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Jmjd3-mediated epigenetic regulation of inflammatory cytokine gene expression in serum amyloid A-stimulated macrophages

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Abstract

Serum amyloid A (SAA), a major acute-phase protein, has potent cytokine-like activities in isolated phagocytes and synovial fibroblasts. SAA-induced proinflammatory cytokine gene expression requires transcription factors such as NF- κ B; however, the associated epigenetic regulatory mechanism remains unclear. Here we report that Jmjd3, a histone H3 lysine 27 (H3K27) demethylase, is highly inducible in SAA-stimulated macrophages and plays an important role in the induction of inflammatory cytokine genes. SAA-induced Jmjd3 expression leads to reduced H3K27 trimethylation. Silencing of Jmjd3 expression significantly inhibited SAA-induced expression of proinflammatory cytokines including IL-23p19, G-CSF and TREM-1, along with up-regulation of H3K27 trimethylation levels on their promoters. Depletion of Jmjd3 expression also attenuated the release of proinflammatory cytokine genes in a peritonitis model and ameliorated neutrophilia in SAA-stimulated mice. Finally, we observed that Jmjd3 is essential for SAA-enhanced macrophage foam cell formation by oxidized LDL. Taken together, these results illustrate a Jmjd3-dependent epigenetic regulatory mechanism for proinflammatory cytokine gene expression in SAA-stimulated macrophages. This mechanism may be subject to therapeutic intervention for sterile inflammation and atherosclerosis.

Keywords

Serum amyloid A; Jmjd3; histone demethylase; macrophages; sterile inflammation

1. Introduction

Serum amyloid A (SAA), a major acute-phase protein, is produced by hepatocytes during acute-phase response and released to the blood circulation [1, 2]. SAA is also produced in

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inflammatory tissues in response to microbial infection, tissue injury, neoplastic growth and immunological disorders [3, 4]. Elevation in plasma SAA concentration is a clinical indication for inflammatory disorders and is associated with the pathogenesis of chronic diseases such as secondary amyloidosis [5], atherosclerosis [6], obesity [7], diabetes [8] and rheumatoid arthritis [4]. Accumulating evidence supports the notion that SAA also actively participates in the inflammatory process due to its cytokine-like activity. Innate immune cells such as macrophages respond to SAA with changes in their gene expression profile, including the up-regulation of a large number of proinflammatory cytokines such as IL-1 β , matrix metalloproteinase 9 [9], granulocyte colony stimulated factor (G-CSF) [10], IL-8 [11], IL-12 and IL-23 [12]. Recent studies have shown that the cytokine-like activities of SAA are mediated by cell surface receptors including formyl peptide receptor 2 (FPR2), Toll-like receptors 2 (TLR-2) and TLR-4 [11, 13, 14]. These receptors activate the transcription factor NF- κ B, leading to the induced expression of inflammatory cytokines [11, 12]. However, the epigenetic pathways involved in the modulation of SAA-induced inflammatory cytokine gene expression have not been characterized.

Epigenetic modification in response to environmental stimuli plays a fundamental role in inflammatory gene expression [15]. LPS, for example, regulates the transcription of inflammatory cytokine genes in macrophages by altering histone deacetylase (HDAC) expression [16]. HDACs, along with histone acetyltransferases (HATs), are essential for viral infection [17, 18]. In eukaryotic cells, chromatin is organized in nucleosomes with DNA wrapped around histone octamers, each assembled with two copies of the H2A, H2B, H3 and H4 subunits [19]. Transcriptional activators typically recruit enzymes that modify the tails of histone through phosphorylation, acetylation, ubiquitination, SUMOylation, methylation, and ADP-ribosylation [20]. These modifications produce different results in transcriptional regulation. While lysine acetylation of histone by histone acetyltransferases usually increases transcriptional activity, lysine methylation of histone H3 regulates nuclear processes dedicated to the maintenance of active or silent states of gene expression depending on the levels and sites of methylation [21, 22].

The broad and potent effect of SAA on the induction of inflammatory cytokine genes suggests the presence of epigenetic regulatory mechanisms. Using PCR-array expression profiling, we found that Jmjd3 (Kdm6b) was markedly induced in SAA-stimulated macrophages. Jmjd3 contains a C-terminal Jumonji C (JmjC) domain and is a demethylase that catalyzes site-specific demethylation of the trimethylated lysine 27 in histone H3 (H3K27me3), resulting in di- and mono-methylated histone H3 (H3K27me2, H3K27me1) [23]. Since its discovery, Jmjd3 has been found to play important roles in regulating polycomb-mediated gene silencing during embryonic development and cell reprogramming [24, 25]. A link between Jmjd3 and inflammation has been suggested based on the function of Jmjd3 in controlling macrophage differentiation and cell identity [26] and on the induction of Jmjd3 in macrophages exposed to bacterial products [27]. Since Jmjd3 expression is markedly up-regulated by SAA, we speculated that it might play a role in SAA-induced inflammatory gene transcription. In this report, we show that Jmjd3 reduces the level of H3K27me3, thus promoting the transcription of a variety of inflammatory cytokine genes in SAA-stimulated macrophages.

2. Materials and Methods

2.1 Mice

C57BL/6 mice were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China). *Tlr2*^{-/-} mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Age- and sex-matched littermates were used in the experiments. The procedures involving mice were carried out using protocols approved by the Institutional Animal Care and Use Committee at the Shanghai Jiao Tong University. Mice that underwent bone marrow transplantation were housed in sterile filter-top cages and supplied with SulfaTrim water for at least 14 days before and up to 42 days after irradiation.

2.2 Reagents

The Epigenetic Chromatin Modification Enzymes PCR Array Kit was purchased from QIAGEN (PAMM-085A; SABiosciences, Venlo, The Netherlands). Recombinant human SAA was obtained from PeproTech (Rocky Hill, NJ). The content of bacterial endotoxin is less than 0.1 ng/μg protein. LPS from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich (St Louis, MO). The inhibitors for protein kinases MEK (U0126) and PI3K (LY294002) were purchased from Calbiochem (San Diego, CA). The anti-Jmjd3 antibody was obtained from Abcam (Cambridge, MA). Antibodies for H3K27me3 and Jmjd3 (C-terminus) were obtained from Millipore (Billerica, MA). Antibodies for HDAC1, β-actin, the anti-rabbit and anti-mouse IgG HRP linked antibodies were obtained from Cell Signaling Technology (Danvers, MA).

2.3 Cells preparation and culture

Mouse macrophages (BMDMs and PMs) were prepared from WT or knockout C57BL/6 mice as described [45]. Human monocytic THP-1 cells (TIB-202), mouse RAW264.7 macrophages (TIB-71), the viral packaging cell line BOSC23 (CRL-11270) were all obtained from ATCC (Manassas, VA). The cells were maintained in RPMI 1640 supplemented with 2 mM of L-glutamine, 10% of FBS (GIBCO), 25 mM HEPES, 100 U/ml penicillin, and 100 mg/ml streptomycin. All cell cultures were kept in a humidified atmosphere with 5% CO₂ at 37°C.

2.4 siRNA interference

Mouse peritoneal macrophages were transfected with specific siRNA using Silencer® siRNA Transfection II Kit (Ambion) according to the manufacturer's instructions. The cells were then recovered for 48 h before stimulation. The siRNA oligonucleotide were designed and synthesized by Shanghai RIBOBIO Co., LTD (Guangzhou, China). Sequence of MyD88-specific siRNA1 and 'Nonsense' siRNA were shown in Supplementary table 1.

2.5 Plasmid constructs

Mouse cDNA coding for Jmjd3, JmjC (a.a. 1141-1614) and JmjC carrying an Ala mutation at His-1388 (Mut. JmjC) were a gift from Prof. Gioacchino Natoli (European Institute of Oncology, Milan, Italy) as described in [24]. The cDNAs were subcloned into the multi-cloning sites of a retrovirus-based expression plasmid, MigR1, which also contains an

internal ribosome entry site (IRES) for GFP expression (Addgene, Cambridge, MA). Oligonucleotides targeting mouse Jmjd3 were annealed and ligated into the RNAi-Ready pSIREN-RetroQ ZsGreen vector (Clontech, Mountain View, CA). All sequences for Jmjd3 cloning and shRNA were shown in Supplementary table 1.

2.6 Retrovirus-mediated gene transfer

BOSC23 cells were co-transfected with 6 µg of the constructed plasmid plus 1.5 µg of the pVSV-G plasmid (Clontech, Mountain View, CA) using HG TransGene Reagent (Health & Gene, China). After 6 h, the medium was removed and replaced with fresh medium. Viral supernatants were collected, passed through a filter and concentrated. For infection, cells were incubated with serially diluted retroviral supernatants in the presence of 8 µg/ml Polybrene (Sigma, St. Louis, MO), centrifuged at 2,000 rpm for 90 min at 30°C, followed by incubation at 37°C for an additional 6 h. The media was replaced with fresh RPMI 1640 supplemented containing 10% FBS. After 48 h, the cells were treated with SAA for the indicated times, and then harvested for different assays.

2.7 Immunofluorescence

RAW264.7 cells were grown on Microscope cover glass (Thermo-Fisher) and fixed with 4% paraformaldehyde at 4°C. After washing and permeabilization, cells were inversed on the dilution of an anti-H3K27me3 antibody (10 µg/ml) for overnight at 4°C. The cells were then repeatedly washed with PBS and incubated with 20 µg/ml of Alexa Fluor® 568 Goat Anti-Rabbit IgG (H+L) Antibody (Life technologies, Carlsbad, CA) for 60 min. Nuclei were stained with DAPI (10 µg/ml) for 5 min. The cover glass was washed with PBS and examined under a Leica TCS SP UV confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.8 Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Millipore) according to the manufacturer's with minor modifications. Briefly, after SAA stimulation, RAW264.7 cells were cross-linked and then washed and resuspended in SDS Lysis Buffer. Nuclei were fragmented by sonication. Chromatin fractions were cleared with protein A-agarose beads followed by immunoprecipitation overnight with an anti-H3K27me3 antibody (Millipore) or with control IgG. Cross-linking was reversed, followed by proteinase K digestion. The primer sequences were shown in Supplementary Table 1. Data are presented as the amount of DNA recovered relative to the input control.

2.9 Bone marrow transplantation

To induce chimeras, male C57BL/6 mice (6 to 8 weeks of age) were exposed to a split exposure of 7 Gy total body irradiation, using an RS 2000 X-ray Irradiator (Rad Source Technologies, Suwanee, GA). One day before transplantation, bone marrow-derived cells were isolated from C57BL/6 mice, followed by infection with retrovirus expressing either scrambled shRNA or Jmjd3-specific shRNA (jmjd3sh2). After 24 h, bone marrow-derived cells were harvested and injected into the tail vein of the irradiated recipients (n = 5-7 per group). The mice were kept for six weeks before experiments.

2.10 Intracellular staining and FACS Analysis

The following antibodies were used for FACS analysis: PerCP-Cy5.5-conjugated anti-CD11b, eFluor®660-conjugated antibodies against TREM-1, IL-23p19 and G-CSF (eBioscience, San Diego). For intracellular staining, peritoneal macrophages were incubated with Golgiplug (BD Bioscience) for 4 h at 37°C. Subsequently, the cells were washed and incubated with surface antibody at 4°C for 30 min. The cells were fixed with Fixation and Permeabilization solution (BD, Franklin Lake, NJ) at 4°C for 20 min, washed with PBS, and stained with the above intracellular antibody at 4°C for 30 min. After washing twice, the samples were analyzed on a BD LSRFortessa™ flow cytometer using CellQuest Pro software (BD Bioscience) and Flowjo 7.2.2 (Treestar, Ashland, OR, USA).

2.11 SAA-induced neutrophilia

SAA was injected subcutaneously into mice that had undergone bone marrow transplantation. The injection was performed daily for 7 consecutive days, with SAA used at 500 ng/kg per day in 200 µl sterile PBS. BSA was used as a control. Blood samples were collected once a day immediately before and during the experiment. Blood was collected by eye puncture using heparinized capillaries. Total white blood cell (WBC) count and WBC differential count were determined using an automatic hematology analyzer (Nihon Kohden, Japan).

2.12 Foam cell formation and oil red O staining

After retrovirus infection for 24 h, RAW264.7 cells were stimulated with LDL (50 µg/ml) plus vehicle or SAA for another 24 h. The cells were stained with oil Red O (Sigma) according to the manufacturer's instruction. Hematoxylin staining was used to visualize cells. The stained cells were detected by light microscopy, and total cells as well as foam cells were counted.

2.13 Statistic analysis

Each experiment was performed on at least three separate occasions. Data analysis was carried out using paired student's *t*-test. A *p* value less than 0.05 is considered statistically significant. The Prism software (version 5.0, GraphPad, San Diego, CA) was used for statistic analysis and graphing.

3. Results

3.1. Jmjd3 expression is induced by SAA in macrophages

To identify components for epigenetic regulation of SAA-induced cytokine gene expression, PCR array analysis was conducted using mRNA from mouse bone marrow-derived macrophages (BMDM) that were treated with human SAA or vehicle for 4 h. Of the 84 genes examined that code for DNA- and histone-modifying enzymes, 16 genes were differentially expressed after SAA stimulation, including up-regulation of those for the histone demethylases Jmjd2 (*Kdm4a*), Jmjd3 (*Kdm6b*) and LSD1 (*Kdm1a*), and down-regulation of the genes for several histone deacetylases (HDAC4, 5, 6, 7, 10 and 11) (Fig. 1A). Among these genes, *Jmjd3* showed the most robust induction following SAA

stimulation (9.8-fold up). Real-time PCR results showed that SAA treatment increased the level of the Jmjd3 transcript in the concentration range between 50 nM and 1 μ M (Fig. 1B). A relatively fast induction of Jmjd3 peaking at 2 h was followed by a prolonged induction lasting for at least 12 h (Fig. 1C). In comparison, the expression level of another closely related Kdm6 subfamily demethylase, Utx (Kdm6a) [22, 28, 29], was not changed. Consistent with the PCR results, Western blot showed an up-regulation of Jmjd3 at the protein level by SAA (Fig. 1D and 1E).

Previous reports have shown that LPS can induce the expression of Jmjd3 [27]. To exclude the effect of contaminating LPS in the induction of Jmjd3, polymyxin B, an amphiphilic cyclic polycationic peptide and potent LPS inhibitor [30], was added to some of the samples. As shown in Supplementary Fig. S1, pre-treatment with polymyxin B (1 μ g/ml) did not significantly alter SAA-induced Jmjd3 expression while effectively blocking the LPS-induced Jmjd3 expression. Taken together, these results support the notion that SAA induces Jmjd3 expression in macrophages.

3.2. The SAA-induced Jmjd3 expression requires MyD88, PI3K and ERK

We examined the signaling components involved in SAA induction of Jmjd3. Our previous study has shown that TLR2 plays an important role in the cytokine-like activity of SAA [13]. Peritoneal macrophages from *Tlr2*^{-/-} mice were prepared to detect its role in SAA-induced Jmjd3 expression. As shown in Fig. 2A, genetic deletion of *tlr2* reduced the level of the Jmjd3 transcript in SAA-stimulated macrophages. Silencing of MyD88 expression by siRNA (Fig. 2B, 2C) also reduced the levels of the Jmjd3 transcript (Fig. 2D) and its protein (Fig. 2E), suggesting that MyD88 is involved in optimal induction of Jmjd3 by SAA. Since SAA is also known to activate TLR4 [14], which signals in part through MyD88, the results suggest that these two TLRs may contribute to SAA-induced expression of Jmjd3.

We previously reported that SAA could activate phosphatidylinositol 3-kinase (PI3K) and extracellular signal-related kinase (ERK) [12]. The potential involvement of these kinases in SAA-induced Jmjd3 expression was examined. As shown in Fig. 2F, treatment of peritoneal macrophages with the MEK inhibitor U0126 and the PI3K inhibitor LY294002 reduced SAA-induced Jmjd3 mRNA expression, with U0126 having a stronger effect. These inhibitors also reduced the expression of the Jmjd3 protein (Fig. 2G). These results show that the SAA-induced Jmjd3 up-regulation requires signaling pathways downstream of MyD88, including the activation of PI3K and ERK.

3.3. SAA down-regulates histone methylation through Jmjd3

To study the functions of Jmjd3 in SAA-activated macrophages, retrovirus-based shRNA delivery was used to specifically knock down Jmjd3 in RAW264.7 cells. Of the two different shRNAs prepared, one (Jmjd3sh2) significantly reduced Jmjd3 expression at both mRNA and protein levels compared to the scrambled shRNA (Figs. 3A and 3B). This shRNA was subsequently used for investigation of the relationship between SAA-induced Jmjd3 expression and histone H3 trimethylation at Lys-27 (H3K27me3). RAW 264.7 cells that were incubated with SAA for 2, 4 and 8 h displayed a time-dependent increase in Jmjd3 protein production, along with a decrease in H3K27me3 level between 2 and 4 h (Fig. 3C).

This change was consistent with the time course of Jmjd3 induction by SAA. The reduction in H3K27me3 level was abrogated in the presence of the shRNA2 Jmjd3sh2, suggesting that SAA-induced Jmjd3 contributes to the down-regulation of the H3K27me3 level.

Mouse Jmjd3 contains 1641 amino acids with one recognizable domain, the JmjC domain [23]. Crystal structure and biochemical studies have shown that the JmjC domain is the catalytic center for demethylation of H3K27me3 [23, 31]. Expression of the C-terminal fragment (a.a. 1141-1614) containing the JmjC domain was sufficient to down-regulate the H3K27me3 level [24]. Several amino acids within the JmjC domain, including His-1388, have been identified as being critical to the demethylase activity [24]. To examine the gene modulation effect of Jmjd3 in more detail, we conducted retrovirus-mediated transduction to express the JmjC-containing fragment (a.a. 1141-1614) in RAW264.7 cells (Fig. 3D). As expected, expression of the JmjC domain caused an up-regulation of selected cytokine genes (Supplementary Fig. S2A) due to its constitutive demethylase activity [24]. Retroviral transduction was also performed with an enzyme-dead Jmjd3 (His-1388 to Ala mutation; Mut. JmjC) or with vector alone (MigR1). Based on the expression levels of GFP as determined by confocal microscopy (Fig. 3E), approximately 50% of the RAW264.7 cells expressed high levels of the exogenous protein (solid arrows); the other 50% of the cells expressed the protein at low levels (open arrows). Histone H3 methylation was detected using an antibody specific to H3K27me3 (red). In vector-only cells, SAA stimulation led to a reduction in the level of H3K27me3 after 4 h, which could result from the endogenous Jmjd3. In cells over-expressing the mutant JmjC domain, SAA stimulation did not alter the levels of H3K27me3, suggesting that the mutant JmjC domain produced a dominant negative effect over the endogenous Jmjd3.

3.4. Jmjd3 modulates inflammatory gene expression in SAA-activated macrophages

Previous reports have shown that stimulation of monocytes and macrophages with SAA led to the expression of multiple inflammatory cytokines [11-13, 32]. We sought to determine whether Jmjd3 plays a role in the transcription of these inflammatory cytokine genes. Based on the qRT-PCR profiling results, SAA potently induced the expression of IL-23p19 and IL-12p40 in RAW264.7 cells, with no significant effect on the expression of IL-12p35 (Fig. 4A). The induction was significantly less in cells expressing the Jmjd3-specific shRNA2, suggesting that the SAA-induced expression of IL-23p19 and IL-12p40 require Jmjd3. Likewise, the induction of G-CSF by SAA was abolished in the Jmjd3 knockdown cells (Fig. 4B). Jmjd3 also regulated the SAA-induced expression of several other inflammatory cytokine genes, including CXCL1, IL-1 β , IL-8 and TNF α (Supplementary Fig. S2B). These results imply that Jmjd3 has a broad effect in the induced expression of inflammatory cytokines and chemokines in SAA-stimulated macrophages.

Triggering receptor expressed on myeloid cell-1 (TREM-1) is a cell surface receptor found in neutrophils, monocytes and macrophages. TREM-1 serves to amplify inflammatory response by promoting the secretion of inflammatory mediators [33]. Enhanced expression of TREM-1 on circulating monocytes has been found in several non-infectious inflammatory diseases [34], suggesting that it might facilitate sterile inflammatory response. In SAA-stimulated mouse peritoneal macrophages, the transcripts of TREM-1 increased significantly

(Fig. 4C). Conversely, the transcripts of TREM-2, a triggering receptor that negatively regulates inflammatory response [35], decreased slightly following SAA stimulation. As shown in Fig. 4D, the increase in SAA-induced TREM-1 expression was significantly inhibited in cells treated with Jmjd3 shRNA2, suggesting a role for Jmjd3 in SAA-induced expression of TREM-1.

In Fig. 3, we showed that JmjC carrying the His-1388 to Ala mutation (Mut. JmjC) served a dominant negative function over the endogenous Jmjd3. Experiments were conducted to determine whether Mut. JmjC could affect inflammatory cytokine gene expression. As shown in Fig. 4E, RAW264.7 cells expressing the mutant JmjC had reduced levels of the IL-23p19, G-CSF and TREM-1 transcripts compared to vector-only cells following SAA stimulation. In contrast, wild type JmjC promoted the expression of these genes in RAW264.7 cells even without SAA stimulation (Supplementary Fig. S2A). Previous reports showed that Jmjd3, acting as a H3K27 demethylase, could be recruited to its target genes for removal of the H3K27me3 marks on chromatin, thus enabling or enhancing transcriptional activation. To verify the exact role of Jmjd3 in the control of H3K27me3 levels at target genes in SAA-activated macrophages, we investigated the extent of H3K27me3 modification in the upstream promoter regions of IL-23p19, G-CSF and TREM-1 using chromatin immunoprecipitation (ChIP). Jmjd3 recruitment paralleled decreased H3K27me3 levels at the IL-23p19, G-CSF and TREM-1 promoters after SAA stimulation, shown as promoter enrichment in Fig. 4F. This effect was reversed in cells treated with the Jmjd3 shRNA2, which silenced Jmjd3 expression. It is concluded, based on these findings, that Jmjd3 could reduce the level of H3K27me3 after SAA stimulation, thus removing transcriptional suppression of these genes.

3.5. Silencing of Jmjd3 abrogates SAA-induced neutrophilia and cytokine gene expression *in vivo*

To further examine the *in vivo* functions of Jmjd3 in SAA-induced inflammatory response, retroviral transduction was performed with bone marrow cells from C57BL/6 mice, using the pSIREN-RetroQ ZsGreen vector containing either a Jmjd3-specific shRNA (Jmjd3sh2) or a scrambled shRNA. These cells were then intravenously injected into irradiated recipient mice of the same genetic background (Fig. 5A). Six weeks after bone marrow transplantation, peritoneal macrophages were collected by lavage and qRT-PCR was conducted to determine the levels of the Jmjd3 transcript. As shown in Fig. 5B, mice receiving Jmjd3sh2-transduced bone marrow cells displayed significantly reduced levels of the Jmjd3 transcript compared to those treated with scrambled shRNA. Silencing Jmjd3 also affected the SAA-induced cytokine expression. Following peritoneal administration of SAA at 1mg/kg for 6 h, macrophages were collected for qRT-PCR measurement of the transcripts (Fig. 5C) and intracellular staining of the proteins (Fig. 5D) for IL-23p19, TREM-1 and G-CSF, respectively. Whereas macrophages from mice receiving scrambled shRNA responded to SAA with elevated levels of the IL-23p19, TREM-1 and G-CSF transcripts, those receiving Jmjd3sh2 did not show a significant induction.

We previously showed that SAA could induce neutrophilia in mice through elevation of G-CSF production [10]. The chimera mice were used to detect the effect of Jmjd3 in SAA-

induced neutrophilia. Six weeks after bone marrow transplantation, SAA was injected subcutaneously into the chimeric mice at a daily dose of 500 ng/kg in 200 μ L PBS, for 7 consecutive days. Blood neutrophil count was determined daily and BSA-injected mice were used as controls. Compared with the BSA controls, blood neutrophil count went up significantly in SAA-treated mice that received scrambled shRNA (Fig. 5E). The chimeras receiving Jmjd3sh2 exhibited a significantly reduced response to SAA in peripheral blood neutrophil count. Taken together, these results show an *in vivo* function of Jmjd3 in regulating SAA-induced inflammatory responses including cytokine production and neutrophilia.

3.6. Role of Jmjd3 in SAA potentiation of oxidized LDL-induced macrophage foam cell formation

SAA is involved in the metabolism of high density lipoproteins (HDL) through its displacement of ApoA-I. As a result, SAA binding to HDL alters its protective function against the development of atherosclerosis [6, 36]. Previous studies have reported that SAA could enhance oxidized LDL-induced foam cell formation, which plays an important role in the pathogenesis of atherosclerosis [37]. Although the epigenetic regulation of this process remains unclear, Jmjd3 expression was increased in patients with familial hypercholesterolemia (FH) compared with healthy individuals [38] (Supplementary Fig. S3A). To investigate whether Jmjd3 plays a role in SAA-enhanced foam cell formation, its expression was silenced by retrovirus-mediated shRNA delivery to RAW264.7 cells. The cells were then stimulated with or without SAA in the presence of oxLDL for 24 h. As shown in Fig. 6A, SAA enhanced the oxLDL-induced foam cell formation compared with oxLDL alone. oxLDL uptake by these cells was lower in the Jmjd3 shRNA2 (Jmjd3sh2) group, compared with scrambled shRNA (Scrsh) treated group. As expected, the cells expressing JmjC domain showed a higher percentage of foam cell formation, while the Mut. JmjC cells behaved similarly to the Jmjd3sh2 cells with a much lower percentage of foam cells (Fig. 6B; Supplementary Fig. S3B and S3C). These data suggest that Jmjd3 contributes to the enhancement effect of SAA on oxLDL-induced foam cell formation.

To further elucidate the potential effect of Jmjd3 in the pathogenesis of atherosclerosis, we examined the expression of inflammatory cytokine genes in RAW264.7 cell treated with oxLDL and SAA. As shown in Fig. 6C, oxLDL induced the expression of IL-23p19, TNF α and TREM-1, and SAA potentiated the induction of these genes by oxLDL. Silencing of Jmjd3 by shRNA (Jmjd3sh2) led to a significant inhibition of the oxLDL-induced expression of these genes as well as the potentiation effect of SAA. Taken together, these results suggest a potential role for Jmjd3 in oxLDL-induced foam cell formation and the expression of inflammatory cytokine genes in these cells.

4. Discussion

Damage Associated Molecular Patterns (DAMPs) are molecules that can initiate and perpetuate non-infectious (sterile) inflammatory response. DAMPs include cytoplasmic and nuclear proteins that are normally not assessable to the receptors on immune cells [39]. These “alarmins” include high mobility group box-1 (HMGB1), S100A8 (MRP8, calgranulin A), cathelicidins and Hsp60/70. Similar to these alarmins, SAA is present at

very low levels in normal condition but its plasma levels increase by up to 1,000-fold in response to tissue injury, neoplastic growth, surgery and immunological disorders. Localized synthesis of SAA contributes to its elevated levels in inflammatory tissues and organs. Like HMGB1, SAA utilizes endogenous pattern recognition receptors for its potent cytokine-inducing activities [13, 14, 40]. In this study, we present experimental evidence that the histone H3 demethylase Jmjd3 is up-regulated by SAA and provides epigenetic regulation of SAA-induced expression of inflammatory cytokine genes.

There is considerable evidence suggesting that changes in chromatin structures lead to the regulation of immune response by pathogen-associated molecular patterns (PAMPs). The resulting production of cytokines and chemokines is accompanied by drastic changes in the epigenetic profiles. The effects of bacterial and viral stimuli on epigenetic regulation of gene expression have been well studied [16-18]. In comparison, much less is known about the profile of epigenetic marks associated with endogenous alarmins. We speculated that SAA could have an impact on epigenetic modifications such as arginine- and lysine-methyl transferases, DNA methyltransferase, histone acetyltransferases and histone deacetylases, which could be revealed by examination of these epigenetics-related enzymes using PCR arrays. The profile changes, showing decreased expression of a number of HDAC genes, are consistent with the notion that histone acetylation promotes inflammatory gene expression, whereas histone deacetylation results in transcriptional repression of inflammatory genes [41]. Interestingly, among the significantly up-regulated genes, the histone demethylase Jmjd3 has the highest inducibility, followed by other Kdm family genes including LSD1 (Kdm1a) and Jmjd2 (Kdm4a). We further confirmed the induction of Jmjd3 at both mRNA and protein levels, while the closely related H3K27 demethylase Utx was not induced by SAA. These findings show that SAA selectively activates the Jmjd3-dependent epigenetic regulation pathway.

SAA activation of TLR2 leads to the induction of a large number of inflammatory cytokine genes [13]. However, *Tlr2*^{-/-} deficiency only led to a modest reduction in Jmjd3 expression in SAA-stimulated peritoneal macrophages. Since transfection of MyD88-specific siRNA into mouse peritoneal macrophages resulted in a more complete inhibition of SAA-induced Jmjd3 expression, other receptors might be involved in the SAA-induced Jmjd3 expression. To date, TLR2 and TLR4 are the only two receptors for SAA within the TLR family [14]. Therefore, it is likely that SAA uses both TLR2 and TLR4 for epigenetic regulation of inflammatory cytokine gene expression. This is consistent with published reports showing that Jmjd3 contributes to gene expression in LPS-activated macrophages [27]. The potential involvement of FPR2 in SAA-induced Jmjd3 expression cannot be ruled out. Based on these findings, it is speculated that SAA triggers concerted signaling mechanisms that lead to epigenetic modulation as well as activation of transcription factors. SAA differs from most other PAMPs in that it can activate multiple receptors, thus maximizing its potency in the induction of inflammatory cytokine genes.

It has become increasingly apparent that epigenetic regulation plays important roles in inflammatory diseases. In the present study we showed that the SAA-induced neutrophilia is subject to modulation by Jmjd3. Moreover, Jmjd3 is also involved in the oxLDL-induced foam cell formation, which is enhanced by SAA. Other published studies have shown that

Jmjd3 is up-regulated in several types of tumors such as Hodgkin's lymphoma and renal cell carcinoma. In these situations, Jmjd3 may participate in the development and metastasis of cancer cells [42, 43]. For the effect of Jmjd3 in sterile inflammation, it is reported that Jmjd3 could mediate IL-6 gene regulation in endothelial cells following spinal cord injury [44]. Previously, it was shown that up-regulation of SAA in the blood circulation could enhance the induction of foamy macrophage formation by oxLDL and thus implicates SAA as a potential causal agent in atherogenesis [37]. In this study, we showed that attenuated macrophage foam cell formation could result from dampened proinflammatory gene induction in cells with Jmjd3 silencing. These observations are consistent with the notion that Jmjd3 is involved in the pathogenesis of atherogenesis. Targeting Jmjd3 may provide a therapeutic option in the intervention of sterile inflammation and atherosclerosis.

In summary, the results from this study provide evidence that SAA-induced inflammatory cytokine gene expression is accompanied with the induction of Jmjd3, which in turn provides an epigenetic regulatory mechanism for the cytokine-like activity of SAA. We also showed that *in vivo* gene silencing of Jmjd3 is accompanied by abrogated expression of inflammatory cytokine genes and neutrophilia, highlighting the importance of Jmjd3 in sterile inflammation. In addition, Jmjd3 deficiency *in vitro* seems to reduce inflammatory cytokine gene expression and protects against macrophage foam cell formation. To the best of our knowledge, this is the first report on the Jmjd3 involvement in SAA-induced gene expression. A better understanding of the epigenetic regulatory mechanism will help designing new therapies for the treatment of inflammatory diseases.

5. Conclusion

- SAA induces the expression of Jmjd3 at both mRNA and protein levels.
- SAA-induced Jmjd3 accompanies the reduction in the level of H2K27me3.
- Silencing of Jmjd3 abrogates SAA-induced cytokine gene expression and neutrophilia.
- MyD88, ERK and PI3K are involved in the SAA-induced Jmjd3 expression.
- Silencing of Jmjd3 in macrophages reduces foam cell formation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

SAA	serum amyloid A
H3K27me3	trimethylation of histone H3 at lysine 27
HDACs	histone deacetylases
FPR2	formyl peptide receptor 2
G-CSF	granulocyte colony-stimulating factor
IL-23p19	interleukin-23 alpha subunit p19
IL-12p40	interleukin-12 p40
IL-12p35	interleukin-12 p35
TREM-1/2	triggering receptor expressed on myeloid cell-1/2
TGF-β	transforming growth factor beta
CXCL1	chemokine (C-X-C motif) ligand 1
IL-1β	interleukin-1 beta
IL-8	interleukin-8
TNF-α	tumor necrosis factor alpha
PMs	peritoneal macrophages

BMDMs	bone marrow-derived macrophages
PI3K	phosphatidylinositide 3-kinases
MAPK	mitogen-activated protein kinase
ERK	extracellular signal-regulated kinase
HATs	histone acetyltransferases
oxLDL	oxidized low density lipoproteins
DAMPs	damage associated molecular patterns
PAMPs	pathogen associated molecular patterns
HMGB1	high mobility group box-1

Highlights

- SAA induces Jmjd3 expression in macrophages
- Elevated Jmjd3 expression leads to reduced trimethylation at Lys-27 in Histone H3
- Silencing of Jmjd3 abrogates SAA-induced cytokine gene expression and neutrophilia
- Jmjd3 provides an epigenetic regulatory mechanism for SAA-induced gene expression

Fig. 1.

Jmjd3 is induced in SAA-activated macrophages. (A) Genes expression heat map depicting epigenetic-related genes differentially expressed ($p < 0.01$) in mouse BMDM treated with buffer (Con) or 0.5 μM SAA for 4 h. (B, C) qRT-PCR analysis of Jmjd3 and Utx transcripts in mouse peritoneal macrophages (PMs) treated for 2 h with buffer or SAA at indicated concentrations (B) or for different periods of time as indicated (C). Data shown are means $\pm$ SEM of three experiments. *, $p < 0.05$; **, $p < 0.01$. (D, E) Western blot analysis of Jmjd3 in mouse PMs stimulated for 4 h with SAA at the indicated concentrations (D) or with 0.5 μM of SAA for different periods of time as indicated (E). The blots shown are representative of three experiments.

Fig. 2.

Optimal induction of Jmjd3 by SAA requires MyD88, PI3K and ERK. (A) PMs from *Tlr2*^{-/-} mice and wild type (WT) littermate controls were stimulated with 0.5 μ M of SAA for 2 h. The abundance of the transcript for Jmjd3 was determined by qRT-PCR. (B, C) PMs were transfected with MyD88-specific siRNA (siMyD88) or negative control siRNA (NC) as described in *Materials and Methods*. After 48 h, the knockdown efficiency was determined by qRT-PCR (B, means $\pm$ SEM of three experiments) as well as by Western blotting (C, representative blots from three experiments). (D, E) After siRNA transfection for 48 h, PMs were stimulated with 0.5 μ M of SAA for 2 h (D, for detection of Jmjd3 transcript) or 4 h (E, for detection of Jmjd3 protein). (F, G) PMs were pretreated with the MEK inhibitor U0126 (10 μ M) and the PI3K inhibitor LY294002 (10 μ M) for 1 h and then stimulated with SAA (0.5 μ M) for another 2 h (F, for qRT-PCR detection of Jmjd3 transcript) or 4 h (G, for Western blotting of Jmjd3 protein). Data are representative of three experiments (blots in C, E, G) or are presented as the means $\pm$ SEM of at least three experiments (bars graphs A, B, D, F). *, $p < 0.05$, **, $p < 0.01$.

Fig. 3.

Down-regulation of histone methylation by SAA. (A, B) RAW264.7 cells were infected with retrovirus expressing shRNAs targeting Jmjd3 mRNA (Jmjd3sh1 and -Jmjd3sh2) or scrambled shRNA (Scrsh). Results of qRT-PCR (A) and Western blotting analysis (B) of Jmjd3 expression are shown. (C) Western blotting analysis of the levels of Jmjd3 and nuclear H3K27me3 in RAW264.7 cells infected with retrovirus expressing Jmjd3 shRNA2 (Jmjd3sh2) or scrambled shRNA (Scrsh) for 48 h. The cells were stimulated with SAA (0.5 μ M) for the indicated time points. HDAC1 was used as a nuclear marker. (D) Western blots showing the expression of JmjdC-containing fragment (a.a.1141-1614; WT JmjdC) or one that carries the His to Ala mutation at a.a. 1388 (Mut. JmjdC), after retroviral transduction of RAW264.7 cells with the respective vectors or with empty vector (MigR1). The expression of these fragments was detected with an antibody recognizing the C-terminus of mouse Jmjd3. (E) RAW264.7 cells were similarly infected for 48 h as in (D) and then stimulated with 0.5 μ M of SAA for 4 h and fixed. The H3K27me3 level was detected with a specific antibody for H3K27me3 (red, with Alexa Fluor® 568-conjugated secondary antibody), and the nuclei were stained with DAPI (blue). GFP expression under IRES of the bicistronic vector (green) indicates cells that are infected. The results are presented as the means $\pm$ SEM of at least three experiments (A) or representative blots (B, C, D) and images (E) from three independent experiments. *, $p < 0.05$.

Fig. 4. Jmjd3 modulates proinflammatory genes expression in SAA-activated macrophages. (A, B) After infection with shRNAs for 48 h, RAW264.7 cells were treated with 0.5 μ M SAA for 2 h. The transcript induction of IL-23p19, IL-12p40 and IL-12p35 (A) as well as G-CSF (B) was determined by qRT-PCR. (C) PMs were stimulated with or without 0.5 μ M SAA for the indicated periods of time, qRT-PCR was then conducted for TREM-1 and TREM-2 transcript. (D) After infection with shRNA for 48 h, RAW264.7 cells were treated with 0.5 μ M SAA for 2 h. The transcript induction of TREM-1 was determined by qRT-PCR. (E) RAW264.7 cells were infected with an enzyme-dead Jmjd3 expression vector (Mut. 1141-1614) or the empty MigR1 vector (MigR1). After 48 h, cells were treated with 0.5 μ M SAA for 2 h. The transcript induction of IL-23p19, G-CSF, TREM-1 genes was determined by qRT-PCR. (F) SAA-induced changes in H3K27me level at the promoters of selected proinflammatory cytokine genes. RAW264.7 cells were infected with retrovirus carrying

shRNA targeting the Jmjd3 mRNA (Jmjd3sh2) or scrambled shRNA (Scrsh). After 48 h, the cells were treated with 0.5 μ M of SAA for 1 h, followed by ChIP using an anti-H3K27me3 antibody or matching IgG for immunoprecipitation. PCR amplification of the respective genomic fragment (binding sites) on the promoters of IL-23p19 (A), G-CSF (B) and TREM-1(C) was presented as fold enrichment relative to the control (unstimulated cells). Data represented are means $\pm$ SEM of three independent experiments. *, $p<0.05$, **, $p<0.01$.

Fig. 5.

Gene-silencing of Jmjd3 inhibits SAA-induced inflammatory cytokine gene expression *in vivo* and SAA-induced neutrophilia. (A) Schematic diagram for bone marrow transplantation, conducted using a procedure described in *Materials and Methods*. (B) Jmjd3 transcript level, determined by qRT-PCR, in peritoneal macrophages collected by lavage 6 weeks after bone marrow transplantation ($n=7$ per group). (C) Six weeks after bone marrow transplantation, SAA (1mg/kg body weight) or PBS was administered intraperitoneally ($n=7$ per group) for 6 h. Peritoneal macrophages were harvested and qRT-PCR was conducted to

determine the transcript levels for IL-23p19, TREM-1 and G-CSF. Each symbol represents an individual mouse (n=7), small horizontal lines indicate the mean. *, p<0.05. (D) Detection of IL-23p19 and TREM-1 at the protein level by flow cytometry. Peritoneal macrophages were harvested by lavage from mice that received SAA or PBS. Intracellular IL-23p19, TREM-1 and G-CSF proteins were detected by staining with eFluor®660-conjugated anti-IL-23p19, anti-TREM-1 and anti-G-CSF antibodies, respectively. Data shown are histogram from one experiment, representative of three independent experiments with similar results. (E) SAA (500 ng/kg body weight) was injected subcutaneously into the chimeric mice (n=7 per group) that underwent bone marrow transplantation for 6 weeks. The injection was carried out once daily for 7 consecutive days, using BSA as a control. Blood neutrophil counts were determined daily using an automatic hematology analyzer. Data shown are means $\pm$ SEM of three independent experiments.

Fig. 6.

Jmjd3 is required for the SAA enhancement of oxidized LDL-induced macrophage foam cell formation. (A, B) After infection with shRNA targeting the Jmjd3 mRNA (Jmjd3sh2) or scrambled shRNA (Scrsh) for 24 h, RAW264.7 cells were stimulated with or without SAA in the presence of oxLDL for another 24 h, as described in *Materials and Methods*. (A) Oil Red O staining for foam cells, viewed by light microscopy (40x). (B) The stained foam cells were counted and its percentage of total cells is shown. (C) After retrovirus infection for 24 h, RAW264.7 cells were treated with oxLDL for 18 h and then with SAA for another 6 h. The cells were collected for determination of IL-23p19, TNFα and TREM-1 transcripts. Data shown are representative images from three experiments (A) or are presented as the means $\pm$ SEM from three independent experiments (B, C). *, $p < 0.05$, **, $p < 0.01$.