Institut für Tierwissenschaften, Abteilung Tierzucht und Tierhaltung

der Rheinischen Friedrich-Wilhelms-Universität Bonn

Epigenetic regulation of CD14 in TRIF pathway in pulmonary alveolar macrophages of German Landrace pigs

Inaugural-Dissertation

zur

Erlangung des Grades

Doktorin der Agrarwissenschaften

(Dr. agr.)

der

Landwirtschaftlichen Fakultät

der

Rheinischen Friedrich-Wilhelms-Universität Bonn

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Tag der mündlichen Prüfung:	13. Mai 2016
Erscheinungsjahr:	2016

Dedicated to

My beloved family

Epigenetic regulation of CD14 in TRIF pathway in pulmonary alveolar macrophages of German Landrace pigs

Diseases of the respiratory system are a main problem in pig production. Pulmonary alveolar macrophages (PAMs) are key players to defend these respiratory diseases like lung inflammation caused by bacterial infection. Cluster of differentiation 14 (CD14) is the pattern recognition receptor (PRR) involved in the recognition of bacterial component lipopolysaccharide (LPS) through the MyD88-dependent and TRIF pathway of innate immunity. Gene expression regulation of CD14 and downstream genes may prevent LPS-induced inflammation in pigs. However, epigenetic modulation on gene expression of *CD14* in response to infection is poorly understood. As a histone deacetylase (HDAC) inhibitor, sulforaphane (SFN) shows an anti-inflammatory activity and suppresses the DNA methylation. To identify the epigenetic changes of *CD14* mediated with SFN in LPS-induced TRIF pathway, a PAMs model *in vitro* was investigated. For this, mRNA expression of CD14 and downstream genes of TRIF pathway were quantified using qPCR. The cytokine level of tumor necrosis factor-α (TNFα) and interleukin-1β (IL-1β) were measured by enzyme - linked immunosorbent assay (ELISA). The protein level of NF-κB was analyzed by Western blot. In addition, gene expression of the epigenetic enzymes DNA methyltransferase-1 (DNMT1) and DNMT3a were quantified. Furthermore, the DNA methylation alterations of *CD14* at promotor and gene body (CDS region) were analyzed using bisulfite sequencing in SFN and LPS treated PAMs.

We found that CD14 gene expression was induced by 5 μg/ml LPS in time dependent manner. At time point 12 h, the gene expression of CD14 and downstream genes in TRIF pathway including TRIF, TRAF6, NF_YB, TRAF3, IRF7 and cytokines such as TNF-α, IL-1β, IL-6 and IFN-β were significantly induced by LPS. The LPS induced gene expression was suppressed by SFN in a dose dependent manner. The LPS-induced cytokine level of TNFα, IL-1β and NF-κB were also inhibited by SFN. Similarly, the DNMT3a mRNA expression was increased by LPS and down regulated by SFN at a dose of 5 μM. Furthermore, the bisulfite sequencing results presented that gene body methylation of *CD14* was positively associated with gene expression of LPS treated PAMs and this methylation status was inhibited by SFN in a dose dependent manner. This *in vitro* study suggests that CD14 is involved in TRIF pathway including TRIF-TRAF6 and TRIF-TRAF3 pathway by LPS induction. Further, this LPS-CD14 activation was suppressed by SFN via the epigenetic regulation of CD14 gene body methylation associated with DNMT3a.

This study provided novel insights into SFN-mediated epigenetic downregulation of CD14 gene in LPS induced-TRIF pathway inflammation and may open new avenues of approaches to prevent and mitigate the LPS-induced inflammation in pigs.

Epigenetische Regulation von CD14 des TRIF-Signalwegs in Lungen Alveolarmakrophagen bei Schweinen der Deutschen Landrasse

Krankheiten des Atmungssystems sind ein Hauptproblem in der Schweineproduktion. Pulmonale Alveolarmakrophagen (PAMs) spielen bei der Verteidigung des Körpers gegen Erkrankungen der Atemwege, wie Lungenentzündung, durch bakterielle Infektion eine Schlüsselrolle. Das „Cluster of differentation“ 14 (CD14) agiert als „pattern recognition receptor“ (PRR) und ist damit an der Identifizierung von bakteriellen Komponenten wie Lipopolysacchariden (LPS), durch die MyD88-abhängigen und dem TRIF Signalweg der angeborenen Immunität, beteiligt. Die Regulation der Genexpression von CD14 und den nachgeschalteten Genen können die LPS-induzierte Entzündung verhindern. Allerdings, bisher jedoch sind epigenetische Modulationen von CD14 in Bezug auf Infektionen kaum untersucht. Als ein Histon-Deacetylase (HDAC)-Inhibitor, zeigt Sulforaphan (SFN) eine entzündungshemmende Wirkung und unterdrückt die DNA-Methylierung. Um die epigenetischen Veränderungen von CD14 vermittelt durch SFN in LPS-induziertem TRIF Signalweg zu identifizieren, wurden PAMs *in vitro* untersucht. Hierzu wurden die mRNA-Expression von CD14 und nachgeschalteten Gene des TRIF Signalwegs mit qPCR quantifiziert. Die Zytokinkonzentrationen von Tumornekrosefaktor-α (TNF) und Interleukin-1β (IL-1β) wurden mittels Enzym-linked Immunosorbent Assay (ELISA) gemessen. Der Proteingehalt von NF-κB wurde durch Western-blot bestimmt. Ebenfalls wurde die Genexpression der epigenetischen Enzyme DNA-Methyltransferase-1 (DNMT1) und DNMT3a quantifiziert. Des Weiteren, wurde der DNA-Methylierungsstatus von CD14 sowohl in der Promotor als auch in der intragenic Region (CDS Region) unter Verwendung von Bisulfit-Sequenzierung in SFN und LPS behandelten PAMs analysiert. Es konnte festgestellt werden, dass die Genexpression von CD14 durch 5 µg/ml LPS im Zeitverlauf angeregt wird. Zum Zeitpunkt 12 h nach LPS Behandlung zeigte sich ein signifikanter Genexpressionsunterschied von CD14 und nachgeschalteten Gene des TRIF Signalweges einschließlich TRIF, TRAF6, NFYB, TRAF3, IRF7 und von Zytokinen wie TNF-α, IL-1β, IL-6 und IFN-ß. Der LPS-induzierte Genexpressionsunterschied wurde durch SFN dosisabhängig unterdrückt. Ebenfalls war das durch LPS-induzierte Zytokin-Level, einschließlich TNF, IL-1β und NF-κB, durch SFN gehemmt. Weiterhin konnte auch bei der mRNA-Expression von DNMT3a durch LPS eine erhöhte und durch SFN eine nach unten regulierte Expression

bei einer Dosis von 5µM festgestellt werden. Die Bisulfit-Sequenzierungsergebnisse der intragenic Region von CD14 ergab eine positive Assoziation mit der Gene-Expression von LPS-behandelten PAMs und dieser Methylierungsstatus wurde von SFN dosisabhängig gehemmt. Diese in vitro Studie legt nahe, dass CD14 nach LPS Behandlung an den TRIF Signalwegen, einschließlich TRIF-TRAF6 und TRIF-TRAF3 beteiligt ist. Ferner wurde diese LPS-CD14-Aktivierung durch SFN über die epigenetische Regulation der Methylierung der CD14 intragenic Region assoziiert mit DNMT3a unterdrückt. Diese Studie gibt einen neuen Einblick in die SFN-vermittelte epigenetische Herunterregulation vom CD14-Gen in durch LPS induzierten TRIF Signalweg Entzündungen und kann neue Verfahrensweisen eröffnen die LPS-induzierte Entzündung bei Schweinen zu verhindern und zu mildern.

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List of abbreviations

Ac-H3	acetyl-histone H3
Ac-H4	acetyl-histone H4
AD	Alzheimer's disease
ADP	adenosine diphosphate
AIM2	absent in melanoma 2
ALRs	AIM2 like receptors
AM(s)	alveolar macrophage(s)
ARF6	ADP-ribosylation factor 6
BAL	bronchoalveolar lavage
BMS	burning mouth syndrome
bp	base pair (s)
CD	Crohn's disease
CD14	cluster of differentiation 14
cDNA	complementary DNA
CDS	coding sequence
CGIs	CpG islands
COX-2	cyclooxygenase-2
CpG	C-phosphate-G
CT	cytosine-thymidine
dATP	2'-deoxyadenosine 5'-triphosphate
DCs	dendritic cells
ddH_2O	double-distilled water
dGTP	deoxyguanosine triphosphate
DMSO	dimethyl sulfoxide

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DNMTs	DNA methyltransferases
dNTP	deoxyribonucleoside triphosphate
DPBS	dulbecco's phosphate-buffered saline
dsRNA	double-stranded RNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
EGGR	glycyrrhiza glabra L.
ELISA	enzyme-linked immunosorbent assay
F	forward primer
FBS	fetal bovine serum
FSL-1	fibroblast stimulating ligand-1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic DNA
GL	German Landrace
GPI	glycosyl-phosphatidylinositol
HATs	histone acetyltransferases
HDAC	histone deacetylation
HDACs	histone deacetylases
HPDL	human periodontal ligament
hTERT	human telomerase reverse transcriptase
IFN	interferon
IKK	I_YB kinase

IL	interleukin
iNOS	nitric oxide synthase
IPTG	isopropylthio-β-galactoside
IRAK	IL-1 receptor-associated kinase
IRF	interferon regulatory factor
JNK	Jun amino-terminal kinase
kb	kilobase
kDa	kilodalton
Kdo	3-deoxy-D-*manno*-oct-2-ulosonic acid
LPS	lipopolysaccharide
LRRs	leucine-rich repeats
LTA	lipoteichoic acid
LY96	lymphocyte antigen 96
M13	messier 13
mAbs	monoclonal antibodies
Mal	myD88-adaptor-like
mCD14	membrane CD14
MCs	mast cells
MDO	membrane-derived oligosaccharides
MHC	major histocompatibility complex
miRNAs	microRNAs
moDCs	monocyte-derived dendritic cells
MyD88	myeloid differentiation primary-response protein 88
Na2HPO4·2H_2O	xylencyanol, di-Sodium Hydrogen Phosphat 2 Hydrate
NAL	nacystelyn

NBN	nobiletin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NKs	natural killer cells
NLRs	nucleotide-binding oligomerization domain (NOD)-like receptors
Nrf2	nuclear factor erythroid-derived 2-like 2
nt	nucleotide (s)
OLP	oral lichen planus
PAMPs	pathogen associated molecular patterns
PAM(s)	pulmonary alveolar macrophage(s)
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
Pen-Strep	penicillin - streptomycin
PG	peptidoglycan
PI (4,5) P2	phosphatidylinositol 4,5-bisphosphate
PPEtN	phosphate-pyrophosphorylethanolamine
PrdxV	mammalian peroxiredoxin V
PRRs	pattern recognition receptors
PTMs	post-translational modifications
QDs	quantum dots
qRT-PCR	quantitative real-time reverse transcriptase PCR
R	reverse primer
RBC	red blood cell
RIP(K)	receptor-interacting serine/threonine-protein (kinase)
R-LPS	rough lipopolysaccharide

RLRs	retinoid acid-inducible gene I (RIG-I)-like receptors
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rounds per minute
RPMI	roswell Park Memorial Institute
sCD14	soluble CD14
SDS-PAGE	sodium dodecyl sulfate - polyacrylamide gel electrophoresis
SFN	sulforaphane (1-isothiocyanato-4-(methylsulfinyl)-butane)
S-LPS	soluble-lipopolysaccharide
SOC	super optimal broth with catabolite repression
SOCS1	suppressors of cytokine signaling1
ssRNA	single-stranded RNA
TAB	TAK1-binding protein
TAE	tris-acetate-EDTA
TAK	TGF-β-activated kinase
TBK	TANK-binding kinase
TET	ten-eleven translocation
TGF-β	transforming growth factor- β
THP-1	human macrophage-derived cell line
TIR	toll-interleukin 1 (IL-1) receptor
TLRs	toll-like receptors
TM6	TRAM-derived decoy peptide
TNF	tumor necrosis factor
TRAF	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule

TRIF	TIR-domain-containing adaptor protein inducing IFNβ
UWS	unstimulated whole saliva
WST-1	water-soluble tetrazolium salt-1
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside
× *g*	times gravity
↓	decrease
5caC	5-carboxylcytosine
5fC	5-formylcytosine
5hmC	5-hydroxymethylcytosine
5mC	5-methylcytosine

1 Introduction

Inflammation is the body's reaction to injuries including trauma, hypersensitivity and infection, which aims to defend against pathogens and repair tissue (Ferrero-Miliani et al. 2007, Moldoveanu et al. 2009). Unlike the adaptive immunity which needs more time for the defense against antigens, the innate immunity always takes a quick response to inflammatory-related stimulations (Sheu et al. 2013). Lipopolysaccharide (LPS) is the component of the outer membrane of gram-negative bacteria and one of the most predominant microbial stimulators of inflammation (Dobrovolskaia and Vogel 2002). It activates the innate immune response in monocytes (Dobrovolskaia and Vogel 2002), dendritic cells (DCs) (Dat et al. 2015) and macrophages (Zhao et al. 2015). Macrophages are phagocytes which develop and differentiate from tissue monocytes, with pivotal functions in host defense and inflammation (Ginhoux 2014, Gordon and Taylor 2005). In macrophages, LPS induces the production of pro-inflammatory cytokines including tumor necrosis factor-α (TNFα), interleukin1-β（IL-1β), IL-6 and IL-8 and anti-inflammation mediators such as IFN-a and IL-10 (Dobrovolskaia and Vogel 2002, Mosser and Edwards 2008, Rogler et al. 1998). Pulmonary alveolar macrophages (PAMs) or alveolar macrophages (AMs) reside in the pulmonary alveolus, constitute a vital component of the alveolar spaces and play a central role in pulmonary innate immunity (Hoppstadter et al. 2010, Sato-Nishiwaki et al. 2013).

Cluster of differentiation 14 (CD14), a glycosyl-phosphatidylinositol (GPI)-linked protein is a pattern recognition receptor (PRR) which binds directly to LPS (Wright et al. 1990) and transfers LPS molecules in a co-expressed way to Toll-like receptor 4 (TLR4) and lymphocyte antigen 96 (LY96, also know as MD-2) (da Silva Correia et al. 2001). It enhances the inflammatory response and induced LPS-dependent production of IL-8 in vitro (He et al. 2014). CD14 was discovered as a myeloid differentiation antigen (Griffin et al. 1981) and human monocytes antigen (Todd et al. 1981) by reactivity of monoclonal antibodies (mAbs) depending on antigen-antibody reaction. There are two forms of CD14, the membrane CD14 (mCD14) (Todd et al. 1981) and the soluble CD14 (sCD14) (Maliszewski et al. 1985). It is already known that mCD14 is expressed in spleen macrophages and Kupffer cells in granuloma (Hancock et al. 1983, Ziegler-Heitbrock and Ulevitch 1993). CD14 knockdown in macrophages inhibited the secretion of inflammatory cytokines like IL-6 and LPS induced TNFα strongly. This suggested that CD14 was critical for LPS binding to macrophages (Ma et al. 2015).

CD14 is also expressed in AMs (Ziegler-Heitbrock and Ulevitch 1993) and it binds with LPS and delivers LPS to the TLR4-MD2 complex on AMs (Kuronuma et al. 2009). After LPS stimulation, CD14-TLR4 activates the myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathway (TRIF pathway) (Akira and Takeda 2004). The TLR4-TRIF (TIR-domain-containing adaptor protein inducing interferon-β) interaction requires for the adaptor TRIF-related adaptor molecule (TRAM) and TRIF activates both TNF receptor associated factor 3 (TRAF3) and TRAF6 by a binding domain present on its N-terminus (Brown et al. 2011, Wang et al. 2001, Yamamoto et al. 2003b, Yamamoto et al. 2003a). The activation of TRAF6 or receptor-interacting serine/threonine-protein kinase (RIPK)-1 by TRIF initiates the NF-κB signaling which is similar to the MyD88-dependent pathway (Qian and Cao 2013). While the activation of TRAF3 initiates the interferon (IFN) regulatory factor (IRF)-3 through IKKi and TANK-binding kinase (TBK)-1, the phosphorylation of IRF3 induces the production of IFN-β (Brown et al. 2011, Doyle et al. 2002). TRAF3 regulates the production of the anti-inflammatory cytokine IL-10 and the activation of the IFN (Hacker et al. 2006). It is well known that CD14 works in the LPS-MyD88 signaling (Haghparast et al. 2011, Jiao et al. 2013, Tachado et al. 2010, Tsai et al. 2011, Zhang et al. 2009) but its activation in the TRIF pathway is argumentative. It has been shown that LPS initiates TRIF signaling independent of CD14 (Watanabe et al. 2013) and CD14 is required for the TRIF-independent signaling (Jiang et al. 2005, Lloyd-Jones et al. 2008, Regen et al. 2011) through IRF3 (Roy et al. 2014). Thereby, CD14 may be involved in the TRIF pathway by directly binding with LPS. This LPS inducing CD14-TRIF pathway genes including CD14, TRIF-TRAF6 sub-pathway genes and TRIF-TRAF3 sub-pathway genes may be further epigenetically regulated by DNA methylation.

DNA methylation is one of the key epigenetic performers which interact with regulatory proteins and non-coding RNAs (Delcuve et al. 2009). It refers to the addition of methyl groups to the adenine or cytosine bases of the DNA (Plongthongkum et al. 2014). DNA methylation is associated with gene regulation (Wilson et al. 2014) by DNA methyltransferases (DNMTs) including DNMT1 and DNMT3. DNMT1 has maintenance methylation activity (Ronemus et al. 1996) and DNMT3 has the role in *de novo* methylation (Robertson et al. 2000). DNA methylation of gene promotor regions is associated with gene silencing (Bird 2002, Huang et al. 2014, Jones and Baylin 2002). Oppositely, the gene body methylation is positively correlated with gene

expression in humans (Ball et al. 2009, Hellman and Chess 2007, Huang et al. 2014, Klose and Bird 2006, Laurent et al. 2010, Lister et al. 2009).

Sulforaphane (SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane), is a natural member of the isothiocyanate family which is mainly found in vegetables from consumed cruciferous vegetables such as broccoli, cabbage and kale (Juge et al. 2007, Meeran et al. 2010, Singh et al. 2005). It has anticancer (Ho et al. 2009, Meeran et al. 2010, Singh et al. 2005, Xiao et al. 2009), anti-inflammatory (Ko et al. 2013), antioxidant and antidiabetic (de Souza et al. 2012) effects. It has been shown that SFN also leads to the demethylation of gene promoter region. SFN treatment caused the demethylation of the first 5 CpGs in the promoter region of the nuclear factor erythroid-derived 2-like 2 (Nrf2) gene, thereby it increased messenger RNA (mRNA) and protein expression of Nrf2 and Nrf2 downstream target genes, while it decreased the protein levels of DNMT1 and DNMT3a (Zhang et al. 2013).

Reports on CD14 function in TRIF pathway are controversial. Furthermore, the CD14 gene expression regulation which may be controlled by the epigenetic factor SFN has not been shown. In this study, we have performed the first epigenetic analysis of porcine CD14 gene with the histone deacetylase HDAC inhibitor SFN, using LPS treated PAMs of German Landrace (GL) pigs. The aim of this study is to investigate the potential of SFN suppression for CD14 gene expression in TRIF pathway through the epigenetic modification in pig PAM cells.

2 Literature review

2.1 Inflammation and innate immunity

Inflammation is the body's reaction to injuries and stimulations including trauma, hypersensitivity and infection, which aim to defend the body against pathogens and repair tissue (Ferrero-Miliani et al. 2007, Moldoveanu et al. 2009). Lung inflammation is the vigorous response to the aggressors including pollutants, irritants, allergens, toxins and pathogens which easily may reach to lung because it supplies a huge surface for gas exchange and pathogen exposure (Moldoveanu et al. 2009).

Innate immunity and adaptive immunity are the two major defenses of the host against micro-bacterial, viral, or other antigenic stimulations. Unlike the adaptive immunity which needs time to against the antigen, the innate immunity reaction always takes quick response to the inflammatory or antigen-related stimulation (Sheu et al. 2013), thereby it first identifies the specific antigens and then launches inflammatory cells to target that designated antigens (Moldoveanu et al. 2009).

2.2 Cells in the innate immune response

As cells of the innate immune system, mast cells (MCs) not only have the role of inducing inflammation by producing pro-inflammatory mediators, but also can act as key regulators of tolerance (Jungraithmayr 2015). Phagocyte such as macrophage, neutrophil and dendritic cell displays an early and crucial event in host defenses against pathogens (Henneke and Golenbock 2004). Basophil is an innate immune cell which has expression of a functional TLR2 receptor, while eosinophil is the innate immune inducer which lead the production of cytokines including transforming growth factor-β (TGF-β), interleukin (IL)-3, IL-4, IL-5, IL-8, IL-10, IL-12, IL-13, IL-16, IL-18 and tumor necrosis factor (TNF)-α (Stone et al. 2010). Natural killer cells (NKs) are also important contributors to innate defense against a number of different infectious agents (Biron et al. 1999).

2.3 Pulmonary alveolar macrophages and inflammation

Macrophages are phagocytes which developed and differentiated from monocytes in the tissues, with pivotal functions in development, tissue remodeling, repair or homeostasis, host defense and inflammation (Ginhoux 2014, Gordon and Taylor 2005). They are antigen-presenting cells since they express the major histocompatibility complex (MHC)

class II molecules, therefore they work not only in innate immunity but also in adaptive immunity (Hoppstadter et al. 2010). Macrophages are the main source of cytokines, chemokines and other inflammatory mediators that disseminate or inhibit the immune response (Moldoveanu et al. 2009). Furthermore, macrophages can secret quite a few cytokines including the pro-inflammatory cytokines such as TNF, IL-1, IL-6 and IL-8 and anti-inflammatory mediators such as IL10, thus they play a crucial role in inflammation (Mosser and Edwards 2008, Rogler et al. 1998).

Pulmonary alveolar macrophages (PAMs), or alveolar macrophages (AMs) are a type of macrophages, which reside in the pulmonary alveoli (Fig. 1) (Wissinger et al. 2008). They constitute a vital component of the alveolar spaces and play a central role in pulmonary innate immunity (Hoppstadter et al. 2010, Sato-Nishiwaki et al. 2013).

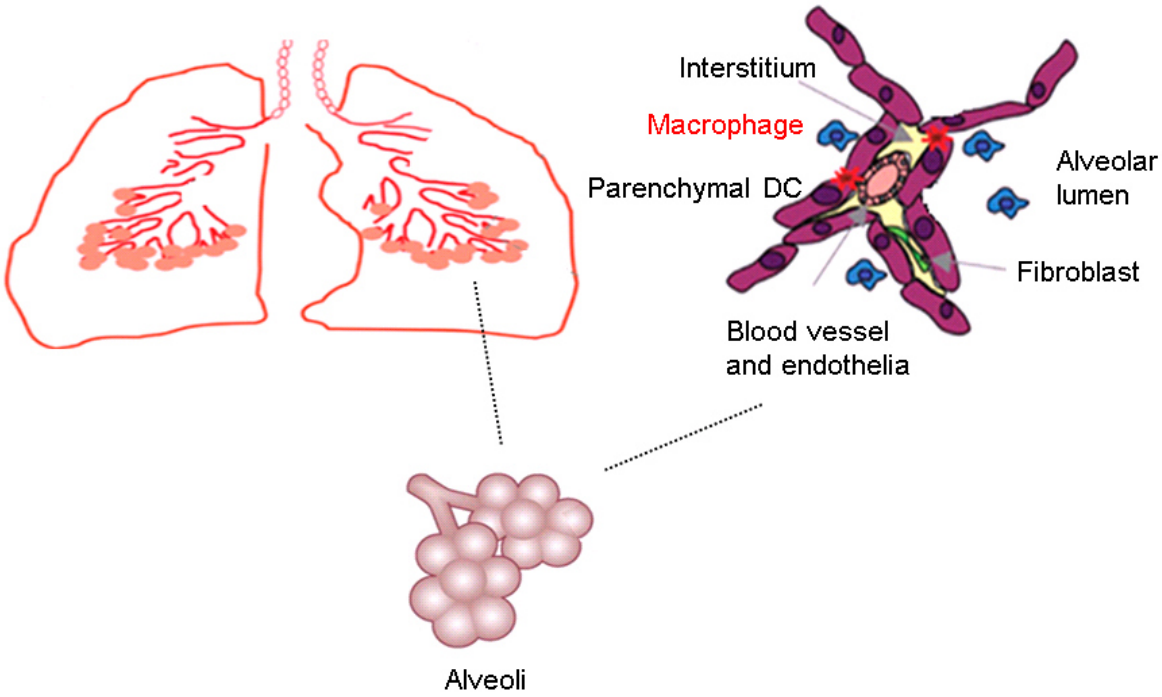

Fig. 1 PAMs are located in the alveoli (Wissinger et al. 2008, modified)

2.4 Pathogen recognition by innate immunity

Unlike the adaptive immunity, innate immunity has a role in the nonspecific recognition of pathogens, by which it is activated via pathogen associated molecular patterns (PAMPs) linking to pattern recognition receptors (PRRs) (Maciejewska Rodrigues et al. 2009). PAMPs are derived from microorganisms such as bacteria, fungi, parasites and viruses. Bacteria include gram-negative bacteria lipopolysaccharide (LPS), gram-positive bacteria

peptidoglycan (PG), lipoteichoic acid (LTA), flagellin and C-phosphate-G (CpG)-DNA. Viruses comprise DNA, double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) (Akira et al. 2006). They are recognized by Toll-like receptors (TLRs) and other PRRs, such as nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), retinoid acid-inducible gene I (RIG-I)-like receptors (RLRs) and absent in melanoma 2 (AIM2) like receptors (ALRs) (Akira et al. 2006, Creagh and O'Neill 2006, Janeway and Medzhitov 2002, Tang et al. 2012). Several classes of PRRs, including TLRs and cytoplasmic receptors like NLRs and RLRs, recognize different microbial components and activate immune cells directly (Akira et al. 2006).

2.4.1 LPS

Lipopolysaccharide (LPS) endotoxin is an outer membrane component of gram-negative bacteria and one of the most predominant microbial stimulator of inflammation (Dobrovolskaia and Vogel 2002). Peripheral stimulation of the innate immune system with LPS causes an exaggerated neuro-inflammatory response in aged BALB/c mice (Henry et al. 2009). The structure of LPS includes a hydrophobic domain named lipid A, a distal polysaccharide (O-antigen) and a core oligosaccharide (Fig. 2) (Raetz and Whitfield 2002). LPS activates the innate immune response with monocytes (Dobrovolskaia and Vogel 2002), dendritic cells (DCs) (Dat et al. 2015) and macrophages (Zhao et al. 2015). In macrophages, LPS induces the production of inflammation cytokines, such as TNF-α and IL1-β (Beutler and Cerami 1988, Carson et al. 2011, Dinarello 1991) and anti-inflammation mediator IL-6 (Dobrovolskaia and Vogel 2002).

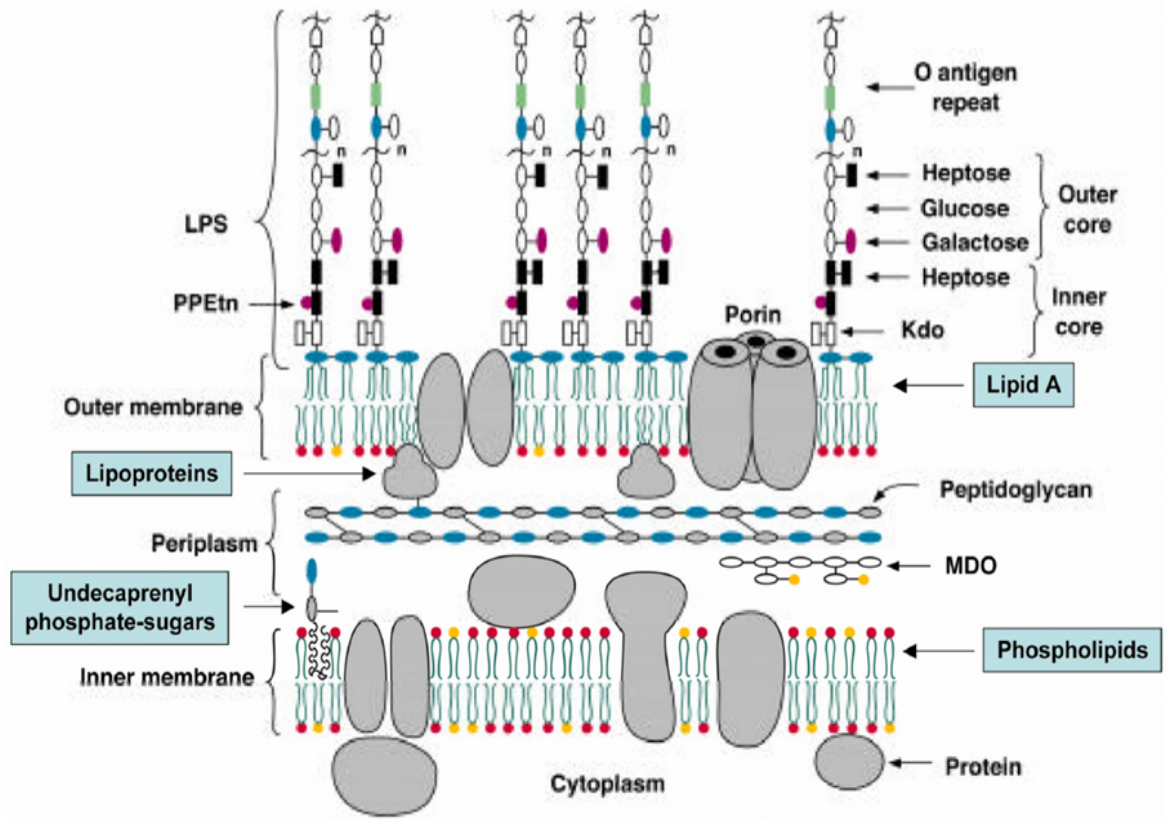

Fig. 2 LPS structure and membrane structure of *E. coli* (Raetz and Whitfield 2002) (PPEtN: phosphate-pyrophosphorylethanolamine, Kdo: 3-deoxy-D-*manno*-oct-2-ulosonic acid, MDO: membrane-derived oligosaccharides)

2.4.2 TLRs

TLRs are type I transmembrane proteins. They have three parts with ectodomains, transmembrane domains and intracellular toll-IL-1 receptor (TIR) domains. The ectodomains contain leucine-rich repeats (LRRs) that mediate the recognition of PAMPs, while the TIR domain is the part which is required for downstream signal transduction (Kawai and Akira 2010). So far, 12 members of the TLRs family have been identified in mammals (Akira et al. 2006), while 10 functional TLRs have been identified in humans (Kawai and Akira 2010). TLRs recognize PAMPs including proteins, lipids (TLR1, TLR2 and TLR6), lipoproteins and nucleic acids (TLR8 and TLR9) derived from a wide range of microbes such as viruses, bacteria, fungi and parasites (Akira et al. 2006, Kawai and Akira 2010).

Based on their cellular localization and respective PAMP ligands, TLRs can be divided into 2 subfamilies, one is composed of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11

which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins; the other one is composed of TLR3, TLR7, TLR8 and TLR9 which are expressed only in intracellular vesicles such as the lysosomes, endosomes, endolysosomes and endoplasmic reticulum (ER), where they recognize microbial nucleic acids (Kawai and Akira 2010).

TLR1 recognizes the microbial components of triacyl lipopeptides from the species of bacteria and mycobacteria (Table 1) (Akira et al. 2006). TLR2 can recognize several microbial components including diacyl lipopeptides (mycoplasma), triacyl lipopeptides (bacteria and mycobacteria), LTA (group B Streptococcus), peptidoglycan (PG, gram-positive bacteria), porins (Neisseria), lipoarabinomannan (mycobacteria), zymosan (Saccharomyces cerevisiae), phospholipomannan (Candida albicans), glucuronoxylomannan (cryptococcus neoformans), tGPI-mutin (Trypano-soma), hemagglutinin protein (measles virus) (Table 1) (Akira et al. 2006). TLR4, the typical member of the TLR family which is the only receptor utilizing all four TIR-domain-containing adaptors (Kagan and Medzhitov 2006). It was the first PRR discovered to be expressed in mammalian innate immune cells (Medzhitov and Janeway 1997). Two pathways including the myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathways are activated when TLR4 is stimulated with LPS (Akira and Takeda 2004).

Table 1 TLR recognition of microbial components (Akira et al. 2006)

Microbial components	Species	TLR usage
Bacteria		
LPS	Gram-negative bacteria	TLR4
Diacyl lipopeptides	*Mycoplasma*	TLR6/TLR2
Triacyl lipopeptides	Bacteria and mycobacteria	TLR1/TLR2
LTA	Group B Streptococcus	TLR6/TLR2
Peptidoglycan	Gram-positive bacteria	TLR2
Porins	*Neisseria*	TLR2

Lipoarabinomannan	Mycobacteria	TLR2
Flagellin	Flagellated bacteria	TLR5
CpG-DNA	Bacteria and mycobacteria	TLR9
ND (not determined)	Uropathogenic bacteria	TLR11
Fungus		
Zymosan	*Saccharomyces cerevisiae*	TLR6/TLR2
Phospholipomannan	*Candida albicans*	TLR2
Glucuronoxylomannan	Cryptococcus neoformans	TLR2 and TLR4
Parasites		
tGPI-mutin	*Trypanosoma*	TLR2
Glycoinositolphospholipids	*Trypanosoma*	TLR4
Hemozoin	*Plasmodium*	TLR9
Profilin-like molecule	*Toxoplasma gondii*	TLR11
Viruses		
DNA	Viruses	TLR9
dsRNA	Viruses	TLR3
ssRNA	RNA viruses	TLR7 and TLR8
Envelope proteins	RSV, MMTV	TLR4
Hemagglutinin protein	Measles virus	TLR2
Host		
Heat-shock protein 60, 70		TLR4
Fibrinogen		TLR4

2.4.3 The TLR signaling pathway

Depending on ligand stimulation, TLRs attract different adaptor proteins including MyD88, myD88-adaptor-like (Mal), TIR-domain-containing adaptor protein inducing IFNβ (TRIF) and TRIF-related adaptor molecule (TRAM) by TIR-TIR interactions (Qian and Cao 2013). All TLRs, with the exception of TLR3, activate the MyD88-dependent signaling pathway by utilizing MyD88 while TLR4 and TLR3 initiate the TRIF-dependent (MyD88-independent pathway) signaling pathway (Qian and Cao 2013). The whole TLR signaling pathway is shown in Fig. 3.

(http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map04620&keyword=myd88)

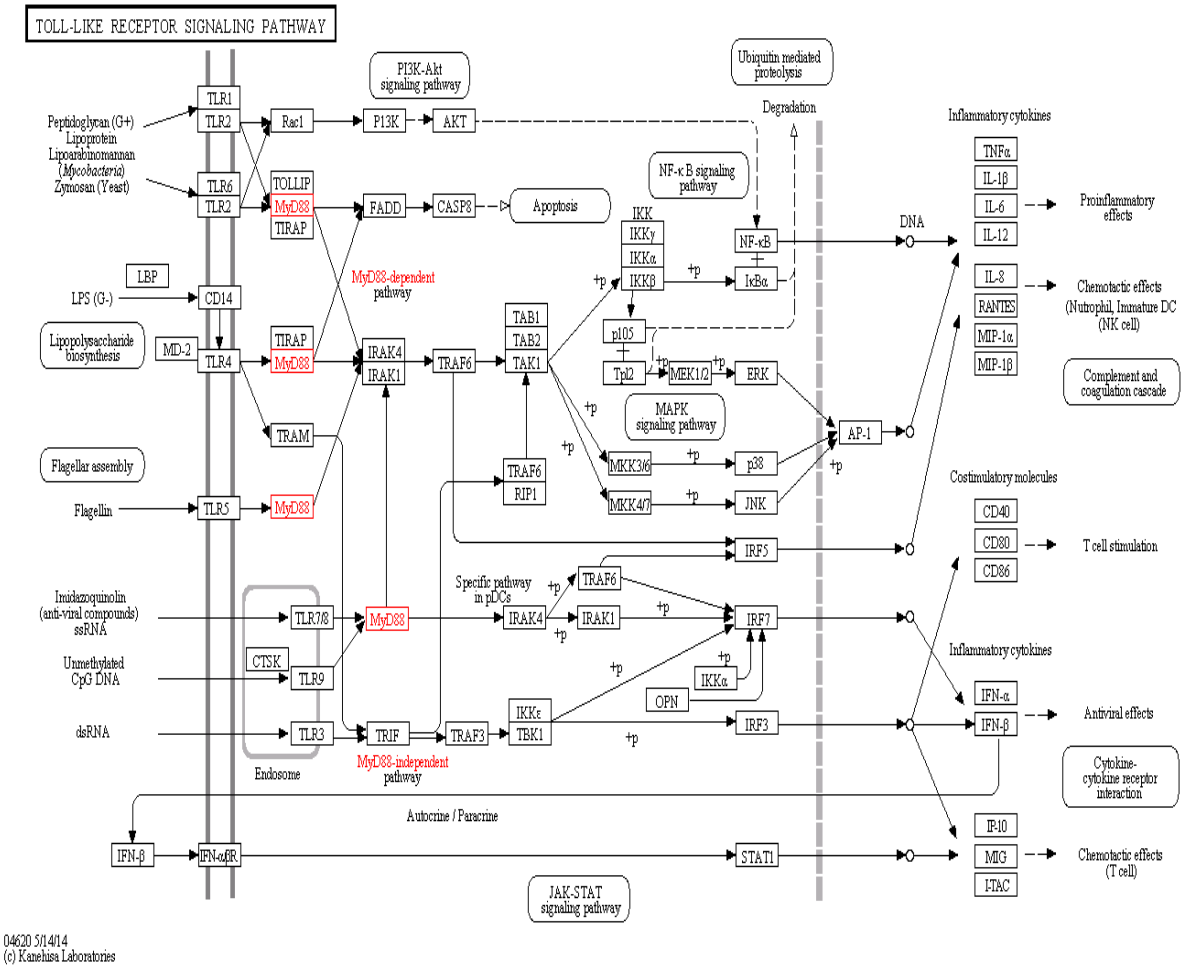

Fig. 3 TLR signaling pathway (KEGG 20150205)

2.4.3.1 The MyD88-dependent pathway

The TLR4/MyD88 signaling pathway is primarily used to induce the expression of pro-inflammatory cytokines (Brown et al. 2011). MyD88 activates IL-1 receptor-associated kinase (IRAK)-1 though the interaction with IRAK-4 by the death domain part of IRAK-4. Then the activation of TNF receptor associated factor (TRAF)-6 is promoted with other E2 ubiquitin protein ligases which activate a compound containing TGF-β-activated kinase (TAK)-1, TAK1-binding protein (TAB)-1, TAB-2 and TAB-3 (Brown et al. 2011, Cao et al. 1996, Chen 2005, Li et al. 2000, Li et al. 2002, Muzio et al. 1997). The TAK1/TABs complex induces the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) signaling pathway by activating I_YB kinase (IKK) and the mitogen-activated protein kinases (MAPK) signaling pathway by Jun amino-terminal kinase (JNK) (Wang et al. 2001).

2.4.3.2 The TRIF-dependent pathway

The TRIF-dependent (MyD88 independent) pathway is initiated by the TLR3 and TLR4 (Qian and Cao 2013). The TLR4-TRIF interaction requires for the adaptor TRAM and TRIF activates both TRAF3 and TRAF6 by a binding domain present on its N-terminus (Brown et al. 2011, Wang et al. 2001, Yamamoto et al. 2003a, Yamamoto et al. 2003b). In spite of the similar structure, TRAF3 and TRAF6 have different functions in the TRIF signaling pathway (Hacker et al. 2006). The activation of TRAF6 or receptor-interacting serine/threonine-protein kinase (RIPK)-1 by TRIF initiates the nuclear factor 'kappa-light-chain-enhancer' of activated B-cells (NF-κB) signaling which is similar to the MyD88-dependent pathway (Qian and Cao 2013). While the activation of TRAF3 initiates the interferon (IFN) regulatory factor (IRF)-3 through IKKi and TANK-binding kinase (TBK)-1, the phosphorylation of IRF3 induces the production of IFN-β (Brown et al. 2011, Doyle et al. 2002). TRAF3 regulates the production of anti-inflammatory cytokine IL-10 and the activation of the IFN (Hacker et al. 2006). The TRIF pathway is shown in Fig. 4 (KEGG http://www.kegg.jp/kegg-bin/highlight_pathway?scale=1.0&map=map04620&keyword=myd88).

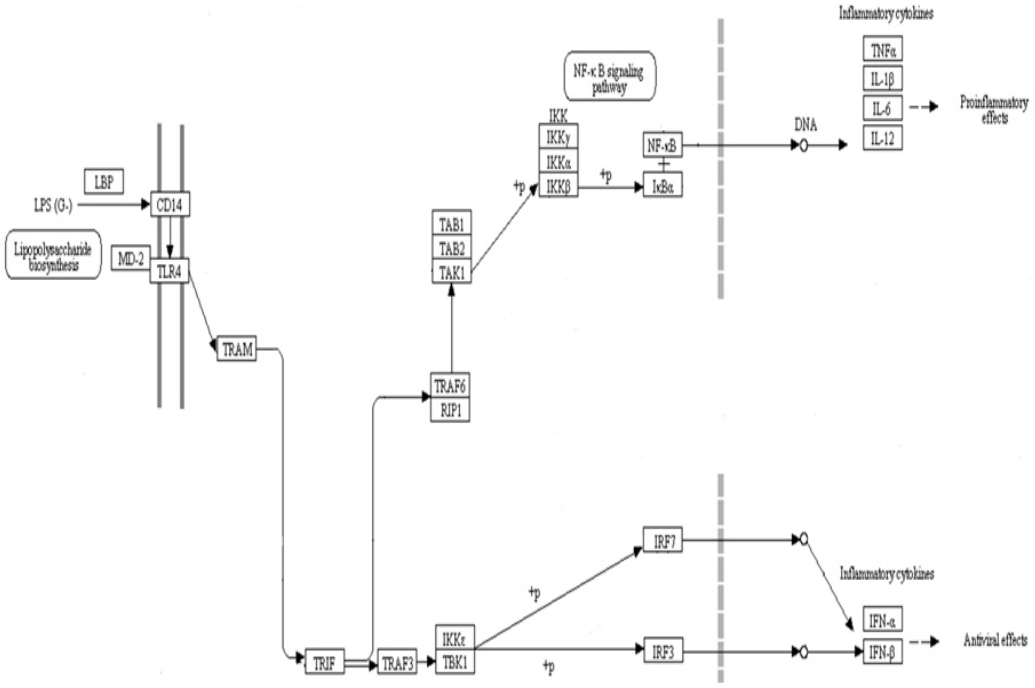

Fig. 4 TRIF signaling pathway (KEGG 20150205 with modification)

2.5 CD14 gene and its function

2.5.1 Molecular structure, forms and distribution of CD14

Cluster of differentiation 14 (CD14) was discovered as a myeloid differentiation antigen (Griffin et al. 1981) and human monocytes antigen (Todd et al. 1981) by reactivity of monoclonal antibodies (mAbs) depending on antigen-antibody reaction. CD14 is a 53-55 kDa glycoprotein (Gregory and Devitt 1999) with 356 amino acids (Goyert et al. 1988), it contains the leucine-rich repeats which are incorporated into the plasma membrane *via* a glycosyl-phosphatidylinositol (GPI) anchor (Gregory and Devitt 1999). The CD14 gene is located on chromosome 5q 23-q31 in human (Goyert et al. 1988) and chromosome 18 in mouse (Ferrero et al. 1990). In addition to human and mouse, CD14 gene was identified in other species. Bovine CD14 gene encodes 373 amino acids and the coding sequence is separated by a 90 nt intron (Ikeda et al. 1997). In rat, CD14 gene contains 883 bp of 5′-flanking region, a 93 bp exon-1, an 87 bp intron followed after the ATG translation start codon and a 377 bp partial exon-2 (Liu et al. 2000). The porcine CD14 gene is located to chromosome 2q21 which includes two exons and a short intron (80 bp) following after the ATG translation start codon (Qiu et al. 2007).

CD14 exists in two forms, the membrane CD14 (mCD14) (Todd et al. 1981) and the soluble CD14 (sCD14) (Maliszewski et al. 1985). mCD14 is expressed in spleen macrophages and kupffer cells in granuloma (Hancock et al. 1983, Ziegler-Heitbrock and Ulevitch 1993). It is also expressed in alveolar macrophages and microglia cells (Ziegler-Heitbrock and Ulevitch 1993). sCD14 was detected in cellular supernatant of monocytes and normal plasma (Maliszewski et al. 1985). The porcine CD14 gene was expressed in various tissues, such as spleen, liver, thymus, skeletal muscle and white matter (Qiu et al. 2007).

2.5.2 Functional properties of CD14

2.5.2.1 CD14 as biomarker of disease

CD14 is an acute phase inflammatory biomarker. In older adults, it predicts cardiovascular disease. It also strongly and independently predicts all-cause mortality in older adults (Reiner et al. 2013). Periodontitis, a chronic infectious disease of tooth supporting tissues which is leading to inflammation and loosing teeth subsequently, is associated with elevated levels of sCD14 (Nicu et al. 2009). sCD14 is a marker of disease activity in Crohn's disease (CD) (Lakatos et al. 2011). It is also a potential marker for pneumonia in children (Marcos et al. 2010). Microglia-derived sCD14 is a candidate cerebrospinal fluid biomarker for Alzheimer's disease (AD) and Parkinson's disease (PD) (Yin et al. 2009). It is also shown that the sCD14 level is associated with inflammation and protein-energy wasting in hemodialysis patients (Raj et al. 2009). sCD14 was higher in unstimulated whole saliva (UWS) and it was used as potential salivary biomarker for oral lichen planus (OLP) and burning mouth syndrome (BMS) (Srinivasan et al. 2008). It’s suggested that the elevated sCD14 concentration may be one of the host-response components of the periodontal disease clinical manifestations (Isaza-Guzman et al. 2008). Inhibition of CD14 may be a novel treatment strategy in melioidosis because it plays a detrimental role in the host response against *burkholderia pseudomallei* although it is involved in the recognition of *burkholderia pseudomallei* by innate immune cells (Wiersinga et al. 2008). Bottema et al. indicates that atopy is importantly influenced by CD14 in interaction with pet exposure at the age of 4 and 8 years childen (Bottema et al. 2008).

2.5.2.2 CD14 is responsible for immunity and TLR activities

Soluble CD14 is associated with human dendritic cell cytokine production and increased T cell responses (Liu et al. 2012). CD14-159C/T could effect the T cell-mediated immunity in extensively burned patients (Dong et al. 2009). The CD14-159C>T polymorphism is associated with soluble CD14 gene expression, which might influence the balance of pro- and anti-inflammatory immune responses in healthy term neonates (Hartel et al. 2008). CD14 expression in tonsils and adenoids implies that CD14 takes part in an important immunological sentinel function in the innate immunity of the upper airway (Ben-Yaakov et al. 2010).

CD14 plays a role in the stimulation of TLRs and it acts as a co-receptor for endosomal TLR activation (Baumann et al. 2010). CD14-mediated TLR activation might be related with the cardiovascular and metabolic complications of obesity (Roncon-Albuquerque et al. 2008). CD14 was necessary for the microbe-induced endocytosis of TLR4 while CD14 controls the trafficking and signalling functions of TLR4. This innate immune trafficking cascade illustrates how pathogen detection systems operate to induce both membrane transport and signal transduction (Zanoni et al. 2011). CD14 gene expression is critical for TLR2-mediated recognition of LTA in human peripheral blood (Bunk et al. 2010). CD14 is responsible for the internalization of TLR2 ligand fibroblast stimulating ligand-1(FSL-1) via the clathrin-dependent endocytic pathway (Shamsul et al. 2010). CD14 is a co-receptor of TLR7 and TLR9, it is required for TLR7- and TLR9-dependent induction of pro-inflammatory cytokines in vitro and for TLR9-dependent innate immune responses in mice (Baumann et al. 2010). TLR9 immune response of A, B and C-classes oligodeoxy-nucleotides was upregulated by CD14 (Weber et al. 2012). Taken all together, the CD14 function in the TLRs can be explained in three panels: (1) CD14 activates NF-κB - dependent cytokine production with low-LPS doses challenge; (2) CD14 promotes TLR4 endocytosis and type-I IFN expression in TRIF pathway; (3) CD14 has the role to lead to the activation of nuclear factor of activated T-cells, cytoplasmic (NFATc) transcription factor family members (Fig. 5) (Zanoni and Granucci 2013).

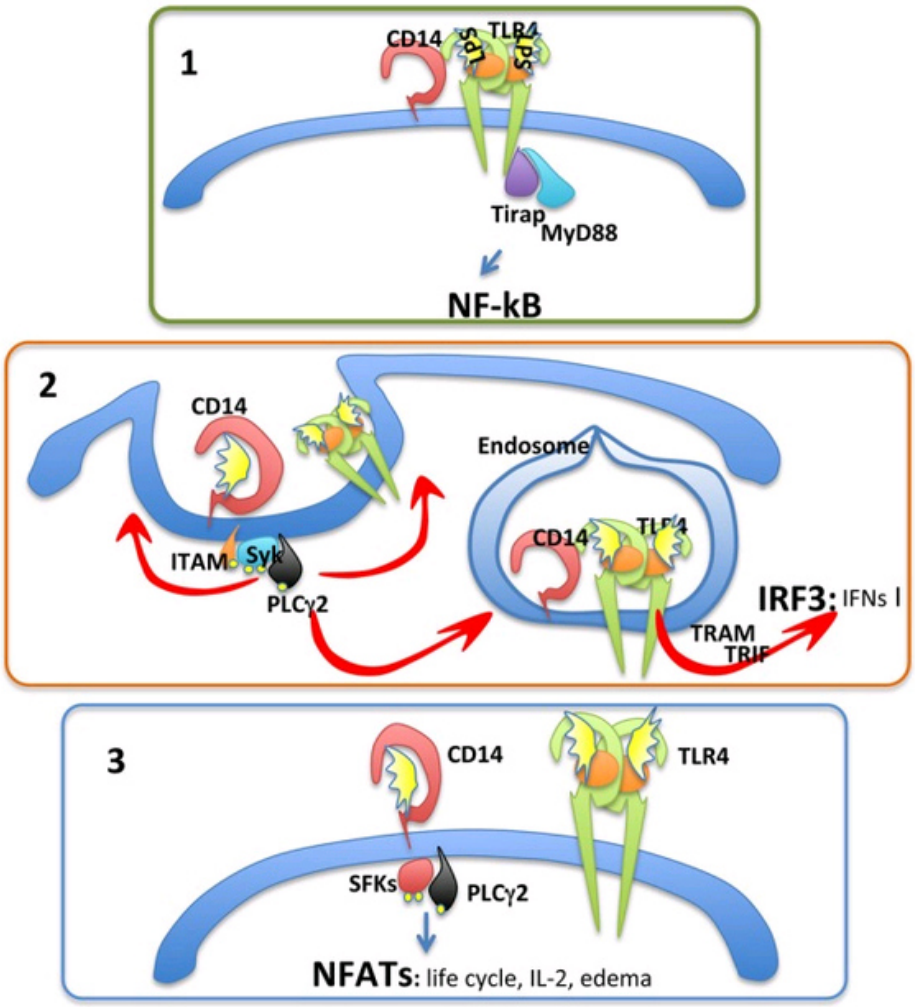

Fig. 5 CD14 fundamental functions in the TLRs (Zanoni and Granucci 2013)

2.5.3 CD14 is required for LPS - induced TRIF pathway

CD14 is the PRR which binds directly to LPS (Wright et al. 1990) and transfers LPS molecules in a co-expressed way to TLR4 and lymphocyte antigen 96 (LY96 or MD-2) (da Silva Correia et al. 2001). It is shown that CD14 is critical for LPS binding to macrophages, CD14 knockdown in macrophages inhibited the secretion of inflammatory cytokines including IL-6 and TNFα induced by LPS (Ma et al. 2015). CD14 enhanced the inflammatory response and LPS-dependent production of IL-8 induced by type 1 pili enterotoxigenic *E. coli* (ETEC) in vitro (He et al. 2014). It has been well known that CD14 is involved in the LPS-MyD88 signaling pathway (Haghparast et al. 2011, Jiao et al. 2013, Tachado et al. 2010, Tsai et al. 2011, Zhang et al. 2009) but its involvement in the TRIF pathway is argumentative. It was shown that LPS initiates the TRIF pathway independent

of CD14 (Watanabe et al. 2013), but evidence also displayed that CD14 is required for the TRIF-independent signaling (Jiang et al. 2005, Lloyd-Jones et al. 2008, Regen et al. 2011) through IRF3 (Roy et al. 2014). Thereby, it is not only important for the MyD88-dependent pathway to express TNFa, but is also required for the TRIF pathway to mediate IFNs expression (Jiang et al. 2005) via regulation of TLR4 endocytosis and facilitation of TRIF-mediated signal transduction (Zanoni et al. 2011).

2.6 Epigenetics

Epigenetics is the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence (Russo et al. 1996). The epigenetic mechanisms regulate all biological processes including genome reprogramming during early embryogenesis and gametogenesis, cell differentiation and maintenance of a committed lineage (Delcuve et al. 2009). Key epigenetic performers are DNA methylation, RNA interference and histone modifications which interact with regulatory proteins and non-coding RNAs (Fig. 6) (Delcuve et al. 2009, Kim et al. 2011).

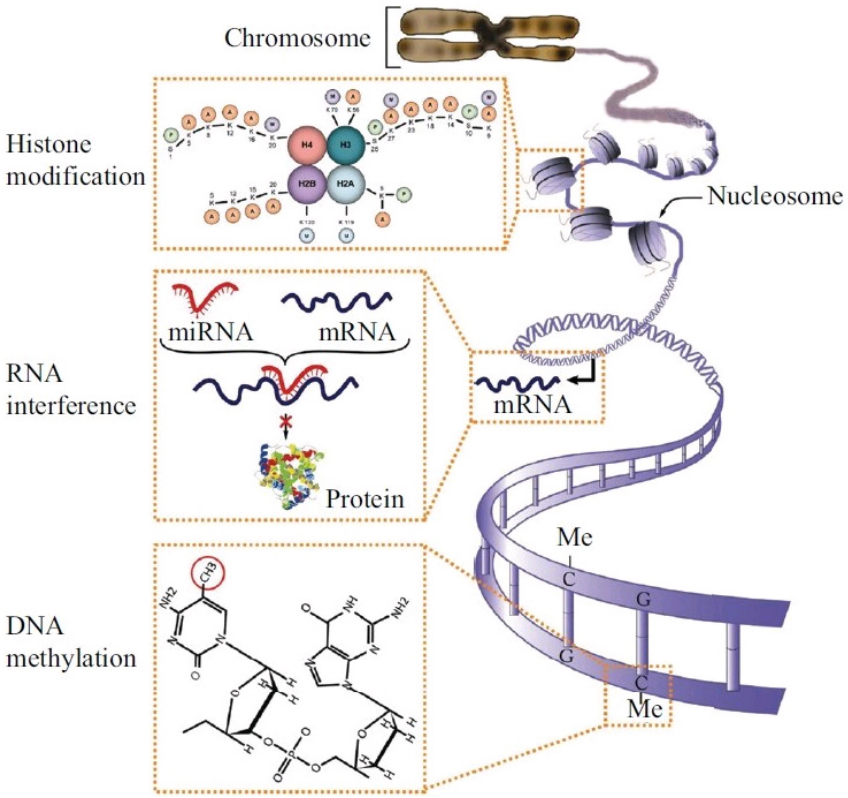

Fig. 6 Scheme of epigenetic mechanisms (Kim et al. 2011)

2.6.1 DNA Methylation

DNA methylation refers to the addition of methyl groups to the adenine or cytosine bases of DNA (Plongthongkum et al. 2014) and is associated with gene regulation (Wilson et al. 2014). It has inhibitory influence on gene expression by recruitment of methyl binding proteins, interference with transcription factor binding and the establishment and maintenance of an oppressive chromatin structure (Curradi et al. 2002, Havlis and Trbusek 2002, Liu et al. 2004). The most common mark of DNA methylation is the 5-methylcytosine (5mC) (Plongthongkum et al. 2014). Most of the DNA methylation analysis work in animals has focused on 5-methylcytosine (5mC) in the CpG sequence context. In vertebrate genomes, more than half of the genes contain short (approximately 1 kb) CpG-rich regions known as CpG islands (CGIs) (Jones 2012).

DNA methylation can occur in the promoter and gene body regions of genes. DNA methylation of gene promotor is associated with gene silencing (Bird 2002, Huang et al. 2014, Jones and Baylin 2002), oppositely, gene body methylation is positively correlated with gene expression in humans (Ball et al. 2009, Hellman and Chess 2007, Huang et al. 2014, Klose and Bird 2006, Laurent et al. 2010, Lister et al. 2009).

2.6.1.1 Gene body methylation

Gene body methylation is the DNA methylation of transcription units (Sarda et al. 2012). It occurs not only in human genomes but also in the genomes of animal and plant species (Sarda et al. 2012, Yu et al. 2015). Recent researches show the increasing support of the gene body methylation as the ancestral pattern of DNA methylation in animal genomes (Feng et al. 2010, Zemach et al. 2010). Although the promoter DNA methylation is known as a silencing mechanism, the gene body has recently been recognized as a major mechanism for regulating gene expression in many tissues (Baylin and Jones 2011). Unlike the negative effect of promotor methylation on gene expression, gene body methylation displays a positive role (Huang et al. 2014). The strongest correlation between the age-related changes in gene expression and methylation is not confined to the promoter region; oppositely, the high densities of hypo-methylated CpG-rich regions crossing the gene body are preferentially associated with gene downregulation (Yu et al. 2015). It is displayed that methylation in the gene body region does not prevent transcription elongation in genome-wide approaches (Ball et al. 2009). However, the underlying mechanisms of gene body

methylation in regulating gene expression and the functional roles of gene body methylation is still not clear.

2.6.1.2 DNA modification enzymes

DNA methyltransferases (DNMTs) are the family of enzymes which catalyze the methyl reaction via transferring a methyl group from S-adenosylmethionine to the 5 position of cytosine on CpG dinucleotides (Arand et al. 2012, Teerawanichpan et al. 2004). DNMT1 was the first discovered DNMTs in mammals (Bestor 2000), then DNMT2 (Yoder and Bestor 1998) and DNMT3 (DNMT3a and DNMT3b) families were found respectively (Okano et al. 1998). It's has been shown that DNMT1 class has maintenance methylation activity in vivo (Ronemus et al. 1996), DNMT2 class has only a methyltransferase domain therefore it lacks significant role in DNA methylation (Okano et al. 1998) while DNMT3 class contributes the role in *de novo* methylation (Robertson et al. 2000).

In addition to DNMTs, a class of enzymes named ten-eleven translocation (TET) including TET1, TET2 and TET3 have been discovered to produce epigenetic modifications like 5-carboxylcytosine (5caC), 5-formylcytosine (5fC) and 5-hydroxymethylcytosine (5hmC) (Plongthongkum et al. 2014).

2.6.1.3 Bisulfite sequencing

Bisulfite genomic sequencing is a effective technique to discern the 5-methylcytosine position for the accurate mapping of methylation sites on individual DNA molecules by using sodium bisulfite to convert un-methylated cytosine residues to uracil with a very small amounts of genomic DNA (gDNA) (Clark et al. 1994). The technical steps of bisulfite sequencing are (1) bisulfite conversion of gDNA, (2) polymerase chain reaction (PCR) by using bisulfite PCR primers for bisulfite-converted DNA and (3) direct sequencing or cloning of the purified bisulfite PCR and then sequencing (Frommer et al. 1992, Liu et al. 2004) (Fig. 7).

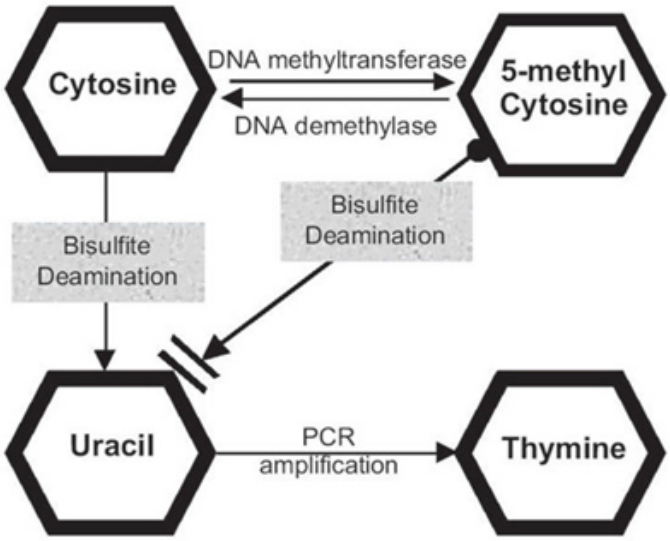

Fig. 7 Bisulfite genomic sequencing (Liu et al. 2004)

2.6.2 Histone modifications

Histone modification is another key epigenetic factor which is typically controlled by the dynamic processes including histone acetylation and histone deacetylation (HDAC) regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively (Meeran et al. 2010). Histone acetylation like acetylation of histone H3 and acetylation of histone H4 causes the transcriptional activation throughout chromatin remodeling. Conversely, histone deacetylation results in transcriptional silencing (Meeran et al. 2010). HDACs are enzymes that eliminate the acetyl modification from histones (Delcuve et al. 2009). HDAC inhibitors induce the HAT co-activator complexes to transfer acetyl groups to lysine residues in histones, therefore an open chromatin structure which facilitates the binding of transcription factors to gene promoters for regulation of gene expression (Meeran et al. 2010, Murakami et al. 2005, Struhl 1998). In addition to acetylation, histones also have reversible post-translational modifications (PTMs) including ubiquitination, phosphorylation, adenosine diphosphate (ADP) - ribosylation and methylation (Kouzarides 2007).

2.6.3 RNA interference

MicroRNAs (miRNAs) are small RNA molecules, about 22 nucleotides long, that can suppress their target gene expression post-transcriptionally. They were described first in

Caenorhabditis elegans in 1993. miRNAs have been shown to inhibit translation or decrease mRNA stability by binding to specific sites usually in the 3' untranslated region (3′UTR) of target messages, thus providing another way of gene expression controlling (Kim et al. 2011).

2.7 SFN as a regulator of anti-inflammation and epigenetics

Sulforaphane (SFN; 1-isothiocyanato-4-(methylsulfinyl)-butane), a naturally occurring member of the isothiocyanate family mainly found in cruciferous vegetables such as broccoli, broccoli sprouts, cauliflower, radish, cabbage and kale (Juge et al. 2007, Meeran et al. 2010, Singh et al. 2005), has anticancer (Ho et al. 2009, Meeran et al. 2010, Singh et al. 2005, Xiao et al. 2009), anti-inflammatory (Ko et al. 2013), antioxidant and antidiabetic (de Souza et al. 2012) effects.

The anticancer function of SFN is considered to be associated to the inducing of phase-II enzymes of xenobiotic transformation or detoxifying and transcription increment of tumor suppressor proteins perhaps by inhibitory effects on histone deacetylase (Brandenburg et al. 2010). It was shown that SFN also can lead to the demethylation of gene promoter region. SFN treatment caused the demethylation of the first 5 CpGs in the promoter region of the nuclear factor erythroid-derived 2-like 2 (Nrf2) gene, thereby it increased messenger RNA (mRNA) and protein expression of Nrf2 and Nrf2 downstream target genes, while it decreased the protein levels of DNMT1 and DNMT3a (Zhang et al. 2013).

SFN has been shown to have an anti-inflammatory role. SFN regulates LPS-induced innate immune responses of monocyte-derived dendritic cells (moDCs) in pigs (Qu et al. 2015). It modulates TLR4 activation and inhibits LPS-induced inflammatory responses (Koo et al. 2013). Functions of SFN in the modulation of TLR4 signaling have been investigated in different cell lines. SFN reduced LPS-induced TNF-α expression in macrophage cells (Youn et al. 2010). It also suppressed NFκB in LPS stimulated RAW macrophages (Heiss et al. 2001) and endothelial cells (Liu et al. 2008). SFN and nobiletin (NBN) inhibit synergistically LPS-induced inflammation in RAW 264.7 cells (Guo et al. 2012). It has been shown that SFN suppressed LPS-induced inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and TNF-α expression in mouse macrophages (Heiss et al. 2001).

2.8 Hypothesis and Objectives

Hypothesis:

LPS-induced CD14 expression can be modified by SFN through epigenetic mechanisms in the TRIF pathway in pulmonary alveolar macrophages of German Landrace pigs

Objectives:

(1) To investigate the effect of LPS on CD14 gene expression in PAMs of German Landrace pigs

(2) To find out the epigenetic effect of SFN on LPS-induced CD14 expression in PAMs of German Landrace pigs

3 Material and methods

3.1 Material

3.1.1 Reagents

Chemical	Cat.no.	Manufacturer/Supplier
Ketamin	98046	Pharmazeutische, Handelgesellschaft GmbH, Garbsen - Berenbostel
T61	03542	Pharmazeutische Handelgesellschaft GmbH, Garbsen - Berenbostel
Meliseptol®	1110493	Labomedic GmbH, Bonn, Germany
NaCl	P029.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
$Na_2HPO_4.2H_2O$	T877.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
KCl	6781.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
KH_2PO_4	3904.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
DPBS (1×)	14190-094	Life Technologies GmbH, Darmstadt, Germany
Fungizone® antimycotic	15290-026	Life Technologies GmbH, Darmstadt, Germany
Penicillin-streptomycin (10,000 U/ml)	15140-122	Life Technologies GmbH, Darmstadt, Germany
NH_4Cl	K298.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
$KHCO_3$	P748.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

EDTA	8043.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
RPMI 1640 medium	61870-044	Life Technologies GmbH, Darmstadt, Germany
FBS	10270-106	Life Technologies GmbH, Darmstadt, Germany
Gentamicine (10 mg/ml)	15710-049	Life Technologies GmbH, Darmstadt, Germany
Trypan blue stain (4%)	93595	Sigma-Aldrich (Fluka), St. Louis, USA
LPS-EB	tlrl-3pelps	InvivoGen, California, USA
R,S-sulforaphane	4478-93-7	Biomol GmbH, Hamburg, Germany
DMSO (≥ 99.9%)	154938	Sigma-Aldrich, St. Louis, USA
Anti-CD163 antibody	MCA2311F	Bio-Rad Laboratories GmbH, München, Germany
WST-1 Cell Proliferation Assay Kit	10008883	Biomol GmbH, Hamburg, Germany
AllPrep®DNA/RNA/Potein Mini Kit	80004	Qiagen GmbH, Hilden, Germany
First Strand cDNA Synthesis Kit	K1612	Thermo Fisher Scientific Inc. St. Leon-Roth, Germany
iTaq™ Universal SYBR® Green Supermix	172-5120	Bio-Rad Laboratories GmbH, München, Germany
EZ DNA Methylation-Direct™ Kit	D5020	Zymo Research Europe GmbH, Freiburg, Germany
Ethanol (≥ 99,8%)	9065.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
ZymoTaq™ DNA Polymerase Kit	E2002	Zymo Research Europe GmbH, Freiburg, Germany

QIAquick PCR Purification Kit	28106	Qiagen GmbH, Hilden, Germany
Tris base	4855.3	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Agarose	N3101-0500	STARLAB GmbH, Ahrensbur, Germany
Ethidium bromide (1%)	2218.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
pGEM®-T Vector System II	A3610	Promega GmbH, Mannheim, Germany
Tryptone/peptone	8952.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Yeast extract	2363.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
NaOH	6771.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Agar	2266.2	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Ampicillin	HP62.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
IPTG	2316.3	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
X-B-gal	2315.3	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
D-(+)-glucose	16325	Sigma-Aldrich, St. Louis, USA
Taq DNA polymerase	786-447	G-Biosciences, St Louis, USA
Anti-CD14 antibody	ab27545	Abcam, Cambridge, UK
Anti-acetyl-histone H3 antibody	06-599	Merck Millipore, Darmstadt, Germany
Anti-acetyl-histone H4 antibody	06-866	Merck Millipore, Darmstadt, Germany

Anti- NF-κB p65 antibody	ab72555	Abcam, Cambridge, UK
Anti- NF-κB p105/p50	ab47336	Abcam, Cambridge, UK
Anti-β-actin (C4) antibody	sc-47778	Santa Cruz Biotechnology, Inc., Heidelberg, Germany
Goat anti-mouse IgG-HRP	sc-2005	Santa Cruz Biotechnology, Inc., Heidelberg, Germany
Clarity™ ECL Western Blotting Substrate	170-5060	Bio-Rad Laboratories GmbH, München, Germany
TNFα Pig ELISA Kit	Ab100756	Abcam, Cambridge, UK
IL-1β Pig ELISA Kit	Ab100754	Abcam, Cambridge, UK

3.1.2 Buffer, media and gels

1× PBS (without Ca^{2+} and Mg^{2+}, pH 7.4)	(g/L)
NaCl	8
$Na_2HPO_4.2H_2O$	1.44
KCl	0.20
KH_2PO_4	0.24
PAMs washing PBS	**Percentage (%)**
1× DPBS (without Ca^{2+} and Mg^{2+}, pH 7.4)	90
Fungizone® antimycotic	5
Pen-Strep	5
1× RBC lysis buffer	**(g/L) or (ml/L)**
NH_4Cl	8.30 g/L
$KHCO_3$	1 g/L
5% EDTA	1.80 ml/L
ddH_2O added to	1 L
PAMs culture medium	**Percentage (%)**
RPMI 1640 medium	87
FBS	10
Pen-Strep (100× concentrate)	1
Fungizone® antimycotic	1
Gentamicine (10 mg/ml)	1
Cell count medium	**ul**
Cell suspension	10
4% Trypan blue stain	50

PAMs culture medium	40
Tris-acetate-EDTA (TAE) (10×)	ml/L or g/L
Acetic acid (100%)	571 ml
Tris base	242 g
EDTA	100 ml
ddH_2O	added to 1 L
Agarose gel	2%
Agrose	4 g
TAE (1×)	200 ml
Ethidium bromide	9 µl
LB medium with ampicillin	
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Adjust pH to 7.0 with NaOH	
Agar	15 g
Autoclave, cool to 50 °C, adding	
Ampicillin	5 ml
SOC medium	500 ml
Tryptone	10 g
Yeast extract	2.50 g
1 M NaCl	5 ml
1 M KCl	1.25 ml
dd H_2O	485 ml

Autoclave, cool to room temperature,				
Adjust pH to 7.0 with NaOH				
2 M Mg^{2+} stock (filter-sterilized)			5 ml	
2 M glucose (filter-sterilized)			5 ml	
dd H_2O			Adding to 500 ml	
dNTP solution			400 ml	
dATP (100 mM)			10 µl	
dGTP (100 mM)			10 µl	
dTTP (100 mM)			10 µl	
Add ddH_2O to			400 ml	
Acrylamide gradients SDS-PAGE	8%	10%	12%	15%
Acrylamide (30%)	1.33 ml	1.67 ml	2.00 ml	2.50 ml
Separating gel buffer	1.25 ml	1.25 ml	1.25 ml	1.25 ml
ddH_2O	2.32 ml	2.00 ml	1.67 ml	1.15 ml
APS (Aliquot in -20 °C)	25 µl	25 µl	25 µl	25 µl
Temed	5 µl	5 µl	5 µl	5 µl
Sample gel			5.6%	
Acrylamide (30%)			0.725 ml	
Sample gel buffer			0.500 ml	
ddH_2O			2.825 ml	
APS			38 µl	
Temed			5 µl	
10× blotting buffer			1 L	
Tris			30.3 g	

Glyzin	144.0 g
ddH_2O	1 L
pH 8.3	

1× blotting buffer	1 L

10× blotting buffer	100 ml
Methanol	200 ml
ddH_2O	700 ml

3.1.3 Equipment and consumables

Equipment/consumables	Manufacturer/Supplier
Cell strainer (70 µm)	BD Biosciences, Heidelberg
Rotilabo® filter 0.22 µm	Carl Roth GmbH + Co. KG, Karlsruhe
Centrifuge tubes (15 ml, 50 ml)	SARSTEDT AG & Co., Nümbrecht
Centrafuge (5810R, 5424, 5416, 5415R)	Eppendorf AG, Hamburg
Haemocytometer	Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen
Microscope (ECLIPSE TS100)	Nikon GmbH, Düsseldorf
Digital camera system for microscopy digital sight series (DS-Fi1)	Nikon GmbH, Düsseldorf
Memmert CO_2 incubator	Fisher Scientific UK Ltd, Loughborough
6 - well cell culture plates	STARLAB GmbH, Hamburg
24 - well cell culture plates	STARLAB GmbH, Hamburg
96 - well cell culture plates	STARLAB GmbH, Hamburg
Serological pipettes (1,2, 5, 10, 25 ml)	Greiner Bio-One GmbH, Frickenhausen
Aspirating pipette	Greiner Bio-One GmbH, Frickenhausen
Cell scrapers	Greiner Bio-One GmbH, Frickenhausen
Plate reader synergy™ 2	BioTek Instruments GmbH, Bad Friedrichshall
FACSCanto flow cytometer	BD Biosciences GmbH, Heidelberg

StepOnePlus™ real time PCR system	Life Technologies GmbH, Darmstadt (Applied Biosystems®)
MicroAmp® fast optical 96-well reaction plate with barcode, 0.1 ml	Life Technologies GmbH, Darmstadt (Applied Biosystems®)
CEQ8000 sequencer system (CEQ8000)	Beckman Coulter, Inc., Fullerton, CA
Water-bath 1083	GFL Gesellschaft für Labortechnik mbH, Burgwedel
Nanodrop 8000 spectrophotometer	Thermo Fisher Scientific Biosciences GmbH, St. Leon-Roth
Universal high speed centrifugation (Z300 K, Z300, Z200 M/H, Z233 MK, Z323 K)	HERMLE Labortechnik GmbH,Wehingen
ELV fully automatic autoclave (3870)	Tuttnauer Europe B.V., Netherlands
Multi®-ultra tubes 0.65 ml	Carl Roth GmbH + Co. KG,Karlsruhe
SafeSeal® tubes 1.5 ml	Carl Roth GmbH + Co. KG, Karlsruhe
SafeSeal® tubes 2.0 ml	Carl Roth GmbH + Co. KG,Karlsruhe
PCR® strip tubes	VWR International GmbH, Darmstadt (Axygen®)
Pipette tips (10 µl, 200 µl, 1000 µl)	Labomedic GmbH, Bonn
Pipettes (0.5 - 10, 2 - 20, 20 - 200, 100 - 1000 µl)	Eppendorf AG, Hamburg

3.1.4 Software programs and statistical packages

Software	Using for	Sources
Primer 3	qRT-PCR primer design	http://simgene.com/Primer3
BLAST	Check alignment specificity	http://blast.ncbi.nlm.nih.gov/Blast.cgi
MethPrimer	Bisulfite PCR primers design, CpG islands prediction	http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi
BiQ Alalyzer	Bisulfite sequence analysis	http://biq-analyzer.bioinf.mpi-inf.mpg.de/index.php
FlowLogic® software	Flow cytometry analyses	BD Biosciences, Germany

3.2 Methods

3.2.1 Experimental animals

A total of three 35-days-old female GL piglets (after weaning) were used in this study. Animals were fed in the same environmental conditions in the teaching and research station of Frankenforst, University of Bonn, Germany. The feeding and husbandry practices of the animals followed the husbandry regulations and standard guidelines (ZDS, 2003). The experiment was approved by the Veterinary and Food Inspection Office, Siegburg, Germany (the number of permission: 39600309-547115). The piglets were selected from one litter and all of them were free from all major pig diseases. At the age of 28 days, piglets were weaned and placed in collective pens.

3.2.2 PAMs cells isolation from lungs of the GL piglets

Animals were humanely euthanized with a dose of ketamine and afterwards T61 (adjusted to the individual body weight) through the vena cava cranialis. After the animals had been euthanized, the chest and abdominal skin was disinfected with meliseptol. The thorax was opened gently and the whole lung was removed with trachea carefully. Afterwards, the lungs were washed with ice-cold sterile calcium-magnesium-free phosphate buffered saline (PBS) (1×, pH 7.4), then transported on ice to laboratory.

At the laboratory, lungs were taken out from the ice box, washed with 1× PBS (4 °C) again and put on the aluminium foil on ice. The PAMs from porcine lungs were obtained by bronchoalveolar lavage (BAL) using ice-cold PBS medium, as described by Islam (Islam et al. 2012), with some modifications. Sterile PBS medium (300 - 400 ml) was poured into the lungs with plastic dropper for 6 - 8 times and gently massaged 30 sec, The lavage fluid was collected in a sterile bottle and filtered by using sterile gauze. Afterwards, it was filtered by 70 µm cell strainer. The PAMs were precipitated by centrifuge with 1,500 rpm for 10 min at 4 °C. After centrifugation, the supernatant was completely and carefully removed. To remove the red blood cells (RBCs), the isolated cells were treated with 5 ml 4 °C red blood cell (RBC) lysis buffer solution for 5 min incubation at room temperature, aiming to prevent the contamination of red blood cells. The reaction of lysis buffer was stopped with 20 ml sterile Dulbecco's phosphate buffered saline (DPBS, 1×, pH 7.4, Life Technologies, Darmstadt, Germany) and a cell pellet was produced by centrifugation (1,500 rpm for 10 min, 4 °C).

The RBC lysis reaction process was repeated as often as required until all the red blood cells were removed completely. Supernatant was removed carefully and cell pellet was washed with sterile DPBS (1×, 4 °C, pH 7.4, Life Technologies, Darmstadt, Germ-any), then suspended by PAMs culture medium (37 °C) including 87% Roswell Park Memorial Institute (RPMI) 1640 medium (GlutaMAX™) (Life Technologies, Darmstadt, Germany), 10% fetal bovine serum (FBS), 1% penicillin-streptomycin (Pen-Strep) (100× concentrate, Life Technologies, Darmstadt, Germany), 1% fungizone® antimycotic (Life Technologies, Darmstadt, Germany) and 1% gentamicine (10 mg/ml, Life Technologies, Darmstadt, Germany). Cells were counted in a haemocytometer with the final cell suspension in 10 times dilution using 4% trypan blue stain and PAMs culture medium and the viability of cells also was determined on the basis of trypan blue dye exclusion. Then cells were cultured in PAMs culture medium in a 5% CO_2 humidified cell culture incubator at 37 °C.

3.2.3 PAMs cell morphology detection

PAMs were seeded in a poly-D-lysine-coated 6 - well cell culture plates (2×10^6 cells/ml) to observe the cell morphology. The medium was changed every second day. Morphology of PAMs on day 3, 5, 7, 12, 18 and 29 was evaluated by Microscope (ECLIPSE TS100, Nikon GmbH, Düsseldorf).

3.2.4 Cell characterization by flow cytometry analyses

Cell characterization were done by flow cytometry assay using FACSCanto flow cytometer (BD Biosciences, Heidelberg, Germany) and *FlowLogic*® software (BD Biosciences, Germany) with one cell marker CD163 (Abdserotec, cat. MCA2311F). PAMs were harvested, washed with sterile DPBS and cell numbers were counted by hemocytomer. Afterwards, 1×10^6 cells were incubated with 10 µL of conjugated antibody CD163 for 30 min at 4 °C. Cells were stained according to the manufacturer. Cells were washed two times and re-suspended in 1 ml and 400 µl flow cytometry staining buffer, respectively. Assay was performed by FACSCanto flow cytometer and the results were analyzed by using *FlowLogic*® software.

3.2.5 Treatment of PAMs with LPS and SFN

PAMs were seeded in poly-D-lysine-coated 24 - well cell culture plates (5×10^5 cells/ml) with 1 ml medium for LPS treatment in different time points to isolate total RNA. Cells were also seeded in 96 - well (1×10^5 cells/ml) plates with 100 µl medium for 48 h to perform cell viability assays. Thereafter, cell culture medium was changed every two days. On day 9, cells were treated with 5 µg/ml of LPS (LPS from E.coli 0111:B4, Invivogen, USA) for 1, 3, 6, 12, 24, 36 and 48 h (Fig. 8AB). Cells without LPS treatment served as a vehicle control (Fig. 8AB).

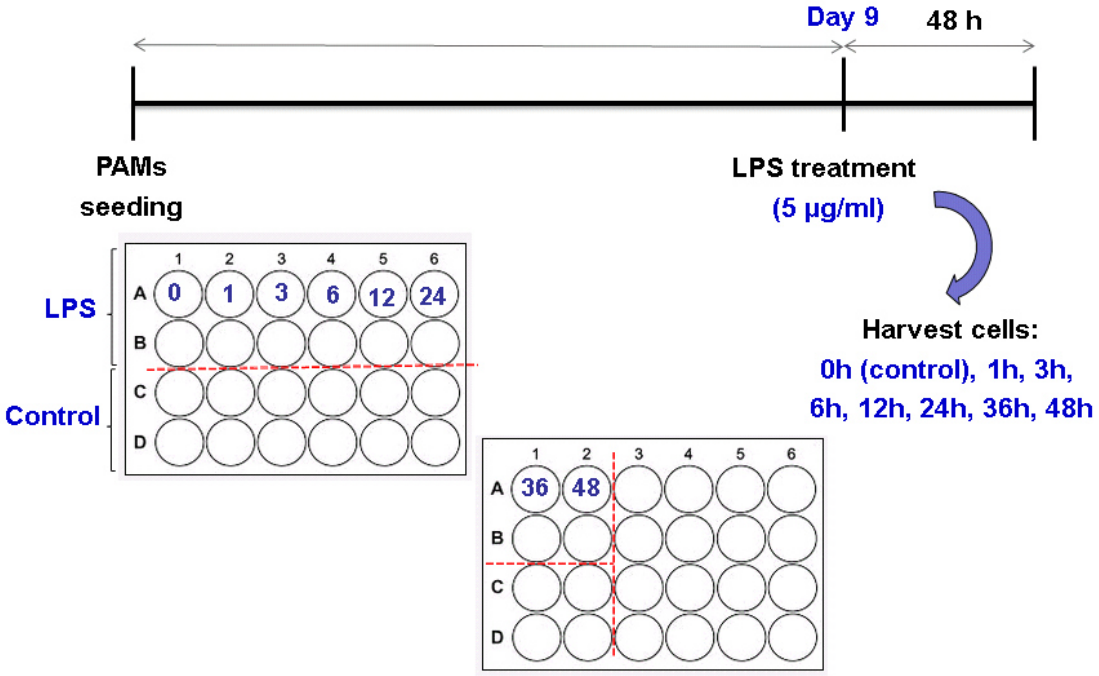

Fig. 8 (A) PAMs with LPS treatment in different time points in 24-well plates

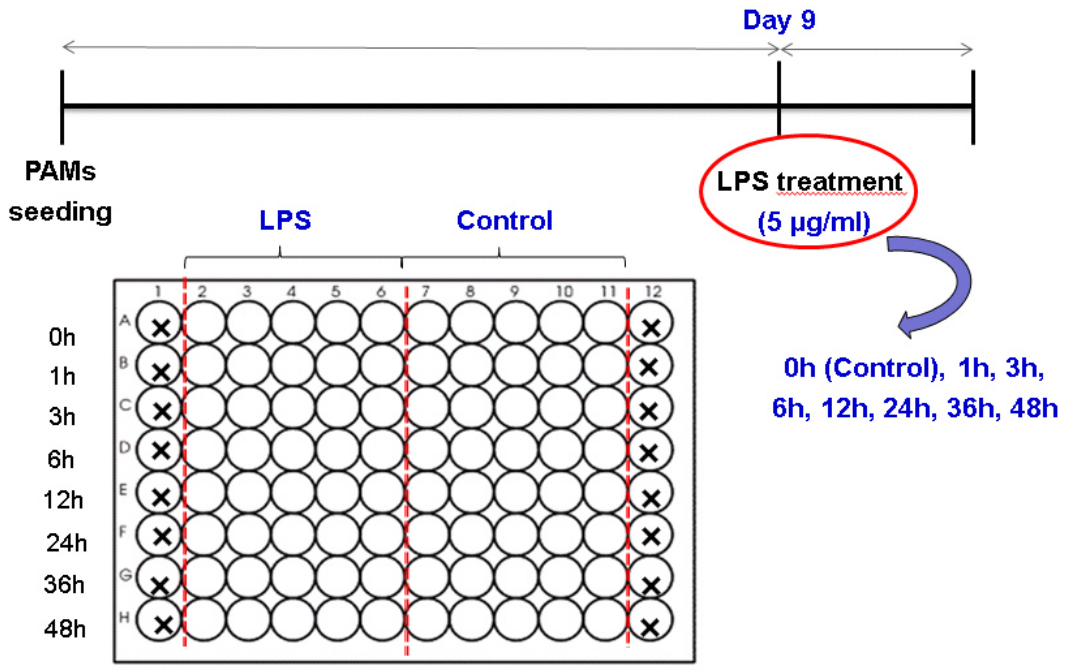

Fig. 8 (B) PAMs with LPS treatment in different time points in 96-well plates for cell viability test

(×: means wells were not used)

S - SFN (Biomol GmbH, Hamburg, Germany) was prepared in dimethyl sulfoxide (DMSO) and stored as a stock concentration of 50 mmol/L at -20 °C. PAMs were seeded in the poly - D - lysine - coated 6 - well cell culture plates (2×10^6 cells/ml) with 3 ml medium to isolate total RNA. Cells also were seeded in 96 - well (1×10^5 cells/ml) plates with 100 µl medium for 48 h to perform cell viability assays. On day 7, cells were treated with 0, 5, 10 µM SFN respectively (the maximum concentration of DMSO was 0.05% (v/v) in the medium) for 48 h. Cells treated only with DMSO served as control. Cells were treated with 5 µg/ml of LPS for 12 h subsequently. PAMs were also treated with SFN (0, 5, 10 µM) on day 7 for 48 h and LPS (0 or 5 µg/ml) on day 9 for 12 h in six groups (SFN0-LPS0; SFN0-LPS5; SFN5-LPS0; SFN5-LPS5; SFN10-LPS0; SFN10-LPS5) in 6-well plates, group SFN0-LPS0 served as a control (Fig. 9AB).

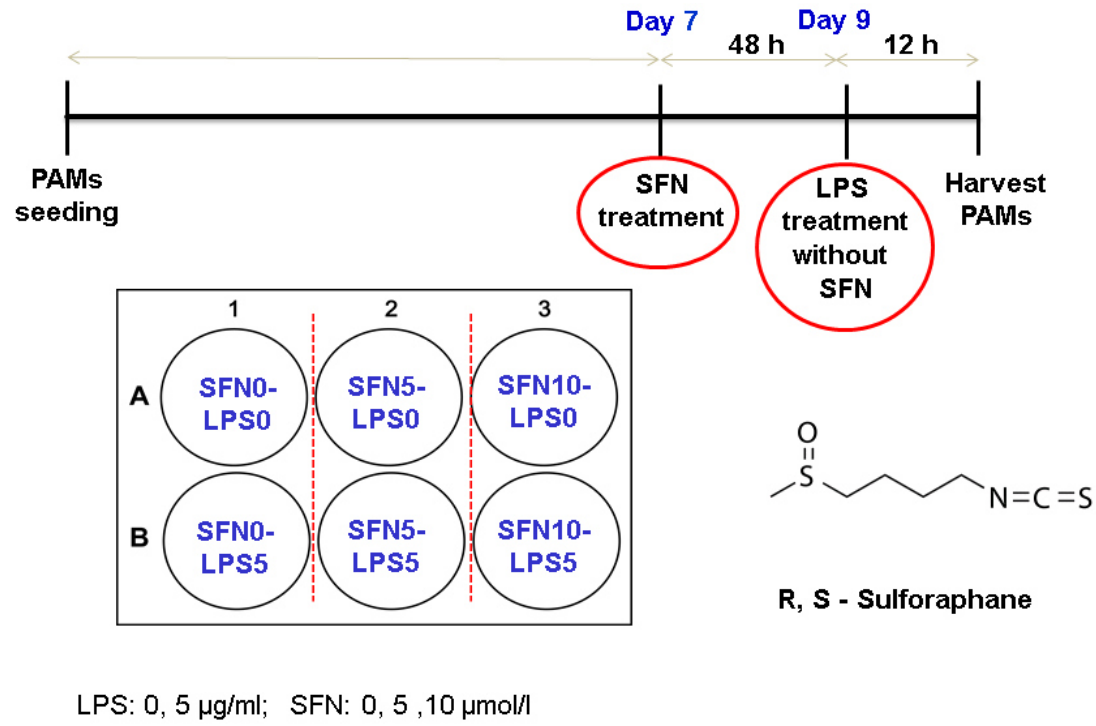

Fig. 9 (A) PAMs with SFN and LPS treatment in 6-well plates (3 plates)

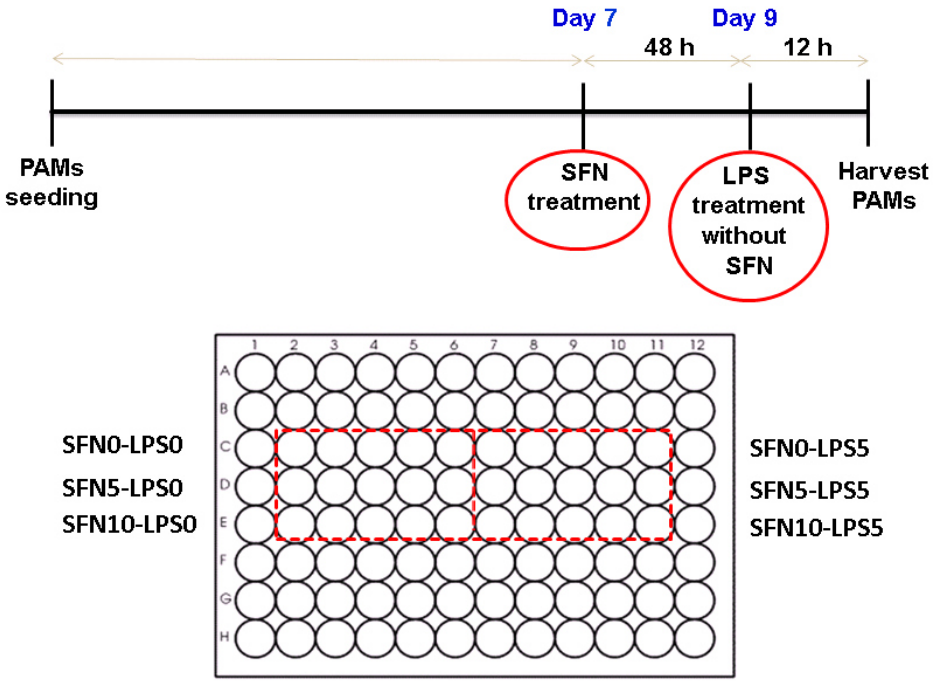

Fig. 9 (B) PAMs with SFN and LPS treatment in 96-well plate for cell viability test

3.2.6 Cell viability assay

The cell viability of PAMs was measured with water - soluble tetrazolium salt - 1 (WST-1) Cell Proliferation Assay Kit (Biomol GmbH, Hamburg, Germany). Cells were seeded in a 96 - well plates at a density of 1×10^5 cells/well in 100 µl of PAMs culture medium (Fig. 8B and 9B). For PAMs with/without LPS treatment, cells were challenged with 5 µg/ml LPS for 48, 36, 24, 12, 6, 3, 1 h on day 9 (Fig. 8B). For PAMs with/without LPS-SFN treatment, cells were challenged with SFN (0, 5, 10 µM) on day 7 for 48 h and LPS (0, 5 µg/ml) on day 9 for 12 h (Fig. 9B). At the end of stimulation, the old cell supernatant was aspirated and the fresh PAMs culture medium was added. A volume of 10 µl of the reconstituted WST-1 solution was added to each well. Cells were incubated for two h at 37 °C in the 5% CO_2 incubator. Then the supernatant was transferred to a new 96 - microplate and the absorbance of each sample was measured at a wavelength of 450 nm on the plate reader Synergy™ 2 (BioTek Instruments GmbH, Bad Friedrichshall, Germany).

Quantitative cell viability data was expressed as means ± SD, n = 3. The statistical analysis of data was investigated with SAS (version 9.3) using the general linear model containing the LPS and SFN treatments as fixed effects. The $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ were set as statistically significant.

3.2.7 RNA extraction, cDNA synthesis and real-time PCR

Total cellular RNA was isolated from PAMs using the AllPrep® DNA/RNA/Protein Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. Cells were disrupted by buffer RLT and the cell lysate was homogenized by a vortex mixer. RNA was bound with RNeasy spin column and washed with 700 µL buffer RW1 and 500 µL buffer RPE (2 times). Then the RNA was eluted with 30 µl ribonuclease (RNase) - free water and the concentration was measured by Nanodrop 8000 spectrophotometer.

200 ng of total RNA were reverse transcribed into first strand cDNA using the First Strand cDNA Synthesis Kit (Thermo Fisher Scientific Biosciences GmbH, St. Leon-Rot , Germany). The reaction system, which included 0.5 µl oligo $(dT)_{18}$ primer (100 µM, 0.5 µg/µl), 0.5 µl random hexamer primer (100 µM, 0.2 µg/µl), nuclease-free water and total RNA (200 ng) up to 11 µl, was mixed gently, centrifuged briefly and incubated at 65 °C for 5 min and then quick chilled on ice. Afterwards, 4 µl 5 × reaction buffer (250

mM Tris-HCl (pH 8.3), 250 mM KCl, 20 mM $MgCl_2$, 50 mM DTT), 1 µl Ribolock RNase inhibitor (20 U/µl), 2 µl dNTP Mix (10 mM) and 2 µl M-MuLV reverse transcriptase (20 U/µl) were added to the reaction tube with a total volume of 20 µl. Reaction tube was mixed gently and centrifuged for 30 sec and incubated at 25 °C for 5 min, 37 °C for 60 min and 70 °C for 5 min. cDNA concentrations were also measured by Nanodrop 8000 spectrophotometer.

The qRT-PCR was performed by using iTaq™ Universal SYBR® Green Supermix (Bio-Rad Laboratories GmbH, Germany). PCR primers (Table 2) were designed by using Primer 3 on line tool (http://simgene.com/Primer3) or chosen from the literature and additionally proofed with BLAST (basic local alignment search tool) (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure specificity. The pig glyceraldehyde - 3 - phosphate dehydrogenase (GAPDH) gene was selected as the house keeping gene. The total volume of 20 µl for each qRT-PCR reaction consisted of 100 ng cDNA, forward (F) and reverse (R) primers, RNase - and deoxyribonuclease (DNase) - free water and 10 µL of iTaq™ Universal SYBR® Green Supermix (2×). qRT-PCR amplification was conducted with 95 °C for 3 min; 95 °C for 15 sec, 60 °C for 45 sec (40 cycles); 95 °C for 15 sec, 62 °C for 1 min, 95 °C for 15 sec (Melt-Curve) by using StepOnePlus™ Real time PCR System (Applied Biosystems®).

Gene-specific expression was measured as relative to the geometric mean of the expression of the housekeeping gene GAPDH. The delta Ct (ΔCt) values were calculated as the difference between target gene and reference gene.

$\Delta Ct = Ct_{target} - Ct_{housekeeping\ gene}$

The relative gene expression was calculated as $2^{(-\Delta Ct)}$ and the fold change in expression between the treated and untreated PAMs was calculated as $2^{(-\Delta Ct_{treated})} / 2^{(-\Delta Ct_{control})}$ (Schmittgen and Livak KJ, 2008) (control: untreated group SFN0-LPS0). Relative gene expression levels were checked for outliers. Outliers were identified when these values deviating more than three standard deviations from the raw mean. These extreme values were eliminated from future analysis. Data was analyzed by SAS (version 9.3) with liner mixed model:

$$Y = LPS + SFN + LPS * SFN + animal + e$$

Y: fold change; *animal*: random effect of the animal; e: residuals

Group means were expressed as least square means (± standard error (SE)). The values were tested by a Dunnett - Hsu test in order to identify significant differences compared

to the control group (SFN0-LPS0). In addition, a Tukey - Kramer test (pair wise comparisons) was performed to compare other classes with each other. The (* and #) $P < 0.05$, (** and ##) $P < 0.01$, (*** and ###) $P < 0.001$ were set as statistically significant.

Table 2 Primers and their sequences for RT-PCR

	F	R
CD14	5'-TGG ACC TCA GTC ACA ACT CG-3'	5'-CCT TTA GGC ACT TGC TCC AG-3'
TRAM	5'-GTC TCC TGT ATG GCG ATG GT-3'	5'-TCT GTT GCA GGG AGG GTA AC-3'
TRIF	5'-CAC CTC CTT GTG GAG GAG AA -3'	5'-GAC GTC CTC CAT GTC CCT AA -3'
TRAF6	5'-CTG GAC GCC CTA AGA CAG AG-3'	5'-AAC CCT CCC TCC GAA GAC TA-3'
RIPK1	5'-AAA CTG ACG AAG GAG GAG CA -3'	5'-CAG ATG GCA TTT TCG TAG GG-3'
TRAF3	5'-CTC CTC CAG CCC AAA ATG TA-3'	5'-TTC TTC AAA TGC ACC AGC AG-3'
NF-κB	5'-TGG GAA AGT CAC AGA AAC CA-3'	5'-CCA GCA GCA TCT TCA CAT CT-3'
TNFa	5'-TCC TCA CTC ACA CCA TCA GC-3'	5'-CCA AAA TAG ACC TGC CCA GA-3'
IL-1β	5'-GTA CAT GGT TGC TGC CTG AA-3'	5'-CTA GTG TGC CAT GGT TTC CA-3'
IL-6	5'-GGC AGA AAA CAA CCT GAA CC -3'	5'-GTG GTG GCT TTG TCT GGA TT-3'
IL-12-p40	5'-ATG CAC CTT CAG CAG CTG GTT G-3'	5'-CTA ATT GCA GGA CAC AGA TGC-3'
IRF7	5'-ACA CTC TAC CCC CGT GTC TG-3'	5'-AGA CCC GTA CAG GAG CAC AC-3'
IRF3	5'-TTC CTG AGC CAG ACA CCT CT-3'	5'-ACT CCC ACT CGT CGT CAT TC-3'
IFN-α	5'-TTC CAG CTC TTC AGC ACA GA-3'	5'-ATG ACA CAG GCT TCC AGG TC-3'
IFN-β	5'-ACC TGG AGA CAA TCC TGG AG-3'	5'-AGG ATT TCC ACT TGG ACG AC-3'
GAPDH	5'-CAA GCA GTT GGT GGT ACA GG-3'	5'-GCT GGT GCT GAG TAT GTC GT-3'
DNMT1	5'-GCG GGA CCT ACC AAA CAT-3'	5'-TTC CAC GCA GGA GCA GAC-3'
DNMT3a	5'-CTG AGA AGC CCA AGG TCA AG-3'	5'-CAG CAG ATG GTG CAG TAG GA-3'

F = Forward, R = Reverse

3.2.8 Protein extraction and Western blot analysis

Total cellular protein purification was performed by using the AllPrep® DNA/RNA/Protein Mini Kit (Qiagen, Germany) according to the manufacturer's instruction. One volume (700 µl) of buffer APP was added to the flow-through from RNA-extraction step. Tubes were mixed vigorously and incubated at room temperature for 10 min to precipitate protein and centrifuged at 12,000 rpm for 10 min. The supernatant was decanted carefully. A volume of 500 µL of 70% ethanol was added to the protein pellet, centrifuged at 12,000 rpm for 1 min. The supernatant was removed and the protein pellet was dried for 5-10 min at room temperature. Afterwards, 50 µl buffer ALO were added and mixed vigorously to dissolve the protein pellet, incubated for 5 min at 95 °C to completely dissolve and denature the protein. Samples were cooled to room temperature, then centrifuged for 1 min at 12,000 rpm to pellet any residual insoluble material. The supernatant of protein was transferred to a new 1.5 ml tube and saved in -80 °C for Western blotting.

Equal amounts of protein extracts from the PAMs were loaded and separated by using 8-12% acrylamide gradients sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE). After running the gel, proteins were transferred from gel to a membrane. The membrane was incubated with primary antibodies (anti-CD14 antibody (1:400, Abcam), anti-acetyl-Histone H3 polyclonal antibody (1:1000, Millipore), anti-acetyl-Histone H4 polyclonal antibody (1:2000, Millipore), anti- NF-κB p105 / p50 (phospho S932) antibody (1:500, Abcam) and anti-β-actin (C4) (1:500, Santa Cruz Biotechnology, Inc.)). Signals were detected by a secondary antibody (goat anti-mouse immunoglobulin G - horseradish peroxidase (IgG-HRP) (1:8000, Santa Cruz Biotechnology, Inc.)). The specific signals of immunoreactive bands were detected by chemiluminescence using Clarity™ ECL Western Blotting Substrate (1:1000, Bio-Rad Laboratories GmbH). Images were acquired by Quantity One 1-D analysis software (Bio-Rad).

3.2.9 ELISA analysis of cytokines

PAMs cells culture supernatants were collected after PAMs stimulation with or without SFN for 48 h and LPS for 12 h (from the arrangement treatment groups Fig. 9A) to measure cytokines by Enzyme-linked immunosorbent assay (ELISA). IL-1β and TNF-α cytokines levels were measured by using porcine specific ELISA kits (Abcam,

Cambridge, UK) with the sensitivity of minimum detectable dose as 6 pg/ml (less than) and 20 pg/ml (less than), respectively. Assays were conducted in 96-well microplates according to the manufacturer's instruction and the absorbance of 450 nm was measured on the plate reader Synergy™ 2 (BioTek Instruments GmbH, Bad Friedrichshall, Germany).

Data was expressed as mean ± SD, n=3. The statistical differences between protein level values were analyzed by SAS (version 9.3) using the general linear model. The $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ were set as statistically significant.

3.2.10 *CD14* CpG islands prediction

The *CD14* promoter region and the complete coding sequence (CDS) (GenBank DQ079063.1, 1762 bp) (Sanz et al. 2007) were submitted to the online program: Methprimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) to identify the CGIs.

3.2.11 Genomic DNA extraction

PAMs were cultured in 6 - well plates at a density of 2×10^6 cells/well and were treated with SFN on day 7 for 48 h followed by 12 h stimulation of LPS on day 9 (Fig. 9A). gDNA was extracted by using buffer EB by AllPrep® DNA/RNA/Protein Mini Kit (Qiagen, Germany). Cells were disrupted by buffer RLT and the cell lysate was homogenized by a vortex mixer. The gDNA was bound with an AllPrep DNA spin column placed in a 2 ml collection tube from the homogenized lysate. The gDNA was washed by 500 µL buffer AW1 and 500 µL buffer AW2, afterwards, eluted by buffer EB (preheated to 70 °C) with 2 min incubation at room temperature (15 - 25 °C) and 1 min centrifugation at 10,000 rpm. The concentration of gDNA was measured by Nanodrop 8000 spectrophotometer.

3.2.12 Bisulfite conversion of gDNA

To assess the methylation status of CD14 gene promoter and complete CDS, sodium bisulfite methylation sequencing was performed. 300 ng purified gDNA were used for bisulfite conversion followed the protocol of EZ DNA Methylation-Direct™ Kit (Zymo Reserch, Freiburg, Germany). Cytosine-thymidine (CT) conversion reagent was prepared by adding 790 µL of M-solubilization buffer and 300 µL of M-dilution buffer

by following 10 min frequent vortex at room temperature, then adding 160 µL of M-reaction buffer and mixing an additional 1 min. M-wash buffer was prepared by adding 24 ml of 100% ethanol. A total amount of 300 ng gDNA and ddH_2O with a volume of 20 µl were added to 130 µl of CT conversion reagent solution in a PCR tube, mixed and then centrifuged briefly. The PCR tubes were placed in a thermal cycler and incubated by 8 min at 98 °C, 3.5 hours at 64 °C and 4 °C storage for up to 20 h. A volume of 600 µl of M-binding buffer was added into a Zymo-Spin™ IC column which was placed into a provided collection tube. The sample of gDNA with CT conversion reagent solution was loaded into the Zymo-Spin™ IC column containing the M - binding buffer after finishing the thermal cycler program, mixed by inverting the column several times, then centrifuged at full speed (> 10,000 × g) for 30 sec. Afterwards, 100 µl of M-wash buffer were added to the column and centrifuged at full speed for 30 sec. Then 200 µl of M-desulphonation buffer were added to the column with 15 - 20 min incubation at room temperature (20 - 30 °C) and 30 sec centrifugation at full speed. A volume of 200 µl of M-wash buffer was added to the column and centrifuged at full speed for 30 sec, this step was repeated one time. The bisulfite conversed gDNA was eluted by 10 µl of M-elution buffer with 30 sec centrifugation at full speed and measured by Nanodrop 8000 spectrophotometer and stored at - 20 °C.

3.2.13 Bisulfite PCR amplification and PCR purification

Bisulfite PCR was performed by ZymoTaq^{TM} DNA Polymerase Kit (Zymo research, Germany) using touchdown PCR. Bisulfite PCR primers (9 primers: CD1401 to CD1409, Table 3) of CD14 promoter region and complete CDS (GenBank DQ079063.1, 1762 bp) were designed by using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi) online software. In the PCR reaction, 12.5 µl 2× reaction buffer, 0.4 µl dNTP Mix (25 mM each dNTP), 0.5 µl Forward Primer (10 µM), 0.5 µl Reverse Primer (10 µM), 1 µl bisulfite gDNA, 0.2 µl ZymoTaq^{TM} DNA polymerase (5 U/µl) and DNase/RNase - Free H_2O were added for a total volume of 25 µl. The reaction program of bisulfite touchdown PCR is displayed in table 4.

Table 3 Primers for bisulfite PCR

Primer	F (5′-3′)	R (5′-3′)	Product size (bp)
CD1401	AGGGAAAAGTTAAGGAAATTTTTTG	CAAAACCTCTAAAATCCTTAACACTAAAC	169
CD1402	GGATTTTAGAGGTTTTGTAAGATTTTTY	ACTCCCTAACTTCCAAACTCCAC	248
CD1403	GGAGTTTGGAAGTTAGGGAGTGT	CAACAAAAACAACAACAACAACAA	219
CD1404	TTGTTGTTGTTGTTGTTTTTGTTG	TTTAAAAAAAAACTCTTCCAAACTCC	194
CD1405	GTTTGGAAGAGTTTTTTTTAAAGAG	ACAAAACCAAAACCAAAATCTAAAC	135
CD1406	GGTTTTTGTTTAGATTTTGGTTTTG	TTAACTAAAACCACTACTACAATTC	212
CD1407	TTTTAGTTAAGTTTTAAGGTATTGAAAGTG	AACAAAAAACTACAATCAACCC	151
CD1408	GGGTTGATTGTAGTTTTTTGTT	ACAATCCTTTAAACACTTACTCCAACT	253
CD1409	TGATTTTGGAAGGGAATTTTTATAT	ATTCCCCTTCCTTAAACCTTAAAC	176

Note: Y-CT

Table 4 Programs of touchdown PCR for bisulfite primers

Primer	Program
CD1401 CD1403	95 °C for 10 min; 95 °C for 30 sec, 0.5 °C↓/58 °C for 1min (20 cycles); 95 °C for 45 sec, 48 °C for 1min, 72 °C for 1 min 30 sec (40 cycles); 72 °C for 10 min; 4 °C for ∞
CD1402	95 °C for 10 min; 95 °C for 30 sec, 0.5 °C↓/59°C for 1min (20 cycles); 95°C for 45 sec, 49 °C for 1 min, 72 °C for 1 min 30 sec (40 cycles); 72 °C for 10 min; 4 °C for ∞
CD1404 CD1405 CD1406 CD1407	95 °C for 10 min; 95 °C for 30 sec, 0.5 °C↓/55 °C for 1min (20 cycles); 95 °C for 45 sec, 45 °C for 1 min, 72 °C for 1 min 30 sec (40 cycles); 72 °C for 10 min; 4 °C for ∞,
CD1408 CD1409	95 °C for 10 min; 95 °C for 30 sec, 0.5 °C↓/56 °C for 1 min (20 cycles); 95 °C for 45 sec, 46 °C for 1 min, 72 °C for 1 min 30 sec (40 cycles); 72 °C for 10 min; 4 °C for ∞

The bisulfite touchdown PCR production was purified by using QIAquick PCR Purification Kit which was used for purification of 100 bp to 10 kb PCR products. Five

volumes of buffer PB were added into 1 volume of bisulfite touchdown PCR samples and mixed by pipette. The QIAquick spin columns were placed in provided 2 ml collection tubes. The PCR sample-PB solution was loaded into the columns, then columns were centrifuged at 12,000 rpm for 1 min. A volume of 750 µl buffer PE was added to the QIAquick spin columns, centrifuged at 12,000 rpm for 1 min. The flow-through was discarded and the column was centrifuged at 12,000 rpm for an additional 1 min. The column was placed in a new 1.5 ml micro-centrifuge tube. A volume of 30 µl buffer EB (10mM Tris-HCl, pH 8.5) was added to the center of the QIAquick membrane. The column was stand for 1 min and centrifuged for 1 min at 12,000 rpm. The purified PCR product (5 µl) was loaded to 2% agarose gel. To check the PCR products, the gel was run following by a step at 120 V for 30 min. The concentration of purified PCR product was measured by Nanodrop 8000 spectrophotometer.

3.2.14 Cloning

The purified bisulfite touchdown PCR products were cloned by using pGEM®-T Easy Vector Systems (Promega GmbH, Germany). The ligation reaction system was set as described below (Table 5). Reaction was mixed by pipette and incubated overnight at 4 °C.

Table 5 Ligation reaction system

Reaction component	Standard reaction	Positive control	Background control
2× Rapid ligation buffer, T_4 DNA ligase	5 µl	5 µl	5 µl
pGEM®-T easy vector (50 ng)	1 µl	1 µl	1 µl
Purified PCR product	3 µl	0 µl	0 µl
Control insert DNA	0 µl	2 µl	0 µl
T_4 DNA ligase	1 µl	1 µl	1 µl
Nuclease-free water	0 µl	1 µl	3 µl
Total reaction volume	10 µl	10 µl	10 µl

The transformation was performed by using JM109 High Efficiency Competent Cells. LB/ampicillin/IPTG/X-Gal plates were prepared. The JM109 High Efficiency Competent Cells were removed from -80 °C freezer and placed in an ice bath until just thawed (about 5 min) and mixed by gently flicking the tubes. The ligation reaction tubes were centrifuged briefly and 5 µl of ligation reaction were put into sterile 1.5 ml tubes on ice, then 100 µl of competent cells were added. The tubes were flicked gently to mix and placed on ice for 20 min. Then cells were heat-shocked in a water bath at exactly 42 °C without shake for 45 - 60 sec and returned immediately to ice for 2 min. A volume of 950 µL SOC medium was added to the ligation reaction transformations and incubated for 1.5 hours at 37 °C with shaking (150 rpm). Cells were centrifuged for 3 min at 1,000 rmp and re-suspend in 200 µl SOC medium. A volume of 100 µL of each transformation cell suspension was poured onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the uncut DNA control, a 1:10 dilution with SOC was recommended. The plates was incubated overnight (18 h) at 37 °C. White clones (positive clones) were selected and picked up for cloning PCR. The blue clones were also picked up for cloning PCR as a negative control.

3.2.15 Cloning PCR

With the picked clones, a PCR was performed. Cloned DNAs were heated by thermal cycler at 95 °C for 15 min. Cloning PCR was performed by Taq DNA polymerase with messier 13 (M13) primers (F 5′-TTG TAA AAG GAG GGC CAG T-3′, R 5′-CAG GAA ACA GCT ATG ACC, Tm-56 °C). The PCR reaction system was set as described below (Table 6) and PCR program was performed with 95 °C for 5 min; 95 °C for 30 sec, 56 °C for 1 min, 72 °C for 1 min, 40 cycles; 72 °C for 10 min; 4 °C for ret. To check the PCR product, 5 µl of each product were loaded to 2% agarose gel. The gel was run following a step at 120 V for 30 min.

Table 6 The reaction system of M13 cloning PCR

Reaction component	Volume (μl)
dd H_2O	5.8
10× PCR buffer (Mg^{2+} plus)	2.0
dNTPs (10 mM each)	1.0
Primer (10 μM) F	0.5
Primer (10 μM) R	0.5
Cloning DNA	10.0
Taq DNA polymerase (5 U/μl)	0.2
Total reaction volume	20.0

* F-Forward primer, R-reverse primer

3.2.16 Bisulfite sequencing and DNA methylation analysis

A minimum of 8 different positive clones (white clones) for each subject were randomly selected for sequencing with M13 primer (forward primer) performed by the CEQ8000 sequencer system (Beckman Coulter, Inc., CA). Bisulfite sequencing data were performed with default parameters using BiQ Analyzer on line tool (http://biq-analyzer.bioinf.mpi-inf.mpg.de/index.php). Sequences with a conversion rate below 90% were excluded from analysis.

4 Results

4.1 PAMs cell phenotypes

4.1.1 PAMs cell morphology

PAMs cell morphology and cell growth state in different time points (day 3, 5, 7, 12, 18 and 29) were recorded in this study. The PAMs grew as time increased (Fig. 10). Cell morphology is mainly characterized by two structures including round shape and dumbbell shape (Fig. 10C). Cell feelers grew around cell membrane on day 5 (Fig. 10B). Cells were confluent on day 12 (Fig. 10D). Most of cells died as cytoplasmic lysis on day 29 (Fig. 10F). From these results, we suggested day 9 was chosen for LPS treatment and day 7 was chosen for SFN pre-treatment.

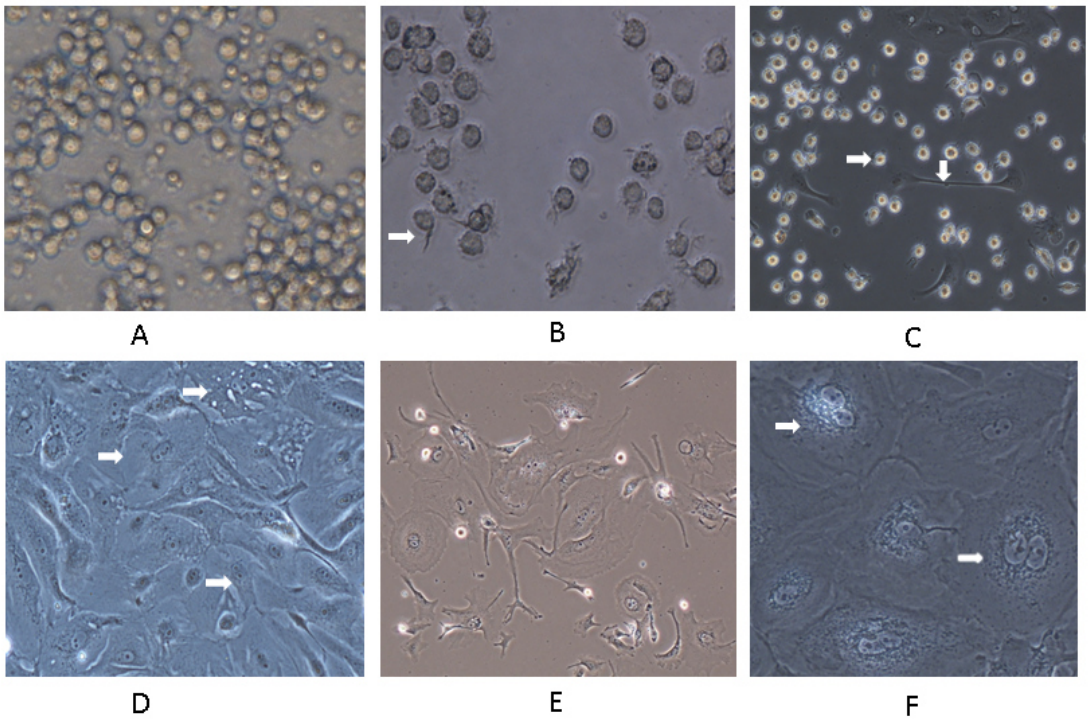

Fig. 10 Morphology of PAMs on different days of culture (ABCDF-20×, E-10× under microscope). A-day 3, B-day 5, C-day 7, D-day 12, E-day 18, F-day 29.

4.1.2 Cell characterization by flow cytometry analyses

Cells were analyzed by flow cytometry, results are shown in Fig. 11. 35% of cells were stained with marker CD163.

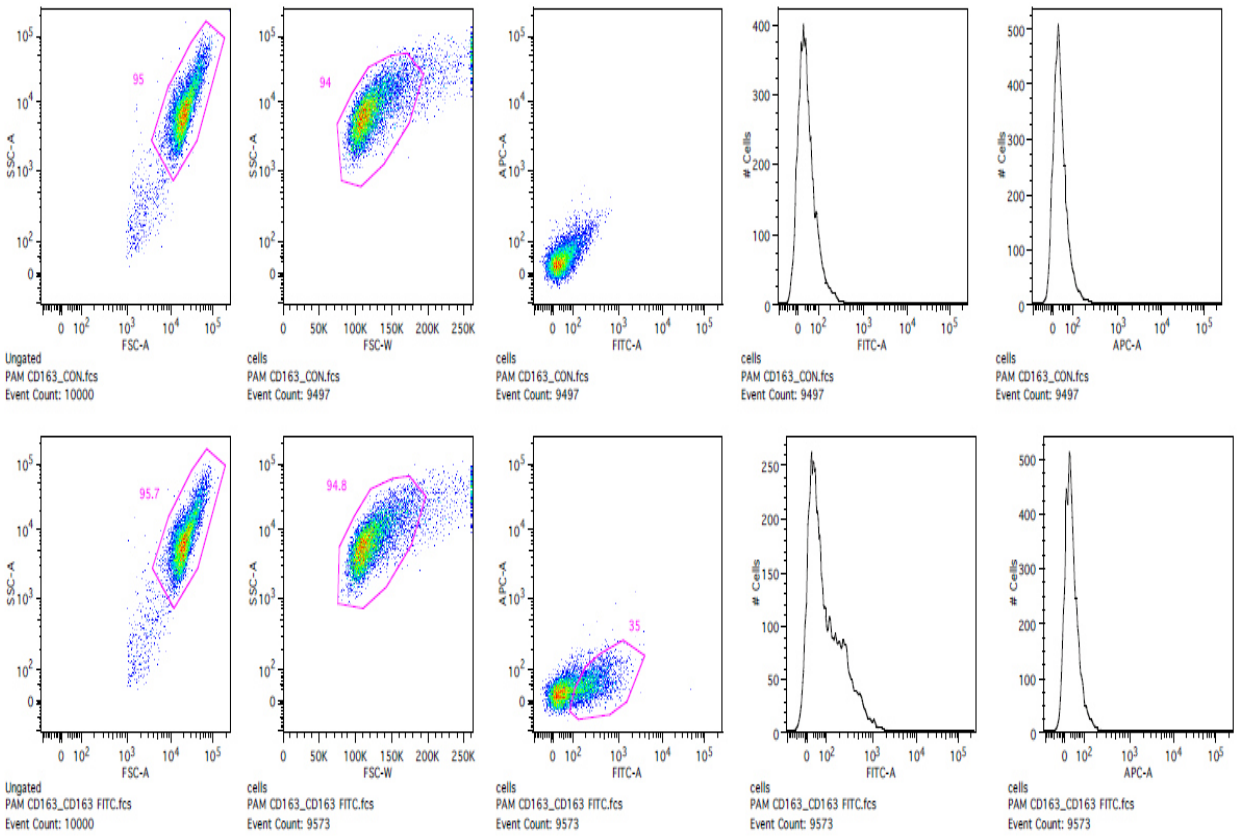

Fig. 11 Flow cytometry results of PAMs with cell marker CD163. The y-axis represents the cell population, the x-axis represents the fluorescence. The upper row is the result of cells without marker (control) and the lower row is the result of cells with marker CD163. Figures with red color mean the rates of cell population.

4.1.3 LPS affects PAMs cell viability in a time dependent manner

PAM cells viability was measured by WST-1 Cell Proliferation Assay Kit (Biomol GmbH, Hamburg, Germany). LPS at a dose of 5 μg/ml was used to induce inflammatory conditions in cultured PAMs. To determine the effective but nontoxic time point of LPS treatment on PAMs, we first performed cell viability assay to evaluate the cell number and activity. The results show that there was no difference between LPS treated cells and control, also between the untreated cells and control at all time points (Fig. 12A). When treated and untreated groups were compared, LPS had no noticeable effect on the cell viability after 1, 12 and 48 h (1 h, 12 h and 48 h of LPS post stimulation, ps) (Fig. 12A). The highest cell viability of both treated and untreated PAMs in these three groups was observed at 12 h LPS ps. Therefore, the time point of 12 h ps was selected for the further study.

4.1.4 SFN affects PAMs cell viability in a dose dependent manner

For the further study, the LPS treatment at time point of 12 h was used as the positive control (SFN0-LPS5) and SFN was pre-added in different doses of 0, 5 and 10 μM at day 7 for determining the effective dose of SFN on PAMs cell viability. Cell viability of SFN5-LPS0 and SFN5-LPS5 had no differences compared to the control group (SFN0-LPS0) (Fig. 12B). There were significant differences between SFN10-LPS0 and SFN0-LPS0 (control), as well as between SFN10-LPS5 and SFN0-LPS0 (control) (Fig. 12B). SFN pre-treatment significantly increased the cell viability in LPS treated PAMs (SFN5-LPS5 and SFN10-LPS5) compared to the positive control (Fig. 12B).

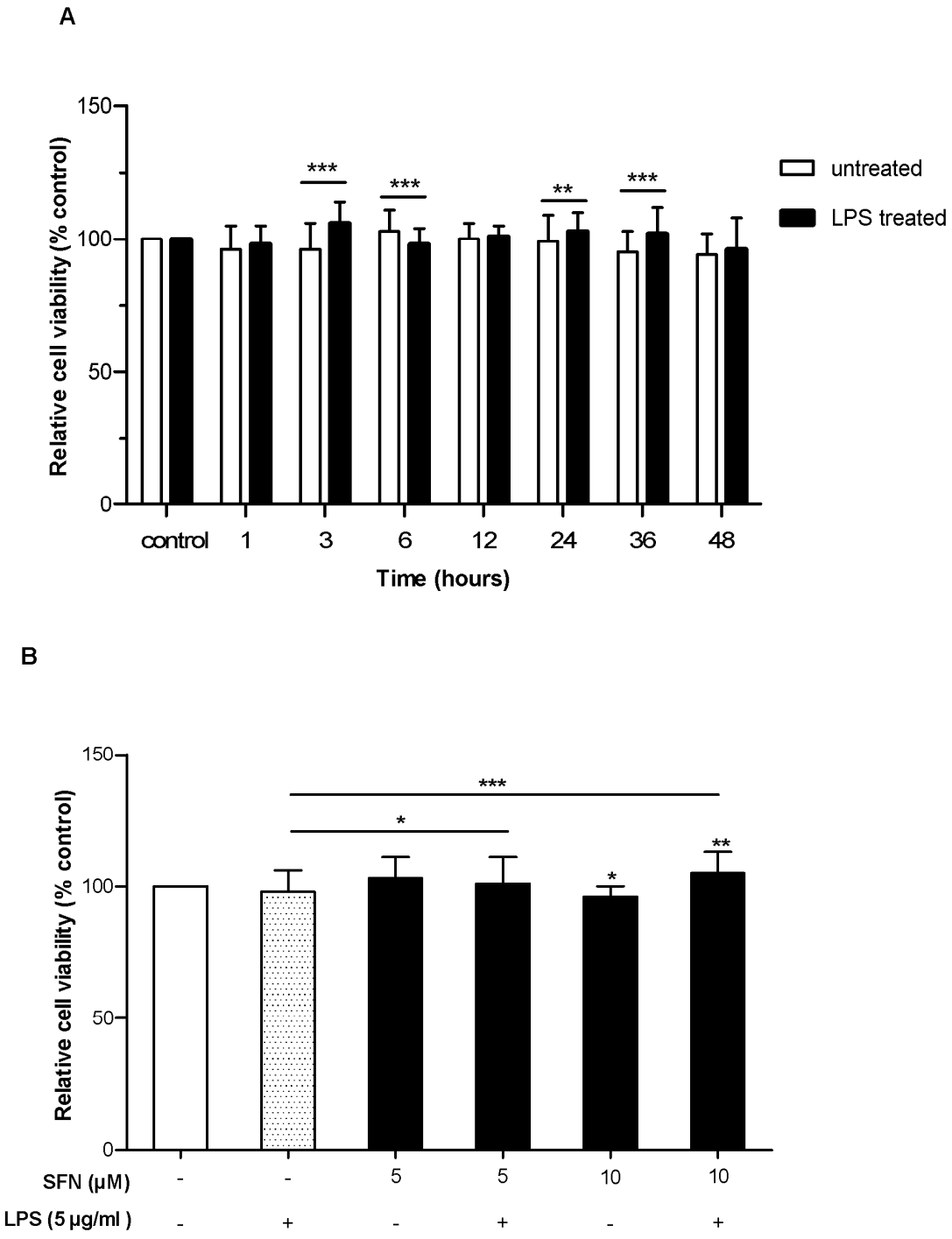

Fig. 12 Results of the PAMs cell viability assay. A) The relative cell viability of PAMs without and with 5 μg/ml LPS treatment (1, 3, 6, 12, 24, 36, 48 h). B) The relative cell viability of PAMs without and with SFN (5, 10 μM) and LPS (5 μg/ml) treatment. All of the data were expressed as mean ± SD, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.2 CD14 mRNA expression was induced by LPS in time dependent manner

To investigate the potential effects of LPS on *CD14* expression in PAMs, quantitative real-time PCR (qPCR) was performed. The result showed that LPS significantly induced CD14 gene expression at time points 1, 6, 12 and 24 h (Fig. 13) while the highest expression was displayed at time point 12 h (Fig. 13). At 12 h, gene expression of CD14 in LPS treated PAMs was significantly higher than in LPS untreated group (LPS0-12 h) and control (LPS0-0 h) (Fig. 13). Theses results indicated that LPS acts on the *CD14* expression at time points 1, 6, 12 and 24 h, but the greatest effect was at 12 h (Fig. 13). The results indicated that LPS induced *CD14* expression in a time dependent manner (Fig. 13).

4.3 TRIF downstream genes were induced by LPS

CD14 directly binds with LPS and activates the MyD88-dependent pathway (Akira and Takeda 2004), it may also be involved in TRIF pathway by binding with LPS. Thereby, we further analyzed the mRNA expression of CD14 downstream TRIF pathway genes including TRAM, TRIF, the TRIF-TRAF6 sub-pathway genes (TRAF6, RIPK1, NF-κB) and the TRIF-TRAF3 sub-pathway genes (TRAF3, IRF7, IRF3) in 5 μg/ml LPS treated PAMs. LPS significantly increased gene expression of CD14, TRIF, TRAF6, NF-κB, TRAF3 and IRF7 (Fig. 14 ABC).

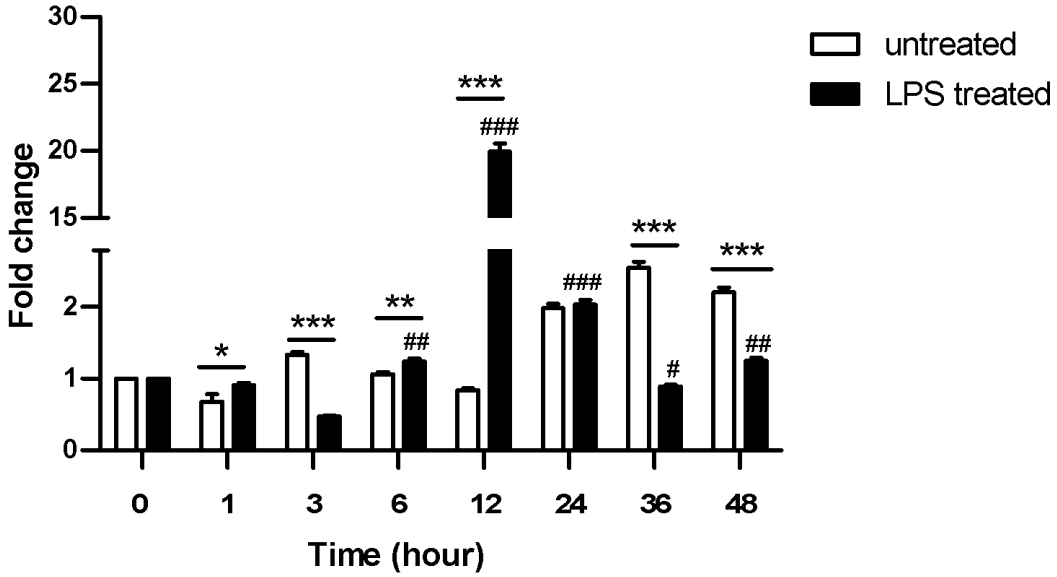

Fig. 13 Effect of LPS on *CD14* mRNA expression in PAMs. Relative *CD14* mRNA expression in PAMs with or without 5 μg/ml LPS treatment in 7 time points (1, 3, 6, 12, 24, 36, 48 h) were assayed. mRNA expression without LPS treatment on 0 h was set as control. Comparison between treated and untreated cells at one time point is indicated as '*'. Comparison between treated group and control (0 h) is indicated as '#'. All of the data are expressed as mean ± SD, n = 3. * (#), ** (##) and *** (###) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.4 mRNA expression of CD14 and downstream LPS-induced TRIF pathway genes were suppressed by SFN in a dose dependent pattern

As a HDAC inhibitor, SFN also has anti-inflammatory effects (Ko et al. 2013). To explore the effect of SFN on LPS induced inflammation, we examined the relative mRNA of *CD14* and downstream genes expression in TRIF pathway as illustrated in Fig. 14. The results clearly demonstrated that SFN pre-treatment significantly inhibited CD14 gene expression (Fig. 14A). We set the SFN0-LPS5 treated cells as the positive control. Compared with the positive control, CD14 expression was decreased significantly ($p < 0.05$) in SFN5-LPS5 group.

SFN suppressed LPS-induced TRAM ($p < 0.05$) and TRIF ($p < 0.001$) mRNA expression comparing with positive control at the dose of 5 μM (Fig. 14 A). In the TRIF-TRAF6 pathway, the mRNA expression of TRAF6 and NF-κB genes were decreased in the SFN5-LPS5 group compared with the positive control, while the expression of LPS-

induced RIPK1 gene was inhibited ($p < 0.05$) by SFN at the doses of 5 μM (Fig. 14 B). In TRIF-TRAF3 pathway, SFN suppressed ($p < 0.01$) LPS-induced *TRAF3* expression at the dose of 5 μM (Fig. 14 C). These results suggest that the inhibitory effects of SFN on LPS-induced TRAM, TRIF, RIPK1 and TRAF3 at the dose of 5 μM might be mediated through decreased *CD14* mRNA expression. The other genes including TRAF6, NF-κB and IRF7 showed the same trend, only IRF3 represented on a contrary trend. Results of additional group comparisons can be found in appendix file 1 and 2 (Fig. 21 and Fig. 22).

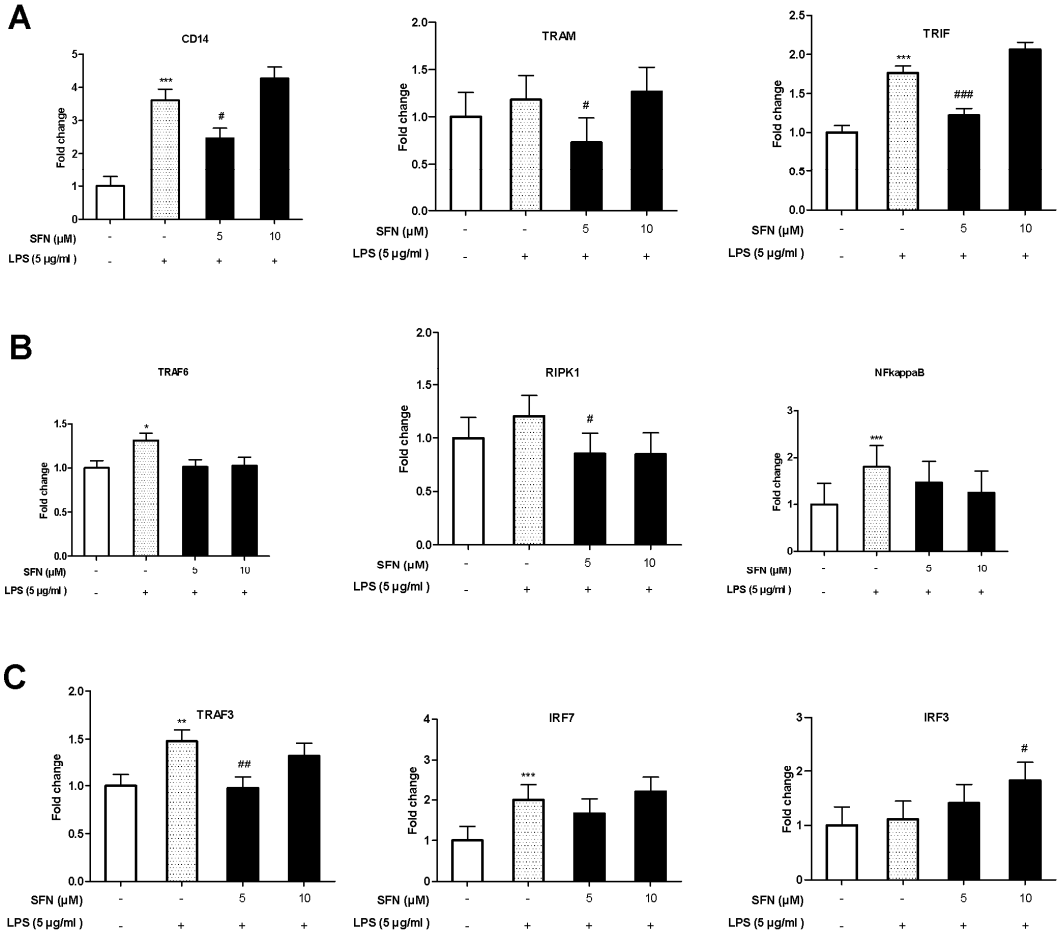

Fig. 14 SFN inhibited *CD14* mRNA expression and effected on TRIF pathway gene expression in PAMs. A) *CD14* and downstream genes TRAM and TRIF mRNA expression in SFN-LPS stimulated PAMs. B) TRIF-TRAF6 pathway genes mRNA expression in SFN-LPS treated PAMs. C) TRIF-TRAF3 pathway genes mRNA expression in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. mRNA

expression in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. mRNA expression in PAMs without SFN treatment but with LPS treatment (SFN0-LPS5) was set as the positive control. Comparison between treated group and control is indicated as '*'. Comparison between SFN treated group and the positive control is indicated as '#'. All of the data were expressed as mean ± SEM, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.5 Gene expression of LPS-induced cytokines in CD14-TRIF pathway were inhibited by SFN in dose dependent manner in PAMs

Cytokines such as TNFα, IL-1β, IL-6, IL-12p40, IFNα and IFNβ were produced in the CD14-TRIF pathway where the first four cytokines are from the TRIF-TRAF6 sub pathway and the last two cytokines are from the TRIF-TRAF3 sub pathway. Thus we measured the mRNA expression of theses cytokines in the same six groups of cells with SFN-LPS treatment. As shown in Fig. 15A, compared with the positive control (SFN0-LPS5), the gene expression of TNFα was significantly decreased in SFN5-LPS5 ($p < 0.05$) group. For IL-1β, the gene expression was suppressed by SFN at both 5 μM ($p < 0.001$) and 10 μM ($p < 0.001$) as compared with the positive control (Fig. 15A). The gene expression of IL-6 was suppressed by SFN at 10 μM ($p < 0.05$) compared with the positive control (Fig. 15B). Gene expression of cytokines released from TRIF-TRAF3 pathway including IFN-α and IFN-β are shown in Fig. 15 C. SFN inhibited LPS-induced *IFN- β* expression at the dose of 5 μM ($p < 0.001$). Results of additional group comparisons can be found in appendix file 3 and 4 (Fig. 23 and Fig. 24).

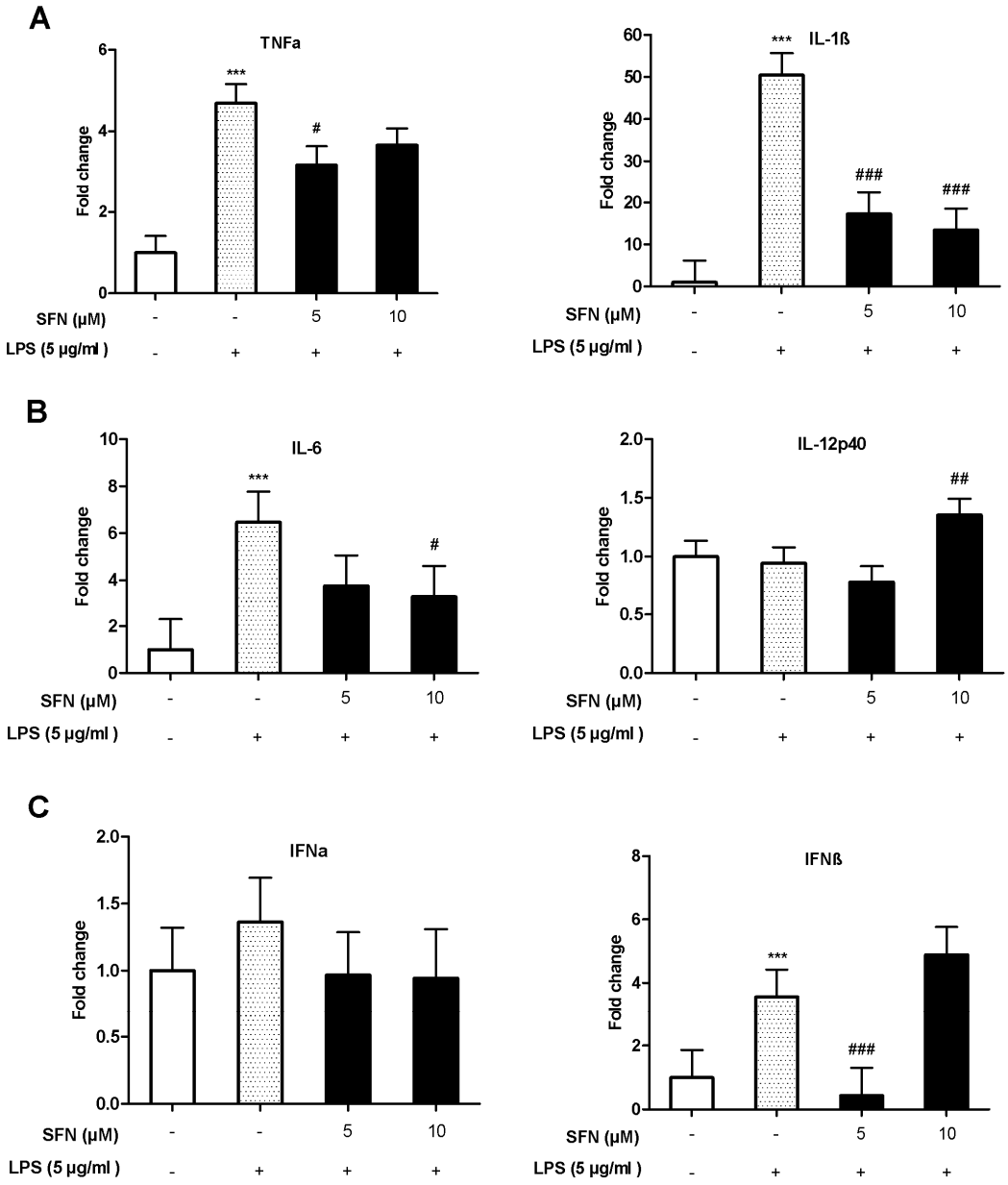

Fig. 15 SFN altered mRNA expression of cytokines in TRIF pathway in PAMs. A and B) Relative gene expression of TRIF-TRAF6 pathway released cytokines in SFN-LPS treated PAMs. C) Relative gene expression of TRIF-TRAF3 pathway released cytokines in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. Relative mRNA expression in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. Relative mRNA expression in PAMs without SFN but with LPS treatment (SFN0-LPS5) was set as the positive control. Comparison between treated group and control is indicated as '*'. Comparison between SFN treated group and the positive

control is indicated as '#'. All of the data were expressed as means ± SEM, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.6 Protein levels of pro-inflammatory cytokines TNFα and IL-1β were suppressed by SFN

Productions of cytokines including TNFα and IL-1β were measured by ELISA (Fig. 16). To our surprise, we found that both LPS-induced TNF-α and IL-1β production was suppressed by 5 μM and 10 μM SFN. The results clearly show that LPS induced TNF-α and IL-1β production. The level of TNF-α in SFN0-LPS5 cells was significantly ($p < 0.001$) increased compared with the control group and the level of IL-1β in the same group was also significantly ($p < 0.05$) higher than the control group. Treatment with SFN reduced the production of TNF-α with significant effects for SFN5-LPS0 ($p < 0.001$), SFN5-LPS ($p < 0.001$), SFN10-LPS0 ($p < 0.01$) and SFN10-LPS5 ($p < 0.01$) as compared with the positive control. SFN also significantly repressed IL-1β production in SFN5-LPS0 ($p < 0.001$), SFN5-LPS ($p < 0.001$) and SFN10-LPS0 ($p < 0.01$) groups as compared to the positive control.

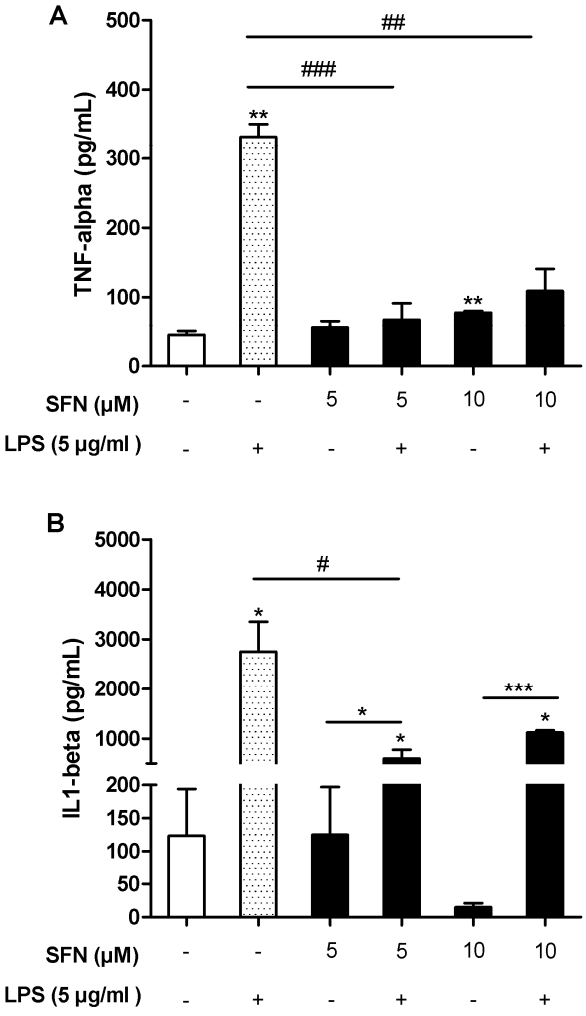

Fig. 16 Levels of pro-inflammatory cytokines TNFα and IL-1β were suppressed by SFN treatment. A) Levels of TNFα were suppressed by SFN, B) Levels of IL-1β were suppressed by SFN. TNF-α and IL-1β were measured by ELISA. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. The cytokine level in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. Cytokine level in PAMs without SFN treatment but with LPS treatment (SFN0-LPS5) was set as the positive control. Comparison between treated group and control is indicated as '*'. Comparison between SFN treated group and the positive control is indicated as '#'. All of the data were expressed as means ± SD, n = 3. * (#), ** (##) and *** (###) indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4.7 NF-κB protein expression was inhibited by SFN

To next examine whether SFN regulates the activation of NF-κB in LPS-treated PAMs, we analyzed the NF-κBp50/105 protein expression. Our results show that NF-κBp50 and p105 were upregulated in LPS treated PAMs (Fig. 17). But SFN pretreatment caused a dose-dependent decrease of LPS-induced P50/105 translation (Fig. 17).

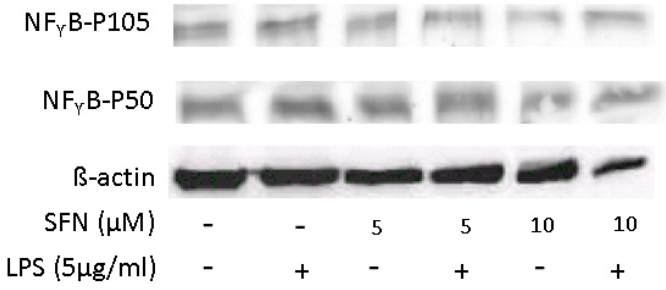

Fig. 17 NF-κB protein expression was inhibited by SFN

4.8 Gene expression of the epigenetic enzymes DNMT1 and DNMT3a were altered by SFN

To further investigate the epigenetic modulations that occurred in CD14 caused by SFN, we assessed the expression of DNMT1 and DNMT3a in SFN-LPS treated PAMs (Fig. 18). To our surprise, we discovered that SFN considerably inhibit DNMT1 and DNMT3a expression in a dose-dependent manner. Compared with the positive control (SFN0-LPS5), the DNMT1 expression was decreased in SFN5-LPS5 group. The LPS-induced *DNMT3a* expression was suppressed ($p < 0.05$) by 5 μM SFN. Results of additional group comparisons can be found in appendix file 5 and 6 (Fig. 25 and Fig. 26).

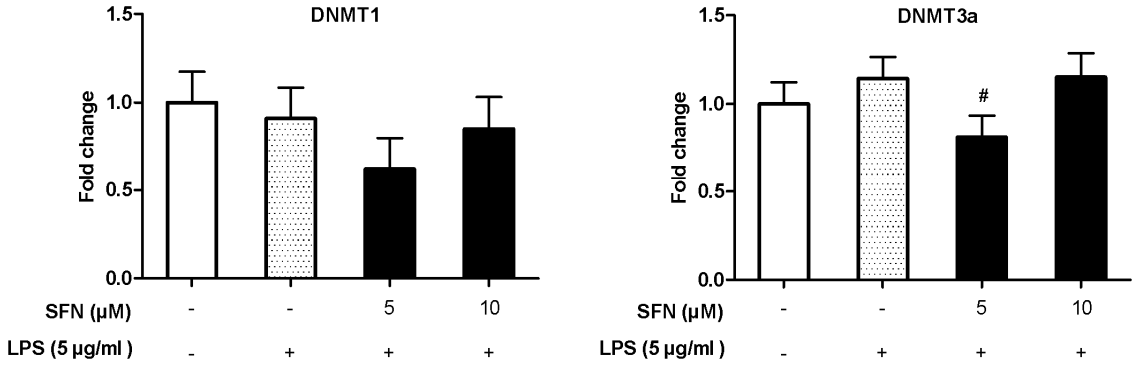

Fig. 18 SFN altered DNMT1 and DNMT3a gene expression. Relative DNMT1 and DNMT3a gene expression were measured in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. Relative mRNA expression in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. Relative mRNA expression in PAMs without SFN treatment but with LPS treatment (SFN0-LPS5) was set as the positive control. Data are expressed as means ± SEM, n = 3, # indicate significant differences at $p < 0.05$.

4.9 CpG islands prediction of CD14 promotor and CDS

To further understand the epigenetic regulation of *CD14*, we analyzed the CpG islands and CpG sites distribution in *CD14* promotor region including the 5'-UTR (1- 492 bp) and CD14 gene body region (CDS) (from 493 bp to1694 bp) (GenBank DQ079063.1, 1762 bp of whole sequence) by using MethPrimer (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Two CpG islands were found in whole sequence including island 1 (196 bp) which starts from 400 bp and ends at 595 bp and island 2 which starts from 601 bp and ends at 1417 bp (Fig 19). 135 CpG sites were found in the whole sequence including 22 CpGs in promotor and 5'-UTR region and the other 113 in the CDS region (Fig. 27).

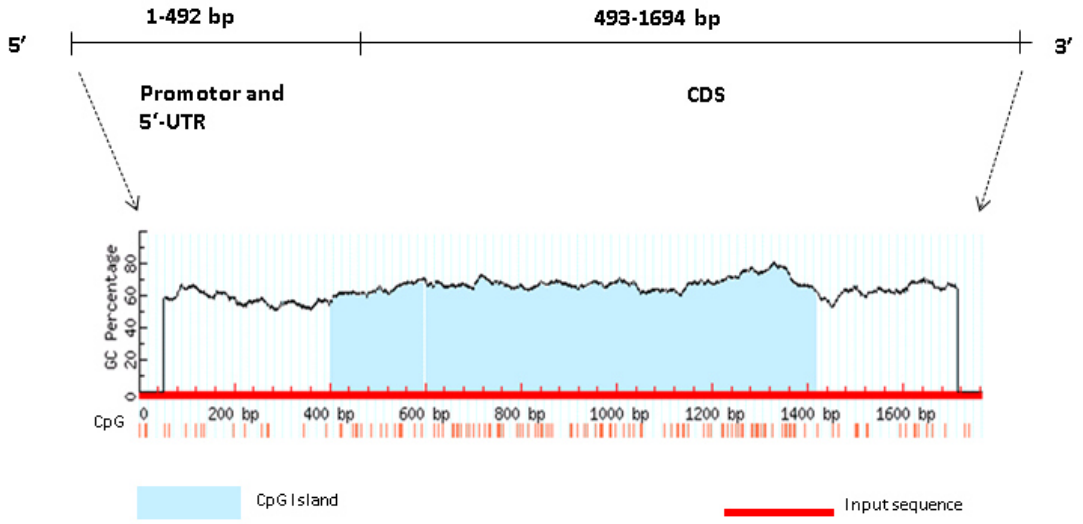

Fig. 19 CpG islands prediction of the *CD14* promotor and CDS
CDS: coding sequence

4.10 Gene body DNA methylation of *CD14* was suppressed by SFN in LPS-induced PAMs

It is well known that DNA methylation plays an important role in gene regulation and gene expression. To further explore the epigenetic regulation of *CD14* in the TRIF pathway in SFN-LPS treated PAMs and the molecular mechanism of SFN-induced repression of *CD14*, we analyzed the DNA methylation status of the *CD14* promotor region and CDS (gene body) region using bisulfite sequencing. CpG islands and CpG sites distribution in *CD14* promotor including the 5'-UTR (1- 492 bp) and CDS (493-1694 bp) (GenBank DQ079063.1, 1762 bp of whole sequence) were predicted by using MethPrimer. Two CpG islands were found in the whole sequence, island 1 with a length of 196 bp starts from 400 bp to 595 bp and the second one from 601 bp to 1417 bp (Fig 19). 135 CpG sites were found in the whole sequence including 22 CpGs in promotor and 5'-UTR region and other 113 in CDS region (Fig. 27). The CpGs in the products of the primers was displayed in table 7. Gene expression is suppressed by DNA methylation of gene promotor, but induced by gene body methylation (Huang et al. 2014, Laurent et al. 2010). The present study showed there were no methylation changes in the *CD14* promoter region (1-492 bp, primer 1 to 3 region) in any SFN-LPS treated PAMs (Fig 20A). Surprisingly, two alterations of the gene body methylation (CDS region) were found in sequence starting from 869 bp to 1081 bp (primer 6) and

from 1537 bp to 1712 bp (primer 9) in PAMs (Fig 20B). For primer 6 region, methylation was increased with 5 µg/ml LPS (16.7%) compared to SFN0-LPS0 (5.9%). On the other hand, methylation was suppressed with 5 µM SFN which showed 0% methylation in both SFN5-LPS0 and SFN5-LPS5 group. In LPS induced PAMs, methylation status of *CD14* in primer 6 with both SFN5-LPS5 and SFN10-LPS5 cells was suppressed. Similar results were found in the primer 9 region, methylation was induced by LPS resulting in 25% methylation in SFN0-LPS5 PAMs but only 12.5% methylation in the control group. Methylation was repressed by SFN that led to 0% methylation in SFN5-LPS0 and SFN10-LPS0 treated cells. In LPS induced PAMs, demethylation was shown in SFN10-LPS5 (12.5% methylation) group but not in SFN5-LPS5 (25% methylation) in this region.

Table 7 CpGs in the products of primers and their positions in the original sequence

Primer	Sequnce sites (bp)	Position	Islands	Product size (bp)	CpGs in product	CpG name in sequnce
CD1401	20 to 188	Promotor and 5′UTR		169	6	CpG3 – CpG8
CD1402	172 to 419	Promotor and 5′UTR		248	7	CpG9 – CpG15
CD1403	399 to 617	Promotor and 5′UTR; and exon	Island 1	219	15	CpG16 – CpG30
CD1404	594 to 787	Exon	Island 2	194	20	CpG31 – CpG50
CD1405	766 to 900	Exon	Island 2	135	13	CpG51 – CpG63
CD1406	869 to 1081	Exon	Island 2	212	17	CpG64 – CpG80
CD1407	1071 to 1221	Exon	Island 2	151	11	CpG81 – CpG91
CD1408	1200 to 1452	Exon	Island 2	253	27	CpG92 – CpG118
CD1409	1537 to 1712	Exon		176	8	CpG126 – CpG133
CD1410	1428 to 1560	Exon	Island 2	133	7	CpG119 – CpG125

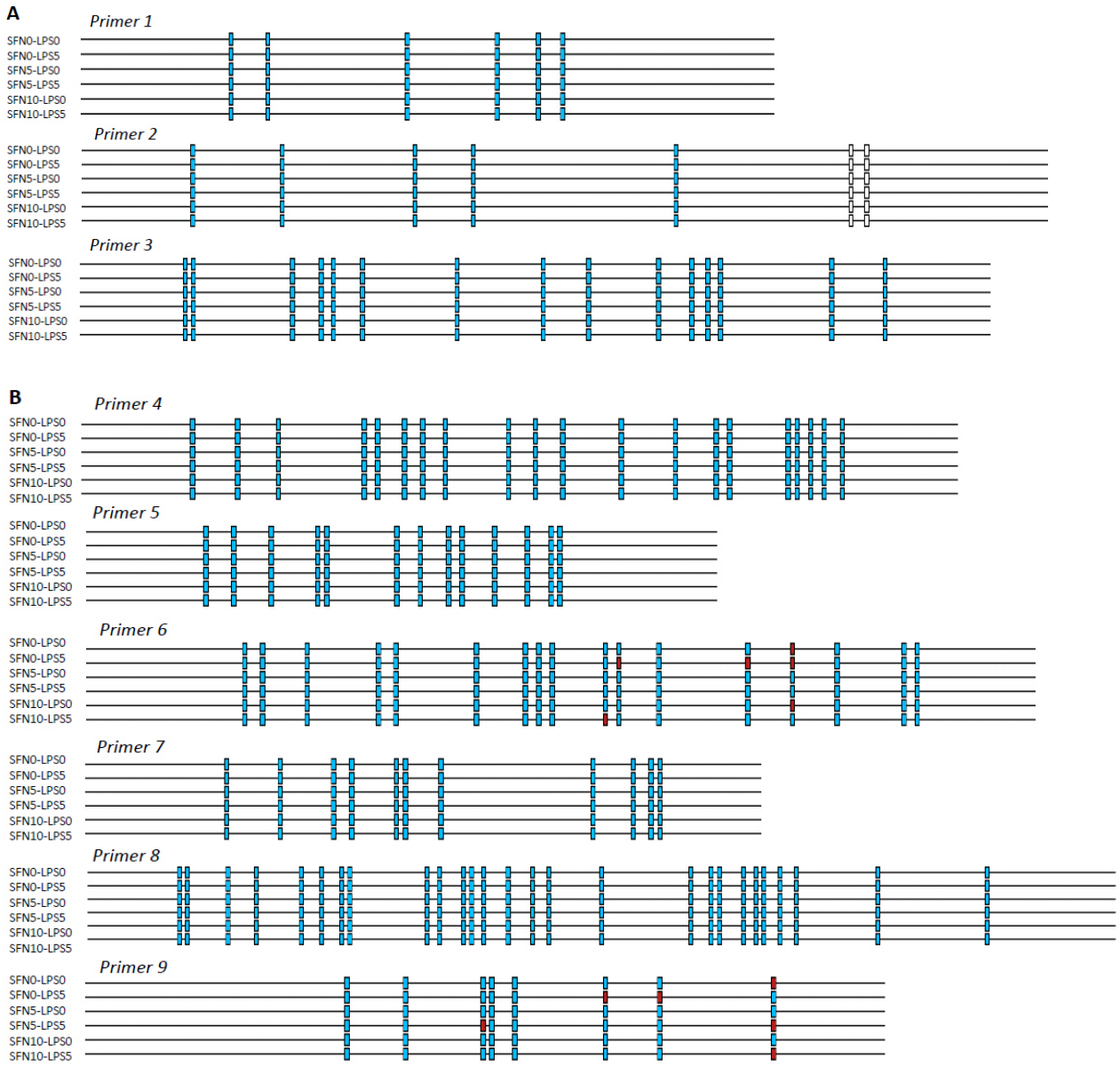

Fig. 20 *CD14* methylation alteration was induced by SFN in PAMs. A) Methylation status of the CD14 promotor and 5'-UTR region of PAMs treated with SFN (0, 5, 10 µM) and LPS (0, 5 µg/ml). Here was no methylation alteration in *CD14* promotor and 5'-UTR. B) Methylation status of the CD14 CDS region of PAMs treated with SFN (0, 5, 10 µM) and LPS (0, 5 µg/ml). At least 8 white cloning PCR products were used for sequencing, the result of one sample shown above is from at least 4 sequences (all of these 4 sequences show the same results). *CD14* methylation in CDS (primer 6 and primer 9) were increased by LPS, but suppressed by SFN. Blue filled (▮) squares: un-methylated; red filled squares (▮): methylated; open squares: unknown; Blank lines (—): sequence of each primer products.

5 Discussion

LPS leads to inflammation in mammals through inducing the production of inflammatory cytokines including TNF-α and IL1-β in macrophages (Beutler and Cerami 1988, Carson et al. 2011, Dinarello 1991, Dobrovolskaia and Vogel 2002). CD14 is a PRR which binds directly to LPS and facilitates the TRIF pathway (Wright et al. 1990, Zanoni et al. 2011). As a HDAC inhibitor, SFN has an anti-inflammatory effect (Liu et al. 2008). In the present study, we demonstrated that *CD14* mediated the LPS-induced TRIF pathway in PAMs to secrete proinflammatory and inflammatory cytokines. Additionaly, SFN inhibited the LPS-induced inflammation in TRIF pathway by regulation CD14 through epigenetic alteration.

5.1 PAMs and their biological characters

Phagocytosis displayed by macrophages, neutrophils and dendritic cells is an early and crucial event in host defense against pathogens (Henneke and Golenbock 2004). Macrophages are phagocytes which develop and differentiate from monocytes in the tissues, with pivotal functions in development, tissue remodeling, repair or homeostasis, host defense and inflammation (Ginhoux 2014, Gordon and Taylor 2005). They are the main source of cytokines, chemokines and other inflammatory mediators disseminating or inhibiting the immune response (Moldoveanu et al. 2009).

PAMs are the central components of lung innate immune system (Murphy et al. 2008). As well known, the alveolar macrophages or PAMs are the bactericidal functionalities of the lungs (Green and Kass 1964). The basic biologic knowledge of PAMs like cell growth, the inherent half-life, cell apoptosis are the premise of the PAMs' functional study. A half-life of 2 weeks (Godleski and Brain 1972) and 30 days (Matute-Bello et al. 2004) for PAMs has been shown. In this study, PAMs were observed growing in a time dependent manner. Viable growth of PAMs was shown between day 3 to day 18. At day 29, most of the cells were degenerated. Thus, the time point of day 9 was chosen for LPS treatment and day 7 was chosen for SFN pretreatment.

Cell markers can be used for recognizing and sorting cell types. The typical macrophage markers are CD11b and D163. CD13 is a marker of mature resident macrophages related to secretion of inflammatory mediators (Dimitrijevic et al. 2013, Held et al. 2013). Flow cytometry and cell sorters were developed aiming to characterize single-

cells, due to cell physical and chemical properties. The principle of the method is based on the laser path. It measures the single-cells in liquid suspension by passing them through an electronic detection apparatus (Metes et al. 2003). In this study, it was shown that 35% of the PAMs were stained with the cell marker CD163 (PAMs without SFN and LPS treatment) by using flow cytometry.

5.2 Cell viability of PAMs with LPS/ SFN-LPS challenge

Cell viability is used for cell vitality and chemical cytotoxicity tests. The enzyme-based WST method relies on a reductive coloring reagent and dehydrogenase in a viable cell to determine cell viability. Cell viability is effected by the cell culture density (Zhuang and Wogan 1997) and experimental chemical concentration.

In this study, PAMs were cultured with a density of $1 * 10^5$ cells/well. LPS at a dose of 5 μg/ml was used to induce inflammatory conditions in cultured PAMs. We showed that LPS treatment had no notable effect on the cell viability at all the 7 time points compared with the control.

Factors like extract of Glycyrrhiza glabra L. (EGGR) and SFN can affect cell viability. EGGR (100 μg/ml) increased cell viability (from 66.6 to 99%) of LPS-treated macrophages (Li et al. 2015). SFN (5 μM) pre-treatment enhanced cell viabilities of cytotoxicity of cadmium selenide (CdSe) quantum dots (QDs)-treated liver cells in mice (Wang et al. 2015). It was also shown that pre-treatment with SFN increased cell viability by decreasing intracellular reactive oxygen species production (Ziaei et al. 2013). In this study, SFN (5, 10 μM) pre-treatment increased LPS-treated PAMs cell viability.

5.3 CD14 mRNA and downstream TRIF pathway genes expression were induced by LPS

Inflammation is the body's reaction to defend against pathogens (Ferrero-Miliani et al. 2007, Moldoveanu et al. 2009). LPS is the major structural component of the outer wall of gram-negative bacteria. It is a potent initiator of inflammatory responses. Therefore, LPS is important in lung inflammation and is regularly used as a model for pulmonary inflammation. Cytokines, the nonstructural and small proteins with molecular weights ranging from 8 to 40,000 d, are primarily involved in host responses to disease or infection like inflammation (Dinarello 2000). They are the marker and regulators

(promoters or suppressors) of the inflammation. In the LPS-TRIF pathway, TRAF6 activated the release of the pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-12, while TRAF3 initiated the production of IFN-α and IFN-β (Fig. 4).

LPS increased IL-8 release in a human macrophage-derived cell line (THP-1) cells at a concentration of 10 μg/ml after 24 h of incubation (Antonicelli et al. 2004). LPS treatment also increased TLR2 mRNA expression in a mouse macrophage cell line (Matsuguchi et al. 2000). It is known to cause inflammation in mammals through induction of the production of inflammation cytokines such as TNF-α and IL1-β in macrophages (Beutler and Cerami 1988, Carson et al. 2011, Dinarello 1991, Dobrovolskaia and Vogel 2002). LPS injection in mice upregulated the levels of LBP, CD14, TNF-α, IL-6 and IFN-γ in spleen (Zhang et al. 2015). LPS induced phosphorylation of IRF3 and the activation of the TRIF signaling pathway in monocytes (Endo et al. 2014).

CD14 is an acute phase inflammatory biomarker. It is the PRR binding directly to LPS and facilitates TRIF pathway (Wright et al. 1990, Zanoni et al. 2011). CD14 was necessary for the microbe-induced endocytosis of TLR4 while CD14 controls the trafficking and signalling functions of TLR4. This innate immune trafficking cascade illustrates how pathogen detection systems operate to induce both membrane transport and signal transduction (Zanoni et al. 2011). In a CD14-dependent manner, soluble-lipopolysaccharide (S-LPS) and rough lipopolysaccharide (R-LPS) at low dose induced acute lung inflammation, while the inflammatory response triggered by high dose S-LPS or R-LPS was reduced by CD14 (Anas et al. 2010).

CD14 expression is essential for the development of LPS-induced systemic inflammation and for activation of circulating neutrophils, but not necessary for the mechanism of LPS detection and activation of neutrophil recruitment in the liver microcirculation (McAvoy et al. 2011). LPS induces CD14-TLR4 pro-inflammatory signaling through triggering the generation of phosphatidylinositol 4, 5-bisphosphate [PI (4, 5) P2]. It was shown that LPS induced a CD14-dependent immobile fraction of TLR4 in the plasma membrane (Klein et al. 2015). This is in accordance with previous findings that CD14 mRNA expression was induced by LPS in alveolar macrophage of pigs (Islam et al. 2013). Exposure of alveolar macrophages to LPS (0.1, 1, 5, 10 μg/ml) increased *CD14* expression compared with control (Islam et al. 2013). In this study, we

found that CD14 gene expression was significantly increased by 5 μM LPS treatment at time point 12 h.

It was shown that LPS initiates the TRIF pathway independent of CD14 (Watanabe et al. 2013), but CD14 is also required for the TRIF-independent signaling (Jiang et al. 2005, Lloyd-Jones et al. 2008, Regen et al. 2011). In the TRIF pathway, TRIF activates both TRAF3 and TRAF6 by a binding domain present on its N-terminus (Brown et al. 2011, Wang et al. 2001, Yamamoto et al. 2003a, Yamamoto et al. 2003b). The activation of TRAF6 initiates NF-κB signaling which leads to the release of the pro-inflammatory cytokines including TNF-α, IL-1β, IL-6, IL-12 (Fig. 4) and the activation of TRAF3 initiates IRF-3 through IKKi and TBK-1, then the phosphorylation of IRF3 induces the production of IFN-β (Brown et al. 2011, Doyle et al. 2002). TRAF3 regulates the production of anti-inflammatory cytokine IL-10 and the activation of the IFN (Hacker et al. 2006). It was shown that CD14 is required for LPS-induced activation of the TLR4/TRAM-TRIF pathway even at very high LPS doses. Both smooth and rough LPS cannot induce TRAM-TRIF-dependent IRF3 activation and type-I IFN production without CD14 (Jiang et al. 2005). In this study, similar as CD14, the mRNA expression of the downstream genes including TRIF, TRAF6, NFκB, TRAF3 and IRF7 also was also enhanced by LPS.

Cytokine genes like *IL-6*, *IL-8* and *TNF-α* are immune associated genes with strong response to LPS (Green and Kerr 2014). TNF-α mRNA expression was induced by LPS (Green and Kerr 2014). LPS induced the release of IL1-β, IL12-β, TNF-α, IL-6, IL-8, IFN-γ and IL-10 in a dose-dependent manner in pig macrophages (Islam et al. 2013). In this study, the mRNA expression of inflammatory cytokines including TNF-α, IL-1β, IL-6 and IFN-β were upregulated in LPS stimulated PAMs.

5.4 mRNA expression of CD14 and downstream LPS-induced TRIF pathway genes were suppressed by SFN in a dose dependent pattern

Epigenetic control including the DNA methylation and histone acetylation may play a crucial role in the revision of immune-responsive genes related to pathogen recognition and subsequent signaling (Green and Kerr 2014). Recent research presented chemical inhibitors such as 5-aza-2-deoxycytidine (AZA), trichostatin A (TSA) and SFN as epigenetic modifiers (Luo et al. 2015, Samiec et al. 2015, Su et al. 2014, Yang et al. 2014). As a HDAC inhibitor, SFN also has anti-proliferative, pro-apoptotic role in

cancer cells (Chu et al. 2009, Ho et al. 2009) and anti-inflammatory activity (Ko et al. 2013, Koo et al. 2013). SFN regulates LPS-induced innate immune responses of porcine moDCs (Qu et al. 2015). In this study, we found that SFN suppresses the gene expression of CD14 and it's down stream genes including TRAM, TRIF, RIPK1 and TRAF3 in the TRIF pathway.

Our results suggest that SFN possesses anti-inflammatory activity, resulting in downregulation of LPS-induced CD14, TRAM, TRIF, RIPK1 and TRAF3 in macrophages in pigs.

5.5 LPS-induced inflammatory cytokines and NF-κB were suppressed by SFN in a dose dependent manner in PAMs

Pro-inflammatory cytokines such as IL-1 and TNF-α play critical roles in the progress of diseases including cancer (Guo et al. 2012). They are the Th1 cytokines to produce the pro-inflammatory responses to fight viruses and other intracellular parasites and to eliminate cancer cells (Moldoveanu et al. 2009). TNF-α concentration in the cell supernatant fluid increased immediately at 1 h after LPS stimulation (Islam et al. 2012). NF-κB is connected with cancer, inflammatory and autoimmune diseases due to its role in modulating transcription of DNA, cytokine production and cell survival (Gilmore 2006).

Inflammation balance is one of important strategies for the prevention and treatment of diseases. Factors like nacystelyn (NAL) can modulate the LPS-induced inflammation. NAL decreased IL-8 release from LPS-stimulated THP-1 cells after 4 h of incubation (Antonicelli et al. 2004). LPS-induced mammary gland histopathologic changes, NF-κB and MAPKs activations and TNF-α, IL-1β and IL-6 production were inhibited by TRAM-derived decoy peptide (TM6) in mice (Zhang et al. 2015). EGGR suppressed LPS-induced iNOS, COX-2 cytokines, TNF-α, IL-1β and IL-6 at both the mRNA and the protein level (Li et al. 2015). ADP-ribosylation factor 6 (ARF6) reduced LPS-induced cytokine production and regulated TRAM/TRIF-dependent TLR4 signaling in mouse macrophages (Van Acker et al. 2014). TF5 (a peptide derived from putative helix B of TRIF TIR) downregulated plasma cytokine levels and protected mice from a fatal LPS challenge in a mouse model of TLR4-driven inflammation (Piao et al. 2013). Mammalian peroxiredoxin V (PrdxV), a multifunctional protein which inhibits stress-induced apoptosisis and protects cells from DNA damage, is the key mediator

contributing to the regulation of LPS/TLR4-induced immune responses. It selectively regulates IL-6 production by modulating the Jak2-Stat5 pathway (Choi et al. 2013).

Epigenetic control including the DNA methylation and histone acetylation may play a crucial role in the regain of immune-responsive genes related to pathogen recognition and subsequent signaling (Green and Kerr 2014). Recent research presented chemical inhibitors such as 5-aza-2-deoxycytidine (AZA), trichostatin A (TSA) and SFN to be epigenetic modifiers (Fan et al. 2012, Luo et al. 2015, Samiec et al. 2015, Su et al. 2014, Yang et al. 2014).

As a HDAC inhibitor, SFN also has anti-proliferative and pro-apoptotic roles in cancer cells (Chu et al. 2009, Ho et al. 2009) and anti-inflammatory activity (Ko et al. 2013, Koo et al. 2013). SFN showed a potent decrease in LPS-induced secretion of pro-inflammatory and pro-carcinogenic signaling factors in cultured Raw 264.7 macrophages (Heiss et al. 2001). SFN regulated the LPS-induced innate immune responses of porcine monocyte-derived dendritic cells (moDCs) though epigenetic mechanisms (Qu et al. 2015). SFN suppressed the LPS-induced secretion of inflammatory mediators like TNFα, IL-1β and IL-6 (Heiss et al. 2001, Wierinckx et al. 2005). Low doses of SFN and NBN significantly reduced LPS-induced IL-1 mRNA expression (Guo et al. 2012).

In this study, we found that SFN suppressed the mRNA expression of LPS-dependent inflammatory cytokines including TNFα, IL-1β, IL-6 and IFNβ in a dose dependent manner. In addition, the protein level of TNFα and IL-1β also were suppressed by SFN. Further, the protein level of NF-κB was inhibited by SFN.

Taken together, our data indicate that SFN possesses anti-inflammatory activity, resulting in downregulation of LPS-stimulated TNFα, IL-1β, IL-6, IFNβ and NF-κB in porcine macrophages.

5.6 DNMT3a gene expression was induced by LPS but suppressed by SFN

It is well known that DNMT1 is the key maintenance methyltransferase, whereas DNMT3a and DNMT3b play the role in *de novo* activity (Robertson et al. 2000, Ronemus et al. 1996). Knockdown of the DNMT1 gene reduced the suppressors of cytokine signaling1 (SOCS1) gene promoter methylation and upregulated the

expression of SOCS1 in activated RAW264.7 cells (Cheng et al. 2014). DNMT1 inhibited SOCS1 expression though the SOCS1 hypermethylation, that caused the suppression of the activation of the JAK2/STAT3 pathway and enhanced the level of LPS-induced pro-inflammatory cytokines such as TNF-α and IL-6 in macrophages (Cheng et al. 2014). Liver cells and spleen cells responded to exposure of LPS with alterations in proteins levels involved in DNA methylation (DNMT1, DNMT3a and 3b) or DNA repair with changes in gene expression (Kovalchuk et al. 2013a, Kovalchuk et al. 2013b).

LPS induced NF-κB activation and decreased levels of histone HDAC1 and DNMTs (Biswas and Yenugu 2013). DNMT1 mRNA expression was significantly increased by LPS treatment in fibroblasts derived from human periodontal ligament (HPDL) (Uehara et al. 2014). LPS downregulated DNMT1 ($p < 0.05$) gene expression in HaCaT cells (de Camargo Pereira et al. 2013), the same result was found in our study. It was shown that DNMT3a gene expression was downregulated in response to LPS in PBMCs (Doherty et al. 2013). However, the DNMT3a gene expression was increased by LPS in this study.

SFN inhibited LPS-induced DNMT3a, HDAC6 and HDAC10 gene expression, whereas it upregulated DNMT1 gene expression (Qu et al. 2015). SFN extremely decreased the protein expression of DNMT1, DNMT3a and DNMT3b (Su et al. 2014). SFN also downregulated the protein levels of DNMT1, DNMT3a, HDACs 1, 4, 5 and 7 while increased the level of active chromatin marker acetyl-Histone 3 (Ac-H3) in prostate cancer TRAMP C1 cells (Zhang et al. 2013). DNMT1 and DNMT3a were decreased in SFN-treated breast cancer cells suggesting that SFN may inhibit human telomerase reverse transcriptase (hTERT) by affecting epigenetic pathways. SFN inhibited the LPS induced HDAC6, HDAC10 and DNMT3a gene expression while upregulated the expression of DNMT1 gene (Qu et al. 2015).

In the present study we found SFN reduced DNMT1 and DNMT3a mRNA expression in a dose dependent manner. Like CD14 gene, DNMT3a gene expression was increased by LPS and suppressed significantly by SFN at the dose of 5 μM. This result suggests that CD14 DNA methylation changes may be related with DNA methylation enzyme DNMT3a in PAMs.

Take together, CD14 may be involved in the TRIF pathway and the suppression of LPS-induced CD14 gene expression was associated with the suppression of CD14 gene body methylation which may be caused by downregulation of DNMT3a gene expression in PAMs post SFN pre-treatment.

5.7 Gene body DNA methylation of *CD14* was suppressed by SFN in LPS-induced PAMs

DNA methylation is a critical epigenetic modification in mammals (Huang et al. 2014). It refers to the addition of methyl groups to the adenine or cytosine bases of DNA and is tightly associated with gene regulation (Plongthongkum et al. 2014, Wilson et al. 2014). Over the past years, most studies showed that DNA methylation modification occurs in gene promoter to be a key molecular mechanism leading to gene expression changes (Curradi et al. 2002, Gao et al. 2007, Kontorovich et al. 2009, Park et al. 2012). Although the promoter DNA methylation is known as a silencing mechanism, the gene body has recently been recognized as a major mechanism for regulating gene expression in many tissues (Baylin and Jones 2011).

Recently, some studies demonstrated that DNA methylation in the gene body is connected with gene expression (Fan et al. 2015, Sarda et al. 2012, Wang et al. 2013, Yang et al. 2014). DNA methylation in the gene body region can alter gene expression and is a therapeutic target in cancer (Yang et al. 2014). The high densities of hypomethylated CpG-rich regions across the gene body are preferentially associated with gene downregulation (Yu et al. 2015). Although several recent studies investigated the role of gene body DNA methylation on gene expression, the function of gene body DNA methylation is poorly understood (Kulis et al. 2012, Lister et al. 2009, Lou et al. 2014, Maunakea et al. 2010, Varley et al. 2013).

In an attempt to identify potential epigenetic changes which mediate the effect of SFN on *CD14* expression, we further assessed the methylation status of CpG islands embedded in the CD14 promotor and the whole CDS region. Surprisingly, our results showed that there were no methylation changes in the *CD14* promotor of non CpG island and also CpG island regions, but methylation alterations were found in the *CD14* CDS region (Fig. 20B). The results showed that CD14 gene body methylation was induced by 5 μM LPS in PAMs (Fig. 20B, primer 6 and primer 9 region). Therefore, gene expression of CD14 was increased in LPS treatment cells. Theses results showed

that gene body DNA methylation was positively connected with CD14 gene expression. Similarly, research suggested that gene body DNA methylation may increase transcriptional activity by blocking the initiation of intragenic promoters or by affecting the activities of repetitive DNAs within the transcriptional unit (Maunakea et al. 2010).

However, CD14 gene body methylation was also decreased by the HDAC inhibitor SFN. Results showed that CD14 gene body methylation was inhibited by both 5 μM SFN and 10 μM SFN (Fig 20B, primer 6 and primer 9 region). It might be considered that the CD14 gene expression was suppressed by SFN depending on the methylation inhibition of CD14 gene body in LPS-induced PAMs. Gene body DNA demethylation induced-gene repression may be due to 3 factors including: 1) whether embedded demethylated functional elements regain activity, 2) whether demethylated regions are located at intron-exon junctions together with destabilized nucleosomes, 3) possible effects on the rates of transcript elongation and splicing (Yang et al. 2014).

In summary, CD14 acts not only as a LPS-receptor, but also as a LPS-induced inflammation regulator that mediates the TRIF pathway genes and cytokines expression. This regulatory role of CD14 may depend on the epigenetic changes in gene body methylation caused by SFN. In addition, this DNA methylation alteration is probably related with DNMT3a expression. The SFN-mediated inhibition of DNMTs especially DNMT3a could be an important contributing factor in CD14 DNA methylation regulation and also in the SFN anti-inflammation role.

6 Summary

Infections of the respiratory system which cause diseases like lung inflammation are main threats in pig production. LPS is the component of the outer membrane of gram-negative bacteria and one of the most predominant microbial stimulators of inflammation. The innate immunity system can recognize and response to bacteria. PAMs constitute a vital component of the alveolar spaces and play a central role in pulmonary innate immunity and functionally work in LPS-induced lung inflammation. Two pathways including the MyD88-dependent and MyD88-independent (TRIF) pathways are activated after TLR4 stimulation with LPS. CD14 is the PRR which binds directly to LPS, transfers LPS molecules in a co-expressed way to TLR4 and initates the MyD88-dependent and TRIF pathways. Therefore, *CD14* may be involved in TRIF pathway cytokines release and downstream genes expression.

As an epigenetic regulator, SFN was also shown to have an anti-inflammation role. Therefore, SFN may inhibit the LPS-induced inflammation through regulation of CD14 gene expression. The epigenetic modulation including DNA methylation and histone modulation of CD14 in response to infection are poorly understood. In the present study, we identified the epigenetic changes of CD14 mediated with SFN in LPS-induced TRIF pathway with an *in vitro* PAMs model in pig. The methylation status of CpG islands embedded in the CD14 promotor and whole CDS region was analyzed.

We found that CD14 gene expression was significantly increased by 5 μM of LPS treatment at 12 h. Likewise, the mRNA expression of the downstream genes TRIF, TRAF6, NF_YB, TRAF3 and IRF7 were also upregulated significantly. In addition, the mRNA expression of inflammatory cytokines including TNF-α, IL-1β, IL-6 and IFN-β were significantly upregulated in LPS stimulated PAMs. It is important to point out that CD14 mediates the TRIF signals and induced the inflammatory cytokines in LPS challenged PAMs.

SFN inhibited the gene expression of CD14 and its downstream genes including TRAM, TRIF, RIPK1, TRAF3 and IRF7 that are involved in TRIF pathway to recognize LPS/bacteria. In addition, SFN also downregulated the protein level of LPS-induced inflammatory cytokines including TNFα, IL-1β, IL-6 and IFNβ in a dose dependent manner.

The CD14 DNA methylation results showed that there was no methylation change occurring in the *CD14* promotor either in non CpG islands or in CpG islands, but methylation alterations were found in *CD14* CDS region. Results showed that *CD14* gene body methylation was induced by 5 μM of LPS in PAMs. Therefore, gene expression of CD14 was increased in LPS treated PAMs. Theses results showed that gene body DNA methylation was positively connected with CD14 gene expression. However, *CD14* gene body methylation was decreased by SFN. Results presented that *CD14* gene body methylation was inhibited by both 5 μM SFN and 10 μM SFN. The surprising result from our study is that gene body regions show sustained DNA demethylation and gene downregulation of expression after SFN withdrawal. It might be considered that the CD14 gene expression suppressed by SFN is depending on the methylation inhibition of *CD14* gene body in LPS-induced PAMs. Therefore, CD14 plays a role not only as a LPS-receptor, but also as a LPS-induced inflammation regulator that mediates the TRIF pathway genes and cytokines expression and also cytokines secretion through the epigenetic changes in gene body methylation by SFN in PAMs.

Another important outcome of this study is that *CD14* DNA methylation changes may be related to expression of DNA methylation enzyme DNMT3a. We found that SFN downregulated DNMT3a mRNA expression in a dose dependent manner. It is suggested that *CD14* is involved in the TRIF pathway in PAMs. The repression of LPS-induced CD14 gene expression was associated with the *CD14* gene body demethylation which might be caused by DNMT3a gene suppression due to SFN regulation.

In summary, in the present *in vitro* study, we have demonstrated not only that *CD14* is involved in the TRIF pathway by LPS treatment in a time dependent manner, but also explored the possible epigenetic mechanism such as demethylation in the gene body region related to *CD14* repression in SFN pre-treated PAMs. In addition, this DNA methylation alteration is probably related with DNMT3a expression. This study provided new insights into SFN-mediated epigenetic downregulation of CD14 gene in LPS induced-TRIF pathway inflammation. It may open new methods to prevent and reduce the LPS-induced inflammation in pigs.

Zusammenfassung

Infektionen der Atemwege, die zu Krankheiten wie Lungenentzündung führen, sind eine der Hauptbedrohungen in der Schweineproduktion. LPS als Komponente der äußeren Membran von gramnegativen Bakterien ist einer der vorherrschenden mikrobiellen Stimulatoren für Entzündungen. Das angeborene Immunsystem kann schnell solche Entzündungen erkennen und darauf reagieren. PAMs bilden eine wichtige Komponente der Alveolarräume und spielen daher eine zentrale Rolle in der angeborenen pulmonalen Immunabwehr sowie bei der funktionellen Arbeit durch LPS-induzierte Lungenentzündung. Zwei wesentliche Signalwege, MyD88-abhängig und MyD88-unabhängig (TRIF) werden aktiviert, nach der Stimulation von TLR4 durch LPS. CD14 ist der PRR, der direkt an LPS bindet, die LPS-Moleküle zum TLR4 transportiert und dadurch die MyD88-abhängigen und den TRIF-Signalweg initiiert. Daher scheint CD14 an der Freisetzung von TRIF Signalweg-Zytokinen und an der Expression von nachgeschalteten Genen beteiligt zu sein.

Als epigenetische Regler hat SFN eine entzündungshemmende Wirkung. Daher ist die Frage, ob SFN die LPS-CD14-induzierte Entzündung durch Regulierung der CD14 Genexpression hemmen kann, von Interesse. Allerdings ist das Verständnis der epigenetischen Modulationen einschließlich DNA-Methylierung und Histon-Modifikation von CD14 in Bezug auf die Reaktion auf eine Infektion gering. In der vorliegenden Studie haben wir daher die epigenetischen Veränderungen von CD14 durch SFN in LPS-induziertem TRIF Signalweg mit einem in vitro-Modell in PAMs beim Schwein, untersucht. Um potentielle Veränderungen des DNA-Methylierungsstatus zu identifizieren, die mit der Wirkung von SFN auf die CD14-Expression assoziiert sind, wurde der Methylierungsstatus der CpG Inseln sowohl der CD14-Promotor Region als auch der intragenetic Region (CDS Region) analysiert.

Es konnte festgestellt werden, dass die CD14 Genexpression signifikant in Zellen mit 5 µM LPS nach 12 h erhöht war. Ebenfalls war die mRNA-Expression der nachgeschalteten Gene, TRIF, TRAF6, NFYB, TRAF3 und IRF7, hochreguliert. Des Weiteren, ergab die mRNA-Expressionsanalyse der Zytokine, TNF-α, IL-1β, IL-6 und IFN-β, ebenfalls eine Hochregulation in LPS stimulierten PAMs. Es ist wichtig darauf hinzuweisen, dass CD14

die TRIF Signale vermittelt und die inflammatorischen Zytokine in LPS behandelten PAMs induziert.

Durch die Zugabe von SFN wurde die Genexpression von CD14 und den nachgeschalteten Genen, TRAM, TRIF, RIPK1, TRAF3 und IRF7, die im TRIF Signalweg an der Erkennung von LPS/Bakterien beteiligt sind, gehemmt. Weiterhin, unterdrückt SFN die Produktion von LPS-induzierten inflammatorischen Zytokinen, TNFα, IL-1β, IL-6 und IFN-ß, in Abhängigkeit von der Dosis.

Die Analyse des Methylierungsprofils ergab überraschenderweise, dass es keine Änderung des Methylierungsstatus in der CD14-Promotor Region gab, weder in CpG-Inseln noch außerhalb von CpG-Inseln. Allerdings zeigte sich eine Veränderung des Methylierungsbildes in der intragenic Region des CD14 Gens. Die Ergebnisse ergaben, dass eine Behandlung mit 5 μM LPS die CD14-Methylierung induziert. Daher war die Genexpression von CD14 LPS behandelten PAMs erhöht. Diese Ergebnisse zeigten, dass die Gen intragenic Region Methylierung positiv mit der CD14 Genexpression assoziiert ist. Jedoch wurde die CD14 Methylierung durch SFN wieder gesenkt. Die inragenic Region Methylierung von CD14 war sowohl bei 5 μM SFN als auch 10 μM SFN gehemmt. Das überraschende Ergebnis unserer Studie ist, dass die Gen inragenic Region eine klare DNA Demethylierung zeigt und das eine Herunterregulation der Genexpression nach SFN Behandlung beobachtet werden kann. Es könnte daher in Betracht gezogen werden, dass die CD14-Gen-Expression, unterdrückt durch SFN, abhängt von der Methylierungshemmung der CD14-Gen intragenic Region in LPS-induzierten PAMs. Deshalb spielt CD14 nicht nur eine Rolle als LPS-Rezeptor, sondern auch als eine Art LPS-induzierter Entzündungsregler, der die TRIF Signalweg-Gene, die Zytokin-Expression und auch die Zytokin-Sekretion durch die epigenetischen Veränderungen in der inragenic Region Methylierung durch SFN in PAMs vermittelt.

Eine weitere wichtige Entdeckung dieser Studie ist, dass die Veränderungen des CD14 DNA-Methylierungsstatus im Zusammenhang mit der Expression des DNA-Methylierungs-Enzyms DNMT3a stehen. Es konnte festgestellt werden, dass SFN die DNMT3a mRNA-Expression in Abhängigkeit von der Dosis herunterreguliert. Daraus ergibt sich, dass CD14 in den TRIF Signalweg involviert zu sein scheint und daher die Hemmung der LPS-induzierten CD14 Genexpression mit der CD14 intragenic Region

Methylierung assoziiert ist, die durch die Herrunterregulation der DNMT3a Genexpression in PAMs aufgrund der SFN Vorbehandlung verursacht sein könnte.

Zusammenfassend konnten wir in dieser in vitro Studie nicht nur zeigen, dass CD14 am TRIF Signalweg nach einer LPS-Behandlung im Zeitverlauf beteiligt ist, sondern auch der mögliche epigenetische Mechanismus wie Demethylierung in der intragenic Region in Verbindung mit der Repression von CD14 in SFN behandelten PAMs steht. Darüber hinaus wird diese DNA Methylierungsänderung wahrscheinlich mit der DNMT3a Expression assoziiert sein. Diese Studie gibt einen neuen Einblick in die SFN-vermittelten epigenetischen Herunterregulationen des CD14-Gens in LPS induzierten TRIF Signalweg Entzündungen. Dadurch ergeben sich neue Wege zur Entwicklung von neuen Ansätzen zur Vermeidung bzw. Milderung von LPS-induzierten Entzündungen bei Schweinen.

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8 Appendix

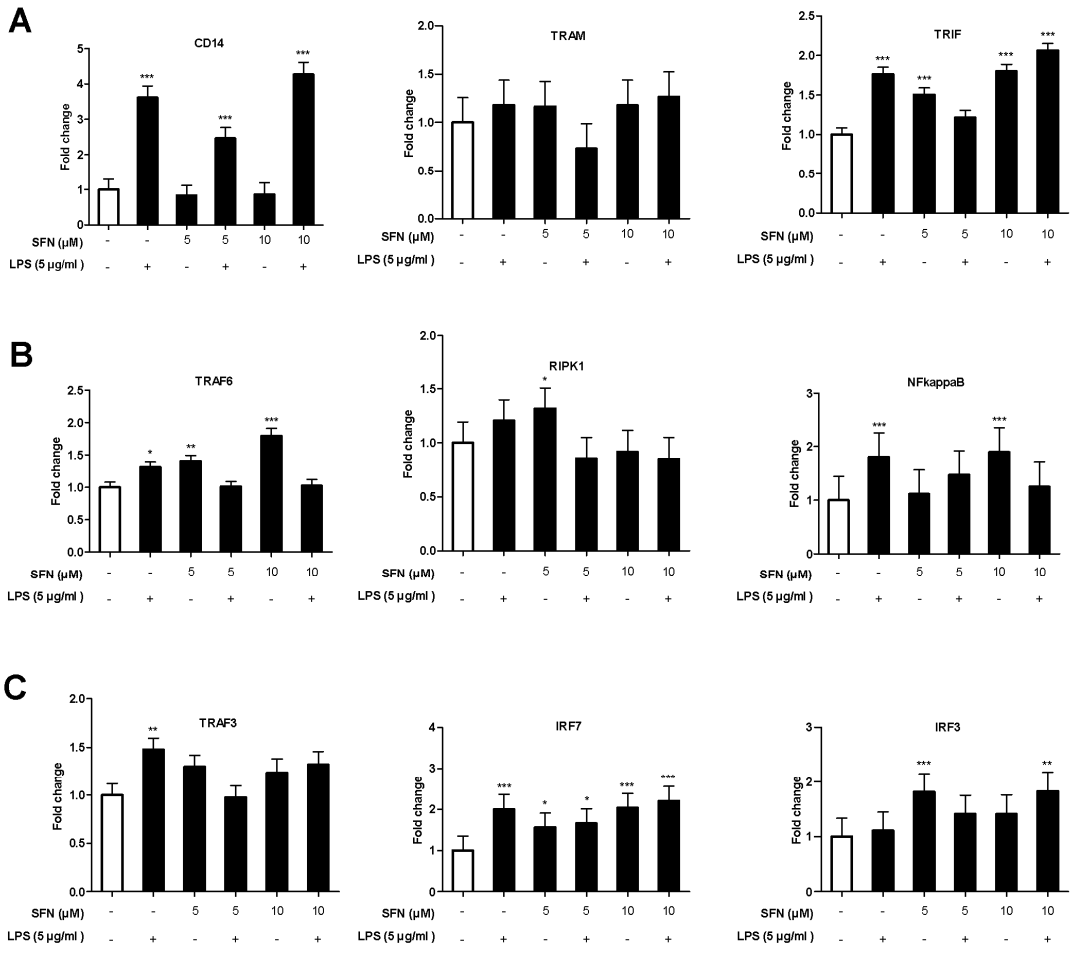

Fig. 21 (Appendix 1) The role of LPS and SFN on gene expression regulation of *CD14* and downstream genes in PAMs. A) *CD14* and downstream genes TRAM and TRIF mRNA expression in SFN-LPS stimulated PAMs. B) TRIF-TRAF6 pathway genes mRNA expression in SFN-LPS treated PAMs. C) TRIF-TRAF3 pathway genes mRNA expression in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. Fold change in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. Comparison between treatment group (black column) and control group (white column) is indicated as '*'. All of the data were expressed as least square means ± SE, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

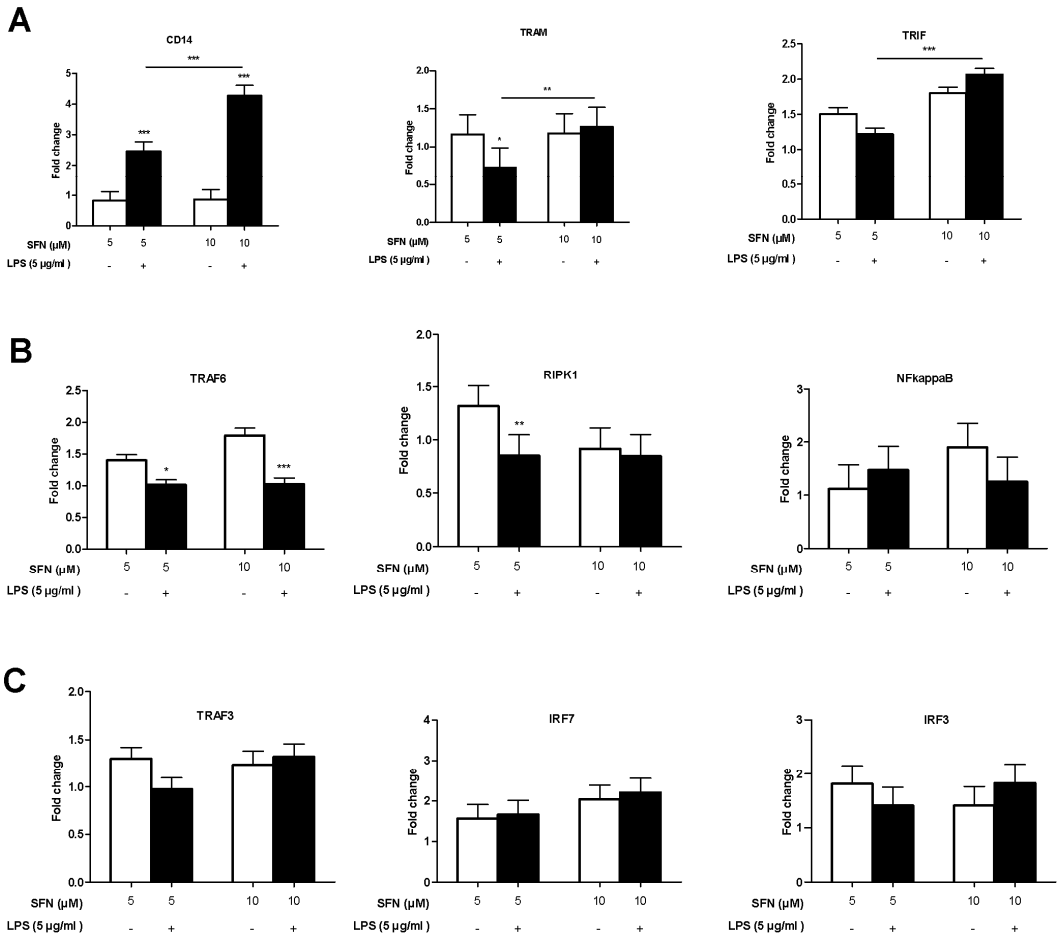

Fig. 22 (Appendix 2) Effect of SFN5 and SFN10 on gene expression regulation of *CD14* and downstream genes in PAMs. A) *CD14* and downstream genes TRAM and TRIF mRNA expression in SFN-LPS stimulated PAMs. B) TRIF-TRAF6 pathway genes mRNA expression in SFN-LPS treated PAMs. C) TRIF-TRAF3 pathway genes mRNA expression in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. All of the data were expressed as least square means ± SE, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

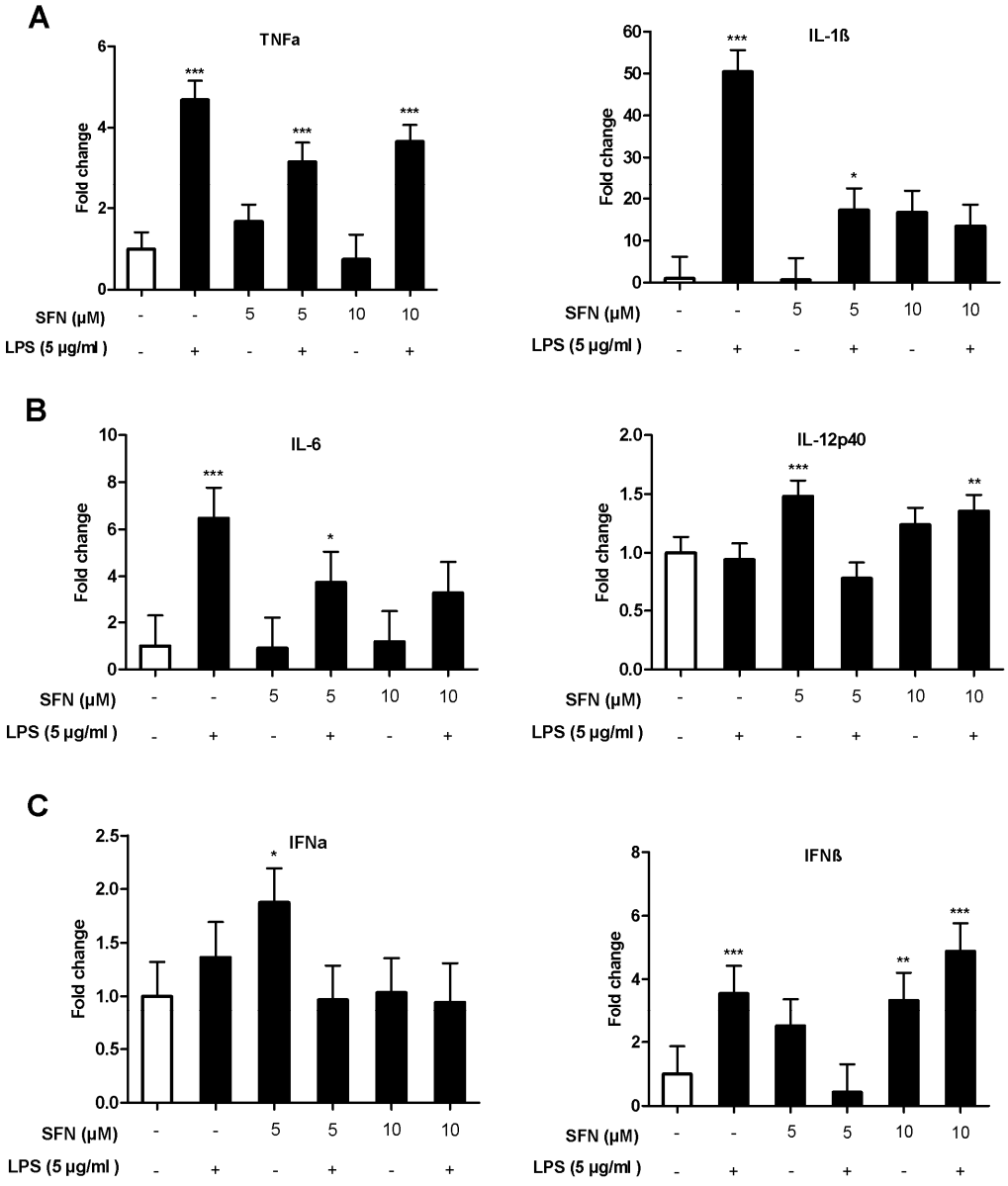

Fig. 23 (Appendix 3) The role of LPS and SFN on gene expression regulation of cytokines in the TRIF pathway in PAMs. A) Relative gene expression of TRIF-TRAF6 pathway released cytokines in SFN-LPS treated PAMs B) Relative gene expression of TRIF-TRAF3 pathway released cytokines in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 µM) for 24 h treatment and LPS (5 µg/ml) for 12 h treatment were used in this assay. Fold change in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. Comparison between treatment group (black column) and control group (white column) is indicated as '*'. All of the data were expressed as least square means ± SE, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

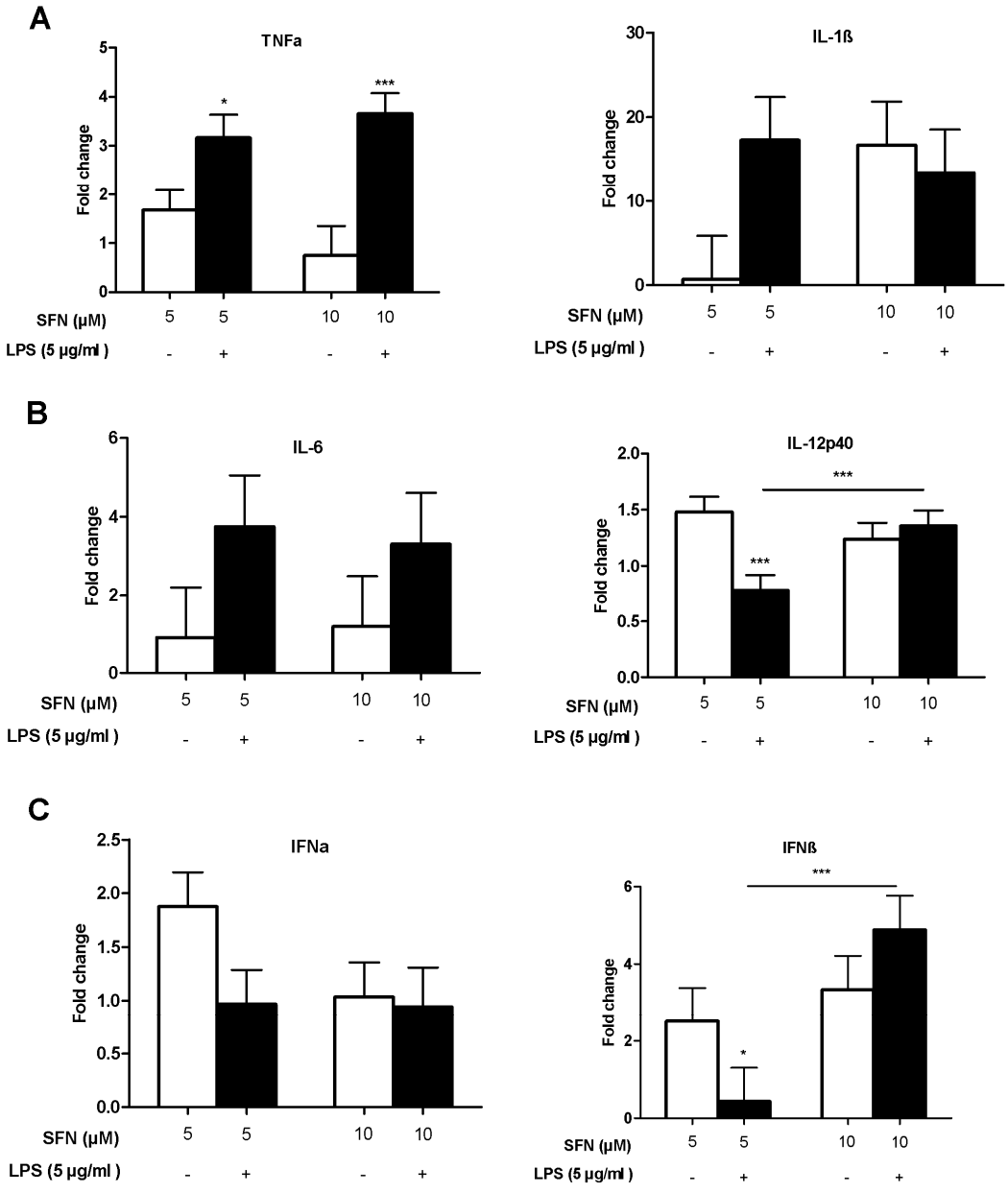

Fig. 24 (Appendix 4) Effect of SFN5 and SFN10 on gene expression regulation of cytokines in the TRIF pathway in PAMs. A) Relative gene expression of TRIF-TRAF6 pathway released cytokines in SFN-LPS treated PAMs B) Relative gene expression of TRIF-TRAF3 pathway released cytokines in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 µM) for 24 h treatment and LPS (5 µg/ml) for 12 h treatment were used in this assay. Comparison between treatment group and control group is indicated as '*'. All of the data were expressed as least square means ± SE, n = 3. *, ** and *** indicate significant differences at $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

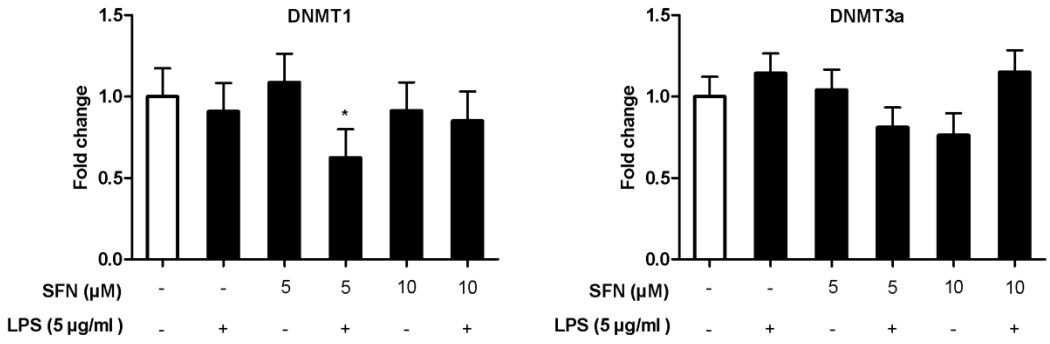

Fig. 25 (Appendix 5) The role of LPS and SFN on gene expression regulation of DNMT1 and DNMT3a. Relative DNMT1 and DNMT3a gene expression were measured in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. Fold change in PAMs without SFN and without LPS treatment (SFN0-LPS0) was set as control. Comparison between treatment group (black column) and control group (white column) is indicated as '*'. All of the data were expressed as least square means ± SE, n = 3. * indicates significant difference at $p < 0.01$.

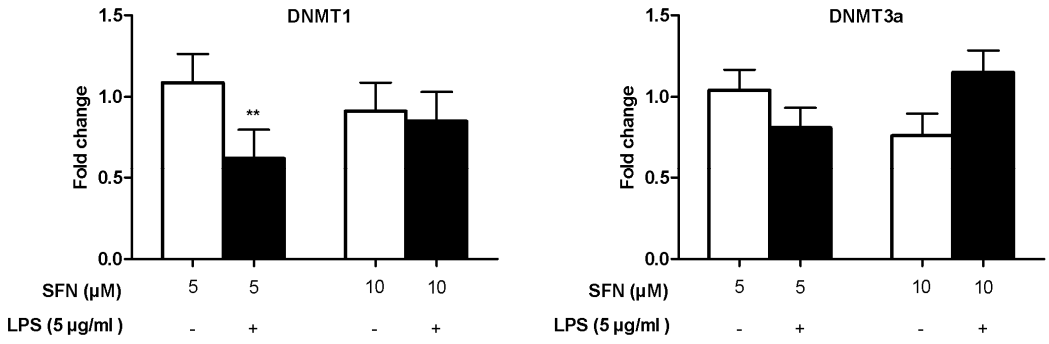

Fig. 26 (Appendix 6) Effect of SFN5 and SFN10 on gene expression regulation of DNMT1 and DNMT3a. Relative DNMT1 and DNMT3a gene expression were measured in SFN-LPS treated PAMs. PAMs with or without SFN (5, 10 μM) for 24 h treatment and LPS (5 μg/ml) for 12 h treatment were used in this assay. Comparison between treatment group and control group is indicated as '*'. All of the data were expressed as least square means ± SE, n = 3. ** indicates significant difference at $p < 0.01$.


Primer 1-F
Primer1-R
Primer2-F
Primer2-R
Primer3-F
Primer3-R
Primer4-F


721 AGTGCGTGGCTGCCGTCGAGGTGGAGATCCGCGGCGGCGGCCGGAGCCTGGAAGAGTTCC
721 AGTGCGTGGTTGTCGTCGAGGTGGAGATTCGCGGCGGCGGTCGGAGTTTGGAAGAGTTTT
43 44 45 46/47 48 49 50<<<<<<<<<<<<<<<<<<Primer4-R
>>>>>>>>>>>>>>>>Primer5-F
781 TTTTAAAGAGCGCCAGCGCCAACCCGAAGCAGTACGCGGACATGCTCAAGGCGCTGCGCT
781 TTTTAAAGAGCGTTAGCGTTAATTCGAAGTAGTACGCGGATATGTTTAAGGCGTTGCGTT
<<<<<<< 51 52 53 54/55 56 57
>>>>>>>>>>
841 TGCGGCGACTCACGGTGGGCGCTGCGCGGGTTCCTGCTCAGATTCTGGCCCTGGTCCTGC
841 TGCGGCGATTTACGGTGGGCGTTGCGCGGGTTTTTGTTTAGATTTTGGTTTTGGTTTTGT
58 59 60 61 62/63 <<<<<<<<<<<<<<<<<<<<<<<<<Primer5-R
>>>>>>>>>>>>>>>>>>>>>>>> Primer6-F
901 ATGCGCTCGGGTTCTCCCGCCTCAAGGAACTGACGCTCGAGGACCTGGAGGTAACCGGCC
901 ATGCGTTCGGGTTTTTTCGTTTTAAGGAATTGACGTTCGAGGATTTGGAGGTAATCGGTT
64 65 66 67 68 69
961 AGGTACCGCCGCCGCTTCAGGAAACGCCGGGGCCTGCGCTCACCACCCTCAGACTCCGTA
961 AGGTATCGTCGTCGTTTTAGGAAACGTCGGGGTTTGCGTTTATTATTTTTAGATTTCGTA
70 71 72 73 74 75 76
1021 ATGTGTCGTGGGCCACCGGAGGTGCCTGGCTCGGCGAACTGCAGCAGTGGCTCCAGCCAA
1021 ATGTGTCGTGGGTTATCGGAGGTGTTTGGTTCGGCGAATTGTAGTAGTGGTTTTAGTTAA
77 78 79 80<<<<<<<<<<<<<<<<<<<<<<<<<Primer6-R
>>>>>>>>>>Primer7-F
1081 GCCTCAAGGTACTGAAAGTGGCGCAAGCAAGCTCGCTTGCCTTTCCGTGCGCCCAGCTTC
1081 GTTTTAAGGTATTGAAAGTGGCGTAAGTAAGTTCGTTTGTTTTTTCGTGCGTTTAGTTTC
< 81 82 83 84 85
>>>>>>>>>>>>>>>>>>>>>
1141 GCGCCTTCCCGGCTCTCACCACCCTGGACCTATCTGACAATCCCGGGCTGGGCGAGCGCG
1141 GCGTTTTTTCGGTTTTTATTATTTTGGATTTATTTGATAATTTCGGGTTGGGCGAGCGCG
86 87 88 89 90/91
<<<<<<<<<<<<<<<<<Primer7-R
>Primer8-F
1201 GGCTGACTGCAGCTCTCTGTCCGCGCAAGTTCCCGGCCCTCGAGGATCTGGCGCTGCGCA
1201 GGTTGATTGTAGTTTTTTGTTCGCGTAAGTTTTCGGTTTTCGAGGATTTGGCGTTGCGTA
<<<<<<<<<<<<<<<<<<<<92/93 94 95 96 97
>>>>>>>>>>>>>>>>>>>>
1261 ACGCGGGGATGGAGACACCCAGCGGCGTGTGCGCGGCGCTGGCGGGGGCGGGCGTGCAAC
1261 ACGCGGGGATGGAGATATTTAGCGGCGTGTGCGCGGCGTTGGCGGGGGCGGGCGTGTAAT
98 99 100/101 102/103 104 105 106 107
1321 CCCACCGCCTGGACCTCAGTCACAACTCGCTGCGCGCCACCGCCGCGGGCGCCCGGGAGT
1321 TTTATCGTTTGGATTTTAGTTATAATTCGTTGCGCGTTATCGTCGCGGGCGTTCGGGAGT
108 109 110/111 112 113/114 115 116
1381 GTGTCTGGCCTGCCGCCCTGAGCTCTCTCAATTTGTCATTCGCTGAGCTGGAGCAAGTGC
1381 GTGTTTGGTTTGTCGTTTTGAGTTTTTTTAATTTGTTATTCGTTGAGTTGGAGTAAGTGT
117 118 <<<<<<<<<<<<<<<Primer8-R
>>>>>>>>>>>>>>>

Fig. 27 (Appendix 7) Sequence, CpG sites and bisulfite primers of CD14 promoter and CDS.

Note: Upper row: Original sequence; Lower row: Bisulfite modified sequence, for display, assume all CpG sites are methylated; ++ CpG sites; :::: Non-CpG 'C' converted to 'T'; >>>>>> Left primer; <<<<<< Right primer

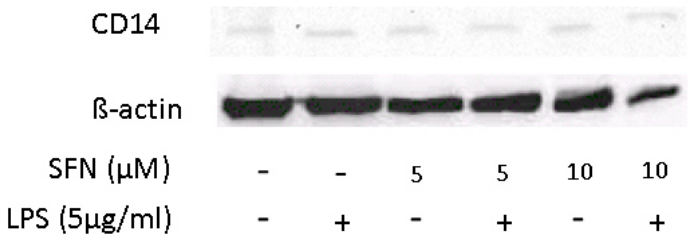

Fig. 28 (Appendix 8) Protein levels of CD14 in SFN-LPS treated PAMs

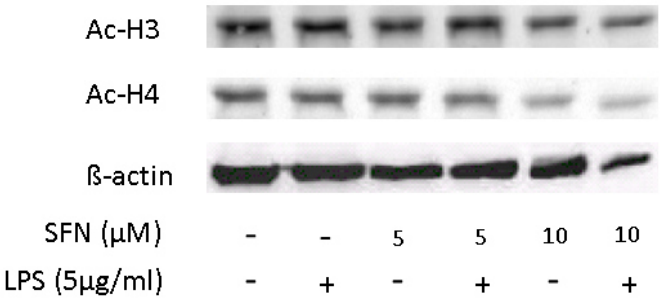

Fig. 29 (Appendix 9) Protein levels of Ac-H3 and Ac-H4 in SFN-LPS treated PAMs

9 Acknowledgement

First of all, I would like to thank Prof. Dr. Karl Schellander for providing me with the opportunity to pursue my doctoral thesis in the Institute of Animal Science and supporting me under all the conditions during these years. I sincerely appreciate Prof. Dr. Karl Schellander's support, guidance, kindness, help and assistance.

I would also like to thank Prof. Dr. Dr. Helga Sauerwein, the professor of Physiology & Hygiene Group in the Institute of Animal Science, University of Bonn. I am grateful for her valuable advices, critical comments and evaluation as my second supervisor of my PhD study. My sincere gratitude also goes to Prof. Dr. Karl-Heinz Südekum, the professor of Animal Nutrition Group in Institute of Animal Science, University of Bonn. I sincerely appreciate for his willingness, valuable advices, critical comments and evaluation as the chairman of my PhD defense.

I would like to express my sincere thanks to Dr. Christiane Neuhoff for her kindness, guidance and great help in designing experiment, collecting samples, ordering chemicals, modifying manuscript and revising my PhD thesis. I also sincerely appreciate Dr. Maren Julia Pröll's help including preparing the documents for coming to Germany, operating experiment, modifying manuscript and revising my PhD thesis. My thanks also go to Dr. Muhammad Jasim Uddin, Dr. Mehmet Ulas Cinar, Dr. Huitao Fan, Dr. Dessie Salilew Wondim, Mr. Rui Zhang and PD Dr. Dawit Tesfaye for their help with experiment design, manuscript modification and my PhD thesis revision.

I would also like to thank Dr. Ernst Tholen and Dr. Christine Große-Brinkhaus for their kind cooperation and great help in statistical analysis.

I want to give my thanks to PD Dr. Michael Hölker and Mr. Ludger Buschen from the experimental research station 'Frankenforst' for their kind help in experimental sample collection at Frankenforst. Thanks to Birgit Koch-Fabritius for Western blot assay, Nadine Leyer for performing sequencing, Helga Brodeßer, Stephanie Fuchs and all Azubis for their kindly help and technical assistance.

I would also like to take this opportunity to thank Ms. Bianca Peters for supporting and helping me with the official and life matters. Thanks also go to Mr. Peter Müller for his really useful help in computer technique.

My thanks also go to all my dear colleagues in the Institute of Animal Science for their

kind help in work and personal life. I want to thanks Dr. Ibrahim Sally Rashad Elsaid, Dr. Dessie Salilew-Wondim, Dr. Mohammad Ariful Islam, Dr. Asep Gunawan, Dr. Mahmudol Hasan Sohel, Dr. Sina Seifi, Dr. Ijaz Ahmed, Dr. Simret Weldenegodguad, Dr. Amin Ahmed Abdel-Samad Zaki, Dr. Hanna Heidt, Dr. Sarah Bergfelder, Dr. Prastowo Sigit, Dr. Xueqi Qu, Mr. Aminul Islam, Ms. Sharmin Aqter Rony, Mr. Samuel Etay, Mr. Zidane, Mohammed, Mr. Eryk Andreas, Mr. Hari Om Pandey, Ms. Julia Welzenbach and Ms. Esther Heuß.

Thanks my dear Chinese friends here for helping me and encourage my in these years. I want to thanks Mr. Rui Zhang, Mr. Fangjun Zhu, Mr. Huitao Fan and his wife Xiaoli Qi, Ms. Lijie Liu, Ms. Huanhuan Tai, Ms. Xinxin Du, Ms. Yingying Zhao and Ms. Xiaomei chen.

I sincerely appreciate China Scholarship Council (CSC) for providing me financial support during my study period.

I owe deepest gratitude to my parents, my dear sister, my brother and my whole family for help and support throughout the years and my life. I also would like to thank Mr. Henkel and his family for their love, care and help during the years.

10 Curriculum vitae

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Ph.D study Major in Animal Science, Sep 2011-present, Dept. of Animal Breeding and Husbandry, Institute of Animal Science, University of Bonn, Germany, Title of research: Epigenetic regulation on CD14 in TRIF pathway in alveolar macrophages of German Landrace Pigs

M. S. Major in Animal Genetics and Breeding, Sep 2008-Jul 2011, Institute of Animal Genetics and Breeding, Sichuan Agricultural University, P. R. China, Title of the thesis: "ELOVL2 and ELOVL5 Gene Cloning and The Expression in Different Tissues in Erlang Mountainous chicken in Different Feeding Methods"

B. S. Major in Animal Science, Sep 2004-Jul 2008, Sichuan Agricultural University, P.R. China

Middle school study Sep 1998-Jun 2004, Liang Shan National Middle school, P.R. China

Primary school study Sep 1992-Jun 1998, Li Zhuang village primary school, P.R. China

►Work experience

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Major in Animal Science, Dept. of Animal Breeding and Husbandry, Institute of Animal Science, University of Bonn, Germany. Title of research: Epigenetic regulation on CD14 in TRIF pathway in alveolar macrophages of German Landrace Pigs

Jul 2008-July 2011

Worked as a research fellow (as a student for M.S.) in institute of Animal Genetics and Breeding (Sichuan Agricultural University). Our research work was focused on breeding a high-quality chicken breed Erlang Mountainous chicken and my thesis is a study on elongation of very long-chain fatty acids (ELOVL) members.

Mar 2008-Jun 2008

Practiced chicken breading in Sichuan Daheng Poultry Breeding Company, Ltd.

Sep 2004-Mar 2008

Study fellow (as a student for BS degree) in College of Animal Science and Technology, Sichuan Agricultural University.

Laboratory and software skills: Cell culture (culture innate immune cell - Pulmonary alveolar macrophages); Cell sorting - flow cytometry; Epigenetic DNA methylation (Bisulfite sequencing); ELISA; DNA & RNA extraction; Electrophoresis; Real-time quantitative PCR; Egg & meat quality analysis; measuring carcass traits of chicken; SAS (statistic software); BISMA (Bisulfite sequencing DNA methylation analysis); Bio-Rad CFX96 (software for real-time quantitative PCR); DNAstar (primer design, DNA/protein sequences merge and align); Primer Premier / Methyprimer (primer design, DNA/protein sequences merge and align); FlowJo (flow cytometry)

►Publication and contributions

Thesis publication

Yang Q, Pröll M, Salilew-Wondim D, et al. (2015): Lipopolysaccharide-induced expression of CD14 in TRIF pathway is epigenetically regulated by sulforaphane in the porcine pulmonary alveolar macrophages. Innate Immunity (Under review)

Yang Q, Neuhoff C, Zhang R, et al. (2014): Sulforaphane inhibits CD14 gene expression in LPS stimulated alveolar macrophages of German Landrace pigs. The German Society for Animal Production Meeting (DGfZ/GfT). 17. / 18. Sep. 2014. Dummerstorf, Germany: A21 (Proc)

Yang Q, Pröll M, Zhang R, et al. (2015): Lipopolysaccharide-induced expression of CD14 is epigenetically regulated by sulforaphane in pulmonary alveolar macrophages of German Landrace Pigs. The German Society for Animal Production Meeting (DGfZ/GfT). 16-17. Sep. 2015. Berlin, Germany: D14 (Proc)

Other publications

Qiu MH, Yang Q, Wu HL, et al. (2011): Cloning and characterization of chicken ELOVL7 gene and comparison with other vertebrates. 5th International Conference Proceedings on Bioinformatics and Biomedical Engineering (iCBBE). 10-12. May 2011. Wuhan, China.

Yang Q, Jiang XS, Du HR, et al. (2009): Analyzing slaughter performance of different strains in the Daheng high-quality chickens, progress in China's poultry science. The 14th Symposium Proceedings of National Poultry Science. 20. Jun. 2009. Haerbing, China.

Yang Q, Liu YP, Zhu Q. The investigation of earthquake disaster reconstruction for layers in Anxian district, Sichuan Animal and Veterinary Sciences. 2009, (12):15-16.

Ren WS, Zhu Q, Yang Q, et al. (2010): Analyzing of IMF and IMP different strains in the Daheng high-quality chickens. China animal husbandry and veterinary medicine, 2011, (6): 230-232.

Long YL, L DY, Yang Q, et al. (2011): Comparative analysis of meat quality and flavor in different strains of Daheng high-quality broiler. Journal of Sichuan Agricultural University. 2011, (2): 266-268.