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5-Azacytidine suppresses RNA polymerase II recruitment to the SLPI gene

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Abstract

Histone methylation is regarded as a stable modification important in the epigenetic regulation of gene expression. Transcriptionally active chromatin is methylated at H3-K4 whereas repressed chromatin is methylated at H3-K9. To investigate the role of histone methylation in an acute inflammatory response, A549 cells were treated with IL-1β and/or the methylase inhibitor 5-azacytidine (5-aza), and histone H3-K4 methylation levels and transcription of secretory leukocyte protease inhibitor (SLPI) were measured. IL-1β stimulation enhanced histone H3-K4 tri-methylation across the SLPI coding region at 24 h. In parallel, IL-1β enhanced recruitment of RNA polymerase II to the SLPI gene. 5-aza attenuated both H3-K4 tri-methylation and RNA polymerase II recruitment to a similar extent resulting in reduced SLPI mRNA and protein levels. These data suggest that in addition to epigenetic regulation of constitutive SLPI expression, H3-K4 tri-methylation may play a role in stimulated SLPI expression by modulating RNA polymerase II recruitment and subsequent gene transcription.

Keywords: Histone methylation; Inflammation; Methylase inhibitor; RNA polymerase II; IL-1β; SLPI

Chronic inflammatory diseases, such as asthma and chronic obstructive pulmonary disease (COPD), are caused by abnormal expression of both pro- and anti-inflammatory genes including GM-CSF, IL-8, and secretory leukocyte protease inhibitor (SLPI) [1–3]. Post-translational modifications of histones including phosphorylation, acetylation, and methylation, and DNA cytosine methylation are associated with the organisation of chromatin structure and the regulation of gene expression: for example, H3-K4 tri-methylation is linked to transcriptional activation, whilst that at H3-K9 and/or DNA hyper-methylation are associated to transcriptional repression [4,5]. This association of histone lysine methylation patterns with transcriptional activity is maintained in the wide range of biologic species from yeast [6] and chicken [7] to humans [8–10]. Although the role of H3-K4 tri-methylation has been reported to play a role in oncogenesis [9], X-chromatin inactivation, and development in human cells [8,10], no evidence for a role in inflammatory responses has been elucidated.

Although histone H3-K4 tri-methylation was observed to distribute over hetero-chromatin [10–12], no association was detected in the process of transcriptional repression between histone H3-K4 hyper-methylation and RNA polymerase II recruitment on genes in yeast [13] and in chicken embryo-erythrocytes [14]. Furthermore, it was shown that H3-K4 hyper-methylation was not related to acute TNF-α-induced gene expression [15]. These results suggest that H3-K4 tri-methylation may contribute to constitutive, but not inducible, gene expression [16].

In this study, we investigated the degree of H3-K4 tri-methylation across the constitutively active SLPI gene [17] and the inducible gene GM-CSF [17]. We also

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investigated the effect of IL-1β and the histone methylase inhibitor 5-azacytidine (5-aza) on H3-K4 tri-methylation and on SLPI transcription. We have investigated SLPI expression because (i) SLPI is constitutively expressed in A549 cells, (ii) the SLPI gene contains no CpG-rich regions so that we could minimize the effect of DNA methylation, and (iii) SLPI is an important molecule involved in airway inflammation [3,18].

Materials and methods

Cell culture and stimulation. A549 were cultured in Dulbecco's modified Eagle's medium (Sigma–Aldrich, Poole, UK) to 60–80% confluency before the medium was replaced with serum-free media and incubated for a further 48–72 h. Cells were pre-treated with 5-aza (Sigma–Aldrich) at the concentrations indicated, 30 min prior to stimulation with recombinant human IL-1β 1 ng/mL (Upstate, Dundee, UK).

ELISA. The concentration of SLPI in the media was determined by sandwich ELISA (R&D System, Oxon, UK).

mRNA extraction and RT-PCR. mRNA was extracted using an RNeasy Kit (Promega, Southampton, UK), and cDNA was synthesised by treating mRNA (1 ng) with reverse transcriptase (Promega) after treatment with DNase I (Promega). SLPI and β-actin mRNA expression was determined by quantitative polymerase chain reaction (QPCR) (Corbett Research, Australia), using SYBR Green (Qiagen, Hilden, German). The primer sets for SLPI were: (forward) 5′-*atggcaggaat caagctttc*-3′; (reverse) 5′-*atgaagtccagcggcctctt*-3′ [19] and for β-actin, (forward) 5′-*tctacaatgagctgcgtgtggctc*-3′ (reverse) 5′-*aggaaggaaggctc caagagttcctc*-3′ [20]. SLPI mRNA expression was corrected for the amount of β-actin mRNA.

Chromatin immunoprecipitation assay. Chromatin immunoprecipitation (ChIP) assays were performed as previously described [17] and Q-PCR was utilised to calculate the amount of immunoprecipitated DNA. The primers used in this study were designed across various regions on the SLPI gene (Fig. 1A): SL-1: (forward) 5′-*tacctacact cagctatgg*-3′ (reverse) 5′-*tgttctgtgcctgacacagg*-3′; SL-2: (forward) 5′-*tcatagccttacctggcatag*-3′ (reverse) 5′-*tggacttcatggtgaaggcagg*-3′; SL-3: (forward) 5′-*agtgcccagtgacttatggcc*-3′ (reverse) 5′-*tcagacacaggctg tgcttg*-3′; and SL-4: (forward) 5′-*ggcacattcctgtgttcacc*-3′ (reverse) 5′-*agcctaggttcctcagtctgg*-3′. The data were normalised to the amount of input DNA, which was obtained at the beginning of the processing the samples.

RNA polymerase II ChIP assay. RNA polymerase II association with transcribed regions of the SLPI gene was quantified by ChIP assay and used as a measure of SLPI gene transcription [21]. An antibody directed against RNA polymerase II (Abcam, Cambridge, UK) and the primer set SL-3 were used.

DNA methylation estimation/HpaII sensitivity assay. *Hpa*II digestion assay was performed to estimate DNA methylation levels [22]. DNA was extracted using phenol extraction and ethanol precipitation before overnight digestion with *Hpa*II, a methylation sensitive DNA endonuclease. The both number of DNA fragments, whose *Hpa*II sites

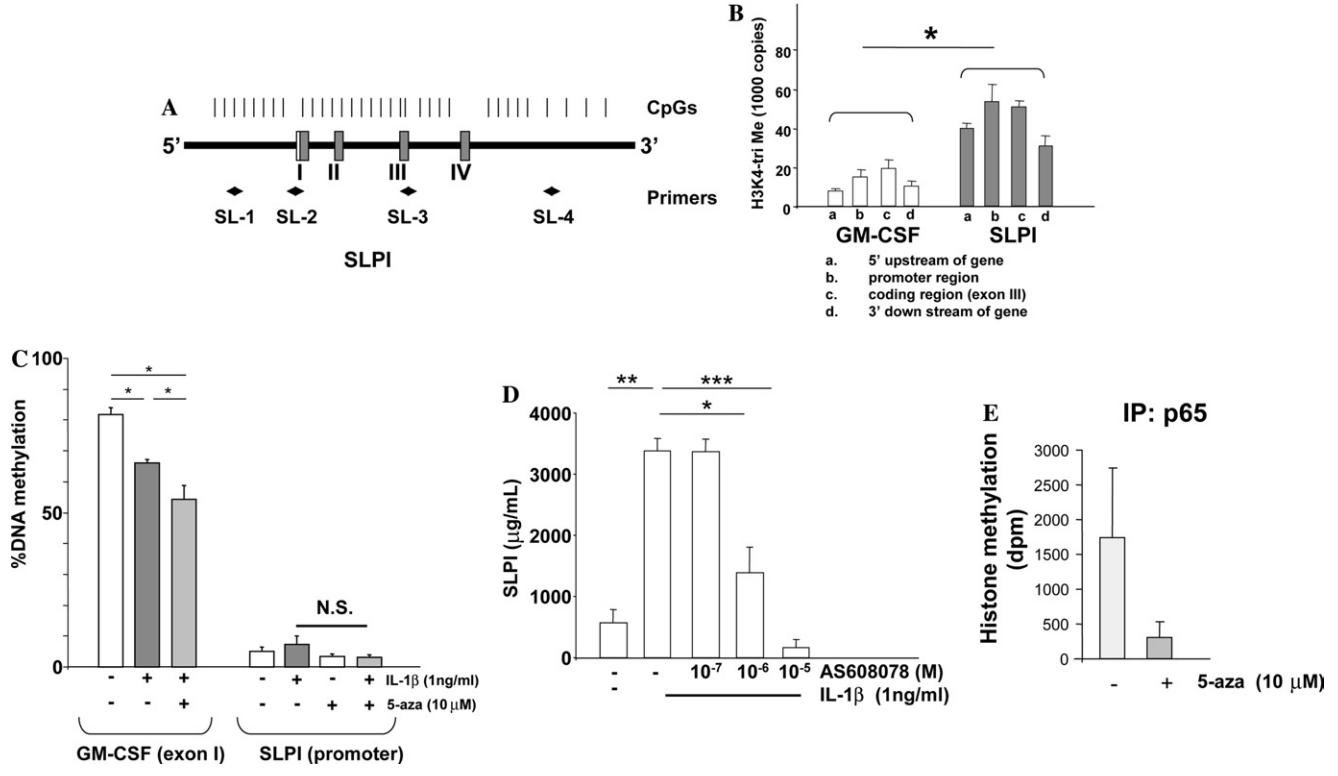

Fig. 1. (A) Structure of SLPI gene, localisation for PCR primers sets (SL-1—SL-2), and the distribution of CpGs di-nucleotides (|). (B) A comparison of the degree of histone H3 methylation at Lys (K) 4 across the GM-CSF gene and across the SLPI gene, determined by chromatin immunoprecipitation assay: a, represents the region of 5′ far upstream region of genes; b, the gene promoter regions including the transcriptional starting site; c, the gene coding region including exon III; and d, the 3′ non-coding downstream region. (C) Comparison of DNA CpG methylation levels on the GM-CSF and SLPI genes following IL-1β (1 ng/mL) and/or 5-azacytidine (5-aza, 10 μM) stimulation. (D) Effect of the IKK2 inhibitor (AS608078) on IL-1β-induced SLPI secretion. (E) The effect of 5-aza on p65-immunoprecipitated histone methylation activity. NS, not significant, $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

were digested, and that of total DNA were estimated by using Q-PCR. The ratio of DNA methylation was estimated by the ratio of undigested DNA fragments compared to total DNA fragments.

Immunoprecipitation. Cell extracts were prepared using 100 μL of immunoprecipitation buffer (10 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.5% NP-40; and complete protease inhibitor cocktail [Boehringer–Mannheim, Berkshire, UK]). The lysis mixture was incubated on ice for 15 min and centrifuged for 10 min at 4 °C. Extracts were precleared with 20 μL A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA) and 2 μg of normal IgG. After microcentrifugation, 20 μL of A/G agarose conjugated with 5 μg of antibody (Santa Cruz) was used to precipitate p65 at 4 °C overnight with rotation. The immune complexes were pelleted by gentle centrifugation and washed three times with 500 μL of immunoprecipitation buffer.

Histone methylation assay. Methylase activity was measured as previously described [23]. Samples were incubated with 20 μg histone (Sigma) and 5 μL [methyl-^{3}H]*S*-adenosyl methionine (NEN, 80 Ci/mmol) in PBS at 30 °C for 1 h. Then, reaction solution was spotted onto p81 cationic exchange paper (Whatman), washed in carbonate buffer, and quantified by scintillation counting.

Statistical analysis. Data are reported as means ± SE. Statistical analysis was performed using Prism 3.02 (GraphPad Software, San Diego, USA), with *t* test and Mann–Whitney test, as appropriate. Significance was defined as p value <0.05.

Results

Histone methylation levels are greater across SLPI than those across GM-CSF

Tri-methylated H3-K4 levels across the constitutively active SLPI gene were significantly enhanced compared to those detected across the inducible GM-CSF gene in the absence of any stimulation (Fig. 1B). Similar results were seen with H3-K4 di-methylation (data not shown). These results supported the hypothesis that H3-K4 hyper-methylation is associated with constitutive gene expression and may act as an epigenetic marker in these cells.

DNA methylation does not play a role in SLPI transcription

The SLPI gene has no CpG-rich regions (Fig. 1A) and, in addition, using an *Hpa*II sensitivity assay we found significantly lower levels of CpG methylation ($5.0 \pm 1.4\%$) within the SLPI promoter compared to that in GM-CSF exon I ($67.7 \pm 3.8\%$). Neither IL-1β nor 5-aza affected CpG methylation levels at 24 h (Fig. 1C). Due to the low DNA methylation levels as well as the lower frequency of CpGs in the SLPI gene, we predicted that 5-aza treatment will have minimal effect on CpG methylation and, therefore, DNA methylation would play a limited role in the subsequent actions of 5-aza on SLPI gene expression. To confirm that 5-aza could modulate histone methylation status in these cells, we showed that IL-1β-induced SLPI expression was regulated by NF-κB as it was suppressed by the IKK2 inhibitor, AS602868 (Fig. 1D) and, in addition, that p65-associated histone methylase activity could be reduced by 5-aza treatment (Fig. 1E).

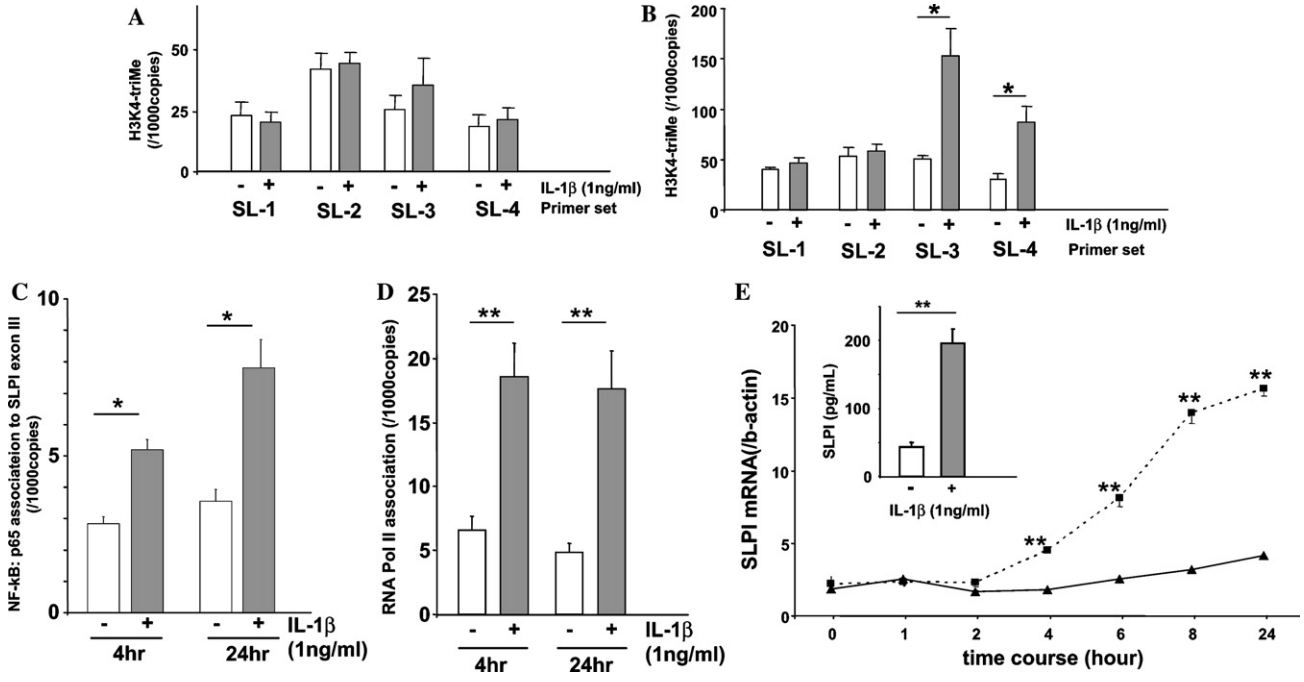

Fig. 2. Effect of IL-1β (1 ng/mL) on histone methylation levels at (A) 4 h and (B) 24 h. (C) The effect of IL-1β on the association of NF-κB to the SLPI coding region determined by chromatin immunoprecipitation. (D) The effect of IL-1β on SLPI transcriptional rate represented by recruitment of RNA polymerase II to SLPI exon III at 4 and 24 h. (E) Effect of IL-1β on the time-course of SLPI mRNA and protein (inset) levels at 24 h. Broken line (–■–) indicates IL-1β-stimulated cells and a solid (–▲–) indicates the control cells. *$p < 0.05$, **$p < 0.01$.

IL-1β enhanced the methylation levels across SLPI gene

We next examined the effect of IL-1β on H3-K4 tri-methylation, RNA polymerase II recruitment to the SLPI coding region (close to the NF-κB binding site) and on SLPI gene expression. IL-1β induced a time-dependent induction of H3-K4 tri-methylation at the coding region and 3′-UTR of the SLPI gene (Figs. 2A and B). This effect was not seen at 4 h but was clearly demonstrable by 24 h where it was increased by 203 ± 55% within the coding region. There was a similar degree of enhanced recruitment of NF-κB: p65 association (237 ± 9%) to the SLPI coding region (Fig. 2C) and RNA polymerase II to the region (260 ± 60%) at 24 h (Fig. 2D). This increase in RNA polymerase II recruitment, as well as p65 association, was associated with increased levels of SLPI mRNA and protein expression (Fig. 2E). This suggested that histone H3-K4 tri-methylation may be involved in IL-1β-stimulated SLPI release at later (24 h) time points. H3-K4 tri-methylation, however, clearly did not play a role in IL-1β-stimulated SLPI release at early (4 h) time points, since SLPI mRNA levels (Fig. 2E) as well as its transcriptional activity (Fig. 2D) were already elevated at 4 h, whilst H3-K4 tri-methylation did not occur at this earlier time point (Fig. 2A).

5-aza reduced the histone methylation level at 24 h

5-aza did not affect the degree of histone H3-K4 tri-methylation at 4 h (Fig. 3A). In contrast, 5-aza significantly attenuated IL-1β-induced H3-K4 tri-methylation at 24 h at the SLPI coding region and 3′-UTR by 37.9 ± 5.8% and 31.3 ± 9.8%, respectively ($p < 0.05$) (Fig. 3A). RNA polymerase II recruitment (42.7 ± 7.1%, $^{**}p < 0.01$) and p65 association (22.8 ± 6.8%, $^{*}p < 0.05$) to the SLPI coding region were also significantly attenuated to a similar extent at 24 h but not at 4 h (Fig. 3B), suggesting a reduction in transcriptional activity. There was a good correlation at 24 h between H3-K4 tri-methylation and RNA polymerase II recruitment to SLPI and GM-CSF (Fig. 3C). Our results, where 5-aza is acting predominantly as a histone methylase inhibitor at the SLPI gene, indicate that H3-K4 methylation can act as a dynamic regulator of IL-1β-stimulated SLPI gene expression.

This is confirmed by the significant reduction in IL-1β-stimulated SLPI protein (Fig. 4A) and mRNA expression (Fig. 4B), following pre-treatment with 5-aza. This occurred in a concentration-dependent manner (Fig. 4A) and was not seen at the earliest time-point investigated (4 h) but was associated with the later more marked effects on SLPI expression (Fig. 4C). In addition, IL-1β stimulated histone H4 acetylation across the SLPI coding region (SL-3) at 24 h and this was attenuated by further 5-aza addition (Fig. 4D). A similar effect was seen at 4 h but this did not reach significance (Fig. 4E), thereby emphasising the delayed effect of 5-aza on SLPI transcription.

Discussion

We report that the constitutively active SLPI gene has a greater degree of H3-K4 tri-methylation than the inducible gene GM-CSF in human lung epithelial cells. Furthermore, we have shown that IL-1β enhanced H3-K4 tri-methylation at the SLPI coding region, whilst 5-aza attenuated inducible methylation. Our study further demonstrated that the changes in histone H3-K4 tri-methylation were correlated with the recruitment of RNA polymerase II to the SLPI coding region and to SLPI mRNA expression. Since neither IL-1β nor 5-aza affected SLPI gene DNA methylation and we could demonstrate reduced NF-κB-associated histone methylase activity following 5-aza treatment, it is likely that changes in SLPI gene expression were mediated through histone methylation. Thus, histone H3-K4 tri-methylation levels were associated with SLPI gene induction following an acute

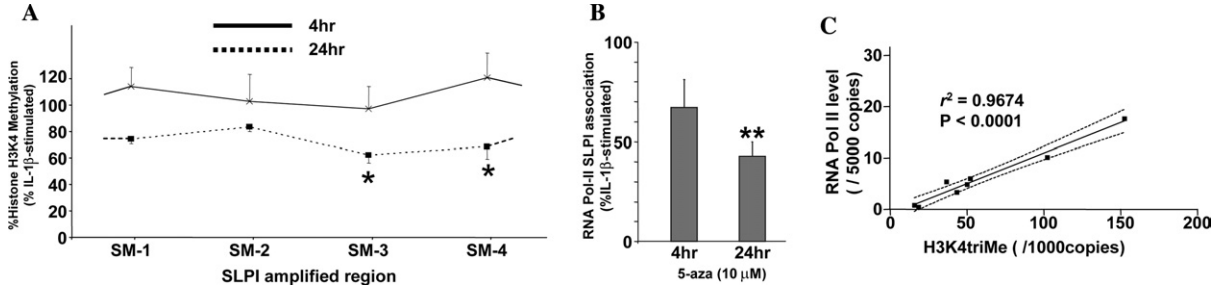

Fig. 3. (A) The inhibitory effect of 5-aza (10 μM) on histone H3 K4 methylation in IL-1β-stimulated cells at 4 h (solid lines: –×–) and 24 h (broken lines: –■–). The data obtained were adjusted to those of IL-1β-stimulated cells set as 100%. (B) Inhibitory effect of 5-aza on IL-1β-induced recruitment of RNA polymerase II on SLPI gene. (C) Linear correlation between histone H3 K4-tri-methylation and the RNA polymerase II recruitment to the GM-CSF and SLPI genes. Dots represent cells that were stimulated either with IL-1β, 5-aza or both, or control cells. $^{*}p < 0.05$ and $^{**}p < 0.01$.

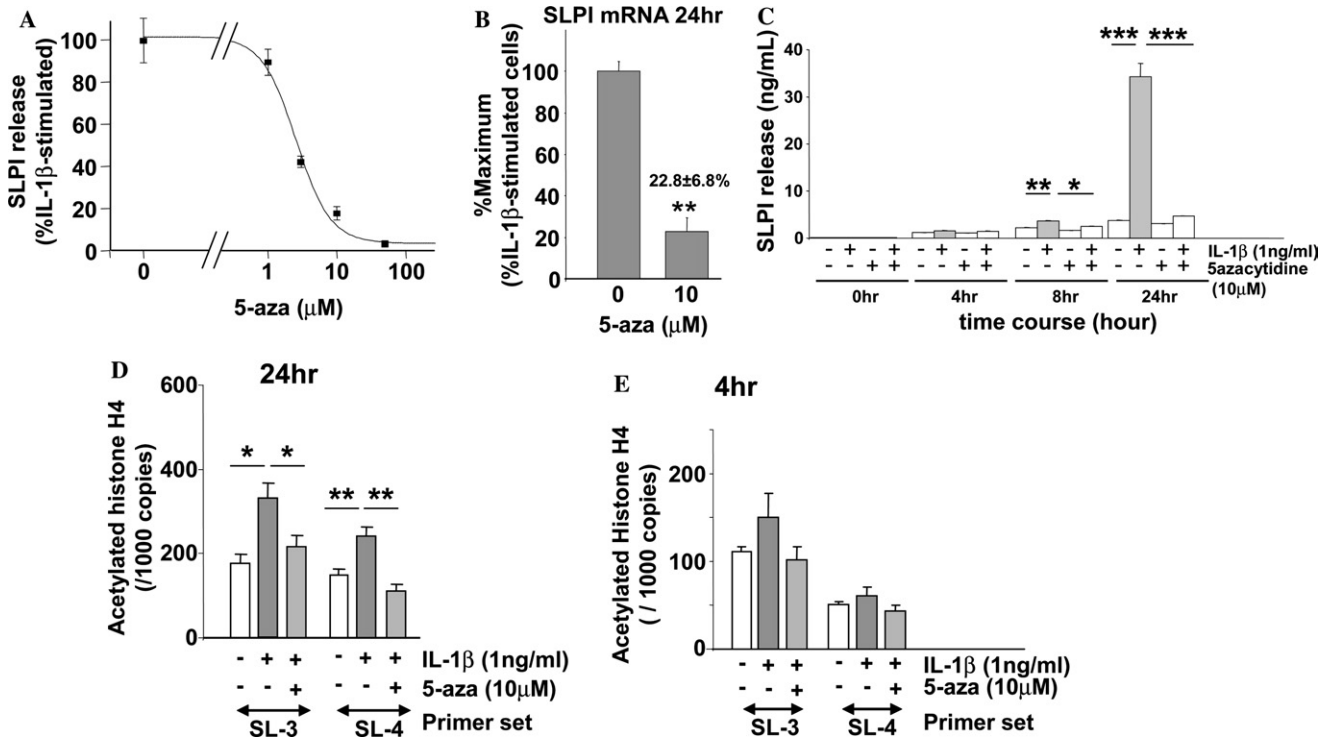

Fig. 4. (A) Inhibitory effect of 5-aza (10 μM) on SLPI mRNA levels. (B) Concentration-dependent inhibitory effect of 5-aza on the IL-1β-enhanced SLPI released protein levels. (C) Time-course of SLPI released protein levels under the condition indicated. (D) Pan-histone H4 acetylation levels across the SLPI gene at 24 and 4 h (E). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

inflammatory stimulus. In contrast, we did not find any changes in H3-K4 tri-methylation levels at 4 h confirming previous work showing that TNF-α stimulation did not alter H3-K4 tri-methylation levels on a distinct subset of inducible genes [15]. These data suggest that H3-K4 tri-methylation may not only play a role in the epigenetic regulation of SLPI but may also be involved in acute inflammatory mediator-stimulated induction of SLIPI gene expression.

Previous studies have reported that H3-K4 methylation, induced by the Set-1-containing complex COMPASS, can attract RNA polymerase II to coding region of genes and thereby contribute to the maintenance of transcription [24–27]. This process requires the Paf1 protein complex, which is associated with the elongating RNA polymerase II, to enable recruitment of the COMPASS methylase [27]. Whether Paf1 recruits COMPASS or other histone methylases such as Set-1 was not investigated in this study but the data raise the question as to whether 5-aza can directly modulate the activity of Paf1. Alternatively, RNA polymerase II might hyper-methylate histone H3-K4. In our study, the increase in RNA polymerase II involvement precedes histone H3-K4 hyper-methylation, since the histone H3-K4 methylation levels were not altered by IL-1β stimulation at 4 h, while the mRNA levels as well as the transcriptional activity were already increased. Several reports have demonstrated that RNA polymerase II formed a complex with histone methylases Set-1 [13], Set-2 [28] or SMYD-3 [29] and, during transcriptional elongation, the RNA polymerase II complexes with Set-1 or with SMYD-3 could methylate specifically histone H3-K4 [13,28–30]. Furthermore, Set-1 has been reported to be essential to maintain the expression of promoter-acetylated and coding region-methylated genes in part through protecting active coding regions from deacetylation by histone deacetylases [24]. In support of this concept, it was shown that IL-1β was able to induce histone H4 acetylation in the SLPI coding region and that addition of 5-aza reduced histone acetylation across the SLPI coding region at 24 h, and not at 4 h. This change was in parallel to the changes in H3-K4 methylation.

We have not identified the key methylase or de-methylase involved in IL-1β stimulation or 5-aza treatment. However, sequence analyses showed that there were three potential binding sites for SMYD-3 around the SLPI exon III, while no such site was found across GM-CSF gene. The histone methylation occurred exclusively on the SLPI coding region or 3′ downstream of the SLPI gene, supporting the possibility of the involvement of the histone methylase SMYD-3.

We have shown that, following IL-1β-stimulation, the degree of histone H3-K4 tri-methylation of the

SLPI coding region correlates with RNA polymerase II recruitment and SLPI gene expression. We also provide further evidence that H3-K4 tri-methylation may act as an epigenetic marker of gene expression since the levels of histone methylation are consistently higher across the whole SLPI gene than those seen across the highly inducible GM-CSF gene. Furthermore, we have shown that 5-aza has an inhibitory effect on histone H3-K4 tri-methylation and results in a reduction in SLPI gene expression. Further experiments particularly in clinical samples from patients with chronic inflammatory disease will elucidate the role of histone H3-K4 tri-methylation in modulating the inflammatory response.

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