Death Domain-associated Protein 6 (Daxx) Selectively Represses IL-6 Transcription through Histone Deacetylase 1 (HDAC1)-mediated Histone Deacetylation in Macrophages*

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Background: The role and underlying mechanism of Daxx in innate immunity remain to be investigated.

Results: Daxx interacts with HDAC1 and binds to the promoter of IL-6. Daxx selectively represses IL-6 transcription through HDAC1-mediated histone deacetylation.

Conclusion: Daxx selectively represses IL-6 transcription in TLR-triggered macrophages.

Significance: Discovering a new negative regulator of IL-6 and providing insight to epigenetic mechanism of IL-6 production.

As a multifunctional nuclear protein, death domain-associated protein 6 (Daxx) regulates a wide range of biological processes, including cell apoptosis and gene transcription. However, the function of Daxx in innate immunity remains unclear. In our study, we show that Daxx is highly expressed in macrophages and localized in nucleus of macrophages. The expression of Daxx is significantly up-regulated by stimulation with TLR ligands LPS and poly(I:C). Silence of Daxx selectively represses IL-6 expression at transcription level in LPS-activated macrophages. Upon stimulation of LPS, Daxx specifically binds to the promoter of IL-6 and inhibits histone acetylation at IL-6 promoter region. Further mechanism analyses show that histone deacetylase 1 (HDAC1) interacts with Daxx and binds to the promoter of IL-6. Daxx silencing decreases the association of HDAC1 to IL-6 promoter. Therefore, our data reveal that Daxx selectively represses IL-6 transcription through HDAC1-mediated histone deacetylation in LPS-induced macrophages, acting as a negative regulator of IL-6 during innate immunity and potentially preventing inflammatory response because of overproduction of IL-6.

Daxx knock-out mouse is embryonic lethal, which suggests that Daxx is essential for early development (2). Daxx predominantly localizes in nucleus acting as a component of promyelocytic leukemia protein-nuclear bodies (PML-NBs) (3, 4). Daxx does not contain known DNA-binding domains; however, Daxx, as a transcriptional regulator, possesses transcriptional repression activity and interacts directly with several transcription factors, such as Pax3/5 and Smad4 (5, 6). Daxx can also recruit histone deacetylase (HDAC) and transcriptional regulator (ATRX) to the promoter region through interacting with specific transcription factors and subsequently suppress transcription of target genes (7–9). More and more recent studies show that the apoptosis-related genes, such as FADD, caspase-1, in inhibitor of apoptosis (IAP) proteins, are involved in the pathogenesis of inflammation and regulation of immune response (10–12); however, the role of Daxx in the inflammatory innate immune response remains unknown up to now.

The regulation of gene expression is associated with alterations in chromatin structure mediated by enzymatic modifications (acetylation, methylation, and phosphorylation) of the core histones (13–15). Histone acetylation is in general associated with active promoters and open chromatin (16), and plays important roles in transcriptional regulation for establishing cell-specific function states (17). While the effects of histone methylation may be associated with either transcriptional activation or repression, depending on which lysyl residue is modified (18, 19) and whether this residue is mono, di, or trimethylated. It has been shown that lipopolysaccharide (LPS)-stimulated macrophages undergo extensive transcriptional reprogramming that is mediated partially by histone acetylation (20). Acetylation of histone 3 (H3Ac) is one of the widely studied histone modifications. It has been reported to play essential roles in regulating the accessibility of DNA in promoter regions for protein binding and determining the transcription activity of a given gene (21). As enzymes for histone acetylation, histone acetyltransferases and histone deacetylases are at the center of transcription regula-

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3 The abbreviations used are: Daxx, death domain-associated protein 6; HDAC, histone deacetylase; IAP, inhibitor of apoptosis; LPS, lipopolysaccharide; TLR, Toll-like receptor; PML, promyelocytic leukemia protein; Q-PCR, quantitative RT-PCR.
tion in variety of biological and pathological processes. Recruited by corepressors or multi-protein transcriptional complexes to target gene promoters, nuclear HDACs repress gene transcription through removing acetyl groups on histones transferred by histone acetyltransferases. Although histone acetylation and its enzymes have been reported to precisely regulate transcription of inflammatory factors during innate immune response (22, 23), the mechanisms for the epigenetic regulation of inflammatory innate immune response need to be further investigated.

As a component of PML-nuclear bodies, Daxx interacts with PML in the mediation of the antiviral defense (24, 25). However, the role of Daxx in inflammatory innate immune response is still unknown. Thus, in our study, we investigated the role of Daxx in the inflammatory innate response in macrophages. Our data show that Toll-like receptor (TLR) ligands such as LPS can significantly up-regulate Daxx expression in macrophages. Daxx is predominantly enriched in the nucleus of macrophages and can selectively repress pro-inflammatory cytokine IL-6 transcription. Mechanistic study showed that Daxx decreases histone acetylation via HDAC1 in this genomic region, thus selectively regulating IL-6 at the transcriptional level. Therefore, we discovered Daxx as a new negative regulator of IL-6 production, providing a new epigenetic mechanism for the regulation of IL-6 transcription in inflammatory innate immune response.

MATERIALS AND METHODS

**Mice and Reagents**—C57BL/6 mice with 6–8 weeks were obtained from Joint Ventures Siper BK Experimental Animal Co, Shanghai, China. LPS (0111:B4) was from Sigma-Aldrich. Poly(I:C) and CpG oligodeoxynucleotide (ODN) was from Calbiochem. Primary antibodies for phosphorylated forms of ERK1/2 (Thr202/Tyr204), JNK1/2 (Thr183/Tyr185), p38 (Thr180/Tyr182), phosphorylated p65 (Ser536), Myc tag, and Flag tag were obtained from Cell Signaling Technology. Antibody against Daxx, β-actin, GAPDH, and HRP-coupled secondary Ab were from Santa Cruz Biotechnology.

**Construction of Expression Plasmids**—The Flag-tagged recombinant vectors encoding mouse Daxx, and Myc-tagged recombinant vectors encoding HDAC1 and HDAC2 were constructed by PCR-based amplification and then subcloned into the pcDNA3.1 eukaryotic expression vector (Invitrogen), respectively. The clones were confirmed by DNA sequencing.

**Cell Culture and Transfection**—Murine macrophage cell line RAW264.7 and human HEK293T cell line were obtained from American Type Culture Collection and cultured as described previously (26). The cells were transfected with JetPEI (PolyPlus) for plasmid DNA. Thioglycolate-elicited mouse primary peritoneal macrophages were prepared and cultured as described previously (26).

**Real-time Quantitative PCR**—Total RNA was extracted using TRIzol reagent (Invitrogen) and reverse-transcribed using the First Strand cDNA Synthesis kit (Toyobo) following the manufacturer’s instructions. Real-time quantitative RT-PCR (Q-PCR) analysis was performed with Light Cycler (Roche Diagnostics) and the SYBR RT-PCR Kit (Takara, Kyoto, Japan). The primers used for Daxx were 5’-CACCTTCCCCGATTAGGAGA-3’ and 5’-CGCTCTGTAAACCTGAG-3’.

**RNA Interference Assay**—For transient transfection, Daxx small interfering RNA (siRNA) was synthesized as follows: 5’-GACUUAAACACUGAAATT-3’, 5’-UCCGGCAGUGAUUUAAATT-3’. The control small RNA sequence was 5’-UUCUCGGACGUGUCAGUTT-3’. siRNA duplexes were transfected into mouse peritoneal macrophages using INTERFERin (PolyPlus) according to the standard protocol.

**Assay of IL-6 Production**—IL-6 in the supernatants was measured by an ELISA Kit (R&D Systems).

**Immunofluorescence Staining and Confocal Microscopy**—Mouse macrophages were cultured on coverslips overnight. After LPS stimulation, cells were washed with PBS twice before being fixed with 4% paraformaldehyde in PBS for 15 min at room temperature, and then permeabilized and blocked with PBS containing 1 mg/ml saponin and 5 mg/ml BSA for 1 h. Staining with primary Daxx Abs was performed for 1 h at room temperature in blocking buffer. After washing, samples were incubated with appropriate secondary Abs (1:200). Slides were finally examined by confocal microscopy as previously described (27).

**Immunoblot and Immunoprecipitation Analysis**—Cells were lysed with cell lysis buffer (Cell Signaling Technology) and extracted protein was measured by the BCA protein assay reagent kit (Pierce). Immunoblot and immunoprecipitation analysis were performed with indicated antibodies as described previously (28).

**ChIP Assay**—ChIP was performed as previously described (28), with some modifications: 1 × 10^7 cell were fixed for 20 min at 37 °C with 1% formaldehyde and then lysed using 1% SDS lysis buffer. Target protein-DNA complexes in chromatin lysates were, respectively, immunoprecipitated with anti-Daxx, anti-HDAC1, or anti-immunoglobulin G (Abcam) as control. DNA was extracted with a DNA-purification column (Qiagen) and was subjected to Q-PCR analysis. Data were normalized by input DNA for each sample. Some samples were also analyzed by standard semi-quantitative PCR with primer pairs specific for the indicated promoter regions. For binding to endogenous mouse IL-6 promoter, we used forward 5’-GGCTGTGTA- ATCTGGTCACTG-3’ and reverse 5’-GCTTAGTGCTCA- TTGAGGC-3’. The primers for mouse IFN-β were 5’-GCCAGAGCTTGAAATAGGA-3’ (forward) and 5’-CTGTCA- AGGGCTGACTGAG-3’ (reverse). The primers for mouse IL-12p40 were 5’-TCCCCCAGATACTTTTGCA-3’ (forward) and 5’-TGATGGAAAAACCCAAATGAGAA- CTG-3’ (reverse).

**Statistical Analysis**—The statistical significance was determined by Student’s t test between two groups, with a p value < 0.05 considered to be statistically significant. All experiments were independently performed three times in triplicate.

**RESULTS**

Up-regulation of Daxx Expression in Macrophages by TLR Ligands—First, we examined the expression patterns of Daxx in mouse tissues. As shown in Fig. 1A, Daxx was ubiquitously expressed in mouse normal tissues, especially highly expressed in immune organs such as thymus and spleen. To understand the role of Daxx in the TLR-triggered innate immune response,
we further detected the expression of Daxx in murine immune cells, and found that it was highly expressed in primary macrophages, macrophage-like cell line RAW264.7 (Fig. 1B).

According to previously reported microarray data, transcription of Daxx increased significantly in LPS-stimulated murine peritoneal macrophage (29). Therefore, we wondered whether Daxx might play important role in innate immune response. So, we stimulated the murine peritoneal macrophages with various TLR ligands such as LPS, the synthetic RNA duplex poly(I:C) or the CpG oligodeoxy, and then found that LPS (100 ng/ml) and poly(I:C) (10 μg/ml) significantly increased expression of Daxx, with a peak at 4 h after LPS stimulation and 8 h after poly(I:C) stimulation, but CpG ODN (0.33 μM) had no substantial effect on the Daxx expression (Fig. 1C). Furthermore, the protein level of Daxx was confirmed to be increased in LPS-stimulated macrophages (Fig. 1D).

**Daxx Selectively Represses IL-6 Expression in LPS-stimulated Macrophages**—Considering that TLR signals induced up-regulation of Daxx in macrophages, we wondered that whether or not Daxx was involved in the regulation of TLR-triggered innate immune response. To reveal the role of Daxx in TLR-triggered pro-inflammatory cytokines and type I interferon production, we silenced the murine peritoneal macrophages with various TLR ligands such as LPS, the synthetic RNA duplex poly(I:C) or the CpG oligodeoxy, and then found that LPS (100 ng/ml) and poly(I:C) (10 μg/ml) significantly increased expression of Daxx, with a peak at 4 h after LPS stimulation and 8 h after poly(I:C) stimulation, but CpG ODN (0.33 μM) had no substantial effect on the Daxx expression (Fig. 1C). Furthermore, the protein level of Daxx was confirmed to be increased in LPS-stimulated macrophages (Fig. 1D).

**Daxx Is Predominantly Located in the Nucleus of Macrophages**—Beside regulation of signal transduction in cytoplasm, Daxx has been reported to mainly reside in nucleus to regulate gene transcription (30). To investigate the subcellular location of Daxx in macrophages, we treated peritoneal macrophages with LPS for 4 h, then fixed and stained with anti-Daxx antibody for confocal immunofluorescence microscopy analysis. Predominant nuclear-localization of Daxx with a speckled pattern was visualized before and after LPS stimulation (Fig. 4). This indicates that Daxx may function mainly in the nucleus of macrophages to regulate IL-6 transcription.

**Daxx Binds to Proximal Promoter of IL-6 and Decreases Histone Acetylation in Macrophages**—As a multifunctional nuclear protein, Daxx plays important roles in transcription regulation. Our observation that Daxx selectively repressed LPS-induced IL-6 transcription independent of MAPKs and NF-κB pathways implied that Daxx may mediate gene-specific transcription regulation in gene locus of IL-6. Through chromatin immunoprecipitation (ChIP) assay, we found that Daxx directly bound to proximal promoter of IL-6, but not to the promoter of IL-12p40 and interferon-β, in macrophages (Fig. 5A).

Histone acetylation and methylation has been reported to play central roles in transcriptional regulation near transcription start site (13, 31). These important histone modifications are implicated in regulating transcription of inflammatory cytokines, such as IL-6, during innate immune response (32). Therefore, we propose that Daxx may inhibit IL-6 transcription by regulating these epigenetic modifications at promoter of IL-6. We silenced Daxx in mouse peritoneal macrophages, and tested whether histone acetylation and methylation at the IL-6 promoter were affected during LPS response. As the important positive roles of histone acetylation and H3K4me3 in IL-6 tran-

**FIGURE 1.** TLR ligands significantly induce Daxx expression in macrophages. A and B, Q-PCR analysis of Daxx mRNA expression in mouse tissues and immune cells. β-actin was control. C and D, mouse peritoneal macrophages were stimulated with LPS (100 ng/ml), CpG-ODN (0.33 μM) or poly(I:C) (10 μg/ml), respectively, for the indicated time, and then mRNA, protein expressions of Daxx were detected by Q-PCR and Western blot. Data are from three independent experiments with similar results (mean ± S.D.).

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As previously reported (33, 34), we focused on these two histone modifications. Chromatin was precipitated using anti-acetyl H3 and anti-H3K4me3 antibodies and analyzed by Q-PCR. When Daxx was silenced, level of H3Ac at IL-6 promoter increased in macrophages. However, level of H3K4me3 was not affected when Daxx was silenced. These results demonstrate that Daxx represses IL-6 transcription, at least partially, via specifically repressing histone acetylation at IL-6 promoter.

**Daxx Decreases Histone Acetylation via HDAC1 at IL-6 Promoter**—As a transcription repressor, Daxx can interact directly with epigenetic modifiers including HDACs, which mediate histone deacetylation (20, 33, 35). Thus, we speculate that Daxx might decrease histone acetylation via HDAC1 at IL-6 promoter.

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**FIGURE 2. Daxx selectively represses LPS-induced IL-6 expression in macrophages.** A, mouse peritoneal macrophages were transiently transfected with two specific siRNA of Daxx for 48 h and then stimulated with LPS (100 ng/ml) for the indicated time, the relative mRNA expressions of Daxx, IL-6, IL-12p40, and IFN-β were analyzed by Q-PCR. B, IL-6, IL-12p40, and IFN-β in the supernatants of the LPS-stimulated macrophages with or without silencing of Daxx were measured by ELISA. Similar results were obtained from three independent experiments. **, p < 0.01. Data are shown as mean ± S.D. of three independent experiments.

**FIGURE 3. Selective repression of IL-6 expression by Daxx does not depend on MAPK and NF-κB pathways in LPS-stimulated macrophages.** A and B, mouse peritoneal macrophages were transfected with Daxx siRNA for 48 h, and then stimulated with LPS (100 ng/ml) for the indicated time. Phosphorylated ERK1/2, JNK1/2, p38 and p65 were detected by Western blot. Total ERK1/2, JNK1/2, p38, and p65 were used as a loading control.
We first detected the interaction between Daxx and histone deacetylases primarily enriched in nucleus (HDAC1 and -2). We performed coimmunoprecipitation analysis of Daxx and HDACs in nuclear extracts of peritoneal macrophages, and found that the endogenous Daxx protein associated with HDAC1 in vivo (Fig. 6A). Furthermore, we transfected Flag-tagged Daxx and Myc-tagged HDAC1 into HEK293T cells and immunoprecipitated each other for Western blot analysis to further convincingly confirm their direct interaction in vitro. As shown in Fig. 6B, Flag-tagged Daxx interacted with HDAC1.

Together, these experiments provide evidence that Daxx and HDAC1 interact with each other both in vitro and in vivo, suggesting that they may form a complex to co-regulate gene transcription. Thus, we wondered whether HDAC1 could bind to the promoter of IL-6, just as Daxx did. The chromatin immunoprecipitation (ChIP) assay was performed with HDAC1 antibody. We found that HDAC1 bound to the same region with Daxx in promoter of IL-6 in macrophages (Fig. 6C), and then silencing Daxx decreased the association of HDAC1 to IL-6 promoter (Fig. 6D). These results suggest that Daxx decreases histone acetylation via HDAC1 at IL-6 promoter.

**DISCUSSION**

In this study, we have revealed that Daxx is a negative regulator of IL-6 during innate immune response. Daxx of which expression increased in LPS-activated macrophages specifically bound to the promoter of IL-6 and repressed TLR signal-induced IL-6 transcription. In mechanism analyses of Daxx-mediated repression, we found that Daxx interacted with HDAC1, which also specifically bound to IL-6 promoter and repressed histone acetylation at IL-6 promoter through HDAC1 Fig. 7. Therefore, our study identified two new negative regulators of IL-6, Daxx and HDAC1, but also provided a new transcription regulation model of Daxx-mediated target-specific repression through epigenetic modifier HDAC1 for IL-6 expression at transcription level in innate immune response.

Previous studies have shown that Daxx can inhibit p65 transcription, correlating with the inhibition of p65 acetylation in HeLa cells (36). However, in our study, we found expression of IL-12p40, also a target of p65, was not affected when Daxx was silenced. On the basis of our observations that nucleus-localized Daxx represses IL-6 independent of MAPK, NF-κB pathways, we ignored the effect of Daxx on the TLR signaling pathways and went further to investigate the mechanism underlying the selective repression of LPS-induced TLR signaling by Daxx as a nucleus protein in macrophages through interacting epigenetic modifiers.
For other important cytokines implicated in inflammation, we found LPS-induced TNF-α expression was also significantly increased when Daxx was silenced. However, ChIP assay showed no binding of Daxx to TNF-α promoter during LPS response (data not shown). These results indicate an indirect regulation of TNF-α expression by Daxx. Additional experiments should be performed to identify other proteins involved in Daxx-mediated repression of TNF-α expression in TLR-triggered inflammatory innate response.

Histone deacetylases are often recruited by corepressors or multi-protein transcriptional complexes to gene promoters, in which they regulate transcription through histone modification without directly binding of DNA. Daxx was purified as a component of a multiprotein repression complex that includes HDAC1 and HDAC2 (11, 14, 39); and recent studies have shown that HDAC1 and HDAC2 are essential for maintaining genomic stability in mice (35). HDACs were also involved in regulating cytokines cooperating with transcriptional repressors both in innate and adaptive immune response (37–39). Therefore, we performed coimmunoprecipitation analysis of Daxx and HDACs in nuclear extracts of peritoneal macrophages, and found that the endogenous Daxx protein associated with mouse HDAC1 both in vivo and in vitro. But we did not find the interaction between Daxx and HDAC2 possibly due to a low level of HDAC2 in macrophages (20). Meanwhile, HDAC1 is highly expressed and play important regulatory roles in macrophages (40). Furthermore, other studies show that HDAC inhibitors impair innate immune responses and inhibit expression of cytokines to TLR agonists (38, 41). As we know, these inhibitors have widely inhibitory effects for all the HDAC family members. Some of these HDACs may be implicated in regulating TLR pathways, which regulate production of various inflammatory factors. While our study just revealed HDAC1-mediated selective repression of IL-6 at chromatin level.

Our data identified a new selective nuclear repressor of IL-6 expression in the inflammatory innate response, thus providing a new mechanistic insight to the epigenetic regulation of IL-6 transcription. As an important pleiotropic cytokine, IL-6 is involved in the regulation of immune response, hematopoiesis, inflammation, and oncogenesis. Especially, IL-6 plays a critical role in inflammation initiation and maintenance of chronic...
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inflammatory states. Notably circulating IL-6 levels elevate in several autoimmune diseases, such as rheumatoid arthritis, systemic lupus erythematosus, Crohn disease, and psoriasis, and correlate with markers of disease activities (42, 43). Thus, IL-6 is now an important target for the prevention and treatment of inflammatory autoimmune diseases. Deeply revealing the regulation of IL-6 by Daxx in various physiopathological processes will provide new clues to better understand the molecular mechanisms of initiation and development of these inflammatory diseases and further develop new therapeutic approaches.

In summary, we have identified Daxx as a transcription repressor of cytokine IL-6 in inflammatory innate response. Selective suppression of IL-6 expression by Daxx might have general importance to the prevention and treatment of inflammatory autoimmune diseases.

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