Aus dem Institut für Physiologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

Potential role of Smyd1 and PML in human endothelial cells during LPS-induced sepsis

Charakterisierung der Funktion von Smyd1 und PML bei LPS-abhängigen Entzündungsreaktionen in Endothelzellen

zur Erlangung des
akademischen Grades
Doctor medicinae
(Dr. med.)

vorgelegt der Medizinischen
Fakultät Charité –
Universitätsmedizin Berlin

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Datum der Promotion: 25.06.2023

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List of abbreviations

ABs	Antibodies
Akt	A serine/threonine protein kinase
APS	Ammoniumperoxydisulfate
BCA	Bicinchoninic acid
cDNA	Complementary deoxyribonucleic acid
CT	Cycle threshold
ddH_2O	Double-distilled water
DMEM	Dulbecco's Modified Eagle Medium
dNTP	Deoxynucleotide
DTT	Dithiotreitol
ECs	Endothelial cells
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ESL	Endothelial surface layer
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCl	Hydrochloric acid
HDACs	Histonedeacetylases
ICU	Intensive care unit
IFN-γ	Interferon gamma
IκB	Inhibitor of nuclear factor kappa-B
IL	Interleukin
IgG	Immunoglobulin G
JAK/STAT3	Janus kinase/signal transducer and activator of transcription
LB	Luria-Bertani
LPS	Lipopolysaccharide
M-MuLV	Moloney Murine Leukemia Virus

MEFs	Mouse embryonic fibroblasts
MFI	mean Fluorescence intensity
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
NF-κB	Nuclear factor kappa-B
PDTC	Pyrrolidine Dithiocarbamate
PI3K	Phosphatidylinositol 3-kinase
PML	Promyelocytic leukemia
PML-NBs	PML nuclear bodies
PMN	Neutrophilic granulocytes
Psi	Pounds per square inch
RAR	Retinoic acid receptor
RIPA	Radioimmunoprecipitation assay
RPM	Rotations per minute
RT	Room temperature
RT-qPCR	Real time semi-quantitative PCR
SCCM	Society of Critical Care Medicine
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium-dodecyl-sulfate polyacrylamide gel-electrophoresis
SEM	Standard error of mean
skNAC	Skeletal muscle nascent polypeptide associated complex
SMYD	SET and MYND domain containing protein
TBS	Tris-buffered saline
TBST	Tris buffered Saline with Tween 20
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNF α	Tumor necrosis factor alpha
TRIM	Tripartite motif
VCAM-1	Vascular cell adhesion molecule-1

Abstract English

Introduction: Sepsis is a global life-threatening condition associated with destructive effects on the organs due to exaggerated immune response. It commonly occurs after the exposure to Lipopolysaccharide (LPS) following infection with Gram-negative bacteria particularly in severely ill patients. LPS leads frequently to endothelial cell dysfunction and plays an important role in the progression of sepsis leading to one or multiple organs failure. Smyd1 is a histone methyltransferase that affects chromatin remodeling and is involved in regulation of cellular development and cancer. PML protein is involved in the oncogenesis of acute promyelocytic leukemia and has a prominent role in many physiological and pathological processes such as tumor suppression, angiogenesis and inflammatory responses. Both Smyd1 and PML were found to be expressed in endothelial cells but little is known about their role in sepsis and inflammation. The aim of this study was to determine the contribution of Smyd1 and PML to the immune response under septic conditions in ECs.

Methods: EA.hy926 cells were treated with LPS. The expression of Smyd1 and PML was detected, after reverse transcription of mRNA, via Real time semi-quantitative PCR (RT-qPCR) and on the protein level using Fluorescence-activated cell sorting (FACS). Then, EA.hy926 cells were transfected with Smyd1 and PML carrying plasmids to study their effect on the expression of IL-1, IL-6, IL-8 and NF-κB on the mRNA level with RT-qPCR and/or on the protein level via immunoblotting,Enzyme-linked immunosorbent assay (ELISA) and FACS.

Results: Incubation with LPS increased Smyd1 and PML, while incubation with IL-6 increased PML only. Overexpression of Smyd1 increased the expression of IL-1, IL-6, and IL-8, while PMLIV did not induce IL-8. Although Smyd1 and PMLIV appeared to increase the amount and activity of NFκB, the Smyd1-induced expression of IL-6 was not completely dependent on NFκB.

Conclusions: It was shown in this work that Smyd1 and PML are involved in inflammatory reactions of the endothelial cells. Thereby, we reported an increase of IL-6, IL-8 and IL-1 by Smyd1 which could be in part due to Smyd1 dependent activation of NFκB signaling pathway. We also confirmed the stimulatory effect PML on IL-6 expression in endothelial cells and revealed an IL-6-dependent increase of PML as well. A heuristic model is being developed in which, after an amplification phase of IL-6 production, the degradation of Smyd1 after its PML-dependent SUMOylation helps to end the acute inflammatory reaction.

Zusammenfassung

Einleitung: Die Sepsis als ein Erkrankungszustand mit überschießenden Abwehrreaktionen und daraus resultierendem Multiorganversagen ist weltweit verbreitet und lebensbedrohlich. Hervorgerufen wird sie besonders häufig durch die Freisetzung von Lipopolysacchariden (LPS) aus Gram-negativen Bakterien. Neben ihrer Wirkung auf Abwehrzellen im engeren Sinne wirken LPS auf vaskuläre Endothelzellen. Es kommt zur charakteristischen, inflammatorischen Veränderung des Endothels, die für den Krankheitsverlauf entscheidend ist. Smyd1 ist eine H3K4 Histon-Methyltransferase mit SET Domäne und spielt u.a. eine Rolle bei der Proliferation von Karzinomen. PML-Proteine, ebenfalls im Zusammenhang mit Karzinomen untersucht, bilden Kernkörperchen, in denen die Aktivität von Histonmethyltransferasen wie auch von Histon-Deacetylasen durch SUMOylierungsreaktionen reguliert wird. Sowohl Smyd1 wie PML konnten in Endothelzellen nachgewiesen werden. Ob sie bei inflammatorischen Reaktionen des Endothels eine Rolle spielen können, ist bisher unbekannt. Es war das Ziel dieser Arbeit, Smyd1 und PML in LPS-exponierten Endothelzellen zu untersuchen.

Methoden: EA.hy926 Zellen wurden mit LPS oder IL-6 inkubiert oder es wurde Smyd1 oder PMLIV mit Hilfe von Expressionsplasmiden überexprimiert. Für Smyd1, PML, IL-1, IL-6, IL-8 und NFκB kodierende mRNA wurde durch semi-quantitative real time RT-PCR bestimmt. Zugehörige Proteine wurden durch Immunoblot, FACS oder ELISA bestimmt.

Ergebnisse: Inkubation mit LPS erhöhte Smyd1 und PML, während Inkubation mit IL-6 lediglich PML erhöht wurde. Überexpression von Smyd1 erhöhte IL-1, IL-6 und IL-8, während durch PMLIV IL-8 nicht erhöht wurde. Obwohl Smyd1 und PMLIV Menge und Aktivität von NFκB zu erhöhen schienen, war die Smyd1-induzierte Expression von IL-6 nicht vollständig von NFκB abhängig.

Schlussfolgerungen: Es wurde in dieser Arbeit gezeigt, dass Smyd1 und PML an den inflammatorischen Reaktionen von Endothelzellen beteiligt sind. Dabei berichteten wir über einen Anstieg von IL-6, IL-8 und IL-1 durch Smyd1, der zum Teil auf eine Smyd1-abhängige Aktivierung des NFκB-Signalwegs zurückzuführen sein könnte. Wir bestätigten auch die stimulierende Wirkung von PML auf die IL-6-Expression in Endothelzellen und zeigten ebenfalls einen IL-6-abhängigen Anstieg von PML. Es wird ein heuristisches Modell entwickelt,in dem nach einer Amplifikationsphase der IL-6 Produktion die Degradation von Smyd1 nach seiner PML- abhängigen SUMOylierung die akute Entzündungsreaktion zu beenden hilft.

Introduction

Sepsis is a severe clinical condition which has been identified according to the 2016 guidelines, through the Society of Critical Care Medicine (SCCM) and the European Society of Intensive Care Medicine, as a life-threatening organ dysfunction because of improper regulation of host response to infection (1, 2). As a matter of fact, sepsis is a universal major healthcare problem; it is the most prevalent cause of mortality in critically ill patients admitted to the intensive care units (ICU) and it has a huge burden on health system costs (3, 4). Global assessment showed that about one third of ICU admitted patients of sepsis died before leaving hospital (1,5). In the developing world, sepsis accounts for 60-80% of mortality cases annually (6). In USA, the number of cases hospitalized with sepsis in 2008 was beyond the double compared to 2000 reaching about 1,140,000 cases (7) and the number of deaths from sepsis increased byabout 30% from 2000 to 2007 (8). Previous literature indicated that sepsis usually follows gram-negative bacterial infection in critically ill patients (9, 10).

The vascular endothelium represents a highly selective barrier between the blood and all other tissue compartments. It has an important role regarding the regulation of vascular tone, haemostasis and inflammatory reactions (11, 12). The endothelium has a fundamental role in the pathogenesis of sepsis as it is a main target for inflammatory agents under septic conditions (13, 14). During sepsis, cytokines stimulate shedding of the glycocalyx, (sugar- conjugated protein) that surrounds the endothelial cells (ECs), thus baring of the adhesion molecules which, in turn, stimulate leukocyte adhesion to the ECs. The leukocytes (neutrophils and monocytes in particular), have a significant role in the pathogenesis of sepsis; after their adhesion to the ECs, they sneak into the tissue by a special movement called “diapedesis” where they secrete numerous inflammatory mediators in response against pathogens on one hand, but lead to organ impairment and/or failure of the respective body system on the other hand (15). In addition to their interacting with activated leukocytes, ECs themselves secrete inflammatory cytokines (16). At this point, it is worth mentioning that regarding the cellular structure and function, it was found that the endothelial cell lining displays extreme heterogeneity between distinct vessels of different body organs and therefore responds variably to sepsis (17).

The bacterial endotoxin, Lipopolysaccharide (LPS), is an integral constituent of the outer membrane in gram-negative bacteria. It dissociates from the membrane during growth or as a result of the lysis of the bacterial cell following exposure to antimicrobials or the immune-mediated defense mechanisms (18, 19). LPS can initiate an over active inflammatory response if large amounts enter circulation as in cases of endotoxemia (20). It was reported that LPS inevitably interacts with the ECs and is considered as one of the most important inflammatory stimuli that causes endothelial cell injury through evocation of multiple consequences, such as breakdown of the vascular barrier (21), apoptosis of ECs and detachment from the vessel basement membrane, stimulation of the coagulation process, with further progression of microcirculatory injury and development of sepsis-associated dysfunction of different organs (22-24). Such effects of LPS are mostly exerted through the secretion of different inflammatory factors such as IL-6, IL-8 and tumor necrosis factor alpha (TNF α) (25). Despite the broad understanding of the ECs-heterogeneity, the mechanism(s) regarding the molecular regulation of the LPS-induced diverse responses are still unclear (26).

The SET and MYND domain containing protein (Smyd)1 is one of the Smyd family members (Smyd1 to Smyd5) which exert different biological roles being involved in the regulation of cellular development and cancer (27). Smyd1, called muscle (m)-Bop1 as well, is a histone methyltransferase that plays an important role in chromatin remodeling. The expression of Smyd1 was thought, for a long time, to be limited to muscles (skeletal and cardiac muscles) where it has a crucial transcriptional regulatory role during myogenesis and myocyte differentiation through methylation of histone H3 at lysine 4 and thereby modulating the structure of chromatin conformation (28-33). In addition, a previous study showed that Smyd1 is expressed in endothelial cells, and plays a role in migration and tube formation in human endothelial cells (34). Smyd1 is composed of three domains; MYND zinc finger domain, a SET domain and, in addition, a C-terminal domain which on its absence the methyltransferase activity of Smyd1 protein is impaired and consequently the Smyd1 function in myofibrils is affected (35).

Smyd1 protein methylates cytosolic proteins, like myosin, and thus controls muscle filament assembly (36). However, rather than its main presence in cytoplasm, nuclear localization of Smyd1 protein has been denoted as well in myoblasts *in vitro* (37) and cardiac muscles (35)

where it is involved in many interactions with different proteins in the nucleus and thereby contributing in the regulation of gene expression (38, 39).

Growing data indicate a significant function of Smyd proteins in immunity (40, 41). Specifically, Smyd1 was found to be profoundly expressed in cytotoxic T cells (42, 43). Previous studies denoted also that Smyd1 can be induced by interferon gamma (IFN-γ) most likely because of IFN-γ-stimulated response elements. Thus, Smyd1 is engaged in the regulation of T-cells and the immune response in general (43). Moreover, the nascent polypeptide associated complex (skNAC), which has been observed to form a protein dimer with Smyd1, has been implied recently in the regulation of cytokine secretion thereby also denoting a possible impact of Smyd1 in the immune response (44, 45).

Several mechanisms were postulated explaining Smyd1 interactions, functions and the different phenotypes induced upon its absence. It was denoted that Smyd1 could have both; a histone modification based transcriptional stimulatory role which is accomplished by the SET domain that mainly functions as a methyltransferase through assisting in trimethylation of H3K4 (32) or a down regulatory effect via the MYND zinc finger domain which recruits co-repressors such as histonedeacetylases (HDACs) (46). In addition, Smyd1 regulates sarcomere assembly by binding to skNAC in skeletal and heart muscle cells (37, 47). A Prior study at our lab showed the colocalization of skNAC with promyelocytic leukemia (PML) and it was indicated that the co-binding of skNAC and Smyd1 might act in complex with PML at the onset of myogenesis (48).

PML protein is a member of tripartite motif (TRIM)-containing proteins and has seven isoforms (PMLI to PMLVII) which are similar in their N-terminal regions but have different C-termini (49, 50). *PML* gene, as a fusion partner of the retinoic acid receptor (RAR), is causally involved in the oncogenesis of acute promyelocytic leukemia resulting from a chromosomal translocation between chromosomes 15 and 17. The protein products of *PML* gene form subnuclear very fine structures, called PML nuclear bodies (PML-NBs), which are sized from 0.2 to 1 μm and their count ranges from 1 to 30 PML-NBs per cell dispersed between the chromatin (51, 52). PML and PML-NBs play a central-point role through which various up and/or down regulatory pathways merge (53). PML-NBs serve as nuclear stores fora diverse of proteins, such as p53 and HDAC7, which travel in and out depending upon cellular signaling

according to different stimuli (54). In addition, PML-NBs can induce acetylation and/or sumoylation of proteins owing to their post-translational modificatory role (55, 56).

Many studies on the PML protein denoted its pivotal role in regulation of growth suppression, transcription, viral infection, cellular senescence as well as apoptosis either in physiological status or pathological circumstances such as in tumors (51, 54, 57-59). In the same context, several studies showed that down regulation of *PML* was denoted in diverse human cancers and proved that its knockout leads to cellular proliferation and induction of tumorigenesis (60-62).

Rather than former data focusing on PML and its role in apoptosis, controlling of cell cycle and tumor suppression, it has also been suggested that PML has a prominent role in other physiological and pathological processes such as angiogenesis and inflammatory responses (53, 63). Microarray analysis showed that *PML* is profoundly expressed in endothelium, suggesting its involvement in angiogenesis and vascular biology (53). Additionally, a body of work has illuminated the coregulatory role of PML in innate immune defence through its implication in the production of a broad spectrum of proinflammatory cytokines such as IFNs, interleukin 1 (IL-1) and IL-6 (63, 64). During viral infections, interferons interact with specific elements on the PML promoting their transcription and stimulate enormous production of NBs with enhancement of their correlated transcriptional activity which ends by inhibiting viral genes expression (65). Previous literature reported also that knockout of *PML* in mice causes dysfunction of macrophages, prominent decline of IL-6 production and spread of abscesses in almost all organs in response to LPS stimulation (63, 64).

The Nuclear factor kappa-B (NF-κB) belongs to a family of transcription factors which regulate the expression of genes involved in multiple biological processes such as cellular proliferation, invasion, apoptosis, immune response, and inflammation (66, 67). NF-κB proteins involve homo or heterodimeric partners from a pool of transcriptional activator domains (p50, p52, RelA, C-Rel, and RelB) (68). NF-κB could be considered as a signal integrator that regulates the process of vascular inflammation. LPS triggers Toll-like receptor-4 receptors of immune cells and induces the phosphorylation and then degradation of the inhibitor of nuclear factor kappa-B (IκBα and IκBβ) (69, 70). As a result, NF-κB is translocated from the cytoplasm into the nucleus, where it binds to DNA and exerts its transcriptional regulatory effects (71, 72).

Activated NF-κB has a central role in ECs during inflammation; it elicits the production of proinflammatory cytokines and stimulates cell adhesion molecules such as VCAM-1 and ICAM-2 as well as chemotaxins with further enhancement of leukocyte infiltration (69, 72). In cases of bacterial septicemia, hyperactivated NF-κB represents a central station in the cascade of events in ECs which usually elicits an overwhelming immune response that ends with ECs and organ dysfunction (71, 73-75). Accordingly, NF-κB serves apparently as an optimum spot to which the treatment of septic shock should be directed (76, 77).

IL-6 is produced by many cell types, including ECs (78) and it is exceptionally important for its effective pro-inflammatory and chemotactic activities during the progress of vascular inflammation (79). It exerts its crucial role in inflammation through induction of inflammatory acute- phase reactions, proliferation and differentiation of lymphocytes as well as activation of macrophages (25, 78, 80, 81). IL-6 is one of the most NF-κB-dependent induced cytokines which has an axial role in acute immune response, beside its main function during the inflammatory process in ECs through the NF-κB–IL-6 signaling pathway (72).

Upon the aforementioned literature, we hypothesized that Smyd1 is incorporated in the inflammatory response during sepsis in ECs. We tried to reveal such role and to get insight into the possible mechanism(s) through which it works. The role of PML was established regarding its engagement in LPS-induced immune response in different cell lines but not in ECs. Therefore, we investigated also in this study the impact of PML following LPS stimulation to confirm its role in ECs from one side and support the validity of Smyd1 results,acting as a positive control, from the other side.

Materials and Methods

2.1 Materials

2.1.1 Chemicals:

Biochrom, Berlin, Germany:

- Fetal Bovine Serum (FBS)
- Trypsin/ Ethylenediaminetetraacetic acid (EDTA) solution (0.05 %/0.02 %)

Carl Roth GmbH + Co. KG, Karlsruhe, Germany:

- 1,4-Dithiothreitol (DTT)
- 6-amino-n-caproic acid
- Acetic acid
- Acrylamid (30 %)
- Ammoniumperoxydisulfate (APS)
- Bromophenol blue
- Ethanol (≥ 99, 8 %)
- Glycine
- Hydrochloric acid (HCL)
- Kanamycin sulphate
- Methanol (≥ 99, 9 %)
- Milk powder, 0.9 % fat
- Pyrrolidine Dithiocarbamate (PDTC)
- Sodium chloride
- Tricine
- Tris base / Tris-HCL

Cell Signaling Technology, Danvers, MA, USA:

- Anti-NFκB p65 antibody (Cat.No: 8242S; rabbit)
- Anti-IκB-α antibody (Cat.No: 4814S; mouse)

Eurofins, Hamburg, Germany:

- Primers for *IL-1* and *IL-8*.

EURx, Gdańsk, Poland:

- GeneMATRIX Universal RNA Purification kits EURX®)

GE Healthcare, Chicago, IL, USA:

- ECL™ Prime Western Blotting System
- Nitrocellulose membranes
- Whatman™ filter papers

Gibco, Carlsbad, CA, USA:

- 0.5% Trypsin-EDTA (10x)
- Dulbecco's Modified Eagle Medium (DMEM): with 4.5 g/L D-Glucose, L- Glutamine - without Sodium Pyruvate.
- Opti-MEM

Life Technologies, Darmstadt, Germany:

- Alexa Flour 488 and 594

MWG Biotech AG, Ebersberg, Germany:

- Primers for *PML, Smyd1, IL-6 and GAPDH*

PeproTech, Hamburg, Germany:

- Recombinant human IL-6

Proteintech, Rosemont, IL, USA:

- Anti-GAPDH antibody: (Cat.No: HRP-60004; mouse)

Santa Cruz Biotechnologies, Heidelberg, Germany:

- Anti-PML antibody: (Cat.No: sc-377390 ; mouse)
- Anti-Smyd1 antibody: (Cat.No: sc-514805; mouse)
- Anti-IL-6 (Cat.No: sc-28343; mouse)
- Secondary antibodies:
  - goat anti-rabbit IgG-HRP: sc-2004
  - rabbit anti-mouse IgG-HRP: sc-358914
- Mono-clonal IgG Abs:

- mouse IgG1: sc-3877
- mouse IgG2a: sc-3878

Sigma Aldrich, St. Louis, MO, USA:

- Glycerol
- LPS from *Escherichia coli*
- Nonidet P-40
- Sodium deoxycholate
- Sodium dodecyl sulfate (SDS)
- Tryptone
- Tween 20
- Yeast extract

Sino Biological Inc., Beijing, China:

- Human expression plasmid pCMV2-Smyd1-flag

Thermo Fisher Scientific, Waltham, MA, USA:

- 10,000 IU/mL penicillin - 10 mg/mL streptomycin
- Phosphate-buffered saline (PBS)
- Fisher BioReagents™ EZ-Run™ Prestained *Rec* Protein Ladder.
- Anti-Smyd1 antibody: (Cat.No: PA5-31482; rabbit)
- TurboFect reagent

2.1.2 Buffers:

Blocking Buffer:

1X TBST with 5% skimmed milk; for 200 mL: 10 g skimmed milk were added to 200 mL 1X TBST and mixed thoroughly.

Gel preparation:

12 % separating gel (10 mL)		4 % stacking gel (5 mL)	
ddH_2O	3.8 mL	ddH_2O	3 mL
1.5 M Tris-HCl pH 8.8	2.6 mL	0.5 M Tris-HCl pH 6.8	1.25 mL
30 % (w/v) acrylamide	3.4 mL	30 % (w/v) acrylamide	0.7 mL
10 % (w/v) SDS	100 µL	10 % (w/v) SDS	50 µL
10 % (w/v) APS	100 µL	10 % (w/v) APS	50 µL
TEMED	10 µL	TEMED	5 µL

Laemmli buffer 5x:

0.25 M Tris-HCl pH 6.8, 0.5 M DTT, 10 % SDS, 50 % Glycerol & 0.5 % Bromo-phenol blue.

LB medium:

For 1 L : Tryptone 10 g, NaCl 10 g, Yeast extract 5 g & ddH_2O up to 1 L followed by autoclaving for 20 min at 15 psi on liquid cycle.

Radioimmunoprecipitation assay (RIPA) buffer:

5 mL Tris-HCl (1 M, pH 8.0), 3 mL NaCl (5 M), 1 mL nonidet P-40, 5 mL sodium deoxycholate (10 %) and 1 mL SDS (10%) per 100 mL ddH_2O.

Running buffer (1X SDS buffer):

28.8 g Glycine, 6.04 g Tris base, 20 mL of 10% SDS & ddH_2O to a final volume of 2 L.

Tris-buffered saline (TBS) 10X:

For 1 L: 24 g Tris base, 88 g NaCl, HCl & ddH_2O to a final volume of 1 L with adjustment of pH to 7,6.

Tris-buffered saline, 0.1% Tween 20 (TBST):

For 500 mL: 50 mL of TBS 10X, 450 mL of ddH_2O & 500 µL Tween 20

Western blotting; Semi-dry Solutions:

- Anode1: 0.3 M Tris - 20% Methanol
- Anode2: 25 mM Tris - 20% Methanol
- Cathode: 25 mM Tris - 20% Methanol – 40 mM 6-amino-n-caproic acid

2.1.3 Equipment:

Applied Biosystems, Foster City, CA, USA:

- QuantStudio 5 Real-Time PCR System

Amersham Bioscience, Buckinghamshire, UK:

- Ultraspec 2100 pro UV/Visible spectrophotometer

BD Biosciences, Franklin Lakes, NJ, USA:

- BD FACSCalibur™

Biometra, Göttingen, Germany:

- UNO Thermoblock PCR Thermocycler
- Shaker
- Standard Power Pack P25

Biorad, Munich, Germany:

- Gel electrophoresis equipment
- Mini Protean 3 System: glass plates, spacer plates
- Trans-Blot SD semi-dry electrophoretic transfer cell

Biostep, Burkhardtsdorf, Germany:

- Dark Hood DH-50 with digital camera

Eppendorf, Hamburg, Germany:

- Centrifuge 5417R
- Safe-Lock Microcentrifuge Tubes

Heidolph, Schwabach, Germany:

- Titramax 1000, rocking platform

Heraeus, Hanau, Germany:

- Biofuge stratos centrifuge
- Incubator B 5060 EK/CO_2

Invitrogen, Waltham, MA, USA:

- IL-6 -ELISA- kit

Leitz, Wetzlar, Germany:

- Ortholux microscope

MACHEREY-NAGEL GmbH & Co, Germany:

- NucleoBond® Xtra Midi

Promega, Madison, Wisconsin, USA:

- GoTaq qPCR Master Mix (Promega®)

Sarstedt, Nürnbrecht, Germany:

- 6 / 12 well plates
- 96-well microplate
- Tissue culture flasks

Systec, Linden, Germany:

- Autoclave (model Vx65)

Tecan Sunrise, Männedorf, Switzerland:

- Microplate reader for 96-well plates

Thermo Fisher Scientific, Waltham, MA, USA:

- First Strand cDNA Synthesis Kit
- Pierce™ BCA Protein Assay Kit

2.2 Methods

2.2.1 Cell culture:

EA.hy926 cells (Elabscience, Biotechnology Inc., Houston, TX, USA) were cultured in cell culture flasks at 37°C in 10 mL DMEM supplemented with 10% FBS, 1% of Antibiotics (10,000 IU/mL penicillin - 10 mg/mL streptomycin) and incubated in humidified atmosphere with 5% CO_2 until confluence. Then, EA.hy926 cells were seeded on 6- or 12-well culture plates for further experiments. The cell culture medium was exchanged 24 h before all experiments with fresh medium.

2.2.2 Cell stimulation with LPS and IL-6:

An experimentally mimicked septic condition was created in vitro using LPS. EA.hy926 cells were stimulated for 3 h with various dilutions of LPS ranging from 1 ng/mL to 10 μg/mL. According to the intial results, LPS was used in a final concentration of 1 μg/mL in the further experiments. Regarding the study of IL-6 effect on Smyd1 or PML expression in EA.hy926 cells, the recombinant human IL-6 was used in a final concentration of 10 ng/mL. Both LPS and IL-6 were applied directly on cell cultures with gentle mixing and cells were then incubated for 3 h or 24 h. The control cells were treated with the vehicle alone.

2.2.3 Cell treatment with PDTC:

Pyrrolidine dithiocarbamate (PDTC) in a final concentration of 10 μM; dissolved in dimethyl sulfoxide (DMSO), was added to the medium. DMSO alone served as a negative control.

2.2.4 RNA isolation and reverse transcription:

Total cellular RNA was isolated by GeneMATRIX Universal RNA Purification kit according to the manufacturer's protocol. Shortly: cells were lysed with denaturing buffer then homogenized to eliminate DNA fragments. Samples were after that added onto a binding-spin-column and centrifuged so that all RNA molecules are adsorbed to the matrix column. After several washing steps to remove the contaminants, the RNA was eluted with RNase-free water (30-40 μL). The resultant RNA amount was measured by spectrophotometry at 260 nm. To perform reverse transcription, we used First Strand cDNA Synthesis Kit (Thermo Fisher Scientific®) according to the manufacturer's instructions. Briefly, in ice cooled nuclease free tubes, 1 μg of total RNA from each sample and 1μL of oligo-dT-18-primer were added to nuclease-free water up to a total volume of 11 μL and heated at 65°C for 5 min. in UNO-Thermoblock to denaturate the RNA and then quickly chilled on ice. After spinning down, we added 4 μL 5X Reaction Buffer, 1 μL RiboLock RNase Inhibitor, 2 μL 10mM dNTP Mix and 2 μL (40 U) from M- MuLV reverse transcriptase to each of the samples, placed on ice with gentle mixing. Thenthe samples were incubated in the thermo-block for 60 min at 37°C followed by heating at 70°C for 5 min. to terminate the reaction.

2.2.5 Real-time quantitative PCR (RT-qPCR):

2 μL of cDNA template were added to primers (in a final concentration of 0.3 μmol/L), 5 μL master mix and nuclease-free water to a total volume of 10 μL. The PCR reaction was performed using QuantStudio 5 PCR machine; the run was started at 95°C for 10 min. before proceeding for 45 cycles, each individual cycle is about 90 sec. and involves a sequel of denaturation (95 °C for 30 sec.), annealing (temperatures were as listed in Table1) for 30 sec. and elongation (72°C for 30 sec.). In each RT-qPCR run, non-reverse transcriptase samples as well as non-template controls were tested. The melting curve analysis was performed for every single experiment to assure the production of a single transcript. The relative quantification levels of gene expression were assessed by the comparative CT ($2^{-\Delta\Delta CT}$) method with normalization of expression to glyceraldehyde 3- phosphate dehydrogenase (GAPDH). The ΔΔCT of the $2^{-\Delta\Delta CT}$ method is calculated as follows:

ΔΔCT = ΔCT (a target sample) – ΔCT (a reference sample)

where ΔCT is the difference of threshold cycles between the target and reference genes in each sample (82).

Table 1) Primers used for RT-qPCR:

Gene	Primer pairs	Annealing temp.	Product size
Smyd1	5′ CTG GAG AAG CAG GAG CCA GTG TT3′ 5′ GCA TAG GCT TTG CAG ATC CC3′	60°C	257 bp
PML	5′ CCG CAA GAC CAA CAT CTT 3′ 5′ CAG CGG CTT GGA ACA TCC T 3′	54°C	90 bp
IL-6	5′ TGC CAG CCT GCT GAC GAA G 3′ 5′ AGC TGC GCA GAA TGA GAT GAG 3′	56°C	90 bp
IL-1	5’ CCA GCT ACG AAT CTC CGA CC 3’ 5’ CAT GGC CAC AAC TGA CG 3’	58°C	70 bp
IL-8	5’ TAG CAA AAT TGA GGC CAA GG 3’ 5’ TCC TGG CTA GCA GAC TAG GG 3’	58°C	194 bp
GAPDH	5′ ATG ACC TTG CCC ACA GCC TT3′ 5′ AAC TGC TTA GCA CCC CTG GC3′	60°C	200bp

2.2.6 Protein extraction and measurement:

Cell culture plates were placed on ice and the cells were washed with ice-cold PBS. After removal of PBS, adherent cells were scrapped with a cell scraper in the presence of ice-cold RIPA buffer (100 µL) then the suspension was gently transferred into pre- cooled 1.5 mL eppendorf tubes and left on ice with shaking on a vigorous shaker for 30 min. Next, the cells were further disrupted by passing the lysates through a 25-gauge needle attached to a 1 mL syringe and the resultant homogenates were spun at 14,000 rotations/minute (rpm) for 12 min at 4°C. The supernatants were carefully aspirated and placed in fresh tubes on ice while pellets were discarded. Afterwards, protein concentrations were determined using Bicinchoninic acid (BCA) protein assay following the manufacturer's guide lines. In brief, duplicates of 10 µL from each of test samples andstandards were pipetted into a 96 well microplate and 200 µL of the working reagent wereadded to each well and mixed gently for 30 sec. on a plate shaker. Then the plate was incubated at 37°C for 30 min. and the absorbance was detected at 562 nm on a plate readerand the protein concentration was estimated using the standard curve produced by formerlyidentified protein concentrations.

2.2.7 Antibodies:

Primary antibodies were used for immunoblotting in the following dilutions: Anti- Smyd1, Anti- NFκB p65 and Anti-IκB-α Antibodies (ABs) were used in a dilution of 1:1000, Anti-PMLand Anti-IL-6 ABs in a dilution of 1:500 while Anti-GAPDH antibody in a dilution of 1:10000. Secondary ABs were diluted at a ratio of 1:1000. A blocking buffer (1X TBST with 5% skimmed milk) was used to dilute the ABs.

2.2.8 SDS PAGE and immunoblotting:

Proteins were separated using the sodium-dodecyl-sulfate polyacrylamide gel-electrophoresis (SDS-PAGE) according to Laemmli (83). In short, acrylamide gels were prepared as mentioned above including 12% resolving gels (to separate the proteins based on their molecular weight) and 4% stacking gels (to line up all proteins of each sample, so that they enter the resolving gel at the same time). About 2-3 mL of isopropanol were

applied to remove air bubbles, then gels could polymerize for 30-45 min. at room temperature (RT). Protein samples, consisting of 40 µg of cell extracts, were mixed with ¼ volume of5 x Laemmli buffer and heated at 95°C for 10 min to denature proteins followed by incubation for 5 min. on ice. Afterwards, the gel was placed in the electrophoresis systemand samples of equal amount of protein (20-30 µL) were loaded on the gel wells with thefirst well always used for the standard molecular weight marker. The chamber was closedand the run, performed in 1x SDS running buffer, was started at 80 V for 30 min, then raised to at 120 V for an additional 1 h.

The semi-dry electrotransfer technique followed using the "Trans-Blot SD semi-dry transfer cell" apparatus from Bio-Rad. Briefly, proteins were transferred to the nitrocellulose membrane after placing the resolving gel on it between the filter paper layers below and above which were pre-soaked in anode or cathode buffers respectively and running the blot for 45 min at 25 V. The membrane was then blocked for one hour using the blocking buffer at 4°C and after that incubated with primary ABs over night at 4°C and on the next day for about 1 h at RT before the washing step (3 times x 10 min. each). Then the membrane was incubated with the conjugated secondary antibody, in a dilution of 1:10000, for 2 h at RT followed by washing again (3 times x 10 min. each), toremove unbound secondary ABs and avoid high background signals. For developing of immunoblots, ECL detection solutions were mixed in a ratio of 1:1 to prepare a sufficient final volume of detection reagent (0.1 mL/cm^2 membrane), then added on the membrane placed on a clean sheet of plastic wrap. After incubation for 3-5 min. at RT, the chemiluminescence was visualized by digital imaging.

2.2.9 Enzyme-linked immunosorbent assay (ELISA):

ELISA was performed to study the level of IL-6 protein secreted into the cell culture supernatant after overexpression of *Smyd1 or PML* in EA.hy926 cells. Briefly, the cells could grow until about 70-90% confluence in 6 well plates, and became transfected as stated below in 2.2.11, with Smyd1- or PML-expressing DNA plasmids while the control wells were treated with an empty vector. After 24 h, supernatants were collected and analyzed with ELISA kit in accordance with the manufacturer's instructions. The concentration of IL-6 in the samples was assessed depending on a standard curve produced with known amounts of IL-6.

2.2.10 Fluorescence-activated cell sorting (FACS):

Treated or untreated cells were harvested and centrifuged at 1500 rpm. Pelleted cells were resuspended and washed with PBS and centrifuged again at 1500 rpm. Methanol 99% was added to pelleted cells (1 mL/tube) and tubes were kept then at -20°C for 20 min. Afterwards, cell pellets were resuspended, washed and incubated, after brief vortexing, at 4°C with the respective primary non-conjugated ABs or isotype-matched mono-clonal IgG control Abs, all diluted in 1% FBS in PBS (1:100). After 2 h, cells were centrifuged and washed twice with 1% FBS in PBS followed by incubation with the proper conjugated secondary ABs (Alexa Flour 488 and 594), diluted in 1% FBS in PBS (1:1000), at 4°C for 1 h. After centrifugation of the tubes, pelleted cells were washed two times with 1% FBS in PBS, the samples were resuspended in 500 µL of the fixation solution (0.3% paraformaldehyde in PBS) and then analyzed with the FACS (BD Calibur) using CellQuest Software.

2.2.11 Plasmids isolation:

The Plasmids expressing Smyd1, PML and the empty vectors were isolated from *E. coli* using NucleoBond xtra midi column kit according to the manufacturer's protocol. A small amount of the bacteria-glycerol stock was scraped with a pipette tip which was directly dropped in a flask containing 400 mL of Luria-Bertani (LB) and Kanamycin (50 µg/mL) followed by incubation at 37°C for 16 h with continuous shaking at 220 rpm. After this incubation, the LB medium was centrifuged at 6000 *x g* for 15 min. and the supernatant was discarded. The pellet was re-suspended in 8 mL of re-suspension (RES) buffer then 8 mL of lysis (LYS) buffer were added, tubes were gently inverted and the mixture was incubated at RT for 5 min. before adding 8 mL of neutralization (NEU) buffer and mixingby inverting. Meanwhile, the NucleoBond® Xtra column filter was equilibrated with 12 mL of equilibration (EQU) buffer. The whole neutralized homogeneous lysate was thenapplied onto the equilibrated filter. Afterwards, the filter and column were washed with 5mL of EQU then the filter was removed while the column was washed once againwith 8 mL of WASH buffer. Next, the plasmid DNA was eluted by addition of 5 ml of preheated(50°C) elution (ELU) buffer to the column and precipitated by addition of 3,5 mL isopropanol to the sample which was then centrifuged at 15000 rpm

for 30 min. and the supernatant was discarded while the pellet was washed with 2 mL of 70% ethanol followed by centrifugation again at 15000 rpm for 5 min. at RT. After removal of ethanol,the DNA pellet was left for about 45 min to dry and then dissolved, with gentle pipetting,in 300 µL TE buffer. Lastly, the sample was stored at -20°C after determination of the DNA concentration using spectrometry.

2.2.12 Plasmid DNA transfection:

Many studies investigated the function of PML-IV and revealed its role in tumor suppression, resistance against viral infections and also in regulation of intrinsic and innate immunity (84-86). Therefore, the PML expressing plasmid used in this study contained the insert PML isoform IV. The transfection of EA.hy926 cells was performed using the expressing plasmids of PML-IV and Smyd1, pEGFP-C1-PML-IV and pCMV2-Smyd1-flag, respectively. The pEGFP-C1-PML-IV plasmid was a kind gift from Peter Hemmerich, Jena. Germany (87). The cells were transfected with eukaryotic *Smyd1 or PML* expressing plasmids using Lipofectamine 2000 (Invitrogen®) according to the manufacturer's recommendations. A transfection efficiency of 80-90% of the cells was achieved after optimizing the transfection conditions by changing DNA and Lipofectamine 2000 concentrations. For 12 well plates, the cells were seeded in 1 mL of DMEM, supplemented as mentioned in 2.2.1, and incubated overnight till reaching about 70% of confluence. For every single sample, the transfection complex was prepared as follows: each of the plasmid DNA (1.6 µg) and Lipofectamine 2000 (3 µL) were diluted separately in 100 µL of DMEM without FBS and mixed slowly and incubated for 5 min. at RT. Afterwards, both dilutions were gently mixed together and incubated again at RT for a duration of 20 min. then the transfection complex (200 µL) was added to the medium in each well and mixed carefully while rocking the plate. For 6 well plates, double amounts were used from each of DNA, transfection reagent as well as serum freeDMEM and then added to the well containing 2 mL of supplemented medium. The plateswere incubated after transfection at 37°C in 5% CO_2 for 18-24 h while the medium was changed after 6 h of transfection. After the incubation period, the cells were harvested for further assessment of mRNA or protein expression levels regarding target genes. To exclude the effect of the transfection process itself on the results, control cells were transfected with the respective empty vector which lacks the specific gene insert.

2.2.13 Statistical analysis:

Unless otherwise stated, all measurement results are expressed as mean ± the standard error of mean (SEM) of, at least, three independent experiments ("*n*" refers to the number of independent experiments). Before their statistical analysis, all data sets were tested for normal distribution using the Shapiro-Wilk test. The results were then tested for significant differences between the different groups using a one-way ANOVA followed by Turkey´smultiple comparison test or with unpaired t-test using Prism 9 software (GraphPad Software, San Diego, CA, USA). The statistical significance was set at values of $p \leq 0.05$(*), $p \leq 0.01$ (**), $p \leq 0.001$ (***), $p \leq 0.0001$ (****).

3 Results

3.1 Smyd1 expression upon treatment of EA.hy926 cells with LPS

To investigate the effect of sepsis on smyd expression, EA.hy926 cells were stimulated with different concentrations of LPS ranging from 1 ng/mL to 10 μg/mL for 3 h. As indicated in figure (1 A), there was a dose-dependent increase of Smyd1 expression which reached the maximum level in resonse to 1 μg/mL final concentration of LPS. This concentrtion was applied to the culture medium and the level of mRNA expression of Smyd1 in EA.hy926 cells was determined using RT-qPCR after 3 h and 24 h of incubation time. As shown in figure (1B), LPS stimulation upregulated the level of *Smyd1* mRNA to about 3.5-fold after 3 h of incubation. Following 24 h of incubation, *Smyd1* mRNA was still increased but to a much smaller extent. These results were further confirmed through FACS which showed significantly increased Smyd1protein levels following 3 h and 24 h of incubation time with LPS (figure 1 C-D). The increase observed by FACS was nearly of the same extent as on the mRNA level. Although Smyd1 was still increased after 24 h of incubation, there was a significant decline from 3 h to 24 h.

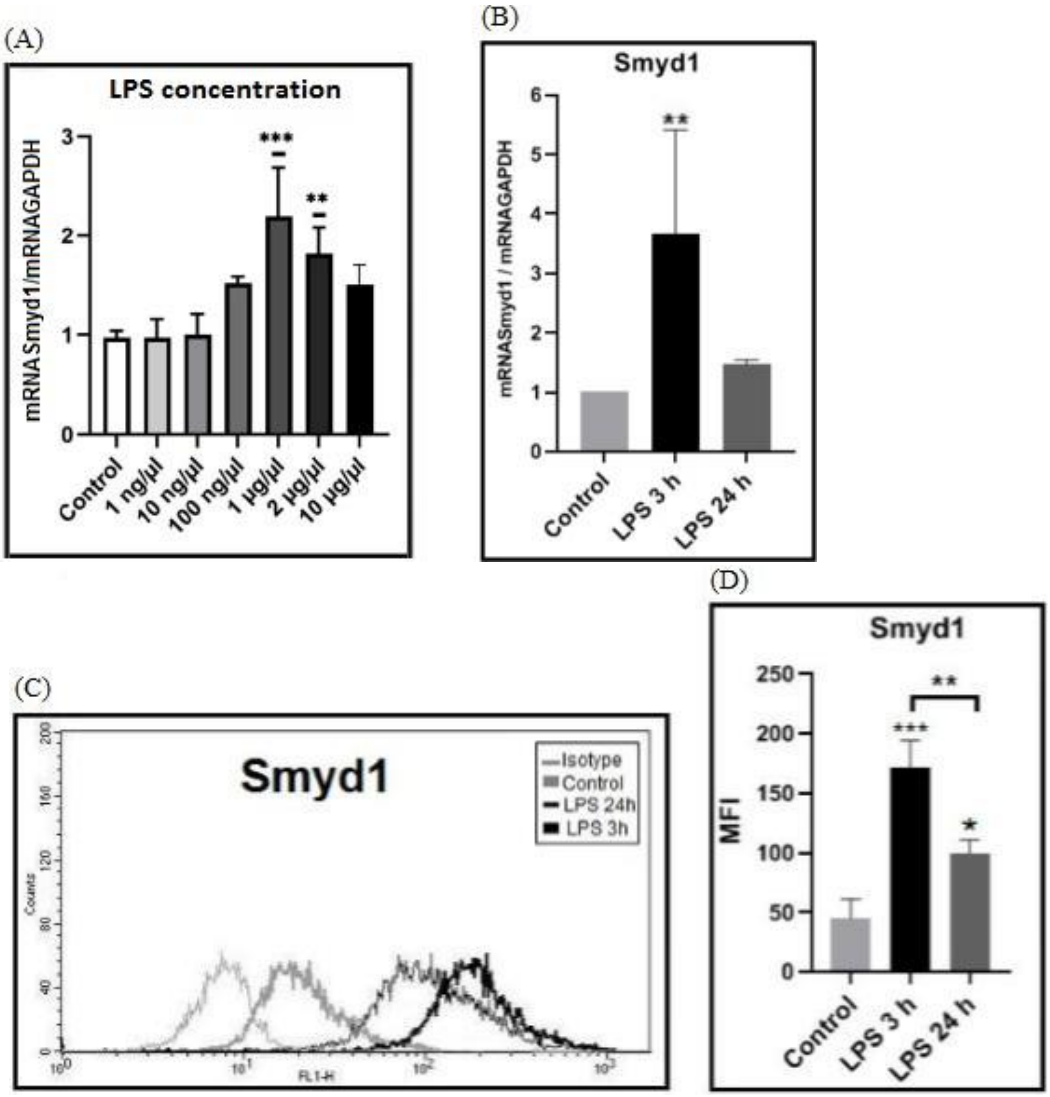

Figure 1) Effect of LPS stimulation on Smyd1. (A) RT-qPCR for the quantification of Smyd1 mRNA levels in EA.hy926 cells that were stimulated with different concentrations of LPS ranging from 1 ng/mL to 10 µg/mL for 3 h relative to control (no LPS supplement). Data are presented as mean ±SD, n=3, **p≤0.01, ***p≤0.001 using one-way ANOVA [109]. EA.hy926 cells were then treated with LPS (1 µg/mL) or with vehicle alone for 3 h and 24 h; **(B)** Quantification of *Smyd1* mRNA levels using RT-qPCR with normalization to vehicle-treated controls. Data are presented as mean ± SEM, n=3, **p≤0.01 using one-way ANOVA [109]. **(C)** Representative FACS histogram of Smyd1 after staining with anti-Smyd1 antibody compared with cells stained with isotype matched non-binding control antibodies [109]. **(D)** Mean Fluorescenceintensity (MFI) of Smyd1 protein levels in vehicle- and LPS-treated EA.hy926 cells. Data are presented as mean ± SEM, n=3, *p ≤ 0.05, **p≤0.01, ***p≤0.001 using one-way ANOVA.

3.2 PML expression upon treatment of EA.hy926 cells with LPS

The LPS (1 µg/mL, final concentration)-challenged EA.hy926 cells were analyzed after 3 h and 24 h concerning PML expression. On the mRNA level, *PML* was slightly increased in 24 h LPS-treated samples but, like *Smyd1*, it was increased dramatically in those assessed after 3 h

(figure 2 A). The FACS results showed increased PML protein levels 3 h and 24 h post LPS stimulation (figure 2 B). The increase of PML at 3 h was significantly different from the control cells, then it started obviously to decline, but without reaching the control level within 24 h (figure2 C).

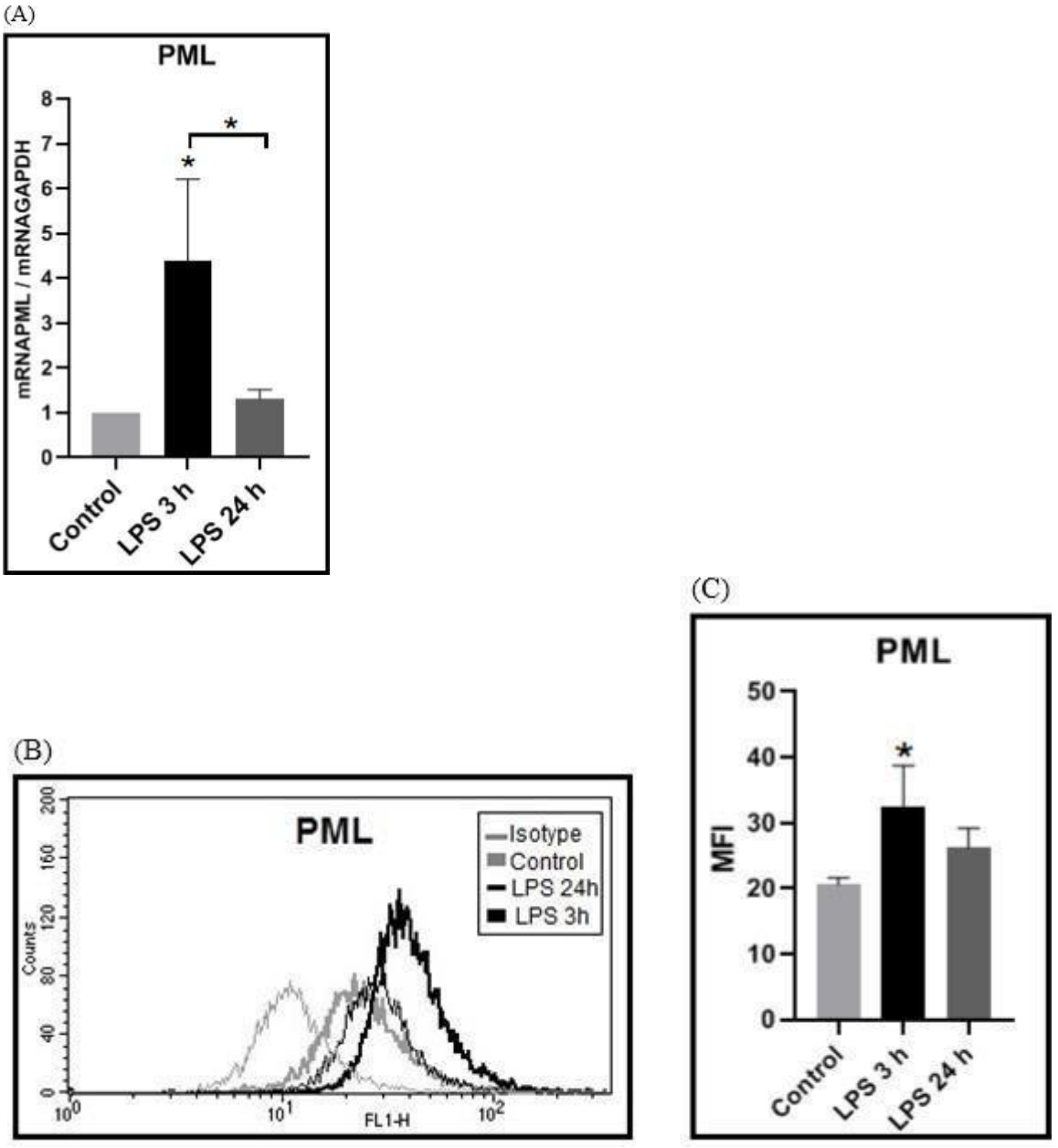

Figure 2) Effect of LPS stimulation on PML. EA.hy926 cells were treated with LPS (1 µg/mL) or with vehicle alone for 3 h and 24 h. **(A)** Quantification of *PML* mRNA levels using RT-qPCR with normalization to vehicle-treated controls. Data are presented as mean ± SEM, n=4 and *$p \leq 0.05$ using one-way ANOVA. **(B)** Representative FACS histogram showing the regulation of PML protein levels after staining with anti-PML antibody compared with cells stained with isotype matched non-binding control antibodies. **(C)** The bar charts represent MFI of PML protein levels in vehicle- and LPS-treated EA.hy926 cells. Data are presented as mean ± SEM, n=3 and *$p \leq 0.05$ using one-way ANOVA.

3.3 IL-6 expression upon overexpression of *Smyd1* or *PML*

The pCMV2-Smyd1-Flag or pEGFP-C1-PML-IV expression plasmids were used for transfection of EA.hy926 cells to investigate the potential role of these genes on the expression of IL-6 as compared to the transfection of the respective empty vector (control). Results of RT-qPCR showed a significant increase of *IL-6* mRNA after overexpression of either Smyd1 or PML (figure 3 A). These results were confirmed on the protein level by immunoblotting and ELISA. Along the same line, ELISA results (figure 3 B) showed significantly increased levels of IL-6 in supernatants collected 24 h after overexpression of PML or Smyd1 compared to empty vector-treated controls. In accordance with the previous data, western blotting, performed after 24 h of transfection, revealed a notably upregulation of IL-6 through either Smyd1 or PML relative to control samples transfected with the empty vector (figure 3 C).

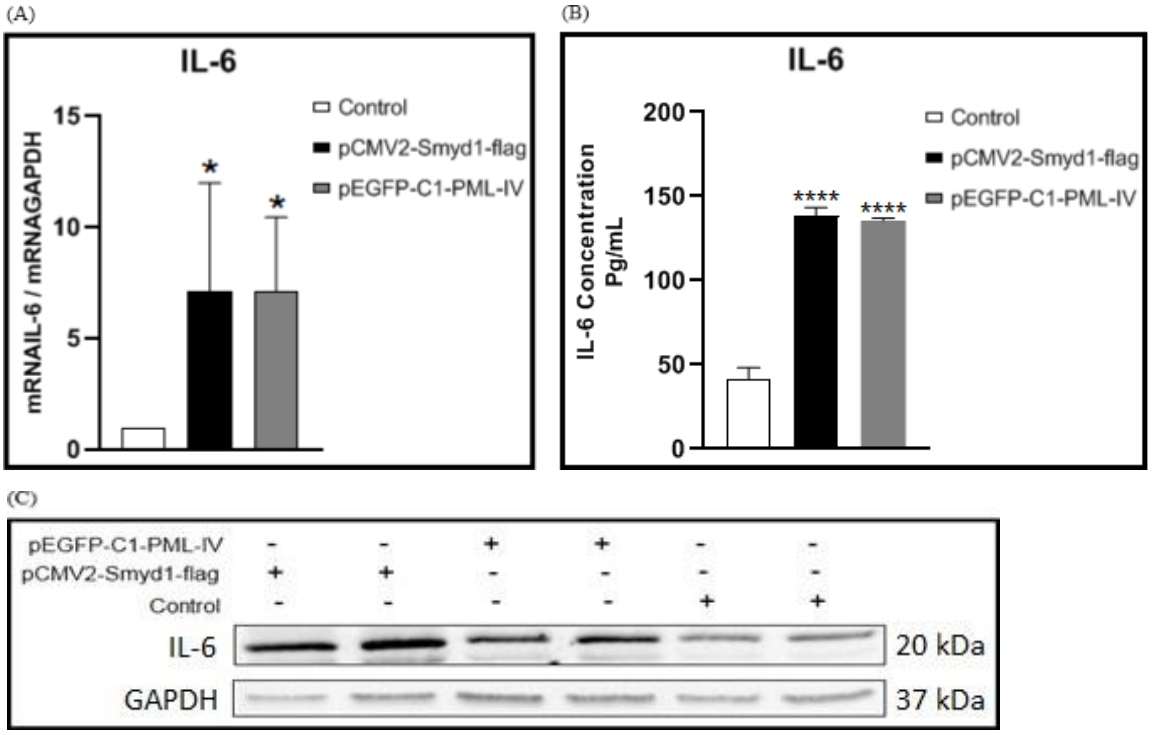

Figure 3) Effect of *Smyd1 or PML* overexpression on IL-6. EA.hy926 cells were analyzed for IL-6 expression after 24 h from transfection with the expression plasmids encoding *Smyd1* [109] *and PML or the empty vector* (control). **(A)** RT-qPCR showing the mRNA expression level of *IL-6* which was normalized to the respective empty vector-treated control cells. Data are shown as mean ± SEM, (n= 5),*p ≤ 0.05 using one-way ANOVA. **(B)** Bar chart showing the mean concentration of IL-6 in the supernatant from pCMV2-Smyd1-flag-, pEGFP-C1-PML-IV- and empty vector-transfected cells using a specific ELISA kit. Data are expressed as mean ± SEM, (n=4), ****p≤0.0001 using one-way ANOVA. **(C)** Immunoblot of IL-6 from whole lysates of Smyd1- and PML-overexpressing EA.hy926 and empty vector-treated cells. Equal loading was confirmed by immunoblotting of the housekeeping gene GAPDH.

3.4 IL-1 and IL-8 expression upon overexpression of *Smyd1 or PML*

Next to verification of their role in IL-6 production, we also studied the influence of Smyd1 and PML on the expression of *IL-1 and IL-8*. Figure 4 (A) shows that the mRNA levels of *IL-1* were significantly elevated upon the overexpression of *Smyd1 and PML*, however, in figure 4 (B) *IL-8* was only significantly upregulated with *Smyd1* overexpression. These results support, at least, the possible involvement of Smyd1 in the provoked inflammatory mediated cytokine response in ECs.

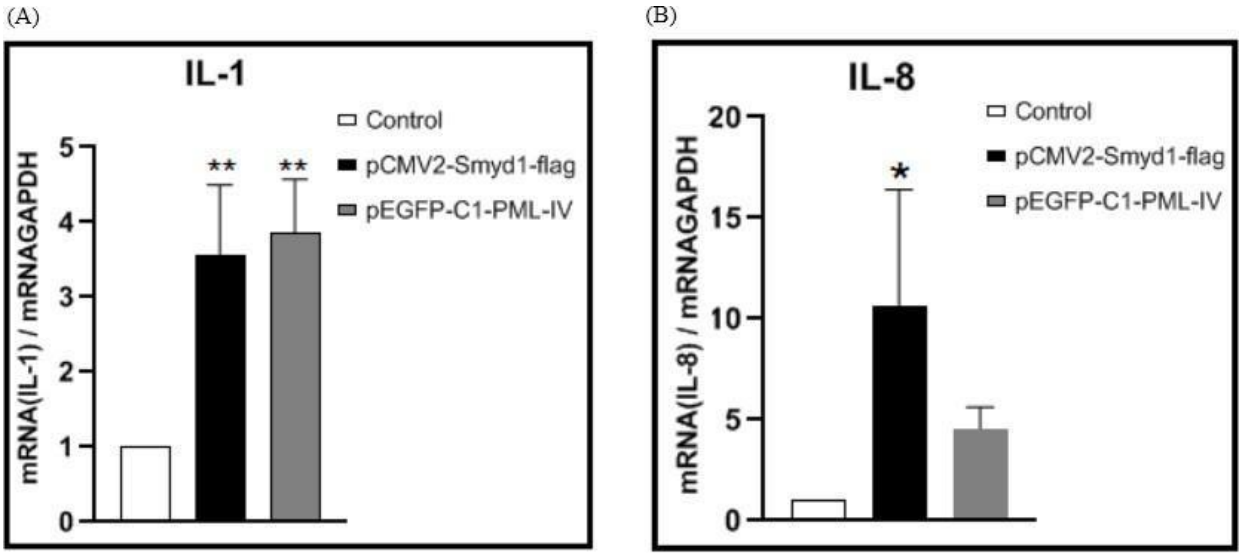

Figure 4) Effect of *Smyd1 or PML* overexpression on *IL-1 and IL-8*. Quantification of the mRNA expression levels of (A) *IL-1 and* (B) *IL-8* in EA.hy926 cells was determined using RT-qPCR after 24 h of transfection with *Smyd1 and PML* expressing plasmids an related to transfection of the empty vector (controls). Data are shown in (A) and (B) as mean ± SEM, (n=3),*P ≤ 0.05 and **p≤0.01 using one-way ANOVA.

Due to limited resources, we could not assess the protein levels of IL-1 and IL-8. Further studies are for sure required to confirm and explain the inductive link between Smyd1 or PML and these cytokines.

3.5 Smyd1 and PML expression upon treatment of EA.hy926 cells with IL-6

To determine the presence of a possible feedback between IL-6 and Smyd1 or PML, the effect of IL-6 (10 ng/mL, final concentration) stimulation on the expression of Smyd1 and PML in EA.hy926 was tested at two time points (3 h and 24 h). RT-qPCR results indicated that IL-6 had no significant impact on *Smyd1* (figure 5 A), however it exerted strong upregulation of *PML* mRNA

which occurred early and transiently after 3 h of stimulation (figure 5 B). With regard to the protein level, immunoblotting revealed also no change in Smyd1 level (figure 5 C), while PML was increased compared to unstimulated controls after 3 h and 24 h of IL-6 stimulation (figure 5 D).

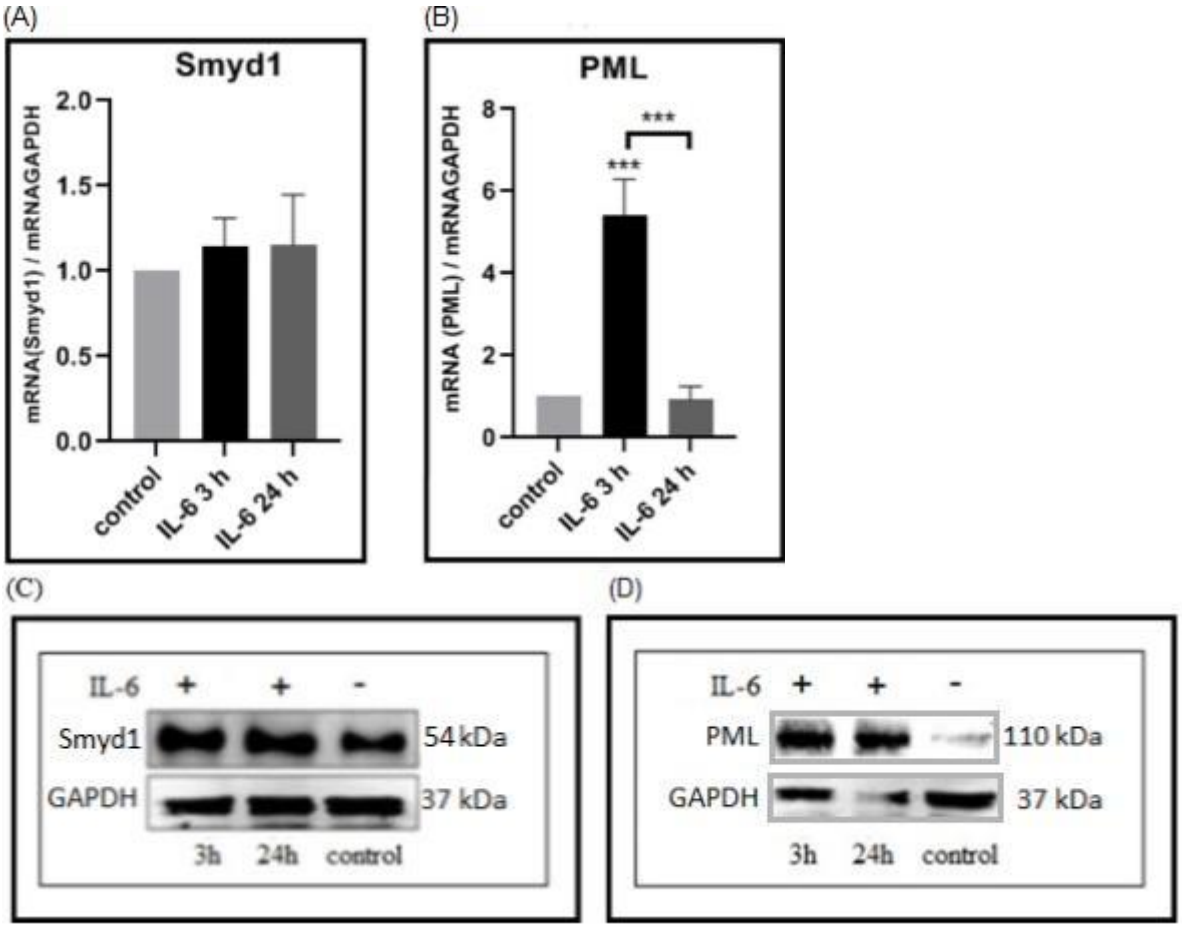

Figure 5) Effect of IL-6 stimulation on Smyd1 and PML. EA.hy926 cells were either treated with vehicle (control) or stimulated with IL-6 (10 ng/mL) for 3 h and 24 h. **(A)** Transcription levels of **(A)** *Smyd1* [109] *and* **(B)** *PML* were analyzed 3 h and 24 h after IL-6 stimulation using RT-qPCR. The mRNA expression levels were normalized to the vehicle-treated controls. Data are shown in (A) and (B) as mean ± SEM, (n=3), ***p≤0.001 using one-way ANOVA. Western blotting of **(C)** Smyd1 [109] and **(D)** PML after 3 and 24 hof stimulation of EA.hy926 cells with IL-6 in contrast to vehicle-treated controls. Equal loading was confirmed by immunoblotting of the housekeeping gene GAPDH.

3.6 NF-κB expression upon overexpression of *Smyd1*

We tried to figure out the role of Smyd1 on the expression level of NF-κB which is a main activator of the transcription of the *IL-6* gene and other cytokines during inflammation and sepsis (72). It was found through FACS analysis that cells transfected with pCMV2-Smyd1-Flag showed a significant increase of NF-κB when correlated to control cells treated with the empty vector (figure 6).

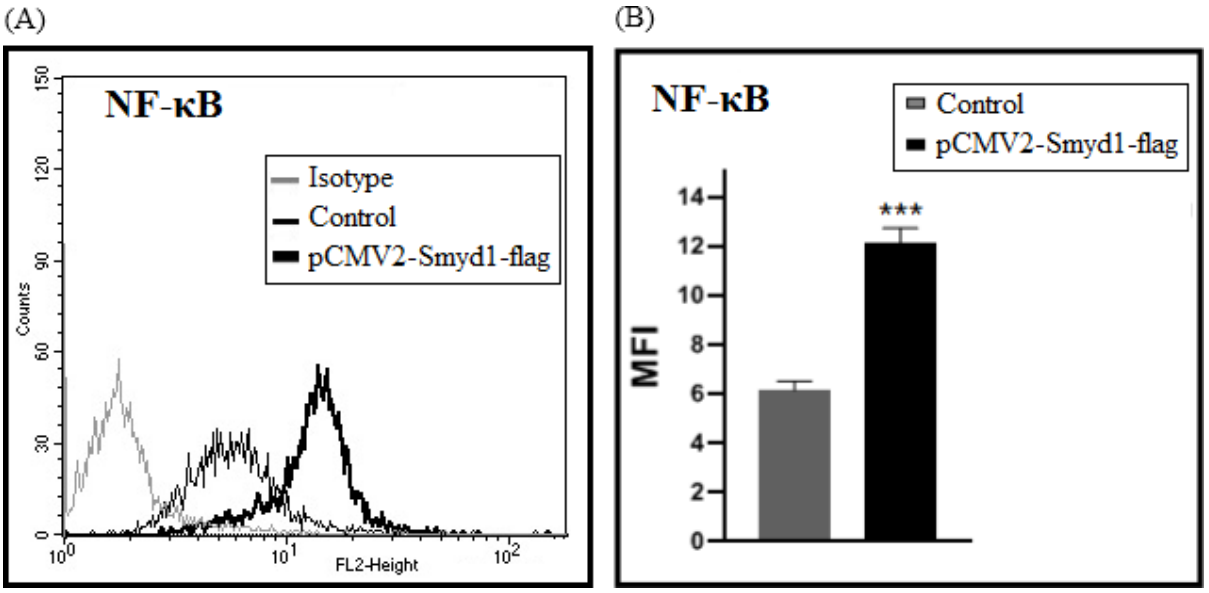

Figure 6) Effect of *Smyd1* overexpression on NF-κB. EA.hy926 cells were analyzed 24 h following transfection either with empty vector (control) or pCMV2-Smyd1-Flag. **(A)** Histogram shows FACS analysis of NF-κB expression after staining with anti-NF-κB antibody compared with cells stained with isotype matched non-binding control antibodies [109]. **(B)** MFI of NF-κB in cells which were transfected either with pCMV2-Smyd1-Flag or with the empty vector (control). The data are shown as mean ± SEM, n= 3, ***p≤0.001 using unpaired t-test.

3.7 NF-κB expression upon overexpression of *PML*

NF-κB was assessed in EA.hy926 cells using FACS 24 h after transfection with the PML expressing vector. The results showed a significant increase of NF-κB level compared to empty vector-transfected control samples (figure 7).

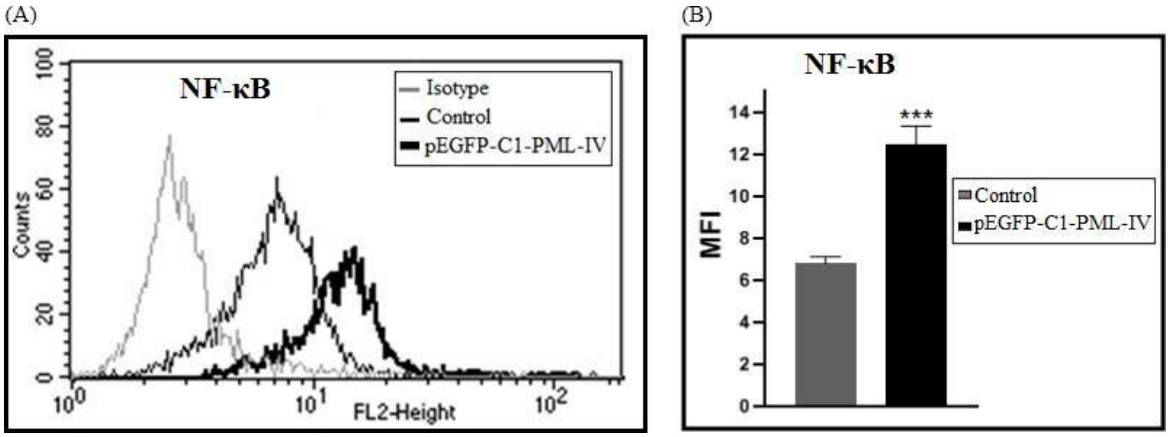

Figure 7) Effect of *PML* overexpression on NF-κB. EA.hy926 cells were analyzed 24 h following transfection either with empty vector (control) or pEGFP-C1-PMLIV. (**A**) Representative FACS histogram shows NF-κB expression after staining with anti-NF-κB antibody compared with cells stained with isotype matched non-binding control antibodies. (**B**) MFI of NF-κB in cells which were transfected either with pEGFP-C1-PMLIV or just with the empty vector (control). The data are shown as mean ± SEM, n= 3, ***p≤0.001 using unpaired t-test.

3.8 IκBα expression upon overexpression of *PML and Smyd1*

Western blotting was done to investigate IκBα, the NF-κB inhibitory protein. In agreement with the previous results obtained by FACS, the immunoblotting showed that, in opposition to empty vector treated control cells, IκBα was reduced in EA.hy926 upon overexpression of *PML* and *Smyd1* (figure 8).

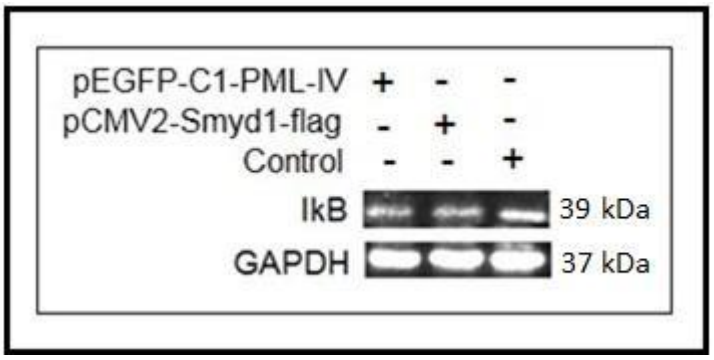

Figure 8) Effect of *Smyd1 and PML* overexpression on IκBα. Immunoblot showing the expression of IκBα 24 h after transfection of EA.hy926 cells by Smyd1 or PML expressing vectors compared to the control empty vector-treated cells. Equal loading was confirmed by immunoblotting of the housekeeping gene GAPDH [109].

3.9 Involvement of NF-κB in Smyd1-dependent IL-6 expression

In order to study a putative role of NF-κB as a possible intermediate in Smyd1-dependent IL-6 upregulation, RT-qPCR and ELISA analysis were performed to detect the effect of Smyd1 with or without pyrrolidine dithiocarbamate (PDTC), an inhibitor of NF-κB, on IL-6. The results negated the entire dependency of Smyd1 on NF-κB in its IL-6 expression promoting action. As shown in figure 9, meanwhile a lower level of expression and secretion of IL-6 was noticed in PDTC-treated samples after transfected with pCMV2-Smyd1-Flag than in transfected untreated samples, yet it was higher in correlation to those treated with PDTC alone.

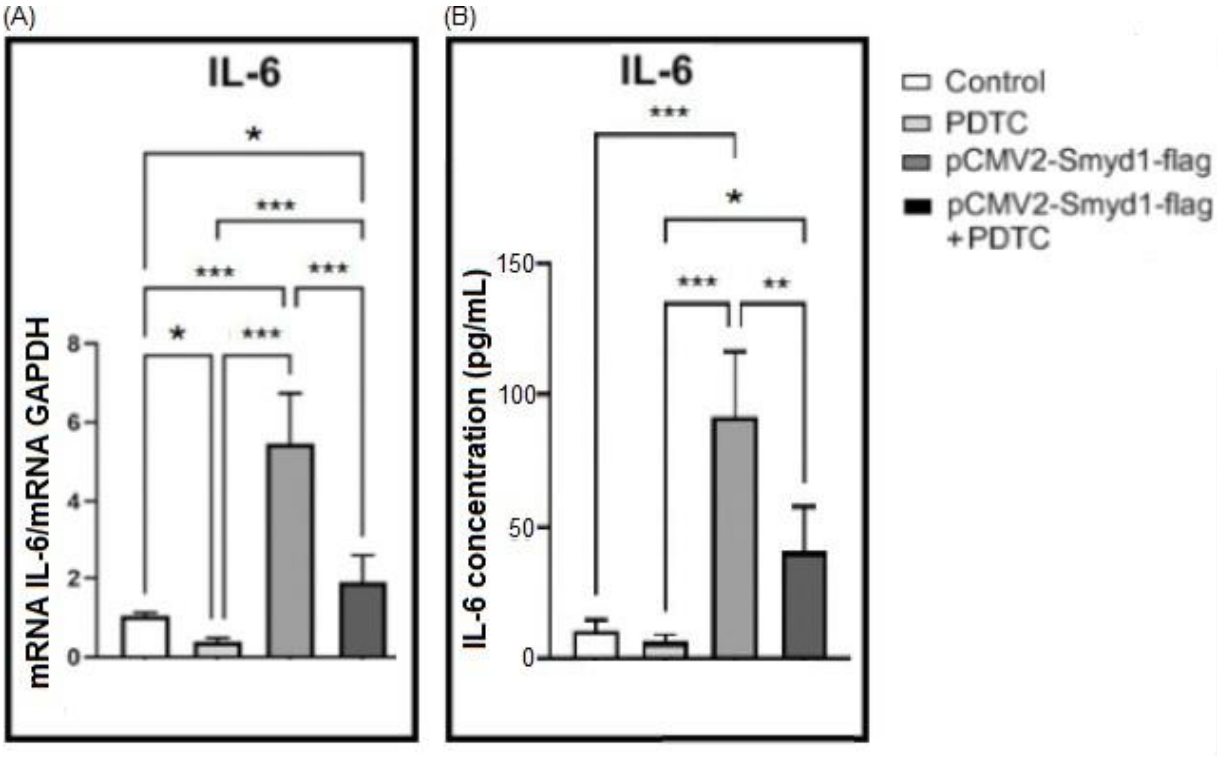

Figure 9) Evaluation of NF-κB role in Smyd1-induced *IL-6* upregulation. **(A)** Bar chart shows RT-qPCR quantification of IL-6 transcriptional level in EA.hy926 cells after 24 h from treatment with either PDTC (10 μM) or Smyd1 expressing plasmid or both. IL-6 expression level was normalized relative to the empty vector-treated control cells. Data are shown as mean ± SD, (n=3), *$p \leq 0.05$ and ***$p \leq 0.001$ using two-way ANOVA [109]. **(B)** Bar chart showing the mean concentration of IL-6 in the supernatants collected from EA.hy926 cells after 24 h of treatment with either PDTC (10 μM) or Smyd1 expressing plasmid or both using a specific ELISA kit. The level of IL-6 secretion was normalized relative to the empty vector-treated control cells. Data are expressed as mean ± SD, (n=3), *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$ using two-way ANOVA [109].

Discussion:

Although sepsis has been addressed by many studies and approaches, it is still representing a fatal condition that leads to multi-organ failure and is considered to be one of the 10 most frequent causes of death (1). The gram-negative bacteria are very frequently involved in the pathogenesis of sepsis or septic shock (88). The endothelial cell lining has a central role in the immunological response being one of the earliest cells which detect extrinsic microbial pathogens and react to them (89). The release of LPS from the outer membrane of gram-negative bacteria into the circulation activates the ECs during sepsis (69). The ECs, in turn, stimulate the innate immunity through secretion and induction of signaling pathways for different pro-inflammatory cytokines (as IL-1, IL-6, and IL-8) which, consequently, augment the recruitment of other immune cells to the inflammation spots leading to endothelial cell dysfunction (79, 90).

In the present study, we simulated the sepsis status, *in vitro*, by treating EA.hy926 cells with LPS and studied its effect on Smyd1 and PML. Then we investigated the possible involvement of Smyd1 and PML in the sepsis-mediated immune response of ECs through regulation of inflammatory pathways and mediators.

4.1. Evidence of increased expression of Smyd1 and PML during sepsis

While most of studies on Smyd1 were focused upon its restriction in cardiac or skeletal muscle (28, 32, 35, 91-93), a recent study denoted a novel role for Smyd1 in migration of ECs and angiogenesis (34). It is already established that both inflammation and angiogenesis are linked together; the immune cells produce pro-angiogenic elements necessary for an increase in vascularization by the outgrowth of capillary sprouts during inflammatory processes (94). Therefore, here in our study, we examined the potential function of Smyd1 in ECs during sepsis. The expression of Smyd1 in EA.hy926 was found to be increased, on the mRNA and protein levels, at 3 h and 24 h post LPS stimulation. These results resembled former observations about the fifth member of Smyd family (Smyd5) whose expression increased significantly in macrophages after LPS stimulation (43). The expression level after 3 h was significantly higher than after 24 h. Similar time-dependent changes of Smyd1 upreulation was denoted after incubation of ECs with INF-γ for 3 h and 24 h (95). We also consider our data to be assuring of previous findings regarding the expression of Smyd1 in ECs and its involvement in angiogenesis (34).

Regarding PML, our results indicated its upregulation on mRNA and protein levels in LPS challenged EA.hy926 after stimulation for 3 h and 24 h. These data agree with those obtained by Yu-Hsun et al. who showed that LPS strongly increases PML in macrophages on the transcript and protein levels (64). Also, our results may indirectly support previous observations of increased resistance to sepsis in $PML^{-/-}$ mice (63). However, until the entire interaction network of PML has been examined quantitatively and over time, it is still too early to draw such far-reaching conclusions. Taken together, our data potentiate the evidence that PML in ECs is implicated in the inflammatory processes and the correlated immune response.

4.2. Upregulation of inflammatory cytokines upon overexpression of Smyd1 or PML

ECs are main stations to which different cytokine signals originating from immune cells are delivered to mediate inflammation (96). IL-6, IL-8 and IL-1 are exceptionally important cytokines due to their effective pro-inflammatory and chemotactic activities during the progress of vascular inflammation (78, 79, 90). In the present study, we showed that overexpression of both Smyd1 and PML strongly increased IL-1 and IL-6 on the transcriptional level in ECs, while IL-8 was significantly increased only by Smyd1. Among the three cytokines tested, IL-8 is a chemokine, especially for neutrophils. Regarding the internal validity of this study, this means that at least the observed effect of Smyd1 on IL-8 is even more likely to be specific. In a broader, speculative context, it could be an indication that Smyd1 and PML are important for either different types of inflammatory reactions or for different time phases of inflammatory reactions. A function of Smyd1 could lie in an early inflammatory phase, in which the recruitment of neutrophils is crucial.

We proceeded then with investigating the effect of Smyd1 as well as PML on the expression of IL-6. Confirming of the RT-qPCR results, western blot analysis showed upregulation of IL-6 on the protein level upon overexpression of Smyd1 or likewise PML. Similarly, ELISA revealed an increased concentration of IL-6 in the collected cell culture supernatants after transfection by eitherof Smyd1 or PML expressing plasmids. The various measurements thus consistently showed increased expression, translation, and secretion of IL-6 due to the overexpression of either Smyd1 or PML-IV. The signal path is of course not yet sufficiently clear at this point.

However, Smyd1 or PML-IV may be enough to start the signaling pathway, as upstream, downstream, or independent of NFκB.

At least we can postulate here a new role for Smyd1 in terms of its possible contribution to the regulation of cytokine mediated immune responses during inflammation and sepsis. With respect to PML, our findings confirmed previous studies which indicated PML-dependent induction of IL-6 expression in mouse embryonic fibroblasts (MEFs) (97) and also in plasma cells collected frompatients with multiple myloma (98). Furthermore, it was found by Lunardi et al. that IL-6 production was strongly reduced in PML knockout mice with subsequent resistance to LPS stimulation, which additionally supports our results (63). However, our data do not agree with Reibet et al. whofound no significant difference in the expression of IL-6 with overexpression or knockout of PMLin MEFs following infection with gram-positive Listeria bacterial pathogens (100). Regarding systemic defense reactions, it should be kept in mind, that in contrast to IL-8, which acts preferentially on neutrophils,IL-6 supports B and T lymphocytes in particular.

Our data are consistent concerning IL-1, but not regarding IL-8, with a previous study which reported the upregulation of IL-1β and IL-8 in PML-transfected human mesenchymal stem cells (99). The results with respect to IL-1 were verified by a recent study in 2019 (64) which showed a decrease in IL-1β production in PML deficient macrophages. On the other hand, another study, by Fausti et al., reported an upregulation of IL-8 after PML knockdown in human colorectal carcinoma cell line (101). Most likely, these differences might be due to the different cell types and/or different time points used to analyze PML-dependent effects in the cited studies.

4.3. Positive feedback of IL-6 on PML but not on Smyd1

We next examined whether there is a feedback from IL-6 on the expression of Smyd1 and PML in ECs. To do this, we incubated the endothelial cells with IL-6 and analyzed them for Smyd1 and PML. Interestingly, we found that medium supplemented with IL-6 exerted almost no effect on Smyd1 expression or secretion in EA.hy926 cells while, on the contrary, it boosted the PML level remarkably compared to the control cells. As for PML, similar findings were settled by Sona Hubackova, et al. who reported that supplementation of culture media with IL-6 induced PML and the number of PML-NBs in HeLa (cervix carcinoma) and U2OS (osteaosarcoma)

cell lines through induction of the Akt/NF-κB pathway as well as activation of Janus kinase/signal transducer and activator of transcription (JAK/STAT3) signaling and binding of STAT3 to the PML regulatory region. Furthermore, they showed that diminution of IL-6 in culture medium or knock-down of STAT3 leads to a reduction of PML and PML-NBs (102). This is further supported by for instance Kato et al. who used IL-6 to activate STAT3 (103), which in turn has been shown to increase PML (104).

Altogether, the presented data in agreement with the literature reflect the presence of a positive feedback loop between IL-6 and PML which would steadily augment the cellular response to existent inflammatory stimuli. Nevertheless, Norihiko Ohbayashi et al. reported conflicting effects; they showed that IL-6 causes desumoylation with further inactivation of PML (105) whichwas shown in previous literature to be a suppressor of STAT3 activation (103, 106, 107). However,it seems reasonable to propose that both results could be complementary considering that IL-6 induces the expression of PML via the PI3K/Akt/NFkB- and JAK/STAT3-pathway during sepsis or inflammation until a certain limit at which PML itself stops such activation in a negative feedback mechanism. If those inflammatory conditions persist or reoccur, IL-6 will deSUMOylate PML and thus prohibit its counteraction switching JAK/ STAT3 signaling on again.

4.4. Smyd1 and PML possibly stimulate IL-6 by promoting NF-κB expression

Since it is well established that IL-6 expression is directly regulated by NF-κB (72, 108), we decided to investigate the effect of transfection with Smyd1- or PML-expressing plasmids on NF-κB expression levels in EA.hy926 cells in order to be able to build up a possible persuasive frame explaining how Smyd1 works during inflammation on one hand and to confirm the previously described observations about the effect of PML on NF-κB, however here in ECs, on the other hand. After performing three independent experiments to determine the NF-κB protein level using FACS, we denoted an upregulatory role of both Smyd1 and PML on NF-κB expression. This effect was checked out by assessing IκBα expression in ECs upon the overexpression of either Smyd1 or PML. Our results showed that IκBα was downregulated in both cases explaining the stimulatory impact of Smyd1 and PML on NF-κB after releasing it from the inhibitory domination elicited by IκBα.

Thus, we uncovered another role of Smyd1 during sepsis being an amplificator of the NF-κB-pathway. One possible assumption would be that Smyd1 in sepsis, induced by LPS-stimulated pathways, contributes to the inactivation of IkB and thus supports the production of IL-6. Against this assumption, we found that the IL-6 level remained significantly increased in Smyd1 plasmid-transfected EA.hy926 cells even with repressing NF-κB activity by PDTC. Therefore we excluded the whole attribution of the stimulatory effect of Smyd1 on IL-6 only to NF-κB. In our recently published study (109) we confirmed a dominant parallel stimulatory mechanism of Smyd1 on IL-6 expression and secretion depends on its enzymatic activity as an epigenetic modulator of gene expression through enhanced methylation of H3K4me3 (data are not shown).

Regarding the experiments studying the effect of PML on NF-κB expression, our data were consistent with a recent study which showed that PML has a key role in enhancing NF-κB transcriptional activity and affirmed that by denoting negative regulation of NF-κB in $PML^{-/-}$ MEFs and macrophages after LPS stimulation (97). Also Lunardi et al. showed in their study that $PML^{-/-}$ mice were resistant to LPS triggered septic shock due to improper activation of the Toll-like receptor (TLR)/NF-κB signaling pathway, thus hindering the production of the dependent cytokines (63). However, other previous studies implied that PML adversely affects NF-κB expression in different cells (like bone marrow-derived macrophages and U2OS through binding of PML with NF-κB and hindering its ability to bind its specific target (64, 110). The explanation of such disagreement between results is difficult but could be attributed to different experimental conditions (*in vivo or in vitro*) and cell types.

4.5. Conclusion and future work

Many researches focus nowadays on the defining and understanding of sepsis and its underlying mechanisms to establish better treatment plans that can prevent or minimize organs dysfunction associated with sepsis. In this study, it was revealed for the first time that LPS-induced sepsis increases the expression of Smyd1 in ECs. Furthermore, we could successfully identify a novel role of Smys1 in stimulation of IL-6 and NF-κB expression. Our study also confirmed data from previous studies about the engagement of PML in the inflammatory process and sepsis, however, according to our knowledge, this is the first study performed on ECs regarding the influence of LPS on PML and the upregulatory effect of PML on IL-6 and NF-κB. In addition, we denoted a positive feedbackloop between IL-6 and PML which potentiates the key role played by PML in the sepsis-mediated immune response by ECs.

Finally, and totally unexpected, two negative results of the study were of particular importance for our developing understanding of the systemic relationships. This is, that PML did not induce IL-8, and that IL-6 did not induce Smyd1. This could represent a time path in the interplay between Smyd1 and PML during acute inflammation such as sepsis. Based on and at the end of the study, the following heuristic model is presented:

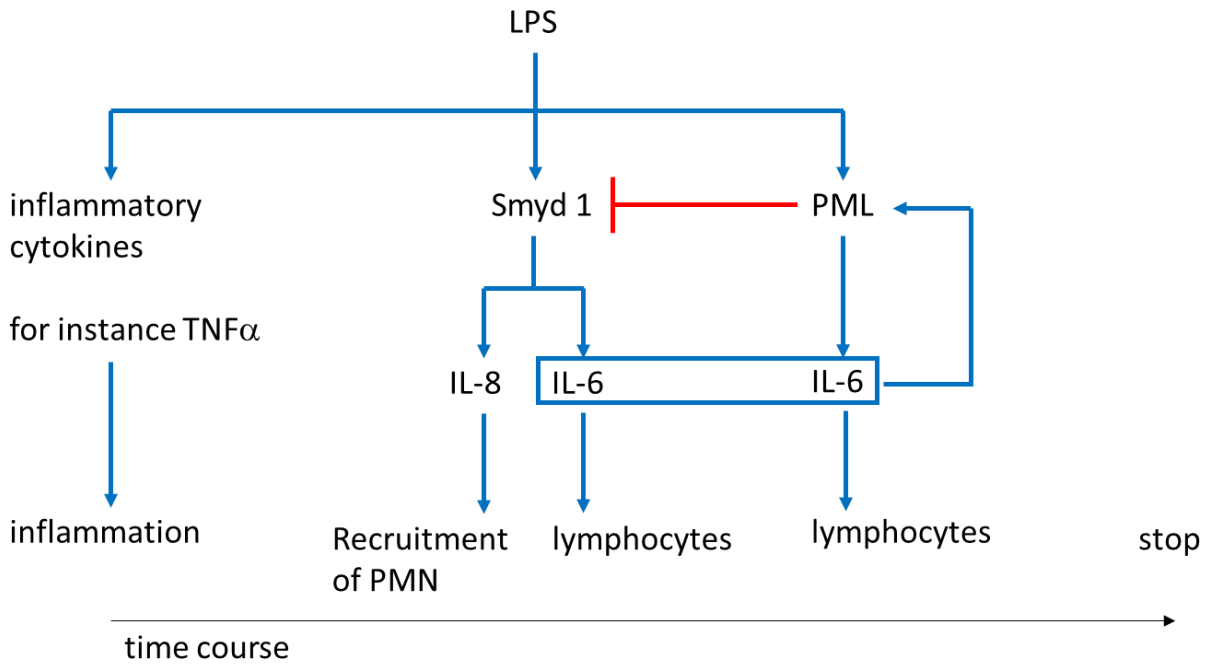

Figure 10) Heuristic model of the interplay between Smyd1 and PML during acute inflammation. *For details please refer to the text.*

Without considering the signaling pathways discussed above in detail, we assume that LPS induces inflammatory cytokines also and initially independently of Smyd1 and PML. Even if Smyd1 and PML should also be induced immediately after the binding of LPS to TLR4, their own effect on cytokine expression will only be delayed due to longer signal and reaction pathways. At this point in time, the production of IL-8 and IL-6 in particular increases. This may especially promote the recruitment of neutrophilic granulocytes (PMN). Since IL-6 induces PML, an amplification phase of IL-6 production now follows. The further increase in PML leads to increased SUMOylation of Smyd1, whereupon Smyd1 is degraded proteasomally, as was recently shown in our group (95). This leads to a predominance of the PML effect and, in the case of the cytokines, to a shift in the functional balance from IL-8 to IL-6. At the level of the immune cells, the support of the adaptive

defense by the lymphocytes takes the place of the PMN. Finally, the disruption of the STAT3 pathway by PML may help to stop the inflammatory response.

Although this model is very simplistic, it indicates that relevant statements can only be derived if time courses are examined much more closely. In addition, even smaller changes in concentrations of the respective reaction partners would have to be investigated. Only then could biological model calculations be carried out in a meaningful way.

This thesis represents just a small piece of puzzle in the research area of the innate immune signaling during sepsis. Further studies should follow to assure our results, however, on primary ECs specially regarding the role of Smyd1 in inflammation and sepsis which is still illusive and needs to be well clarified.

Reference List:

1. Plevin R, Callcut R. Update in sepsis guidelines: what is really new? Trauma Surg Acute Care Open. 2017;2(1):e000088.
2. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Marshall JC, Martin GS, Opal SM, Rubenfeld GD, van der Poll T, Vincent JL, Angus DC. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016 Feb 23;315(8):801-10. doi: 10.1001/jama.2016.0287. PMID: 26903338; PMCID: PMC4968574. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315(8):801-10.
3. Fleischmann C, Scherag A, Adhikari NK, Hartog CS, Tsaganos T, Schlattmann P, Angus DC, Reinhart K; International Forum of Acute Care Trialists. Assessment of Global Incidence and Mortality of Hospital-treated Sepsis. Current Estimates and Limitations. Am J Respir Crit Care Med. 2016;193(3):259-72.
4. Shankar-Hari M, Phillips GS, Levy ML, Seymour CW, Liu VX, Deutschman CS, Angus DC, Rubenfeld GD, Singer M; Sepsis Definitions Task Force. Developing a New Definition and Assessing New Clinical Criteria for Septic Shock: For the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). JAMA. 2016;315(8):775-87.
5. Liu V, Escobar GJ, Greene JD, Soule J, Whippy A, Angus DC, Iwashyna TJ. Hospital deaths in patients with sepsis from 2 independent cohorts. JAMA. 2014;312(1):90-2.
6. Kissoon N, Carcillo JA, Espinosa V, Argent A, Devictor D, Madden M, Singhi S, van der Voort E, Latour J; Global Sepsis Initiative Vanguard Center Contributors. World Federation of Pediatric Intensive Care and Critical Care Societies: Global Sepsis Initiative. Pediatr Crit Care Med. 2011;12(5):494-503.
7. Hall MJ, Williams SN, DeFrances CJ, Golosinskiy A. Inpatient care for septicemia or sepsis: a challenge for patients and hospitals. NCHS Data Brief. 2011(62):1-8.
8. Lagu T, Rothberg MB, Shieh MS, Pekow PS, Steingrub JS, Lindenauer PK. Hospitalizations, costs, and outcomes of severe sepsis in the United States 2003 to 2007. Crit Care Med. 2012;40(3):754-61.
9. Yi L, Huang X, Guo F, Zhou Z, Chang M, Tang J, Huan J. Lipopolysaccharide Induces Human Pulmonary Micro-Vascular Endothelial Apoptosis via the YAP Signaling Pathway. Front Cell Infect Microbiol. 2016;6:133.

10. Wendel M, Paul R, Heller AR. Lipoproteins in inflammation and sepsis. II. Clinical aspects. Intensive Care Med. 2007;33(1):25-35.
11. Yuan SY. Protein kinase signaling in the modulation of microvascular permeability. Vascul Pharmacol. 2002;39(4-5):213-23.
12. Grandel U, Grimminger F. Endothelial responses to bacterial toxins in sepsis. Crit Rev Immunol. 2003;23(4):267-99.
13. Matsuda N, Teramae H, Yamamoto S, Takano K, Takano Y, Hattori Y. Increased death receptor pathway of apoptotic signaling in septic mouse aorta: effect of systemic delivery of FADD siRNA. Am J Physiol Heart Circ Physiol. 2010;298(1):H92-101.
14. Trepels T, Zeiher AM, Fichtlscherer S. The endothelium and inflammation. Endothelium. 2006;13(6):423-9.
15. Weinbaum S, Tarbell JM, Damiano ER. The structure and function of the endothelial glycocalyx layer. Annual review of biomedical engineering. 2007;9:121-67.
16. Krishnaswamy G, Kelley J, Yerra L, Smith JK, Chi DS. Human endothelium as a source of multifunctional cytokines: molecular regulation and possible role in human disease. Journal of interferon & cytokine research: the official journal of the International Society for Interferon and Cytokine Research. 1999;19(2):91-104.
17. Aird WC. Endothelial cell heterogeneity. Cold Spring Harb Perspect Med. 2012;2(1):a006429.
18. Dofferhoff AS, Nijland JH, de Vries-Hospers HG, Mulder PO, Weits J, Bom VJ. Effects of different types and combinations of antimicrobial agents on endotoxin release from gram-negative bacteria: an in-vitro and in-vivo study. Scand J Infect Dis. 1991;23(6):745- 54.
19. Evans ME, Pollack M. Effect of antibiotic class and concentration on the release of lipopolysaccharide from Escherichia coli. J Infect Dis. 1993;167(6):1336-43.
20. Mayeux PR. Pathobiology of lipopolysaccharide. J Toxicol Environ Health. 1997;51(5):415-35.
21. Yi L, Huang X, Guo F, Zhou Z, Dou Y, Huan J. Yes-associated protein (YAP) signaling regulates lipopolysaccharide-induced tissue factor expression in human endothelial cells. Surgery. 2016;159(5):1436-48.
22. Hotchkiss RS, Tinsley KW, Swanson PE, Karl IE. Endothelial cell apoptosis in sepsis. Crit Care Med. 2002;30(5 Suppl):S225-8.
23. Li Y, Zhang XS, Yu JL. Acanthoic acid inhibits LPS-induced inflammatory response by activating LXRalpha in human umbilical vein endothelial cells. Int Immunopharmacol. 2016;32:111-5.

24. Song Y, Zhao H, Liu J, Fang C, Miao R. Effects of Citral on Lipopolysaccharide- Induced Inflammation in Human Umbilical Vein Endothelial Cells. Inflammation. 2016;39(2):663- 71.
25. Weglarz L, Dzierzewicz Z, Skop B, Orchel A, Parfiniewicz B, Wiśniowska B, Swiatkowska L, Wilczok T. Desulfovibrio desulfuricans lipopolysaccharides induce endothelial cell IL-6 and IL-8 secretion and E-selectin and VCAM-1 expression. Cellular & molecular biology letters. 2003;8(4):991-1003.
26. Dayang EZ, Plantinga J, Ter Ellen B, van Meurs M, Molema G, Moser J. Identification of LPS-Activated Endothelial Subpopulations With Distinct Inflammatory Phenotypes and Regulatory Signaling Mechanisms. Frontiers in immunology. 2019;10:1169.
27. Leinhart K, Brown M. SET/MYND Lysine Methyltransferases Regulate Gene Transcription and Protein Activity. Genes (Basel). 2011;2(1):210-8.
28. Gottlieb PD, Pierce SA, Sims RJ, Yamagishi H, Weihe EK, Harriss JV, Maika SD, Kuziel WA, King HL, Olson EN, Nakagawa O, Srivastava D. Bop encodesa muscle-restricted protein containing MYND and SET domains and is essential for cardiacdifferentiation and morphogenesis. Nature genetics. 2002;31(1):25-32.
29. Kawamura S, Yoshigai E, Kuhara S, Tashiro K. smyd1 and smyd2 are expressed in muscle tissue in Xenopus laevis. Cytotechnology. 2008;57(2):161-8.
30. Li D, Niu Z, Yu W, Qian Y, Wang Q, Li Q, Yi Z, Luo J, Wu X, Wang Y, Schwartz RJ, Liu M. SMYD1, the myogenic activator, is a direct target of serum response factor and myogenin. Nucleic acids research. 2009;37(21):7059-71.
31. Olson EN. Gene regulatory networks in the evolution and development of the heart. Science. 2006;313(5795):1922-7.
32. Tan X, Rotllant J, Li H, De Deyne P, Du SJ. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. Proceedings of the National Academy of Sciences of the United States of America. 2006;103(8):2713-8.
33. Shilatifard A. Chromatin modifications by methylation and ubiquitination: implications in the regulation of gene expression. Annual review of biochemistry. 2006;75:243-69.
34. Ye X, Qian Y, Wang Q, Yuan W, Mo X, Li Y, Jiang Z, Xu W, Deng Y, Wan Y, Fan X, Wu X, Wang Y. SMYD1, an SRF-Interacting Partner,Is Involved in Angiogenesis. PloS one. 2016;11(1):e0146468.
35. Just S, Meder B, Berger IM, Etard C, Trano N, Patzel E, Hassel D, Marquart S, Dahme T, Vogel B, Fishman MC, Katus HA, Strähle U, Rottbauer W. The myosin-interacting protein SMYD1 is essential for sarcomere organization. Journal of cell science. 2011;124(Pt 18):3127-36.

36. Du SJ, Tan X, Zhang J. SMYD proteins: key regulators in skeletal and cardiac muscle development and function. Anatomical record. 2014;297(9):1650-62.
37. Sims RJ, 3rd, Weihe EK, Zhu L, O'Malley S, Harriss JV, Gottlieb PD. m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart- and muscle-specific transcription factor. The Journal of biological chemistry. 2002;277(29):26524-9.
38. Sirinupong N, Brunzelle J, Ye J, Pirzada A, Nico L, Yang Z. Crystal structure of cardiac-specific histone methyltransferase SmyD1 reveals unusual active site architecture. The Journal of biological chemistry. 2010;285(52):40635-44.
39. Brown MA, Sims RJ, 3rd, Gottlieb PD, Tucker PW. Identification and characterization of Smyd2: a split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase that interacts with the Sin3 histone deacetylase complex. Molecular cancer. 2006;5:26.
40. Stender JD, Pascual G, Liu W, Kaikkonen MU, Do K, Spann NJ, Boutros M, Perrimon N, Rosenfeld MG, Glass CK. Control of proinflammatory gene programs by regulated trimethylation and demethylation of histoneH4K20. Molecular cell. 2012;48(1):28-38.
41. Nagata DE, Ting HA, Cavassani KA, Schaller MA, Mukherjee S, Ptaschinski C, Kunkel SL, Lukacs NW. Epigenetic control of Foxp3 by SMYD3 H3K4 histone methyltransferase controls iTreg development and regulates pathogenic T-cell responses during pulmonary viral infection. Mucosal immunology. 2015;8(5):1131-43.
42. Hwang I, Gottlieb PD. Bop: a new T-cell-restricted gene located upstream of and opposite to mouse CD8b. Immunogenetics. 1995;42(5):353-61.
43. Doughan M, Spellmon N, Li C, Yang Z. SMYD proteins in immunity: dawning of a new era. AIMS biophysics. 2016;3(4):450-5.
44. Hradetzky S, Roesner LM, Balaji H, Heratizadeh A, Mittermann I, Valenta R, Werfel T. Cytokine effects induced by the human autoallergen alpha-NAC. The Journal of investigative dermatology. 2014;134(6):1570-8.
45. Berkholz J, Orgeur M, Stricker S, Munz B. skNAC and Smyd1 in transcriptional control. Experimental cell research. 2015;336(2):182-91.
46. Lutterbach B, Westendorf JJ, Linggi B, Patten A, Moniwa M, Davie JR, Huynh KD, Bardwell VJ, Lavinsky RM, Rosenfeld MG, Glass C, Seto E, Hiebert SW. ETO, a targetof t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors. Molecularand cellular biology. 1998;18(12):7176-84.

47. Park CY, Pierce SA, von Drehle M, Ivey KN, Morgan JA, Blau HM, Srivastava D. skNAC, a Smyd1-interacting transcription factor, is involved in cardiac development and skeletal muscle growth and regeneration. Proc Natl Acad Sci USA 2010;107(48):20750-5.

48. Berkholz J, Michalick L, Munz B. The E3 SUMO ligase Nse2 regulates sumoylation and nuclear-to-cytoplasmic translocation of skNAC-Smyd1 in myogenesis. Journal of cell science. 2014;127(Pt 17):3794-804.

49. Nisole S, Maroui MA, Mascle XH, Aubry M, Chelbi-Alix MK. Differential Roles of PML Isoforms. Frontiers in oncology. 2013;3:125.

50. Hsu KS, Kao HY. PML: Regulation and multifaceted function beyond tumor suppression. Cell & bioscience. 2018;8:5.

51. Bernardi R, Pandolfi PP. Structure, dynamics and functions of promyelocytic leukaemia nuclear bodies. Nature reviews Molecular cell biology. 2007;8(12):1006-16.

52. Dellaire G, Bazett-Jones DP. PML nuclear bodies: dynamic sensors of DNA damage and cellular stress. BioEssays: news and reviews in molecular, cellular and developmental biology. 2004;26(9):963-77.

53. Gurrieri C, Capodieci P, Bernardi R, Scaglioni PP, Nafa K, Rush LJ, Verbel DA, Cordon-Cardo C, Pandolfi PP. Loss of the tumor suppressor PML in human cancers of multiple histologic origins. Journal of the National Cancer Institute. 2004;96(4):269-79.

54. Lallemand-Breitenbach V, de The H. PML nuclear bodies. Cold Spring Harbor perspectives in biology. 2010;2(5):a000661.

55. Lapi E, Di Agostino S, Donzelli S, Gal H, Domany E, Rechavi G, Pandolfi PP, Givol D, Strano S, Lu X, Blandino G. PML, YAP, and p73 are components of a proapoptotic autoregulatory feedback loop. Molecular cell. 2008;32(6):803-14.

56. Van Damme E, Laukens K, Dang TH, Van Ostade X. A manually curated network of the PML nuclear body interactome reveals an important role for PML-NBs in SUMOylation dynamics. International journal of biological sciences. 2010;6(1):51-67.

57. Gamell C, Jan Paul P, Haupt Y, Haupt S. PML tumour suppression and beyond: therapeutic implications. FEBS letters. 2014;588(16):2653-62.

58. Sahin U, Lallemand-Breitenbach V, de The H. PML nuclear bodies: regulation, function and therapeutic perspectives. The Journal of pathology. 2014;234(3):289-91.

59. Miki T, Xu Z, Chen-Goodspeed M, Liu M, Van Oort-Jansen A, Rea MA, Zhao Z, Lee CC, Chang KS. PML regulates PER2 nuclear localization and circadian function. The EMBO journal. 2012;31(6):1427-39.

60. Ding W, Tong Y, Zhang X, Pan M, Chen S. Study of Arsenic Sulfide in Solid Tumor Cells Reveals Regulation of Nuclear Factors of Activated T-cells by PML and p53. Scientific reports. 2016;6:19793.

61. Wang ZG, Delva L, Gaboli M, Rivi R, Giorgio M, Cordon-Cardo C, Grosveld F, Pandolfi PP. Role of PML in cell growth and the retinoic acid pathway. Science. 1998;279(5356):1547-51.

62. Rego EM, Wang ZG, Peruzzi D, He LZ, Cordon-Cardo C, Pandolfi PP. Role of promyelocytic leukemia (PML) protein in tumor suppression. The Journal of experimental medicine. 2001;193(4):521-29.

63. Lunardi A, Gaboli M, Giorgio M, Rivi R, Bygrave A, Antoniou M, Drabek D, Dzierzak E, Fagioli M, Salmena L, Botto M, Cordon-Cardo C, Luzzatto L, Pelicci PG, Grosveld F, Pandolfi PP. A Role for PMLin Innate Immunity. Genes & cancer. 2011;2(1):10-9.

64. Lo YH, Huang YW, Wu YH, Tsai CS, Lin YC, Mo ST, Kuo WC, Chuang YT, Jiang ST, Shih HM, Lai MZ. Selective inhibition of the NLRP3 inflammasome by targeting to promyelocytic leukemia protein in mouse and human. Blood. 2013;121(16):3185-94.

65. Geoffroy MC, Chelbi-Alix MK. Role of promyelocytic leukemia protein in host antiviral defense. Journal of interferon & cytokine research: the official journal of the International Society for Interferon and Cytokine Research. 2011;31(1):145-58.

66. Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nature reviews Immunology. 2008;8(11):837-48.

67. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nature reviews Immunology. 2005;5(10):749-59.

68. Vallabhapurapu S, Karin M. Regulation and function of NF-kappaB transcription factors in the immune system. Annu Rev Immunol. 2009;27:693-733.

69. Dauphinee SM, Karsan A. Lipopolysaccharide signaling in endothelial cells. Lab Invest. 2006;86(1):9-22.

70. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. Cell. 1997;91(2):243-52.

71. Böhrer H, Qiu F, Zimmermann T, Zhang Y, Jllmer T, Männel D, Böttiger BW, Stern DM, Waldherr R, Saeger HD, Ziegler R, Bierhaus A, Martin E, Nawroth PP. Role of NFkappaB in the mortality of sepsis. The Journal of clinical investigation. 1997;100(5):972-85.

72. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. Cardiovascular research. 2010;86(2):211-8.

73. Abraham E. Nuclear factor-kappaB and its role in sepsis-associated organ failure. J Infect Dis. 2003;187 Suppl 2:S364-9.

74. Ding J, Song D, Ye X, Liu SF. A pivotal role of endothelial-specific NF-kappaB signaling in the pathogenesis of septic shock and septic vascular dysfunction. Journal of immunology. 2009;183(6):4031-8.

75. Orfanos SE, Mavrommati I, Korovesi I, Roussos C. Pulmonary endothelium in acute lung injury: from basic science to the critically ill. Intensive Care Med. 2004;30(9):1702-14.

76. Christman JW, Holden EP, Blackwell TS. Strategies for blocking the systemic effects of cytokines in the sepsis syndrome. Crit Care Med. 1995;23(5):955-63.

77. Zeni F, Freeman B, Natanson C. Anti-inflammatory therapies to treat sepsis and septic shock: a reassessment. Crit Care Med. 1997;25(7):1095-100.

78. Chi L, Li Y, Stehno-Bittel L, Gao J, Morrison DC, Stechschulte DJ, Dileepan KN. Interleukin-6 production by endothelial cells via stimulation of protease-activated receptors is amplifiedby endotoxin and tumor necrosis factor-alpha. Journal of interferon & cytokine research: the official journal of the International Society for Interferon and Cytokine Research. 2001;21(4):231-40.

79. Mantovani A, Bussolino F, Introna M. Cytokine regulation of endothelial cell function: from molecular level to the bedside. Immunology today. 1997;18(5):231-40.

80. Liu HT, He JL, Li WM, Yang Z, Wang YX, Yin J, Du YG, Yu C. Geniposide inhibits interleukin-6and interleukin-8 production in lipopolysaccharide-induced human umbilical vein endothelial cells by blocking p38 and ERK1/2 signaling pathways. Inflammation research Official journal of the European Histamine Research Society [et al]. 2010;59(6):451-61.

81. Tilg H, Dinarello CA, Mier JW. IL-6 and APPs: anti-inflammatory and immunosuppressive mediators. Immunology today. 1997;18(9):428-32.

82. Rao X, Huang X, Zhou Z, Lin X. An improvement of the 2^(-delta delta CT) method for quantitative real-time polymerase chain reaction data analysis. Biostatistics, bioinformatics and biomathematics. 2013;3(3):71-85.

83. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970;227(5259):680-5.

84. Bischof O, Nacerddine K, Dejean A. Human papillomavirus oncoprotein E7 targets the promyelocytic leukemia protein and circumvents cellular senescence via the Rb and p53 tumor suppressor pathways. Molecular and cellular biology. 2005;25(3):1013-24.

85. Chen D, Feng C, Tian X, Zheng N, Wu Z. Promyelocytic Leukemia Restricts Enterovirus 71 Replication by Inhibiting Autophagy. Frontiers in immunology. 2018;9:1268.
86. El Asmi F, Maroui MA, Dutrieux J, Blondel D, Nisole S, Chelbi-Alix MK. Implication of PMLIV in both intrinsic and innate immunity. PLoS pathogens. 2014;10(2):e1003975.
87. Giovannoni F, Ladelfa MF, Monte M, Jans DA, Hemmerich P, Garcia C. Dengue Non-structural Protein 5 Polymerase Complexes With Promyelocytic Leukemia Protein (PML) Isoforms III and IV to Disrupt PML-Nuclear Bodies in Infected Cells. Front Cell Infect Microbiol. 2019;9:284.
88. Rhodes A, Evans LE, Alhazzani W, Levy MM, Antonelli M, Ferrer R, Kumar A, Sevransky JE, Sprung CL, Nunnally ME, Rochwerg B, Rubenfeld GD, Angus DC, Annane D, Beale RJ, Bellinghan GJ, Bernard GR, Chiche JD, Coopersmith C, De Backer DP, French CJ, Fujishima S, Gerlach H, Hidalgo JL, Hollenberg SM, Jones AE, Karnad DR, Kleinpell RM, Koh Y, Lisboa TC, Machado FR, Marini JJ, Marshall JC, Mazuski JE, McIntyre LA, McLean AS, Mehta S, Moreno RP, Myburgh J, Navalesi P, Nishida O, Osborn TM, Perner A, Plunkett CM, Ranieri M, Schorr CA, Seckel MA, Seymour CW, Shieh L, Shukri KA, Simpson SQ, Singer M, Thompson BT, Townsend SR, Van der Poll T, Vincent JL, Wiersinga WJ, Zimmerman JL, Dellinger RP. Surviving Sepsis Campaign: International Guidelines for Management of Sepsis and Septic Shock: 2016. Intensive Care Med. 2017;43(3):304-77.
89. Mai J, Virtue A, Shen J, Wang H, Yang XF. An evolving new paradigm: endothelial cells--conditional innate immune cells. Journal of hematology & oncology. 2013;6:61.
90. Anand AR, Cucchiarini M, Terwilliger EF, Ganju RK. The tyrosine kinase Pyk2 mediates lipopolysaccharide-induced IL-8 expression in human endothelial cells. Journal of immunology. 2008;180(8):5636-44.
91. Li H, Randall WR, Du SJ. skNAC (skeletal Naca), a muscle-specific isoform of Naca (nascent polypeptide-associated complex alpha), is required for myofibril organization. FASEB journal: official publication of the Federation of American Societies for Experimental Biology. 2009;23(6):1988-2000.
92. Li H, Xu J, Bian YH, Rotllant P, Shen T, Chu W, Zhang J, Schneider M, Du SJ. Smyd1b_tv1, a key regulator of sarcomere assembly, is localized on the M-line of skeletal muscle fibers. PloS one. 2011;6(12):e28524.
93. Li H, Zhong Y, Wang Z, Gao J, Xu J, Chu W, Zhang J, Fang S, Du SJ. Smyd1b is required for skeletal and cardiac muscle function in zebrafish. Molecular biology of the cell. 2013;24(22):3511-21.

94. Mueller M. Inflammation and angiogenesis: innate immune cells as modulators of tumor vascularization. Tumor angiogenesis: Springer; 2008. p. 351-62.

95. Becker S, Steinemann G, Karle W, Roos K, Liem CH, Muralikumar S, Volkamer A, Munz B, Zakrzewicz A, Berkholz J. Stability of Smyd1 in endothelial cells is controlled by PML-dependent SUMOylation upon cytokine stimulation. The Biochemical journal. 2021;478(1):217-34.

96. Sprague AH, Khalil RA. Inflammatory cytokines in vascular dysfunction and vascular disease. Biochemical pharmacology. 2009;78(6):539-52.

97. Ahmed A, Wan X, Mitxitorena I, Lindsay AJ, Paolo Pandolfi P, McCaffrey MW, Keeshan K, Regulation of NF-kappaB by PML and PML-RARalpha. Scientific reports. 2017;7:44539.

98. Ohgiya D, Matsushita H, Onizuka M, Nakamura N, Amaki J, Aoyama Y, Kawai H, Ogawa Y, Kawada H, Ando K. Associationof promyelocytic leukemia protein with expression of IL-6 and resistance to treatment in multiple myeloma. Acta haematologica. 2012;128(4):213-22.

99. Sun J, Fu S, Zhong W, Huang H. PML overexpression inhibits proliferation and promotes the osteogenic differentiation of human mesenchymal stem cells. Oncology reports. 2013;30(6):2785-94.

100. Ribet D, Lallemand-Breitenbach V, Ferhi O, Nahori MA, Varet H, de Thé H, Cossart P. Promyelocytic Leukemia Protein (PML) Controls Listeria monocytogenes Infection. mBio. 2017;8(1).

101. Fausti F, Di Agostino S, Cioce M, Bielli P, Sette C, Pandolfi PP, Oren M, Sudol M, Strano S, Blandino G. ATM kinase enablesthe functional axis of YAP, PML and p53 to ameliorate loss of Werner protein-mediated oncogenic senescence. Cell death and differentiation. 2013;20(11):1498-509.

102. Hubackova S, Krejcikova K, Bartek J, Hodny Z. Interleukin 6 signaling regulates promyelocytic leukemia protein gene expression in human normal and cancer cells. The Journal of biological chemistry. 2012;287(32):26702-14.

103. Kato M, Muromoto R, Togi S, Iwakami M, Kitai Y, Kon S, Oritani K, Matsuda T. PML suppresses IL-6- induced STAT3 activation by interfering with STAT3 and HDAC3 interaction. Biochem Biophys Res Commun. 2015;461(2):366-71.

104. Martín-Martín N, Piva M, Urosevic J, Aldaz P, Sutherland JD, Fernández-Ruiz S, Arreal L, Torrano V, Cortazar AR, Planet E, Guiu M, Radosevic-Robin N, Garcia S, Macías I,

Salvador F, Domenici G, Rueda OM, Zabala-Letona A, Arruabarrena-Aristorena A, Zúñiga-García P, Caro-Maldonado A, Valcárcel-Jiménez L, Sánchez-Mosquera P, Varela-Rey M, Martínez-Chantar ML, Anguita J, Ibrahim YH, Scaltriti M, Lawrie CH, Aransay AM, Iovanna JL, Baselga J, Caldas C, Barrio R, Serra V, Vivanco Md, Matheu A, Gomis RR, Carracedo A. Stratification and therapeutic potential of PML in metastatic breast cancer. Nature communications. 2016;7:12595.

105. Ohbayashi N, Kawakami S, Muromoto R, Togi S, Ikeda O, Kamitani S, et al. The IL-6 family of cytokines modulates STAT3 activation by desumoylation of PML through SENP1 induction. Biochem Biophys Res Commun. 2008;371(4):823-8.

106. Ohbayashi N, Kawakami S, Muromoto R, Togi S, Ikeda O, Kamitani S, Sekine Y, Honjoh T, Matsuda T. Opposingeffects of PML and PML/RAR alpha on STAT3 activity. Blood. 2003;101(9):3668-73.

107. Hsu KS, Zhao X, Cheng X, Guan D, Mahabeleshwar GH, Liu Y, Borden E, Jain MK, Kao HY. Dual regulation of Stat1 and Stat3 by the tumor suppressor protein PML contributes to interferon alpha- mediated inhibition of angiogenesis. The Journal of biological chemistry. 2017;292(24):10048-60.

108. Libermann TA, Baltimore D. Activation of interleukin-6 gene expression through the NF-kappa B transcription factor. Molecular and cellular biology. 1990;10(5):2327-34.

109. Shamloul A, Steinemann G, Roos K, Liem CH, Bernd J, Braun T, Zakrzewicz A, Berkholz J. The methyltransferase Smyd1 mediates LPS-triggered up-regulation of IL-6 in endothelial cells. Cells. 2021;10(12):3515.

110. Wu WS, Xu ZX, Hittelman WN, Salomoni P, Pandolfi PP, Chang KS. Promyelocytic leukemia protein sensitizes tumor necrosis factor alpha-induced apoptosis by inhibiting the NF-kappaB survival pathway. The Journal of biological chemistry. 2003;278(14):12294- 304.

Statutory Declaration

“I, Ahmed Shamloul, by personally signing this document in lieu of an oath, hereby affirm that I prepared the submitted dissertation on the topic [“Potential role of Smyd1 and PML in human endothelial cells during LPS-induced sepsis”] – [“Charakterisierung der Funktion von Smyd1 und PML bei LPS-abhängigen Entzündungsreaktionen in Endothelzellen"], independently and without the support of third parties, and that I used no other sources and aids than those stated.

All parts which are based on the publications or presentations of other authors, either in letter or in spirit, are specified as such in accordance with the citing guidelines. The sections on methodology (in particular regarding practical work, laboratory regulations, statistical processing) and results (in particular regarding figures, charts and tables) are exclusively my responsibility.

Furthermore, I declare that I have correctly marked all of the data, the analyses, and the conclusions generated from data obtained in collaboration with other persons, and that I have correctly marked my own contribution and the contributions of other persons (cf. declaration of contribution). I have correctly marked all texts or parts of texts that were generated in collaboration with other persons.

My contributions to any publications to this dissertation correspond to those stated in the below joint declaration made together with the supervisor. All publications created within the scope of the dissertation comply with the guidelines of the ICMJE (International Committee of Medical Journal Editors; www.icmje.org) on authorship. In addition, I declare that I shall comply with the regulations of Charité – Universitätsmedizin Berlin on ensuring good scientific practice.

I declare that I have not yet submitted this dissertation in identical or similar form to another Faculty.

The significance of this statutory declaration and the consequences of a false statutory declaration under criminal law (Sections 156, 161 of the German Criminal Code) are known to me.”

Date	Signature

Declaration of contribution to the publications

Ahmed Shamloul contributed the following to the below listed publications:

Ahmed Shamloul, Gustav Steinemann, Kerrin Roos, Celine Huajia Liem, Jonathan Bernd, Thorsten Braun, Andreas Zakrzewicz and Janine Berkholz. The Methyltransferase Smyd1 Mediates LPS-Triggered Up-Regulation of IL-6 in Endothelial Cells. Cells. 2021 Dec; 10(12): 3515. doi: 10.3390/cells10123515.

Contribution:
Ahmed Shamloul participated equally as, Gustav Steinemann, in the first Co-authorship of the above-mentioned publication.

He was responsible for the investigations and formal data analysis correlated to the following figures in the publication:
Fig 1 B/E
Fig. 2 A/C/D/G
Fig. 3 A
Fig. 4 A/B
Fig. 5 A/C

Moreover Ahmed Shamloul had the same contribution in the writing and preparation of the original draft.

* Please note: Ahmed Shamloul was responsible alone for all the additional work related to the studies on “PML” included in the mantel text.

_____
Signature of doctoral candidate

Journal Data Filtered By: **Selected JCR Year: 2020** Selected Editions: SCIE, SSCISelected Categories: **"CELL BIOLOGY"** Selected Category Scheme: WoS

Gesamtanzahl: 195 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	NATURE REVIEWS MOLECULAR CELL BIOLOGY	58,477	94.444	0.075480
2	NATURE MEDICINE	114,401	53.440	0.184050
3	CELL	320,407	41.582	0.526960
4	CANCER CELL	50,839	31.743	0.081040
5	NATURE CELL BIOLOGY	52,554	28.824	0.070950
6	Cell Metabolism	52,192	27.287	0.091000
7	Journal of Extracellular Vesicles	8,485	25.841	0.011820
8	CELL RESEARCH	24,108	25.617	0.034400
9	Cell Stem Cell	32,147	24.633	0.062780
10	TRENDS IN CELL BIOLOGY	19,007	20.808	0.030120
11	Signal Transductionand Targeted Therapy	3,848	18.187	0.005730
12	MOLECULAR CELL	86,299	17.970	0.161840
13	Science Translational Medicine	45,509	17.956	0.103780
14	Autophagy	25,343	16.016	0.027970
15	CELL DEATH AND DIFFERENTIATION	27,701	15.828	0.028730
16	NATURE STRUCTURAL & MOLECULAR BIOLOGY	32,038	15.369	0.051210
17	Protein & Cell	5,352	14.870	0.009500
18	Annual Review of Cell and Developmental Biology	11,884	13.827	0.011100
19	DEVELOPMENTAL CELL	36,177	12.270	0.058350
20	TRENDS IN MOLECULAR MEDICINE	13,213	11.951	0.014720

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
21	EMBO JOURNAL	76,189	11.598	0.055000
22	MATRIX BIOLOGY	8,972	11.583	0.011010
23	GENES & DEVELOPMENT	61,885	11.361	0.048660
24	PLANT CELL	64,794	11.277	0.036260
25	AGEING RESEARCH REVIEWS	10,264	10.895	0.013510
26	Cell Discovery	3,492	10.849	0.006770
27	CURRENT BIOLOGY	78,289	10.834	0.116100
28	JOURNAL OF CELL BIOLOGY	79,173	10.539	0.057070
29	Cell Systems	5,813	10.304	0.035330
30	Cold Spring Harbor Perspectives in Biology	22,738	10.005	0.030460
31	Wiley Interdisciplinary Reviews-RNA	3,743	9.957	0.008220
32	ONCOGENE	77,576	9.867	0.059180
33	Cell Reports	73,442	9.423	0.254400
34	AGING CELL	13,890	9.304	0.017950
35	CELLULAR ANDMOLECULAR LIFE SCIENCES	34,003	9.261	0.033790
36	EMBO REPORTS	19,502	8.807	0.027490
37	Cell Death & Disease	40,835	8.469	0.063770
38	JOURNAL OF BIOMEDICA LSCIENCE	6,621	8.410	0.007330
39	CURRENT OPINION IN CELL BIOLOGY	15,784	8.382	0.019750
40	Science Signaling	15,954	8.192	0.023910
41	Stem Cell Reports	10,762	7.765	0.029290

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Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
42	SEMINARS IN CELL & DEVELOPMENTAL BIOLOGY	14,105	7.727	0.021440
43	CYTOKINE & GROWTH FACTOR REVIEWS	7,650	7.638	0.005850
44	AMERICAN JOURNAL OF RESPIRATORY CELL AND MOLECULAR BIOLOGY	15,280	6.914	0.015050
45	Stem Cell Research & Therapy	13,356	6.832	0.018900
46	CELL PROLIFERATION	5,130	6.831	0.005130
47	CELL CALCIUM	6,842	6.817	0.006250
48	International Reviewof Cell and Molecular Biology	3,057	6.813	0.004320
49	CURRENT OPINION IN STRUCTURAL BIOLOGY	12,448	6.809	0.018970
50	CELLULAR ONCOLOGY	2,462	6.730	0.002430
51	CELL BIOLOGY AND TOXICOLOGY	2,298	6.691	0.001370
52	Frontiers in Cell and Developmental Biology	7,731	6.684	0.015420
53	Cells	18,802	6.600	0.026970
54	Oxidative Medicine and Cellular Longevity	27,913	6.543	0.036150
55	Tissue Engineering Part B-Reviews	4,536	6.389	0.003040
56	JOURNAL OF CELLULAR PHYSIOLOGY	39,997	6.384	0.041830
57	MOLECULAR MEDICINE	6,239	6.354	0.004460
58	STEM CELLS	23,967	6.277	0.017860
59	Journal of Molecular Cell Biology	3,144	6.216	0.004700
60	TRAFFIC	7,808	6.215	0.007630
61	MOLECULAR CANCER RESEARCH	11,253	5.852	0.013250

Selected JCR Year: 2020; Selected Categories: "CELL BIOLOGY"

Article

The Methyltransferase Smyd1 Mediates LPS-Triggered Up-Regulation of IL-6 in Endothelial Cells

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Abstract: The lysine methyltransferase Smyd1 with its characteristic catalytic SET-domain is highly enriched in the embryonic heart and skeletal muscles, participating in cardiomyogenesis, sarcom- ere assembly and chromatin remodeling. Recently, significant Smyd1 levels were discovered in endothelial cells (ECs) that responded to inflammatory cytokines. Based on these biochemical prop- erties, we hypothesized that Smyd1 is involved in inflammation-triggered signaling in ECs and therefore, investigated its role within the LPS-induced signaling cascade. Human endothelial cells (HUVECs and EA.hy926 cells) responded to LPS stimulation with higher intrinsic Smyd1 expression. By transfection with expression vectors containing gene inserts encoding either intact Smyd1, a catalytically inactive Smyd1-mutant or Smyd1-specific siRNAs, we show that Smyd1 contributes to LPS-triggered expression and secretion of IL-6 in EA.hy926 cells. Further molecular analysis revealed this process to be based on two signaling pathways: Smyd1 increased the activity of NF-κB and promoted the trimethylation of lysine-4 of histone-3 (H3K4me3) within the IL-6 promoter, as shown by ChIP-RT-qPCR combined with IL-6-promoter-driven luciferase reporter gene assays. In summary, our experimental analysis revealed that LPS-binding to ECs leads to the up-regulation of Smyd1 expression to transduce the signal for IL-6 up-regulation via activation of the established NF-κB pathway as well as via epigenetic trimethylation of H3K4.

Keywords: Smyd1; lipopolysaccharide; sepsis; IL-6; NF-κB; histone methylation; endothelial cells

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Citation: Shamloul, A.; Steinemann, G.; Roos, K.; Liem, C.H.; Bernd, J.; Braun, T.; Zakrzewicz, A.; Berkholz, J. The Methyltransferase Smyd1 Mediates LPS-Triggered Up-Regulation of IL-6 in Endothelial Cells. *Cells* **2021**, *10*, 3515. https://doi.org/10.3390/cells10123515

Academic Editor: Gerda Egger

Received: 8 September 2021
Accepted: 7 December 2021

Published: 13 December 2021

1. Introduction

Like most other lysine methyltransferases, the members of the Smyd gene family express a characteristic SET domain, which contains the active site required for protein methylation using *S*-adenosyl methionine (SAM) as a methyl group donor [1,2]. Remark- ably, only in the case of the Smyd proteins is the catalytic SET domain split by a MYND domain [3]. The five proteins of the Smyd gene family were originally discovered in embry- onic heart and skeletal muscles, where they were found to participate in cardiomyogenesis and sarcomere assembly [4]. However, it is now clear that their tissue distribution and their functional relevance is much more complex than initially reported, as Smyd proteins are involved in many physiological processes, such as chromatin remodeling, transcrip- tion, signal transduction and cell cycle control, due to multiple interactions with other molecules [3,4].

In principle, five different types of molecular mechanisms of the Smyd proteins are distinguishable. (1) By the joint action of the two spatially separated SET half domains, four

of the five Smyd family members methylate lysines (K) in histones (H) at selected positions, such as H3K4, H3K36 or H4K5 [4–6]. The complexity of these histone modifications is increased by the fact that most of the lysines can be methylated once (me1), twice (me2) or even three times (me3). These posttranslational modifications of histones cause conformational changes of the nucleosome, which might directly alter the accessibility of the promoter sequences for the transcription apparatus [7]. Alternatively, according to the histone code [8], methylated histones might be recognized by factors that then indirectly trigger changes in the transcription frequency of the downstream target genes [9].
(2) The SET domain-related methyltransferase activity of Smyd proteins methylates other proteins than histones in order to influence their activity/function [10]. (3) Smyd proteins adhere directly to/interact with DNA promoter sequences for the steric inhibition of the transcription apparatus or increasing their methyltransferase activity [11,12]. (4) With their MYND-type zinc finger domain, Smyd proteins interact with other proteins, e.g., to recruit transcription co-repressors [13,14] or transcription factors [9]. (5) The C-terminal domain (CTD) of Smyd proteins inhibits their methyltransferase activity as an intrinsic autoinhibitory loop [15].

Among the members of the Smyd gene family, Smyd1 is the only protein identified so far that is expressed in endothelial cells (ECs) under non-pathological conditions [16,17].ECs outline the inner surface of the blood vessels and, therefore, represent important barriers between the bloodstream and extravascular tissue. Smyd1 influences the migration activity and tube formation of ECs and thus might have an impact on angiogenesis [16]. Furthermore, the Smyd1 expression in ECs is influenced by the cytokines IFN-γ and TNF-α [17], suggesting that this member of the Smyd family is sensitive to inflammation.

Inflammatory reactions in humans are frequently triggered by lipopolysaccharides (LPS). Physiologically, LPS are large molecules of the outer cell membrane (as part of the cell wall) of Gram-negative bacteria. However, when these bacteria are pathologically released into the circulation of a human, LPS may shed from the cell wall to be released in the circulatory system of the host, where they finally interact with specific receptors on the EC surface of all blood vessels [18]. As a result, molecular signaling cascades are initiated, which include the induction/suppression of numerous relevant downstream molecules in these ECs, e.g., adhesion molecules, such as E-selectin and ICAM1 [19,20], or proinflammatory cytokines, such as IL-1β and IL-6 [21,22]. Taken together, LPS act as endotoxins in hosts, evoking several pathophysiological reactions in the vascular system, e.g., the breakdown of the barrier function [23], apoptosis of ECs [24] and stimulation of coagulation cascades [25] with further progression of organ dysfunctions, which can ultimately lead to the development of fatal sepsis [26,27].

Given that the inflammatory-sensitive signaling molecule Smyd1 is expressed in ECs, which are reactive to LPS exposition, we hypothesized that Smyd1 is integrated into the LPS signaling cascade and is therefore involved in the regulation of IL-6 expression. To validate this hypothesis, we systematically examined the relationship between LPS, Smyd1 and IL-6 in human EC cultures. By molecular analysis of EA.hy926 cells transfected with Smyd1 vectors or Smyd1-specific siRNAs, we found that LPS induced Smyd1-dependent trimethylation and activation of the IL-6 promoter, suggesting an upstream effect of Smyd1 on IL-6 expression in ECs.

2. Materials and Methods

2.1. Cell Culture

EA.hy926 cells (Elabscience, Biotechnology Inc., Houston, TX, USA) were cultured in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (growth medium). Human umbilical vein endothelial cells (HUVECs) were isolated as described [28] with approval of the Ethics Committee of the Charité—Universitätsmedizin Berlin, Germany (EA4/107/17), and cultured in Endothelial Cell Growth Medium (PromoCell, Heidelberg, Germany). Umbilical cords were obtained from

healthy mothers with written informed consent in accordance with the Declaration of Helsinki. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.2. Cell Stimulation Experiments with LPS or IL-6

LPS stimulation experiments, EA.hy926 cells and HUVECs were incubated with different concentrations of LPS (in the range of 1 ng/mL to 10 µg/mL; Sigma Aldrich, St. Louis, MO, USA). LPS stock solutions (1 mg/mL) were dissolved in PBS, pH 7.4 containing 1% bovine serum albumin (BSA) as carrier. For the study of the effect of IL-6 on Smyd1 expression in EA.hy926 cells, recombinant human IL-6 (PeproTech, Hamburg, Germany) diluted in PBS, pH 7.4 containing 1% BSA was used in a final concentration of 10 ng/mL. Both LPS and IL-6 were added to the cells with gentle mixing, to be then incubated for 3 h or 24 h, respectively. The control cells were treated with PBS/BSA.

2.3. Treatment of Cells with PDTC or Bay 11-7082

Pyrrolidine dithiocarbamate (PDTC, final concentration: 10 µM; Roth, Karlsruhe, Germany), and Bay 11-7082 (final concentration: 10 µM; Callbiochem, San Diego, CA, USA), both dissolved in dimethyl sulfoxide (DMSO), were added to the medium. DMSO alone served as a negative control.

2.4. Transfection with Human Expression Vectors

The transfection of EA.hy926 cells with expression plasmids was performed as previously described [29]. For transient transfection of the full-length, active Smyd1 expression plasmid into EA.hy926 cells, the pCMV2-Smyd1-flag (Sino Biological, Beijing, China) plas- mid and of the methyltransferase-silent Smyd1 variant, the pBK-CMV-Smyd1$_{HMTasemutant}$ (↑Smyd1 SET Mut) plasmid in which the Smyd1 gene insert contained a point mutation within the SET domain were used in combination with the TurboFect reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, EA.hy926 cells were transfected with 1 µg DNA diluted in 100 µL Opti-MEM (Gibco) for 24 h when cells reached ~70% confluence. Transfection efficiency was tested by RT-qPCR and by immunoblot analysis. pBK-CMV-Smyd1$_{HMTase\ mutant}$ was a kind gift from Haley O. Tucker, Department of Molecular Biosciences, University of Texas at Austin, U.S.A. The term "control cells" refers to cells transfected with the corresponding vector lacking a specificgene insert.

2.5. Transfection with Specific siRNAs

The transfection of EA.hy.926 cells was performed, using a mixture of four unrelated siRNA species (25 nM final concentration) directed against the Smyd1 nucleotide sequence (Dharmacon, Lafayette, CO, USA; Cat.No: L-022738-01-0005). A non-gene-specific "scrambled" siRNA was used as a negative control. EA.hy926 cells were transfected using the transfection reagent Interferin (Polyplus Transfection, Illkirch-Graffenstaden, France), according to the manufacturer's instructions. Knockdown of the target mRNA was monitored 24 h after transfection with siRNAs by RT-qPCR and immunoblotting.

2.6. RNA Isolation, Reverse Transcription and Real Time qPCR

RNA was extracted from EA.hy926 and HUVECs using the GeneMATRIX Universal RNA Purification kit from EURx according to the manufacturer's instructions. Semi-quantitative RT-qPCR analysis was carried out using the QuantStudio 5 system (Applied Biosystems, Foster City, CA, USA). Amounts of specific cDNAs were determined using the GoTaq qPCR Master Mix (Promega, Madison, WI, USA). Primer sequences, product sizes and annealing temperatures are listed in Table 1. In each experiment, melting curve analysis was performed to verify that a single transcript was produced. RT-qPCR relative mRNA levels were calculated using the comparative CT ($2^{-\Delta\Delta C_T}$) method, with GAPDHas a reference. Non-RT- and non-template controls were run for all reactions.

Table 1. Primer sequences, product sizes and annealing temperatures used to amplify the corresponding cDNA templates.

Template	Forward Primer	Reverse Primer	Product Size	Annealing Temperature
GAPDH	5′-ATG ACC TTG CCC ACA GCC TT-3′	5′-AAC TGC TTA GCA CCC CTG GC-3′	200 bp	60 °C
IL-6	5′-TGC CAG CCT GCT GAC GAA G-3′	5′-AGC TGC GCA GAA TGA GAT GAG-3′	90 bp	56 °C
Smyd1	5′-CTG GAG AAG CAG GAG CCA GTG TT-3′	5′-GCA TAG GCT TTG CAG ATC ATC CC-3′	257 bp	60 °C
ICAM1	5′-GGC CGG CCA GCT TAT ACA C-3′	5′-TAG ACA CTT GAG CTC GGG CA-3′	166 bp	58 °C
VCAM1	5′- TCA GAT TGG AGA CTC AGT CAT GT-3′	5′-ACT CCT CAC CTT CCC GCTC-3′	109 bp	62 °C
CCL2	5′-GAG AGG CTG AGA CTA ACC CAG A-3′	5′-ATC ACA GCT TCT TTG GGA CAC T -3′	259 bp	62 °C

2.7. Immunoblotting

Cells were homogenized in RIPA buffer (Santa Cruz, Biotechnology, Dallas, TX, USA) containing standard protease inhibitors (Sigma-Aldrich) and 20 mM N-ethylmaleimide (Sigma-Aldrich) for 15 min at 4 °C. Cell lysates were collected after centrifugation to be subjected to protein concentration determination using the BCA Protein Assay Kit (Thermo Fisher Scientific). Total protein extracts (20 µg) were resolved by SDS-PAGE gel electrophoresis and transferred to nitrocellulose membranes (GE Healthcare, Chicago, IL, USA) for immunoblotting. Cytoplasmic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Primary antibodies were diluted as follows: anti-Smyd1 (1:1000, Thermo Fisher Scientific, Cat.No: PA5-31482; rabbit), anti-IL-6 (1:500, Santa Cruz Biotechnology, Cat.No: sc-28343; mouse), anti- NF-κB p65 (1:1000, Cell Signaling Technology, Danvers, MA, USA; Cat.No: 8242S; rabbit), anti-IκBα (1:1000, Cell Signaling Technology, Cat.No: 4814S; mouse), anti-GAPDH (1:10,000, Proteintech, Rosemont, IL, USA; Cat.No: HRP-60004; mouse), anti-Emerin (1:2000, Abcam, Cambridge, UK; Cat.No: ab153718; rabbit). A peroxidase-conjugated secondary antibody (1:1000, Santa Cruz) was used to detect antibodies bound to the blot matrix and visualized using a chemiluminescence kit (Bio-Rad, Hercules, CA, USA).

2.8. Immunofluorescence Analysis

Cellular localization of Smyd1 and NF-κB -p65 was assessed on fixed EA.hy926 cells by immunocytochemistry combined with confocal laser scanning microscopy. Cells were fixed with 4% paraformaldehyde in PBS, pH 7.4 for 15 min at room temperature and permeabilized by PBS, pH 7.4, containing 0.5% Triton X-100. Nuclei were stained with Draq5 (1:1000, Thermo Fisher Scientific). After blocking with 10% FCS in PBS, pH 7.4, fixed cells were incubated overnight at 4 °C with an anti-Smyd1 (1:100, Thermo Fisher Scientific, Cat.No: PA5-31482; rabbit), an anti-NF-κB-p65 antibody (1:100, Cell Signaling, Cat.No: 8242S; rabbit) and/or an anti-alpha Tubulin-1 (1:100, D-11, Santa Cruz, Cat.No: sc-32293; mouse) antibody in blocking buffer. Alexa Fluor-conjugated secondary antibodies (Life Technologies, Carlsbad, CA, USA) were diluted in blocking buffer containing 5 µM Draq5. Cells were incubated with the respective secondary antibody mix for 1 h at room temperature, washed 3 times with PBS, pH 7.4, and covered with fluorescence mounting medium (1:2000, Agilent Technologies, Santa Clara, CA, USA). Isotype-matched, non-binding primary IgG antibodies of the same species in the same concentration as the specific primary antibodies served as negative controls. Stained cells were then assessedwith aconfocallaser microscope (Leica DMI 6000, Wetzlar, Germany) equipped with 20× and 63× oil immersion lens, a single-photon argon laser, a solid-state laser and a helium-neon laser. The Leica LAS AF Lite software was used to process the digital images and analyze the mean fluorescence intensity (MFI). For MFI quantification, all images were analyzed using the same optical settings. The MFI was measured in 20 regions of interest (ROIs) per experiment belonging to either the nucleus or the cytoplasmic area. A non-fluorescent region of the same image was analyzed as background MFI and then subtracted from each of the MFI in the specific ROIs.

2.9. ELISA Analysis

IL-6 and IL-8 protein concentrations were determined in cell culture supernatants using commercial human ELISA kits (Invitrogen, Waltham, MA, USA) according to the manufacturer's guidelines. Samples were analyzed in triplicate.

2.10. Luciferase Assay Reporter Gene Assay

EA.hy926 cells were transiently transfected with pBABE lucIL6 reporter or NF-κB luciferase reporter vector (BPS Bioscience, San Diego, MA, USA) and partially transfected in parallel with the wild-type expression plasmid pCMV2-Smyd1-flag (Sino Biological) or the pBK-CMV-Smyd1$_{\text{HMTase mutant}}$ plasmid using TurboFect transfection reagent (Thermo Fisher Scientific) according to the manufacturer's instructions. pBABE lucIL6 was a kind gift from Sheila Stewart (Addgene plasmid # 52884) [30]. The vector contains the human IL-6 promoter region upstream of the luciferase-encoding sequence of plasmid pBABE hygro. NF-κB luciferase reporter vector contains a firefly luciferase gene under the control of a multimerized NF-κB responsive element located upstream of a minimal promoter. Stimulation with LPS (1 μg/mL) served as the positive control. After 24 h, the cells were harvested in lysis buffer and assayed for firefly activity as described by the manufacturer (Promega). The amount of luciferase activity in each sample was quantified in a Varioskan Flash Multimode Reader (Thermo Fisher Scientific).

2.11. Flow Cytometry

After centrifugation (1000 rpm for 5 min at 4 °C), pelleted EA.h926 cells were resuspended in PBS and then fixed in methanol for 20 min at− 20 °C. Subsequently, cells were blocked in 1% FCS in PBS for 1h and then incubated with the primary non-conjugated antibodies (anti-Smyd1, Santa Cruz; anti-NF-κB-p65, Cell Signaling) or isotype-matched monoclonal IgG control antibodies, all diluted in 1% FCS in PBS at 4 °C for 2 h. Subsequently, the cells were washed twice with 1% FCS in PBS and stained with the secondary antibodies conjugated to Alexa Flour 488 or 594, diluted in PBS (1:1000) with 1% FCS at 4 °C for 1 h. The flow cytometric analysis was carried out after two additional washing steps with a FACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) using CellQuest software.

2.12. ChIP RT-qPCR Assays

ChIP-RT-qPCR assays with transfected or LPS-stimulated EA.hy926 cells were performed using the EpiQuik Chromatin Immunoprecipitation Kit (Epigentek, Farmingdale, NY, USA) following the protocol supplied by the manufacturer. For the ChIP assays, 1×10^6 cells were fixed in 1% formaldehyde for 10 min. Lysed samples were sonicated to fragment the DNA. The DNA-bound proteins were immunoprecipitated with antibodies directed against H3K4me1, H3K4me2 and H3K4me3 (Epigentek) or Smyd1 (Thermo Fisher Scientific). The collected DNA and input samples were analyzed for associated DNA fragments using quantitative RT-qPCR. Previous work by other groups [31-33] showed that the transcription frequency of IL-6 is related to the state of different regions present in the IL-6 promoter active in different cell types, in particular to that of three distinct CpG islands: a cluster at approximately −1500/−1300 bp (8 CpG sites), a cluster at approxi- mately −670/−400 bp (7 CpG sites) and a cluster at approximately 100/150 bp relative to the transcription start (10 CpG sites). It was shown, for example, that the qualitative and quantitative modification patterns of histone H3 on each of these three CpG islands had a significant influence on the IL-6 transcription rates in synovial fibroblasts [33]. Therefore, ChIP RT-qPCR analysis with primer pairs that cover DNA sequences in the vicinity of these three CpG islands, primer pair 1 at −1522 bp to −1291 bp, primer pair 2 at −691 bp to −399 bp and primer pair 3 at 96 bp to 293 bp of the IL-6 gene, were carried out. The primer sequences, PCR product sizes and position within the IL-6 gene used in ChIP-RT-qPCR are listed in Table 2.

Table 2. Sequences, PCR product sizes and position within the IL-6 gene of the primer pairs used in the ChIP-RT-qPCR assays. The information on the primer positions refers to the NCBI reference sequence for human interleukin 6: NG_011640.1.

Template	Forward Primer	Reverse Primer	Product Size	Position (for/rev)
IL-6 region 1	5′-TTT TCA CAC CAA AGA ATC CC-3′	5′-CTT ATT TAC CAA ACA TGG TGT-3′	231 bp	3532/3763
IL-6 region 2	5′-CAG GTG AAG AAA GTG GCA GA-3	5′-GAC CAG ATT AAC AGG CTA GAA-3′	292 bp	4363/4655
IL-6 region 3	5′-TCC TTA GCC CTG GAA CTG CC-3′	5′-AGG CAA CAC CAG GAG CAG CCC C-3′	197 bp	5150/5347

2.13. Statistical Analysis

Data were analyzed with GraphPad Prism software 8.0 (Graph-Pad Software, San Diego, CA, USA) and presented as mean ± SD (n ≥ 3). All data sets were tested by Shapiro– Wilk for their normality of distribution prior to statistical analysis and the Brown–Forsythe test was performed to test the equality of variance. Comparisons between the two groups were performed by Student *t*-test (2-tailed unpaired), between >2 groups by one-way ANOVA followed by the Dunnett test or by two-way ANOVA followed by the Bonferroni post hoc test.

3. Results

3.1. LPS Stimulation of Endothelial Cells Leads to Higher Smyd1 Expression

To investigate whether LPS has an effect on Smyd1 expression in endothelial cells (ECs), HUVECs and EA.hy926 cells were incubated without LPS (control) or with different concentrations of LPS (in the range of 1 ng/mL to 10 µg/mL) for 3 h. This LPS treatment led to a dose-dependent increase in Smyd1 expression at the mRNA level ($p < 0.01$), which peaked at 1 µg/mL (Figure 1A; HUVECs: +321%; EA.hy926 cells: +123%). Higher Smyd1 mRNA concentrations ($p < 0.01$) were expressed after 3 h (HUVECs: +397%; EA.hy926 cells: +290%) than 24 h in both HUVECs and EA.hy926 cells during stimulation with 1 µg/mL LPS (Figure 1B). Corresponding results (peak in Smyd1 expression at 1 µg/mL (Figure 1C and Figure S1A)) after 3 h of LPS stimulation (Figure 1D and Figure S1B) were found at the protein level when EA.hy926 cell lysates were subjected to quantitative immunoblotting. Accordingly, flow cytometry analysis demonstrated higher intracellular Smyd1 levels in EA.hy926 cells after 3 h and 24 h of LPS stimulation than in non-stimulated cells (Figure 1E). Strong anti-Smyd1 immunoreactivity was observed in both the nuclear and cytoplasmic compartments by immunocytochemistry when EA.hy926 cells remained non-stimulated or were stimulated with LPS for 3 h (Figure 1F). However, the immunocytochemical signal was higher in the nucleus and the cytoplasmic compartment of LPS-stimulated compared to non-stimulated EA.hy926 cells (Figure 1F,G).

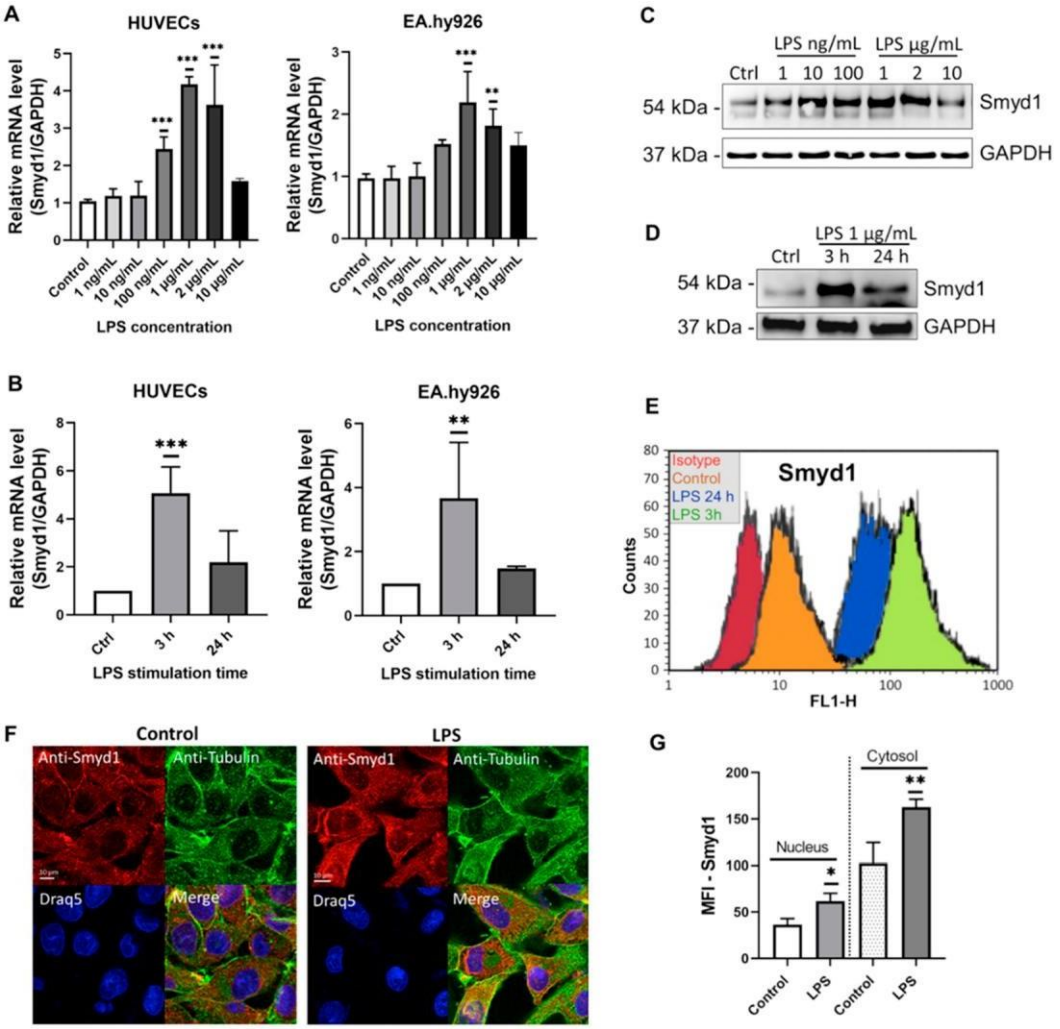


Figure 1. LPS stimulation increases Smyd1 expression in endothelial cells. (**A**) RT-qPCR for the quantification of Smyd1 mRNA levels in HUVECs and EA.hy926 cells that were stimulated with different concentrations of LPS ranging from 1 ng/mL to 10 µg/mL for 3 h. Expression values relative to control (no LPS supplement). $n = 3$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA. (**B**) RT-qPCR for the quantification of Smyd1 mRNA levels in HUVECs and EA.hy926 cells that were stimulated with 1 µg/mL LPS for 3 h or 24 h. Expression values relative to control (Ctrl, no LPS supplement). $n = 3$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA. (**C**) Immunoblotting for the determination of Smyd1 protein levels in total lysates of EA.hy926 cells incubated with different concentrations of LPS ranging from 1 ng/mL to 10 µg/mL for 3 h in comparison to no LPS supplement (Ctrl). Representative immunoblot of $n = 3$. (**D**) Immunoblotting for the determination of Smyd1 protein levels in total lysates of EA.hy926 cells after incubation with 1 µg/mL LPS for 3 h or 24 h in comparison to no LPS supplement (Ctrl). Representative immunoblot of $n = 3$. (**E**) Representative flow cytometry histograms of EA.hy926 cells stimulated without LPS or with 1 µg/mL LPS for 3 h or 24 h and incubated with a monoclonal antibody recognizing Smyd1 and, subsequently, fluorescence-labeled secondary antibodies. An isotype-matched monoclonal IgG was used as control. $n = 3$. (**F**) Immunocytochemistry with anti-Smyd1 (red) and anti-Tubulin (green) antibodies on EA.hy926 cells incubated for 3 h with vehicle only (Control) or LPS (1 µg/mL). Draq5 staining for labeling of cell nuclei (blue). Representative images of $n = 3$ experiments. (**G**), Mean fluorescence intensity (MFI) of Smyd1 immunoreactivity, as shown in F, was densitometrically determined in the nucleus (left two columns) and the cytosol (right two columns). $n = 3$ independent experiments each with 20 cells evaluated. * $p < 0.05$, ** $p < 0.01$ using Student *t*-test. All graphs reported as mean ± SD.

3.2. Smyd1 Increases IL-6 Levels in Endothelial Cells

Because LPS is an established trigger of IL-6 production and released in many cells, we next evaluated whether Smyd1 influences the expression of IL-6 in ECs. Therefore, the IL-6 expression levels were determined in EA.hy926 cells that were transfected with either a Smyd1 gene-containing vector or a vector lacking a specific gene insert (Figures 2A, B

and S1C). lL-6 expression was higher ($p < 0.001$) in the Smyd1-overexpressing cells than the control cells at both the mRNA (+344%; Figure 2C) and protein (+47%; Figures 2D and S1D) level.

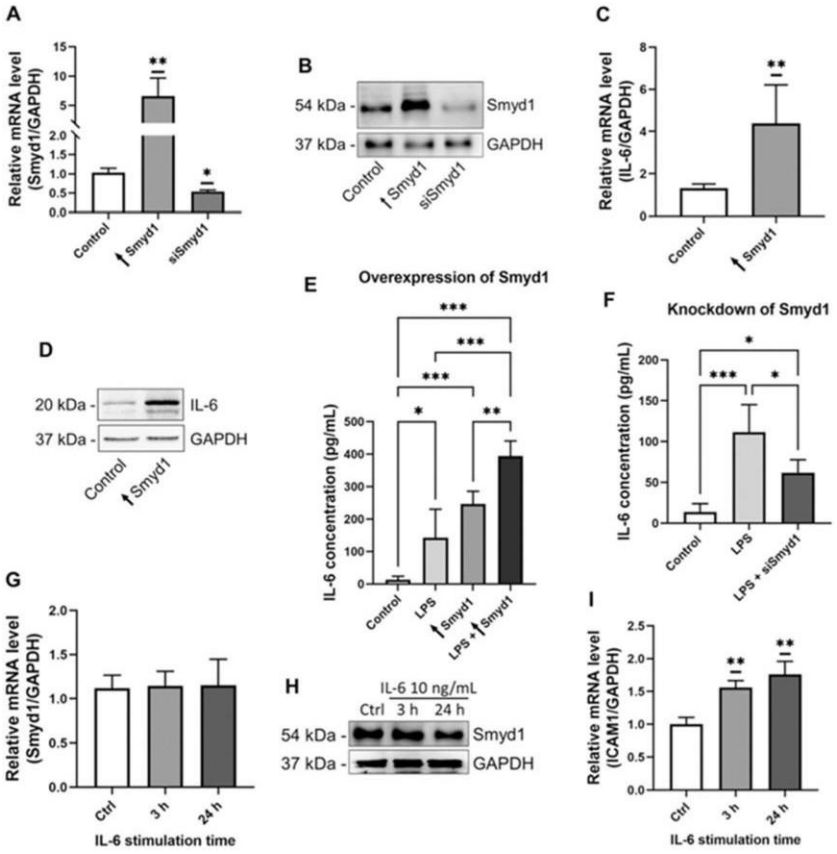


Figure 2. Smyd1 increases IL-6 expression and secretion in endothelial cells. (**A**,**B**) RT-qPCR to quantify Smyd1 mRNA levels (**A**) and immunoblotting for the determination of Smyd1 protein levels (**B**) in pCMV2-Smyd1-flag vector (↑Smyd1) or Smyd1-specific siRNA transfected EA.hy926 cells. Expression values relative to control (transfection with vector lacking gene insert). $n = 3$, * $p < 0.05$, ** $p < 0.01$ using one-way ANOVA. Representative immunoblot of $n = 3$. (**C**,**D**) RT-qPCR for the quantification of IL-6 mRNA levels (**C**) and immunoblotting for the determination of IL-6 protein levels (**D**) in total lysates of EA.hy926 cells, which were transfected with either a pCMV2-Smyd1-flag vector (↑Smyd1) or a vector without specific gene insert (Control). Expression values relative to control. $n = 3$, ** $p < 0.01$ using Student *t*-test. Representative immunoblot of $n = 3$. (**E**) ELISA for the quantification of IL-6 protein concentrations in supernatants of EA.hy926 cells transfected with a pCMV2-Smyd1-flag vector (↑Smyd1) or a vector lacking a specific gene insert (Control) without or with LPS (1 µg/mL) stimulation for 24 h. $n = 7$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using two-way ANOVA. (**F**) ELISA for the quantification of IL-6 protein concentrations in supernatants of EA.hy926 cells simultaneously transfected with Smyd1-specific siRNA and stimulated with LPS (1 µg/mL) for 24 h or scrambled siRNA (Control). $n = 5$, * $p < 0.05$, *** $p < 0.001$ using two-way ANOVA. (**G**,**H**) RT-qPCR to quantify mRNA levels (**G**) and immunoblotting for the determination of protein levels (**H**) of Smyd1 in EA.hy926 cells that were stimulated with 10 ng/mL IL-6 for 3 h or 24 h. Expression values relative to control (Ctrl; no IL-6 stimulation). $n = 3$, * $p > 0.05$ using one-way ANOVA and representative immunoblot of $n = 3$. (**I**) RT-qPCR to quantify ICAM1 mRNA levels in EA.hy926 cells that were stimulated with 10 ng/mL IL-6 for 3 h or 24 h. Expression values relative to control (Ctrl;no IL-6 stimulation). $n = 3$, ** $p < 0.01$ using one-way ANOVA. All graphs reported as mean ± SD.

As shown in Figure 2E,F, ELISA was carried out to quantify IL-6 concentrations in supernatants of EA.hy926 cells that were collected 24 h after transfection with a vector

containing either the intact Smyd1 gene or without the specific gene insert (control) or Smyd1-specific siRNA or scrambled siRNA (control), each with simultaneous LPS stimulation. Compared to the supernatants of the control cells, IL-6 concentrations were higher ($p < 0.05$) in the supernatants of EA.hy926 cells either stimulated with LPS (+910%) or overexpressing Smyd1 (+1790%). The highest IL-6 levels were found in the supernatants of the EA.hy926 cells that were simultaneously LPS-stimulated and Smyd1-transfected ($p < 0.001$; +2990%; Figure 2E). In contrast, LPS stimulation led to lower IL-6 secretion ($p < 0.05$;− 45%; Figure 2F) if the EAhy926 cells were treated with Smyd1-siRNA compared to experimentally non-manipulated cells stimulated with LPS.

To uncover a possible feedback regulation of IL-6 on Smyd1 expression, we quantified the Smyd1 levels in EA.hy926 cells after 3 h and 24 h of IL-6 stimulation, respectively. As shown in Figure 2G,H, RT-qPCR and immunoblotting (Figure S1E) revealed that IL-6 did not influence ($p \geq 0.05$) Smyd1 expression levels (3 h: +7%, 24 h: +13%). In contrast, the expression of the established downstream target ICAM1 was up-regulated ($p < 0.01$) in IL-6 stimulated EA.hy926 cells (3 h: +56%, 24 h: +73%; Figure 2I).

3.3. Smyd1 Induces IL-6 Expression via Activation of NF-κB

To test the hypothesis that Smyd1 increases IL-6 expression via NF-κB, the role of Smyd1 in the activation of NF-κB was examined. Flow cytometry analysis revealed that EA.hy926 cells transfected with a Smyd1-containing vector expressed higher levels of the NF-κB subunit RelA/p65 (NF-κB-p65) than control EA.hy926 cells that were transfected with a vector without gene insert (Figure 3A).

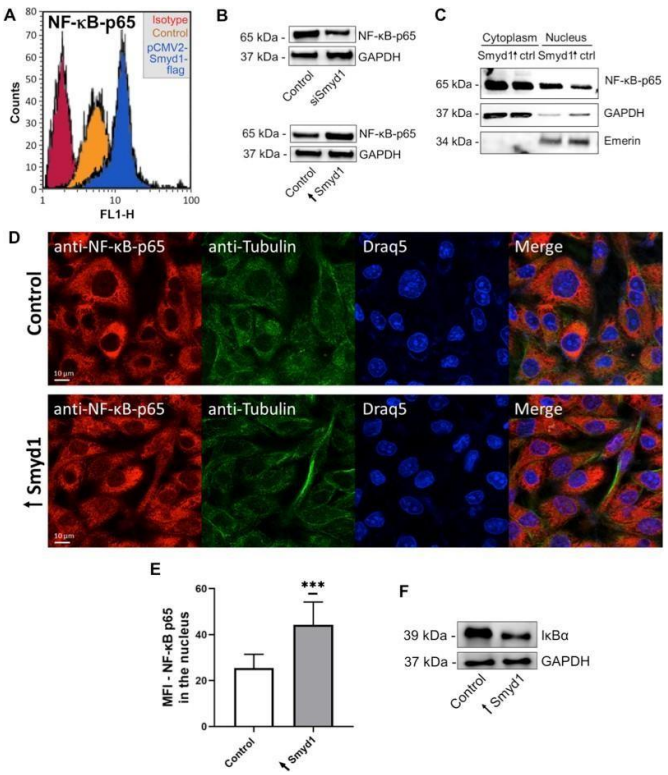


Figure 3. Smyd1 up-regulates and activates nuclear translocation of NF-κB in EA.hy926 cells. (**A**) Representative flow cytometry histograms of EA.hy926 cells transfected with a pCMV2-Smyd1- flag vector or a vector without gene insert (control) and incubated with a monoclonal antibody recognizing the p65 subunit of NF-κB and, subsequently, fluorescence-labeled secondary antibodies. An isotype-matched monoclonal IgG control antibody was used as control. (**B**) Immunoblotting for

To characterize the role of Smyd1 in modulating IL-6 expression in more detail, EA.hy926 cells were transfected with an IL-6 promoter-driven luciferase reporter gene in combination with Smyd1 expression constructs (Figure 5F). LPS stimulation or transfection with the active Smyd1 gene resulted in higher luciferase activity (LPS stimulation: $p < 0.05$; +81%; Smyd1 transfection: $p < 0.001$, +202%), whereas overexpression of the Smyd1 mutant was not associated with higher luciferase activity rates ($p \geq 0.05$, +35%). The effect of Smyd1 on NF-κB promoter activity was evaluated in transient transfection experiments with an NF-κB luciferase reporter construct (Figure 5G). If these EA.hy926 cells were exposed to1 µg/mL LPS for 24 h, the promoter activity of NF-κB was strongly induced ($p < 0.001$; +247%; Figure 5G). Strikingly, co-transfection with the Smyd 1 vector or the Smyd1 mutant also increased the NF-κB promoter activity compared to the control transfected cells (Smyd1 transfection: $p < 0.01$, +130%; Smyd1 SET Mut transfection: $p < 0.05$, +88%; Figure 5G).

In addition, the expression levels of established pro-inflammatory, NF-κB-dependent mediators were quantified in Ea.hy926 cells subjected to Smyd1 by RT-qPCR transfection. The experimental overexpression of Smyd1 and the methyltransferase-inactive mutant resulted in higher mRNA levels of ICAM1 (Smyd1 transfection: $p < 0.01$, +131%; Smyd1 SET Mut transfection: $p < 0.05$, +85%; Figure 5H), CCL2 (Smyd1 transfection: $p < 0.01$, +250%; Smyd1 SET Mut transfection: $p < 0.05$, +187%; Figure 5I) and VCAM1 (Smyd1 transfection: $p < 0.01$, +214%; Smyd1 SET Mut transfection: $p < 0.05$, +160%; data not shown).

3.6. Smyd1 Affects the H3K4me3 Methylation Pattern of the IL-6 Promoter

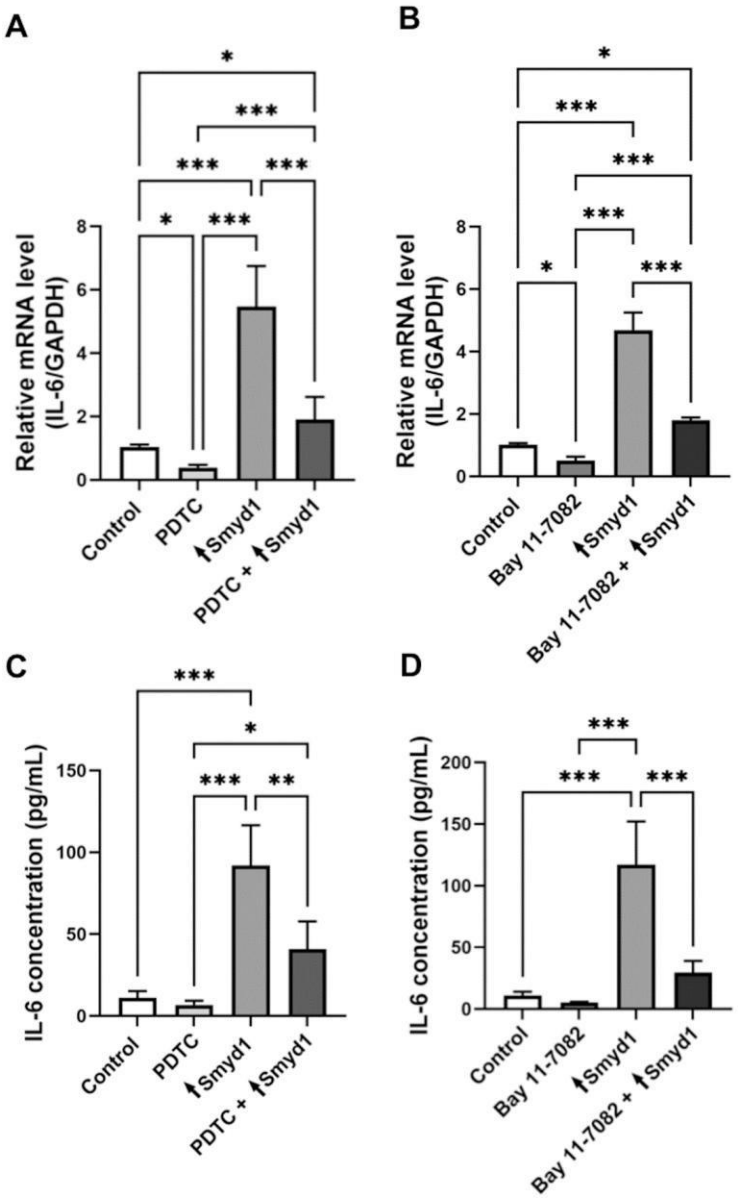
Since Smyd1 acts as an H3K4 histone methyltransferase, we next examined whether Smyd1 affects the methylation pattern of H3K4 within the IL-6 promoter region. Therefore, ChIP assays were used to evaluate the degree of H3K4 mono-, di- and trimethylation at three selected regions within the IL-6 promoter (IL-6 1, 2 or 3). EA.hy926 cells were transfected with an expression vector containing either the wild-type Smyd1 gene, a modified Smyd1 gene exhibiting a point mutation within the SET domain (extinguishing the histone methyltransferase activity) or without the specific gene insert as a negative control. Additional negative control experiments were performed using non-immune IgG. In addition, a representative active chromatin region (GAPDH) was included as a control in the ChIP RT-qPCR experiments (data not shown). Differences in the patterns of immunoprecipitated methylated proteins were not observed using the primers specific for region 1 (Figure 6A) within the distal region of the IL-6 promoter and region 3 (Figure 6C) downstream of the IL-6 downstream promoter element (DPE). In contrast, more H3K4 trimethylation events ($p < 0.001$, +572%; Figure 6B) were observed within the proximal IL-6 promoter region 2 (Figure 6B) in Smyd1-overexpressing cells compared with cells transfected with the Smyd1-SET mutant or the control vector. Co-stimulation of Smyd1-transfected EA.hy926 cells with LPS (1 µg/mL) for 24 h resulted likewise in an enrichment of H3K4 trimethylation within the proximal IL-6 promoter region 2 (LPS: $p < 0.05$, +150%; Figure 6E) but not in region 1 or region 3 ($p \geq 0.05$; Figure 6D,F). In addition, region 2 showed weak binding of Smyd1, which was slightly increased after LPS stimulation and also after simultaneous transfection of Smyd1 (LPS: $p < 0.05$, +88%; Figure 6G). Only a very weak interaction of Smyd1 was found with region 1 (data not shown) and no interaction with region 3 (data not shown).

the determination of NF-κB-p65 levels in total lysates of EA.hy926 cells transfected either with a vector lacking a specific gene insert (Control) or a pCMV2-Smyd1-flag vector (↑Smyd1) and scrambled siRNA (Control) or Smyd1-specific siRNAs (siSmyd1). Representative immunoblots of n = 3. (**C**) Immunoblotting for the determination of NF-κB-p65 subunit levels in nuclear and cytoplasmic fractions of EA.hy926 cells (isolated by NE-PER extraction kit) that were transfected with a pCMV2-Smyd1-flag vector (↑Smyd1) or a vector without gene insert (Ctrl) for 24 h. Blot matrices were probed with antibodies against NF-κB p65, Emerin (nuclear marker) and GAPDH (cytoplasmic marker). Representative immunoblot of n = 3. (**D**) Immunocytochemistry of EA.hy926 cells with anti-NF-κB subunit (red) and anti-Tubulin (green) antibodies 24 h after transfection with a vector without gene insert (Control; upper panel) or a pCMV2-Smyd1-flag vector (↑Smyd1; lower panel). Draq5 (blue) was used to counterstain the cell nuclei. Representative images of n = 3. (**E**) Mean fluorescence intensity (MFI) of NF-κB p65 cytochemical immunoreactivity (as shown in **D**) was densitometrically determined in the nucleus. n = 3 independent experiments each with 20 cell nuclei evaluated. *** $p < 0.001$ using Student t-test. (**F**) Immunoblotting for the determination of IκBα protein levels in total lysates of EA.hy926 cells transfected with a pCMV2-Smyd1-flag vector (↑Smyd1) or a vector without specific gene insert (Control). Representative immunoblot of n = 3. All graphs reported as mean ± SD.

The flow cytometry findings were validated by immunoblotting on total lysates from Smyd1-transfected EA.hy926 cells. As shown in Figures 3B and S1F,G, the knockdown of Smyd1 led to lower NF-κB p65 immunoblotting levels, whereas the overexpression of Smyd1 resulted in higher NF-κB p65 levels compared to the NF-κB p65 levels in the control cells. Interestingly, NF-κB p65 was particularly enriched in the nuclear fraction (Figures 3C and S1H) when lysates of EA.hy926 cells transfected with the Smyd1-gene-containing vector or the corresponding vector without gene insert were subjected to subcellular fractionation. Quantitative image analysis of immunocytochemical stainings also confirmed the NF-κB p65 nuclear translocation in Smyd1-overexpressing EA.hy926 cells (Figure 3D,E). Furthermore, Smyd1-overexpressing EA.hy926 cells showed lower IκBα protein levels than the control cells (Figures 3F and S1I).

3.4. Smyd1 Induces IL-6 Expression also Independently of NF-κB

In order to explore whether the influence of Smyd1 on IL-6 is exclusively mediated via NF-κB, EA.hy926 cells were transfected with vectors containing either the Smyd1 gene or without the specific gene insert in the presence/absence of the chemical NF-κB inhibitors PDTC and Bay-11-7082. Real-time RT-qPCR on lysates (Figure 4A,B) and ELISA analysis using supernatants (Figure 4C,D) revealed lower IL-6 expression ($p < 0.001$) and secretion ($p < 0.01$) in Smyd1-overexpressing cells after treatment of the cells with PDTC (real-timeRT-qPCR: 39.5%; ELISA: 43.3%) or Bay 11-7082 (real-time RT-qPCR: 56.9%; ELISA: 33.6%) compared to the untreated Smyd1-overexpressing cells. Accordingly, lower IL-6 expression ($p < 0.05$) and secretion were found in EA.hy926 cells not subjected to Smyd1 transfection but treated with PDTC (real-time RT-qPCR: 45.5%; ELISA: 60.5%; Figure 4A,C) or Bay 11-7082 (real-time RT-qPCR: 46.4%; ELISA: 48.4%; Figure 4B,D).



≥Figure 4. The inhibition of NF-κB activity does not completely abolish the increase of Smyd1 expression in EA.hy926 cells. (**A**,**B**) RT-qPCR for the quantification of IL-6 mRNA levels in EA.hy926 cell lysates transfected with a pCMV2-Smyd1-flag vector (↑Smyd1) and/or treated with the NF-κB inhibitors PDTC (10 μM) (**A**) or Bay 11-7082 (10 μM) (**B**). Expression values relative to the untreated control cell extracts $n = 3$, * $p < 0.05$, *** $p < 0.001$ using two-way ANOVA. (**C**,**D**) ELISA for the quantification of IL-6 concentrations in supernatants of EA.hy926 cells transfected with the pCMV2-Smyd1-flag vector (↑Smyd1) and/or treated with PDTC (10 μM) (**C**) or Bay 11-7082 (10 μM) (**D**). $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using two-way ANOVA. All graphs reported as mean ± SD.

3.5. The Methyltransferase Activity of Smyd1 Is Involved in the Regulation of IL-6 Expression

To investigate whether the methyltransferase activity of Smyd1 is involved in the regulation of IL-6 expression, EA.hy926 cells were transfected with a vector containing a gene insert that encodes a Smyd1 variant without methyltransferase activity due to a point mutation within the SET domain (Figures 5A,B and S1J). In contrast to the active Smyd1-overexpressing EA.hy926 cells, this methyltransferase-inactive mutant had only a non-significant ($p \geq 0.05$) impact on IL-6 expression, both at the mRNA level (Figure 5C) and the amount of secreted IL-6 (Figure 5D). Similar results were obtained for cells that were simultaneously Smyd1-transfected and LPS-stimulated (Figure 5E).

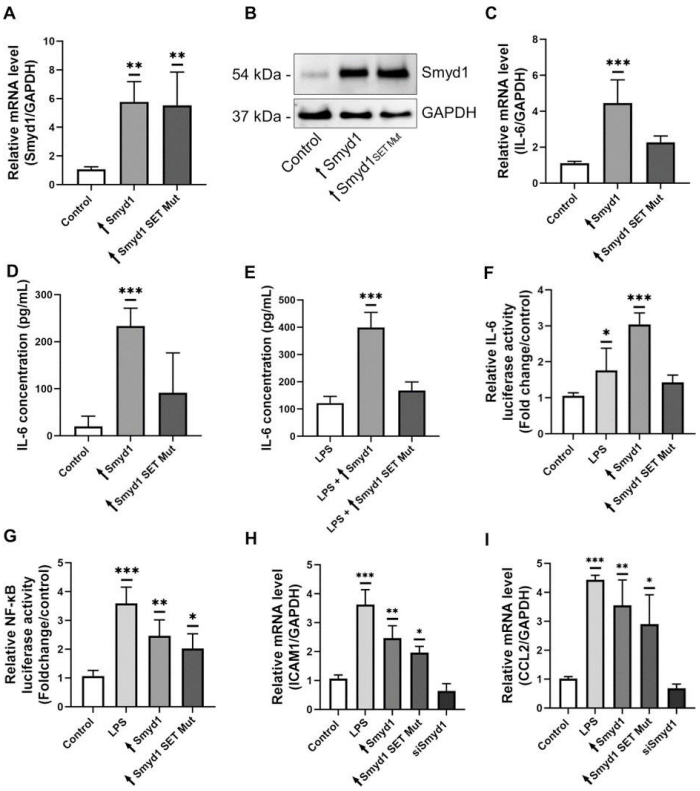


Figure 5. The methyltransferase activity of Smyd1 regulates IL-6 expression. (**A**,**B**) RT-qPCR for the quantification of Smyd1 mRNA levels (**A**) and immunoblotting for determination of Smyd1 protein levels (**B**) in total lysates of EA.hy926 cells transfected with the pCMV2-Smyd1-flag vector (↑Smyd1) or the Smyd1-SET-mutant vector (↑Smyd1 SET Mut). Expression values relative to control (transfection with vector lacking gene insert). $n = 3$, ** $p < 0.01$ using one-way ANOVA. Representative immunoblot of $n = 3$. (**C**) RT-qPCR for the quantification of IL-6 mRNA levels in EA.hy926 cells transfected with the pCMV2-Smyd1-flag vector (↑Smyd1) or the vector with a Smyd1 gene insert expressing a point mutation within the SET domain (↑Smyd1 SET mutant). Expression values relative to control (transfection with vector lacking gene insert). $n = 5$, *** $p < 0.001$ using one-way ANOVA. (**D**,**E**) ELISA for the determination of IL-6 concentrations in supernatants of non-stimulated (**D**) and

1 µg/mL LPS-stimulated (**E**) EA.hy926 cells transfected with a pCMV2-Smyd1-flag vector (↑Smyd1) or Smyd1-SET-mutant vector (↑Smyd1 SET Mut). $n = 5$, *** $p < 0.001$ using one-way ANOVA. (**F**) Relative luciferase activity of EA.hy926 cells transfected with a pBABE lucIL-6 reporter alone (Control), in combination with LPS stimulation (LPS) or co-transfected with the pCMV2-Smyd1-flag (↑Smyd1) or the vector with a mutated Smyd1 gene insert within the SET domain (↑Smyd1 SET Mut). Expression values relative to control. $n = 3$, * $p < 0.05$, *** $p < 0.001$ using one-way ANOVA. (**G**)

Relative luciferase activity of EA.hy926 cells transfected with a NF-κB luciferase reporter gene alone (Control), in combination with LPS stimulation (LPS) or co-transfected with a pCMV2-Smyd1-flag (↑Smyd1) or a Smyd1-SET-mutant vector (↑Smyd1 SET Mut) vector. Expression values relative to control. $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA. (**H**) RT-qPCR for the

quantification of ICAM1 mRNA levels in EA.hy926 cells stimulated with LPS (1 µg/mL) for 24 h or transfected with Smyd1-specific siRNA, a pCMV2-Smyd1-flag vector (↑Smyd1) or the Smyd1-SET-mutant vector (↑Smyd1 SET mutant). Expression values relative to control. $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA. (**I**) RT-qPCR for the quantification of CCL2 mRNA levels in EA.hy926 cells stimulated with LPS (1 µg/mL) for 24 h or transfected with Smyd1-specific siRNA, a pCMV2-Smyd1-flag vector (↑Smyd1) or the Smyd1-SET-mutant vector (↑Smyd1 SET mutant). Expression values relative to control (transfection with vector lacking gene insert). $n = 3$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ using one-way ANOVA. All graphs reported as mean ± SD.

As recently published [17], Smyd1 is detected in both the nucleus and cytoplasm of ECs. Whereas the nuclear fraction of the Smyd1 pool probably exerts transcriptional control mainly through epigenetic regulation (histone methyltransferase activity/recruitment of HDACs), Smyd1-dependent methylation of non-histone targets in the cytoplasm may play a role in cell regulation [10]. Because Smyd1protein levels were increased in both cellular compartments in response to LPS stimulation, exposition of ECs to this bacterial fragment might lead to changes in transcriptional activity as well as cell signaling.

RT-qPCR and immunoblot analyses revealed more IL-6 expression at the mRNA and the protein levels in Smyd1-transfected than in vector-transfected EA.hy926 cells. Furthermore, IL-6 was enriched in supernatants of EA.hy926 cells that were transfected with the vector encoding Smyd1 compared to those transfected with the vector lacking a gene insert. In contrast, the knockdown of Smyd1 expression caused less secretion of IL-6. Accordingly, the genetic loss of Smyd1 expression skeletal muscles resulted in a reduced expression of IL-6 in fetal mice prior to perinatal death [34]. Taken together, these findings imply that Smyd1 is an upstream regulator of IL-6 expression. Interestingly, IL-6 had no effect on Smyd1 expression, excluding a feedback interaction between these two proteins.

ChIP analysis demonstrated more H3K4 trimethylation, commonly associated with the activation of transcription of nearby genes, within the region 2 of the IL-6 promoter in Smyd1-overexpressing EA.hy926 cells than in cells transfected with a mutant Smyd1 form exhibiting a point mutation within the SET domain (abolishing the histone methyl-transferase activity) or in cells transfected with the vector lacking a specific gene insert. Strikingly, higher enrichment of H3K4 trimethylation within region 2 of the IL-6 promoter was detected in EA.hy926 cells stimulated with LPS. These data suggest that LPS triggers IL6-related immune responses, at least in part via up-regulation of Smyd1. A relationship between Smyd1 and the degree of methylation of H3K4 was previously demonstrated in heart and skeletal muscle cells [35–39]. Accordingly, SMYD1 elevates the levels of PGC-1α, a major regulator of mitochondrial biogenesis, in the adult heart of mice [39], and Isl1, a transcription factor important for embryogenesis, in the embryonic heart of mice [38] via H3K4me3 methylation in the corresponding promoter region.

EA.hy926 cells overexpressing the Smyd1 form with mutated SET domain showed lower IL-6 mRNA and protein levels as well as lower IL-6 promoter activity when compared to active Smyd1-overexpressing EA.hy926 cells. These data also suggest that the methyltransferase activity of Smyd1 is involved in the increase in IL-6 expression in ECs, which was not clearly the case for other LPS-induced pro-inflammatory mediators testedin this study, such as ICAM1, VCAM1 and CCL2.

The findings of the ChIP RT-qPCR assays suggest that Smyd1-formed H3K4me3 interacts with region 2 (but not with regions 1 and 3) of the IL-6 promoter to induce higher I-6 transcription rates. However, analysis using the UCSC Genome Browser revealed that regions 2 and 3 represent potential binding sites for nucleosomes trimethylated on histone-3. There are different possibilities to explain this contradiction. (1) The rate of H3K4 trimethylation is directly related to the local degree of methylation of the DNA [40]. For example, it was found that H3K4me3 is preferentially located at non-methylated CpG sites in CpG islands of promoters, which causes subsequent induction of the transcription of downstream genes. It is unclear how the UCSC Genome Browser takes into account that the DNA in the three regions can be dynamically methylated. (2) The H3K4me3/CpG island interaction involves other molecules, such as methyl-CpG-binding protein-2 [32] or Cfp1 [41]. If these proteins were located differently in the three DNA regions within the IL-6 promoter, it would be possible that more H3K4me3-modified nucleosomes are bound in region 2 and fewer in region 3 than predicted. (3) The stability of the H3K4me3 posttranslational modification depends on the activity of several histone deacetylases (HDACs; reviewed in 2019 [42]). If the concentration/activity of the HDACs was different between the three IL-6 regions, the stability and turnover of the H3K4 trimethylation label at the promoter would differ, which consequently could lead to variations in the IL-6 transcription rate. (4) Nucleosomes with H3K4me3 modification lead, depending on

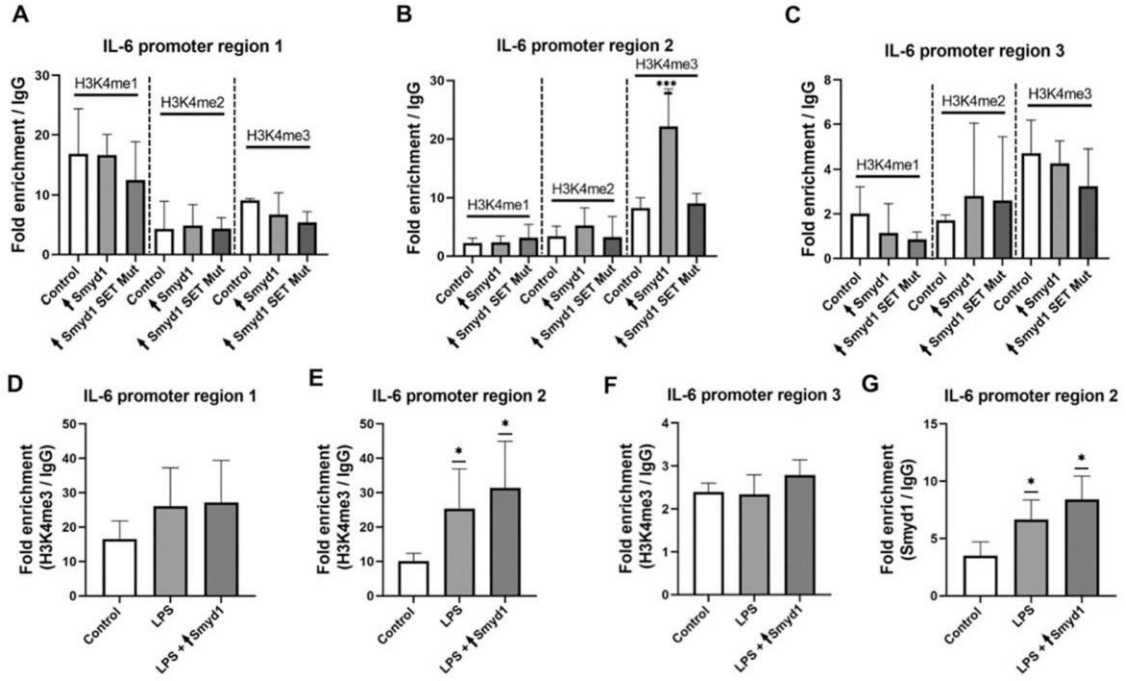


Figure 6. Smyd1 increases the H3K4me3 methylation status in the region 2 upstream of the transcription start site (TSS) within the IL-6 promoter. (**A**–**C**) ChIP assays were used to assess the degree of methylation of histone H3 atK4 (H3K4me1, H3K4me2 and H3K4me3) within the regions 1-3 of the IL-6 promoter in EA.hy926 cells that were transfected with either a pCMV2-Smyd1-flag vector (↑Smyd1) or a vector with a mutant Smyd1 gene lacking methylation activity due to a point mutation within the SET domain (↑Smyd1 SET mutant). An unrelated methylation-susceptible chromatin region with the GAPDH gene was included as a control in the ChIP-qPCR experiments. Three regions were selected: region 1 (−1469 to −1238; (**A**)), region 2 (−638 to −346; (**B**)) and region 3 (+150 to +347; (**C**)). The results were normalized to the negative (*IgG*) sample. $n = 4$. *** $p < 0.001$ using one-way ANOVA. (**D**–**F**) ChIP assays were used to assess the enrichment of the methylation site H3K4me3 within the IL-6 promoter in EA.hy926 cells that were transfected with a pCMV2-Smyd1-flag vector alone (↑Smyd1) or in combination with LPS (1 µg/mL) stimulation for 24 (LPS + ↑Smyd1). $n = 3$. * $p < 0.05$ using one-way ANOVA. (**G**), ChIP-qPCR for the Smyd1 level within the region 2 of the IL-6 promotor. $n = 3$. * $p < 0.05$ using one-way ANOVA. Data are shown as mean ± SD.

4. Discussion

This study aimed to investigate whether LPS mediates IL-6 up-regulation in ECs by involving the methyltransferase Smyd1. Major observations were as follows: (1) LPS stimulation of HUVECs and EA.hy926 cells resulted in higher Smyd1 mRNA and protein levels, (2) Smyd1 increased the expression of IL-6 in EA.hy926 cells and its secretion without a feedback effect of IL-6 on the Smyd1 expression, (3) Smyd1 influenced IL-6 expression partly via NF-κB and partly independently of NF-κB, and (4) the methyltransferase activity of Smyd1 led to more H3K4me3 methylation events within the IL-6 promoter.

The intrinsic Smyd1 expression at the mRNA and the protein levels was higher in HUVECs and EA.hy926 cells stimulated with LPS for 3 h and 24 h than in non-stimulated cells. These findings indicate that LPS induced Smyd1 up-regulation, which is, therefore, a downstream target of the LPS signaling cascade in ECs. Using a similar methodological approach, Smyd5 was previously identified to be part of the LPS signaling cascade in macrophages [13].

Interestingly, the Smyd1 expression level was higher after 3 h than after 24 h of LPS stimulation. A comparable time course in Smyd1 up-regulation was observed when ECs were incubated with the proinflammatory cytokine INF-γ for 3 h and 24 h [17]. We suggest that there is a molecular mechanism that terminates the induction of Smyd1 expression after a few hours, which is mediated by immunomodulatory molecules.

the location within the three regions of the IL-6 promoter, to the activation of different subsets of transcription factors and thus, possibly different IL-6 transcription rates. The promoter region of the human IL-6 gene is very complex [33]: it contains a TATA box in the core promoter region, several binding sites for transcription factors, such as NF-κB, SP (specificity protein)1, AP-1 (activator-protein-1), CREB (cyclic AMP-responsive element-binding protein), and C/EBP (CCAAT-enhancer-binding protein) in the proximal promoter region and binding sites for methylated histones and MeCPs in the distal promoter region. A summarizing list of transcription factors predicted by the UCSC Genome Browser is added as Table S1.

The transcription factor nuclear factor-kappa B (NF-κB) is a molecule that has an established impact on IL-6 expression and secretion in ECs [43]. To give an overview, stimulation with LPS leads to the activation of a receptor complex consisting of Toll-like receptor (TLR) 4, soluble CD14 and MD2 in ECs [44,45]. As a result, intracellular signaling pathways are up-regulated, leading to the activation of NF-κB in a complex process that is realized in two phases in most cells [44,45]. In an early activation process dependent on MyD88, LPS binding to the receptor complex induces the recruitment of several adaptor molecules (TIRAP, IRAK1 and IRAK4) that then activates the IKK complex, which finally leads to the proteolysis of members of the inhibitor of the NF-κB (IκB) family [44,46]. If these NF-κB inhibitors are absent due to their degradation, NF-κB is translocated to the nucleus, where it subsequently accumulates [47]. As a result, the transcriptional activity of NF-κB is enhanced to produce high mRNA levels of pro-inflammatory mediators, such as IL-6. After translation and secretion, these mediators can ultimately cause cell injury and sepsis [45]. A second pathway that leads to a late-phase, MyD88-independent activation of NF-κB in response to LPS binding, has not yet been observed in ECs [45]. It therefore remains to be clarified whether, and if so, at which step, Smyd1 converges with this NF-κB signal cascade.

To investigate whether Smyd1 influences IL-6 induction via NF-κB, we quantified the NF-κB levels in EA.hy926 cells transfected with Smyd1-expressing plasmids or Smyd1- specific siRNAs. Actually, both NF-κB expression and IL-6 transcriptional activity were higher in the Smyd1-overexpressing and lower in the Smyd1-downregulated (siRNA) cells when compared to the control vector-transfected cells. Furthermore, the nuclear accumulation and promoter activity of NF-κB were higher in the Smyd1-overexpressing ECs and lower in the Smyd1-downregulated ECs than in the control vector-transfected ECs. In contrast, IkBα levels were lower in the Smyd1-overexpressing ECs. These ex- perimental data consistently suggest that Smyd1 is an upstream signaling molecule to increase the nuclear concentration and the transcriptional activity of NF-κB in ECs. How- ever, because the promoter activity of NF-κB was also higher in cells transfected with the methyltransferase-inactive Smyd1 mutant, the effect on NF-κB appears to be independent of the methyltransferase activity of Smyd1.

Strikingly, the expression and secretion of IL-6 in EA.hy.926 cells incubated with two established chemical NF-κB blockers (PDTC and Bay 11-7082) was lower than in Smyd1-overexpressing ECs but still higher than in transfected control ECs. These findings imply that the effect of Smyd1 on IL-6 expression was not completely abolished by the chemical NF-κB inhibition and suggest that there is an additional molecular mechanism without NF-κB contribution by which Smyd1 affects IL-6 expression in ECs. In summary, our experimental findings allow the conclusion that Smyd1 up-regulates IL-6 transcription in ECs simultaneously by an NF-κB-dependent and an NF-κB-independent mechanism.

In other cell systems, several molecular signaling pathways were identified through which IL-6 expression is induced without NF-κB involvement. (1) The transcription factor CCAAT-enhancer-binding protein delta (C/EBPδ) increased the IL-6 transcription in breast cancer cells [48] presumably by binding to one or both C/EBP binding sites within the IL-6 promoter region [49]. (2) The NF-κB-independent transcription factors CREB, AP-1and C/EBP induced strong IL-6 mRNA up-regulation in mouse calvarial osteoblasts [50]. (3) The zinc-finger transcription factor Kruppel-like factor 4 (KLF4) has a dual function

in the up-regulation of IL-6 in murine bone marrow-derived dendritic cells: in order to facilitate transcription, KLF4 binds to specific binding sites within the IL-6 promoter and also plays a role in chromatin remodeling at the IL-6 promoter region [51]. (4) Several examples demonstrated that the density of DNA methylation events within the IL-6 promoter contributes to the activation of IL-6 mRNA production. In rat hepatoma cells, loss of methylation events in the IL-6/STAT3 promoter was related to higher IL-6 levels and higher proliferation rates [52]. DNA hypomethylation was observed in the IL-6 promoter regions in synovial fibroblasts from osteoarthritis patients compared with those from non-diseased study participants [34]. The degree of DNA methylation influences the binding of methyl CpG binding proteins (MeCPs), which mediate the interaction with methylated histones [53]. (5) Histone modifications are also known to be involved in IL- 6 gene expression [32,34,54,55]. In macrophages, epigenetic histone acetylation [54] and H3K36 di-methylation [55] regulate the transcription frequency of the IL-6 gene. Two mechanisms have been identified as to how these posttranslational modifications of histones alter transcriptional activity: (a) The chemical group attached to one histone can change the conformation of the complete nucleosome by directly increasing/reducing the accessibility of a DNA promoter for the binding of the RNA polymerase transcription complex. (b) Alternatively, an interaction of a modulating factor with the modified histone can indirectly influence transcription frequency, e.g., H3K4me3 was found to activate a large number of transcription factors, such as AP-1 [56], via interaction with the nucleosomeremodeling factor (NURF) complex [57,58]. Considering the fact that the trimethylation of lysine 4 in histone 3 (H3K4me3) increased the IL-6 transcription rates in our study, we speculate that the histone methyltransferase is crucial for the mediation of the NF-κB- independent influence on the IL-6 expression in ECs. However, it is currently not possible to determine whether H3K4me3 directly or indirectly changes the IL-6 transcriptional activity in this cell system.

In summary, our results reveal that Smyd1, as a previously unknown epigenetic modulator in human ECs, affects LPS-induced IL-6 expression and secretion in two ways: Firstly, through enhanced methylation of H3K4me3 and secondly through activation of the NF-κB signaling pathway (Figure 7). Since the IL-6 is crucial for the progression of acute inflammatory diseases, such as sepsis, this cytokine represents an important molecule within the LPS-signaling cascade. Our study, in addition to the emerging role in heart disease, suggests that Smyd1 may be of therapeutic interest. In addition to developing a specific Smyd1 inhibitor, future research should, therefore focus on uncovering additional target genes and identifying specific histone methylation sites of Smyd1.

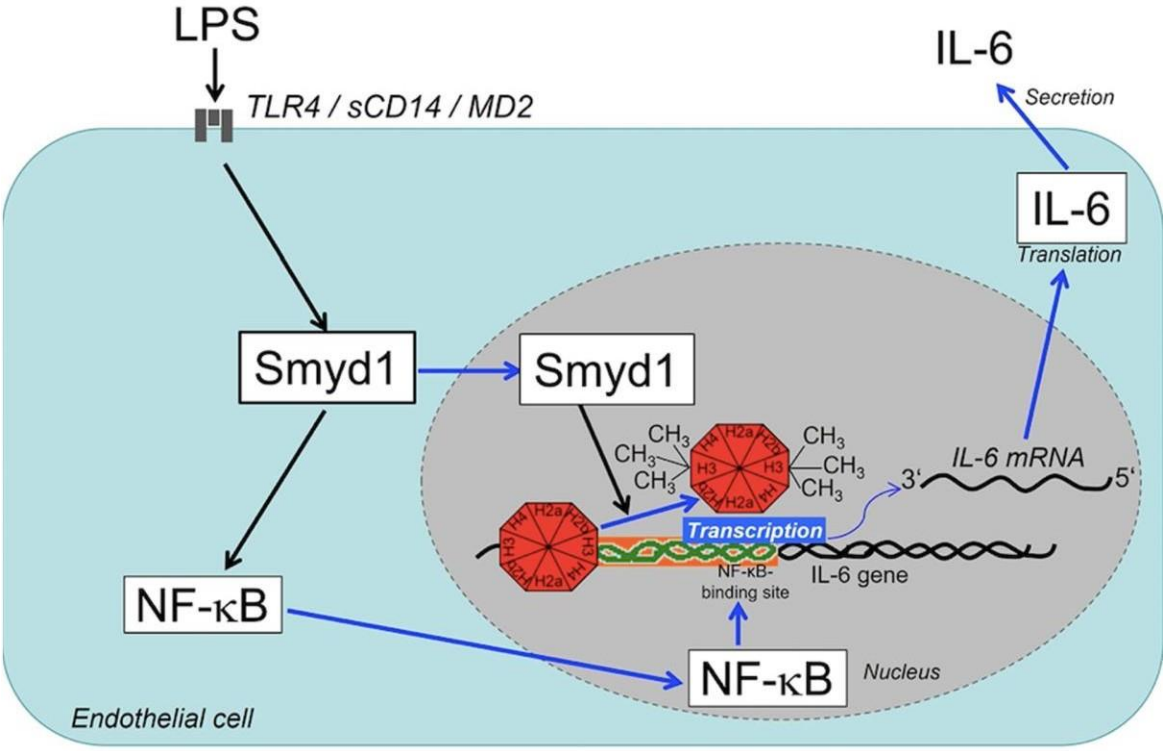


Figure 7. Schematic summary of the molecular model deduced from the experimental findings of this study. By bindingto its specific receptor complex on the cell surface, LPS induces an up-regulation of Smyd1 in ECs. The corresponding intracellular increase of Smyd1 levels results in the activation of two signaling cascades. (1) More Smyd1 presence in the cytoplasm causes more frequent translocation of Smyd1 molecules into the cell nucleus. Enrichment of the methyltransferase Smyd1 in the nucleus results then in trimethylation of lysine 4 in histone 3 (H3K4me3) at region2 of the IL-6 promoter. This epigenetik modification leads to better accessibility of the promoter for the RNA polymerase transcription complex, so that the IL-6 transcription rate is increased. 2. More Smyd1 presence in the cytoplasm accompanied by IκBα down-regulation also up-regulates NF-κB expression leading to a larger cytoplasmic NF-κB pool. Subsequently, more NF-κB molecules diffuse into the nucleus, where NF-κB binds to a specific site within the promoter sequence (green-orange) of the IL-6 gene.As a consequence of the double activation of the promoter region, IL-6 transcription, as well as subsequent translation and secretion of the IL-6 protein, are accelerated resulting in higher IL-6 bioavailability. Black arrows indicate up-regulation, while blue arrows stand for intracellular transport.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cells10123515/s1, Figure S1: Densitometric quantification of tested protein expression levels on immunoblots, Supplementary Table S1: Compilation of the transcription factors that bind to the human IL-6 promoter sequences of the three DNA regions analyzed in the ChIP analysis, as calculated in the UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38).

Author Contributions: Conceptualization, J.B. (Janine Berkholz) and A.Z.; validation, G.S., C.H.L., K.R. and J.B. (Jonathan Bernd); formal analysis, A.S., G.S. and J.B. (Janine Berkholz); investigation, A.S., G.S., C.H.L. and J.B. (Janine Berkholz); resources, T.B.; writing—original draft preparation, A.S., G.S., K.R. and J.B. (Janine Berkholz); writing—review and editing, K.R. and J.B. (Janine Berkholz); visualization, J.B. (Janine Berkholz); supervision, J.B. (Janine Berkholz) and A.Z.; project administra- tion, J.B. (Janine Berkholz); funding acquisition, J.B. (Janine Berkholz). All authors have read and agreed to the published version of the manuscript.

Funding: This project was supported by the German Research Foundation to J.B. (Janine Berkholz) (6697/2-1) and to T.B. (BR2925/10-1 and BR2925/11-1). Work in the authors' laboratory is furthermore supported by the Sonnenfeld Stiftung.

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Charité—Universitätsmedizin Berlin, Germany (EA4/107/17).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: All data generated or analyzed during this study are included in this published article.

Acknowledgments: We thank Angela Becker for excellent technical assistance, PD Oliver Baum for helpful discussions and Haley O. Tucker for pBK-CMV-Smyd1$_{HMTase\ mutant}$.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Qian, C.; Zhou, M.-M. SET domain protein lysine methyltransferases: Structure, specificity and catalysis. *Cell Mol. Life Sci.* **2006**, *63*, 2755–2763. [CrossRef]
2. Dillon, S.C.; Zhang, X.; Trievel, R.C.; Cheng, X. The SET-domain protein superfamily: Protein lysine methyltransferases. *Genome Biol.* **2005**, *6*, 1–10. [CrossRef]
3. Spellmon, N.; Holcomb, J.; Trescott, L.; Sirinupong, N.; Yang, Z. Structure and function of SET and MYND domain-containing proteins. *Int. J. Mol. Sci.* **2015**, *16*, 1406–1428. [CrossRef] [PubMed]
4. Tracy, C.M.; Warren, J.S.; Szulik, M.; Wang, L.; Garcia, J.; Makaju, A.; Russell, K.; Miller, M.; Franklin, S. The Smyd familyof methyltransferases: Role in cardiac and skeletal muscle physiology and pathology. *Curr. Opin. Physiol.* **2018**, *1*, 140–152. [CrossRef]
5. Huang, L.; Xu, A.-M. SET and MYND domain containing protein 3 in cancer. *Am. J. Transl. Res.* **2017**, *9*, 1.
6. Brown, M.A.; Sims, R.J.; Gottlieb, P.D.; Tucker, P.W. Identification and characterization of Smyd2: A split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase that interacts with the Sin3 histone deacetylase complex. *Mol. Cancer* **2006**, *5*, 1–11. [CrossRef] [PubMed]
7. Bannister, A.J.; Kouzarides, T. Regulation of chromatin by histone modifications. *Cell Res.* **2011**, *21*, 381–395. [CrossRef]
8. Strahl, B.D.; Allis, C.D. The language of covalent histone modifications. *Nature* **2000**, *403*, 41–45. [CrossRef]
9. Sims, R.J.; Weihe, E.K.; Zhu, L.; O'Malley, S.; Harriss, J.V.; Gottlieb, P.D. m-Bop, a repressor protein essential for cardiogenesis, interacts with skNAC, a heart-and muscle-specific transcription factor. *J. Biol. Chem.* **2002**, *277*, 26524–26529. [CrossRef] [PubMed]
10. Rasmussen, T.L.; Ma, Y.; Park, C.Y.; Harriss, J.; Pierce, S.A.; Dekker, J.D.; Valenzuela, N.; Srivastava, D.; Schwartz, R.J.; Stewart, M.D. Smyd1 facilitates heart development by antagonizing oxidative and ER stress responses. *PLoS ONE* **2015**, *10*, e0121765. [CrossRef]
11. Franklin, S.; Kimball, T.; Rasmussen, T.L.; Rosa-Garrido, M.; Chen, H.; Tran, T.; Miller, M.R.; Gray, R.; Jiang, S.; Ren, S. The chromatin-binding protein Smyd1 restricts adult mammalian heart growth. *Am. J. Physiol. Heart Circ. Physiol.* **2016**, *311*, H1234–H1247. [CrossRef]
12. Xu, S.; Wu, J.; Sun, B.; Zhong, C.; Ding, J. Structural and biochemical studies of human lysine methyltransferase Smyd3 reveal the important functional roles of its post-SET and TPR domains and the regulation of its activity by DNA binding. *Nucleic Acids Res.* **2011**, *39*, 4438–4449. [CrossRef] [PubMed]
13. Stender, J.D.; Pascual, G.; Liu, W.; Kaikkonen, M.U.; Do, K.; Spann, N.J.; Boutros, M.; Perrimon, N.; Rosenfeld, M.G.; Glass, C.K. Control of proinflammatory gene programs by regulated trimethylation and demethylation of histone H4K20. *Mol. Cell* **2012**, *48*, 28–38. [CrossRef]
14. Thompson, E.C.; Travers, A.A. A Drosophila Smyd4 homologue is a muscle-specific transcriptional modulator involved in development. *PLoS ONE* **2008**, *3*, e3008. [CrossRef] [PubMed]
15. Sirinupong, N.; Brunzelle, J.; Ye, J.; Pirzada, A.; Nico, L.; Yang, Z. Crystal structure of cardiac-specific histone methyltransferase SmyD1 reveals unusual active site architecture. *J. Biol. Chem.* **2010**, *285*, 40635–40644. [CrossRef] [PubMed]
16. Ye, X.; Qian, Y.; Wang, Q.; Yuan, W.; Mo, X.; Li, Y.; Jiang, Z.; Xu, W.; Deng, Y.; Wan, Y. SMYD1, an SRF-interacting partner, is involved in angiogenesis. *PLoS ONE* **2016**, *11*, e0146468.
17. Becker, S.; Steinemann, G.; Karle, W.; Roos, K.; Liem, C.H.; Muralikumar, S.; Volkamer, A.; Munz, B.; Zakrzewicz, A.; Berkholz, J. Stability of Smyd1 in endothelial cells is controlled by PML-dependent SUMOylation upon cytokine stimulation. *Biochem. J.* **2021**, *478*, 217–234. [CrossRef]
18. Medzhitov, R. Toll-like receptors and innate immunity. *Nature Rev. Immunol.* **2001**, *1*, 135–145. [CrossRef]
19. Lush, C.W.; Cepinskas, G.; Kvietys, P.R. LPS tolerance in human endothelial cells: Reduced PMN adhesion, E-selectin expression, and NF-κB mobilization. *Am. J. Physiol. Heart Circ. Physiol.* **2000**, *278*, H853–H861. [CrossRef]
20. Wong, D.; Dorovini-Zis, K. Upregulation of intercellular adhesion molecule-1 (ICAM-1) expression in primary cultures of human brain microvessel endothelial cells by cytokines and lipopolysaccharide. *J. Neuroimmunol.* **1992**, *39*, 11–21. [CrossRef]
21. Bozza, F.A.; Salluh, J.I.; Japiassu, A.M.; Soares, M.; Assis, E.F.; Gomes, R.N.; Bozza, M.T.; Castro-Faria-Neto, H.C.; Bozza, P.T. Cytokine profiles as markers of disease severity in sepsis: A multiplex analysis. *Crit. Care* **2007**, *11*, 1–8. [CrossRef] [PubMed]
22. Jirik, F.; Podor, T.; Hirano, T.; Kishimoto, T.; Loskutoff, D.; Carson, D.; Lotz, M. Bacterial lipopolysaccharide and inflammatory mediators augment IL-6 secretion by human endothelial cells. *J. Immunol.* **1989**, *142*, 144–147.

23. Bannerman, D.D.; Sathyamoorthy, M.; Goldblum, S.E. Bacterial lipopolysaccharide disrupts endothelial monolayer integrity and survival signaling events through caspase cleavage of adherens junction proteins. *J. Biol. Chem.* **1998**, *273*, 35371–35380. [CrossRef]
24. Hotchkiss, R.S.; Tinsley, K.W.; Swanson, P.E.; Karl, I.E. Endothelial cell apoptosis in sepsis. *Crit. Care Med.* **2002**, *30*, S225–S228. [CrossRef] [PubMed]
25. Pawlinski, R.; Mackman, N. Tissue factor, coagulation proteases, and protease-activated receptors in endotoxemia and sepsis. *Crit. Care Med.* **2004**, *32*, S293–S297. [CrossRef]
26. Pinsky, M.R. Dysregulation of the immune response in severe sepsis. *Am. J. Med Sci.* **2004**, *328*, 220–229. [CrossRef] [PubMed]
27. Volk, T.; Kox, W. Endothelium function in sepsis. *Inflamm. Res.* **2000**, *49*, 185–198. [CrossRef]
28. Maroski, J.; Vorderwülbecke, B.J.; Fiedorowicz, K.; Da Silva-Azevedo, L.; Siegel, G.; Marki, A.; Pries, A.R.; Zakrzewicz, A. Shear stress increases endothelial hyaluronan synthase 2 and hyaluronan synthesis especially in regard to an atheroprotective flow profile. *Exp. Physiol.* **2011**, *96*, 977–986. [CrossRef]
29. Berkholz, J.; Kuzyniak, W.; Hoepfner, M.; Munz, B. Overexpression of the skNAC gene in human rhabdomyosarcoma cells enhances their differentiation potential and inhibits tumor cell growth and spreading. *Clin. Exp. Metastasis.* **2014**, *31*, 869–879. [CrossRef] [PubMed]
30. Alspach, E.; Flanagan, K.C.; Luo, X.; Ruhland, M.K.; Huang, H.; Pazolli, E.; Donlin, M.J.; Marsh, T.; Piwnica-Worms, D.; Monahan, J. p38MAPK plays a crucial role in stromal-mediated tumorigenesis. *Cancer. Discov.* **2014**, *4*, 716–729. [CrossRef]
31. Dandrea, M.; Donadelli, M.; Costanzo, C.; Scarpa, A.; Palmieri, M. MeCP2/H3meK9 are involved in IL-6 gene silencing in pancreatic adenocarcinoma cell lines. *Nucleic Acids Res.* **2009**, *37*, 6681–6690. [CrossRef]
32. Poplutz, M.K.; Wessels, I.; Rink, L.; Uciechowski, P. Regulation of the Interleukin-6 gene expression during monocytic differentia- tion of HL-60 cells by chromatin remodeling and methylation. *Immunobiology* **2014**, *219*, 619–626. [CrossRef]
33. Yang, F.; Zhou, S.; Wang, C.; Huang, Y.; Li, H.; Wang, Y.; Zhu, Z.; Tang, J.; Yan, M. Epigenetic modifications of interleukin-6 in synovial fibroblasts from osteoarthritis patients. *Sci. Rep.* **2017**, *7*, 1–11. [CrossRef]
34. Rasmussen, T.L.; Tucker, H.O. Loss of SMYD1 Results in Perinatal Lethality via Selective Defects within Myotonic Muscle Descendants. *Diseases* **2019**, *7*, 1. [CrossRef] [PubMed]
35. Gottlieb, P.D.; Pierce, S.A.; Sims, R.J.; Yamagishi, H.; Weihe, E.K.; Harriss, J.V.; Maika, S.D.; Kuziel, W.A.; King, H.L.; Olson, E.N. Bop encodes a muscle-restricted protein containing MYND and SET domains and is essential for cardiac differentiation and morphogenesis. *Nat. Genet.* **2002**, *31*, 25–32. [CrossRef] [PubMed]
36. Tan, X.; Rotllant, J.; Li, H.; DeDeyne, P.; Du, S.J. SmyD1, a histone methyltransferase, is required for myofibril organization and muscle contraction in zebrafish embryos. *Proc. Nat. Acad. Sci. USA* **2006**, *103*, 2713–2718. [CrossRef]
37. Berkholz, J.; Orgeur, M.; Stricker, S.; Munz, B. skNAC and Smyd1 in transcriptional control. *Exp. Cell Res.* **2015**, *336*, 182–191. [CrossRef] [PubMed]
38. Wang, Z.; Schwartz, R.J.; Liu, J.; Sun, F.; Li, Q.; Ma, Y. Smyd1 Orchestrates Early Heart Development Through Positive and Negative Gene Regulation. *Front. Cell Dev. Biol.* **2021**, *9*, 500. [CrossRef] [PubMed]
39. Warren, J.S.; Tracy, C.M.; Miller, M.R.; Makaju, A.; Szulik, M.W.; Oka, S.-i.; Yuzyuk, T.N.; Cox, J.E.; Kumar, A.; Lozier, B.K. Histone methyltransferase Smyd1 regulates mitochondrial energetics in the heart. *Proc. Nat. Acad. Sci. USA* **2018**, *115*, E7871–E7880. [CrossRef] [PubMed]
40. Rose, N.R.; Klose, R.J. Understanding the relationship between DNA methylation and histone lysine methylation. *Biochim. Biophys. Acta* **2014**, *1839*, 1362–1372. [CrossRef]
41. Thomson, J.P.; Skene, P.J.; Selfridge, J.; Clouaire, T.; Guy, J.; Webb, S.; Kerr, A.R.; Deaton, A.; Andrews, R.; James, K.D. CpG islands influence chromatin structure via the CpG-binding protein Cfp1. *Nature* **2010**, *464*, 1082–1086. [CrossRef]
42. Porter, N.J.; Christianson, D.W. Structure, mechanism, and inhibition of the zinc-dependent histone deacetylases. *Curr. Opin. Struct. Biol.* **2019**, *59*, 9. [CrossRef]
43. Brasier, A.R. The nuclear factor-κB–interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc. Res.* **2010**, *86*, 211–218. [CrossRef]
44. Pålsson-McDermott, E.M.; O'Neill, L.A. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* **2004**, *113*, 153–162. [CrossRef] [PubMed]
45. Dauphinee, S.M.; Karsan, A. Lipopolysaccharide signaling in endothelial cells. *Lab. Investig.* **2006**, *86*, 9–22. [CrossRef] [PubMed]
46. Chen, Z.; Hagler, J.; Palombella, V.J.; Melandri, F.; Scherer, D.; Ballard, D.; Maniatis, T. Signal-induced site-specific phosphorylation targets I kappa B alpha to the ubiquitin-proteasome pathway. *Genes. Dev.* **1995**, *9*, 1586–1597. [CrossRef]
47. Brown, K.; Park, S.; Kanno, T.; Franzoso, G.; Siebenlist, U. Mutual regulation of the transcriptional activator NF-kappa B and its inhibitor, I kappa B-alpha. *Proc. Nat. Acad. Sci. USA* **1993**, *90*, 2532–2536. [CrossRef]
48. Balamurugan, K.; Mendoza-Villanueva, D.; Sharan, S.; Summers, G.H.; Dobrolecki, L.E.; Lewis, M.T.; Sterneck, E. C/EBPδ links IL-6 and HIF-1 signaling to promote breast cancer stem cell-associated phenotypes. *Oncogene* **2019**, *38*, 3765–3780. [CrossRef] [PubMed]
49. Hungness, E.S.; Luo, G.j.; Pritts, T.A.; Sun, X.; Robb, B.W.; Hershko, D.; Hasselgren, P.O. Transcription factors C/EBP-β and-δ regulate IL-6 production in IL-1β-stimulated human enterocytes. *J. Cell Physiol.* **2002**, *192*, 64–70. [CrossRef]

50. Persson, E.; Voznesensky, O.S.; Huang, Y.-F.; Lerner, U.H. Increased expression of interleukin-6 by vasoactive intestinal peptide is associated with regulation of CREB, AP-1 and C/EBP, but not NF-κB, in mouse calvarial osteoblasts. *Bone* **2005**, *37*, 513–529. [CrossRef] [PubMed]
51. Rosenzweig, J.M.; Glenn, J.D.; Calabresi, P.A.; Whartenby, K.A. KLF4 modulates expression of IL-6 in dendritic cells via both promoter activation and epigenetic modification. *J. Biol. Chem.* **2013**, *288*, 23868–23874. [CrossRef]
52. Jung, J.; Moon, J.W.; Choi, J.-H.; Lee, Y.W.; Park, S.-H.; Kim, G.J. Epigenetic alterations of IL-6/STAT3 signaling by placental stem cells promote hepatic regeneration in a rat model with CCl4-induced liver injury. *Int. J. Stem. Cells* **2015**, *8*, 79. [CrossRef] [PubMed]
53. Lee, W.; Kim, J.; Yun, J.-M.; Ohn, T.; Gong, Q. MeCP2 regulates gene expression through recognition of H3K27me3. *Nat. Commun.* **2020**, *11*, 1–16. [CrossRef] [PubMed]
54. Hu, L.; Yu, Y.; Huang, H.; Fan, H.; Hu, L.; Yin, C.; Li, K.; Fulton, D.J.; Chen, F. Epigenetic regulation of interl eukin 6 by histone acetylation in macrophages and its role in paraquat-induced pulmonary fibrosis. *Front. Immunol.* **2017**, *7*, 696. [CrossRef]
55. Xu, G.; Liu, G.; Xiong, S.; Liu, H.; Chen, X.; Zheng, B. The histone methyltransferase Smyd2 is a negative regulator of macrophage activation by suppressing interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α) production. *J. Biol. Chem.* **2015**, *290*, 5414–5423. [CrossRef] [PubMed]
56. Alkhatib, S.G.; Landry, J.W. The nucleosome remodeling factor. *FEBS Lett.* **2011**, *585*, 3197–3207. [CrossRef]
57. Wysocka, J.; Swigut, T.; Xiao, H.; Milne, T.A.; Kwon, S.Y.; Landry, J.; Kauer, M.; Tackett, A.J.; Chait, B.T.; Badenhorst, P. A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. *Nature* **2006**, *442*, 86–90. [CrossRef]
58. Santos-Rosa, H.; Schneider, R.; Bernstein, B.E.; Karabetsou, N.; Morillon, A.; Weise, C.; Schreiber, S.L.; Mellor, J.; Kouzarides, T. Methylation of histone H3 K4 mediates association of the Isw1p ATPase with chromatin. *Mol. Cell* **2003**, *12*, 1325–1332. [CrossRef]

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LIST OF PUBLICATIONS

1- Ahmed Shamloul, Gustav Steinemann, Kerrin Roos, Celine Huajia Liem, Jonathan Bernd, Thorsten Braun, Andreas Zakrzewicz and Janine Berkholz. The Methyltransferase Smyd1 MediatesLPS-Triggered Up-Regulation of IL-6 in Endothelial Cells. Cells. 2021 Dec; 10(12): 3515. doi: 10.3390/cells10123515. Impact Factor: 6.6

2- Shady, Ebtessam Ahmed Abou; Bahgat, Nehal M.; Abdelaziz, Enas A.; **Eldeen, Ahmed M. Salah.** Study of the changes in gastric emptying and motility in aged rats. Medical Research Journal:2012 Jun; 11(1), p 20–26. doi:10.1097/01.MJX.0000415059.97519.34.

Acknowledgments:

First and last of all, all praises to God.

I am truly grateful to Prof. Dr. Axel R. Pries, Dr. med. Andreas Zakrzewicz and Dr. rer. nat. Janine Berkholz for their close supervision, valuable suggestions and sincere help in every aspect of this project a well as their input and patience throughout my laboratory work.

I would like to thank the staff members and colleagues in the Institute of Physiology at Charite Medical University Berlin for their help and support during my study in Berlin.

My particular thanks go to Dr. rer. nat. Mohammed Yassen (Institute of Virology at Charite Medical University Berlin) for providing advice and sharing his scientific expertise.

Last but not the least; I would like to express my deepest gratitude to my wife, children and my parents for all their love and encouragement.