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RESEARCH PAPER

Low activity of LSD1 elicits a pro-inflammatory gene expression profile in riboflavin-deficient human T Lymphoma Jurkat cells

Dandan Liu · Janos Zempleni

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Abstract Mono- and dimethylation of lysine (K)-4 in histone H3 (H3K4me1, H3K4me2) create epigenetic gene activation marks that are enriched near the transcription start site of genes. Lysine-specific demethylase 1 (LSD1) is a flavin adenine dinucleotide (FAD)-dependent demethylase that catalyzes the demethylation of H3K4me1 and H3K4me2, thereby mediating gene repression. This study tested the hypothesis that LSD1 activity depends on the concentrations of the FAD precursor, riboflavin, in cell culture media, and that riboflavin deficiency causes derepression of pro-inflammatory cytokines. Human T lymphoma Jurkat cells were cultured in riboflavin-defined media, representing plasma levels of riboflavin in moderately deficient, sufficient, and supplemented humans. The expression of LSD1 mRNA and protein followed the pattern riboflavin-deficient > riboflavin-sufficient > riboflavin-supplemented cells. However, the increase in LSD1 expression was insufficient to compensate for FAD depletion, and LSD activities were more than 30 % higher in riboflavin-supplemented cells compared with the other treatment groups. The enrichment of H3K4me2 marks was 11–137 % greater in riboflavin-deficient cells compared with sufficient cells in exon 1 of genes coding for the pro-inflammatory cytokines interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor-α. Consistent with the enrichment of gene activation marks, the expression of mRNA coding for pro-inflammatory cytokines was 62–487 % higher in riboflavin-deficient cells compared with sufficient cells. These findings support the hypothesis that riboflavin deficiency contributes toward a pro-inflammatory gene expression pattern through a loss of LSD1 activity.

Keywords LSD1 · Demethylation · FAD · H3K4me2 · Pro-inflammatory cytokines · TSS

Abbreviations

ChIP	Chromatin immunoprecipitation
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
H3K4me1	K4-monomethylated histone H3
H3K4me2	K4-dimethylated histone H3
H3K4me3	K4-trimethylated histone H3
IL-1α	Interleukin-1α
IL-1β	Interleukin-1β
IL-6	Interleukin-6
K	Lysine
LSD1	Lysine-specific demethylase 1
PMA	Phorbol 12-myristate 13-acetate
qRT-PCR	Quantitative real-time PCR
TNF-α	Tumor necrosis factor-α
TSS	Transcription start site

Introduction

Epigenetic marks play key roles in the regulation of gene expression. Posttranslational methylation of histones is one such epigenetic event that contributes to gene regulation (Greer and Shi 2012). Remarkable progress has been made in characterizing the distribution of histone methylation marks and assessing the roles these marks in gene

D. Liu · J. Zempleni (✉)
Department of Nutrition and Health Sciences, University of Nebraska-Lincoln, Lincoln, NE 68583, USA
e-mail: jzempleni2@unl.edu

regulation on a genome-wide scale in yeast and several mammalian cell lines (Barski et al. 2007; Bernstein et al. 2002; Koch et al. 2007; Lee and Mahadevan 2009; Nair et al. 2012). These studies implicate methylation of lysine (K)-4 in histone H3 in gene activation, whereas methylation of K-9 in histone H3 is implicated in gene repression. Within actively transcribed genes, some methylation marks, e.g., K4-monomethylated histone H3 (H3K4me1), K4-dimethylated histone H3 (H3K4me2), and K4-trimethylated histone H3 (H3K4me3), are enriched near transcription start sites (TSS) in genes (values denote basepairs relative to the TSS): −900 and +1,000 for H3K4me1, −500 and +700 for H3K4me2, and −300 and +100 for H3K4me3 (Barski et al. 2007; Heintzman et al. 2007).

Histone methyltransferases and demethylases are enzymes that create, maintain, and erase histone methylation marks. Lysine-specific demethylase 1 (LSD1, also known as KDM1A, BHC110, and AOF2) is the first histone demethylase that was discovered; it represses transcription by removing methyl groups from H3K4me1/2 (Karytinos et al. 2009; Shi et al. 2004). LSD1 is unique among multiple demethylases in that it belongs to the flavin-containing amine oxidase family and utilizes flavin adenine dinucleotide (FAD) as an essential cofactor for catalytic activities (Forneris et al. 2005). FAD, produced by adenylation of the vitamin riboflavin, serves as a coenzyme in a large number of redox reactions and a small number of reactions with no net redox change in mammalian metabolism (Bornemann 2002; Henriques et al. 2010; Pinto and Rivlin 2013). LSD1 is enriched in gene promoter regions as part of multiprotein gene repression complex (Foster et al. 2010; Wang et al. 2009; Whyte et al. 2012). LSD2 (also known as KDM1B and AOF1), the only mammalian homolog of LSD1, is also a member of the flavin-containing amine oxidase family. Like LSD1, LSD2 may also catalyze demethylation through the removal of methyl groups from H3K4me1/2 in an FAD-dependent reaction (Zhang et al. 2012). Unlike LSD1, LSD2 is enriched in coding regions other than those adjacent to the TSS and forms protein complexes that regulate gene expression independent of LSD2 demethylase activity (Fang et al. 2010; Yang et al. 2010).

Previous studies suggest that stimulation of pro-inflammatory genes causes changes in histone methylation patterns in promoter regions, consistent with a role of histone demethylases in the regulation of pro-inflammatory cytokines (El Gazzar et al. 2007; Foster et al. 2007; Saccani and Natoli 2002). LSD1 may synergize with histone deacetylase 1 to repress pro-inflammatory cytokines in breast cells and hepatocytes (Janzer et al. 2012). Studies in diabetic mouse models specifically implicate LSD1 in the regulation of pro-inflammatory gene expression in vascular smooth muscle cells (Reddy et al. 2008; Wierda et al. 2010).

It is widely recognized that nutritional factors play a role in methylation events, albeit the majority of previous studies focused on methyl donors (Zempleni et al. 2012). Importantly, in a recent publication, we expanded that point of view and demonstrated that FAD-dependent demethylation events, mediated by LSD1, cause an aberrant increase in the expression of albumin in human liver cells (Liu and Zempleni 2014). That publication established a rationale for further studies of the importance of FAD-dependent LSD1 in gene regulation. Aberrant expression of albumin, with a few minor exceptions, does not establish an unambiguous line between FAD supply and human disease. We pose that aberrant regulation of genes that play major roles in human diseases would make a stronger case for the biological importance of our previous observations. Therefore, in this study, we tested the hypothesis that LSD1 activity depends on the concentrations of the FAD precursor, riboflavin, in cell culture media, and that riboflavin deficiency causes derepression of pro-inflammatory cytokines in human T lymphoma Jurkat cells. In addition, we assessed the distribution of H3K4me2 marks in regions upstream and downstream of the TSS in pro-inflammatory genes. Jurkat cells were chosen as model, because riboflavin homeostasis has been well characterized in this cell line (Camporeale and Zempleni 2003), and the cells express the pro-inflammatory cytokines interleukin (IL)-1α, IL-1β, IL-6, and tumor necrosis factor-α (TNF-α) when stimulated with phorbol esters (Khalaf et al. 2010; Wano et al. 1987).

Materials and methods

Cell cultures

Human T lymphoma Jurkat cells (ATCC, Manassas, VA, USA) were cultured in riboflavin-defined RPMI-1640 medium (HyClone, Logan, UT, USA) as described previously (Camporeale and Zempleni 2003; Manthey et al. 2006). Riboflavin concentrations in the culture media were adjusted to 3.1 nmol/L (denoted deficient, "DEF"), 12.6 nmol/L (denoted sufficient, "SUF"), and 301 nmol/L (denoted supplemented, "SUP"), taking into account the residual concentrations of riboflavin, flavin mononucleotide (FMN), and FAD in dialyzed fetal bovine serum. Cells were cultured in riboflavin-sufficient medium for 7 days before transfer into the riboflavin-defined media and continued culture for 7 days before analysis. This protocol was chosen based on previous and preliminary studies, suggesting abnormally slow cell growth after 10 days of culture in riboflavin-deficient medium (Manthey et al. 2005; Werner et al. 2005). The production of pro-inflammatory cytokines was induced by treatment with 100 μg/L phorbol

12-myristate 13-acetate (PMA) for 8 h before analysis (Khalaf et al. 2010). To confirm the direct involvement of LSD1, Jurkat cells were treated with an LSD1 inhibitor, tranylcypromine (Santa Cruz Biotechnology, Santa Cruz, CA, USA), at a final concentration of 2 μM for 24 h before analysis.

Glutathione metabolism

The activity of glutathione reductase and the level of reduced glutathione were used as markers of FAD status in Jurkat cells (Camporeale and Zempleni 2003; Manthey et al. 2006). After 7 days of culture in riboflavin-defined media, Jurkat cells were harvested and lysed for assessment of glutathione metabolism. Glutathione reductase activity was quantified in cell lysates containing 0.5 mg protein as described previously (Sauberlich et al. 1972). One unit of glutathione reductase activity is presented by the change of absorbance at 340 nm per 0.5 mg protein in 10 min of incubation. The concentration of reduced glutathione in lysates was determined colorimetrically using the 5,5-dithiobis(2-nitrobenzoic acid) reduction assay as described previously (Tietze 1969). The assay was calibrated using chemically pure reduced glutathione.

Western blot analysis

Expression of LSD1 protein and global abundance of H3K4me2 marks in whole cell extracts were quantified by Western blot as described previously (Manthey et al. 2006), using rabbit polyclonal anti-human LSD1 (Abcam, Cambridge, MA, USA) and rabbit polyclonal anti-H3K4me2 (Abcam), respectively. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and histone H3 were used as loading controls. Data were quantified by gel densitometry analysis.

LSD demethylase activity

The assessment of LSD demethylase activity was conducted in Jurkat cells after 7 days of culture in riboflavin-defined media with demethylase (LSD-type) activity assay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. One unit of LSD activity is defined as the ratio of sample fluorescence to background fluorescence. This assay does not distinguish between the two LSDs in the human proteome: LSD1 and LSD2 (Culhane and Cole 2007).

Chromatin immunoprecipitation (ChIP) assay

The enrichment of H3K4me2 marks near the TSS of human pro-inflammatory genes such as *IL-1α*, *IL-1β*, *IL-6*, and *TNF-α* was assessed by ChIP assay as described previously (Dahl and Collas 2008). Immunoprecipitations were performed with specific antibodies to H3K4me2 (Abcam), H3 (Abcam), and rabbit IgG (Santa Cruz Biotechnology). Nuclear chromatin extracts without immunoprecipitation were used as input control. Precipitation of chromatin with nonspecific rabbit IgG was used as negative control and produced signals less than 10 % compared with those produced by target-specific antibodies. Precipitations with H3 antibody were used to normalize for H3K4me2 occupancy in pro-inflammatory genes.

Quantitative real-time PCR (qRT-PCR)

The abundance of mRNA coding for LSD1 and pro-inflammatory cytokines was quantified by qRT-PCR using Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA) as described previously (Gralla et al. 2008). The relative amount of each gene was normalized using housekeeping gene *GAPDH*.

Amplicons from ChIP assay were analyzed using the PerfeCTa SYBR Green FastMix (Quanta Biosciences, Gaithersburg, MD, USA) as described previously (Pestinger et al. 2011). The relative occupancy of H3K4me2 in pro-inflammatory gene regulatory regions was calculated as described previously (Wei et al. 2006), and values are reported as the ratio of H3K4me2 to H3 occupation. To investigate the enrichment of H3K4me2 marks surrounding the TSS of genes coding for pro-inflammatory cytokines, primers were designed to amplify sequences upstream (denoted "promoter") or downstream (denoted "exon 1") of the TSS (Fig. 1), using ChIP samples as template (Barski et al. 2007; Heintzman et al. 2007) (Table 1).

Statistical analysis

Data exhibited normal distributions and homogenous variances, as assessed by Komolgorov–Smirnov normality test and Bartlett's test, respectively. Statistical significance

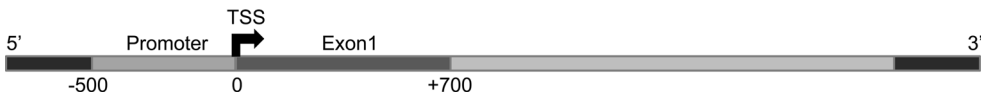

Fig. 1 Schematic of the region adjacent to the TSS in pro-inflammatory genes. Primers were designed to amplify sequences upstream ("promoter") or downstream ("exon 1") of the TSS, respectively, in ChIP assay

Table 1 Oligonucleotide primers used for quantitative real-time PCR

Gene[a]	Sequence (5′–3′)	F/R[b]	Template
GAPDH	TCCACTGGCGTCTTCACC	F	cDNA
	GGCAGAGATGATGACCCTTT	R	
GAPDH	ATGACAAGCATGAGGCAGAG	F	gDNA
	CAACCAGGACCGTTAACCCTTTCT	R	
IL-1α	ACAAAAGGCGAAGAAGACTGA	F	cDNA
	GGAACTTTGGCCATCTTGAC	R	
IL-1α (−319 to −56)	CCAACTCACACAAGCTGCTTT	F	gDNA
	GGTGGTAGAACACCAGACTCTT	R	
IL-1α (+128 to +416)	CTTCTTCTACAGAAGACACACCTT	F	gDNA
	CTGAAGAGGGAAGTTTGCTTGATT	R	
IL-1β	CTGTCCTGCGTGTTGAAAGA	F	cDNA
	TTGGGTAATTTTTGGGATCTACA	R	
IL-1β (−235 to −67)	ATATTTGCATGGTGATACATTTGC	F	gDNA
	CTCTGTTGAATACCTGATTTCACAA	R	
IL-1β (+127 to +338)	GCCTCTTTGTGTGTATGCATATT	F	gDNA
	GAGAGCTGGAGCAGAGGCTT	R	
IL-6	TCCAGAACAGATTTGAGAGTAGTG	F	cDNA
	GCATTTGTGGTTGGGTCAGG	R	
IL-6 (−279 to −93)	CTTCGTGCATGACTTCAGCTTT	F	gDNA
	GATTGTGCAATGTGACGTCCTTT	R	
IL-6 (+132 to +398)	CACAAGTAAGTGCAGGAAATCCTT	F	gDNA
	CGGCTACATCTTTGGAATCTT	R	
LSD1	ACCACAACAGACCCAGAAGG	F	cDNA
	GGTGCTTCTAATTGTTGGAGAG	R	
TNF-α	GCTCTTCTGCCTGCTGCACTT	F	cDNA
	GATGGCACCACCAGCTGGTT	R	
TNF-α (−399 to −192)	CCTGCATCCTGTCTGGAAGTT	F	gDNA
	GGGACACACAAGCATCAAGGAT	R	
TNF-α (+103 to +374)	GCTGCCAGGCAGGTTCTCTT	F	gDNA
	GGCCAGGCACTCACCTCTT	R	

[a] Genbank entries: GAPDH = *Homo sapiens glyceraldehyde-3-phosphate dehydrogenase* (NM_002046.4 for complementary DNA, NG_007073.2 for genomic DNA); IL-1α = *Homo sapiens interleukin-1 alpha* (NM_000575.3 for complementary DNA, NG_008850.1 for genomic DNA); IL-1β = *Homo sapiens interleukin-1 beta* (NM_000576.2 for complementary DNA, NG_008851.1 for genomic DNA); IL-6 = *Homo sapiens interleukin-6* (NM_ NM_000600.3 for complementary DNA, NG_011640.1 for genomic DNA); LSD1 = *Homo sapiens lysine (K)-specific demethylase 1* (NM_001009999.2); TNF-α = *Homo sapiens tumor necrosis factor-alpha* (NM_000594.3 for complementary DNA, NG_007462.1 for genomic DNA)

[b] *cDNA* complementary DNA (for gene expression analysis), *F* forward, *gDNA* genomic DNA (for ChIP assays), *R* reverse

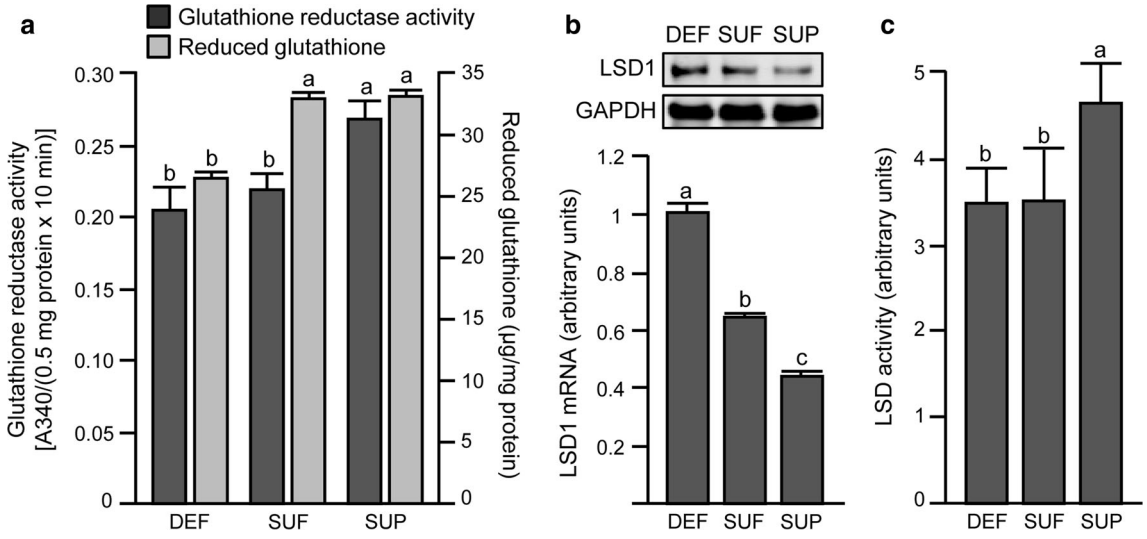

Fig. 2 Glutathione metabolism (**a**), *LSD1* expression (**b**), and LSD demethylase activity (**c**) depend on the concentrations of riboflavin in Jurkat cell culture media. The insert depicts a representative immunoblot of LSD1 protein. Values are mean ± SD, $n = 3$. [a,b,c]Means not sharing a common letter are significantly different for the same variable, p value <0.05. *DEF* deficient, *SUF* sufficient, *SUP* supplemented

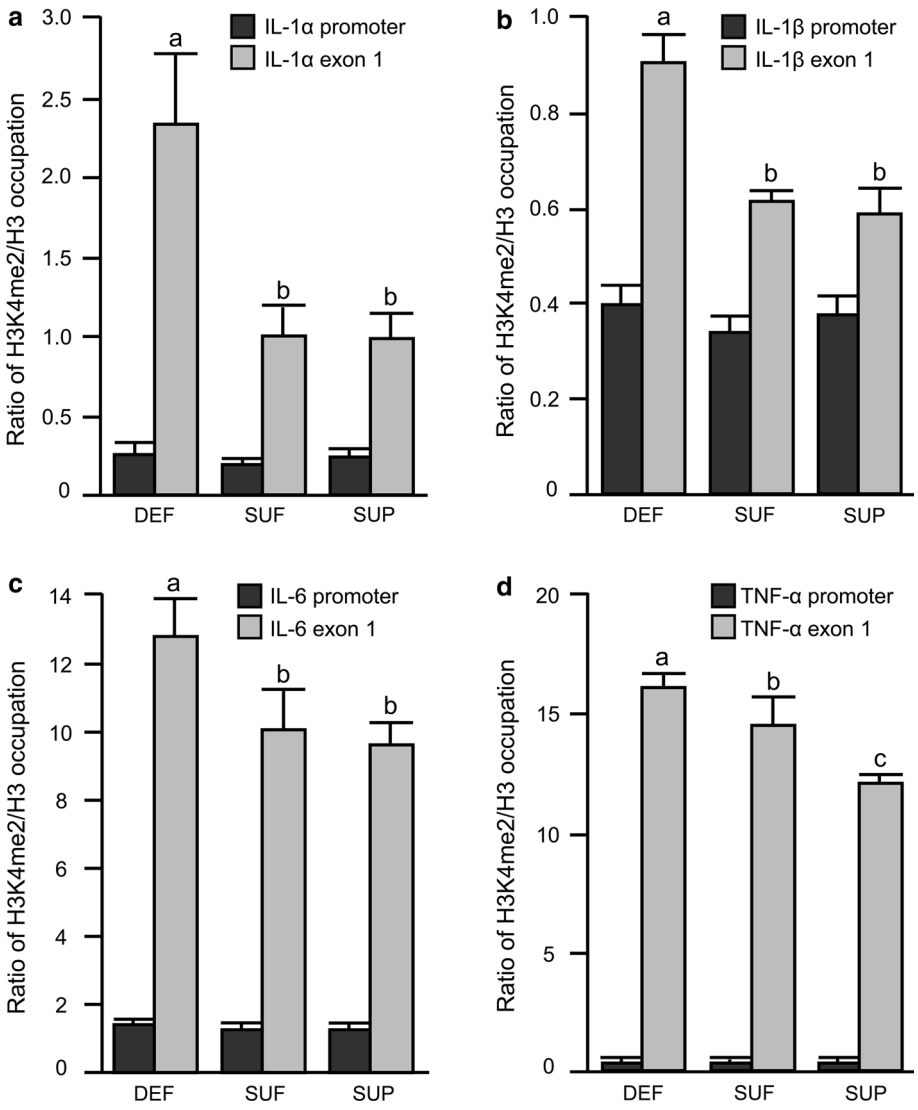

Fig. 3 Enrichment of H3K4me2 marks in *IL-1α* (**a**), *IL-1β* (**b**), *IL-6* (**c**), and *TNF-α* (**d**) gene promoter and exon 1. Values are mean ± SD, $n = 5$. [a,b,c]Means not sharing a common letter are significantly different for the same variable, p value <0.01. *DEF* deficient, *SUF* sufficient, *SUP* supplemented

was assessed by one-way ANOVA and Fisher's protected least significant difference *post hoc* test (SAS Institute 1998). StatView 5.0.1 (SAS Institute, Cary, NC) was used to perform all calculations. Differences were considered significant if p value <0.05. Data are expressed as mean ± SD.

Results

Determination of flavin status

Glutathione reductase activity was 23 % higher in riboflavin-supplemented Jurkat cells compared with riboflavin-sufficient cells, and the levels of reduced glutathione were 20 % lower in riboflavin-deficient cells compared with riboflavin-sufficient cells (Fig. 2a). These observations are consistent with the notion that concentrations of riboflavin in culture media affected the intracellular flavin status, suggesting that treatment was effective (Camporeale and Zempleni 2003).

Expression of LSD1

The expression of *LSD1* mRNA was about 57 % greater in riboflavin-deficient cells and 24 % less in riboflavin-supplemented cells compared with riboflavin-sufficient cells (Fig. 2b). The protein abundance of LSD1 followed a similar pattern (arbitrary units of gel densitometry): 8.72 ± 0.96 for riboflavin-deficient cells versus 6.31 ± 0.42 for riboflavin-sufficient cells versus 2.98 ± 0.56 for riboflavin-supplemented cells (p value <0.05 for all possible comparisons; $n = 3$).

LSD demethylase activity

LSD demethylase activity depended on the concentration of riboflavin in culture media. LSD activity was 33 %

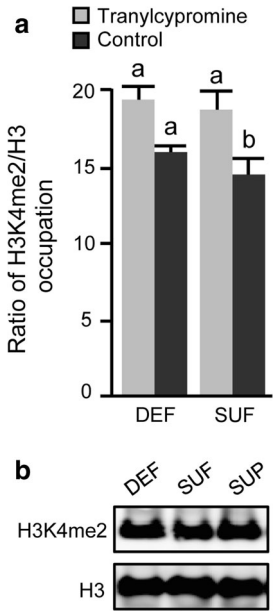

Fig. 4 H3K4me2 occupations in *TNF*-α exon 1 with the treatment of LSD1 inhibitor (**a**) and global abundance of H3K4me2 in whole cell extracts (**b**). Histone H3 was used as a loading control. Values are mean ± SD, $n = 5$. [a,b]Means not sharing a common letter are significantly different for the same variable, p value <0.01. *DEF* deficient, *SUF* sufficient, *SUP* supplemented

greater in riboflavin-supplemented cells compared with riboflavin-sufficient cells after 7 days of culture (Fig. 2c). No significant difference was observed between riboflavin-deficient and -sufficient cells.

Enrichment of H3K4me2 activation marks

The abundance of H3K4me2 marks was higher in exon 1 in riboflavin-deficient cells compared with the other treatment groups. For example, the abundance of H3K4me2 marks was 137, 49, and 27 % higher in exon 1 in the *IL-1α*, *IL-1β*, and *IL-6* genes, respectively, in riboflavin-deficient cells compared with riboflavin-sufficient cells (Fig. 3a–c). Effects were comparably moderate for exon 1 in the *TNF*-α gene; the enrichment of H3K4me2 marks in exon 1 of the *TNF*-α gene was 11 % higher in riboflavin-deficient cells and 17 % lower in riboflavin-supplemented cells compared with riboflavin-sufficient cells (Fig. 3d). In contrast, effects of riboflavin on the abundance of H3K4me2 in the promoter region, as opposed to exon 1, of the four genes were not biologically meaningful (Fig. 3a–d). When treated with tranylcypromine, differences of H3K4me2 enrichment in *TNF*-α exon 1 region between riboflavin-deficient and -sufficient groups were no longer detectable (Fig. 4a). Riboflavin deficiency caused no meaningful change in the global abundance of H3K4me2 marks in whole cell extracts (Fig. 4b), i.e., the observed effects might be locus specific.

Expression of pro-inflammatory cytokines

The levels of mRNA coding for pro-inflammatory cytokines were 62–487 % greater in riboflavin-deficient cells compared with riboflavin-sufficient cells (Fig. 5a–d). In addition, mRNA levels of *TNF*-α were 19 % lower in riboflavin-supplemented cells compared with riboflavin-sufficient cells (Fig. 5d).

Discussion

This is the first report to implicate riboflavin deficiency in the aberrant up-regulation of pro-inflammatory cytokines through loss of LSD1 activity. Our observations suggest that loss of LSD1 demethylase activity specifically affects the abundance of H3K4me2 marks in exon 1 regions of pro-inflammatory genes whereas the abundance of these marks in promoter regions was largely unaffected by riboflavin. Although higher *LSD1* expression was observed in riboflavin-deficient cells, this up-regulation was not sufficient to compensate for the depletion of the flavocoenzyme FAD and holo-LSD1, as judged by the accumulation of H3K4me2 activation marks in exon 1 of pro-inflammatory genes and increased pro-inflammatory gene expression in riboflavin-deficient cells.

This paper has important implications for human health. Lack of riboflavin availability might cause a loss of LSD1 activity, resulting in an increased expression of pro-inflammatory cytokines. Inflammation plays roles in diseases that affect a large percentage of the population, e.g., arthritis and inflammatory bowel disease. For example, 22 % of US adults suffer from doctor-diagnosed arthritis, and 9 % have arthritis-attributable activity limitations (Centers for Disease Control and Prevention 2010). Evidence suggests that pro-inflammatory cytokines IL-1β, IL-6, and TNF-α, produced by synovial cells and infiltrating cells, actively participate in synovitis and joint destruction and have been linked with rheumatoid arthritis (Arend and Dayer 1995; Brennan and McInnes 2008; Corvaisier et al. 2012).

We propose that LSD1 rather than LSD2 mediates the effects of riboflavin deficiency on gene expression. Although peptide demethylation assays cannot distinguish between the two LSD homologs, previous studies demonstrate that LSD2 specifically associates with the coding region of its target genes where it synergizes with euchromatic histone methyltransferases EHMT1/2 and NSD3 in the regulation of transcriptional elongation (Fang et al. 2010). In addition, LSD2 may repress genes through its LSD2-specific Zf-CW domain (Yang et al. 2010), suggesting that LSD2 may be functional in states of riboflavin deficiency. However, we did not formally

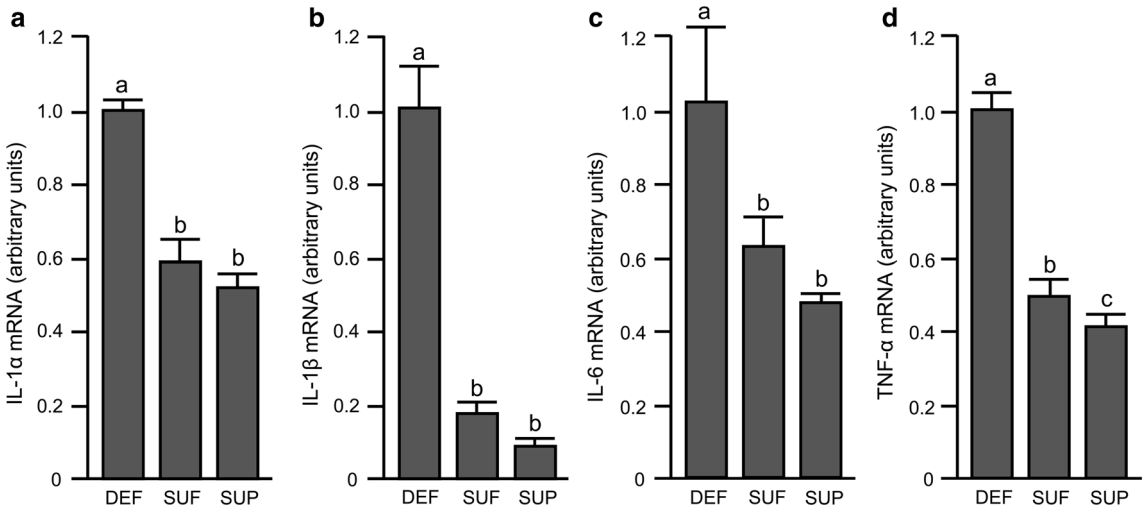

Fig. 5 The expression of *IL-1α* (**a**), *IL-1β* (**b**), *IL-6* (**c**), and *TNF-α* (**d**) mRNA depends on the concentrations of riboflavin in Jurkat cell culture media. Values are mean ± SD, $n = 3$. [a,b,c]Means not sharing a common letter are significantly different for the same variable, p value <0.05. *DEF* deficient, *SUF* sufficient, *SUP* supplemented

exclude the possibility that loss of LSD2 might contribute to the deregulation of pro-inflammatory cytokines in Jurkat cells. Therefore, loss of LSD2 is considered an unlikely, yet possible explanation for the effects reported here.

A few uncertainties remain and need further investigation. First, we did not assess the actual binding of LSD1 around TSS, based on the rationale that such studies would need to be performed using ChIP assay and antibodies that distinguish between apo- and the holo-LSD1. No such antibodies are currently available. Second, the impaired H3K4me2 demethylation in riboflavin-deficient cells might be rescued by FAD-independent histone demethylases, such as H3K4me2 demethylases JARID1A, JARID1B, JARID1C, and JARID1D, which belong to Jumonji AT-rich interactive domain subfamily of Jumonji C domain containing proteins (Christensen et al. 2007; Iwase et al. 2007; Klose et al. 2007; Lee et al. 2007; Tahiliani et al. 2007; Yamane et al. 2007).

Future work is required to determine the underlying mechanism of LSD1-mediated repression of pro-inflammatory cytokines by riboflavin and its implication in human health. A logical next step would be to assess effects of riboflavin in a mouse feeding study, e.g., treating mice on a riboflavin-supplemented diet with an LSD1 inhibitor and then monitor for changes in pro-inflammatory cytokines.

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Conflict of interest D. Liu and J. Zempleni declare no conflicts of interest.

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