Central Role for Endothelial Human Deneddylase-1/SENP8 in Fine-Tuning the Vascular Inflammatory Response

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Central Role for Endothelial Human Deneddylase-1/SENP8 in Fine-Tuning the Vascular Inflammatory Response


A deeper understanding of the mechanisms that control responses to inflammation is critical to the development of effective therapies. We sought to define the most proximal regulators of the Cullin (Cul)-RING ligases, which play a central role in the stabilization of NF-κB and hypoxia-inducible factor (HIF). In these studies, we identify the human deneddylase-1 (SENP8) as a key regulator of Cul neddylation response in vitro and in vivo. Using human microvascular endothelial cells (HMECs), we examined inflammatory responses to LPS or TNF-α by assessing Cul neddylation status, NF-κB and HIF-1α stabilization, and inflammatory cytokine secretion. HMECs with an intact neddylation pathway showed a time-dependent induction of Cul-1 neddylation, nuclear translocation of NF-κB, stabilization of HIF-1α, and increased NF-κB/HIF-α promoter activity in response to LPS. HMECs lacking SENP8 were unable to neddylate Cul-1 and subsequently were unable to activate NF-κB or HIF-1α. Pharmacological targeting of neddylation (MLN4924) significantly abrogated NF-κB responses, induced HIF-1α promoter activity, and reduced secretion of TNF-α-elicted proinflammatory cytokines. MLN4924 stabilized HIF and abrogated proinflammatory responses while maintaining anti-inflammatory IL-10 responses in vivo following LPS administration. These studies identify SENP8 as a proximal regulator of Cul neddylation and provide an important role for SENP8 in fine-tuning the inflammatory response. Moreover, our findings provide feasibility for therapeutic targeting of the Culs during inflammation. The Journal of Immunology, 2013, 190: 392–400.
an isopeptidase capable of directly deneddylating Cul proteins (17, 27), provides new insights into how Cul neddylation might be regulated. SENP8 appears to be able to deneddylate hyperneddylated Cul proteins, thus offering a cleavage pathway beyond the COP9 signalosome (28, 29).

In this study, we define the role of SENP8-mediated deneddylation during inflammation using several approaches, including knockdown and overexpression of SENP8. Furthermore, we use the small molecule inhibitor of neddylation, MLN4924 (30), which has been shown to impact NF-κB inhibition via Cul-mediated IκB degradation (31), to evaluate its benefits for modulating the inflammatory response following LPS or TNF-α stimulation in vitro and in vivo. We identify a previously unappreciated role for SENP8 as a central regulator of the inflammatory process. Furthermore, we show that under physiological conditions, SENP8 functions to make mature Nedd8 available for conjugation to Culs, impacting on two transcription factors (i.e., NF-κB and HIF) important for balancing the inflammatory response.

Materials and Methods

Cell culture

Human microvascular endothelial cell (HMEC)-1 cells were a gift of Francisco Candal (Centers for Disease Control, Atlanta, GA). HMEC-1 cells were cultured in molecular, cellular, and developmental biology-131 medium supplemented with heat-inactivated FBS, penicillin, streptomycin, L-glutamine, epidermal growth factor, and hydrocortisone, as described previously (32). Stable knockdown of SENP8 was performed using short hairpin RNA (shRNA) with the following sequences: 5'-CCG-GCCTAAACTTCATCAAGACCTACTCGAGTAGGTCTTGAATGATTAGGTTTTTG-3' (TRCN0000073338, referred to as clone 38 in figures) or 5'-CCGGGACTGTGGGATGTACGTGATACTCGAGTATCACGTACATCCCACAGTCTTTTTG-3' (TRCN0000073342, referred to as clone 42 in figures; Sigma-Aldrich, St. Louis, MO) introduced using lentiviral particles. Cells transfected with scrambled shRNA were used as control. Knockdown cells were kept in cell culture medium with a maintenance dose of 0.25 mg/ml puromycin. Transient overexpression of SENP8 was achieved by transfecting 1 μg FLAG-tagged SENP8 (Addgene plasmid 18066) (33) construct under a CMV promoter into control HMEC-1 cells. Cells were cultured at 37˚C in an atmosphere of 95% air and 5% CO2 in a humidified incubator. Where indicated, HUVECs (Promocell, Heidelberg, Germany) were obtained and cultured according to the manufacturer’s recommendations.

Real-time PCR

To initially assess the level of SENP8 knockdown by lentiviral knockdown, SENP8 transcript levels were measured by relative real-time PCR. mRNA was isolated using the thiocyanate–phenol-chloroform method. Relative real-time PCR was then performed using Power SYBR Master Mix (Applied Biosystems, Carlsbad, CA) and the following primer sequences:

FIGURE 1. Influence of LPS and adenosine on Cul-1 neddylation status. (A) LPS stimulation (10 μg/ml medium) of HMECs increases neddylation of Cul-1 protein in a time-dependent manner. (B) Similar to previous findings in mice treated with hypoxic preconditioning, adenosine reduced the LPS-induced Cul-1 neddylation in a dose-dependent manner. Representative blots of three experiments per group.

FIGURE 2. Generation and validation of lentiviral SENP8 knockdown in endothelia. (A) Efficiency of lentiviral gene silencing on SENP8 transcript and protein levels was assessed by analysis of two different functional hairpin vectors (clones 38 and 42) compared with a scrambled vector (ctrl). Real-time PCR analysis showed different efficiency levels for both functional vectors, mirrored by immunoprecipitation/Western blot analysis of SENP8. (B) Knockdown of SENP8 was further ascertained in the 42 clone by immunofluorescence (original magnification ×20), indicating loss of positive staining in the knockdown cell line. (C) Direct detection of Cul-conjugated Nedd8 in HMEC SENP8-KD cells in response to LPS over the time course of 6 h is indicated by Western blot against Nedd8. Cells lacking functional SENP8 (KD) show a diminished neddylation response to LPS compared with empty vector (EV)-transfected controls. (D) This impaired general neddylation response was mirrored by abrogated neddylation of Cul-1 in SENP8 knockdown cells. Representative blots are from three or more experiments.
**Cell treatments**

For Western blot experiments and luciferase assays the following treatment regimen was used. Six hours prior to harvesting, cells were treated with 10 μg/ml medium of *Escherichia coli*–derived LPS (E. coli 055:B5; List Biological Laboratories, Campbell, CA), plus MLN4924 (0.33 μM; Millenium Pharmaceuticals, Boston, MA), TNF-α (1 ng/ml medium), TNF-α plus MLN4924, or vehicle (DMSO; Sigma-Aldrich) for 0.5, 1, 2, 4, and 6 h under normoxic conditions. Adenosine (Calbiochem) was used as a 30-min pretreatment at concentrations from 100 μM to 100 nM prior to LPS stimulation.

**Western blotting**

Cytosolic and nuclear proteins were isolated using the NE-PER Nuclear and Cytoplasmic Protein Extraction kit (Thermo Fisher Scientific, Rockford, IL). Protein concentration was determined by Lowry assay. Proteins were resolved under reducing conditions on SDS-PAGE gels followed by transfer onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Protein lysate (100 μg) was then used as input on a 10% SDS-PAGE gel, and Western blot analysis using murine anti-SENP8 (Abnova, Taipei, Taiwan), murine anti–Cul-1 (1:500; Invitrogen, Carlsbad, CA), rabbit anti-Nedd8, NF-κB p50, p65 (1:500; Cell Signaling Technology, Danvers, MA), murine HIF-1α (1:500; BD Transduction Laboratories, San Diego, CA), rabbit hydroxylated HIF-1α (1:500; Novus Biologicals, Littleton, CO), murine anti-TATA box–binding protein (1:1,000; Abcam, Cambridge, MA), rabbit anti–β-actin (1:10,000), and subsequently with a 1:10,000 dilution of HRP-linked anti-rabbit or mouse IgG (MP Biomedicals, Solon, OH). Ab staining was detected using LumiGLO chemiluminescence detection system (KPL, Gaithersburg, MD).

**Immunoprecipitation**

Protein lysate (100 μg) was precipitated on protein A μMACS protein beads (Miltenyi Biotec, Auburn, CA) precoated with 2 μl rabbit anti-SENP8 (Abnova). Following incubation, protein beads were washed and protein was eluted according to the manufacturer’s protocol. Protein (40 μg) was then used as input on a 10% SDS-PAGE gel, and Western blot analysis using murine anti-SENP8 (1:500; Abcam) was performed as described above.

**Immunofluorescence**

Control and SENP8 knockdown cells were seeded on collagen-coated coverslips in a 24-well dish format. After 24 h growth, cells were fixed and stained with an anti-SENP8 primary Ab and stained with an anti-SENP8 primary Ab (1:100; Abnova) overnight, washed, and stained with secondary Ab (Alexa Fluor 555, donkey anti-rabbit, 1:500; Invitrogen). Cells were washed and counterstained with DAPI (1:25,000; Invitrogen). Images were recorded from coverslips using a Zeiss Axiom Imager A.1 microscope with an AxioCam MRc 5 at ×20 magnification (Zeiss EC Plan-Neofluar ×20/0.5) utilizing Zeiss Axiosvision software version 4.6.3. SENP8 expression was quantified by assessing the fluorometric SENP8/DAPI ratio.

**FIGURE 3.** Functional influence of SENP8 on LPS-induced activation of NF-κB and HIF: (A) Following LPS stimulation, control HMECs exhibit an increased translocation of the NF-κB subunits p50 and p65 to the nucleus. This effect is abrogated in cells lacking SENP8. (B) Cells transfected with a constitutive SENP8 overexpressing (OE) vector showed baseline NF-κB–luciferase levels comparable to LPS treated control cells transfected with empty vector (EV) only. (C) MLN4924 (structure homolog of AMP) significantly quenched LPS-induced NF-κB–luciferase response. (D) LPS induced HIF-1α stabilization in the nucleus of empty vector cells after 4 and 6 h stimulation. This effect is not observable in SENP8 knockdown cells. All numerical data are means ± SEM from three or more experiments. **p < 0.01, ***p < 0.001.
Luciferase assay of HIF and NF-κB promoter activity

The promoter constructs for p-NF-κB-luciferase and p-hypoxia response element (HRE)–luciferase (34) were described previously. Transfection of HMEC-1 cells was performed using FuGENE 6 (Roche Diagnostics, Indianapolis, IN) in addition to 0.5 (HRE) or 1.5 μg (NF-κB) DNA, following overnight transfection, luciferase activity was measured after LPS for 6 (for NF-κB activity) or 24 h (for HRE response) using the luciferase reporter assay (Promega, Madison, WI). Observed firefly luciferase activity was normalized to the total protein amount of each sample determined by Lowry assay.

Endothelial permeability assay

FITC-dextran flux over an endothelial monolayer was measured as previously described by Lennon et al. (35).

Animal experiments

Twelve-week-old ΔODD mice (FVB background) of both genders received i.p. LPS doses of 100 μg/kg body weight. A respective control cohort received vehicle i.p. only. Six hours after treatment mice were sacrificed under tribromoethanol anesthesia and blood samples were collected for measuring systemic cytokine release. All animal experiments have been reviewed by the Institutional Animal Care and Use Committee and are in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals.

Cytokine measurements in serum and HIF-luciferase in kidney tissue

Proinflammatory cytokines in murine blood and organ samples were harvested following i.p. 6 h treatment with either 100 μg/kg LPS, vehicle, 3 mg/kg MLN4924, or LPS plus MLN4924 (1 h MLN4924 pretreatment), snap frozen in liquid nitrogen, and stored at −80°C until further analysis. Serum was collected by centrifugation and organ proteins were isolated using a tissue homogenizer. Serum cytokines were measured using the murine inflammatory 7-Plex assay (Meso Scale Discovery, Gaithersburg, MD). Luciferase activity in the kidney was measured using the luciferase reporter assay (Promega).

Data analysis

All raw and calculated data are expressed as means ± SEM of n observations, with n being the number of biological replicates, and analyzed using Prism 5.0 (GraphPad Software, San Diego, CA). Changes in transcript and protein levels and changes in NF-κB and HIF promoter activity levels were compared using a Student unpaired t test or one-way ANOVA with Newman–Keuls post hoc test where appropriate. A p value <0.05 was considered significant.

Results

Model to study Cul neddylation

Neddylation of Cul-1, an E3 ligase critical for the negative regulation of NF-κB through ubiquitination of IkB (36, 37), has been strongly implicated in inflammation (21). To establish a model of inflammation-induced Cul neddylation, we determined the ability of LPS to modulate endothelial Cul-1. As shown in Fig. 1A, LPS increased endothelial Cul-1 neddylation (reflected as an increase in Nedd8-associated band shift upward in the gel) in a time-dependent manner. Densitometry analysis of Nedd8/loading control ratios revealed a 1 ± 0.07-, 1.3 ± 0.54-, 2.7 ± 0.51-, 2.1 ± 0.52-, 1.7 ± 0.38-, and 3 ± 0.67-fold increase for 0, 0.5, 1, 2, 4, and 6 h stimulation, respectively (n = 3 experiments).

Our previous work showed that extracellular adenosine promotes the deneddylation of Cul-1 (26). We therefore determined whether adenosine might influence LPS induced Cul-1 neddylation. As depicted in Fig. 1B, the addition of extracellular adenosine (range, 10 nM–100 μM) attenuated LPS-induced Cul-1 neddylation in a concentration-dependent manner with nearly complete loss of LPS-induced Cul-1 neddylation at concentrations >1 μM compared with untreated control. Densitometry analysis revealed a 41 ± 3.3, 75 ± 8.2, and 86 ± 6.7% decrease relative to LPS alone at 10 nM, 1 μM, and 100 μM adenosine, respectively (n = 3 experiments).

SENP8 as a critical regulator of Cul neddylation

Having profiled endothelial LPS-induced Cul-1 neddylation, we turned our attention to defining molecular regulation. Our previous studies provided a role for the COP9 signalosome in the deneddylation of Cul-1 (26), but they revealed little with regard to inflammation-associated neddylation of the Culs. Moreover, our previous work implicated targets upstream of the COP9 signalosome in control of Cul deneddylation. This turned our attention to SENP8, and in this study we demonstrate a role for isopeptidase in LPS-mediated Cul-1 neddylation. To demonstrate this point, we used lentiviral-mediated shRNA knockdown to generate stable endothelial cell lines with reduced SENP8 expression. Efficiency of gene silencing was determined by real-time PCR and indicated a >50% loss of transcript in cells transfected with clone 42 shRNA targeting SENP8 compared with nontarget control and a less efficient clone 38 (Fig. 2A). Analysis of SENP8 protein levels by a combination of immunoprecipitation and Western

FIGURE 4. Influence of neddylation inhibition on HIF stabilization, HIF hydroxylation, Cul-1 neddylation, and HIF activity. (A) Pretreatment with MLN4924 increases HIF-1α protein in nuclear lysates of HMECs stimulated with LPS to higher levels than MLN4924 alone, indicative of synergistic effects of both compounds. LPS-induced Cul-1 neddylation was lost when cells were pretreated with MLN4924. (B) Treatment with MLN4924 stabilized HIF-1α in its hydroxylated form and allowed for its translocation to the nucleus, implicating effects of MLN4924 on Cul-2 neddylation. (C) Luciferase activity of HRE-luciferase following 24 h LPS stimulation mirrored protein results. All numerical data are means ± SEM from three or more experiments. *p < 0.05, **p < 0.01, ***p < 0.001.
blotting revealed an ~85% loss of SENP8 in clone 42 knockdown cells compared with control and clone 38 (Fig. 2A, insert). Likewise, localization of SENP8 by immunofluorescence (Fig. 2B) revealed perinuclear cytoplasmic distribution of SENP8 and a significant loss in SENP8 clone 42 cells (SENP8/DAPI ratio in empty vector controls 2.5 ± 0.42 versus SENP8 knockdowns 1.3 ± 0.07; p < 0.03). The secondary Ab-only control showed no detectable staining for SENP8 (data not shown).

To further confirm altered neddylation status of Culs, we assessed the conjugation of Nedd8 to Cul by probing directly with anti-Nedd8 Ab. LPS increased the abundance of Nedd8 protein in empty vector cells but not in SENP8 knockdown cells (Fig. 2C). Nedd8 detected in this manner (i.e., directly) likely reflects Nedd8 conjugation to several Cul proteins (e.g., Cul-1 and Cul-2, which have different molecular masses), and thus the diffuse nature of the bands on the blots shown in Fig. 2C. These same cells, when treated with LPS in a similar fashion to control cells, showed a blunted neddylation response of nuclear Cul-1 protein, indicating that SENP8 is necessary for LPS-induced Cul-1 neddylation (Fig. 2D).

To define the influence of endothelial SENP8 on functional inflammatory responses, we evaluated the NF-κB response in control and SENP8 knockdown cells following LPS exposure. As shown in Fig. 3A, LPS induced a profound translocation of the p50 and p65 subunit of NF-κB from the cytoplasm to the nucleus. Within 30 min, control cells showed a robust increase in the nuclear fraction of p50 and p65, which persisted to time points beyond 4 h (Fig. 3A). Both p50 and p65 responses were markedly reduced in cells lacking SENP8 (Fig. 3A). In SENP8 knockdown cells, both the kinetics and the magnitude of the p50/p65 response, that is, the nuclear translocation of said heterodimer, was attenuated compared with those observed in control cells, indicating a central role for SENP8 for the immediate NF-κB response following LPS exposure.

Conversely, overexpression of SENP8 in HMEC-1 cells using a SENP8 plasmid on a heterologous promoter significantly increased NF-κB responses. Indeed, as shown in Fig. 3B, compared with empty plasmid transfection controls, overexpression of SENP8 increased basal NF-κB reporter plasmid activity by as much as 2.8 ± 0.5-fold (p < 0.01), approaching that of maximal LPS-induced NF-κB activity. LPS treatment of SENP8 overexpressing cells did not further increase NF-κB activity compared with empty plasmid controls, suggesting that a maximum level of NF-κB activity had been achieved with these conditions. Nearly identical results were observed in primary endothelial cell cultures (HUVECs), where overexpression of SENP8 increased baseline and LPS-induced TNF-α transcript (Supplemental Fig. 1). Thus, both loss- and gain-of-function studies strongly implicate SENP8 as a critical component to vascular endothelial NF-κB responses.

Influence of the neddylation inhibitor MLN4924 on NF-κB and HIF activity

Recently, a small molecule inhibitor (MLN4924) targeting Nedd8 conjugation to the Culs became commercially available. Given our findings that adenosine (via metabolism from AMP) deneddylates Cul-1 (26), it is interesting that MLN4924 is a structural AMP analog (Fig. 4C) that functions to inhibit Nedd8-activating enzyme and results in the deneddylation of Cul-1 and Cul-2 (30, 38). As shown in Fig. 3C, the NF-κB activity elicited by LPS was quenched to nearly control levels in HMEC-1 cells that had been pretreated with MLN4924 (p < 0.01). Similar results were
obtained in cultured HUVECs, where MLN4924 abrogated LPS-induced Cul-1 neddylation and LPS-induced TNF-α transcript (Supplemental Fig. 1).

Given the importance of Cul-2 neddylation for pVHL activity and thus in the degradation of HIF protein (39), we next addressed the impact of MLN4924 on HIF function. As shown in Fig. 4, in stark contrast to the inhibition of NF-κB (Fig. 3D), MLN4924 stabilized HMEC-1 HIF-1α protein (Fig. 4A), and more specifically the hydroxylated isoform of HIF-1α (Fig. 4B). This observation was significantly enhanced by the presence of LPS, which is consistent with previous studies indicating that LPS stabilizes HIF activity (40, 41). Moreover, HIF activity (as measured using a hypoxia-response element reporter plasmid) was significantly increased by MLN4924 (p < 0.05). These findings indicate that the status of Cul-2 neddylation strongly influences HIF activity.

We next addressed whether Cul neddylation in vascular inflammatory responses was specific for LPS. For these purposes, we selected TNF-α–elicited cytokine/chemokine induction in the presence and absence of MLN4924 as primary endpoints. As shown in Fig. 5, TNF-α potently induced HMEC-1 IL-1β, IL-6, and IL-8 mRNA and protein (all p < 0.05 or p < 0.01). In each case, the neddylation inhibitor MLN4924 significantly inhibited mRNA induction (p < 0.01 or p < 0.05) as well as IL-1β and IL-6 protein induction (p < 0.01). Moreover, MLN4924 significantly inhibited TNF-α–elicited IFN-γ protein (83 ± 8% inhibition, p < 0.05) and IL-12p70 protein (76 ± 5% inhibition, p < 0.01).

FIGURE 6. Impact of MLN4924 on LPS-mediated HIF activity and cytokine production in vivo. ΔODD mice received vehicle, MLN4924 (3 mg/kg body weight), LPS (100 μg/kg body weight), or MLN4924 plus LPS i.p. for 6 h. Inflammatory cytokines were measured in serum and HIF-luciferase was measured in renal lysates. (A) LPS plus MLN4924 induced a significant increase of renal HIF cytokines. (B, C, D, E, F, G, and H) Mice pretreated with the neddylation inhibitor MLN4924 prior to LPS showed no significant upregulation of all proinflammatory serum cytokines while maintaining increased levels of the anti-inflammatory cytokine IL-10 (F). All values are means ± SEM with four or more animals per group. *p < 0.05, **p < 0.01, ***p < 0.001.
Furthermore, MLN4924 inhibited TNF-α–mediated induction of ICAM-1 transcript (Fig. 5D; 79 ± 9% decrease, p < 0.05) and completely abrogated barrier dysfunction elicited by LPS (measured by FITC-dextran flux; see Fig. 5E). Such findings place Cul neddylation as a central regulator of the vascular inflammatory response and clearly implicate Cul deneddylation as a target to inhibit this phenotype.

Cul neddylation and LPS-induced inflammation in vivo

Reagents to define specific aspects of Nedd8/Cul conjugation pathways in vivo have been limiting owing to the embryonic lethality of the gene-targeted mouse lines (42). Thus, we elected to employ MLN4924, which is well tolerated for use in murine tumor models (30). Initially, we profiled whether MLN4924 influenced HIF expression in vivo. For these purposes, we used the HIF reporter ΔODD-luciferase mouse model in combination with LPS (100 μg/kg body weight) exposure in the presence and absence of MLN4924 pretreatment. As shown in Fig. 6A, the combination of LPS and MLN4924 synergistically increased HIF stabilization (reflected as luciferase activity) in kidneys from ΔODD-luciferase mice (p < 0.01). Further analysis of serum cytokines in these mice revealed prominent increases in proinflammatory cytokines and chemokines compared with vehicle-treated mice (Fig. 6B–H). LPS also induced the anti-inflammatory cytokine IL-10 (Fig. 6E). Administration of MLN4924 30 min prior to LPS significantly attenuated the induction of each of the proinflammatory cytokines, including IL-1β, IL-6, TNF-α, IL-12p70, and IFN-γ (Fig. 6B–H). Interestingly, mice that received MLN4924 and LPS showed a further, albeit not statistically significant, increase in the anti-inflammatory cytokine IL-10 (Fig. 6F). Taken together, these in vivo observations reveal a potent anti-inflammatory role for MLN4924.

Discussion

In productive inflammatory responses, a number of transcription factors, including NF-κB and HIF, are tightly regulated by posttranslational modifications that control the kinetics of expression via proteasomal degradation pathways (reviewed in Ref. 43). The polyubiquitination of components within these pathways (e.g., IκB and HIF) is mediated through a multiprotein E3 ligase complex, for which one of these components is a member of the Cul family of proteins (44). E3 ligase activity is controlled through its neddylation status, that is, the conjugation of a Nedd8 moiety to the Cul subunit (45). Regulation of Cul neddylation is achieved through the COP9 signalosome and/or the human deneddylyase SENP8 (27, 29). Work by Mendoza et al. (17) identified the isopeptidase SENP8 to primarily deneddylate Cul5, thereby inactivating the E3 ligase.

Limited information is available regarding the direct role of Cul regulation in inflammation. Amir et al. (21) demonstrated that lack of functional E2 or E3 ligases results in reduced breakdown of the NF-κB precursor p105, thereby limiting the induced inflammatory response. Their work, however, focused on the E1 and E2 ligases of the neddylation pathway, whereas the present studies address the function of upstream targets of said ligases. To address the role of Cul neddylation in inflammation, we initially established a model using HMECs and LPS to evaluate the biochemical features of Cul-1 neddylation. This model demonstrated prominent Cul-1 neddylation that was inhibited by adenosine, which we have previously shown to potently deneddylate the Cul5 (26).

Based on these observations and our previous work suggesting that targets upstream of the COP9 signalosome should control Cul neddylation (26), we targeted SENP8 as a central regulator for the proinflammatory phenotype. Loss- and gain-of-function studies strongly implicated SENP8 as a central regulator of the inflammatory response. Indeed, lentiviral-mediated shRNA knockdown of SENP8 resulted in a loss of the neddylated Cul-1 that was reversed by constitutive overexpression of SENP8 (33). These observations are likely due to the dual protease actions of SENP8. For instance, Wu et al. (27) have described SENP8 as capable of not only removing Nedd8 from target proteins, but also cleaving

![FIGURE 7. Neddylation pathways influencing NF-κB and HIF-α. Left, NF-κB pathway. Proinflammatory stimuli, such as LPS, facilitate the phosphorylation of IκB and result in the recognition of p-IκB by the Cul-1-Nedd8-Skp-βTRCP complex, culminating in its polyubiquitination and proteasomal degradation. The rate-limiting step for this is the conjugation of Nedd8 to Cul-1. Neddylation is achieved through a multienzyme process wherein SENP8 enables cleavage of the Nedd8 precursor and promotes Nedd8 conjugation to the Cul5. Loss of SENP8, or pharmacological inhibition of Nedd8 conjugation by MLN4924, prevents activation of Cul-1 and thus prevents liberation of NF-κB from IκB, resulting in quenched proinflammatory signaling. Right, HIF-α pathway. In contrast to NF-κB, HIF-α in its hydroxylated form is degraded by the proteasome after ubiquitination through the pVHL. pVHL in its activated form contains neddylated Cul-2, thereby controlling cellular HIF-α levels. Use of MLN4924 prevents Cul-2 neddylation and, as shown in this study, leads to higher levels of the hydroxylated HIF-α isoform.](http://www.jimmunol.org/DownloadedFrom)
the precursor of Ned8 to its mature form. Immature Ned8 is incapable of coupling to the E1 and E2 ligases, thus making it unavailable for conjugation to the E3 ligase. Thus, our observations in the SENP8 knockdown line are likely due to a lack of mature Ned8 available for conjugation. The molecular mechanism of Cul-RING ligase activity relies on the conjugation of mature Ned8 to the Cul-RING ligase.

We have shown in the past that adenosine actively deneddylates Cul-1 and results in an inhibition of NF-κB signaling (26). Likewise, we have recently demonstrated that adrenomedullin deneddylates Cul-2 (46), a critical component of the HIF-α E3 Skp-Cul-1-Fbox ubiquitin ligase (47). Cul-2 neddylation should result in diminished pVHL activity and subsequently an accumulation of HIF-1α protein, an effect we did not observe with SENP8 knockdown. It is therefore likely that permanent loss of SENP8 triggers adaptive pathways for HIF-1α control independent of pVHL. In contrast, MLN4924 increased HIF-1α levels and HIF activity, suggesting that short-term inhibition of neddylation influences both Cul-1 and Cul-2 activity. This was also indicated by our in vivo data revealing that HIF levels increase in the kidney in response to LPS and that MLN4924 synergistically increases this response. Important in this regard, HIF stabilization has been proven beneficial in a number of murine disease models (12), including the kidney (48). The underlying mechanisms for HIF-mediated renoprotection remain unresolved but HIF-mediated induction of the ectonucleotidase CD73 (49), a transmembrane protein critical for the generation of extracellular adenosine, has been strongly implicated. For example, Grenz et al. (50) showed induction of the ectonucleotidase CD73 (49), a transmembrane protein critical for the generation of extracellular adenosine, has been strongly implicated.

We conclude that 1) SENP8 is necessary for inflammatory activation of NF-κB, 2) inflammation-induced stabilization of HIF in part relies on functional SENP8, and 3) pharmacological inhibition of SENP8 using the structural AMP analog MLN4924 might serve as a suitable therapeutic target for modulating inflammatory diseases.