Transforming growth factor-β differentially inhibits MyD88-dependent, but not TRAM- and TRIF-dependent, lipopolysaccharide-induced TLR4 signaling

Yoshikazu Naiki, Kathrin S. Michelsen, Wenxuang Zhang, Shuang Chen, Terence M. Doherty, and Moshe Arditi

Transforming growth factor-β1 (TGF-β1) is a multifunctional, potent anti-inflammatory cytokine produced by many cell types that regulates cell proliferation, apoptosis, and immune responses. Toll-like receptors (TLRs) recognize various pathogen-associated molecular patterns and are therefore a pivotal component of the innate immune system. In this study we show that TGF-β1 blocks the NF-κB activation and cytokine release that is stimulated by ligands for TLRs 2, 4, and 5. We further show that TGF-β1 can specifically interfere with TLR2, -4, or -5 ligand-induced responses involving the adaptor molecule MyD88 (myeloid differentiation factor 88) but not the TRAM/TRIF signaling pathway by decreasing MyD88 protein levels in a dose- and time-dependent manner without altering its mRNA expression. The proteasome inhibitor epoxomicin abolished the MyD88 degradation induced by TGF-β1. Furthermore, TGF-β1 resulted in ubiquitination of MyD88 protein, suggesting that TGF-β1 facilitates ubiquitination and proteasomal degradation of MyD88 and thereby attenuates MyD88-dependent signaling by decreasing cellular levels of MyD88 protein. These findings importantly contribute to our understanding of molecular mechanisms mediating anti-inflammatory modulation of immune responses by TGF-β1.

Because transforming growth factor-β1 (TGF-β1) plays a fundamental role in regulation of cell growth, intense interest has focused on understanding the molecular details of the signaling pathway, how it controls cellular proliferation and differentiation and how it interacts with other signaling pathways to affect transcriptional responses (1). TGF-β interacts with only one type II and three type I receptors that activate downstream signaling proteins called Smads, which then translocate to the nucleus and influence transcription of numerous target genes (2, 3). The simplicity of the combinatorial receptor-ligand possibilities and the canonical Smad-mediated pathway belies the tremendously variable and dynamic biological responses elicited by TGF-β. How specificity is achieved while maintaining diversity in response possibilities is not yet known but is at least partly attributable to cross-talk between cytoplasmic effectors of TGF-β signaling components and those of a number of other signaling pathways.

In addition to its established role in cell growth, TGF-β mediates a plethora of other functions and participates in disorders involving fibrosis and acute or chronic inflammation (4). Genetic loss of function studies also indicate an essential role for TGF-β in normal immune function. TGF-β-null mice that survive to adulthood exhibit severe multifocal inflammation, particularly involving the heart, lungs, and salivary glands, but also causing activation of lymph nodes and hyperproliferation of lymphoid cells (5, 6). More recent data have provided intriguing new insights into TGF-β function in disease that suggest an important interaction with immune-mediated defense mechanisms. For example, TGF-β signaling appears to play a role in the suppression of antigen-activated T-cells (7) and attenuates antigen receptor signaling, in part by inducing inhibitors of Toll-like receptors (TLRs) (8).

TLRs activate proinflammatory innate immune mechanisms in response to pathogen-associated molecular patterns such as those originating from foreign microorganisms (9). To transmit their signal, TLRs recruit adaptor proteins such as MyD88 (myeloid differentiation factor 88) that leads to phosphorylation of IκB (inhibitor of NF-κB), allowing nuclear translocation of NF-κB. Work from our laboratory has centered on defining in molecular terms how TLRs participate in chronic inflammatory diseases, particularly cardiovascular disorders such as atherosclerosis. TLR4 is expressed in atherosclerotic plaque, and macrophages up-regulate expression of TLR4 in response to oxidized low density lipoprotein, a proatherogenic lipoprotein (10). Very recently, we reported that TLR4 signaling, particularly via the common downstream signaling molecule MyD88, has a direct role in development and structural stability of atherosclerotic plaque (11). Together, these studies establish a direct link between atherogenesis, inflammation, and innate immunity (12).

Since TGF-β appears to play a role in atherogenesis (13, 14), we sought to define how TGF-β might modulate the molecular signaling linking innate immunity and proatherogenic vascular responses. In addition, since TGF-β has immunomodulatory...
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(4, 15), we reasoned that there might be important regulatory interactions between TGF-β and TLR signaling pathways. Here we report that TGF-β signaling interacts directly with TLR signaling in a mechanism dependent upon MyD88 but not dependent upon TRIF (Toll/IL-1 receptor (TIR) domain-containing adaptor inducing IFN-β), an adaptor molecule utilized by TLR3 and an alternative downstream adaptor for TLR4 (16). We report that TGF-β does not affect MyD88 mRNA levels but decreases MyD88 protein by a mechanism that involves ubiquitin-dependent proteolysis of MyD88.

The effect of TGF-β was specific to MyD88 protein, as protein expression of other TIR domain-containing adaptors like TRIF or TRAM was not decreased. Collectively, our data identify MyD88 as a specific target of TGF-β immune regulation, reveal novel signaling cross-talk interactions between TGF-β and TLR signaling, and provide mechanistic insights into how TGF-β signaling modulates LPS-induced NF-κB activation, cytokine release, and suppression of inflammation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Human dermal microvessel endothelial cells (HMEC) (kindly provided by F. J. Candal, Center for Disease Control and Prevention, Atlanta, GA) (17) were cultured as described previously (18). RAW264.7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg/ml penicillin and streptomycin.

**Reagents and Antibodies—**Recombinant human TGF-β1 was obtained from R&D Systems (Minneapolis, MN). Highly purified phenol-water extracted protein-free Escherichia coli K3235 LPS was obtained from Dr. Stefanie N. Vogel (Uniformed Services University, Bethesda, MD). The purity of this LPS preparation has been demonstrated previously (19). The TLR2 ligand palmitoyl-Cys-(RS)-2,3-di(palmitoyl-oxylpropyl)-Ala-Gly-OH (Pam3Cys) was obtained from Bachem Biotechnology (Santa Cruz, CA), and anti-p38 MAP kinase antibody was purchased from Cell Signaling Technology (Beverly, MA). The proteasome inhibitor epoxomicin was obtained from Calbiochem. NF-κB activity was determined by TransAM™ NF-κB p65/NF-κB p50 transcription factor assay kits (Active Motif, Carlsbad, CA). Anti-ubiquitin monoclonal antibody was purchased from Chemicon (Temecula, CA), and anti-p38 MAP kinase antibody was purchased from Cell Signaling Technology (Beverly, MA). The proteasome inhibitor epoxomicin was obtained from Calbiochem.

**Constructs—**Plasmid DNA used in transient transfection experiments was isolated with an endothoxin-free plasmid maxi kit (Qiagen, Valencia, CA). A human IFN-β promoter luciferase reporter plasmid was obtained from Dr. J. Hiscott (20).

**Transfection and Luciferase Assays—**HMEC were transfected as reported elsewhere (18). The total amount of cDNA transfected was kept constant. After overnight transfection, HMEC were stimulated for 5 h with 20 ng/ml LPS with or without pretreatment (for 60 min) with TGF-β1 (3 ng/ml). Following 5 h of incubation, cells were washed once in phosphate-buffered saline and lysed, and luciferase activity was measured as described previously (18). The data shown are the mean values of three independent experiments.

**Real-time PCR Analysis—**Total RNA was isolated from HMEC cells using a Qiagen RNAeasy mini kit following the manufacturer’s instructions and treated with RNase-free DNase. Reverse transcription reaction was carried out using the SuperScript™ preamplification system (Invitrogen). Quantitative real-time PCR (Q-PCR) was performed with an iCycler Thermocycler (Bio-Rad) using a SYBR Green PCR kit, 10 μM fluorescent, TaqDNA polymerase (Invitrogen), 20 ng of template cDNA, and 20 μM gene-specific primers. Real-time PCR primers were as follows: 5′-ATG ACC CAT ATG TTT CAT TAT-3′ (the antisense, 5′-TAC GAC CAG AGG CAT ACA G-3′). Q-PCR was run under the following conditions: 30 s, 95 °C; 20 s, 55 °C; 10 s, 72 °C for 50 cycles. MyD88 sense (CTC CTC CAC ATC CCT TCC) and MyD88 antisense (CCG CAC GTC CAA GAA CAG AGA) were used. Q-PCR were run under the following conditions: 30 s, 95 °C; 20 s, 60 °C; 10 s, 72 °C for 50 cycles. cDNA samples were standardized based on the expression of β-actin cDNA as housekeeping gene. Q-PCR data are representative of two independent experiments.

**Immunoprecipitation and Western Blot—**Stimulated HMEC were lysed for 30 min on ice with lysis buffer (50 mM Hepes (pH 7.9), 1% Nonidet P-40, 1 mM EDTA, 250 mM NaCl, 20 mM β-glycerophosphate, 1 mM orthovanadate, and protease inhibitors). Following lysis, cell debris was removed by centrifugation. Protein concentrations were determined by Bradford assay. 130 μg of total protein were subjected to SDS-PAGE. For immunoprecipitation, 2 μg of the corresponding Ab was added to 540 μg of cellular extracts and incubated at 4 °C for 3 h on rotator. Then 30 μl of protein G Plus/protein A-agarose (Calbiochem) was added to each sample, followed by incubation for 2 h at 4 °C. The samples were washed three times in lysis buffer, subjected to SDS-PAGE, and blotted onto polyvinylidene difluoride filter transfer membranes (Invitrogen). Membranes were probed for 3 h with antibodies against MyD88, p38 MAP kinase, TRAM, TRIF, or ubiquitin. Bands were detected using ECL reagents (Amer sham Biosciences) according to the manufacturer’s recommendations.

**TNFα and RANTES Measurements—**RAW264.7 cells were pretreated with TGF-β1 for 1 h before stimulation with LPS (10 ng/ml), polyIC (10 μg/ml), flagellin (250 ng/ml), or Pam3Cys (10 μg/ml) for 6 h. Supernatants were harvested, and cytokine and chemokine concentrations were assayed using OptEIA mouse TNF-α ELISA (BD Biosciences, San Diego, CA) and Quantikine M Mouse RANTES ELISA kits (R&D Systems).

**Statistics—**Data are reported as mean values ± S.D. The statistical significance of differences between mean values was determined by Student’s t test. A P value of less than 0.05 was considered significant.

**RESULTS**

**TGF-β1 Inhibits TLR2, TLR4, and TLR5 Ligand-induced NF-κB Activation and TNFα Release—**Interaction of TLRs with their corresponding ligands triggers MyD88- or TRIF-dependent NF-κB activation and the release of proinflammatory cytokines such as TNFα. To determine whether TGF-β1 affects TLR2, TLR4, and TLR5 signaling and if this might be mediated via effects upon NF-κB activation, we stimulated RAW264.7 cells with LPS (10 ng/ml), Pam3Cys (10 μg/ml), flagellin (250 ng/ml), or polyIC (10 μg/ml) with or without pretreatment of cells with TGF-β1 (3 ng/ml). TGF-β1 pretreatment significantly inhibited NF-κB activation (Fig. 1A) and production of TNF-α (Fig. 1B) by RAW264.7 cells in response to LPS, flagellin, and Pam3Cys but not polyLC1. These results suggest that at least part of the effects of TGF-β1 may result from suppression of NF-κB activation that would normally occur in response to MyD88-dependent ligand-induced stimulation of TLRs.

**TGF-β1 Does Not Affect LPS-induced, TRAM/TRIF-dependent Activation of IFN-β and RANTES Secretion—**In addition to the MyD88-dependent NF-κB activation pathway that is required for LPS-induced inflammatory cytokine gene induction, there exists an MyD88-independent signaling pathway that utilizes another adaptor molecule, TRIF and TRAM. Both function in TLR4 signaling to regulate the MyD88-independent pathway during the innate immune response to LPS (21). Our results above indicate that TGF-β1 affects MyD88-dependent signaling, as reflected by expression of TNFα upon stimulation with several MyD88-dependent ligands. To determine whether TGF-β1 also modulates MyD88-independent TLR4 signaling pathways, we assessed the effects of TGF-β1 on expression of TLR4 gene targets (IFN-β and RANTES) that are induced in a MyD88-independent, but TRAM- and TRIF-dependent, manner after stimulation with LPS. We transfected IFN-β promoter-luciferase in HMEC and measured luciferase activity after LPS stimulation with or without TGF-β1 pretreatment. TGF-β1 had no effect on LPS-induced IFN-β promoter-luciferase activation, suggesting that TGF-β1 does not affect TRAM- and TRIF-dependent TLR4 signaling pathway after LPS stimulation but selectively suppresses MyD88-dependent TLR4 signaling (Fig. 1C). We next examined the effect of TGF-β1 pretreatment on LPS-induced RANTES production by RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS.
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Effect of TGF-β1 on MyD88 mRNA and Protein Expression—To confirm the influence of TGF-β1 treatment on MyD88 mRNA and protein expression, HMEC were incubated with or without TGF-β1 (15 ng/ml) for 4 h. TGF-β1 had no effect on MyD88 mRNA expression measured by Q-PCR (Fig. 2A). HMEC were stimulated with TGF-β1 in a dose- (Fig. 2B) and time-dependent fashion (Fig. 2C). Cellular lysates were immunoprecipitated and then analyzed by Western blotting using an antibody against MyD88. MyD88 protein levels decreased following treatment with TGF-β1 in a dose- and time-dependent manner. The lowest levels of MyD88 were observed after treatment with 30 ng/ml for 8 h (Fig. 2C). These results indicate that TGF-β1 decreases protein levels of MyD88 without affecting MyD88 mRNA expression.

TGF-β1 Does Not Decrease TRIF and TRAM Protein Expression—We next examined the specificity of the effect of TGF-β1 on MyD88 protein expression by determining its effect on other TIR domain containing adaptor molecules, including TRIF and TRAM. HMEC were incubated with or without TGF-β1 in a time-dependent fashion (Figs. 2E), and cellular lysates were immunoprecipitated and then analyzed by Western blotting using antibodies against TRIF or TRAM. TGF-β1 did not decrease either TRIF or TRAM protein levels, suggesting that the mechanism by which TGF-β1 inhibits TLR4-dependent NF-κB activation and TNF-α secretion was due to specific effects on MyD88 protein levels.

TGF-β1 Augments Proteasome-mediated Degradation of MyD88—MyD88 protein was rapidly degraded in HMEC treated with TGF-β1. The ubiquitin-proteasome pathway is known to regulate the stability of many proteins (22) and plays an important role in a number of processes critical to immune function (23). We therefore reasoned that ubiquitination-dependent proteolysis of MyD88 may explain our observation that TGF-β1 specifically decreased MyD88 protein levels but not TRIF or TRAM protein. To address this possibility, we preincubated HMEC with the proteasome inhibitor epoxomicin (24). Treatment with epoxomicin abolished the TGF-β1 induced MyD88 degradation in a dose-dependent manner (Fig. 2D). These results indicate that reduction in MyD88 protein levels after TGF-β1 treatment is due to augmentation of proteasomal degradation of MyD88.

TGF-β1 Causes Direct Ubiquitination of MyD88 Protein—Epoxomicin is a general inhibitor of proteasomal degradation that can be expected to alter cellular levels of numerous proteins. It is possible that treatment with epoxomicin affected protein levels in a way that spuriously or secondarily led to a reduction in MyD88 protein levels. We therefore sought to determine whether TGF-β1 directly facilitated the rate of polyubiquitination and subsequent proteasomal proteolysis. To address this question, we incubated HMEC with or without TGF-β1 (30 ng/ml) for 1, 2, 4, and 6 h and performed immunoprecipitation of cellular lysates with anti-MyD88 antibodies followed by Western blotting using anti-ubiquitin or anti-MyD88 antibodies. These experiments revealed an increase in ubiquinated MyD88 that appeared to roughly correspond temporally to decreased MyD88 protein levels. These results appear most consistent with the interpretation that TGF-β1 enhanced polyubiquitination of MyD88, which in turn led to proteasomal degradation and a concomitant decrease in cellular levels of MyD88 protein (Fig. 3). As anticipated, TGF-β1 induced a time-dependent ubiquitination of MyD88, and ubiquitin co-migrated with MyD88 after TGF-β1 treatment in cellular lysates (Fig. 3D). These results suggest that in both HMEC and macrophages, TGF-β1 regulates MyD88-dependent but not TRAM/TRIF-dependent TLR4 signaling in response to LPS stimulation.
A number of studies suggest that signaling by TGF-β superfamily ligands may interact significantly with TLR signaling at multiple levels, but the molecular mechanisms are unknown (25, 26). The adaptor protein Ecsit constitutes a node intersecting bone morphogenetic protein (BMP) (a TGF-β superfamily ligand) and TLR signaling (27), and because TGF-β has anti-inflammatory properties, this suggests the possibility that TGF-β itself might directly attenuate proinflammatory aspects of TLR signaling.

Here we provide further evidence for TGF-β/TLR cross-talk that does indeed implicate a critical role for TGF-β signaling in suppressing the proinflammatory response instigated by TLR signaling. We report that TGF-β potently inhibits LPS-induced NF-κB activation and TNF-α release from RAW 264.7 cells, but had no effect on IFN-β promoter activation or RANTES release, consistent with a specific effect on the MyD88-dependent but not the TRAM/TRIF-dependent pathway downstream of TLRs. TGF-β also decreased MyD88 protein levels in a dose- and time-dependent manner. Studies with a specific proteasome inhibitor abolished this effect, suggesting that TGF-β-mediated reduction of MyD88 protein levels may be due to polyubiquitination and enhanced proteasomal degradation of MyD88. Immunoprecipitation experiments using anti-ubiquitin and anti-MyD88 antibodies confirmed this interpretation. Collectively, our data identify MyD88-dependent TLR signaling as a specific target of TGF-β signaling, reveal novel signaling crosstalk between TGF-β and TLRs, and provide a mechanistic interpretation for modulation and suppression of TLR-induced inflammation by the TGF-β signaling pathway that involves alterations of MyD88 protein levels by stimulation of polyubiquitination and proteasomal degradation of MyD88.

All TLRs except TLR3 can utilize the MyD88 adaptor protein to induce inflammatory cytokine production (16). However, TLR4 can also signal in a MyD88-independent manner by using additional adaptor molecules such as TRIF, which is the sole adaptor molecule for TLR3-induced signaling. We observed that TGF-β1 blocked LPS-induced cytokine release but had no effect on TRIF-dependent readouts such as LPS/TLR4- or polyI:C/TLR3-mediated RANTES production and LPS/TLR4-induced IFN-β promoter activation. Furthermore, TGF-β1 stimulation decreased levels of MyD88 protein, but not those of TRIF or TRAM, and did not affect MyD88 mRNA levels. Collectively these observations suggest that the inhibitory effects of TGF-β1 specifically target the MyD88-dependent pathway of TLR signaling, which is involved in proinflammatory gene ac-

**FIG. 2.** TGF-β1 treatment decreases MyD88 protein but not mRNA levels. A, MyD88 mRNA expression was determined by q-PCR. HMEC were incubated with or without TGF-β1 (30 ng/ml) for 4 h, and MyD88 expression was measured by real-time PCR. There was no significant change in MyD88 mRNA with TGF-β1 treatment compared with untreated conditions. B, HMEC were stimulated with TGF-β1 (30 ng/ml) for different time durations as indicated. Western blotting showed a time-dependent decrease in MyD88 protein levels. D, pretreatment with 10–1000 nM epoxomicin 1 h before TGF-β1 stimulation blocked the decrease in MyD88 protein levels induced by TGF-β1. E, TGF-β1 treatment did not cause a decrease in TRIF or TRAM protein levels. Following TGF-β1 stimulation (30 ng/ml) for various time durations, cell lysates were prepared and immunoprecipitated for 3 h with anti-MyD88 (HFL-296), anti-TRIF, anti-TRAM or anti-p38 MAPK Ab followed by addition of 30 μl of protein G Plus/protein A-agarose suspension and incubated for an additional 2 h. Samples were separated by SDS-PAGE and then analyzed by Western blotting using anti-MyD88 (N-19), anti-TRIF, anti-TRAM, or anti-p38 MAPK antibodies.

**FIG. 3.** Ubiquitination of MyD88 increases after TGF-β1 treatment. HMEC were stimulated with TGF-β1 (30 ng/ml) for various time durations as indicated. Cell lysates were then prepared and immunoprecipitated for 3 h with anti-MyD88 (HFL-296) or anti-p38 MAPK Ab followed by addition of 30 μl of protein G Plus/protein A-agarose suspension and incubated for an additional 2 h. Samples were separated by SDS-PAGE and then analyzed by Western blotting using antibodies against MyD88 (N-19), ubiquitin, or p38 MAPK. Immunoblot results are consistent with the interpretation that TGF-β1 treatment resulted in a time-dependent increase in ubiquitinated MyD88 that preceded the decrease in MyD88 protein levels.

**FIG. 4.** Effects of TGF-β on LPS Signaling. A, Immunoprecipitation experiments using anti-ubiquitin and anti-MyD88 antibodies confirmed this interpretation. Collectively, our data identify MyD88-dependent TLR signaling as a specific target of TGF-β signaling, reveal novel signaling crosstalk between TGF-β and TLRs, and provide a mechanistic interpretation for modulation and suppression of TLR-induced inflammation by the TGF-β signaling pathway that involves alterations of MyD88 protein levels by stimulation of polyubiquitination and proteasomal degradation of MyD88.
tivation. This interpretation is consistent with the well known inhibitory effects of TGF-β1 on inflammatory cytokine production, as TGF-β1 treatment and MyD88 deficiency both lead to diminished IL-6 and IL-12 production. Our findings thus identify an important direct role for TGF-β in limiting the proinflammatory response instigated by TLR-dependent innate immune mechanisms and provide insight into how TGF-β might modulate host defense.

TLRs and MyD88 are central to the host response to Mycobacterium tuberculosis infection (28, 29), and serum and tissue levels of TGF-β are elevated in patients with tuberculosis and correlate with disease severity (30). Our results may be relevant to host defense against M. tuberculosis and may also suggest a molecular basis for at least part of the severe, eventually lethal inflammation observed in TGF-β-null mice; uncontrolled inflammation in these mice may result directly from lack of inhibition of TLR signaling that in turn would be expected to cause persistent hyperactivation of NF-κB and sustained induction of its proinflammatory gene targets. Recently reported data support this interpretation (31) and taken together are consistent with the interpretation that TGF-β signaling directly modulates innate immune mechanisms by suppressing NF-κB activation, thus preventing uncontrolled inflammation.

Animal studies implicate a role for TGF-β signaling in the chronic arterial inflammation that characterizes atherosclerosis (13, 14, 32). A common finding in these studies is exacerbation of plaque inflammation when TGF-β signaling is inhibited or abolished. It has been suggested that markedly increased expression of IFN-γ in plaques of atherosclerosis-prone mice after inhibition of TGF-β signaling could lead to a number of effects that would exacerbate plaque inflammation (33). Our data show that TGF-β decreases MyD88 protein levels and inhibits TLR-dependent activation of NF-κB-dependent proinflammatory gene targets. Inhibition of TGF-β signaling might therefore be expected to enhance NF-κB activation by TLR ligands. However, the validity of this notion must await the results of direct investigations.

Much of the interest in TGF-β signaling has been driven by the known role of TGF-β in cellular proliferation and the attendant realization that TGF-β pathway components represent attractive potential anticancer targets. TGF-β1 has well known immunosuppressive effects thought to be important in cancer (34, 35). It is interesting to note that in many cancers, particularly in patients undergoing chemotherapy, a major contributing or even precipitating cause of death is infection (36). It is tempting to speculate that in such patients, TGF-β signaling may alter TLR signaling in such a way that innate immunity is severely and, eventually lethally, compromised, perhaps by an inappropriate degree of inhibition of the MyD88-dependent pathway and suppression of normal NF-κB activation in response to microbial stimuli. If this notion is correct, therapeutic interventions that target TGF-β signaling in a manner that enhances (or at least does not compromise) TLR-mediated signaling may be an attractive goal that could improve clinical outcomes in patients with cancer or those with heightened vulnerability to infection. However, much more remains to be learned about the nature of TGF-β/TLR interactions in molecular terms, how these may depend upon cellular context and input from other signaling pathways, and particularly how specific alterations in expression and/or activity of TGF-β signaling components might impact TLR-dependent responses.

Ubiquitination and de-ubiquitination are increasingly implicated not just in protein degradation but also in regulatory processes, including functions critical to the immune system (23, 37). An important goal of future studies will be to define in molecular terms precisely how TGF-β1 affects TLR-mediated signaling by altering ubiquitination reactions. This would necessitate a more comprehensive systematic investigation into the role of ubiquitination and de-ubiquitination of all TLR-related signaling components and the subsequent proteolytic and non-proteolytic molecular and functional sequelae.
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