

Histone Methyltransferase Ash1l Suppresses Interleukin-6 Production and Inflammatory Autoimmune Diseases by Inducing the Ubiquitin-Editing Enzyme A20

Meng Xia,^{1,2,5} Juan Liu,^{2,5} Xiaohui Wu,^{3,5} Shuxun Liu,² Gang Li,³ Chaofeng Han,² Lijun Song,² Zhiqing Li,¹ Qingqing Wang,¹ Jianli Wang,¹ Tian Xu,³ and Xuetao Cao^{1,2,4,*}

¹Institute of Immunology, Zhejiang University School of Medicine, Hangzhou 310057, China

²National Key Laboratory of Medical Immunology and Institute of Immunology, Second Military Medical University, Shanghai 200433, China

³Institute of Developmental Biology and Molecular Medicine, School of Life Science, Fudan University, Shanghai 200433, China

⁴National Key Laboratory of Medical Molecular Biology & Department of Immunology, Chinese Academy of Medical Sciences, Beijing 100730, China

⁵These authors contributed equally to this work

*Correspondence: caoxt@immunol.org

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SUMMARY

Histone modifications play important roles in multiple physiological processes by regulating gene expression. However, the roles of histone modifications in immunity remain poorly understood. Here we report that Ash1l, a H3K4 methyltransferase, suppressed interleukin-6 (IL-6), and tumor necrosis factor (TNF) production in Toll-like receptor (TLR)-triggered macrophages, protecting mice from sepsis. Ash1l-silenced mice were more susceptible to autoimmune disease as a result of enhanced IL-6 production. Ash1l enhanced A20 expression through induction of H3K4 modification at the *Tnfaip3* promoter via H3K4 methyltransferase activity of Ash1l SET (Su[var]3-9, E[z] and trithorax) domain. Ash1l suppressed NF-κB, mitogen-activated protein kinase (MAPK) pathways, and subsequent IL-6 production via facilitating A20-mediated NF-κB signal modulator NEMO and transducer TRAF6 deubiquitination. Therefore, Ash1l-mediated H3K4 methylation at the *Tnfaip3* promoter is required for controlling innate IL-6 production and suppressing inflammatory autoimmune diseases, providing mechanistic insight into epigenetic modulation of immune responses and inflammation.

INTRODUCTION

Toll-like receptors (TLR) are critical for pathogen recognition and host defense against infections. Innate immune cells such as macrophages sense invading microbial pathogens via TLRs to induce downstream signaling cascades, subsequently leading to production of proinflammatory cytokines and interferons (IFNs) (Takeuchi and Akira, 2010; Kawai and Akira, 2010). The physiological importance of TLR signaling is underscored by the involvement of TLRs and their major signaling components

in human diseases, including cancer and immunological diseases (Netea et al., 2012; Ben-Neriah and Karin, 2011). Regarding the latter, aberrant TLR signals have been associated with various inflammatory diseases, such as endotoxin shock, rheumatoid arthritis (RA), and inflammatory bowel disease (Marshak-Rothstein, 2006). Thus, negative regulation of TLR signaling is essential for avoiding excessive inflammatory immune responses and maintaining immune homeostasis. To date, a number of intracellular molecules and inhibitory receptors have been identified to be involved in the control of TLR signaling, such as the adaptor molecule TANK and the myeloid surface receptor CD11b (Kawagoe et al., 2009; Han et al., 2010; Liew et al., 2005).

Accumulating studies have suggested the critical involvement of ubiquitination and its regulatory enzymes in the regulation of TLR signaling (Hu et al., 2013). In particular, a ubiquitin-editing enzyme, A20, has emerged as a potent TLR-negative regulator that functions by removing ubiquitin chains from NF-κB essential transducer TRAF6 (Boone et al., 2004). The pivotal regulatory role of A20 has been confirmed by its involvement in protecting hosts from endotoxin shock and TLR4-dependent erosive polyarthritis (Matmati et al., 2011; Ma and Malynn, 2012). Considering the essential role of negative regulators in immune balance, identification, and characterization of the unknown regulators of TLR signals will better elucidate the regulatory mechanism of TLR-triggered inflammatory responses and will provide important clues for prevention and treatment of inflammatory and autoimmune diseases.

Epigenetic modifications are heritable transcriptional regulation of genes without altering genome DNA sequence and play essential roles in a variety of biological and pathological processes (Portela and Esteller, 2010), including DNA methylation, histone modification and chromatin remodeling. In particular, aberrant histone modifications are tightly associated with the pathogenesis of multiple human diseases (Feinberg, 2007), and some of them have been proven to be potential diagnostic biomarkers or therapeutic targets for inflammatory diseases, such as marked histone H3 deacetylation in oligodendrocytes within early-stage multiple sclerosis lesions (Koch et al., 2013) and aberrant histone methylation and acetylation mediated by

methyl-CpG-binding protein 2 in the pathogenesis of RA (Miao et al., 2013). Lysine methylation is one of the most characterized histone modifications to date. In particular, H3K4 methylation associated with transcriptional activation plays critical developmental roles by mediating mitotic inheritance of lineage-specific gene expression such as Hox and Tbx families (Schuettengruber et al., 2007).

Up to now, 14 H3K4 methyltransferases and demethylases have been identified (Allis et al., 2007), some of which have been associated with immune cell differentiation and function (Krvitsov and Armstrong, 2007). For example, Mll1 can specifically accumulate at the *Gata3* locus of memory T helper 2 (Th2) cells, but not naive T cells, and is therefore essential for the expression of *Gata3* and Th2 cell-associated cytokines such as interleukin-4 (IL-4) (Nakata et al., 2010). However, the detailed roles of H3K4 methyltransferases and demethylases in immunity, especially in TLR-mediated innate immune responses, remain poorly understood. Better understanding of the function of these enzymes in innate immunity and the pathophysiology of relevant diseases, as well as the underlying mechanisms, will provide new insight into the epigenetic regulation of immune responses.

The primary goal of the current study is to understand the role of H3K4 methyltransferases and demethylases in regulating innate inflammatory immune responses. For this purpose, we performed a screening test by silencing the 14 kinds of enzymes involved in H3K4 methylation in mouse peritoneal macrophages with specific small interfering RNAs (siRNAs) and then measured lipopolysaccharide (LPS)-induced IL-6 production. Through this systematic study, we found that siRNA targeting Ash1l ([absent, small, or homeotic]-like [*Drosophila*]) increased LPS-induced production of IL-6. Ash1l is the mammalian homolog of *Drosophila* Ash that contains a conserved SET (Su[var]3-9, E[z] and trithorax) domain and acts as an activator of various genes such as *Ubx* and *HOXA10* via its H3K4 methylation activity of the SET domain (Byrd and Shearn, 2003; Gregory et al., 2007). Ash1l may regulate early T cell development via its plant homeo-domain-like zinc (PHD) finger (Tanaka et al., 2008). So far, there is no report about the role of Ash1l in innate inflammatory immune responses. To study the biological role of Ash1l in immunological process in vivo, we generated Ash1l-silenced mice via piggy-back (PB) transposon insertion. Interestingly, Ash1l-silenced mice were more susceptible to endotoxin shock, sepsis, and the development of autoimmune diseases. We demonstrated that Ash1l suppressed TLR-triggered IL-6 production by directly enhancing A20 expression via inducing H3K4 modification at the *Tnfaip3* promoter. The negative regulatory role of Ash1l in innate inflammatory immune response was dependent on its H3K4 methyltransferase activity of the SET domain. Our results elucidate a crucial role of Ash1l in the negative regulation of TLR-triggered innate inflammatory immune response and suppression of autoimmune diseases, providing an insight into the mechanisms of epigenetic modulation of immune responses.

RESULTS

Silencing of Ash1l Increases TLR4- and TLR3-Triggered Proinflammatory Cytokine Production in Macrophages

TLR-induced proinflammatory cytokines are critical pathological mediators of various autoimmune diseases. In particular, IL-6

blockage has been verified as effective treatment for human RA (Tanaka and Kishimoto, 2012). Fourteen kinds of enzymes that modify H3K4 methylation have been identified and classified as methyltransferases and demethylases. To screen for the enzymes that might be involved in innate inflammatory immune responses, we transfected mouse peritoneal macrophages with specific siRNAs respectively designed for these enzymes and then measured LPS-induced IL-6 production. Among the siRNAs test (see Table S1 available online), the one silencing Ash1l (Figure 1A) increased the production of IL-6 and tumor necrosis factor (TNF) in macrophages stimulated by the TLR3 and TLR4 ligands, poly(I:C) and LPS (Figures 1B and 1C). However, the Ash1l silencing did not affect IFN- β production induced by LPS or poly(I:C) (Figure S1A). Silencing of several other methyltransferases, such as Mll1, Mll2, and Mll4 and the demethylase Kdm5a also altered IL-6 production, but this result was much milder compared to that obtained from the Ash1l silencing and expected from the previously proposed roles of some methyltransferases and demethylases in the activation or inhibition of gene expression (Wang et al., 2012; Austenaa et al., 2012) (Table S1). We hence selected Ash1l for further investigation for its unexpected effects in inhibiting LPS-induced IL-6 production as a histone methyltransferase.

On the basis of the screening system, we next investigated the expression profiles of Ash1l in immune system. Ash1l was widely expressed in multiple organs, with particular abundant in brain, kidney, and heart (Figure S1B). Ash1l was also widely expressed in various immune cells, with preferential expression in CD4 $^{+}$ T cells, NK cells, and peritoneal macrophages (Figure S1C). Moreover, LPS upregulated Ash1l and induced its accumulation in both cytoplasm and nucleus of macrophages (Figures S1D and S1E).

To further investigate the role of Ash1l in TLR-triggered innate inflammatory response, we generated Ash1l-silenced mice via inserting the PB transposon between exons 15 and 16 of Ash1l allele (Figure S2A) and confirmed the successful silence of Ash1l expression in Ash1l-silenced mouse peritoneal macrophages (Figures S2B and S2C). The frequency of T cells, B cells, dendritic cells, NK cells, neutrophils, and F4/80 $^{+}$ CD11b $^{+}$ macrophages in the splenocytes was similar between Ash1l-silenced mice and wild-type (WT) littermate mice (Figure S2D). The proportion and total number of peritoneal macrophages and in vitro generated bone marrow-derived macrophages (BMDMs) were also normal in Ash1l-silenced mice (Figure S2E). Though Ash1l was shown to regulate certain genes involved in cell-fate determination in K562 cells (Tanaka et al., 2011), we did not observe any difference in those genes, including integrin alpha 2b (*Itga2b*) and integrin beta 3 (*Itgb3*), in LPS-stimulated RAW264.7 cells after Ash1l silencing (Figure S2F). Therefore, silencing of Ash1l does not affect differentiation of lymphoid and myeloid immune cell subsets.

Notably, in response to LPS or poly(I:C) stimulation, peritoneal macrophages and BMDMs from Ash1l-silenced mice produced more IL-6 and TNF than those derived from the WT mice (Figures 1D and 1E), thus confirming the results obtained from the in vitro Ash1l-silencing studies. Therefore, silencing of Ash1l enhances TLR-triggered production of proinflammatory cytokines in macrophages, whereas it has no effect on macrophage differentiation.

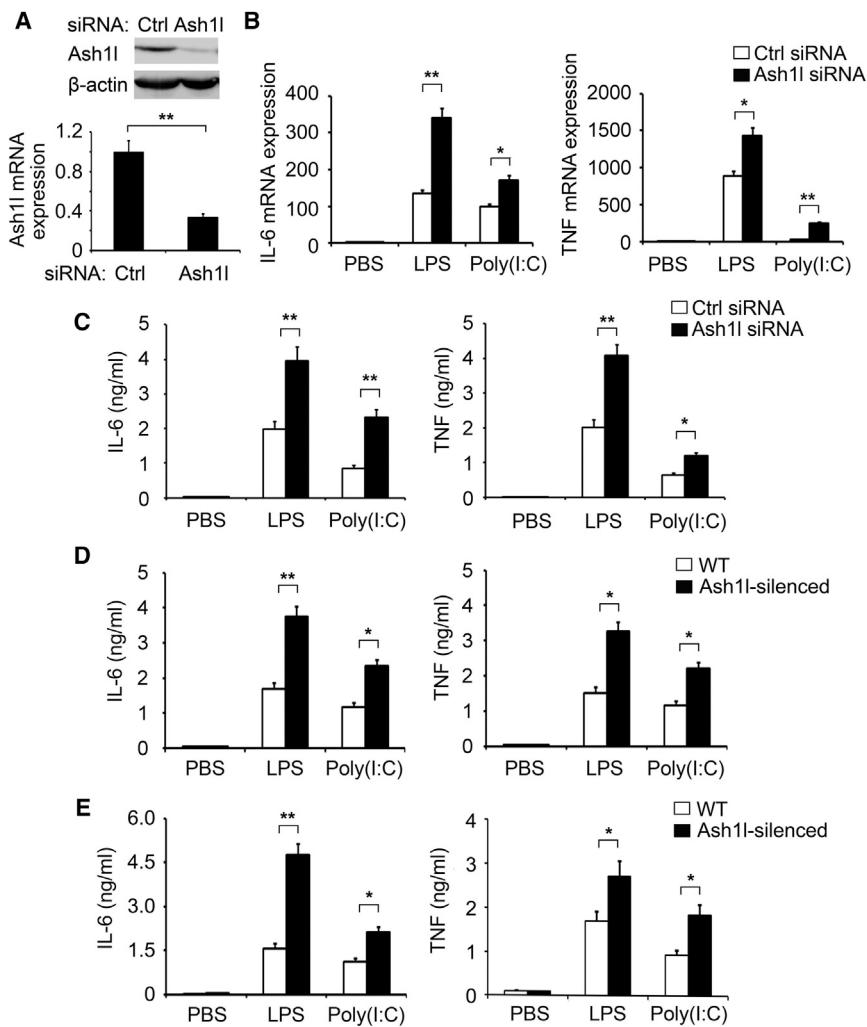


Figure 1. Silencing of Ash1l Enhances TLR-Triggered Production of Proinflammatory Cytokines in Macrophages

(A) Protein (top) and mRNA (bottom) amounts of Ash1l in peritoneal macrophages 48 hr after transfection with control siRNA (Ctrl siRNA) or siRNA specific for Ash1l (Ash1l siRNA).

(B) IL-6 and TNF mRNA amounts of macrophages transfected as in (A), then stimulated for 4 hr with PBS, LPS (100 ng/ml), or poly (I:C) (10 µg/ml).

(C) CBA assay of IL-6 and TNF in supernatants of macrophages transfected as in (A), stimulated for 8 hr with PBS, LPS, or poly (I:C).

(D and E) CBA assay of IL-6 and TNF in supernatants of WT and Ash1l-silenced peritoneal macrophages (D) and BMDMs (E) stimulated as in (C). Error bars represent SD. *p < 0.05, **p < 0.01.

These data sets are representative of three independent experiments. Related to Figure S1 and Table S1.

cate that Ash1l negatively regulates the innate inflammatory response, including suppression of innate IL-6 production, both *in vitro* and *in vivo*.

Ash1l-Silenced Mice Have Increased Susceptibility to Pathogenesis of Autoimmune Disease

A previous study found that *Ash1l* gene is located in the murine type I diabetes locus *Idd17* and becomes upregulated in NOD mice, suggesting a potential role of Ash1l in self-tolerance (Zucchelli et al., 2005). We thus wondered whether Ash1l might also be involved in the path-

ogenesis of autoimmune disease. We detected higher immunoglobulin M (IgM), IgG1, IgG2a, IgG2b, and IgA, but intact IgG3 in serum of 8-month-old Ash1l-silenced mice compared with littermate WT mice (Figure 3A). The concentration of IL-6 in serum of 8-month-old Ash1l-silenced mice was also elevated (Figure S3A). Histological analysis revealed more severe infiltration of mononuclear cells in various organs of aged Ash1l-silenced mice (Figure 3B). Moreover, the aged Ash1l-silenced mice exhibited increased susceptibility to membranous glomerulonephritis with more thickened and prominent capillary loops, which can be linked to a chronic infectious diseases or systemic lupus erythematosus (SLE). In addition, we also observed more deposition of immune complexes of autoantibodies in the glomerulus of aged Ash1l-silenced mice (Figure 3C). These data indicate that aged Ash1l-silenced mice were more prone to spontaneous autoimmune disease, suggesting Ash1l has functional roles in protecting mice from spontaneous autoimmune disease.

We further investigated the effect of Ash1l on autoimmunity development by comparing the incidence and severity of collagenII-induced arthritis (CIA) in Ash1l-silenced and control mice. CIA is a well-established animal model for human RA, both involving IL-6 as a critical mediator of the initiation and

Ash1l-Silenced Mice Exhibit Increased Acute Inflammation in Response to TLR-Ligands Challenge and *E. coli* Infection

To further confirm the effect of Ash1l on TLR-triggered innate inflammatory response, we challenged Ash1l-silenced mice with TLR ligands or Gram-negative bacteria *Escherichia coli* (*E. coli*) *in vivo*. Compared to WT mice, Ash1l-silenced mice produced significantly higher IL-6 and TNF in serum after being challenged with LPS or poly(I:C) (Figure 2A). Upon *E. coli* infection, the Ash1l-silenced mice also produced more IL-6 and TNF (Figure 2B) and had a higher bacterial load in the blood than WT mice (Figure 2C), which is in accordance with previous studies showing that proinflammatory cytokines such as TNF promote the dissemination of *E. coli* (Han et al., 2010). Following sublethal challenge with *E. coli*, the Ash1l-silenced mice displayed markedly reduced survival (Figure 2D). Furthermore, we observed more severe inflammatory hyperemia in the lungs of Ash1l-silenced mice as characterized by increased infiltration of mononuclear cells and red blood cells 6 hr after challenge with *E. coli* or LPS (Figure 2E). Thus, Ash1l-silenced mice developed a more severe innate inflammatory response and were more susceptible to endotoxin shock and *E. coli*-induced sepsis. These data indi-

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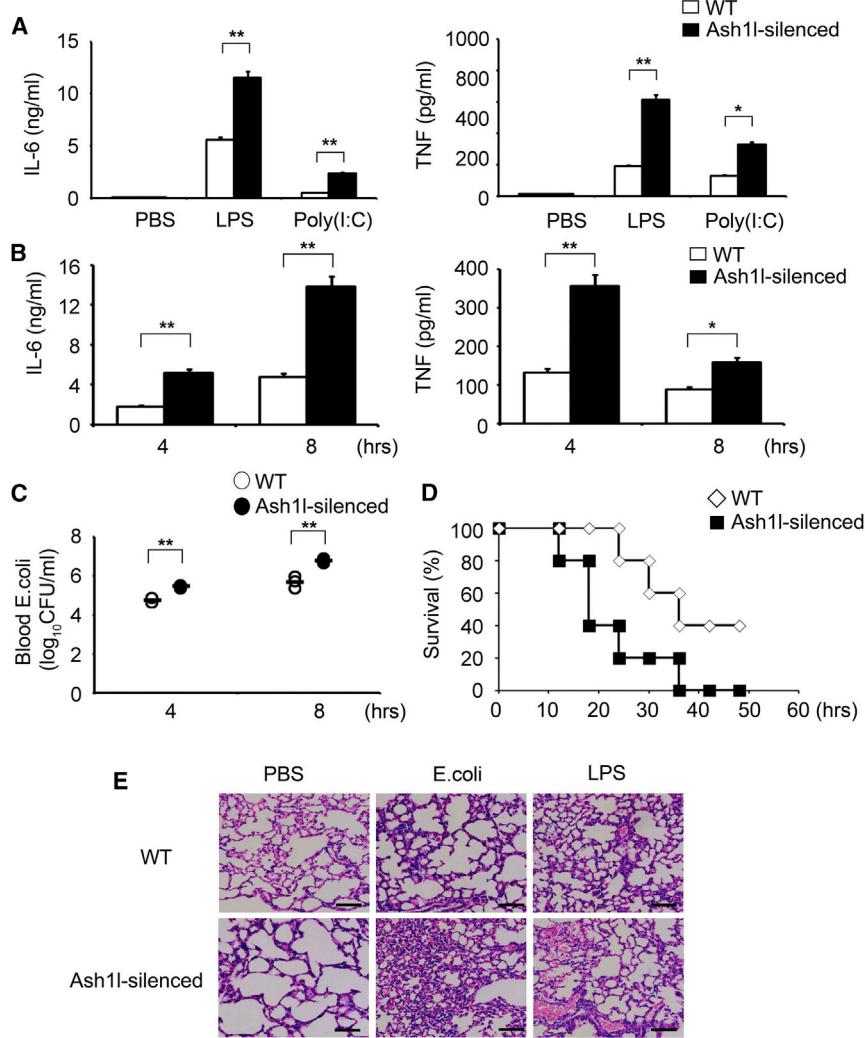


Figure 2. Ash1l-Silenced Mice Are More Susceptible to TLR Ligands Challenge and *E. coli*-Induced Sepsis

(A) CBA assay of IL-6 and TNF in serum of WT and Ash1l-silenced mice ($n = 5$ per genotype) 3 hr after intraperitoneal injection of LPS (15 mg per kg body weight) or poly (I:C) (20 mg per kg body weight).

(B and C) CBA assay of IL-6 and TNF in serum (B) and bacterial load in blood (C) of WT and Ash1l-silenced mice ($n = 5$ per genotype) treated intraperitoneally with 1×10^8 *E. coli* (strain O111:B4) for 4 hr and 8 hr, respectively. Error bars represent SD (B). Each symbol represents an individual mouse, small horizontal lines for the mean (C). * $p < 0.05$, ** $p < 0.01$.

(D) Survival of WT and Ash1l-silenced mice ($n = 10$ per genotype) infected with 5×10^7 *E. coli*. $p < 0.05$ (Wilcoxon test).

(E) Hematoxylin and eosin staining of lungs from WT and Ash1l-silenced mice 6 hr after challenge with PBS or LPS as in (A), or with *E. coli* as in (B). Scale bars represent 20 μ m. Original magnification is $\times 100$. These data sets are representative of three independent experiments. Related to Figure S2.

development of the diseases. Upon monitoring of disease development, we observed higher incidence of Ash1l-silenced mice to CIA accompanied by increased thickness in hind ankle joints and more severe swelling in the paws, as compared to their WT littermates (Figures 4A–4C; Figure S3B). The concentration of IL-6 in serum of Ash1l-silenced mice with CIA was also elevated (Figure S3C). Histological analysis of knee joints also depicted more severe cartilage and bone destruction and mononuclear cells infiltration in joint capsule in Ash1l-silenced mice upon CIA induction (Figure 4D). Together, these data suggest that Ash1l-silenced mice are more susceptible to CIA induction.

Ash1l Attenuates the Pathogenesis of Autoimmune Disease Partially by Controlling IL-6 Production

Given the excessive IL-6 in serum of Ash1l-silenced mice at 8 months of age or under CIA induction, as well as the certified correlation between IL-6 production and arthritis severity, we surmised that the excessive IL-6 might contribute to the more susceptibility of Ash1l-silenced mice to autoimmune disease. To examine this possibility, we generated Ash1l-silenced $\text{IL6}^{-/-}$ double-mutant mice and observed that the severe infiltration of

mononuclear cells in various organs of aged Ash1l-silenced mice was reduced in aged Ash1l-silenced $\text{IL6}^{-/-}$ double-mutant mice (Figure S3D). We also further investigated the role of IL-6 in the regulation of CIA by Ash1l. Consistent to the previous studies, we observed that the $\text{IL6}^{-/-}$ mice were significantly more resistant to CIA induction, as shown by the lack of disease incidence and the normal joints during the whole monitoring process (Figures 4E–4H; Figure S3E). Ash1l-silenced mice developed the most serious

arthritis, showing the highest incidence and clinical scores, thickest ankle joints, and most serious cartilage and bone destruction. Importantly, these severe pathological symptoms of the Ash1l-silenced mice were substantially reduced after they were crossed with the $\text{IL6}^{-/-}$ mice (Ash1l-silenced $\text{IL6}^{-/-}$ double-mutant mice) (Figures 4E–4H; Figure S3E). These data suggest that IL-6 is necessary for the development of CIA and Ash1l potentially prevents the development of CIA via the suppression of IL-6. Interestingly, Ash1l-silenced $\text{IL6}^{-/-}$ double-mutant mice were still more susceptible to CIA than $\text{IL6}^{-/-}$ mice, which indicate that some other factors besides IL-6 might be also involved in the exaggerated CIA development in Ash1l-silenced mice, which needs further investigation.

Ash1l Suppresses TLR- and TNF-Triggered NF- κ B and MAPK Pathways

NF- κ B and MAPK signaling pathways are critical for IL-6 production in response to TLR and TNF stimulation. To investigate the molecular mechanism by which Ash1l negatively regulates TLR-triggered IL-6 production, we examined the key signaling molecules in NF- κ B and MAPK pathways in TLR-triggered

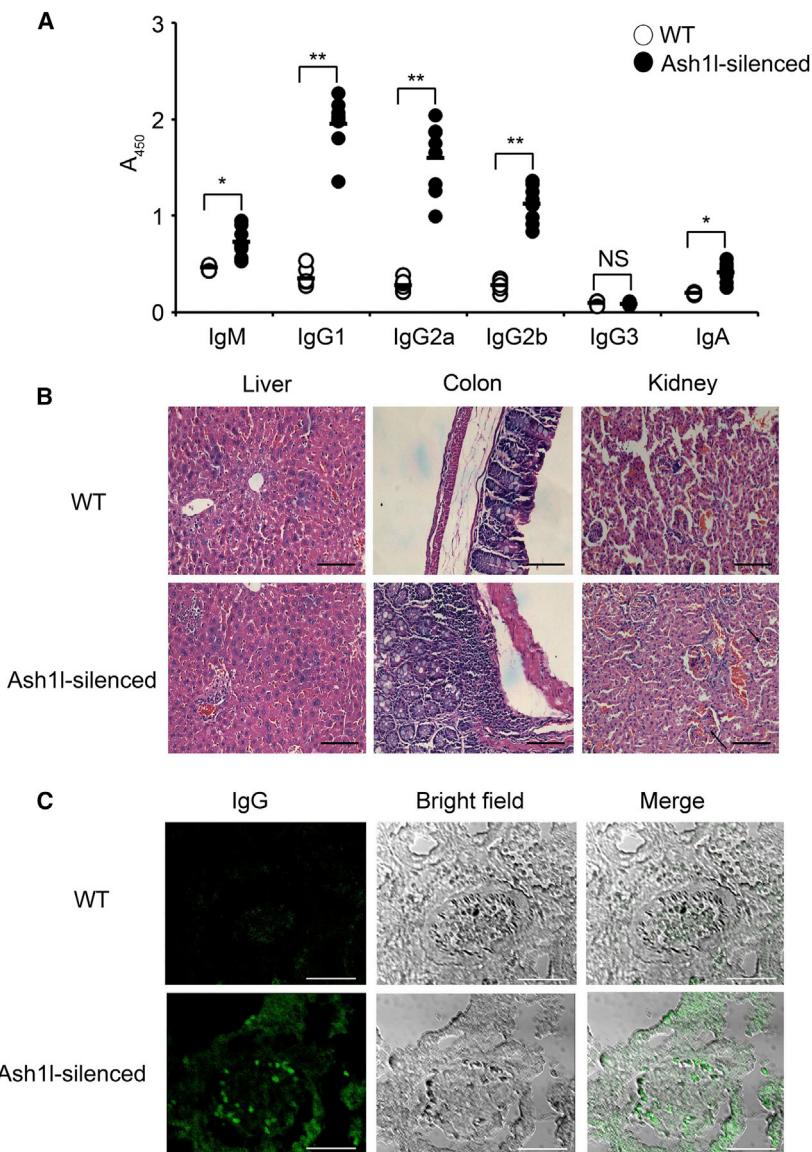


Figure 3. Ash1l-Silenced Mice Are More Prone to Spontaneous Systemic Autoimmune Disease

(A) ELISA assay of IgM, IgG1, IgG2a, IgG2b, IgG3, and IgA in serum of 8-month-old WT and Ash1l-silenced mice ($n = 8$ per genotype). A_{450} represented absorbance at 450 nm. Each symbol represents an individual mouse and the small horizontal lines for the mean. * $p < 0.05$, ** $p < 0.01$; NS, not significant.

(B) Hematoxylin and eosin staining of indicated organ sections from 8-month-old WT and Ash1l-silenced mice. Arrowheads indicate thickened capillary loops. Scale bars represent 50 μ m.

(C) Kidney sections from 8-month-old WT and Ash1l-silenced mice, stained with fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG. Scale bars represent 30 μ m. Original magnification is $\times 200$ (B and C). These data sets are representative of three independent experiments.

TLR-triggered Ash1l-silenced macrophages (Figure 5C; Figure S4C).

Furthermore, deficiency of Ash1l also resulted in the enhanced NF- κ B and MAPK activation in macrophages in response to TNF stimulation (Figures S4D and S4E). In L929 cells that are sensitive to TNF stimulation, silencing of Ash1l led to the increased apoptosis upon TNF stimulation (Figures S4F). Thus Ash1l is also involved in the negative regulation of TNF-induced immune response and cell death.

Together, these data suggest that Ash1l plays as a suppressor in TLR- and TNF-triggered immune response by controlling the key signal transduction of IL-6 production.

Ash1l Suppresses TLR Signals and IL-6 Production through H3K4 Methyltransferase Activity of Its SET Domain

Next, we confirmed the inhibitory effect of Ash1l on the translocation and activation of

Ash1l-silenced macrophages. We observed enhanced phosphorylation of IKK α , IKK β , and their known substrates, I κ B α and NF- κ B p65, as well as the MAPK kinases ERK, JNK, and p38 in LPS-triggered Ash1l-silenced macrophages (Figure 5A; Table S2). However, phosphorylation of IFN regulatory factor 3 (IRF3) was similar between LPS-triggered Ash1l-silenced and WT macrophages (Figure 5A; Table S2), in accordance with the intact TLR-mediated IFN- β production by macrophages after Ash1l silencing (Figure S1A). Consistently, Ash1l silencing resulted in potently enhanced nuclear translocation of p65 but similar nuclear translocation of IRF3 (Figure 5B). We also obtained similar results with Ash1l-silenced macrophages triggered with poly(I:C) (Figures S4A and S4B; Table S3). It has been shown that conjugation of lysine 63 (K63)-linked polyubiquitination chains to NEMO and TRAF6 is required for the activation of NF- κ B and MAPK resulting in the downstream production of IL-6 (Chen, 2012). Consistent with the enhanced NF- κ B and MAPK signals, we observed increased K63-ubiquitination on NEMO and TRAF6 in

NF- κ B with overexpression analysis. The cDNA length of mouse Ash1l is nearly 9 kb, making it extremely difficult to construct a full-length vector. Instead, we constructed Ash1l-fragment 1, 2, 3 vectors respectively encoding 1–880 amino acids (aa), 881–1,886aa, and 1,886–2,958aa of Ash1l (Figure S4G). Notably, reintroduction of Ash1l-fragment 3 vector containing SET domain into HEK293 cells attenuated NF- κ B and IL-6 luciferase activity, whereas reintroduction of Ash1l-fragment 1 and fragment 2 vectors had no such significant effect (Figure S4H). None of the three vectors had any effect on IFN- β activity (Figure S4H). Therefore, we predicted that Ash1l could suppress TLR-triggered innate inflammatory response via its 1,886–2,958aa fragment, which contains a SET domain.

The SET domain has been considered as the signature motif of lysine-specific histone methyltransferase (HMTases) and tightly associated with Ash1l's function on transcriptional activation. Thus, we next investigated whether Ash1l negatively regulated innate inflammatory response dependent on its H3K4

Immunity

Ash1l Is an Inhibitor of TLR Responses

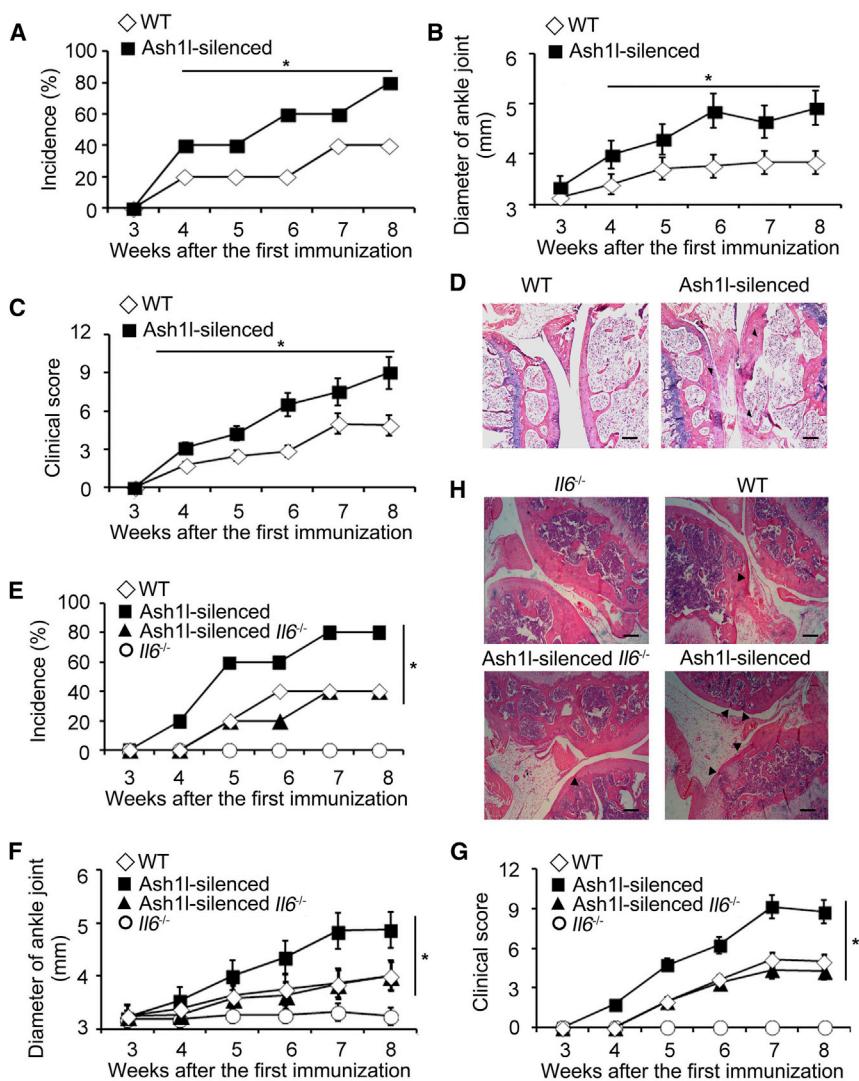


Figure 4. Increased Susceptibility of Ash1l-Silenced Mice to Collagen-II Induced Arthritis

(A–D) CIA was induced in WT and Ash1l-silenced mice ($n = 10$ per genotype) and monitored for disease development 3 weeks after first immunization as follows: (A) Percentage of WT and Ash1l-silenced mice developing arthritis. (B) The average diameter of ankle joints. (C) Clinical scores of WT and Ash1l-silenced mice, evaluated as described in *Supplemental Experiment Procedures*. Error bars represent SD. * $p < 0.05$. (D) Hematoxylin and eosin staining of knee joints from WT and Ash1l-silenced mice. *, mononuclear cells infiltration; ▲, cartilage erosion or new bone formation. Scale bars represent 100 μ m. Original magnification $\times 50$.

(E–H) CIA was induced in WT mice, Ash1l-silenced mice, $II6^{-/-}$ mice, and Ash1l-silenced $II6^{-/-}$ double-mutant mice ($n = 10$ per genotype). The development of diseases was monitored and evaluated as described in (A)–(D). (E) Percentage of mice developing arthritis. (F) The average diameter of ankle joints. (G) Clinical scores of mice from four groups. Error bars represent SD. * $p < 0.05$. (H) Hematoxylin and eosin staining of knee joints. ▲, cartilage erosion or new bone formation. Scale bars represent 100 μ m. Original magnification $\times 50$. These data sets are representative of three independent experiments. Related to Figure S3 and Table S2.

methyltransferase activity of SET domain. In this regard, it has been reported that a single amino acid point mutation Ash1 ΔN^{10} (N1458I) in the SET domain of the *Drosophila* Ash1 abolishes its HMTase activity (Beisel et al., 2002). With homologous comparison, we found that this amino acid residue was equivalent to the amino acid 2212 in the mouse Ash1. We then constructed this single amino acid point mutation Ash1 ΔN (N2212I) (Figure S4G). HMTase assays revealed that H3K4 methyltransferase activity was abolished in the Ash1 ΔN (N2212I) mutant (Figure S4I).

Overexpression of Ash1l-fragment 3 containing the SET domain, but not Ash1l-fragment 1, 2, or mutant vector, attenuated TNF-stimulated nuclear translocation of p65 in HEK293 cells (Figure S4J), which indicated that the SET domain is essential for the suppressive effect of Ash1l on the key signal transduction of IL-6 production. To further confirm that, we transfected Ash1l-silenced and WT macrophages with Ash1l-fragment 3 vector or Ash1l ΔN (N2212I) mutant vector, respectively. We observed that overexpression of Ash1l-fragment 3 vector reduced the phosphorylation of p65 and MAPK

relative to that in mock-transfected control cells in TLR-triggered WT macrophages and reversed the more activated TLR-triggered downstream signals in Ash1l-silenced macrophages, whereas overexpression of Ash1l ΔN (N2212I) mutant vector had no such effect, which indicated that Ash1l indeed suppresses TLR signals dependent on its H3K4 methyltransferase activity of SET domain (Figure 5D; Figure S4K; Tables S4 and S5).

Nuclear p65 serves as a critical transcriptional factor for IL-6 expression, and phosphorylated MAPK is also indispensable for induction of IL-6. We next investigated whether the H3K4 methyltransferase activity of SET domain was required for suppressing the excessive production of IL-6 and TNF in TLR-triggered Ash1l-silenced macrophages. We observed that overexpression of Ash1l-fragment 3 potently suppressed the production of IL-6 and TNF in TLR-triggered WT macrophages relative to that in mock-transfected control cells and substantially reversed the excessive production of IL-6 and TNF in TLR-triggered Ash1l-silenced macrophages (Figure 5E). However, none of Ash1l-fragment 1, fragment 2 vectors, or Ash1l ΔN (N2212I) mutant vector had such effects.

Collectively, these data suggest that Ash1l negatively regulates TLR-triggered IL-6 production through suppressing NF- κ B and MAPK signals dependent on its SET domain-mediated H3K4 methyltransferase activity.

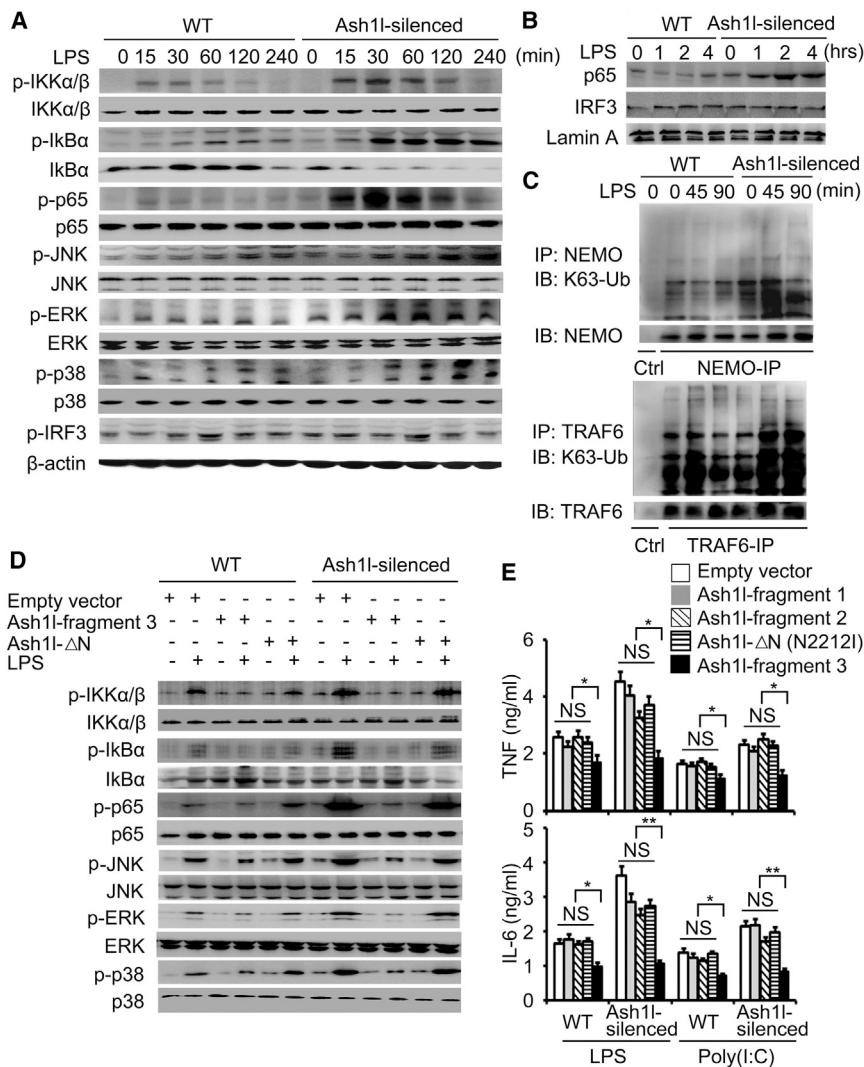


Figure 5. Ash1l Suppresses Activation of TLR Signals and IL-6 Production via H3K4 Methyltransferase Activity of Its SET Domain

(A) Immunoblot analysis (IB) of the phosphorylated (p-) or total protein in lysates of WT and Ash1l-silenced macrophages stimulated for 0–240 min with LPS. Total protein and β -actin serve as loading controls.

(B) IB of p65 and IRF3 in nuclear extracts of WT and Ash1l-silenced macrophages stimulated for 0–4 hr with LPS. Lamin A serves as a loading control.

(C) IB of K63-ubiquitination in lysates of WT and Ash1l-silenced macrophages stimulated for 0–90 min with LPS and immunoprecipitated (IP) with antibody to NEMO (top) and TRAF6 (bottom), respectively. IP with immunoglobulin (IgG) serves as an IP control (Ctrl). IB of NEMO and TRAF6 in total cell lysates serve as a loading control.

(D) IB of phosphorylated or total protein in lysates of WT and Ash1l-silenced macrophages mock-transfected or transfected with Ash1l-fragment 3 (1,886–2,958aa) vector or Ash1l- Δ N (N2212I) mutant vector, respectively, stimulated with LPS for 1 hr.

(E) CBA assay of IL-6 and TNF in supernatants of WT and Ash1l-silenced macrophages transfected with Ash1l-fragment 1 (1–880aa), Ash1l-fragment 2 (881–1,885aa), Ash1l-fragment 3 (1886–2,958aa) vectors or Ash1l- Δ N (N2212I) mutant vector and stimulated for 8 hr with LPS or poly(I:C). Error bars represent SD. *p < 0.05, **p < 0.01, NS, not significant. These data sets are representative of three independent experiments. Related to Figure S4 and Table S4.

Ash1l Suppresses TLR Signaling and IL-6 Production by Facilitating A20 Expression via Its SET Domain

Ash1l acts as a H3K4 methyltransferase that is linked with transcriptional activation of targeted genes. However, our findings above indicated that Ash1l negatively regulates innate IL-6 production. Thus, we propose that Ash1l might be involved in the transcriptional activation of genes encoding certain negative regulators of TLR signals. To confirm this hypothesis, we next selected several well-known inhibitors of TLR signals and analyzed their expression in Ash1l-silenced macrophages. Quantitative PCR revealed that LPS stimulation induced comparable expression of signaling inhibitors such as SOCS1, SOCS3, SHP-1, RKIP, and Mkp1 between Ash1l-silenced and WT macrophages (Figure S5A). Interestingly, A20 expression was reduced in Ash1l-silenced macrophages upon LPS and poly(I:C) stimulation as compared to WT macrophages (Figures S5A; Figure 6A). Nuclear run on experiment showed that nascent Tnfaip3 messenger RNA (mRNA) produced at 30 and 60 min after LPS stimulation was reduced in Ash1l-silenced macrophages, highlighting that Ash1l could directly drive Tnfaip3 transcription (Figure 6B). We also detected the reduced Tnfaip3 mRNA

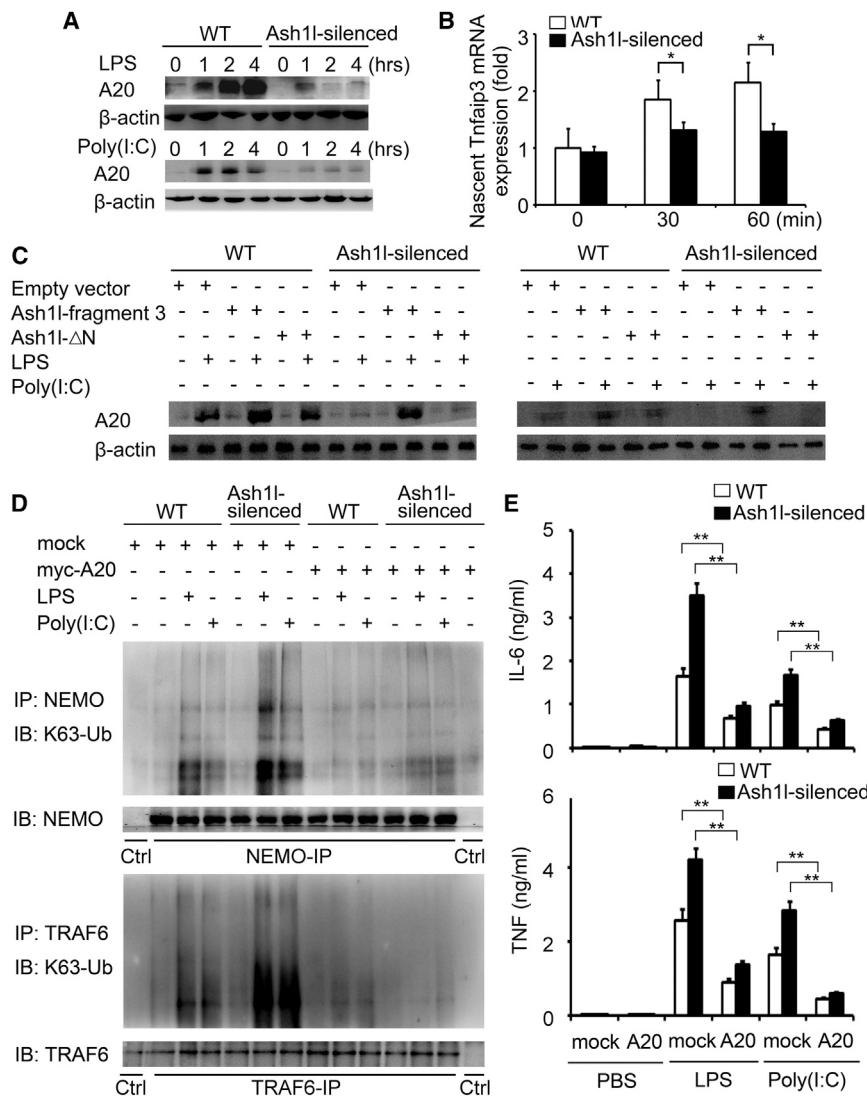
expression in RAW264.7 cells after Ash1l silencing (Figure S5B). However, the expression of PPIA, EEF1A1, and RPLP0, which were proved to be Ash1l-occupied active genes in resting HeLa cells (Gregory et al., 2007), was not affected after silencing of Ash1l (Figure S5B).

To further confirm the involvement of Ash1l in A20 expression, we transfected Ash1l-silenced and WT macrophages with expression vectors encoding Ash1l-fragment 3 or Ash1l- Δ N (N2212I) mutant. Overexpression of Ash1l-fragment 3 potently rescued the attenuated expression of A20 in TLR-triggered Ash1l-silenced macrophages, whereas overexpression of Ash1l- Δ N (N2212I) mutant had no such effect (Figure 6C), indicating that the upregulation of A20 expression was dependent on H3K4 methyltransferase activity of Ash1l SET domain.

A20 inhibits TLR-triggered IL-6 production through deubiquitinating K63-linked polyubiquitination chain on various substrates such as TRAF6 and NEMO (Boone et al., 2004). Given the deficient deubiquitination of K63-linked ubiquitination on NEMO and TRAF6 as well as attenuated expression of A20 in TLR-triggered Ash1l-silenced macrophages, we therefore wondered whether Ash1l suppresses TLR signals by facilitating A20-mediated NEMO and TRAF6 deubiquitination. We found

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that overexpression of A20 indeed reduced the K63-linked ubiquitination on both NEMO and TRAF6 in TLR-triggered WT macrophages relative to that in mock-transfected control cells and substantially restored the impaired K63-deubiquitination in TLR-triggered Ash1l-silenced macrophages to amount similar to that in WT macrophages (Figure 6D). Deubiquitination on NEMO and TRAF6 mediated by A20 serves as a key step for inhibition of IL-6 production. Therefore, we wondered whether the excessive IL-6 production along with the impaired deubiquitination in TLR-triggered Ash1l-silenced macrophages was also due to the reduced A20 expression. Indeed, overexpression of A20 suppressed the production of IL-6 and TNF in TLR-triggered WT macrophages relative to that in mock-transfected control cells and consistently reversed the excessive production of IL-6 and TNF in TLR-triggered Ash1l-silenced macrophages (Figures 6E). Therefore, these data indicate that Ash1l negatively regulates TLR-triggered IL-6 production and innate inflammatory responses by directly facilitating A20 expression via the SET domain.

Figure 6. Ash1l Enhances A20 Expression via SET Domain to Suppresses TLR Signaling and IL-6 Production

(A) Expression of A20 in WT and Ash1l-silenced macrophages stimulated with LPS (top) or poly(I:C) (bottom).

(B) Nascent Tnfaip3 mRNA in WT and Ash1l-silenced macrophages stimulated with LPS for 30 or 60 min. Error bars represent SD. *p < 0.05.

(C) Expression of A20 in WT and Ash1l-silenced macrophages transfected with Ash1l-fragment 3 (1,886–2,958aa) vector or Ash1l Δ N(N221I) mutant vector, and stimulated with LPS or poly(I:C) for 1 hr. β -actin serves as a loading control.

(D) IB of K63-ubiquitination in lysates of WT and Ash1l-silenced macrophages mock-transfected or transfected with plasmid encoding A20, stimulated for 45 min with LPS or poly(I:C) and then immunoprecipitated (IP) with antibody to NEMO (top) and TRAF6. IP with IgG serves as an IP control (Ctrl). NEMO and TRAF6 in total cell lysates serve as a loading control.

(E) CBA assay of IL-6 and TNF in supernatants of WT and Ash1l-silenced macrophages transfected as in (C), and stimulated for 8 hr with LPS or poly(I:C). Error bars represent SD. **p < 0.01. These data sets are representative of three independent experiments. Related to Figure S5.

Ash1l Enhances TLR-Stimulated A20 Expression via Inducing H3K4me3 Modification at the Tnfaip3 Promoter

The data presented above indicate that Ash1l could facilitate A20 expression upon TLR stimulation. Because Ash1l acts as an intrinsic multicatalytic HMTase that can catalyze the conversion of H3K4me1 (monomethylated) to H3K4me3 (trimethylated), we wondered

whether Ash1l could directly target the Tnfaip3 gene and elevate H3K4me3 modification at the Tnfaip3 promoter to enhance A20 expression via its HMTase activity. Chromatin immunoprecipitation (ChIP) experiments with antibody to Ash1l revealed that Ash1l was enriched at the Tnfaip3 promoter region but not I κ B or Tnf promoter region in macrophages upon LPS or poly(I:C) stimulation (Figures 7A and 7B). Furthermore, we did not observe recruitment of Ash1l to the promoter regions of other TLR-induced active genes including NF κ B1, Ifnb1, I κ B α , I κ B β , Mkp1 and Jmjd3, after LPS stimulation, which implied that Ash1l is selectively bound to the Tnfaip3 promoter among those selected genes (Figure S6A). We next tried to investigate how TLR signals induced the selective enrichment of Ash1l to the Tnfaip3 promoter. We wondered whether some transcriptional factors at the Tnfaip3 promoter might be involved in recruiting and interacting with Ash1l. Sp-1 was selected as a candidate because Sp-1 silencing led to the reduced Tnfaip3 mRNA expression in LPS-stimulated macrophages (Figure S6B). However, coIP experiments revealed no interaction between Ash1l and Sp-1 upon LPS stimulation (Figure S6C). Notably, a DNase I sensitivity

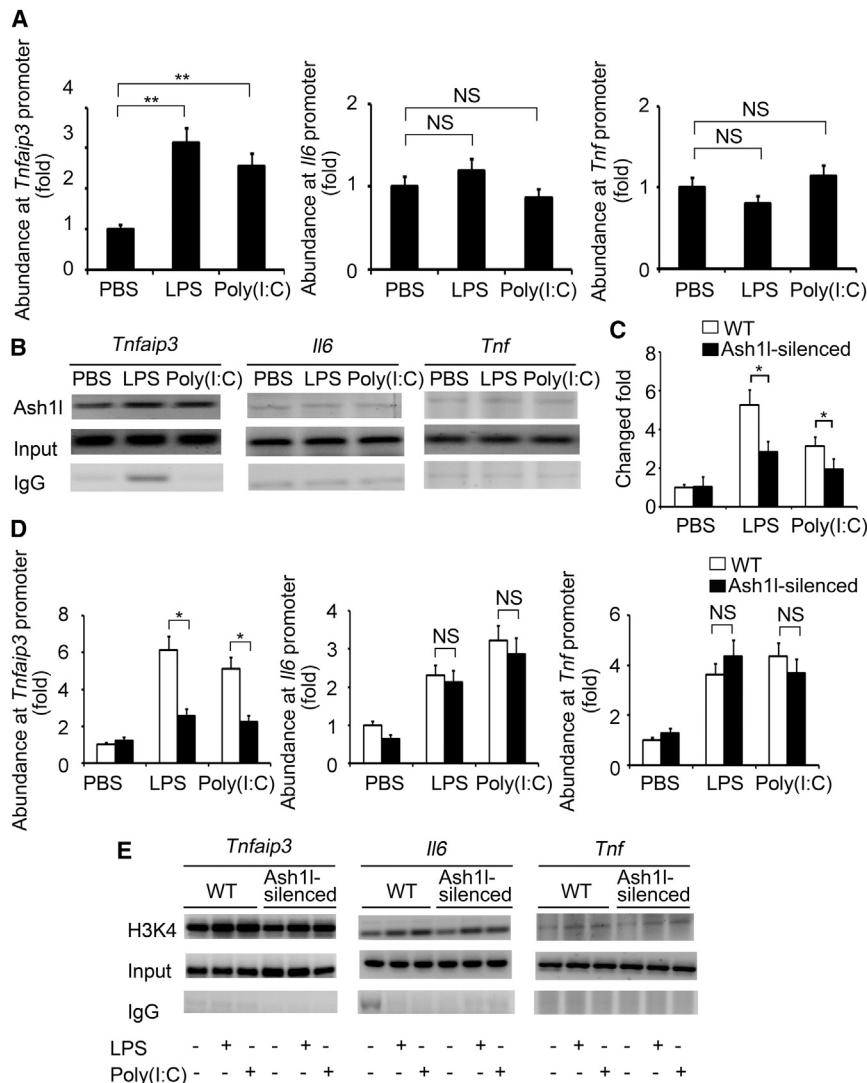


Figure 7. Ash1l Accumulates at *Tnfaip3* Promoter and Elevates Its H3K4me3 Modification in Response to TLR Ligands

(A and B) ChIP analysis of the recruitment of Ash1l to the *Tnfaip3*, *IL6*, and *Tnf* promoter regions with anti-Ash1l antibody in macrophages stimulated for 1 hr with LPS or poly(I:C). *Tnfaip3*, *IL6*, and *Tnf* promoter sequences in input DNA and DNA recovered from antibody-bound chromatin segments were detected by quantitative PCR (A) or semiquantitative PCR (B). IgG serves as a ChIP control and Input as normalized control.

(C) Chromatin accessibility of the *Tnfaip3* promoter region by quantitative RT-PCR with DNase I pretreated nucleus of WT and Ash1l-silenced macrophages stimulated with LPS or poly(I:C) for 1 hr. Results were presented as changed fold concluded with $2^{\Delta\Delta Ct}$, with relative to WT macrophages without stimulation, set as 1.

(D and E) H3K4me3 modifications of the *Tnfaip3*, *IL6*, and *Tnf* promoter regions. ChIP analysis of the trimethylation of histone H3 lysine 4 (H3K4me3) at the *Tnfaip3*, *IL6*, and *Tnf* promoter regions in WT and Ash1l-silenced macrophages stimulated as in (A) and (B). Promoter sequences were detected by quantitative PCR (D) or semiquantitative PCR (E). Error bars represent SD. ** $p < 0.01$, * $p < 0.05$, NS, not significant. These data sets are representative of three independent experiments. Related to Figure S6.

enhances *Tnfaip3* expression through binding to the *Tnfaip3* promoter and increasing its H3K4 modification.

DISCUSSION

Epigenetic modifications such as histone modifications become increasingly involved in the transcriptional regulation of multiple TLR-inducible genes. For

example, H3K4 modification is induced by LPS stimulation at the promoter of *IL12b*, which promotes the production of IL-12p40, a proinflammatory cytokine associated with multiple immunological processes (Kayama et al., 2008). Considering that enzymes modifying H3K4 methylation might be involved in the regulation of TLR-triggered cytokine production, in this study, we performed the siRNA screening test of 14 known H3K4 methyltransferases or demethylases and found that MLL1 and MLL4 could upregulate LPS-induced IL-6 production, consistent with previous studies (Wang et al., 2012; Austenaa et al., 2012). MLL2 was also found to promote LPS signals. On the contrary, we found that Ash1l and Kdm5a could downregulate LPS-triggered signals, which was not reported previously.

Because Ash1l is a well-characterized H3K4 methyltransferase that is associated with transcriptional activation, our discovery of its role in the inhibition of TLR signaling sheds new light on the flexible function of methyltransferases. Ash1l was initially discovered by screening of imaginal disc mutants in *Drosophila melanogaster* (Shearn et al., 1978). A recent study reported the mutations in *Ash1l* gene in 9 of 19 tested cancer cell lines, which

assay showed that the chromatin accessibility of the *Tnfaip3* promoter was more significantly increased in WT macrophages compared to that in Ash1l-silenced macrophages upon TLR stimulation, indicating that Ash1l is involved in loosening of chromatin structure of the *Tnfaip3* promoter (Figure 7C). Hence the selectivity of Ash1l recruitment to the *Tnfaip3* promoter might be due to the regulation of chromatin structure of the *Tnfaip3* promoter by Ash1l, which requires further investigation.

Finally, we assessed the H3K4me3 modification of *Tnfaip3*, *IL6*, and *Tnf* promoter regions in TLR-triggered Ash1l-silenced and WT macrophages. ChIP with antibody to H3K4me3 revealed that TLR ligands induced an increase of H3K4me3 modification at the promoter regions of *Tnfaip3*, *IL6*, and *Tnf*. However, only the promoter region of *Tnfaip3* had a lower concentration of H3K4me3 modification in Ash1l-silenced macrophages compared with WT macrophages (Figures 7D and 7E), suggesting that Ash1l selectively mediated H3K4me3 at *Tnfaip3* promoter regions. These data demonstrate that *Tnfaip3* is one of the Ash1l target genes in macrophages and suggest that Ash1l

indicated involvement of Ash1l in cancer pathogenesis and immunity (Liu et al., 2012). Here we demonstrate that Ash1l negatively regulates TLR-triggered production of proinflammatory cytokines and inflammatory autoimmune disease by suppressing NF- κ B and MAPK pathways via its H3K4 methyltransferase activity of the SET domain.

Furthermore, we identified *Tnfaip3* as a new target gene of Ash1l and showed that Ash1l suppresses TLR-mediated IL-6 and TNF production by promoting A20 expression. Human genetic studies have linked germline single-nucleotide polymorphisms (SNPs) of *Tnfaip3* with susceptibility to multiple human diseases, such as SLE and RA (Shimane et al., 2010). Indeed, Ash1l-silenced mice showed higher susceptibility to spontaneous autoimmune pathogenesis and collagen-II induced arthritis imitating human RA, resembling the phenotype of A20 conditional-deficient mice, indicating that Ash1l indeed maintained immune balance through promoting A20 activity. In this study, we found that A20 overexpression in Ash1l-silenced cells reversed enhancement of IL-6 production and NF- κ B activation. It will be intriguing to investigate whether Ash1l-silenced-Tnfaip3-transgenic mice can rescue the autoimmune phenotype in Ash1l-silenced mice in the future.

Acting as a master regulator in TLR signals, A20 is required to be subtly regulated to achieve assuring inflammatory responses at an appropriate intensity and duration, therefore, it is also necessary to understand the regulation of A20 expression and activity. Various transcription factors and coactivators, such as Sp-1, CBP, and p300, have been suggested to be involved in the rapid induction of the *Tnfaip3* gene upon TNF stimulation (Ainbinder et al., 2002), and various posttranslational modifications of A20, such as phosphorylation and ubiquitylation, serve to restrict its activity (Hutti et al., 2007; Coornaert et al., 2008). However, it remains unclear whether and how epigenetic especially histone modifications are involved in the regulation of A20. In this study, we have demonstrated that the trimethylation concentration at *Tnfaip3* promoter can be enhanced in TLR-triggered macrophages by accumulation of Ash1l, a member of H3K4 methyltransferase. And thus Ash1l directly regulates A20 expression through histone modification, shedding an insight into the regulation of A20 expression. During the study, we also found elevated trimethylation concentrations at both *I6* and *Tnf* promoter upon TLR stimulation, which suggested that histone modifications are also involved in proinflammatory cytokine production. However, TLR-triggered trimethylation of *I6* and *Tnf* promoter were not mediated by Ash1l shown as no difference between WT and Ash1l-silenced macrophages.

The methyltransferase activity of Ash1l has been the subject of controversy. Though some reported that Ash1l could trimethylate H3K4, H3K9, H3K36, and H4K20 (Tanaka et al., 2011), in vivo studies provided evidence that Ash1l is H3K4 selective and acts as a potent transcription activator (Byrd and Shearn, 2003; Gregory et al., 2007). Here in our study, we found that the SET-domain-containing fragment of Ash1l had H3K4 methyltransferase activity. Thus, although Ash1l might have been a candidate enzyme for methylation of H3K4, H3K9, H3K36, and H4K20, we focused on the main and more compelling H3K4 methyltransferase activity of Ash1l. Moreover, we confirmed that Ash1l indeed could activate A20 expression by enhancing the H3K4

modification at *Tnfaip3* promoter, which was dependent on its H3K4 methyltransferase activity.

Collectively, we have demonstrated that Ash1l negatively regulates TLR-triggered innate inflammatory response through suppressing the K63-linked deubiquitination on NEMO and TRAF6 and subsequently activation of NF- κ B and MAPK signals. Ash1l can accumulate at the *Tnfaip3* promoter, enhance its H3K4 methylation modification, and hence activate A20 expression upon TLR stimulation. Thus Ash1l acts as a suppressor of TLR signaling through inducing A20 expression, in a manner dependent on the H3K4 methyltransferase activity of its SET domain. Our study has identified Ash1l as a negative regulator of innate inflammatory immune response and provides mechanistic insight into A20 induction and regulation. More importantly, our findings provide the insightful elucidation of the association of specific histone modification enzymes with TLR-triggered innate immune responses and important clues for the intervention of inflammatory and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies, Measurement of Serum Immunoglobulin, RNA-Mediated Interference, Quantitative RT-PCR, Immunoprecipitation and Immunoblot Analysis, Confocal Microscopy, Plasmid Constructs, Flow Cytometry, DNase I Sensitivity Analysis, Nuclear Run-on Experiment, Assay of Luciferase Reporter Gene Expression, In Vitro Histone Methyltransferase Activity Assay, and detailed experimental procedures can be found in [Supplemental Experimental Procedures](#).

Animal Experiments

Ash1l-silenced mice were generated with the PB transposon insertion (Ding et al., 2005) between exons 15 and 16 of *Ash1l* allele in male germline and bred under pathogen-free conditions. Ash1l-silenced mice on FVB background were consistently crossbred (12 generations) onto a C57BL/6J background, and then were bred with *I6*^{-/-} mice (B6.129S6-*I6*^{tm1Kopf}/J; 002650; Jackson Laboratory) to generate Ash1l-silenced *I6*^{-/-} double-mutant mice. Six- to eight-week-old littermate mice were used. All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai.

Cell Culture and Cytokine Assay

Thioglycolate-elicited mouse peritoneal macrophages were cultured in RPMI-1640 medium with 10% (vol/vol) FCS at a density of 2×10^5 cells per ml for cytokine assay and 1×10^6 cells per ml for immunoblot analysis (Xu et al., 2012). Bone-marrow-derived macrophages (BMDMs) were generated with recombinant mouse GM-CSF (10 ng/ml). After 5 days, cells were replated and stimulated with LPS (100 ng/ml) or poly(I:C) (10 μ g/ml). Amounts of IL-6 and TNF in supernatants and sera were measured by cytometric bead array immunoassay (CBA; BD Biosciences) according to the manufacturer's protocol.

Chromatin Immunoprecipitation Assay

ChIP assays were conducted with a ChIP Assay Kit (Millipore) according to the manufacturer's protocol as described before (Chen et al., 2013). Detailed assay can be found in [Supplemental Experimental Procedures](#).

Establishment of Mouse Models for Endotoxin Shock and Bacterial Infection

WT and Ash1l-silenced mice were injected intraperitoneally with LPS (15 mg per kg body weight), poly(I:C) (20 mg per kg body weight), or 1×10^8 *E. coli* strain O111:B4. Sera were collected and measured by CBA for cytokine concentrations. Colony-forming units (cfu) were measured by counting of viable bacteria on agar plates in blood samples.

Introduction and Evaluation of Collagen-II Induced Arthritis Model

CIA was induced as previously described with minor modifications (Alonzi et al., 1998). Detailed procedures can be found in *Supplemental Experimental Procedures*.

Statistical Analysis

The statistical significance between two groups was determined by Student's t test. The statistical significance of survival curves were estimated with the method of Kaplan-Meier, and curves were compared with the generalized Wilcoxon test. p values of less than 0.05 were considered statistically significant (* $p < 0.05$, ** $p < 0.01$).

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.08.016>.

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