbock), hTLR2 (a gift from Ruslan Medzhitov, Addgene plasmid13082), and TLR4/mTLR9 (a gift from Ruslan Medzhitov, Addgene plasmid 13091) plasmids. Twenty-four hours after transfection, cells were treated with various concentrations of pregnenolone or its metabolic derivatives (Sigma) or DMSO alone (vehicle control) for 1 h at 37 °C. To analyze the degradation of FLAG-TIRAP in the presence of MG132, HEK293T cells overexpressing FLAG-TIRAP were treated with MG132 (30 μm, Sigma) for 3 h, followed by treatment with pregnenolone (100 μm) or DMSO for 1 h. The cells were lysed in radioimmune precipitation assay buffer, and estimation of protein concentrations was performed using Bradford reagent (Sigma). Equal amounts of protein samples were resolved on 4–20% Tris/glycine SDS-PAGE gel (Bio-Rad) and subjected to immunoblotting. The membranes were probed with HRP-conjugated anti-FLAG antibody (Sigma) in 5% milk in TBS-Tween 20 overnight at 4 °C to detect FLAG-tagged proteins. Subsequently, the membrane was washed three times with Tris-buffered saline–Tween 20 for 5 min each and incubated with SuperSignal West Pico chemiluminescent substrate (Pierce) for 5 min, followed by acquisition of the luminescence signal using the Chemi documentation system (Syngene). The membranes were reprobed with HRP-conjugated anti-actin antibody (Sigma) to detect β-actin, which was used as the loading control. Densitometry analyses were performed using ImageJ software.

To analyze the degradation of endogenous proteins, RAW264.7 or N9 microglial cells (0.5×10^6) were seeded into 12-well plates. The next day, the cells were treated with various concentrations of pregnenolone or its derivatives at 37 °C for 1 h, followed by cell lysis and immunoblotting. The membranes were probed with anti-TIRAP or anti-MyD88 (Cell Signaling Technologies) or anti-TLR2 (Santa Cruz Biotechnology), followed by HRP-conjugated anti-mouse/anti-rabbit secondary antibody. Protein bands were detected and captured as mentioned before.

To analyze protein degradation *in vitro*, HEK293T cells (1×10^6) were transfected in a 6-well plate with 4 μg of FLAG-TIRAP or FLAG-TLR2 plasmid. Twenty-four hours after transfection, the cells were collected and resuspended in sonication buffer (7.4 mM HEPES (pH 7.4), 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, and 1.5 mM ATP). Next, the cells were sonicated for 2 min and clarified by centrifugation. The cell lysates were then incubated with 100 μM pregnenolone or DMSO at 30 °C for various times. The samples were then mixed with 2× Laemmli sample buffer and boiled for 10 min, followed by immunoblotting. The membranes were probed with HRP-conjugated anti-FLAG antibody (Sigma) to detect FLAG-tagged proteins.

Co-transfection experiments

RAW264.7 cells (0.5×10^6) were seeded into 12-well plates and co-transfected with 500 ng of FLAG-TIRAP and 1 μg of MYC-CLIP170 plasmids. Twenty-four hours after transfection, cells were treated with pregnenolone (100 μM) or DMSO for 1 h, followed by lysis of cells in radioimmune precipitation assay buffer and immunoblotting. The membranes were probed with HRP-conjugated anti-FLAG antibody (Sigma) to detect FLAG-tagged proteins and HRP-conjugated anti-MYC antibody (Sigma) to detect MYC-CLIP170. The membranes were reprobed with HRP-conjugated anti-actin antibody (Sigma) to detect β-actin, which was used as the loading control.

Ubiquitination assays

HEK293T cells (1×10^6 in 6-well plates) were co-transfected with 1.5 μg of FLAG-tagged TIRAP or TLR2 or MYC-CLIP170 and 1 μg of HA-ubiquitin (a gift from Dr. Shigeki Miyamoto) plasmids in various combinations. Total DNA concentration was maintained at 4 μg using the empty vector. Twenty hours after transfection, the cells were treated with pregnenolone (100 μm) or DMSO for various times. Cells were then washed once with PBS and lysed in 400 μl of lysis buffer containing 20 mM Tris-HCI (pH 7.4) and 1% SDS (19). Cell lysates were transferred into Eppendorf tubes and boiled for 10 min. Lysates were then clarified by centrifugation at 13,000 rpm for 15 min and diluted with buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2% Triton X-100, and 0.5% NP40. The lysates were precleared with protein G Plus–agarose beads (Santa Cruz Biotechnology) and mixed with 5 μg of anti-FLAG antibody (Sigma), followed by incubation overnight at 4 °C on a rotator. Next, protein G Plus–agarose was added to the samples and incubated further for 3 h at 4 °C on a rotator. Subsequently, agarose beads were washed three times with TNT buffer (20 mM Tris (pH8.0), 150 mM NaCl, and 1% Triton X-100), resuspended in 30 μl of SDS sample buffer (Bio-Rad), and boiled for 10 min, followed by SDS-PAGE and immunoblotting. The membrane was probed with HRP-conjugated anti-HA antibody (Sigma) to detect HA-ubiquitin–conjugated proteins. The membranes were reprobed with HRP-conjugated anti-FLAG or anti-MYC antibodies to detect FLAG-tagged and MYC-tagged proteins, respectively.

Silencing of CLIP170

RAW264.7 cells were seeded in a 24-well plate and transfected with CLIP170 esiRNA (Sigma) or nontargeting siRNA (200 ng/ml) using Dharmafect 4 transfection reagent (Dharmacon). 48 h after transfection, cells were treated with pregnenolone, followed by induction with LPS (100 ng/ml) for 1 h. The secreted levels of TNFα were quantified by ELISA.

FRET analysis

HEK293T cells (1×10^6 in 6-well plates) were transfected with eukaryotic expression constructs harboring CFP-YFP, YFP-CLIP-170-CFP, or YFP-CLIP-170-K1-CFP (a gift from Dr. Anna Akhmanova, Utrecht University, Utrecht, Netherlands) using TurboFect transfection reagent. Forty-eight hours after transfection, cells were lysed in buffer containing 20 mM Tris-HCI (pH 7.5), 100 mM NaCl, 1% Triton X-100, and 10% glycerol with a mixture of protease inhibitors (Pierce), followed by clearing the cell lysates by centrifugation at 13,000 rpm for 20 min at 4 °C. Next, the cell lysates were incubated with pregnenolone or DMSO for 15 min, and the emission spectra were measured

using a multimode microplate reader (Tecan) using excitation at 425 nm and 485 nm for CFP and YFP, respectively.

Statistical analysis

Data were analyzed using Sigma Plot software, and statistical significance was determined using a *t* test.

Ethics statement

For collection of bone marrow–derived macrophages and peritoneal macrophages, C57BL-6 mice were housed at the hired animal house facility of the National Institute of Animal Biotechnology at Teena Biolabs Private Limited (Hyderabad, India). Teena Biolabs Private Limited has been registered with the Committee for the Purpose of Control and Supervision of Experiments on Animals, Government of India (registration no. 177/PO/cb/99/CPCSEA). All experimental protocols were approved by the Institutional Animal Ethics Committee of Teena Biolabs Private Limited (approval no. TBPL-NIAB/05/2017).

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