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Innate immune responses of LPS treated porcine monocyte-derived dendritic cells after exposure to the histone deacetylase inhibitor sulforaphane

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Dedicated to my beloved parents and my beloved husband

Yankui

Innate immune responses of LPS treated porcine monocyte-derived dendritic cells after exposure to the histone deacetylase inhibitor sulforaphane

Epigenetic mechanisms are believed to regulate the expression of immune gene and the development of inflammation. Dendritic cells (DCs) are crucial to sense microbial pathogens by their phagocytosis and by altering the expression of essential immune associated genes that are critical for initiation of innate and adaptive immunity. Therefore, it is possible to hypothesize that the DCs could undergo epigenetic modification towards maintaining the immunity homeostasis during inflammation. Thus, this study aimed to understand epigenetic mechanisms and associated gene expression changes in porcine DCs during infection using an in vitro model. For this, initial analysis was performed to select DCs that could exhibit a greater ability to initiate innate immune responses. To achieve this, porcine monocyte-derived DCs (moDCs) and splenic DCs (SDCs) were isolated and challenged with lipopolysaccharide (LPS). Following this, the expression level of immune related genes was analyzed using qRT-PCR and ELISA. The results unveiled that moDCs displayed higher mRNA levels of TLR4-related immune gene, and pro-inflammatory cytokines TNF-α, IL-1ß and IL-8 compared to SDCs. Moreover, the flow cytometry and phagocytosis results indicated that the moDCs displayed a more potent maturation induction and phagocytic maintenance. Therefore, moDCs were chosen to be used for further epigenetic analysis. Moreover, sulforaphane (SFN) which is a natural histone deacetylase (HDAC) inhibitor was used t to induce the epigenetic modifications in moDCs. After pre-incubation with SFN, the moDCs were stimulated with LPS. Indeed, SFN was found to inhibit the global HDAC activity. Both SFN and LPS caused DNA methylation in exon 2 of TLR4 and in promoter region of MHC-SLA genes. Moreover, pre-incubation of SFN suppressed the TLR4 signal pathway and MHC-SLA gene which induced the transduction of inflammatory cytokines (TNF-α, IL-1ß, IL-6 and IL-8) only before 3 to 6 h of LPS stimulation. On the other hand, SFN treatment of moDCs followed by LPS challenge induced DNA demethylation in the promoter region of MHC-SLA gene resulting in up-regulation of TLR4 and MHC-SLA gene expression. Inhibition of HDAC activity and DNA demethylation together by SFN treatment increased the expression level of immune genes after 6 h of LPS stimulation. Addtionally, SFN treatment impaired LPS-induced pro-inflammatory cytokine TNF-α and IL-1ß in the cell culture supernatants, but it induced an accumulation of TNF-α in the cell lysates of moDCs that may contribute to restrict the inflammation spreading. SFN also suppressed moDCs differentiation from immature to mature DCs through down-regulation of CD40, CD80 and CD86 molecules resulted in enhanced phagocytosis of moDCs. Thus, this study manifests a novel insight that HDAC inhibitor, SFN, exerts dynamic modulation on DC phenotypes including differential maturation and production of pro-inflammatory cytokines through epigenetic modifications to positively modify the innate immune responses in porcine moDCs.

Immunreaktion von mit Lipopolysaccharid behandelten porcinen Monozyten abgeleiteken dendritischen Zellen nach Einwirkung des Histonacetylase Inhibtors Sulforahan

Epigenetische Mechanismen regulieren die Expression von Immungenen und beeinflussen somit die Entwicklung von Entzündungen. Dendritische Zellen (DCs) sind entscheidend für die Erkennung von mikrobiellen Pathogenen durch ihre Phagozytose und die Änderung der Expression von Immungenen die für die Einleitung der angeborenen und adaptiven Immunität notwendig sind. Daher ergibt sich die Hypothese, dass epigenetische Veränderungen in DCs zu einer Herstellung der Imunitäts-Homöostase während einer Entzündung führen. Demnach, ist das Ziel dieser Studie epigenetische Mechanismen zu Verstehen und Veränderungen der Genexpressionen in Schweine DCs während einer Infektion in vitro zu untersuchen. Zunachst wuden Analyren durchgeführt, um DCs zu identifizieren, die eine größere Fähigkeit haben die unspezifische Immunantwort zu initiieren. Um dies zu erreichen, wurden Schweine Monozyten DCs (MoDCs) und Milz-DCs (SDCs) isoliert und mit Lipopolysaccharid (LPS) behandelt. Anschließend wurde das Expressionsniveau von immunassoziierten Genen mittels qRT-PCR und ELISA analysiert. Die Ergebnisse ergaben, dass MoDCs im Vergleich zu SDCs ein höheres mRNA-Niveau von TLR4 und ein höheres Niveau von proinflammatorischen Zytokinen TNF-α, IL-1ß und IL-8 aufwiesen. Darüber hinaus, zeigten die Ergebnisse der Durchflusszytometrie und Phagozytose, dass die MoDCs eine stärkere Induktion der Reifung und phagozytische Aktivität hatten. Daher wurden für die weiteren epigenetischen Analysen MoDCs gewählt. Des Weiteren wurde Sulforaphan (SFN), ein natürlicher Histon-Deacetylase Inhibitor (HDAC) verwendet, um die epigenetischen Modifikationen in MoDCs zu induzieren. Nach Vorinkubation mit SFN wurden die MoDCs mit LPS stimuliert. Tatsächlich zeigte sich, dass SFN die globale HDAC-Aktivität hemmt. Sowohl SFN als auch LPS verursachen DNA-Methylierung in Exon 2 des TLR4 und in der Promotorregion des MHC-SLA-Gens. Weiterhin unterdrückt die Vorinkubation mit SFN die TLR4 Signalwege und MHC-SLA, welche die Transduktion von inflammatorischen Cytokinen (TNF-α, IL-1ß, IL-6 und IL-8) nur von Stunde 3 bis 6 der LPS-Stimulation induzierten. Auf der anderen Seite, induziert die SFN Behandlung von MoDCs, gefolgt von LPS, DNA-Demethylierung in der Promotorregion des MHC-SLA-Gens, was zu einer Hochregulierung von TLR4 und MHC-SLA führt. Die Hemmung der HDAC-Aktivität und der DNA-Demethylierung zusammen durch die SFN Behandlung erhöht das Expressionsniveau von Immungenen nach 6 h der LPS-Stimulation. Außerdem, beeinträchtigt die SFN Behandlung die LPS-induzierten proinflammatorische Zytokine TNF-α und IL-1ß in den Zellkulturüberständen, aber es induziert eine Ansammlung von TNF-α im Zellysaten von MoDCs, was evtl. dazu beiträgt die Entzündung zu beschränken. Ebenfalls unterdrückt SFN die MoDCs Differenzierung von unreifen zu reifen DCs durch Herunterregulierung von CD40, CD80 und CD86 Molekülen was eine verbesserte Phagozytose zur Folge hat. So konnte in dieser Studie erstmals gezeigt werden, dass der HDAC-Inhibitor, SFN, eine dynamische Modulation auf DC Phänotypen ausübt einschließlich der differenzierten Reifung und Produktion von pro-inflammatorischen Zytokinen durch epigenetische Modifikationen, um die unspezifische Immunantwort in MoDCs positiv zu verändern.

Contents

List of abbreviations

APC	:	Antigen-presenting cell
APC	:	Allophycocyanin (dye protein)
ANOVA	:	Analysis of variance
BSP	:	Bisulfite sequencing PCR
BSA	:	Bovine serum albumin
cDC	:	Conventional/myeloid DC
CD	:	Cluster of differentiation
cDNA	:	Complementray DANN
CO_2	:	Carbondioxide
Ct	:	Threshold cycle
DC	:	Dendritic cell
DNA	:	Deoxyribonucleic acid
DNase	:	Deoxyribonuclease
DTT	:	Dithiothreitol
DMEM	:	Dulbecco's modified eagle medium
DPBS	:	Dulbecco's Phosphate Buffered Saline
DNMT	:	DNA methyltransferases
ELISA	:	Enzyme-linked immunosorbent assay
EDTA	:	Ethylenediaminetetraacetic acid
FACs		Flow cytometry
FBS	:	Fetal bovine serum
Fig	:	Figure
FITC	:	Fluoresceinisothiocyanat
GM-CSF	:	Granulocyte-macrophage colony-stimulation factor
GAPDH	:	Glyceraldehyde 3-phosphate dehydrogenase
HPRT1	:	Hypoxanthine phosphoribosyltransferase 1
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HDAC	:	Histone deacetylase
IL	:	Interleukin

IFN	:	Interferon
IFN-γ	:	Interferon gamma
IRF-6	:	IFN regulatory factor 6
kDa	:	Kilo Dalton
LPS	:	Lipopolysaccharides
moDC	:	Monocyte-derived DC
MHC	:	Major histocompatibility complex molecules
MAPK	:	Mitogen-activated protein kinases
MCP-1	:	Monocyte chemoattractant protein 1
MIP-3α	:	macrophage inflammatory protein 3 α
miRNAs	:	MicroRNAs
mRNA	:	Messenger RNA
NF-κB	:	Nuclear factor-kappa B
OD	:	Optical density
PAMPs	:	Pathogen-associate molecular patterns
PBMC	:	Peripheral blood monoclear cell
PBS	:	Phosphate-buffered saline
PCR	:	Polymerase chain reaction
pDC	:	Plasmacytoid DC
PE	:	Phycocerythrin
RPMI	:	Roswell park memorial institurte medium
qRT-PCR	:	Quantitative real-time polymerase chain reaction
rp	:	recombinant porcine protein
RNA	:	Ribonucleic acid
ROS	:	Reactive oxygen species
rpm	:	Revoulution per minute
SD	:	Standard deviation
SAHA	:	Vorinostat
SFN	:	Sulforaphane
SDC	:	Splenic DC
siRNA	:	Small interfering RNA
TGFβ1	:	Transforming growth factor, beta 1
TAK1	:	TGF-ß-activated kinase 1

TLR	:	Toll-like receptor
TNF	:	Tumor necrosis factor alpha
TSA	:	Trichostatin A
TRAF6	:	TNF receptor associated factor 6
TRIF	:	TIR domain-containing adaptor protein-including IFN-β

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Chapter 1: General overview

1.1 Introduction

Swine has a great economic importance in livestock industry due to pork production. The pigs are systemically exposed to either clinical or subclinical infections or stressors of diverse microbial pathogens and viruses during the commercial environment. The systemic immune response to the challenge can markedly reduce the potent of production of pigs (Faure et al. 2007, Kim et al. 2012, Litvak et al. 2013, Rakhshandeh and de Lange 2012). The immune system is the defense of host that is composed by a network of cells, tissues and organs that work together to prevent the challenge from infectious organisms and other invaders. Therefore, deciphering of the porcine immune response is very important. The immune system is typically classified into innate immune system and adaptive immune system.

Innate immune system comprises pathways of microbial recognition, inflammation, microbial clearance and cell death to combat infectious microorganisms. Therefore, it constitutes the first line of defense to discriminate very effectively between host cells and pathogens, providing initial defense by induction of inflammatory responses such as antimicrobial pathways and adaptive immunity (Carpenter et al. 2014, Mair et al. 2014). The substantial potential of these responses to drive pathological inflammation and tissue damage gives the prominence need for strict control of the responses. Additionally, the deficiency of innate immune components, which are very rare, can lead to increase the susceptibility to infectious. Therefore, innate immune response or non-specific immune response plays a pivotal role in the host defense during the detection and subsequent triggering of a pro-inflammatory response to invading microorganisms (Ulevitch 2004). Remarkable numbers of genes or multiple pathways are involved in each of the functional categories which could coordinately and promptly regulate in response to distinct external stimulus or pathogens during the initiation of inflammatory response. The multiple layers of regulation collaborate to control those pathogen-induced or danger-induced gene expression programs, which vary depending on the immune cell lineage, which are involved and the specific signal that is encountered. For instance, sentinel cells in the innate immune system implement the detection of microbial pathogens both for lysis and for phagocytosis. The recruited

sentinel cells are well known as antigen-presenting cells (APCs) like dendritic cells (DCs) which are in close contact with the host´s natural environment and rapidly recruit them to the infection site by providing initial defense (Newton and Dixit 2012).

DCs are highly specialized and professional APCs because they are able to induce primary immune responses, thus permitting establishment of immunological memory. DCs play the key role in bridging the innate and adaptive immune response that is one of the most significant advances in field of immunology in the last decades. Indeed, since DCs have been discovered by Steiman and Cohn in the 1970 (Steinman and Cohn 1973), it has become clear that DCs are key inducers and regulators of immune response, as link of innate and adaptive immunity in response to many stimuli (Steinman 2012). Moreover, DCs respond quickly to invading microorganisms and differentiate extensively to become mature or immunogenic accessory cells. Cytokines produced by DC is an initial step in maturation of DC, but numerous other immune cells also produce cytokines in vivo state. DCs are especially distributed in tissues that interface the external environment, such as skin, the gut and spleen, where they can perform a sentinel function for incoming pathogens and have the capacity to recruit and activate cells of innate immune system. So it is meaningful to use a in vitro culture DC model to avoid the microenvironment disturbance (Steinman and Cohn 1973). Moreover, DCs have high phagocytic activity, both in peripheral tissues and secondary organs. Phagocytic DCs are referred to as immature with low levels of MHC class II (MHC II) and costimulatory molecules (CD80, CD86 and CD40) expression which reside in peripheral tissues. Immature DCs migrate to secondary lymph organs after inflammatory stimulation or uptake of pathogenic antigens. During the migration process, immature DCs develop into mature DCs undergoing an up-regulation of surface MHC II and costimulatory molecules and induction of the ability to stimulate T cells (Gehring et al. 2008).

The earliest classical (or conventional) DCs were first discovered as an unique cell type with “stellate” or dendritic morphology in mouse spleen (Steinman and Cohn 1973). Subsequently, a diverse group of functionally specialized remarkable differentiated cell types has been determined with relatedness of phenotypic and functional attributes resembling those of DCs on the basis of cell-surface markers and functional responses, including naïve T cells stimulation. Currently, four major DC types are characterized:

myeloid/conventional DCs (cDCs), monocyte-derived DCs (moDCs), plasmacytoid DCs and Langerhans cells depending on cellular activation status and location in the body (Satpathy et al. 2012). The spleen contains multiple subsets of DCs and several common subsets of splenic DCs (SDCs) have been well categorized. For instance, human spleen resident DCs have been categorized into two major subsets on the basis of their phenotype: the cDCs are characterized by CD11c+HLA-DR+CD123-, they express Toll-like receptor (TLR) 2 and TLR4; plasmacytoid DCs are exhibited the CD11c-HLA-DR+CD123+, and express TLR7 and TLR95 (Velasquez-Lopera et al. 2008).

Although the tissue resident DCs are less arguable, it is difficult to identify the function in a stage-specific (or a differentiation-specific) DC because of the disturbance of microenvironment from in vivo isolation. Fortunately, the high level of CD11c integrin was shown to be useful to enrich DCs (Crowley et al. 1990, Metlay et al. 1990), despite it is not the DC specific marker. However, the identical SDCs might be important to be used as a control to characterize an suitable culture model which could be controlled the identical differential status of DCs. Notably, culture of precursor monocytes with granulocyte-macrophage colony-stimulation factor (GM-CSF) and interleukin-4 (IL-4) led to the development of immature moDCs models in vitro (Grassi et al. 1998, O'Neill and Bhardwaj 2005). These moDCs have the acquired phenotypic features associated with DCs and the development is controlled following the application of maturation stimulus (Romani et al. 1994, Sallusto and Lanzavecchia 1994, Sallusto et al. 1995). These methods to generate huge number of moDCs in vitro have well developed in human and mice and changed the field because the immunologists could more easily study their immunizing properties. However, the relative efficacy of the differences between the identical CD11c+ SDC and moDC has not been compared in pig. To the author´s knowledge, there is lacking information to generate moDCs in vitro and the explanation of the differences of potential ability of SDCs and moDCs to sense the immune response in pig. So it is necessary to sketch the plenty of scarcity knowledge of immune responses between porcine SDC and moDC. Therefore, consistent with the primary idea that maturation is a crucial link between innate and adaptive immunity, lipopolysaccharide (LPS), Gram-negative bacteria wall component, has been studied as a maturation stimulus to initiate the differentiation procession (Sallusto et al. 1995).

DCs can sense the presence of a pathogen by recognizing and detecting the microbial components via a set of pattern-recognition receptors (PRRs) on DCs surface. Presently, it is widely accepted that pathogen-associated molecular patterns (PAMPs), typical components of a microbe and not shared by host cells, are able to activate PRRs such as TLRs, a components of APCs, trigger innate immune responses and antigen-specific adaptive immunity. The activated TLRs trigger several different effector mechanisms to eliminate the infection (Brightbill et al. 1999, Janeway and Medzhitov 2002). So far, 10 functional porcine TLRs (TLR1 to TLR10) have been identified (Uenishi and Shinkai 2009). In fact, the different TLRs expressed on porcine DCs discriminate distinct PAMPs and initiate the distinct signaling pathways to induce DC maturation and activation (Kawai and Akira 2010). The TLR signaling pathways were exhaustively studied after the identification of the TIR domain-containing adaptor molecule MyD88-the first identified member of TIR family. The MyD88 is universally used by all TLRs except TLR3 (Akira et al. 2006). In addition, important studies using MyD88-deficient mice had been shown that MyD88-independent pathway can activate TLRs downstream signaling via IRF3 as well as the delayed NF-κB activation to induce inflammatory cytokines in DCs (Kaisho et al. 2001, Kawai et al. 2001). Subsequently, other three TIR adaptors including TIRAP (also known as MAL), TIR domain-containing adaptor protein-including IFN-β (TRIF) and TRAM were shown to link to TLR4, besides, TIRAP links extent to TLR2 (Hoebe et al. 2003, Horng et al. 2002, Oshiumi et al. 2003, Yamamoto et al. 2002).

Currently, it is widely accepted that LPS, as a major component of inflammation recognized by TLR4 on DCs, leading to the recruitment of sentinel cells (such as DCs, neutrophils, macrophages and mast cells) to the site of infection and elicit strong immune response in body, which is considered as a hallmark of the innate immune response. Notably, all the studies revealed TLR4 to have the most complex signaling arrangement of all the TLRs through either MyD88-dependent pathway to activate NF-κB signaling or the TRAM-TRIF pathway to induce IRF3 signaling. LPS is usually recognized by the crystal structure of TLR4/MD2/CD14 complex to activate the TLR4 signaling pathway. Upon activation, the adaptor proteins including the MyD88 and MyD88 adaptor-like protein, TRIF and TRIF-related adaptor molecule are recruited to the cytoplasmic tail of TLR4 (Egan et al. 2009). Afterwards, subsequent signaling

generates a rapid activation of nuclear factor- kB (NF-kB) and produces pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL-1ß), IL-12p40, IL-6 and chemokines like IL-8 (Egan et al. 2009).

Multiple layers of regulation, including the chromatin structures, DNA methylation, histone modification and the recruitment of transcription factors and the non-coding RNA cooperate together to regulate the pathogen-induced inflammatory gene expression (Carpenter and Fitzgerald 2014). Chromatin structure is a crucial controller in gene expression. Histone acetylation and DNA methylation plays an essential role in epigenetic mechanism in chromatin modification and they serve the epigenetic marks for active or inactive chromatin further to inherit into next generation. DNA methylation, which modifies a cytosine base at the CpG dinucleotide residues with ethyl groups, is catalyzed by DNA methyltransferases (DNMTs) and regulates gene expression patterns by altering chromatin structures (Gonzalo et al. 2006). So far, three active DNMTs have been identified in mammals including DNMT1, DNMT3a, and DNMT3b. DNMT1 is a key maintenance enzyme in mammal cells and therefore responsible for maintaining key methylation patterns in target genes. The DNMT3a and DNMT3b, belong to the DNMT3 family, are de novo DNMTs that can mediate methylation-independent gene repression. Moreover, DNA methylation promotes the recruitment of histone acetyltransferases and histone deacetylases (HDACs) to functional region of target genes thereby repressing genes and their respective products. However, although these DNMTs have an individual role in epigenetic regulation, it is not fully understood how DNMTs mediate the innate immune response to LPS in pig.

Conversely, in mammalian cells, state of histone acetylation is regulated by the opposing actions of HDACs by removing acetyl groups from histone to compact the chromatin structure to further suppress the gene expression. Therefore, HDACs affect diverse biologic functions, principally cell differentiation, growth and survival. So far, 11 different HDACs have been identified and categorized into four classes: Ⅰ, Ⅱ, Ⅲ and Ⅳ based on homology to respective yeast orthologues (Fig. 1) (Gregoretti et al. 2004).

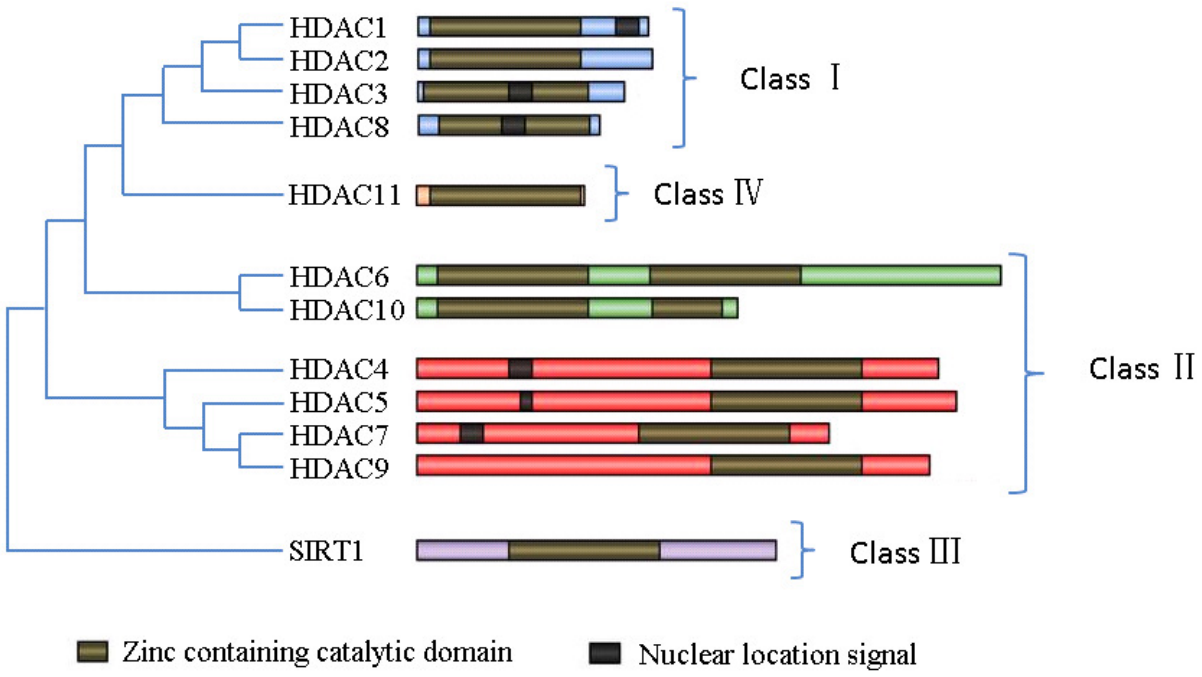

Figure 1. The phylogenetic tree is showing the evolutionary relationship among histone deacetylases (HDACs) family members. The enzymatic domains and the nucleus localization sequences are highlighted in brown and black, respectively (Federico and Bagella 2011).

HDACs attract the researchers´ great interest because of the dysregulation of HDACs activity which has been bridged to the pathogenesis of cancer and inflammation in human and mouse. For instance, it has been reported that the association of NF-κB with class Ⅰ HDAC corepressor proteins could repress the expression of NF-κB-regulated genes and to control the induction of of pro-inflammatory cytokine TNF-α and IL-1ß (Ashburner et al. 2001, Jeong et al. 2014). Meanwhile, HDAC6 of class Ⅱ HDAC plays an essential role in regulation of inflammatory immune response (Shakespear et al. 2011), particularly at the level of the APC/T cell immune response (Serrador et al. 2004), the macrophages response (Halili et al. 2010) and the atypical airway inflammation (Lam et al. 2013). By comparison, HDAC9 and HDAC10 have less established roles than HDAC6 in immune system, although the inhibition of HDAC10 may regulate HSP-90 acetylation (Park et al. 2008). Therefore, according the influences of the HDACs alteration on the immune responses, it provides potential therapeutic targets via modulating the HDACs expression or activity using HDAC inhibitors in inflammatory disease.

Indeed, a class of small-molecule inhibitors namely Ⅰ, Ⅱ, Ⅲ and Ⅳ came into being to exhibit anti-inflammation with safety profiles. So the classic HDAC inhibitors (such as trichostatin A (TSA), vorinostat, valproic, butyrate, and panobinostat) are among the promising anticancer agents under development (Bolden et al. 2006, Marks and Breslow 2007, Prince et al. 2009). Importantly, in recent years, the isothiocyanate sulforaphane (SFN) that naturally occurs in cruciferous vegetable such as broccoli, cauliflower and cabbage is becoming an important group of natural substances that can inhibit of HDAC activity. SFN has been reported to exhibit antioxidative, antimicrobial, anti-inflammatory and antitumoral properties (Cheung and Kong 2010, Mrowietz and Asadullah 2005). Quite a good number of mechanisms have been hypothesized to explain the beneficial effects of SFN on choric inflammation prevention. SFN enhances bacterial clearance by increasing the phagocytic activity of alveolar macrophages and is beneficial against Gram-negative bacteria infection (Fahey et al. 2002, Johnson et al. 2010). A recent study described that SFN has been suggested to inhibit T cell-mediated autoimmune disease in human DCs by impairing the TLR4-induced IL-23 and IL-12 expression and strongly results in the stress response protein heme-oxygenase-1 secretion (Geisel et al. 2014). However, these mechanisms include epigenetic changes resulting from the inhibition of HDAC activity (Dashwood and Ho 2008, Myzak et al. 2007). To our best knowledge, although numerous mechanisms have been studied to explain the beneficial effects of SFN on chronic inflammation prevention, the effects of SFN on cell growth, survival and differentiation in primary cells such as porcine DCs are poorly understood.

1.2 Objectives

To sum up with the background, the thesis was carried out by several experiments to achieve the follow aims:

The first objective aimed to select an appropriate porcine dendritic cells (DCs) that exhibits a greater ability to initiate innate immune responses in vitro compared to splenic DCs (SDCs). For this, lipopolysaccharide (LPS)-induced innate immune responses were evaluated in porcine SDCs and monocyte-derived DCs (moDCs). The impacts of various concentrations of LPS on phagocytosis and cell viability response were examined. In addition, MyD88-dependent and -independent related gene expression in LPS stimulated porcine moDCs and SDCs was investigated in a time-dependent manner. Furthermore, the co-stimulatory molecules, pro-inflammatory cytokines and chemokine production were measured.

The second objective was focused to understand the epigenetic mechanisms and associated gene expression changes during infection of moDCs with LPS in vitro. To achieve this, firstly, the effect of sulforaphane (SFN) on the LPS induced inflammatory response in porcine moDCs was investigated by measuring the phagocytosis, cell viability, cell apoptosis and the relative abundance of essential immune gene expression including inflammatory cytokines. In addition, the effects of LPS stimulation on expression of genes encoding histone deacetylatases (HDACs) and DNA methyltransferases (DNMTs) and histone acetylation levels were analyzed. Furthermore, the modulating effects of SFN on LPS-induced inflammatory response and TLR4 activation were also examined including the differentially expressed functional proteins via the histone acetylation and the DNA methylation patterns of TLR4 and MHC-SAL genes.

1.3 Materials & Methods

1.3.1 Animals

The 35 days old female Pietrain piglets were used for this study. The piglets were housed in the accredited barrier-type livestock facilities at the Teaching and Research Station of Frankenforst, University of Bonn, Germany. All the piglets were clinically healthy and no respiratory or systemic diseases were found according to the clinical history and physical examinations. The feeding, housing and husbandry practices of the animals were approved and followed the guideline (ZDS 2003). The experiment of handling animals was approved by the Veterinary and Food Inspection Office, Siegburg, Germany. All the piglets were euthanatized by intra venous injection of T61 (Intervet) by registered veterinarian after sedation using Ketamine (Intervet) according to German animal protection law. Moreover, this study was carried out in strict accordance with the recommendations in the Guide for Animal Welfare committee of the University of Bonn with proposition number 84-02.05.20.12.075.

1.3.2 Preparation of cells

1.3.2.1 Extraction of Splenic DCs (SDCs)

The porcine spleen was collected within 30 min of euthanasia under sterile conditions and thoroughly flushed with sterile normal 0.9% NaCl saline. The spleen was chopped into 5-7 cm length sections and putted in Hanks-EDTA (cat. H4641; Sigma) buffer on ice before transport to laboratory. The SDCs isolation was performed as described previously (Bimczok 2005). Briefly, the spleen was cut into small pieces and then transferred into collagenase (cat. C2139-100 MG; Sigma) solution (Chapter 1) and incubated in a shaking incubator at 37 °C with 120 rpm for 2-4 h and then stopped the digestion with culture medium containing fetal bovine serum (FBS) (cat. 10270; Invitrogen, Germany). Following the collagenase solution containing released cells was filtered through a 70 µm cell strainer (cat. 352350, BD-Falcon) followed by a filtering through 40 µm cell strainer (cat. 352340, BD-Falcon). Cells were washed two times in Dulbecco's Phosphate Buffered Saline (DPBS) (cat. 14190-094; Invitrogen, Germany) and suspended in RPMI-1640 medium supplemented with 10% FBS, 1000UI /ml penicillin-streptomycin (cat. 15140; Invitrogen, Germany) and 1% fungizone (cat. 15290-026; Invitrogen, Germany). In order to purify the splenic DCs, extracted cells

were cultured for 12-16 h, then washed away the non-adherent cells using warm RPMI-1640 medium (cat. 21875; Invitrogen, Germany). The adherent SDCs were cultured in the indicated time for the further stimulation.

1.3.2.2 Generation of moDC from adherent monocytes of PBMCs

The peripheral blood mononuclear cells (PBMCs) were obtained from porcine blood samples which were collected from the vena cava cranialis in sterilized tubes with ethylenediaminetetraacetic acid (EDTA). The PBMCs were isolated by Ficoll-histopaque (cat. 10771, Sigma, Germany), following the previous description (Seki et al. 2012, Uddin et al. 2012). The moDCs were generated from the adherent monocytes following the procedure described previously (Carrasco et al. 2001, Facci et al. 2006, Raymond and Wilkie 2005). Briefly, first, for the adherent monocytes purification, PBMCs were washed two times in cold DPBS and re-suspended in dulbecco's modified eagle medium (DMEM) (cat. 41966-029; Invitrogen, Germany) supplemented with 2% FBS, 500 IU/ml penicillin-streptomycin and 0.5% fungizone. PBMCs were cultured in DMEM medium for 4 h. After that, the non-adherent cells were discarded by vacuum aspiration and the adherent monocytes were washed two times using pre-warmed (37 °C) DPBS in order to remove the non-adherent cells.

Secondly, for the moDCs generation, the cleaned monocytes were cultured in RPMI-1640 medium supplemented with 10% FBS, 1000UI/ml penicillin-streptomycin, 1% fungizone, 20 ng/ml recombinant porcine (rp) granulocyte-macrophage colony-stimulating-factor (GM-CSF) (cat. 711-PG-010; R&D System, UK) and 20 ng/ml recombinant porcine (rp) interleukin-4 (IL-4) (cat. 654-P4-025; R&D System, UK) for 7 days at 37 °C with 5% CO_2. Half of the medium was replaced every 3rd day with the fresh medium supplemented the rp GM-CSF (20 ng/ml) and rp IL-4 (20 ng/ml) concentration. After 7 days of incubation the adherent moDCs were counted and re-cultured in a new plate for the subsequent assays.

1.3.3 Cell viability assay

In this study, cell viability was investigated using the WST-1 cell proliferation kit (cat. 10008883, Cayman Chemical) following to the manufacturer's instructions (Qu et al. 2014). Regarding to the distinct aims, we investigated the cell viability of either SDCs or moDCs in response to LPS in a dose dependent manner in a 96-well plate (chapter 1).

Additionally, we also investigated the SFN influenced cell viability of moDCs in response to LPS (chapter 2). 10 µl reconstituted WST-1 mixture was added to each well. After 2 h of incubation in a CO_2 incubator at 37 °C, the absorbance of the samples was measured using a microplate reader (Thermo max; Germany) at a wavelength of 450 nm. The cell viability was calculated (%) following the manufacturer's formula.

1.3.4 Flow cytometry

In our experiment, we used FACs to analyze the cell-surface protein expression of CD11c, CD40, CD80 and CD86 using a mouse anti-human CD40 FITC mAb (cat. NB100-77786, Novus Biologicals, UK), a mouse anti-human CD80 PE mAb (cat. FAB140P, R&D systems, Germany) and a mouse anti-human CD86 APC mAb (cat. FAB141A, R&D systems, Germany) antibodies respectively. CD11c PE-Cyanine 7 mAb (cat. 25-0114, BD eBioscience, UK) was used for the DCs isotype control. For the cells staining, the cells from different treatments were harvested and incubated for 30 min with FACs staining buffer (chapter 1) and then washed with the same staining buffer. Cells were stained for 30 min on ice in 100 µl of staining buffer solution in light protected condition. The cells were washed twice using staining buffer and suspended in 400-500 µl staining buffer. The events were acquired on 10,000 cells using a FACscalibur Dual Laser Flow Cytometer (BD Biosciences; USA) and analyzed by FlowLogic software (BD Biosciences; Germany). All these antibodies are IgG1 isotype.

1.3.5 Quantitative real time PCR (qRT-PCR)

Total RNA was isolated using miRNeasy Mini Kit (cat. 217004; Qiagen, Germany) and the RNA concentration was measured by Nanodrop 8000 (Thermo Scientific, Pittsburgh, PA, USA). Complementary DNA (cDNA) was synthesized using miScript II RT kit (cat. 218161; Qiagen, Düsseldorf, Germany) and the cDNA was stored at -20 °C for further use. qRT-PCR was performed in an ABI prism®7000 (Applied Biosystems, Darmstadt, Germany) qRT-PCR system. The transcript of target genes presented in each sample was determined using Maxima SYBR Green/ROX Mix (cat. 218073; Qiagen, Düsseldorf, Germany). The primers (Table 1) were designed using the online Primer3 (version 0.4.0) (Rozen and Skaletsky 2000). The qRT-PCR was conducted with the following program: 95 °C for 3 min, 40 cycles at 95 °C for 15s, 60 °C for 1 min and 95 °C for 1 min in the StepOne Plus qPCR system (Applied Biosystem, Germany). Melting curve analysis was performed to detect the specificity of the PCR reaction. Each

experiment was performed in triplicates and each sample was quantified in triplicate (technical replication) using qRT-PCR. In chapter 1 and chapter 2, gene-specific expression was measured as relative to the expression of the house keeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1). The delta Ct (ΔCt) values were calculated as the difference between target gene and reference gene HPRT1: [$\Delta Ct = Ct_{target} - Ct_{housekeeping\ gene}$]. The average expression values were considered for further analysis. In the chapter 3, relative mRNA expression was normalized to the house keeping genes HPRT1 and glyceraldehyd-3-Phosphat-Dehydrogenase (GAPDH):

[$\Delta Ct = Ct_{target} - \sqrt[2]{CtHPRT1 \bullet CtGAPDH}$]. Gene expression was statistically analysed using the comparative 2-ΔΔCT method (Livak and Schmittgen 2001).

1.3.6 HDAC activity assay

For the nuclear protein isolation (chapter 1 and 2), DCs were harvested using trypsin-EDTA (cat: 25200-072; Invitrogen, Germany). The nuclear extracts were obtained from cultured cells according to the manufacturer´s instruction. Briefly, 500 µl of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, 0.05% NP40 [or 0.05% Igepal or Tergitol] pH 7.9) containing 1% proteinase inhibitor (cat: P8340-1ML; Sigma, Germany) was added to approximate amount of trypsinized cells. Cells were lysed using a mechanical homogenizer on ice for 20 min and centrifuged at 3000 rpm for 10 min at 4 °C. The cell pellets were re-suspended in 374 µl of buffer (5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol [v/v], pH 7.9) containing 26 µl of 4.6 M NaCl and homogenized with 20 abundant strokes on ice. The cell pellets were lysed for 30 min on ice and then centrifuged at 14,000 g for 20 min at 4 °C. The supernatants containing the nuclear extract were removed and stored at -80 °C for subsequent analysis. Protein content was determined using Bradford assay.

For the HDAC activity measurement, in vitro HDAC activity was determined using the Color-de-Lys HDAC colorimetric activity assay kit (BML-AK501-0001, Enzo Life Sciences) according to the manufacturer´s instructions. Briefly, approximately 5 µg of nuclear fraction from each treatment samples was incubated with the HDAC assay buffer and the HDAC colorimetric substrate at 37 °C for 30 min. Then the lysine developer was added and the samples were incubated at 37 °C for another 30 min. At

the end of the incubation period, readings were taken at 405 nm using an ELISA plate reader (ThermoMax, Germany).

1.3.7 Phagocytic activity assay

In the current study, phagocytosis of the cells was investigated using VybrantTM Phagocytosis Assay kit (cat. V-6694, Molecular Probes, Germany). The principle of this analysis is based on the intracellular florescence emitted by the engulfed particles, as well as the effective fluorescence quenching of the extracellular probe by trypan blue (Sahlin et al. 1983). The phagocytosis assay protocol follows the instruction of using five negative controls, five positive controls and four experimental samples. Briefly, cells were cultured in a 6-well cell culture plate for 48 h with RPMI-1640. Cells were scrapped, washed twice with DPBS and then treated in the indicated methods according the different objectives (chapter 1 and 2). For the phagocytosis assay, 50 µl of the LPS in the indicate concentrations were added to the experimental wells containing cells. The cells were incubated for 4 h at 37 °C with 5% CO_2 to allow the cells to adhere on the microplate surface. Afterwards, the RPMI-1640 solution was removed from the microplate wells. Then 100 µl of fluorescent BioParticles suspension was added to all the negative control, positive control and experimental wells. Two hours after incubation at 37 °C in CO_2 incubator, the BioParticles were aspirated from all of the microplate wells. Finally, 100 µl trypan blue was added to the wells and incubated for 1 min at room temperature, and then trypan blue was aspirated. The fluorescence emission was measured in a fluorescence microplate reader (Thermo Eectron, Waltham, MA, USA) using 480 nm excitation and 520 nm emissions. The net phagocytosis of the cells was calculated according to the response of the phagocytosis effector agent following the manufacturer's instructions.

1.3.8 Apoptosis assay

In this study, the caspases assay was based on the spectrophotometric detection of the chromophore p-nitroanilide (pNA) after cleavage from the labelled substrate either LEHD-pNA or DEVD-pNA. The caspases were measured according to the manufacturer´s instruction. Briefly, protein was obtained from the cell lysates by using the Caspase-3/CPP32 Colorimetric Assay Kit (cat. #K106-25; BioVision; CA; USA) or Caspase-9 Colorimetric Assay Kit (cat. #K119-25; BioVision; CA; USA). For this assay, 100 µg protein was added to determine the Caspase-3/CPP32 and Caspase-9

activity. The samples were measured at 405 nm in a microplate reader (Thermo max; Germany).

1.3.9 Enzyme-linked immunosorbent assay (ELISA)

In this study, the cytokine secretion in the cell culture supernatants were collected and measured by commercial porcine specific ELISA kits following the manufacturer´s instruction from R&D Systems. The optical density (OD) values were measured by microplate reader (ThermoMax; Germany) with a setting to 450 nm of wave length and results were calculated according to manufacturer's formula.

1.3.10 Western blotting

In this study, DCs lysates were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with polyclonal antibodies specific for target antibodies, and then revealed with secondary antibody. The secondary antibody was used for the primary antibody detection. Mouse polyclonal anti-ß-actin antibody was used to correct minor differences in protein loading. Finally, the specific signals were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (cat. 34077, Thermo Scientific, Germany). Images were acquired by Quantity One 1-D analysis software (Bio-Rad, Germany).

1.3.11 Bisulfite sequencing PCR (BSP)

In the present study, we used the commercial EZ DNA Methylation Kit (Zymo Research) (chapter 3) to subject the genomic DNA for bisulfit modification. Optimally, the bisulfite PCR products were cleaned-up using QIAquick PCR purification kit (Qiagen). Following the bisulfite PCR products were subcloned into the pGEM T-easy vector (Promega) and then the plasmids were transformed into a competent bacterium E.coli. The positive clones were randomly picked up to amplify the M13 primers PCR for the sequencing performance using CEQ8000 sequencer system (Beckman Coulter). The sequenced results were analyzed by QUMA program. Notably, a confident sequence from QUMA analysis should give the conversion scale above 90%.

1.3.12 Statistical analysis

The data from qRT-PCR, mean inflorescence density of FACs and ELISA analysis were analyzed by SAS software package v. 9.2 (SAS institute, Cary, NC, USA). Pairwise

comparisons were made between the treatment groups and control, using Student's t test and Tukey's multiple comparisons (chapter 1 and chapter 3). Pairwise comparisons were made between treatment groups and the vehicle-treated control, using Student's t test (chapter 2). The data were expressed as means ± standard deviations (SD) and (*) $P < .05$, (**) $P < .01$, (***) $P < .001$ were set as statistically significant.

1.4 General results and discussion

The main purpose of this research was to investigate the amelioration of SFN on the LPS induced acute inflammation majorly through the epigenetic regulation in porcine DCs. Regarding to the major goal of the study, the chapter 2 and chapter 3 have investigated the epigenetic regulation of the DCs mediated inflammatory responses from both histone modifications and DNA methylation of functional immune genes including the gene and inflammatory cytokine expression. According to the high doses of LPS induced acute inflammation, the TLR4 pathway was well investigated.

A growing number of identified sentinel cells have been reported to contribute to the innate immune system including DCs, macrophages, and mast cells. In particular, the DCs play an essential role in innate immune system to functionally initiate the adaptive immune response. Based on the previous description in the introduction, the well-catalogued spleen resident DC are maintaining complex stimulatory and/or differentiation status (Velasquez-Lopera et al. 2008), therefore, it is necessary to establish a DCs culture model which can control the differential stage of DCs. Regarding with this reason, we firstly generated monocyte-derived DCs (moDCs) from the porcine peripheral blood monocytes, and compared functional genes expression response to LPS with the in vivo isolated SDC. Although DCs-mediated inflammatory disease have been well studied in human and other primates (Hoogsteden et al. 1999, Ueno et al. 2007, van den Ancker et al. 2010, Xia et al. 2010), knowledge is lacking about the different reactions between porcine moDCs and SDCs in response to LPS. We determined the LPS-induced SDCs and moDCs in vitro to analyze immune response to dose-dependent LPS treatment in chapter 1.

Phagocytosis, the classic process has been reported to be carried out by macrophages, DCs and other myeloid phagocytes and has been shown to have a crucial role in the first line of host defense. During the phagocytic process, the interaction between phagocytes (such as DCs) and bacteria leads to apoptotic cell death and cell maturation with the up-regulation of maturation markers CD40, CD80, and CD86 on DCs (Gehring et al. 2008). Based on this knowledge, we investigated the impact of dose-dependent LPS treatment on cell viability, maturation and phagocytic activity in chapter 1. The phenotypic results showed that moDCs displayed a greater natural immature phenotype

and LPS induced a greater moDCs phagocytosis, efficient activation and maturation compared to SDCs. The identical functions of immature DCs have been reported to highly internalize diverse bacteria and to rapidly initiate the innate immune response. Regarding to the reports, the finding of this study indicated that moDCs might display a better immature functional phenotype compared SDCs. This study examined the LPS activated TLR4-MyD88-dependent and TLR4-MyD88-independent signaling pathway relative gene expressions and inflammatory cytokine inductions in a time-dependent manner. In chapter 1, I found that LPS induced greater immune response of moDCs through more rapid sensing of the MyD88-dependent signaling as well as more efficient pro-inflammatory cytokine productions. Previous studies showed that high level of pro-inflammatory cytokine induction promoted a strong response of T cell activation in DCs (Abdi et al. 2012, Dearman et al. 2009, Kalupahana et al. 2005). In other words, the greater DCs activation supports the more efficient adaptive immune system initiation. Therefore, regarding with our results, the generation of porcine moDCs in vitro developed an appropriate DCs culture model allowing the study of DCs roles in the host defense system.

Further, based on the results of chapter 1, we focused on the role of the epigenetic modifications of HDAC inhibitor SFN in regulating of innate immune response in porcine moDCs as a culture model in chapter 2 and 3. Epigenetic modifications alter chromatin structure and consequently affect transcription and cellular function. Importantly, DNA methylation and histone acetylation are considered as the major epigenetic mediators, which are directly or indirectly regulated by HDACs in many cell types (Sarkar et al. 2011). DNA methylation leads to recruit HDACs to the promoter region of target genes thereby repressing gene expression (Ghoshal et al. 2005, Sarkar et al. 2011). In contrast, acetylation of histones relaxes the chromatin structure to promote gene transcription, whereas, HDACs conversely regulate the acetylation action to compact the chromatin structure favoring gene silencing. Shortly, HDACs reverse the acetylation modification. Therefore, the HDACs alteration in relation to the aberrant gene expression becomes a critical strategy in epigenetic mechanism to understand the immune system. Indeed, HDAC activity can disturb to inflammatory signal pathways including cytokines, transcription factors to regulate cellular activation, proliferation and differentiation (Toussirot et al. 2013). Increased HDAC activity are common in

antigen induced inflammatory diseases and cancers which can lead to deregulation of a range of cytokine and chemokine expression. Most of HDAC members of HDAC class Ⅰ and class Ⅱ have been well defined. Indeed, a recent study has shown that LPS increased HDAC activity, furthermore, the inhibition of HDAC could decrease LPS-induced TNF-α expression through the NF-κB accumulation at the TNF-α promoter region (Zhu et al. 2010). The findings have been inconsistent and controversial (Ashburner et al. 2001, Aung et al. 2006, Shakespear et al. 2011, Zhu et al. 2010). In chapter 2, our finding coincided to the previous report that LPS-induced down-regulation of class Ⅰ HDAC such as HDAC1 and HDAC2 might contribute to activate the NF-κB dependent inflammatory gene expression (Ashburner et al. 2001). As well the LPS induced induction of HDAC6 of class Ⅱ is implying that HDAC6 plays a crucial role in regulation of inflammatory response, particularly at the level of the APC/T cell immune response (Serrador et al. 2004). In addition, although the HDAC9 and HDAC10 have less established roles than others in immune system, the inhibition of HDAC10 may regulate HSP-90 acetylation (Park et al. 2008).

The class Ⅰ and class Ⅱ HDACs inhibitors such as TSA and SAHA not only have been developed for cancer therapy in an intention to impair cell proliferation and to induce cell cycle arrest and apoptosis (Rosato and Grant 2004), but also have been shown to display anti-inflammatory effects, ameliorating joint inflammation and regulating the production of numerous cytokines in chronic, systemic inflammatory diseases (Toussirot et al. 2013). SFN, a HDAC inhibitor, has previously been reported to inhibit the HDAC activity in human cancer cells (Myzak et al. 2004). SFN treatment could selectively decrease HDAC activity and class Ⅰ and class Ⅱ HDAC protein level as well as increased H3 acetylation in prostate cancer cells (Clarke et al. 2011). In the present thesis of chapter 1, indeed, a dose dependent effect of SFN was showed to inhibit the global HDAC activity in porcine moDCs. Meanwhile, this study observed the effects of SFN on LPS induced HDAC family gene expression, and found that SFN altered LPS induced upregulation of class Ⅱ HDAC, as well as LPS induced DNMT1 and DNMT3a expression in porcine moDCs. Previous studies have been reporting that class II HDAC has been identified as a general mechanism to control the cytokine production (Shakespear et al. 2011). Therefore, the influences of SFN on LPS induced epigenetic encoding enzyme gene expressions demonstrated that the inhibition of

HDAC activity by SFN might be related to the immune gene expressions and protein production.

Although the regulations of SFN on the cellular differentiation, cell survival and immune gene expression have been repeatedly reported, the effects of SFN on innate immune responses via epigenetic modifications have not been well defined. In the present thesis, we demonstrated that SFN has a crucial role in porcine moDC differentiation and potential T-cell activation (phenotype and function). The inhibition of HDAC activity leads to different regulation of co-stimulatory molecules such as CD40, CD80 and CD86 on DCs surface, which are crucially important for antigen presenting and T-cell activation (Song et al. 2011). In chapter 2, in the SFN treated porcine immature moDCs, the LPS induced up-regulation of CD40, CD80 and CD86 molecules was remarkably suppressed. These results are consistent with the previous evidence which have evaluated the impact of HDAC inhibitors on cell surface molecules of human moDCs (Roger et al. 2011). Expectedly, the SFN enhanced the phagocytic activity of moDCs through the maintenance of the DC maturation. That SFN raised the phagocytosis of APCs has been evidenced in the macrophage earlier (Suganuma et al. 2011). In combination with the alteration of both cell surface molecules and phagocytic activity of moDCs, this study demonstrated that SFN may play an important role in DCs maturation as well as in activation of T-cell response.

Moreover, when exposed to pathogens, particularly the well-studied model of LPS activated TLR4 signaling pathway, DC produce a range of pro-inflammatory cytokines through the NF-κB signaling activation. SFN has been reported to affect the activation of LPS-induced TLR4 activation resulting in down-regulation of NF-κB and reduction of cytokine and chemokine production (Koo et al. 2013, Youn et al. 2010). In chapter 2, this study evaluated the effects of SFN TLR4-mediated inflammatory cytokine responses. It was found that SFN pre-treatment of moDCs directly altered the LPS-induced TLR4 and MD2 gene expression and dynamically regulated the TLR4-induced activity of transcription factors NF-κB1 and TBP in the duration of 24 h. SFN showed a protective role in LPS induced cell apoptosis through suppressing the IRF6 and TGF-ß1 production. SFN impaired the pro-inflammatory cytokine TNF-α and IL-1ß secretion into the cell culture supernatants that were induced in moDCs by LPS stimulation, whereas SFN increased the cellular-resident TNF-α accumulation. These results

revealed that through the epigenetic modifications, HDAC inhibitor SFN modulates the LPS induced innate immune responses of porcine moDCs.

Furthermore, SFN epigenetically ameliorated the DC-mediated acute inflammation response to LPS. It is well known that the moDC-mediated LPS-specific innate immune response acts, through both LPS-specific activated TLR4 and pathogen-unspecific activated MHC classic antigen-presenting pathways. In connection with this fact, I have determined SFN-induced DNA methylation of TLR4 gene in exon region and MHC-SAL gene in promoter region in porcine moDC in chapter 3. Surprisingly, I found that both LPS and SFN could induce the TLR4 and MHC-SAL DNA methylation in exon and promoter regions respectively, but SFN could cause DNA demethylation in the promoter region of MHC-SLA gene. It has been evidenced that the Gram negative bacteria Salmonella infection could induce TLR4, TLR2-1 and TLR21 DNA methylation to increase host susceptibility to destroy the pathogen and/or pathogen-infected cells in chicken leukocytes (Gou et al. 2012). The finding of this study is also consistent with another report that down-regulation of TLR4 gene expression by LPS-induced DNA methylation in intestinal epithelial cells might contribute to the maintenance of homeostasis in the intestinal commensal system (Takahashi et al. 2009).

I have further designed the experiment in chapter 3 to evaluate the impact of SFN on the LPS-induced expression of transcriptional factor and kinetics of pro-inflammatory cytokine productions including TNF-α, IL-β, IL-12p40, IL-8, and IFN-γ distribution within a 24 h time-dependent manner. This study found that SFN exerted profound dynamic administration on the moDCs innate immune antimicrobial against response in different pathogen challenging stages such as down-regulating the expression of innate immune receptor, interfering with transcription factors, remodeling and inhibiting the expression of key antimicrobial cytokines and accessory molecules in the early stage after LPS stimulation. Over 24 h LPS stimulation, SFN significantly inhibited the pro-inflammatory cytokine TNF-α, IL-β and IL-8 protein inductions. This pattern of the cytokine production was consistent with the previous finding that HDACs inhibitors impaired the pro-inflammatory cytokine secretion (Roger et al. 2011). Although, on the mRNA level, I found SFN remarkably induced TNF-α, IL-β, IL-8, and IL-12p40 gene expression after 6 h LPS exposure, the western blotting showed that the cellular resident of TNF-α was obviously increased by SFN challenge. These results indicated that

pretreatment with SFN might contribute to restrict the sepsis expansion in the pathogen infected tissue during the acute inflammatory stage.

1.5 General summary and conclusion

In the present study, we mainly described the impact of HDAC inhibitor SFN on LPS-induced inflammatory response, depended on the TLR4-mediated innate immune response, in porcine moDCs. In the porcine immune system, DC populations with possibly distinct differentiating functions are distributed in the whole body of host, and they can migrate from the blood into tissue via lymphatic vessels. In order to minimize the disturbance of physiological development of DC by the environment interfaces of body, we firstly aimed to identify an ideal culture model of DC. With this objective, I evaluated the immune responses of LPS-induced SDCs (in vivo isolated cell model) and moDCs (in vitro generated cell model) in vitro as a model. Following LPS stimulation, I found that although phagocytosis activity, TLR4/MyD88-dependent genes, costimulatory molecules, and pro-inflammatory cytokine including TNF-α, IL-1ß and IL-8 expressions were increased in both the cell subsets, moDCs showed higher levels of these genes and proteins expression compared to SDCs. Interestingly, moDCs showed higher response through the LPS-induced TLR4/TRAF-dependent signaling pathway. In addition, SDCs expressed higher level of IL-12p40 gene and protein, whereas, IFN-γ gene and protein expression were likely to be unchanged after LPS stimulation in both cell subtypes. However, these data demonstrated that porcine moDCs display a greater ability to initiate innate immune responses that could be used as an in vitro model to investigate the pathogen-specific activated immune responses.

Furthermore, we focused on the objective of the study that HDAC inhibitor SFN epigenetically regulated the LPS-activated inflammatory immune response in porcine moDCs. First, we evaluated the impact of SFN on the HDAC encoding gene and enzymes induction. We found that SFN could reverse LPS induced class Ⅱ HDAC gene expression as well as to globally inhibit HDAC activity in a SFN does dependent manner. Interestingly, although the high dose of SFN significantly induced moDCs cell death, 10 μM SFN protected moDCs from LPS-induced cell death, showing a protective role in LPS induced cell apoptosis through suppressing the IRF6 and TGF-ß1 production, kept the maintenance the moDCs in immature status, and maintained the phagocytosis. These results indicated that SFN positively regulated the moDCs immune response might be depending on the HDAC activity. In addition, SFN suppressed the

LPS-induced TLR4 and the cooperation between gene MD2 and transcription factor NF-κB1 and TBP in the early stage of LPS stimulation (within 6 h), whereas rapidly up-regulated those genes expression afterwards (within 24 h). It may demonstrate that SFN contributed to the cell survival and maturation through the dynamic regulation of pathogens challenge. Moreover, SFN impaired the pro-inflammatory cytokine TNF-α and IL-1ß secretion into the cell culture supernatants that were induced in moDCs by LPS stimulation, whereas SFN increased the cellular-resident TNF-α accumulation. The inhibition of the inflammatory cytokine secretion may contribute to restrict the septic inflammation in the local infection. Therefore, regulation of the inflammatory response by histone modifying enzymes may focus on the host-pathogen interactions and disease susceptibility.

More evidences were supplied in the chapter 3, we expanded the longer time distribution of LPS treatment and also evaluated the impact of SFN on TLR4 and MHC-SAL DNA methylation in exon and promoter regions respectively, downstream genes and transcription factors, as well as proinflammtory cytokines induction. We found that HDAC inhibitor SFN induced acetylation of H3 and H4 and firstly reported that SFN demethylated LPS induced MHC-SAL promoter methylation. Based on these data, SFN showed that it exerted profound dynamic administration on the moDCs innate immune antimicrobial response in different pathogen challenging stages, down-regulated the expression of innate immune receptor, interfered transcription factors remodeling and inhibited the expression of key antimicrobial cytokines and accessory molecules in the early stage after LPS stimulation. On the other hand, SFN promoted the immune gene including cytokine expressions after 6 h LPS stimulation. Consistent with these dynamic immune-mediatory effects in LPS induction, it may be postulated that SFN enhances the tolerance of moDCs and protects host moDCs from the inflammatory risk to bacterial infection.

Taken all together, the in vitro generated moDC provides a great physiological immature state as well as extents the efficiency to process and clear the harmful substance (LPS) via phagocytosis and the rapid pro-inflammatory induction. Therefore, the moDC could be considered as a reasonable culture model to understand the immune function of porcine DCs. In the epigenetic study, we firstly report the SFN as an HDAC inhibitor could force moDC to accumulate the inflammatory cytokine. Moreover, the

SFN induced epigenetic regulation of moDC in response to LPS was involved not only by the inhibiting of HDAC activity but also by CpG methylating in porcine moDCs. However, the main finding of this study was HDAC inhibitor SFN treatment could partly restrict spread of inflammatory infection in the host via the epigenetic regulations in porcine in vitro culture model of moDCs.

1.6 References

Abdi K, Singh NJ, Matzinger P (2012): Lipopolysaccharide-activated dendritic cells: "exhausted" or alert and waiting? J Immunol 188, 5981-5989

Akira S, Uematsu S, Takeuchi O (2006): Pathogen recognition and innate immunity. Cell 124, 783-801

Ashburner BP, Westerheide SD, Baldwin AS, Jr. (2001): The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol Cell Biol 21, 7065-7077

Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, Suzuki H, Hayashizaki Y, Hume DA, Sweet MJ, Ravasi T (2006): LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. FASEB J 20, 1315-1327

Bimczok D (2005): Characterisation of porcine intestinal dendritic cell subsets and their role in mucosal immunity using cholera toxin as a model antigen. Dissertation, University of Veterinary Medicine Hannover

Bolden JE, Peart MJ, Johnstone RW (2006): Anticancer activities of histone deacetylase inhibitors. Nat Rev Drug Discov 5, 769-784

Brightbill HD, Libraty DH, Krutzik SR, Yang RB, Belisle JT, Bleharski JR, Maitland M, Norgard MV, Plevy SE, Smale ST, Brennan PJ, Bloom BR, Godowski PJ, Modlin RL (1999): Host defense mechanisms triggered by microbial lipoproteins through toll-like receptors. Science 285, 732-736

Carpenter S, Fitzgerald KA (2014): Transcription of Inflammatory Genes: Long Noncoding RNA and Beyond. J Interferon Cytokine Res

Carpenter S, Ricci EP, Mercier BC, Moore MJ, Fitzgerald KA (2014): Post-transcriptional regulation of gene expression in innate immunity. Nat Rev Immunol 14, 361-376

Carrasco CP, Rigden RC, Schaffner R, Gerber H, Neuhaus V, Inumaru S, Takamatsu H, Bertoni G, McCullough KC, Summerfield A (2001): Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology 104, 175-184

Cheung KL, Kong AN (2010): Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. AAPS J 12, 87-97

Clarke JD, Hsu A, Yu Z, Dashwood RH, Ho E (2011): Differential effects of sulforaphane on histone deacetylases, cell cycle arrest and apoptosis in normal prostate cells versus hyperplastic and cancerous prostate cells. Mol Nutr Food Res 55, 999-1009

Crowley MT, Inaba K, Witmer-Pack MD, Gezelter S, Steinman RM (1990): Use of the fluorescence activated cell sorter to enrich dendritic cells from mouse spleen. J Immunol Methods 133, 55-66

Dashwood RH, Ho E (2008): Dietary agents as histone deacetylase inhibitors: sulforaphane and structurally related isothiocyanates. Nutr Rev 66 Suppl 1, S36-38

Dearman RJ, Cumberbatch M, Maxwell G, Basketter DA, Kimber I (2009): Toll-like receptor ligand activation of murine bone marrow-derived dendritic cells. Immunology 126, 475-484

Egan CE, Sukhumavasi W, Butcher BA, Denkers EY (2009): Functional aspects of Toll-like receptor/MyD88 signalling during protozoan infection: focus on Toxoplasma gondii. Clin Exp Immunol 156, 17-24

Facci MR, Auray G, Buchanan R, van Kessel J, Thompson DR, Mackenzie-Dyck S, Babiuk LA, Gerdts V (2006): A comparison between isolated blood dendritic cells and monocyte-derived dendritic cells in pigs. Immunology 129, 396-405

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002): Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 99, 7610-7615

Faure M, Chone F, Mettraux C, Godin JP, Bechereau F, Vuichoud J, Papet I, Breuille D, Obled C (2007): Threonine utilization for synthesis of acute phase proteins, intestinal proteins, and mucins is increased during sepsis in rats. J Nutr 137, 1802-1807

Federico M, Bagella L (2011): Histone deacetylase inhibitors in the treatment of hematological malignancies and solid tumors. J Biomed Biotechnol 2011, 475641

Gehring S, Gregory SH, Wintermeyer P, San Martin M, Aloman C, Wands JR (2008): Generation and characterization of an immunogenic dendritic cell population. J Immunol Methods 332, 18-30

Geisel J, Bruck J, Glocova I, Dengler K, Sinnberg T, Rothfuss O, Walter M, Schulze-Osthoff K, Rocken M, Ghoreschi K (2014): Sulforaphane Protects from T Cell-Mediated Autoimmune Disease by Inhibition of IL-23 and IL-12 in Dendritic Cells. J Immunol 192, 3530-3539

Ghoshal K, Datta J, Majumder S, Bai S, Kutay H, Motiwala T, Jacob ST (2005): 5-Aza-deoxycytidine induces selective degradation of DNA methyltransferase 1 by a proteasomal pathway that requires the KEN box, bromo-adjacent homology domain, and nuclear localization signal. Mol Cell Biol 25, 4727-4741

Gonzalo S, Jaco I, Fraga MF, Chen T, Li E, Esteller M, Blasco MA (2006): DNA methyltransferases control telomere length and telomere recombination in mammalian cells. Nat Cell Biol 8, 416-424

Gou Z, Liu R, Zhao G, Zheng M, Li P, Wang H, Zhu Y, Chen J, Wen J (2012): Epigenetic modification of TLRs in leukocytes is associated with increased susceptibility to Salmonella enteritidis in chickens. PLoS One 7, e33627

Grassi F, Dezutter-Dambuyant C, McIlroy D, Jacquet C, Yoneda K, Imamura S, Boumsell L, Schmitt D, Autran B, Debre P, Hosmalin A (1998): Monocyte-derived dendritic cells have a phenotype comparable to that of dermal dendritic cells and display ultrastructural granules distinct from Birbeck granules. J Leukoc Biol 64, 484-493

Gregoretti IV, Lee YM, Goodson HV (2004): Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol 338, 17-31

Halili MA, Andrews MR, Labzin LI, Schroder K, Matthias G, Cao C, Lovelace E, Reid RC, Le GT, Hume DA, Irvine KM, Matthias P, Fairlie DP, Sweet MJ (2010): Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. J Leukoc Biol 87, 1103-1114

Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B (2003): Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. Nature 424, 743-748

Hoogsteden HC, Verhoeven GT, Lambrecht BN, Prins JB (1999): Airway inflammation in asthma and chronic obstructive pulmonary disease with special emphasis on the antigen-presenting dendritic cell: influence of treatment with fluticasone propionate. Clin Exp Allergy 29 Suppl 2, 116-124

Horng T, Barton GM, Flavell RA, Medzhitov R (2002): The adaptor molecule TIRAP provides signalling specificity for Toll-like receptors. Nature 420, 329-333

Janeway CA, Jr., Medzhitov R (2002): Innate immune recognition. Annu Rev Immunol 20, 197-216

Jeong Y, Du R, Zhu X, Yin S, Wang J, Cui H, Cao W, Lowenstein CJ (2014): Histone deacetylase isoforms regulate innate immune responses by deacetylating mitogen-activated protein kinase phosphatase-1. J Leukoc Biol 95, 651-659

Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA (2010): The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. Toxicol Sci 114, 237-246

Kaisho T, Takeuchi O, Kawai T, Hoshino K, Akira S (2001): Endotoxin-induced maturation of MyD88-deficient dendritic cells. J Immunol 166, 5688-5694

Kalupahana RS, Mastroeni P, Maskell D, Blacklaws BA (2005): Activation of murine dendritic cells and macrophages induced by Salmonella enterica serovar Typhimurium. Immunology 115, 462-472

Kawai T, Akira S (2010): The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11, 373-384

Kawai T, Takeuchi O, Fujita T, Inoue J, Muhlradt PF, Sato S, Hoshino K, Akira S (2001): Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J Immunol 167, 5887-5894

Kim JC, Mullan BP, Frey B, Payne HG, Pluske JR (2012): Whole body protein deposition and plasma amino acid profiles in growing and/or finishing pigs fed increasing levels of sulfur amino acids with and without Escherichia coli lipopolysaccharide challenge. J Anim Sci 90 Suppl 4, 362-365

Koo JE, Park ZY, Kim ND, Lee JY (2013): Sulforaphane inhibits the engagement of LPS with TLR4/MD2 complex by preferential binding to Cys133 in MD2. Biochem Biophys Res Commun 434, 600-605

Lam HC, Cloonan SM, Bhashyam AR, Haspel JA, Singh A, Sathirapongsasuti JF, Cervo M, Yao H, Chung AL, Mizumura K, An CH, Shan B, Franks JM, Haley KJ, Owen CA, Tesfaigzi Y, Washko GR, Quackenbush J, Silverman EK, Rahman I, Kim HP, Mahmood A, Biswal SS, Ryter SW, Choi AM (2013): Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction. J Clin Invest 123, 5212-5230

Litvak N, Rakhshandeh A, Htoo JK, de Lange CF (2013): Immune system stimulation increases the optimal dietary methionine to methionine plus cysteine ratio in growing pigs. J Anim Sci 91, 4188-4196

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408

Mair KH, Sedlak C, Kaser T, Pasternak A, Levast B, Gerner W, Saalmuller A, Summerfield A, Gerdts V, Wilson HL, Meurens F (2014): The porcine innate immune system: an update. Dev Comp Immunol 45, 321-343

Marks PA, Breslow R (2007): Dimethyl sulfoxide to vorinostat: development of this histone deacetylase inhibitor as an anticancer drug. Nat Biotechnol 25, 84-90

Metlay JP, Witmer-Pack MD, Agger R, Crowley MT, Lawless D, Steinman RM (1990): The distinct leukocyte integrins of mouse spleen dendritic cells as identified with new hamster monoclonal antibodies. J Exp Med 171, 1753-1771

Mrowietz U, Asadullah K (2005): Dimethylfumarate for psoriasis: more than a dietary curiosity. Trends Mol Med 11, 43-48

Myzak MC, Karplus PA, Chung FL, Dashwood RH (2004): A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer Res 64, 5767-5774

Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E (2007): Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med (Maywood) 232, 227-234

Newton K, Dixit VM (2012): Signaling in innate immunity and inflammation. Cold Spring Harb Perspect Biol 4,

O'Neill DW, Bhardwaj N (2005): Differentiation of peripheral blood monocytes into dendritic cells. Curr Protoc Immunol Chapter 22, Unit 22F 24

Oshiumi H, Matsumoto M, Funami K, Akazawa T, Seya T (2003): TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. Nat Immunol 4, 161-167

Park JH, Kim SH, Choi MC, Lee J, Oh DY, Im SA, Bang YJ, Kim TY (2008): Class II histone deacetylases play pivotal roles in heat shock protein 90-mediated proteasomal degradation of vascular endothelial growth factor receptors. Biochem Biophys Res Commun 368, 318-322

Prince HM, Bishton MJ, Harrison SJ (2009): Clinical studies of histone deacetylase inhibitors. Clin Cancer Res 15, 3958-3969

Qu X, Cinar MU, Fan H, Proll M, Tesfaye D, Tholen E, Looft C, Holker M, Schellander K, Uddin MJ (2014): Comparison of the innate immune responses of

porcine monocyte-derived dendritic cells and splenic dendritic cells stimulated with LPS. Innate Immun

Rakhshandeh A, de Lange CF (2012): Evaluation of chronic immune system stimulation models in growing pigs. Animal 6, 305-310

Raymond CR, Wilkie BN (2005): Toll-like receptor, MHC II, B7 and cytokine expression by porcine monocytes and monocyte-derived dendritic cells in response to microbial pathogen-associated molecular patterns. Vet Immunol Immunopathol 107, 235-247

Roger T, Lugrin J, Le Roy D, Goy G, Mombelli M, Koessler T, Ding XC, Chanson AL, Reymond MK, Miconnet I, Schrenzel J, Francois P, Calandra T (2011): Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. Blood 117, 1205-1217

Romani N, Gruner S, Brang D, Kampgen E, Lenz A, Trockenbacher B, Konwalinka G, Fritsch PO, Steinman RM, Schuler G (1994): Proliferating dendritic cell progenitors in human blood. J Exp Med 180, 83-93

Rosato RR, Grant S (2004): Histone deacetylase inhibitors in clinical development. Expert Opin Investig Drugs 13, 21-38

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386

Sahlin S, Hed J, Rundquist I (1983): Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. J Immunol Methods 60, 115-124

Sallusto F, Cella M, Danieli C, Lanzavecchia A (1995): Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. J Exp Med 182, 389-400

Sallusto F, Lanzavecchia A (1994): Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 179, 1109-1118

Sarkar S, Abujamra AL, Loew JE, Forman LW, Perrine SP, Faller DV (2011): Histone deacetylase inhibitors reverse CpG methylation by regulating DNMT1 through ERK signaling. Anticancer Res 31, 2723-2732

Satpathy AT, Wu X, Albring JC, Murphy KM (2012): Re(de)fining the dendritic cell lineage. Nat Immunol 13, 1145-1154

Seki T, Yuasa S, Fukuda K (2012): Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus. Nat Protoc 7, 718-728

Serrador JM, Cabrero JR, Sancho D, Mittelbrunn M, Urzainqui A, Sanchez-Madrid F (2004): HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 20, 417-428

Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ (2011): Histone deacetylases as regulators of inflammation and immunity. Trends Immunol 32, 335-343

Song W, Tai YT, Tian Z, Hideshima T, Chauhan D, Nanjappa P, Exley MA, Anderson KC, Munshi NC (2011): HDAC inhibition by LBH589 affects the phenotype and function of human myeloid dendritic cells. Leukemia 25, 161-168

Steinman RM (2012): Decisions About Dendritic Cells: Past, Present, and Future. Annual Review of Immunology, Vol 30 30, 1-22

Steinman RM, Cohn ZA (1973): Identification of a novel cell type in peripheral lymphoid organs of mice. I. Morphology, quantitation, tissue distribution. J Exp Med 137, 1142-1162

Suganuma H, Fahey JW, Bryan KE, Healy ZR, Talalay P (2011): Stimulation of phagocytosis by sulforaphane. Biochem Biophys Res Commun 405, 146-151

Takahashi K, Sugi Y, Hosono A, Kaminogawa S (2009): Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J Immunol 183, 6522-6529

Toussirot E, Abbas W, Khan KA, Tissot M, Jeudy A, Baud L, Bertolini E, Wendling D, Herbein G (2013): Imbalance between HAT and HDAC activities in the PBMCs of patients with ankylosing spondylitis or rheumatoid arthritis and influence of HDAC inhibitors on TNF alpha production. PLoS One 8, e70939

Uddin MJ, Nuro-Gyina PK, Islam MA, Tesfaye D, Tholen E, Looft C, Schellander K, Cinar MU (2012): Expression dynamics of Toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. Vet Immunol Immunopathol 147, 211-222

Uenishi H, Shinkai H (2009): Porcine Toll-like receptors: the front line of pathogen monitoring and possible implications for disease resistance. Dev Comp Immunol 33, 353-361

Ueno H, Klechevsky E, Morita R, Aspord C, Cao T, Matsui T, Di Pucchio T, Connolly J, Fay JW, Pascual V, Palucka AK, Banchereau J (2007): Dendritic cell subsets in health and disease. Immunol Rev 219, 118-142

Ulevitch RJ (2004): Therapeutics targeting the innate immune system. Nat Rev Immunol 4, 512-520

van den Ancker W, van Luijn MM, Westers TM, Bontkes HJ, Ruben JM, de Gruijl TD, Ossenkoppele GJ, van de Loosdrecht AA (2010): Recent advances in antigen-loaded dendritic cell-based strategies for treatment of minimal residual disease in acute myeloid leukemia. Immunotherapy 2, 69-83

Velasquez-Lopera MM, Correa LA, Garcia LF (2008): Human spleen contains different subsets of dendritic cells and regulatory T lymphocytes. Clin Exp Immunol 154, 107-114

Xia HJ, Zhang GH, Zheng YT (2010): [Roles of dendritic cell in disease progression of AIDS primate models]. Dongwuxue Yanjiu 31, 57-65

Yamamoto M, Sato S, Hemmi H, Sanjo H, Uematsu S, Kaisho T, Hoshino K, Takeuchi O, Kobayashi M, Fujita T, Takeda K, Akira S (2002): Essential role for TIRAP in activation of the signalling cascade shared by TLR2 and TLR4. Nature 420, 324-329

Youn HS, Kim YS, Park ZY, Kim SY, Choi NY, Joung SM, Seo JA, Lim KM, Kwak MK, Hwang DH, Lee JY (2010): Sulforaphane suppresses oligomerization of TLR4 in a thiol-dependent manner. J Immunol 184, 411-419

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion e.V., Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn

Zhu H, Shan L, Schiller PW, Mai A, Peng T (2010): Histone deacetylase-3 activation promotes tumor necrosis factor-alpha (TNF-alpha) expression in cardiomyocytes during lipopolysaccharide stimulation. J Biol Chem 285, 9429-9436

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Comparison of the innate immune responses of porcine monocyte-derived dendritic cells and splenic dendritic cells stimulated with lipopolysaccharide

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Abstract

Dendritic cells (DCs) subsets form a remarkable cellular network that regulates innate and adaptive immune responses. Although pigs are the most approximate model to human, little is known about the regulation of monocyte-derived DCs (moDCs) and splenic DCs (SDCs) in the initiation of immune responses under inflammatory condition. We have investigated the activation and maturation of porcine moDCs and SDCs subpopulations following lipopolysaccharide (LPS) stimulation. Porcine monocytes were isolated which would differentiate into the moDCs. SDCs were isolated directly from the porcine spleen. Following LPS stimulation, phagocytosis activity, TLR4/MyD88-dependent genes, costimulatory molecules, and pro-inflammatory cytokine tumour necrosis factor-α (TNF-α), interleukine-1ß (IL-1ß) and chemokine IL-8 expressions were increased in both the cell subsets. Furthermore, moDCs showed higher levels of these genes and proteins expression compared to SDCs. Interestingly, moDCs were found to be more responsive through the TLR4/TRAF-dependent signaling pathway of activation. Only SDCs expressed higher level of IL-12p40 gene and protein, whereas, IFN-γ gene and protein expression were likely to be unchanged after LPS stimulation in both cell subtypes. These data demonstrate that porcine moDCs display a greater ability to initiate innate immune responses that could be used as a model to investigate the immune responses against antigens.

Introduction

Dendritic cells (DCs) are highly specialized antigen-presenting cell (APCs) and have a critical role in bridging the innate immune and adaptive immune system by recognizing antigens via complex mechanisms that involve decoding and integration of various signals received in a receptor-dependent manner (Jeras et al. 2005, Satpathy et al. 2012). According their stage of functional development, DCs could be divided in two major categories: immature and mature DCs. Immature DCs are characterized by active up-take and processing of antigens through high expression of cell surface pattern recognition receptors (PRRs) and low levels of costimulatory molecules (CD80 and CD86) (Kim et al. 2006) which provides the latter phagocytosis. Upon capture foreign antigens, immature DCs become mature and migrate to secondary lymphoid organs,

where they activate T cell and initiate adaptive immunity (Lanzavecchia and Sallusto 2000). Spleen resident DCs have been categorized into two major subsets on the basis of their phenotype: the conventional/myeloid DCs (cDCs) are characterized by CD11c+HLA-DR+CD123-, they express Toll-like receptor (TLR) 2 and TLR4; plasmacytoid DCs (pDCs) are exhibited the CD11c-HLA-DR+CD123+ in human and mice, and express TLR7 and TLR9 (Nuyts et al. 2013). In order to ignore the involvement of microenvironments in secondary lymphoid tissues that might be important in determining the function of DCs. Notably, culture of precursor monocytes with granulocyte-macrophage colony-stimulation factor (GM-CSF) and interleukin-4 (IL-4) led to the development of immature monocyte-derived DCs (moDCs) models *in vitro* (Grassi et al. 1998, O'Neill and Bhardwaj 2005). Mouse and human splenic CD11c+ immature DCs uptake of LPS or other pathogens could further differentiate into mature DCs, showed up-regulation of MHC-II, CD40, CD80, CD83, and CD86 molecules on DCs surface, which have an impact on the innate immune response (Reis e Sousa 2006). However, the relative efficacy of the different between CD11c+ SDCs and moDCs has not been compared in pig. To the best of author's knowledge, there is little evidence of moDCs generation, activation and their pattern of immune response in pigs.

Notably, the unique immune function of DCs is that the immature DCs become mature DCs by recognition of invasive pathogens through their capacity to detect pathogen-associated molecular patterns (PAMP) using PRRs (Takeuchi and Akira 2010). TLRs are the best characterized PAMPs and are linked to the control of bacterial and viral infection. TLR4 is stimulated by the PAMP lipopolysaccharide (LPS) or endotoxin from Gram-negative bacteria. LPS is one of the best studied immunostimulatory bacterial component that can induce systemic inflammation. LPS interacts with DCs via TLR4/MD2/CD14 receptor complex, induces the release of the essential pro-inflammatory cytokines and chemokines to activate a potent immune response (Chassin et al. 2006). Two signaling pathways, MyD88-dependent and MyD88-independent (TRIF-dependent) associated with TRAF6 have been described for TLR4 activation following LPS stimulation. Activation of MyD88-dependent pathway drives a rapid activation of NF-kB and produces pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), interleukin (IL-)1ß, IL-12p40, IL-6, and chemokines like

monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 3 α (MIP-3α), and IL-8 (Egan et al. 2009, Guzylack-Piriou et al. 2006). On the other hand, LPS activation of MyD88-independent signaling pathway results in the rapid activation and nuclear translocation of interferon regulatory factor 3 (IRF3), which leads to the activation of type 1 interferon (IFN) signaling pathway (Vartanian et al. 2011). It has been reported that IFN-ß of type 1 IFN plays an important role in the cell survival, protein synthesis, and control of inflammatory responses against antigens (Bauerfeld et al. 2012). However, there is no study devoted to distinguish the relevance of immune response of moDCs and SDCs in pigs with regards to the TLR signaling pathway. Therefore, it is important to compare the MyD88-dependent and -independent signaling pathway of immune responses between moDCs and SDCs.

For this purpose, we evaluate the immune responses of LPS-induced SDCs and moDCs in vitro as a model. We analyzed the impact of various concentrations of LPS on phagocytosis and cell viability response, and investigated MyD88-dependent and -independent related gene expressions in LPS stimulated porcine moDCs and SDCs in a time-dependent manner. Furthermore, the co-stimulatory molecules, pro-inflammatory cytokines and chemokine production were measured.

Materials & Methods

Animals

Three 35 days old Pietrain female piglets were used for this study. The piglets were clinical healthy and no respiratory disease was found according to the clinical history and physical examinations. Animals were housed in isolation rooms at the teaching and research station of Frankenforst, University of Bonn, Germany. The feeding, housing and husbandry practices of the animals followed the standard guidelines (ZDS 2003). The experiment of handling animals was approved by the Veterinary and Food Inspection Office, Siegburg, Germany. All the piglets were euthanatized by intra venous injection of T61 (Intervet) by registered veterinarian after sedation using Ketamine (Intervet) according to German animal protection law (ZDS 2003).

Generation of monocyte-derived dendritic cells from porcine peripheral blood monocytes

Peripheral blood mononuclear cells (PBMCs) were isolated from the peripheral blood. Blood samples were collected from the vena cava cranialis in sterilized tubes with ethylenediaminetetraacetic acid (EDTA). The PBMCs were isolated by Ficoll-Paque density gradient (cat. 10771; Sigma) centrifugation as described previously (Seki et al. 2012, Uddin et al. 2012). PBMCs were washed two times in DPBS (cat. 1419-169; Life Technologies) and resuspended in Dulbecco modified Eagle medium (DMEM) (cat. 41966-029; Invitrogen) supplemented with 2% fetal bovine serum (FBS) (cat. 10270; Invitrogen) and 500 IU/ml Penicillin-Streptomycin (cat. 15140; Invitrogen) and 0.5% fungizone (cat. 15290-026; Invitrogen). These PBMCs (5×10^6 cell/ml) were cultured in 6-well plate (2ml/well) for 4 h. The moDCs were generated following the procedure described previously (Carrasco et al. 2001, Facci et al. 2006, Raymond and Wilkie 2005). Briefly, after 4 h of incubation, non-adherent cells were removed and the adherent monocytes were washed two times with pre-warmed (37 °C) DPBS in order to purify the cells. The purified monocytes were cultured in RPMI-1640 medium (cat. 21875; Invitrogen) supplemented with 10% FBS, 1000UI/ml Penicillin-Streptomycin, 1% fungizone, 20 ng/ml recombinant porcine (rp) granulocyte-macrophage colony-stimulating-factor (GM-CSF) (cat. 711-PG-010; R&D System) and 20 ng/ml recombinant porcine (rp) interleukin-4 (IL-4) (cat. 654-P4-025; R&D Systems) for 7 days at 37 °C with 5% CO_2. Half of the medium was replaced every 3rd day with the fresh medium. After 7 days of incubation, the adherent moDCs were counted and re-cultured in a new plate for the subsequent assays.

Splenic DCs (SDCs) isolation

The spleen was collected under sterile conditions within 30 min of euthanasia from the same pigs used for blood sampling. Splenic tissues were cut into 5-7 cm length sections and flushed thoroughly with 0.9% NaCl. The tissues were transported in Hanks-EDTA (cat. H4641; Sigma) solution on ice to the laboratory for DCs isolation (Bimczok 2005). Briefly, the tissue pieces were transferred into collagenase (cat. C2139-100 MG; Sigma) solution (collagenase 1 mg/ml, BSA 1 mg/ml, DNase 10 UI/ml, Penicillin-Streptomycin 500 UI/ml and fungizone 0.25%) and incubated in a shaking incubator at 37 °C with 120 rpm. After 2-4 h of incubation, the collagenase solution containing released cells was filtered through a 70 µm cell strainer (cat. 352350, BD-Falcon) followed by a filtering through 40 µm cell strainer (cat. 352340, BD-Falcon). Cells were washed two

times in DPBS and resuspended in RPMI-1640 medium supplemented with 10% FBS, 1000 UI /ml penicillin-streptomycin and 1% fungizone. In order to purify the splenic DCs, cells were seeded (1 × 107 cells/ml) into a 6-well plate (2 ml per well) and incubated for 12-16 h. Finally, the non-adherent cells were removed by gently washing twice using warm RPMI-1640 medium and then the SDCs were cultured for the further study.

Stimulation of moDCs and SDCs

moDCs and SDCs were seeded separately at 2×10^6 cells/well into 6-well tissue culture plate and incubated over night at 37 °C in CO_2 incubator. Afterwards, both cell types were treated with LPS (cat. # tlrl-3pelps; InvivoGen) at the concentrations of 0.5 µg/ml, 1 µg/ml and 2 µg/ml for 24 h for cells viability assay. For the gene expression study and cytokine measurement, cells were stimulated with 1 µg/ml LPS and harvested at time points 0, 3, 6, 12, and 24 h of post-stimulation. Total RNA was isolated from these cells for gene expression analysis and culture supernatant was used to measure the cytokine levels. SDCs and moDCs stimulated or non-stimulated for 24 h with 1 µg/ml LPS were used for the expression of costimulatory molecules (CD40 and CD86) on these DCs.

Cell viability assay

Cell viability was investigated using the WST-1 cell proliferation kit (cat. 10008883, Cayman Chemical) following to the manufacturer's instructions. Briefly, SDCs and moDCs were cultured in a 96-well plate at a density of 104-105 cells/well in 100 µl of RPMI-1640 medium for 24-48 h in a CO_2 incubator at 37 °C before LPS stimulation. This allowed the cells to adhere on the microplate surface. Cells were cultured un-treated or treated with LPS at the concentration of 0.5 µg/ml, 1 µg/ml and 2 µg/ml for 24 h. 10 µl reconstituted WST-1 mixture was added to each well. After 2 h of incubation in a CO_2 incubator at 37 °C, the absorbance of the samples was measured using a microplate reader (Thermo max; Germany) at a wavelength of 450 nm. The cell viability was calculated (%) following the manufacturer's formula.

DCs maturation and cell type measurement using flow cytometry

For the flow cytometry (FACs) analysis, cells were harvested, washed twice with DPBS and the cell number was counted. The cells were incubated for 30 min with FACs staining buffer (DPBS supplement with 2% FBS, 10 mM NaN3 and 10 mM HEPES)

and then washed with the same staining buffer. Cells were stained either with mouse anti-human CD11c PE-Cyanine 7 Ab (clone N418, 25-0114, BD eBioscience), a mouse anti-human CD40 FITC Ab (clone G28.5, NB100-77786, Novus Biologicals) or a mouse anti-human CD86 APC Ab (clone hB7-2, FAB141A, R&D systems) antibody. All these antibodies are IgG1 isotype. Cells were stained for 30 min on ice in 100 µl of staining buffer solution in light protected condition. The cells were washed twice using staining buffer and resuspended in 400-500 µl staining buffer. The events were acquired on 10,000 cells using a FACscalibur Dual Laser Flow Cytometer (BD Biosciences; USA) and analysed by FlowLogic software (BD Biosciences; Germany).

Gene expression study using quantitative real time PCR (qRT-PCR)

mRNA expression of the genes was quantified using qRT-PCR. Total RNA was isolated using miRNeasy Mini Kit (cat. 217004, Qiagen) and the RNA concentration was measured by Nanodrop 8000 (Nanodrop 8000; Thermo Scientific; USA). Complementary DNA (cDNA) was synthesized using miScript II RT kit (cat. 218161, Qiagen) and the cDNA was stored at -20 °C for further use. qRT-PCR was performed in an ABI prism®7000 (ABI prism®7000; Applied Biosystems; Germany) qRT-PCR system. The transcript of target genes presented in each sample was determined using Maxima SYBR Green/ ROX Mix (cat. 218073, Qiagen). The primers (Table 1) were designed using the online Primer3 Program (version 0.4.0) (Rozen and Skaletsky 2000). The qRT-PCR was conducted with the following program: 95 °C for 3 min, 40 cycles at 95 °C for 15s, 60 °C for 1 min and 95 °C for 1 min in the StepOne Plus qPCR system (StepOne Plus qPCR system; Applied Biosystems; Germany). Melting curve analysis was performed to detect the specificity of the PCR reaction. Each experiment was performed in triplicates and each sample was quantified in duplicate (technical replication) using qRT-PCR. Gene-specific expression was measured as relative to the expression of the house keeping gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Table 1). The delta Ct (ΔCt) values were calculated as the difference between target gene and geometric mean of the reference genes: (ΔCt = Cttarget-Cthousekeeping genes). The average expression values were considered for further analysis.

Phagocytosis assay

Phagocytosis activity of the cells was investigated using Vybrant TM Phagocytosis Assay kit (cat. V-6694; Molecular Probes). The principle of the test is based on the intracellular florescence emitted by the engulfed particles, as well as the effective fluorescence quenching of the extracellular probe by trypan blue (Sahlin et al. 1983). The phagocytosis assay protocol follows the instruction of using five negative controls, five positive controls and four experimental samples. Briefly, cells were cultured in a 6-well plate for 48 h with RPMI-1640. Cells were scrapped from 6-well plate, washed twice with DPBS and cells density was adjusted to 10^6 cells/ml. For the phagocytosis assay, 50 µl of the different concentration of LPS (0.5 µg/ml, 1 µg/ml, 2 µg/ml) in RPMI-1640 were added to the experimental wells containing cells. The cells were incubated for 4 h at 37 °C with 5% CO_2 to allow the cells to adhere on the microplate surface. Afterwards, the RPMI-1640 solution was removed from the microplate wells. Then 100 µl of fluorescent BioParticle suspension was added to all the negative control, positive control and experimental wells. Two hours after incubation at 37 °C in CO_2 incubator, the Bioparticle were aspirated from all of the microplate wells. Finally, 100 µl trypan blue was added to the wells and incubated for 1 min at room temperature, and then trypan blue was aspirated immediately. The fluorescence emission was measured in a fluorescence microplate reader (Thermo Eectron; Waltham; MA, USA) using 480 nm excitation, 520 nm emission. The net phagocytosis of the cells was calculated according to the response of the phagocytosis effector agent following the manufacturer's instructions.

Cytokine measurements by ELISA

ELISA was used to investigate the cytokines secretion differences of moDC and SDCs at different time-points. 1×10^6 cells/ml moDCs and SDCs were stimulated with 1 µg/ml LPS for 0, 3, 6, 12, and 24 h and supernatants were collected for TNF-α (cat. PTA00; R&D Systems), IL-8 (cat. P8000; R&D Systems), IL-1ß (cat. PLB00B; R&D Systems), IL-12p40 (cat. P1240; R&D Systems) and interferon-γ (IFN-γ) (cat. PIF00; R&D Systems) measurement using ELISA according to manufacturer's instructions. The results were determined by microplate reader (ThermoMax; Germany) setting to 450 nm.

Statistical analysis

The data were analyzed by SAS software package v. 9.2 (SAS institute, Cary, NC, USA). Pairwise comparisons were made between the treatment groups and control, using Student's t test and Tukey's multiple comparisons. The data were expressed as means ± standard deviations (SD) and *(*) P* < 0.05, *(**) P* <0.01, *(***) P* <0.001 were set as statistically significant.

Results

Different LPS concentrations affect moDCs and SDCs viability

To determine the oxidative stress and cytotoxicity effects of LPS on porcine DCs, we detected cell viability with different concentrations of LPS (Fan et al. 2012). The viability of both cell types was affected by higher LPS concentration compared to lower LPS concentration (Figure 1A). The viability was significantly decreased in moDCs treated with 1.0 and 2.0 µg/ml LPS. It was found that only in the case of 2.0 µg/ml LPS treatment, the moDCs viability was decreased compared to SDCs (Figure 1B). These results indicated that moDCs were more sensitive to the LPS exposure, and on the basis of these data, 1.0 µg/ml LPS was considered for further studies.

The phagocytic activity of moDCs was higher than SDCs

The phagocytic activity might vary between cell types as well as during the DCs maturation, therefore, the phagocytosis difference between moDCs and SDCs was investigated. The phagocytosis of the moDCs was significantly increased in a concentration-dependent manner (Figure 2A). However, in the case of SDCs, phagocytosis activity was significantly increased in 1.0 and 2.0 µg/ml LPS compared to 0.5 µg/ml LPS (Figure 2A). When moDCs and SDCs were compared, irrespective of LPS concentration, the phagocytic activity of moDCs was significantly higher than in SDCs (Figure 2B).

TLR4/MD2 complex related genes were up-regulated in moDCs and SDCs

To clarify the LPS stimulated regulation of the TLR4/MD2/LPS complex, its associated gene expressions were quantified by qRT-PCR. TLR4 and MD2 mRNAs were significantly (P<0.001) up-regulated 2 h following LPS treatment in moDCs (Figure 3A, B), whereas these genes were increased at 3 h of LPS-stimulation in SDCs (Fig. 3, A, B). When moDCs and SDCs were compared, TLR4 was significantly higher at 1 h and 12 h, whereas MD2 was significantly higher at 1 h and 6 h in moDCs compared to

the SDCs, respectively. We found that CD14 mRNA expression was undetectable for 12 h of LPS-stimulation in moDCs (Figure 3C). The CT value was more than 36 cycles, this was considered as undetectable in this study. CD14 mRNA expression was significantly increased at 6 h of LPS stimulation and remained higher ($P<0.01$) in SDCs (Figure 3C). On the other hand, it was significantly increased at 12 h of LPS stimulation ($P<0.001$) and remained higher in moDCs (Figure 3C). Notably, the CD14 mRNA expression was higher in SDCs at 6 h compared to moDCs, whereas it was higher in moDCs at 24 h compared to SDCs (Figure 3C).

Comparison of NF-kB pathway activation between moDCs and SDCs

In order to further clarify the pathway followed by the DCs subsets, we determined the MyD88-dependent and -independent related gene expression in moDCs and SDCs at different time. To estimate the involvement of MyD88 dependent pathways, we quantified the MyD88 and TRAF6 gene expression. We found that both genes were up-regulated in both cell types and MyD88 was significantly increased from 3 h of LPS stimulation (Figure 3D), whereas TRAF6 was significantly increased from 6 h on SDCs. The expression of TRAF6 was increased only at 12 h on moDCs (Figure 3E). Notably, the MyD88 gene was expressed higher in moDCs compared to the SDCs over the times from 3 h of LPS treatment (Figure 3D).

SDCs responded to LPS stimulation more via TLR4/MyD88-independent pathway

Unlike the MyD88-dependent pathway, MyD88-independent pathway is activated through the IRF3 activation. We found that IRF3 mRNA was significantly increased in SDCs from 6 h of LPS treatment and remained steady to increase up to 24 h (Figure 3F). Interestingly, the IRF3 gene expression in LPS treated moDCs was not different to the untreated control (Figure 3F).

LPS induced moDCs maturation more strongly than SDCs

With the aim to study the effect of LPS on the maturation state of DCs subsets, we examined the production of co-stimulatory molecules CD86 and CD40 proteins on the porcine moDCs and SDCs. Flow cytometric analysis showed that CD86 and CD40 expression were significantly increased on moDCs following LPS stimulation compared to the SDCs (Figure 4). On the other hand, when control group cells were compared with LPS treated cells, both co-stimulatory maker expression were increased on LPS

stimulated DCs indicating that both moDCs and sDCs were responding to the LPS stimulation. CD86 production was significantly up-regulated in moDCs compared to SDCs (Figure 4C). In contrast, CD86 and CD40 molecules were more expressed in SDCs than in moDCs (Figure 4A, B, C), demonstrating that moDCs were more immature than SDCs.

Pro-inflammatory cytokines production were higher in moDCs compared to SDCs

The cytokines mRNA and protein expression was compared between porcine moDCs and SDCs following LPS stimulation. TNF-α mRNA expression was significantly increased in moDCs from 1 h to 12 h of LPS stimulation (Figure 5A). Additionally, TNF-α protein production was remarkably higher in moDCs from 6 h to 24 h following LPS stimulation compared to the SDCs (Figure 5B). In contrast, IL-1ß mRNA expression and protein secretion were significantly higher in SDCs at 1 h and 3 h, whereas both mRNA and protein expressions were remarkably up-regulated in moDCs at 12 h and 24 h (Figure 5C, D) of LPS stimulation.

Chemokine secretion differences between moDCs and SDCs

Our data showed that IL-8 mRNA expression and protein secretion were significantly up-regulated in SDCs compared to moDCs at 1h after LPS stimulation (Figure 5E, F). Following a longer time of LPS stimulation, contrarily, IL-8 mRNA and protein expression was significantly up-regulated in moDCs compared to SDC at 6 h and 12 h (Figure 5E, F). Importantly, IL-8 protein production was remarkably higher in moDC at 3 h and this remained higher till 24 h (Figure 5F) of LPS stimulation compared to the SDCs.

SDCs produce more IL12-p40 and IFN-γ than moDCs

We determined the auto-inflammatory cytokines IL-12p40 and IFN-γ mRNA and protein expression in this study. IL-12p40 mRNA expression was significantly upregulated in SDCs at 6, 12, and 24 h of LPS stimulation compared to the moDCs (Figure 6A). Likewise, the protein secretion was also remarkably higher in SDCs at 3, 6, 12, and 24 h of LPS stimulation compared to the moDCs (Figure 6B). The IL-12p40 mRNA and protein expression remained steady over the times following LPS stimulation (Figure 6A, B). The IFN-γ mRNA expression was significantly higher in SDCs at 1 h compared to moDC, whereas it was significantly higher in moDCs at 3 h

and 24 h compared to SDCs (Figure 6C). In case of protein expression, IFN-γ production was higher at 6 h in moDCs compared to SDCS (Figure 6D).

An enhancement production of pro-inflammatory cytokines by moDCs

In this study, we determined the pro-inflammatory cytokine TNF-α, IL-1ß, IFN-γ, IL-12p40 and chemokine IL-8 production in response to the LPS stimulation at various time points. Here, we analyzed the differences of the protein production by the moDCs and SDCs at different times after LPS stimulation. In order to understand the overview of the cytokines activity over the treatment times in porcine moDCs and SDCs following LPS stimulation, we used the least square mean multiple comparison test (Moskvina and Schmidt 2008). For this analysis all the protein expression of cytokines (Figure 5, 6) were used. We found that TNF-α, IL-1ß, and chemokine IL-8 secretions were significantly higher in moDCs than SDCs (Figure 7). IFN-γ was found to be expressed remarkably higher in moDCs compared to SDCs (Figure 7). Unexpectedly, IL-12p40 was remarkably increased in SDC (Figure 7), although it was not influenced by LPS stimulation in moDCs (Figure 6B).

Discussion

moDCs are more susceptible to LPS and exhibit more mature phenotype than SDCs

Phagocytosis is reported as an important component in the immune system to contribute to the first line of defence (Brown and Neher 2012). Phagocytosis involves the recognition and binding of prey receptors through the PAMPs on the phagocytes surface, in particular to the TLRs and phagocytic receptors. Foreign microbial such as bacteria (LPS) or fungi can be recognized and uptaken from the infection sites by professional phagocytes such as macrophages and DCs. DCs are the key producer of phagocytic receptor and present antigens as major APCs (Bourgeois et al. 2011). DCs represent quite different functional outcomes of phagocytic events according to different class of DCs. In this study, porcine moDCs and SDCs were exposed to various concentrations of LPS. We found that moDCs show more potential phagocytic activity by slight increasing of LPS concentration compare to SDCs. It indicates that phagocytosis is enhanced on in vitro culture model of moDCs. Interaction with bacteria leads to the apoptotic death of phagocytes during the bacterial phagocytosis and degradation stages. Indeed, the moDCs viability display a significantly decrease with

increasing the concentration of LPS. On the basis of phagocytosis and cell viability stimulation of moDCs and SDCs stimulated with various LPS concentrations, we assumed that 1.0 µg/ml was suitable for our further study. It is well known that immature and mature DCs have the distinct morphology, phenotype and function. Importantly, DCs maturation is displayed by the up-regulation of CD40 and CD80/86 molecules in antigenic stimulation (Sansom et al. 2003). CD28 is the most important costimulatory molecule on the naïve T cell surface to clonally expand and acquire effector functions such as controlling of T cell cycle, survival and differentiation (Orabona et al. 2004). It is well-described that CD80/86 through CD28 increases pro-inflammatory cytokine production in T cells (Orabona et al. 2004). CD86 was found to be up-regulated in DCs cell surface suggesting that it interacted with CD28, thereby promoted T cell activation and maturation. In addition, the expression of CD40 on DCs is important for T cell priming and T cell-mediated effector function (Martin-Fontecha et al. 1999). LPS-stimulated human or mice moDCs display a strong expression of maturation marker CD40 and CD86 (Laborde et al. 2007, O'Sullivan et al. 2011) which is in agreement to this study. This study identified that the co-stimulatory molecules were up-regulated on both moDCs and SDCs, whereas, moDCs were induced to be more mature than SDCs following LPS stimulation. Up-regulation of the co-stimulatory molecules in response to LPS stimulation indicated that moDCs display a more mature phenotype and T cell activation compared to SDCs. Immature DCs exhibited a higher phagocytic activity than mature DCs and perform specialized tasks in antigen presentation (Nagl et al. 2002) which is in good agreement to our present study. However, in human and mice it has been reported that immature DCs are immunologically more active with different cell functions than the peripheral circulating waves of tissue-resident DCs (Naik et al. 2007). The differences between the studies could be a result of different DCs cell subsets (moDCs, SDCs, or cord blood DCs).

Comparison of TLR4 signaling pathway activation between moDCs and SDCs

DCs have high phagocytic activity and remove the free microorganisms in the host innate immune system through PRRs, specially TLRs, that bind to PAMPs (Takeuchi and Akira 2010). Importantly, one of the essential roles of DCs, like other host immune cells, is the initiation of the pro-inflammatory response by the regulation of TLRs

activation and cytokines/chemokines secretion (Park et al. 2009). Mounting evidences reveal that LPS-TLR4 interaction is important in clearance of bacteria by up-regulating the phagocytic activity of DCs via these two distinct pathways (Wieland et al. 2005). We have found that LPS up-regulates TLR4 steady-state transcripts in porcine DCs which is in very good agreement with other studies in monocytes, macrophages and PBMCs (Gao et al. 2010b, Islam et al. 2013, Raymond and Wilkie 2005). LPS has been reported to trigger mitogen-activated protein kinases (MAPK), NF-kB, STAT3, IRFs, and activator protein (AP-1) signal-transduction pathway by the activation of TLR4 receptor complex (TLR4/MD2), which evokes the transcription and production of immune genes, including cytokines that are critical for the activation of innate immunity (Auray et al. 2013, Denlinger et al. 1998, Fitzgerald et al. 2003, Prele et al. 2007). In this study, we found that increasing TLR4 and MD2 expression was detected in both types of DCs with higher phagocytic activity, it coincides with the previous reports45. Similar to the other studies in mouse and human, we demonstrated that the TLR4/MD2 complex genes were up-regulated in porcine moDCs and SDCs in a time-dependent manner in response to LPS stimulation (Fitzgerald et al. 2003). TLR4 and MD2 combine into a LPS receptor molecule complex effect at both transcriptional and translational levels. Therefore, the differences of these two genes expression indicated that both DCs subtypes could respond to the pathogens at different inflammatory phases. Additionally, we observed that moDCs have a more sensitive TLR4 receptor complex to recognize LPS following stimulation, and also more efficient in the induction of T cell differentiation and development. One of the most important characteristics of TLR4/MD2 complex activation is the up-regulation of pro-inflammatory cytokines that activate the naïve T cell (Wesa and Galy 2002).

CD14 is the key cell surface protein that is required for the proper functioning of monocytes and DCs (Gao et al. 2010a). Unlike in SDCs, CD14 mRNA was undetectable in moDCs at the early stage of LPS stimulation. This finding is supported by a previous study reporting that during the differentiation of monocytes to moDCs, the cell surface expression of CD14 was silenced in vitro (Bullwinkel et al.). CD14 protein polymorphisms determine the susceptibility of individuals to the microbial infection (Gao et al. 2010a). Additionally, it has been reported that LPS can induce TLR4 pathway independently of CD14, allowing to activate the anti-inflammatory

response (Court et al. 2011). Therefore, our findings postulate that moDCs are more potential immune cells and its activation is flexible to different pathways. It's worthwhile to mention that after LPS stimulation, moDCs displayed a considerably strong TLR4/MyD88-dependent pathway activation, but less likely to induce the TLR4/TRIF-dependent pathway. Application of LPS increased the moDCs potentiality to bacterial infection and showed higher immune responses with remarkably increasing production of the anti-inflammatory cytokines. Moreover, we have also observed that LPS not only induced moDCs maturation more efficiently but also enhanced phagocytosis and migration.

moDCs are more efficient than SDCs in producing inflammatory cytokines and activating NF-kB signaling pathway

Following LPS-induced TLR4 activation, MyD88-denpendent and -independent (or TRIF-dependent) pathways are involved for the further modulation of anti-inflammatory responses (Wieland et al. 2005). Activation of MyD88 coupled to TLR4, activates TRAF6 and then rapidly drives into NF-κB activation resulting in the production of pro-inflammatory cytokines and chemokines such as TNF-α, IL-1β, and IL-8 (Goriely et al. 2006, Kawai et al. 1999). This study identified that LPS after binding to TLR4/MD2 complex, induced pro-inflammatory cytokines expression both at mRNAs and proteins levels in moDC and SDCs, and these responses were more pronounced in moDCs compared to the SDCs. It has been reported that LPS-induced DCs maturation is associated with the up-regulation of co-stimulatory molecules such as MHC Π and CD86 which is dependent on NF-kB activation (Peng et al. 2012). Indeed, delaying LPS addition to moDCs and SDCs abrogated MyD88-mediated up-regulation of LPS-induced TNF-α, IL-1ß, and chemokine IL-8 secretion. Activation of LPS-induced TNF-α, IL-1ß, and IL-8 mRNA expression by LPS requires protein synthesis, which was found in our results and specifically these expression were more rapidly induced in moDCs compared to SDCs. A vast amount of the data indicate that TNF-α induces the immature DCs maturation, and moreover, TNF-α induces transcellular migration of DCs which depends on the IL-8 production (Schlickeiser et al. 2011, Smart and Casale 1994). The migratory DCs activate the T-cells and subsequently initiate the adaptive immune response against the invading bacteria and viruses (Kissner et al. 2011). On the other hand, recruitment of TRIF associated with TLR4 results in the

activation and nuclear translocation of IRF3, an essential transcription factor generally involved in inducing the type I interferon and pro-inflammatory cytokines, such as IL-12, IL-18 and IL-23 in the wild type DCs (Vartanian et al. 2011). Additionally, IRF3 is necessary for the early phase of phagocytosis (Patel et al. 2012). This study found that LPS-induced TLR4/TRIF-dependent signaling pathway activation constitutively expressed IRF3 in SDCs which coincides with other study (Goriely et al. 2006). We found that IRF3 was uninfluenced in moDCs following the LPS stimulation. This finding indicated that TLR4/TRIF-dependent signaling pathway might be not involved in the LPS-induced activation of moDCs.

Beside the positive feature of the moDCs, we also observed some of unexpected findings that moDCs did not satisfactorily release IL-12p40 and IFN-γ following 24 h of LPS stimulation. IL-12p40 displays a critical role in Th17-cell-mediated chronic inflammation by promoting Th17 cell maintenance and by the production of pro-inflammatory cytokines (Guan et al.). It also plays an important role in DCs for IFN-γ production. Expression of IL-12p40 is required for the generation of CD4 T cells and DCs migration, which in turn regulates the level of IFN-γ production after microbial infection (Liu et al. 2012). The IFN-β subunit is involved in the signal transduction and interrupt the IL-12 receptor binding sites that leads to the insufficient IFN-γ production (Cooper and Khader 2007). Indeed, IL-12p40 was strongly released in SDCs over different times following LPS stimulation, and that might affect the IFN-γ secretion. Nevertheless, this increased production of IL-12p40 in SDCs could not overcome the more potential immune activation of moDCs.

Conclusions

Taken together, we found that LPS induced the activation, maturation and phagocytosis of the porcine DCs, and the level of activation of moDCs was greater than that observed in SDCs. The moDCs were also more efficient than SDCs in releasing the pro-inflammatory cytokines after LPS stimulation. Higher levels of cell proliferation, differentiation, and IL-1ß production were observed in moDCs compared to SDCs. The pro-inflammatory cytokines were produced more in moDCs that might activate NF-kB signaling pathway and T cells maturation more efficiently compared to SDCs. The interesting finding in this study is that LPS more strongly activated the TLR4/MyD88-

dependent pathway in moDCs. These results indicate that the generation of porcine moDCs in vitro developed an appropriate DCs culture model allowing the study of DCs roles in the host defense system.

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Reference

Auray G, Facci MR, van Kessel J, Buchanan R, Babiuk LA, Gerdts V (2013): Porcine neonatal blood dendritic cells, but not monocytes, are more responsive to TLRs stimulation than their adult counterparts. PLoS One 8, e59629

Bauerfeld CP, Rastogi R, Pirockinaite G, Lee I, Huttemann M, Monks B, Birnbaum MJ, Franchi L, Nunez G, Samavati L (2012): TLR4-mediated AKT activation is MyD88/TRIF dependent and critical for induction of oxidative phosphorylation and mitochondrial transcription factor A in murine macrophages. J Immunol 188, 2847-2857

Bimczok D (2005): Characterisation of porcine intestinal dendritic cell subsets and their role in mucosal immunity using cholera toxin as a model antigen. Dissertation, University of Veterinary Medicine Hannover

Bourgeois C, Majer O, Frohner IE, Lesiak-Markowicz I, Hildering KS, Glaser W, Stockinger S, Decker T, Akira S, Muller M, Kuchler K (2011): Conventional dendritic cells mount a type I IFN response against Candida spp. requiring novel phagosomal TLR7-mediated IFN-beta signaling. J Immunol 186, 3104-3112

Brown GC, Neher JJ (2012): Eaten alive! Cell death by primary phagocytosis: 'phagoptosis'. Trends Biochem Sci 37, 325-332

Bullwinkel J, Ludemann A, Debarry J, Singh PB Epigenotype switching at the CD14 and CD209 genes during differentiation of human monocytes to dendritic cells. Epigenetics 6, 45-51

Carrasco CP, Rigden RC, Schaffner R, Gerber H, Neuhaus V, Inumaru S, Takamatsu H, Bertoni G, McCullough KC, Summerfield A (2001): Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology 104, 175-184

Chassin C, Goujon JM, Darche S, du Merle L, Bens M, Cluzeaud F, Werts C, Ogier-Denis E, Le Bouguenec C, Buzoni-Gatel D, Vandewalle A (2006): Renal collecting duct epithelial cells react to pyelonephritis-associated Escherichia coli by activating distinct TLR4-dependent and -independent inflammatory pathways. J Immunol 177, 4773-4784

Cooper AM, Khader SA (2007): IL-12p40: an inherently agonistic cytokine. Trends Immunol 28, 33-38

Court N, Rose S, Bourigault ML, Front S, Martin OR, Dowling JK, Kenny EF, O'Neill L, Erard F, Quesniaux VF (2011): Mycobacterial PIMs inhibit host inflammatory responses through CD14-dependent and CD14-independent mechanisms. PLoS One 6, e24631

Denlinger LC, Garis KA, Sommer JA, Guadarrama AG, Proctor RA, Bertics PJ (1998): Nuclear translocation of NF-kappaB in lipopolysaccharide-treated macrophages fails to correspond to endotoxicity: evidence suggesting a requirement for a gamma interferon-like signal. Infect Immun 66, 1638-1647

Egan CE, Sukhumavasi W, Butcher BA, Denkers EY (2009): Functional aspects of Toll-like receptor/MyD88 signalling during protozoan infection: focus on Toxoplasma gondii. Clin Exp Immunol 156, 17-24

Facci MR, Auray G, Buchanan R, van Kessel J, Thompson DR, Mackenzie-Dyck S, Babiuk LA, Gerdts V (2006): A comparison between isolated blood dendritic cells and monocyte-derived dendritic cells in pigs. Immunology 129, 396-405

Fan H, Zhang R, Tesfaye D, Tholen E, Looft C, Holker M, Schellander K, Cinar MU (2012): Sulforaphane causes a major epigenetic repression of myostatin in porcine satellite cells. Epigenetics 7, 1379-1390

Fitzgerald KA, Rowe DC, Barnes BJ, Caffrey DR, Visintin A, Latz E, Monks B, Pitha PM, Golenbock DT (2003): LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. J Exp Med 198, 1043-1055

Gao P, Grigoryev DN, Rafaels NM, Mu D, Wright JM, Cheadle C, Togias A, Beaty TH, Mathias RA, Schroeder JT, Barnes KC (2010a): CD14, a key candidate gene associated with a specific immune response to cockroach. Clin Exp Allergy 40, 1353-1364

Gao Y, Flori L, Lecardonnel J, Esquerre D, Hu ZL, Teillaud A, Lemonnier G, Lefevre F, Oswald IP, Rogel-Gaillard C (2010b): Transcriptome analysis of porcine PBMCs after in vitro stimulation by LPS or PMA/ionomycin using an expression array targeting the pig immune response. BMC Genomics 11, 292

Goriely S, Molle C, Nguyen M, Albarani V, Haddou NO, Lin R, De Wit D, Flamand V, Willems F, Goldman M (2006): Interferon regulatory factor 3 is involved in Toll-like receptor 4 (TLR4)- and TLR3-induced IL-12p35 gene activation. Blood 107, 1078-1084

Grassi F, Dezutter-Dambuyant C, McIlroy D, Jacquet C, Yoneda K, Imamura S, Boumsell L, Schmitt D, Autran B, Debre P, Hosmalin A (1998): Monocyte-derived dendritic cells have a phenotype comparable to that of dermal dendritic cells and display ultrastructural granules distinct from Birbeck granules. J Leukoc Biol 64, 484-493

Guan Q, Ma Y, Hillman CL, Qing G, Ma AG, Weiss CR, Zhou G, Bai A, Warrington RJ, Bernstein CN, Peng Z Targeting IL-12/IL-23 by employing a p40 peptide-based vaccine ameliorates TNBS-induced acute and chronic murine colitis. Mol Med 17, 646-656

Guzylack-Piriou L, Piersma S, McCullough K, Summerfield A (2006): Role of natural interferon-producing cells and T lymphocytes in porcine monocyte-derived dendritic cell maturation. Immunology 118, 78-87

Islam MA, Proll M, Holker M, Tholen E, Tesfaye D, Looft C, Schellander K, Cinar MU (2013): Alveolar macrophage phagocytic activity is enhanced with LPS priming, and combined stimulation of LPS and lipoteichoic acid synergistically induce pro-inflammatory cytokines in pigs. Innate Immun 19, 631-643

Jeras M, Bergant M, Repnik U (2005): In vitro preparation and functional assessment of human monocyte-derived dendritic cells-potential antigen-specific modulators of in vivo immune responses. Transpl Immunol 14, 231-244

Kawai T, Adachi O, Ogawa T, Takeda K, Akira S (1999): Unresponsiveness of MyD88-deficient mice to endotoxin. Immunity 11, 115-122

Kim R, Emi M, Tanabe K (2006): Functional roles of immature dendritic cells in impaired immunity of solid tumour and their targeted strategies for provoking tumour immunity. Clin Exp Immunol 146, 189-196

Kissner TL, Ruthel G, Alam S, Ulrich RG, Fernandez S, Saikh KU (2011): Activation of MyD88 signaling upon staphylococcal enterotoxin binding to MHC class II molecules. PLoS One 6, e15985

Laborde EA, Vanzulli S, Beigier-Bompadre M, Isturiz MA, Ruggiero RA, Fourcade MG, Catalan Pellet AC, Sozzani S, Vulcano M (2007): Immune complexes inhibit differentiation, maturation, and function of human monocyte-derived dendritic cells. J Immunol 179, 673-681

Lanzavecchia A, Sallusto F (2000): Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science 290, 92-97

Liu H, Irwanto A, Tian H, Fu X, Yu Y, Yu G, Low H, Chu T, Li Y, Shi B, Chen M, Sun Y, Yuan C, Lu N, You J, Bao F, Li J, Liu J, Liu D, Yu X, Zhang L, Yang Q, Wang N, Niu G, Ma S, Zhou Y, Wang C, Chen S, Zhang X, Zhang F (2012): Identification of IL18RAP/IL18R1 and IL12B as leprosy risk genes demonstrates shared pathogenesis between inflammation and infectious diseases. Am J Hum Genet 91, 935-941

Martin-Fontecha A, Assarsson E, Carbone E, Karre K, Ljunggren HG (1999): Triggering of murine NK cells by CD40 and CD86 (B7-2). J Immunol 162, 5910-5916

Moskvina V, Schmidt KM (2008): On multiple-testing correction in genome-wide association studies. Genet Epidemiol 32, 567-573

Nagl M, Kacani L, Mullauer B, Lemberger EM, Stoiber H, Sprinzl GM, Schennach H, Dierich MP (2002): Phagocytosis and killing of bacteria by professional phagocytes and dendritic cells. Clin Diagn Lab Immunol 9, 1165-1168

Naik SH, Sathe P, Park HY, Metcalf D, Proietto AI, Dakic A, Carotta S, O'Keeffe M, Bahlo M, Papenfuss A, Kwak JY, Wu L, Shortman K (2007): Development of plasmacytoid and conventional dendritic cell subtypes from single precursor cells derived in vitro and in vivo. Nat Immunol 8, 1217-1226

Nuyts AH, Lee WP, Bashir-Dar R, Berneman ZN, Cools N (2013): Dendritic cells in multiple sclerosis: key players in the immunopathogenesis, key players for new cellular immunotherapies? Mult Scler 19, 995-1002

O'Neill DW, Bhardwaj N (2005): Differentiation of peripheral blood monocytes into dendritic cells. Curr Protoc Immunol Chapter 22, Unit 22F 24

O'Sullivan BJ, Pai S, Street S, An X, MacDonald KP, Wong M, Strutton G, Gerondakis S, Steptoe RJ, de St Groth BF, Hill GR, Thomas R (2011): Immunotherapy with

costimulatory dendritic cells to control autoimmune inflammation. J Immunol, Oct 15, 4018-4030

Orabona C, Grohmann U, Belladonna ML, Fallarino F, Vacca C, Bianchi R, Bozza S, Volpi C, Salomon BL, Fioretti MC, Romani L, Puccetti P (2004): CD28 induces immunostimulatory signals in dendritic cells via CD80 and CD86. Nat Immunol 5, 1134-1142

Park BS, Song DH, Kim HM, Choi BS, Lee H, Lee JO (2009): The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. Nature 458, 1191-1195

Patel AA, Lee-Lewis H, Hughes-Hanks J, Lewis CA, Anderson DM (2012): Opposing roles for interferon regulatory factor-3 (IRF-3) and type I interferon signaling during plague. PLoS Pathog 8, e1002817

Peng H, Guerau-de-Arellano M, Mehta VB, Yang Y, Huss DJ, Papenfuss TL, Lovett-Racke AE, Racke MK (2012): Dimethyl fumarate inhibits dendritic cell maturation via nuclear factor kappaB (NF-kappaB) and extracellular signal-regulated kinase 1 and 2 (ERK1/2) and mitogen stress-activated kinase 1 (MSK1) signaling. J Biol Chem 287, 28017-28026

Prele CM, Keith-Magee AL, Murcha M, Hart PH (2007): Activated signal transducer and activator of transcription-3 (STAT3) is a poor regulator of tumour necrosis factor-alpha production by human monocytes. Clin Exp Immunol 147, 564-572

Raymond CR, Wilkie BN (2005): Toll-like receptor, MHC II, B7 and cytokine expression by porcine monocytes and monocyte-derived dendritic cells in response to microbial pathogen-associated molecular patterns. Vet Immunol Immunopathol 107, 235-247

Reis e Sousa C (2006): Dendritic cells in a mature age. Nat Rev Immunol 6, 476-483

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386

Sahlin S, Hed J, Rundquist I (1983): Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. J Immunol Methods 60, 115-124

Sansom DM, Manzotti CN, Zheng Y (2003): What's the difference between CD80 and CD86? Trends Immunol 24, 314-319

Satpathy AT, Wu X, Albring JC, Murphy KM (2012): Re(de)fining the dendritic cell lineage. Nat Immunol 13, 1145-1154

Schlickeiser S, Stanojlovic S, Appelt C, Vogt K, Vogel S, Haase S, Ritter T, Volk HD, Pleyer U, Sawitzki B (2011): Control of TNF-induced dendritic cell maturation by hybrid-type N-glycans. J Immunol 186, 5201-5211

Seki T, Yuasa S, Fukuda K (2012): Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus. Nat Protoc 7, 718-728

Smart SJ, Casale TB (1994): TNF-alpha-induced transendothelial neutrophil migration is IL-8 dependent. Am J Physiol 266, L238-245

Takeuchi O, Akira S (2010): Pattern recognition receptors and inflammation. Cell 140, 805-820

Uddin MJ, Nuro-Gyina PK, Islam MA, Tesfaye D, Tholen E, Looft C, Schellander K, Cinar MU (2012): Expression dynamics of Toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. Vet Immunol Immunopathol 147, 211-222

Vartanian KB, Stevens SL, Marsh BJ, Williams-Karnesky R, Lessov NS, Stenzel-Poore MP (2011): LPS preconditioning redirects TLR signaling following stroke: TRIF-IRF3 plays a seminal role in mediating tolerance to ischemic injury. J Neuroinflammation 8, 140

Wesa A, Galy A (2002): Increased production of pro-inflammatory cytokines and enhanced T cell responses after activation of human dendritic cells with IL-1 and CD40 ligand. BMC Immunol 3, 14

Wieland CW, Florquin S, Maris NA, Hoebe K, Beutler B, Takeda K, Akira S, van der Poll T (2005): The MyD88-dependent, but not the MyD88-independent, pathway of TLR4 signaling is important in clearing nontypeable haemophilus influenzae from the mouse lung. J Immunol 175, 6042-6049

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion e.V., Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn

Table 1. List of primer sequences used in this study

Gene	Primer set	Anneal temperature (°C)	Amplicon size (bp)	GenBank accession number
TLR4	F:ATCATCCAGGAAGGTTTCCAC R:TGTCCTCCCACTCCAGGTAG	58	235	NM_001097444.1
MD2	F:TGCAATTCCTCTGATGCAAG R:CCACCATATTCTCGGCAAAT	60	226	NM_001104956.1
CD14	F:CTGCCTAGTGCCAAGGATTC R:CAGACTTTGGGGGTTTGTGT	60	177	NM_001097445.2
MyD88	F:CCAGTTTGTGCAGGAGATGA R:TCACATTCCTTGCTTTCGAG	60	185	NM_001099923.1
TRAF6	F:GGGAACGATACGCCTTACAA R:CTCTGTCTTAGGGCGTCCAG	60	174	NM_001105286.1
IRF3	F:CCAGTGGTGCCTACACTCCT R:AGAGGTGTCTGGCTCAGGAA	60	191	NM_213770.1
TNF-α	F:CCACCAACGTTTTCCTCACT R:CCAAAATAGACCTGCCCAGA	60	247	NM_214022.1
IL-1ß	F:GTACATGGTTGCTGCCTGAA R:CTAGTGTGCCATGGTTTCCA	59	137	NM_001005149.1
IL-8	F:TAGGACCAGAGCCAGGAAGA R:CAGTGGGGTCCACTCTCAAT	60	174	NM_213997.1
IL-12p40	F:TCTCGACACGTGGAGATCAG R:TTCACTCCAGGAGGAGCTGT	60	219	NM_214013.1
IFN-γ	F:AGCTCCCAGAAACTGAACGA R:AGGGTTCAAAGCATGAATGG	60	225	NM_213948.1
HPRT1	F:AACCTTGCTTTCCTTGGTCA R:TCAAGGGCATAGCCTACCAC	60	150	NM_001032376.2

F: Forward primer; R: Reverse primer; bp: base pair.

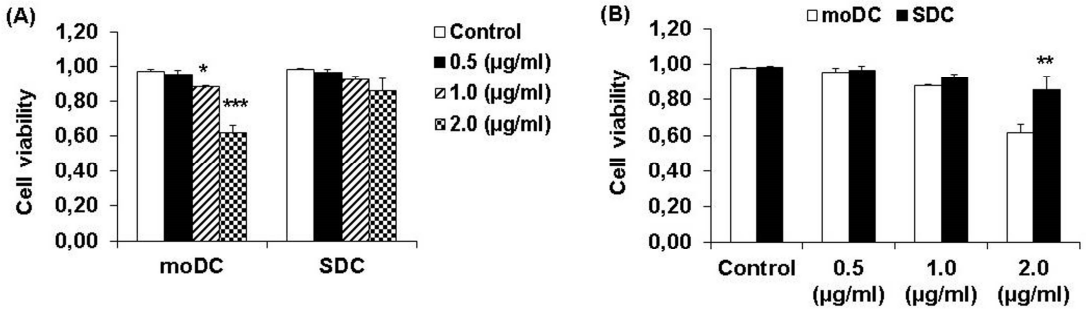

Figure 1. Effects of LPS stimulation on cell viability of moDCs and SDCs. moDCs at day 7 and SDC at day 3 were treated with or without LPS (Control, 0.5, 1.0, and 2.0 µg/ml). Comparison of moDCs or SDCs cell viability after challenge with different LPS concentrations (A) and cell viability between moDCs and SDCs after challenge with LPS (B). The results were combined from three independent experiments and each experiment was performed in triplicate. The data were represented as the mean ± standard deviation (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

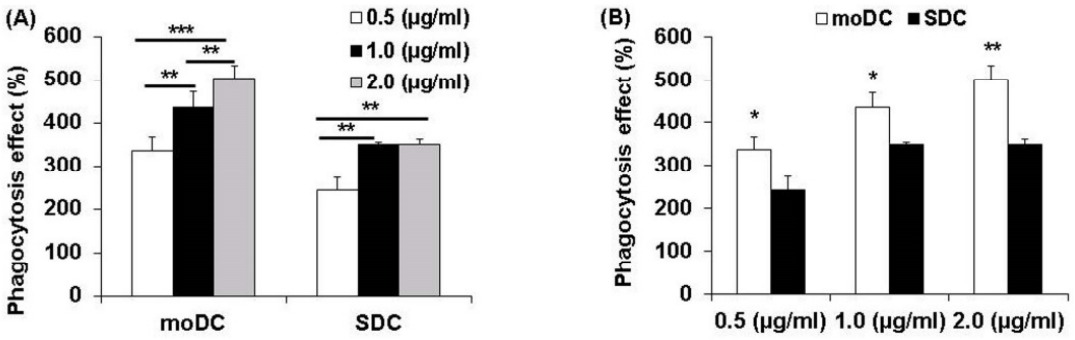

Figure 2. Comparison of phagocytic activity between moDCs and SDCs. moDCs have stronger phagocytic activity than SDCs stimulated by LPS. moDCs at day 7 and SDC at day 3 were scrapped for phagocytosis assay. moDCs and SDCs were incubated with flouorescein-labeled *Escherichia coli* K-12 BioParticles after challenged with LPS (Control, 0.5, 1.0, and 2.0 μg/ml) for 4 h. Phagocytosis of moDCs and SDCs stimulated with different LPS conditions (A) and comparison of moDCs and SDCs phagocytosis in the same LPS condition (B). The results were combined from three independent experiments and each experiment was performed in four replications. The data were represented as the mean ± standard deviation (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

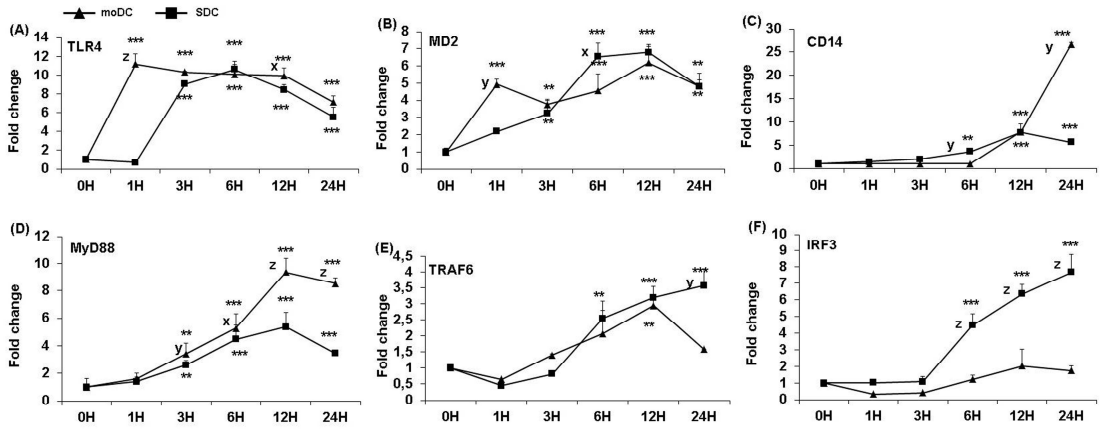

Figure 3. The effect of LPS stimulation on TLR4/MD2/CD14 complex and down-stream genes mRNA expression. The effect of LPS stimulation on TLR4/MD2/CD14 complex and down-stream genes mRNA expression in moDC and SDCs stimulated with LPS (1.0 μl/ml) for the indicated times (0, 1, 3, 6, 12, 24 h). moDCs at day 7 and SDC at day 3 were treated with or without LPS. The TLR4, MD2, CD14, MyD88, TRAF, IRF3 corresponding mRNA expressions were quantified by real-time qRT-PCR after treatments. (A), (B), and (C) show the TLR4/MD2/CD14 complex; (D) and (E) show TLR4/MyD88-dependent and (F) show MyD88-independent pathway related genes expression between moDCs and SDCs subtypes according to the cell-type-dependent and time-dependent manner. The results were combined from three independent experiments and each experiment was performed in triplicate. The data were represented as the mean ± standard deviation (SD). The letter *x (P<0.05), y (P<0.01), and z (P<0.001)* indicate the significant differences of a gene expression between moDCs and SDCs at a time point. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate the time-dependent TLR4/MD2/CD14 complex and down-stream mRNAs expression between LPS stimulation group and LPS control group in moDCs and SDCs.

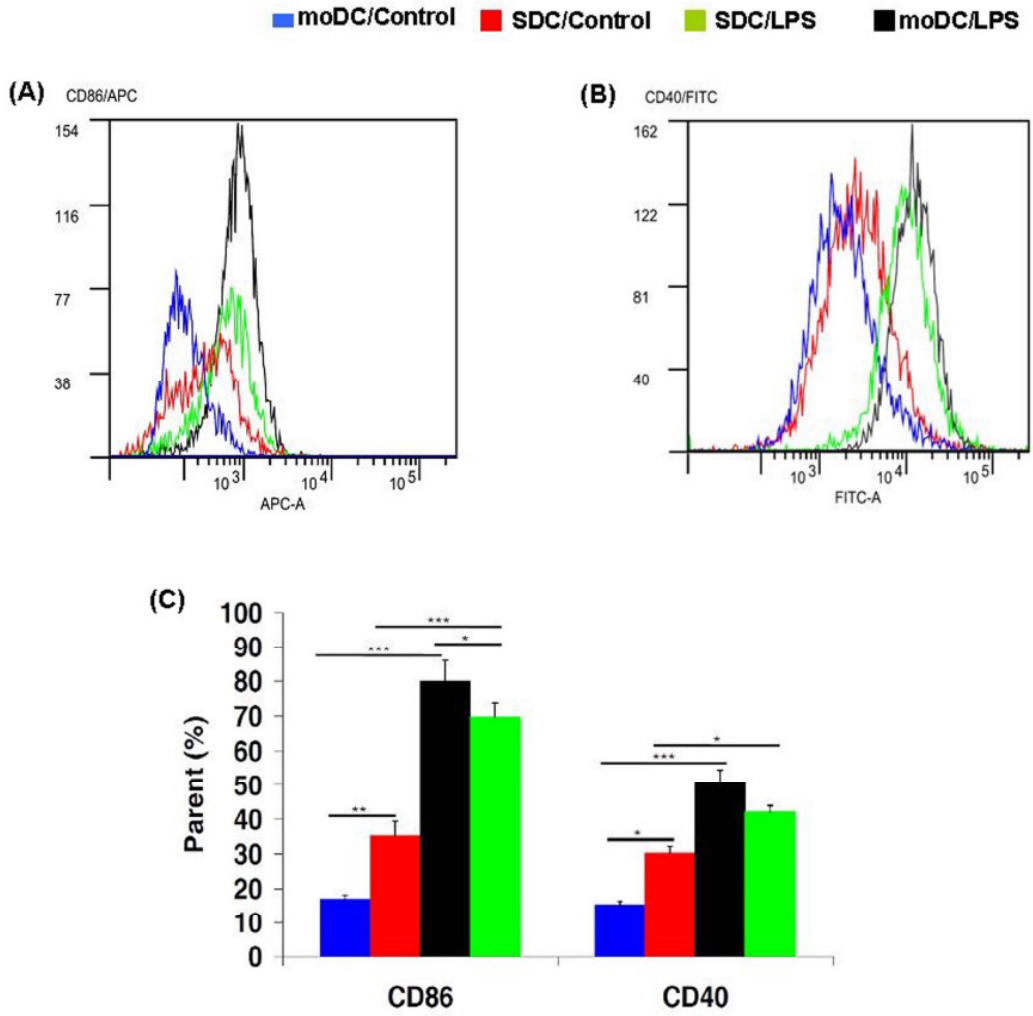

Figure 4. LPS induced moDCs and SDCs maturation. moDCs were harvested at day 7 as adherent immature DCs and adherent SDCs were harvested at day 3. The isotype control of moDCs was from the 7 days immature moDCs, SDCs from the 1 day adherent CD11c positive DCs (data not show). moDCs and SDCs treated with or without LPS (1.0 μg/ml) for 24 h. CD86 (A) and CD40 (B) levels were measured by flow cytometry. Quantification of the parent percentage of mean is shown in (C). The results were combined from two independent experiments and each experiment was performed in duplicate. The data are represented as the mean ± standard deviation (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

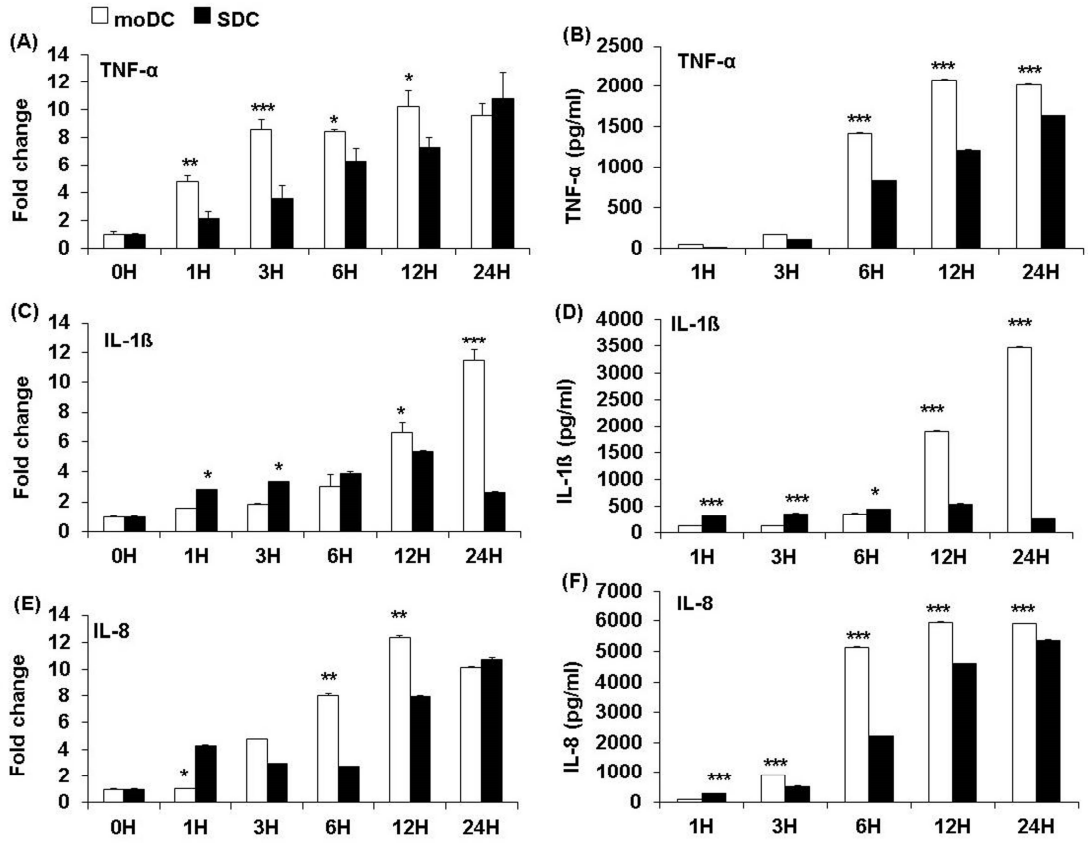

Figure 5. Expression of pro-inflammatory cytokines TNF-α, IL-1ß, and chemokine IL-8 in moDCs and SDCs in response to LPS. mRNA and cell culture supernatants were used from the same sample as TLR4 pathway genes expression. TNF-α, IL-1ß and IL-8 mRNAs expression were quantified by qRT-PCR (A), (C) and (E). The results were combined from three independent experiments and each experiment performed in triplicate. The corresponding proteins secretions were quantified by ELISA (B), (D) and (F). The results were combined from two independent experiments and each experiment was performed in duplicate. The values show the differences between moDCs and SDCs in a cell-subtype-dependent mRNA (A, C, F) and protein (B, D, E) expression manner. The data are represented as the mean ± standard deviation (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

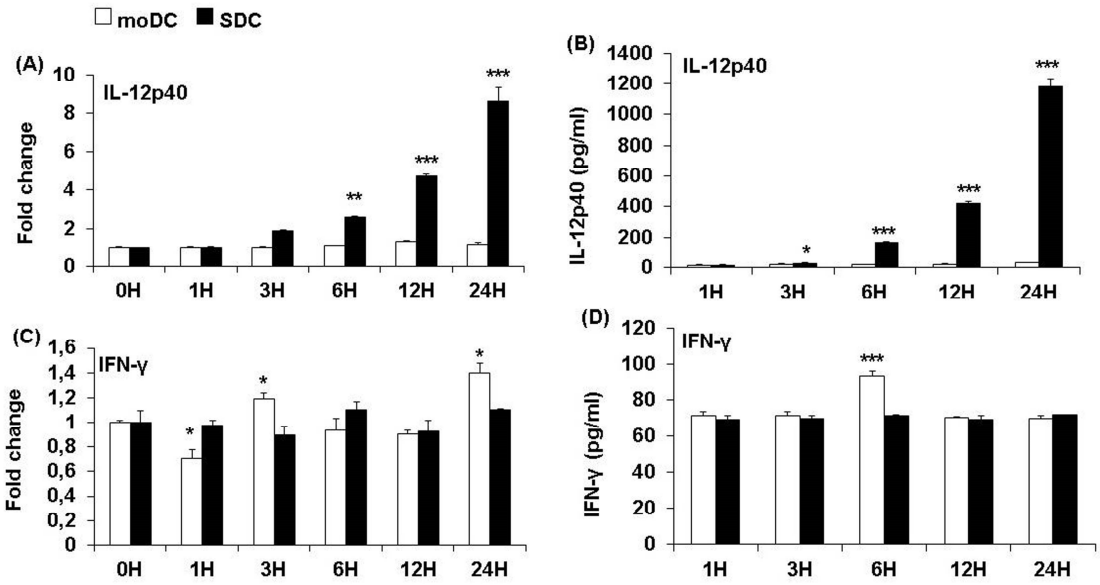

Figure 6. Expression of autoinflammatory cytokines IFN-γ and IL-12p40 in moDCs and SDCs in response to LPS. IL-12p40 and IFN-γ mRNA expression were quantified by qRT-PCR (A) and (C). The results were combined from three independent experiments and each experiment was performed in triplicate. The corresponding proteins secretions were quantified by ELISA (B) and (D). The results were combined from two independent experiments and each experiment was performed in duplicate. The values show the differences between moDCs and SDCs in a cell-subtype-dependent mRNA (A, C) and protein (B, D) expression. The data are represented as the mean ± standard deviation (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

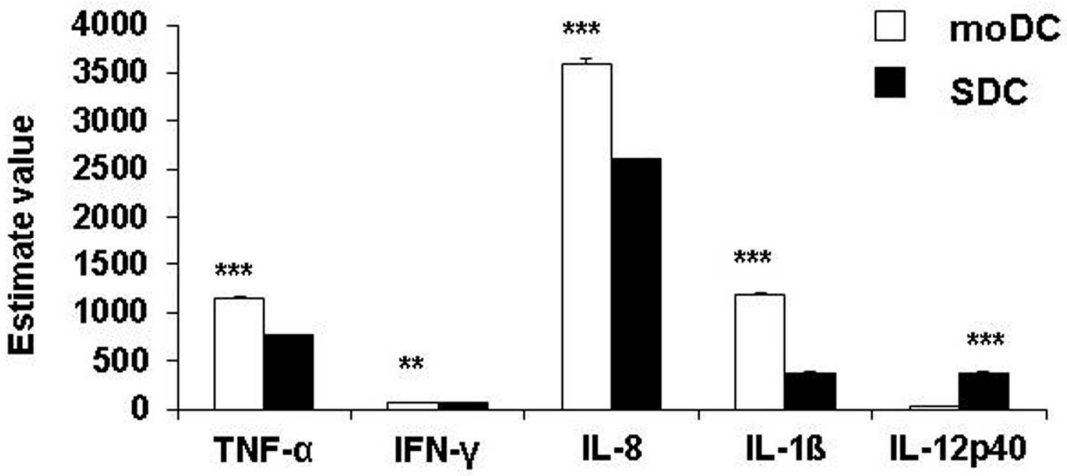

Figure 7. Production differences of overall cytokines and chemokines between moDCs and SDCs. The data comes from the cytokine and chemokine results what we described in Fig 5 and 6. We used the least square mean multiple comparison test to analyze data in order to study the differences of protein secretion between moDCs and SDCs following the LPS stimulation in various times. The values show the differences between moDCs and SDCs in a cell-subtype-dependent protein expression. The data are represented as the mean ± standard deviation (SD). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

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Sulforaphane epigenetically regulates innate immune responses of porcine monocyte-derived dendritic cells induced with Lipopolysaccharide

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Key words: sulforaphane; porcine; antigen-presenting cells; cytokines; phagocytosis; NF-κB

Abstract

Histone acetylation, regulated by histone deacetylases (HDACs) is a key epigenetic mechanism controlling gene expressions. Although dendritic cells (DCs) are playing pivotal roles in host immune responses, the effect of epigenetic modulation of DCs immune responses remains unknown. Sulforaphane (SFN) as a HDAC inhibitor has anti-inflammatory properties, which is used to investigate the epigenetic regulation of LPS-induced immune gene and HDAC family gene expressions in porcine monocyte-derived dendritic cells (moDCs). SFN was found to inhibit the LPS induced HDAC6, HDAC10 and DNA methyltransferase (DNMT3a) gene expression, whereas up-regulated the expression of DNMT1 gene. Additionally, SFN was observed to inhibit the global HDAC activity, and supressed moDCs differentiation from immature to mature DCs through down-regulating the CD40, CD80 and CD86 expression and led further to enhanced phagocytosis of moDCs. The SFN pre-treated of moDCs directly altered the LPS-induced TLR4 and MD2 gene expression and dynamically regulated the TLR4-induced activity of transcription factor NF-κB and TBP. SFN showed a protective role in LPS induced cell apoptosis through suppressing the IRF6 and TGF-ß1 production. SFN impaired the pro-inflammatory cytokine TNF-α and IL-1ß secretion into the cell culture supernatants that were induced in moDCs by LPS stimulation, whereas SFN increased the cellular-resident TNF-α accumulation. This study demonstrates that through the epigenetic mechanism the HDAC inhibitor SFN could modulate the LPS induced innate immune responses of porcine moDCs.

Introduction

Apart from pork production, pig has been used as a major mammalian model in several fields of medical research because of the anatomy, physiology, metabolism, organ development and disease progression similarities to other mammalian species (Lunney 2007). Importantly, pigs are the reservoirs of many zoonotic diseases make them important in the field of immunology. Therefore, deciphering of the porcine immune response is very important. Moreover, availability of numerous cell lines represent a broad range of tissues, further facilitates testing of gene expression and drug susceptibility of host immune system. Therefore, study of the porcine immune response could help to understand the immunological responses of the related mammalian

species. Dendritic cells (DCs) play major roles at multiple layers of immune responses. DCs are professional antigen-presenting cells and primary phagocytic cells of innate immune system that induce both the innate and adaptive immune responses upon the detection of pathogens as well as maintain the immune tolerances. The porcine DC has been frequently used as an experimental model for studying the disease progression and pathogenesis after a highly contagious viral or bacterial infection in either human or swine viral infection physiology (Baumann et al. 2014, Mussa et al. 2011, Mussa et al. 2013, Silva-Campa et al. 2010, Vincent et al. 2003). Such kind of external environmental stimuli can modify the epigenetic profile. This epigenetic modification may vary according to the cell types. We postulated that these epigenetic modifications may cause alterations of gene expression in porcine DCs in case of immune responses. The engagement of toll-like receptors (TLRs) by conserved microbial structures to activate the DCs is essential for initiation of innate immune response. Lipopolysaccharide (LPS), the ligand of TLR4 but not other TLRs, as the most abundant component of Gram-negative bacterial cell wall has been extensively used in studying immune responses of mammalian cells. Because, LPS is the TLR4 agonist that activates nuclear factor-kappa B (NF-κB) and induces high-level of proinflammatory cytokines and chemokines expression after recognition by TLRs (Andreakos et al. 2004, Dearman et al. 2009, Ruud et al. 2007). NF-κB plays an essential role in the regulation of transcription of genes related to rapid responses to stress and pathogens, as well as in the development and differentiation of immune cells (such as DCs and monocytes) (Vanden Berghe et al. 2006). Epigenetic modulation controls multi-layered interplay of NF-κB signalling pathway in achieving appropriate gene expression and transcriptional activity (Ngkelo et al. 2012). Previously, sulforaphane (SFN) has been found to regulate expressions of immune related gene (Kallifatidis et al. 2009, Koo et al. 2013, Liu and Talalay 2013, Youn et al. 2010).

SFN, a natural 1-isothiocyanato-4-(methylsufinyl)-butane compound present in cruciferous vegetables, exhibits anticancer and antimicrobial properties in experimental model (Do et al. 2010, Fahey et al. 2002), but the effects of SFN on cell growth, survival, and differentiation in primary cells are poorly understood. SFN is drawing great attention because of its ability to simultaneously modulate multiple cellular targets involved in cellular protection (Pham et al. 2004) and being suggested to be used in

treatment of bacterial infection (Fahey et al. 2002). Additionally, previous studies reported that SFN had diminished HDAC activity, and both global and localized histone acetylation was increased (Myzak et al. 2004, Myzak et al. 2007). HDAC enzymes remove acetyl groups from lysine residues within histones, which is important in the regulation of gene expression. The HDAC family has 11 (HDAC1-11) members which are catalogued in four classes: HDAC1-HDAC10 belong to class 1 and class 2 (as classic HDACs); a group of nicotinamide adenine dinucleotide (NDA+)-dependent proteins belongs to class 3 (called non classical HDACs), and the sole number of HDAC11 belongs to class 4. HDAC has been reported to affect the pro-inflammatory cytokines production in a range of disease models in mice, including septic shock (Bode et al. 2007, Zhu et al. 2010). Treatment of cells with SFN, as a HDAC inhibitor is regularly being used to investigate the role of histone modifications in the regulation of gene expressions (Fan et al. 2012). Although, epigenetic modifiers, such as HDAC inhibitors have considerable potential as anti-inflammatory and immunosuppressive agents, their effect on porcine DCs has not yet been deciphered. The epigenetic effects of SFN on porcine DCs could extend our knowledge to understand the mechanism of epigenetic regulation in human antigen-presenting cells.

The monocyte-derived dendritic cells (moDCs) have been established in vitro as an ideal culture model to examine the DCs function (Qu et al. 2014). The epigenetic effect of SFN has been studied in various tissues and cells in mice and humans (Myzak et al. 2006, Myzak et al. 2007). Effects of HDAC inhibitors in LPS-induced innate immune response have never been reported in porcine DCs. Therefore, this study aimed to investigate the effect of the HDAC inhibitor, SFN on the LPS induced inflammatory response in porcine moDCs. For this purpose, the effects of LPS stimulation on expression of genes encoding HDACs and DNA methyltransferases and acetylation levels were analysed. Furthermore, the modulations of SFN on LPS-induced inflammatory response and TLR4 activation are also examined.

Materials & Methods

Animals

Three 35 days old Pietrain female piglets were housed at the Teaching and Research Station of Frankenforst, University of Bonn, Germany. All the piglets were clinically

healthy and no respiratory disease was found according to the clinical history and physical examinations. The feeding, housing and husbandry practices of the animals followed the 'Guideline for performance testing of pigs on station for production and carcass traits (ZDS, 2003)'. This experiment was approved and followed the guidenline of 'Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion eV, Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn' (Central Board of the German Pig Producers ev. Committee for Performance Testing, Animal Breeding Value Estimation, Bonn, Germany) (ZDS 2003). Moreover, this study was carried out in strict accordance with the recommendations in the Guide for Animal Welfare committee of the University of Bonn with proposition number 84-02.05.20.12.075.

Generation of moDC from adherent monocytes of PBMCs

Porcine blood samples were collected from the vena cava cranialis in sterilized tubes with ethylenediaminetetraacetic acid (EDTA) which were used to isolated peripheral blood mononuclear cells (PBMCs). The PBMCs were isolated by Ficoll-histopaque (cat. 10771, Sigma, Germany) using density gradient centrifugation as described previously (Seki et al. 2012, Uddin et al. 2012). PBMCs were washed two times in cold Dulbecco's Phosphate Buffered Saline (DPBS) (cat. 14190-094; Invitrogen, Germany) and re-suspended in Dulbecco's modified Eagle medium (DMEM) (cat. 41966-029; Invitrogen, Germany) supplemented with 2% fetal bovine serum (FBS) (cat. 10270; Invitrogen, Germany), 500 IU/ml Penicillin-Streptomycin (cat. 15140; Invitrogen, Germany) and 0.5% fungizone (cat. 15290-026; Invitrogen, Germany). PBMCs (5×10^6 cell/ml) were cultured in 6-well plate (2 ml/well) for 4 h. The moDCs were generated from the adherent monocytes following the procedure described previously (Carrasco et al. 2001, Facci et al. 2006, Raymond and Wilkie 2005). Briefly, PBMCs were incubated for 4 h, non-adherent cells were discarded by vacuum aspiration and the adherent monocytes were washed two times using pre-warmed (37°C) DPBS in order to remove the non-adherent cells. The cleaned monocytes were cultured in RPMI-1640 medium (cat. 21875; Invitrogen, Germany) supplemented with 10% FBS, 1000UI/ml Penicillin-Streptomycin, 1% fungizone, 20 ng/ml recombinant porcine (rp) granulocyte-macrophage colony-stimulating-factor (GM-CSF) (cat. 711-PG-010; R&D System, UK)

and 20 ng/ml recombinant porcine (rp) interleukin-4 (IL-4) (cat. 654-P4-025; R&D System, UK) for 7 days at 37°C with 5% CO_2. Half of the medium was replaced every 3rd day with the fresh medium supplemented the rp GM-CSF (20 ng/ml) and rp IL-4 (20 ng/ml) concentration. After 7 days of incubation the adherent moDCs were counted and re-cultured in a new plate for the subsequent assays.

Stimulation of moDCs

moDCs were seeded separately at 2×10^6 cells/well into 6-well tissue culture plates and incubated over night at 37°C in 5% CO_2 incubator. Afterwards, moDCs were treated with or without SFN (cat. LKT-8044, LKT Laboratories, Inc., Germany) at the concentrations of 5 µM, 10 µM, 15 µM, 20 µM and 50 µM for 24 h for either cells viability or HDAC activity assay. For the gene expression study, inflammatory cytokines and other proteins measurement, cells were pre-incubated with or without 10 µM SFN for 24 h prior to the stimulation with LPS (1µg/ml) (cat. tlrl-3eblps, Invitrogen, France) for additional 24 h or indicated time (such as 0, 1, 3, 6, 12, 24 h). Cells were harvested after 24 h or indicated time of LPS stimulation. Total RNA was isolated from these cells for gene expression analysis and the supernatants were used to measure the cytokine levels. Similarly, for the expression of co-stimulatory molecules (CD40, CD80, and CD86) of cells, moDCs were pre-incubated with or without SFN before stimulating with 1 µg/ml LPS for 24 h. In addition, the effects of SFN (10 µM) on the phagocytic activity of moDCs were determined following stimulation with different concentration of LPS (0.5 µg/ml, 1.0 µg/ml, and 2.0 µg/ml) for 4 h.

Cell viability assay

Cell viability was investigated using the WST-1 cell proliferation kit (cat. 10008883, Cayman Chemical) following to the manufacturer's instructions as described previously (Qu et al. 2014). For the dose dependent SFN effect on cell viability assay, moDCs were cultured with SFN at the concentration of 5 µM, 10 µM, 15 µM, 20 µM and 50 µM for 24 h. In order to study the effect of SFN on LPS induced moDCs death, moDCs pre-incubated with or without SFN (10 µM) for 24 h, were stimulated with or without LPS at the concentration of 1.0 µg/ml for indicated time 1, 3, 6, 12 and 24 h. Then, 10 µl of reconstituted WST-1 mixture was added to each well. After 2 h of incubation in a CO_2 incubator at 37°C, the absorbance of the samples was measured using a microplate

reader (Thermo max; Germany) at a wavelength of 450 nm. The cell viability was calculated (%) following the manufacturer's formula.

DCs maturation measurement using flow cytometry

For the flow cytometry (FACs) analysis, moDCs were pre-cultured with SFN (10 µM) for 24 h before stimulation with LPS (1.0 µg/ml) for 24 h. The cells were harvested and incubated for 30 min with FACs staining buffer (DPBS supplement with 2% FBS, 10 mM NaN_3 and 10 mM HEPES) and then washed with the same staining buffer. Cells were stained with a mouse anti-human CD40 FITC Ab (clone G28.5, NB100-77786, Novus Biologicals), a mouse anti-human CD80 PE Ab (clone 37711, FAB140P, R&D systems) and a mouse anti-human CD86 APC Ab (clone 37301, FAB141A, R&D systems) antibodies. All these antibodies were IgG1 isotype. Cells were stained for 30 min on ice in 100 µl of staining buffer solution in a light protected condition. The events were acquired on 10,000 cells using a FACscalibur Dual Laser Flow Cytometer (BD Biosciences; USA) and analysed by FlowLogic software (BD Biosciences; Germany).

Gene expression analysis

mRNA expression of the genes of interest was quantified using qRT-PCR (quantitative real time PCR). Total RNA was isolated using miRNeasy Mini Kit (cat. 217004; Qiagen, Germany) and the RNA concentration was measured by Nanodrop 8000 (Thermo Scientific, Pittsburgh, PA, USA). Complementary DNA (cDNA) was synthesized using miScript II RT kit (cat. 218161; Qiagen, Düsseldorf, Germany) and the cDNA was stored at -20°C for further use. qRT-PCR was performed in an ABI prism®7000 (Applied Biosystems, Darmstadt, Germany) qRT-PCR system. The transcript of target genes presented in each sample was determined using Maxima SYBR Green/ROX Mix (cat. 218073; Qiagen, Düsseldorf, Germany). The primers (Table 1) were designed using the online Primer3 (version 0.4.0) (Rozen and Skaletsky 2000). The qRT-PCR was conducted with the following program: 95°C for 3 min, 40 cycles at 95°C for 15s, 60°C for 1 min and 95°C for 1 min in the StepOne Plus qPCR system (Applied Biosystem, Germany). Melting curve analysis was performed to detect the specificity of the PCR reaction. Each experiment was performed in triplicates and each sample was quantified in triplicate (technical replication) using qRT-PCR. Gene-specific expression was measured as relative to the expression of the house keeping

gene hypoxanthine phosphoribosyltransferase 1 (HPRT1) (Table 1). The delta Ct (ΔCt) values were calculated as the difference between target gene and reference gene HPRT1: [$\Delta Ct = Ct_{target} - Ct_{housekeeping\ gene}$]. The average expression values were considered for further analysis.

Nuclear extraction and in vitro HDAC activity assay

moDCs were cultured with SFN (0 µM, 5 µM, 10 µM, 15 µM, 20 µM and 50 µM) for 24 h. After that, cells were harvested using trypsin-EDTA (cat: 25200-072; Invitrogen, Germany). The nuclear extracts were obtained from cultured cells according to the manufacturer´s instruction. Briefly, 500 µl of ice-cold hypotonic lysis buffer (10 mM HEPES, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.05% NP40 [or 0.05% Igepal or Tergitol] pH 7.9) containing 1% proteinase inhibitor (cat: P8340-1ML; Sigma, Germany) was added to approximate 4×10^6 trypsinized cells. Cells were lysed using a mechanical homogenizer on ice for 20 min and centrifuged at 3000 rpm for 10 min at 4 °C. The cell pellets were re-suspended in 374 µl of buffer (5 mM HEPES, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol [v/v], pH 7.9) containing 26 µl of 4.6 M NaCl and homogenized with 20 abundant strokes on ice. The cell pellets were lysed for 30 min on ice and then centrifuged at 14,000 g for 20 min at 4 °C. The supernatants containing the nuclear extract were removed and stored at -80 °C for subsequent analysis. Protein content was determined using Bradford assay.

In vitro HDAC activity was determined using the Color-de-Lys HDAC colorimetric activity assay kit (BML-AK501-0001, Enzo Life Sciences) according to the manufacturer´s instructions. Briefly, approximately 5 µg of nuclear fraction from each treatment samples was incubated with the HDAC assay buffer and the HDAC colorimetric substrate at 37 °C for 30 min. Then the lysine developer was added and the samples were incubated at 37 °C for another 30 min. At the end of the incubation period, readings were taken at 405 nm using an ELISA plate reader (ThermoMax, Germany).

Phagocytic activity assay

Phagocytosis of the cells was investigated using Vybrant™ Phagocytosis Assay kit (cat. V-6694, Molecular Probes, Germany). The principle of this analysis is based on the intracellular florescence emitted by the engulfed particles, as well as the effective

fluorescence quenching of the extracellular probe by trypan blue (Sahlin et al. 1983). The phagocytosis assay protocol follows the instruction of using five negative controls, five positive controls and four experimental samples. Briefly, cells were cultured in a 6-well cell culture plate for 48 h with RPMI-1640. Cells were scrapped, washed twice with DPBS and cell density was adjusted to 1×10^6 cells/ml. moDCs were pre-treated with SFN (10 µM) for 24 h before stimulating with LPS. For the phagocytosis assay, 50 µl of the LPS (at final concentration of 0.5 µg/ml, 1 µg/ml, 2 µg/ml in RPMI-1640) were added to the experimental wells containing cells. The cells were incubated for 4 h at 37 °C with 5% CO_2 to allow the cells to adhere on the microplate surface. Afterwards, the RPMI-1640 solution was removed from the microplate wells. Then 100 µl of fluorescent BioParticles suspension was added to all the negative control, positive control and experimental wells. Two hours after incubation at 37°C in CO_2 incubator, the BioParticles were aspirated from all of the microplate wells. Finally, 100 µl trypan blue was added to the wells and incubated for 1 min at room temperature, and then trypan blue was aspirated. The fluorescence emission was measured in a fluorescence microplate reader (Thermo Eectron, Waltham, MA, USA) using 480 nm excitation and 520 nm emissions. The net phagocytosis of the cells was calculated according to the response of the phagocytosis effector agent following the manufacturer's instructions.

Cytokines measurement by ELISA

ELISA was used to investigate the cytokines secretion differences of moDCs in different treatment groups. 1×10^6 cells/ml was cultured in 6-well plates. moDCs were pre-incubated with SFN (10 µM) for 24 hour before stimulating with 1 µg/ml LPS for 24 h. Supernatants were collected for TNF-α (cat. PTA00; R&D Systems, UK) and IL-1ß (cat. PLB00B; R&D Systems, UK) measurements using ELISA kits following manufacturer's instructions. The optical density (OD) values were measured by microplate reader (ThermoMax; Germany) setting to 450 nm of wave length and results were calculated according to manufacturer's formula.

Western blot

Cell lysates were electrophoresed through polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were incubated with polyclonal antibodies specific for NF-κB p65 (cat. ab72555; abcam®; UK), TGFß1 (cat. sc-146; Santa Cruz Biotechnology, Inc; Germany), IRF6 (cat. sc-98829; Santa Cruz Biotechnology, Inc;

Germany), TNF-α (cat. LS-C43037; LSBio; Germany) and ß-actin (cat. sc-47778; Santa Cruz Biotechnology, Inc; Germany), and then revealed with secondary antibody. As a secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit (cat. Sc-2004; Santa Cruz Biotechnology, Inc; Germany) was used for the primary antibody of NF-κB p65, IRF-6, and TNF-α; whereas, peroxidase-conjugated goat anti-mouse IgG (cat. Sc-2005; Santa Cruz Biotechnology, Inc; Germany) was used for ß-actin primary antibody. Mouse polyclonal anti-ß-actin antibody was used to correct minor differences in protein loading. Finally, the specific signals were detected by chemiluminescence using the SuperSignal West Pico Chemiluminescent Substrate (cat. 34077, Thermo Scientific, Germany). Images were acquired by Quantity One 1-D analysis software (Bio-Rad, Germany).

Statistical analysis

The data were analysed by SAS software package ver. 9.2 (SAS institute, Cary, NC, USA). Pairwise comparisons were made between the treatment groups and control, using Student's t test. In addition, to compare multiple treatments groups a variance analysis was followed by Tukey test. The data were expressed as means ± standard deviations (SD) and (*) $P < 0.05$, (**) $P < 0.01$, (***) $P < 0.001$ were set as statistically significant.

Results

LPS treatment differentially influences HDACs gene expression

The expression profiling of genes encoding the four classes of HDAC were quantified by the qRT-PCR. After 24 h LPS exposure, class 1 HDACs such as HDAC1 and HDAC2 mRNA were significantly down-regulated in moDCs (Fig. 1A). On the other hand, HDAC9 and HDAC10, belonging to the class 2 HDAC, were remarkably up-regulated after LPS stimulation for 24 h (Fig. 1B). In addition, the unique HDAC11 belonging to class 4 HDAC was numerically decreased, but did not show statistically significant differences (Fig. 1C). While LPS stimulation altered the expression of five HDAC genes, six HDAC gene expressions remained non-significant in moDCs in response to LPS stimulation (Fig. 1).

Effect of LPS stimulation on DNA methyltransferase (DNMT) gene expressions in moDCs

Expression of genes encoding the enzymes responsible for methylating CpG sites in their DNA recognition elements was also analysed in LPS-induced porcine moDCs which included the maintenance methyltransferase DNMT1 and the de novo methyltransferases DNMT3a and DNMT3b. Expression of all three transcripts (DNMT1, DNMT3a and DNMT3b) were investigated in both untreated and LPS treated cells (Fig. 2). The expression of the DNMT1 was found to be unaffected by LPS treatment, in contrast, the *de novo* methyltransferase genes DNMT3a showed up-regulation in LPS stimulated moDCs. Notably, in this study, DNMT3b was undetectable in porcine moDCs.

Effect of SFN on moDCs viability and LPS-induced cell death

In order to examine the effects of SFN treatment on moDCs viability, cell viability was measured using WST-1 cell proliferation kit as described in earlier section. The moDCs viability was decreased significantly after exposure to the higher dose of SFN (15 µM and 20 µM), while cell viability remained unaffected in response to the lower dose of SFN (5 µM and 10 µM) (Fig. 3A) evidencing a dose-dependent effect of SFN on moDCs viability. In addition, 1 µg/ml LPS dramatically induced cell death after 3 h LPS stimulation (Fig. 3B). Notably, the SFN (10 µM) pre-incubation significantly inhibited the LPS-induced cell death after at 3 h LPS stimulation (Fig. 3B).

Regulation of genes encoding epigenetic enzymes by SFN

We investigated the HDAC activity in different concentration of SFN treatments. The results showed that SFN significantly inhibited HDAC activity in a dose-dependent manner (Fig. 4A). The effects of SFN on genes encoding epigenetic enzyme showed that SFN treatment caused a decrease in mRNA expression of several HDAC genes in LPS treated porcine moDCs (Fig. 4B and C). Our results show that SFN significantly inhibited both HDAC6 and HDAC10 mRNA expression that were induced by LPS in moDCs (Fig. 4B and C). Similarly, SFN treatment significantly enhanced the down-regulation of *de novo* methyltransferase DNMT3a that was induced by LPS treatment (Fig. 4D). The DNMT1 expression was significantly increased in SFN pre-treated moDCs that was induced by LPS treatment (Fig. 4E). However, this trend could not be observed in the case of other (data not shown) DNMT genes.

Effect of SFN treatment on the maturation status and phagocytosis of moDCs

In order to determine the influences of SFN on LPS-induced moDC maturational status, we examined the co-stimulating molecules CD40, CD80/86 expression. The flow cytometry results showed that LPS dramatically induced the expression of CD40 and CD80/86 molecules on with or without SFN pre-incubated moDCs (Fig. 5A and 5B). Furthermore, the SFN pre-incubation significantly inhibited CD80/86 molecule expression on moDCs (Fig. 5A and 5B). Besides, we further performed expression of those molecules at mRNA level, CD40 and CD80/86 mRNA expression was quantified using qRT-PCR. The SFN significantly inhibited the LPS-induced co-stimulatory molecules CD80 and CD86 gene expression in moDCs (Fig. 5C). Additionally, the effects of SFN on the potential of phagocytic activity were also measured. The effects of SFN and LPS on phagocytosis showed a dose-dependent manner (Fig. 5D). The phagocytosis activity of moDCs was increased with an increase of both the SFN and LPS dose (Fig. 5D). SFN pre-treatment significantly increased the phagocytosis of moDCs in response to 2.0 μg/ml of LPS (Fig. 5D) treatment. Moreover, the western blotting result displayed that TGFß1 secretion was significantly increased in response to either SFN and/or stimulation group compared to control group (Fig. 5E). Moreover, the SFN pre-treatment suppressed TGFß1 production in response to LPS stimulation (Fig. 5E).

SFN reversed LPS-induced up-regulation of TLR4 and MD2 gene expression in the early stage of LPS stimulation

To clarify how SFN influences TLR4 activation in the time-dependent manner of LPS stimulation, moDCs were pre-incubated with SFN (10 μM) for 24 h and then stimulated with or without LPS for the indicated times. It was found that SFN significantly up-regulated TLR4 and MD2 mRNA expression following 24 h SFN incubation (Fig. 6). Interestingly, SFN significantly inhibited LPS-induced up-regulation of TLR4 and MD2 within the first 3 h of LPS stimulation (Fig. 6). Surprisingly, after 6 h LPS stimulation, SFN dramatically enhanced LPS-induced up-regulation of TLR4 and MD2 (Fig. 6).

SFN reversed LPS-activated transcription factor expression in a time-dependent manner

In order to further confirm the time-dependent regulation of SFN in LPS induced immune response, we have examined the transcription factor NF-κB1 and TBP expression which are present in most mammalian immune cells such as in moDCs. It

could be found that SFN significantly inhibited LPS-induced up-regulation of NF-κB1 mRNA at 3 h (Fig. 7A). Since the classic NF-κB typically presents as a p50-p65 heterodimer structure in the cytoplasm, we have detected the effects of SFN on LPS-activated p50 and p65 expression at protein level. The western blotting results showed that SFN visibly impaired p65 production, whereas SFN enhanced p50 secretion in moDCs (Fig. 7B). LPS up-regulated p65 was dramatically inhibited at 3 h, but was enhanced at 6 h by SFN pre-incubation in porcine moDCs (Fig. 7B). Additionally, SFN strongly enhanced the LPS up-regulated p50 in the time-dependent manner of LPS stimulation apart from at 12 h (Fig. 7B). Besides, TBP was remarkably up-regulated following 24 h of SFN incubation in moDC (Fig. 7C). Likewise NF-κB1 mRNA expression, SFN significantly impaired LPS-induced TBP expression at 1 h, but SFN enhanced LPS-induced TBP up-regulation at 6 and 12 h (Fig. 7C).

SFN activates the NF-κB signaling and supresses the cytokines secretion in response to LPS treatment, while enhancing the cellular cytokine accumulation in moDCs lysates

The NF-κB transcription factor has a crucial role in the rapid response to pathogens through modification of down-stream immune gene expressions. The effects of SFN on NF-κB and down-stream protein of immune genes (such as IRF6 and TNF-α) secretion in response to LPS treatment were determined using western blotting. The western blotting results displayed that IRF6 secretion was significantly increased in response to either SFN and/or LPS compared to control group (Fig. 8A). Moreover, the SFN pre-treatment suppressed IRF6 production in response to LPS stimulation (Fig. 8A). According to the western blotting data, TNF-α was remarkably increased in response to either SFN or LPS (Fig. 8A). Similarly, SFN pre-treatment further increased the TNF-α production when compared to the moDCs that were not pre-treated with SFN (Fig. 8A). Additionally, several down-stream mRNA expressions of cytokines were quantified using qRT-PCR. SFN significantly up-regulated TNF-α and IL-8 mRNA expression in response to LPS treatment (Fig. 8B). On the other hand, SFN remarkably down-regulate IL-1ß mRNA expression in response to LPS stimulation (Fig. 8B). SFN had no significant effects on IFN-γ expression in moDCs stimulated with LPS (Fig. 8B). Furthermore, TNF-α and IL-1ß protein secretions in moDCs culture supernatant were measured using ELISA. LPS significantly increased cellular TNF-α protein production

(Fig. 8A), while pre-treatment with SFN significantly decreased secretory protein expressions in supernatant (Fig. 8C and 8D). Notably, TNF-α production in cell culture supernatants measured using ELISA, and mRNA expression in cell lysates quantified using qRT-PCR did not coincide (Fig. 8B and 8C). IL-1ß mRNA expression and protein production was significantly decreased in SFN pre-treated moDCs in response to LPS (Fig. 8B and 8D).

Discussion

DCs are the master phagocytes and antigen-presenting cells (APCs) that bridge the innate and adaptive immunity (Banchereau et al. 2000). Because of the unique ability, DCs can identify pathogens directly by expression of a collection of pattern recognition receptors (PRRs) on the cell surface including TLRs (Savina and Amigorena 2007). Although our understanding of the host-pathogen interactions at molecular level has expanded dramatically in recent years, it is believed that the interaction of DCs with pathogens leads to changes in inducible gene expression. The precise molecular and cellular mechanisms of innate immunoregulation through epigenetic changes which responses to pathogens are not yet well understood. SFN represents both anti-inflammatory function and HDAC inhibitory activity (Schwab et al. 2008). Indeed, histone acetylation is critical for regulation of gene expression in different immune processes. moDCs could be an ideal cell model to unravel the immunoregulatory and HDAC inhibitory effects of SFN in response to LPS. This study assayed the ability of SFN to influence the expression of DNMT and HDAC family genes, as well as the effects of SFN on the differentiation and functional properties of moDCs under LPS-induced inflammatory model through the TLR4-dependent signalling pathway.

Initially SFN is best known for its role as an indirect antioxidant to process anti-inflammatory activity (Konwinski et al. 2004). Exposure to different SFN concentrations and exposure periods reported in a transient reactive oxygen species (ROS) burst and caused cell death (Ferreira de Oliveira et al. 2014, Moon et al. 2009). Higher dose of SFN (above 10 μM) was reported to increase ROS levels, which correlated with apoptotic endpoints and cell viability decline (Ferreira de Oliveira et al. 2014). Indeed, our finding is consistent with the above study that the high dose of SFN (such as above 15 μM) significantly induced moDCs cell death. Another potential

mechanism of SFN action via SFN-conjugates is histone deacetylase inhibition, which leads to increase histone acetylation. Results of this present study is coincided with our previous research using similar concentration, demonstrated an inhibition of HDAC activity at 10 μM (Fan et al. 2012). An aim of the present work is to find an advisable SFN concentration to protect the cell death and play the HDAC inhibitory action to increase histone acetylation. Based on the present data and previous results, 10 μM was considered as an ideal concentration to initiate our study. Expectedly, TNF-α, IL-8 and DNMT1 were positively regulated in SFN pre-treated cells in response to LPS stimulation. Similar results have been reported earlier that HDAC inhibitor Trichostatin A enhanced LPS induced COX-2, CXCLl2 and IFIT2 expression in macrophages (Aung et al. 2006). Besides, IL-1ß was found to be negatively regulated in this study. The up-regulation of TNF-α and IL-8 mRNAs by LPS stimulation might be related to the down-regulation of HDAC1 and HDAC2 through NF-κB signal, whereas the subsequent down-regulation of IL-1ß mRNAs in LPS induced moDCs might be related to the up-regulation of HDAC9 and HDAC10 mRNAs (Ashburner et al. 2001). Furthermore, SFN inhibited LPS induced TLR4/MD2 gene complex and the relevant transcription factors expression at the early stage of stimulus (within 6 h). Along with the previous reports, it could be postulated that SFN pre-treatment down-regulated the TLR4 signalling through the impairing of oligomerization process in a dose dependent manner, leading to the suppression of NF-κB activation (Koo et al. 2013, Youn et al. 2010). But, interestingly and unexpectedly, SFN dramatically enhanced the LPS induced relative immune genes expression under longer pathogen stimulation (such as TLR4 and MD2), which is poorly understood.

Epigenetic mechanisms have been shown to play essential roles in the maintenance of gene expression patterns during embryogenesis and cancer (Provenzano and Domann 2007), but little is known about the roles in immune response in pigs. The steady state levels of acetylation of core histones result from the balance between the opposing activities of histone acetyltransferases and HDACs (Wade 2001). HDAC alteration in relation to the aberrant gene expression observed in immune response becomes a critical component in epigenetic mechanism to understand the immune system. In fact, both class I and class II HDACs are involved in regulating proinflammatory response as well as cell proliferation and cell differentiation. HDAC1 and HDAC2 proteins of the class I

HDAC are associated in part with the regulation of the transactivation function of NF-κB. Moreover, the association of NF-κB with the HDAC1 and HDAC2 proteins may supress the expression of NF-κB-regulated genes (Ashburner et al. 2001). The finding of this study coincided with the previous report suggesting that that LPS-induced down-regulation of HDAC1 and HDAC2 might contribute to the activated NF-κB dependent inflammatory gene expression levels (Ashburner et al. 2001). The class II HDAC has been identified as a general mechanism to control the cytokine production (Shakespear et al. 2011). Previous studies have demonstrated that HDAC6 plays an essential role in regulation of inflammatory immune response in APC/T cell (Serrador et al. 2004), macrophages response (Halili et al. 2010) and in the cae of atypical airway inflammation (Lam et al. 2013). Moreover, a recent study has elaborated that HDAC6 inhibition represents a novel molecular target to disrupt the anti-inflammatory STAT3/IL-10 axis in the APC (Park et al. 2008). Along with the previous results, the downregulation of LPS-induced HDAC6 by SFN may postulate anti-inflammatory and anti-tolerance immune response in DCs. Comparatively, the HDAC9 and HDAC10 have less established roles than HDAC6 in immune system, although the inhibition of HDAC10 may regulate HSP-90 acetylation (Park et al. 2008). Besides, HDAC9 and HDAC10 reflect a homologous recombination (Kotian et al. 2011). Although it is not yet clear whether this is by direct participation or transcriptional control, in accordance with other studies, our data suggest that the effect of SFN on LPS-induced upregulation of HDAC10 return to normal phenomenon might be due to the anti-inflammatory function of SFN. Notably, DNA methylation is another key component of epigenetic mechanism that regulates transcriptome levels. In the case of DNA methylation, DNA methyltransferases (DNMTs) are either involved in establishing methylation (i.e., the "*de novo*" methyltransferases DNMT3a and DNMT3b) or copying methylation patterns to the newly synthesized DNA strand during replication (i.e., the "maintenance" methyltransferase DNMT1) (Bierne et al. 2012). DNMT1 is considered to be the key maintenance methyltransferase in mammals (Bierne et al. 2012). In this study, DNMT1 was unaffected by LPS treatment, but it was increased in SFN pre-treated moDCs in response to LPS treatment. These findings indicate that SFN as a HDAC inhibitor might contribute to the suppression of pro-inflammatory cytokines production. In contrast, DNMT3a mRNA was significantly down-regulated by LPS stimulation, and further down-regulated in SFN pre-treated moDCs in response to LPS. DNMT3a encoding the

de novo methyltransferases mediates methylation-independent gene repression (Hu et al. 2012). The findings of this study coincided with the previous study reported that DNMT3a deficiency leaded to increase cytokine gene expression and resulted in higher inflammatory response in a murine model (Mukherjee et al. 2012). We speculate that the regulations of DNMTs methylation might play a role in the immune response to LPS with or without SFN pre-exposure. The expression of inflammatory cytokines and other immune genes has been reported to be dependent on methylation status changes at their promoters in human and mouse (Jones and Chen 2006, Mukherjee et al. 2012). The combined inhibition in the expression of these deacetylases and DNA methyltransferases could facilitate the transcription of genes in response to LPS treatment (Li et al. 2009), which suggested that epigenetic factors might be one of the components involved in the regulation of inflammatory response in porcine immune system.

The HDAC inhibitor SFN supressed the LPS induced of HDAC gene expressions in this study. Additionally, SFN altered the DNMT1 and DNMT3a expression in porcine moDCs. These data indicate that histone deacetylases positively influence the expression of relevant protein-coding genes. In case of HDAC activity, the addition of SFN to moDC cultures globally inhibited the HDAC activity in a dose-dependent manner. This inhibition of HDAC activity by SFN is coinciding with a previous study (Myzak et al. 2004). We speculated that alteration in immune gene expression might be related to the SFN induced inhibition of HDAC activity.

DCs play an essential role in the phagocytosis and antigen-presenting that bridges the innate and adaptive immune response. DCs are currently divided into tolerogenic immature and immunogenic mature stages. After stimulation, the immature DCs transform into immunogenic mature DCs, representing unique inducers ready for primary T-cell responses (Banchereau and Steinman 1998). The foreign antigens can be phagocytized by immature DCs through the interaction of pathogens and the surface receptors on DCs. Immature DC shows the low expression of co-stimulatory molecules CD40, CD80, and CD86. In this study, SFN increased the phagocytic activity in response to LPS stimulation in a dose-dependent and inhibited the expression of mature cell surface markers CD80 and CD86 indicating that SFN inhibited the moDCs maturation. This finding coincided with a previous study reporting that HDAC inhibitor

led to a tolerogenic phenotype of DCs in mice (Misaki et al. 2011). This might indicate that SFN enhanced the maintenance of immature moDCs. In a previous study (Qu et al. 2014), we have shown that phagocytosis of moDCs induced the apoptotic cell death when stimulated with LPS. LPS binds to TLR4 in complex with MD2, and this complex recruits TGF-ß-activated kinase 1 (TAK1), leading to the activation of NF-κB and consequent transcription of a range of genes coding for proinflammatory cytokines, including TNF-α, pro-IL-1ß and IL-8 (Kawai and Akira 2010, Kim et al. 2012). SFN-dose is reported to interrupt the engagement of LPS in TLR4/MD2 complex and a beneficial anti-inflammatory effects of SFN on TLR4 signalling has been reported previously (Koo et al. 2013, Youn et al. 2010). Therefore, we conducted experiments to see whether the SFN influenced LPS-induced TLR4/MD2 complex signalling genes expression on moDCs. For this purpose, we have analysed the expression of TLR4/MD2 complex genes and transcription factor genes in LPS-activated moDCs in time-dependent manner after SFN treatment. Additionally, we have determined the protein levels of transcription factor in different time points. Although it is not completely understood how SFN dynamically regulates the immune gene expression, the inhibition of immune genes in the early hours of LPS stimulation seems to contribute for the anti-inflammatory function in TLR4 signalling.

Apoptotic function of DCs play a critical role in maintaining a balance between tolerance and immune reaction. The immature DCs start apoptosis and subsequently turn into tolerogenic DCs along with TGF-ß1 secretion and $Fox3^{+}$ regulatory T cells induction (Kushwah et al. 2009). TGF-ß1 is a multipotent cytokine that regulates several pathophysiological events and the secretion of TGF-β1 points out the pathophysiological status of DCs (Yamaguchi et al. 1997). In this study, the inhibition of LPS-induced TGF-ß1 production by SFN might demonstrate that SFN supressed the LPS induced apoptotic cell death in moDCs through the suppression of TGF-ß1 signalling. A positive correlation between TGF-ß1 signalling pathway activation and induced cell apoptosis has been reported previously (Ren et al. 2008, Zhang et al. 1999). Besides, TGF-ß1 has been shown to improve early DCs development *in vitro* and suppression of immature DCs activation and maturation through inhibiting the up-regulation of co-stimulatory molecules CD80 and CD86 leads to the induction of tolerance to subsequent immunogens (Yamaguchi et al. 1997, Zhang et al. 1999).

Indeed, this study found that SFN inhibited LPS-induced TGF-ß1 production, DCs maturation, and simultaneously enhanced the phagocytosis activity in porcine moDCs. These results indicate that the SFN treatment partly benefit DCs anti-inflammatory response.

HDAC inhibitors have been reported to interfere the activation of the mitogen-activated protein kinases, IRFs, or NF-κB signal transduction pathways that induced the transcription and production of immune genes (Aung et al. 2006, Cao et al. 2008). On the contrary, HDAC inhibitors (TSA, VPA, and SAHA) have reported to have no effect on ERK1/2 or on NF-κB, IRF3, or IRF7 nuclear translocation induced by LPS or Pam3CSK4 (Roger et al. 2011). These inconsistent findings may be due to the different species or immunogens. In case of porcine moDCs, we found that IRF6 was increased in response to LPS with or without SFN pre-stimulation. Notably, IRF6 protein was supressed in response to LPS in the SFN pre-treated moDCs compared to without SFN pre-treated cells. IRF family members, reported to be involved in the induction of genes that encoded type I IFN, could induce cell differentiation and could regulate gene expression in response to pathogens (Popadin et al. 2013). IRF6 plays functionally diverse roles in the regulation of the immune system. IRF 6 is involved in the immune response process and alters production of serum IFN-γ, IL-10 level and ratio of IFN-γ to IL-10 in pigs (Ingraham et al. 2006). Therefore, we postulated that SFN pre-treatment could influence the LPS induced inflammatory cytokine secretion.

The transcription factor NF-κB plays a crucial role in the transcriptional regulation of genes involved in controlling cell proliferation, differentiation, apoptosis, inflammation and stress responses (Calao et al. 2008). Transcriptional modification mediated by HDAC inhibitor SFN may also rely on the acetylation of NF-κB or on the molecules involved in NF-κB signal transduction pathway to control the extent, potency, and duration of NF-κB-mediated transcriptional activity (Calao et al. 2008). Therefore, we hypothesize that SFN inhibits the expression of NF-κB1 and TBP which acts as a transcription factors for secondary LPS-induced cytokines, such as TNF-α, IL-1ß, and IL-8, in the early stage of inflammation (within 6 h LPS stimulation). In most vertebrate cells, NF-κB presents as two (a homo- and heterodimer) structurally related NF-κB proteins, namely p65, and NF-κB1 (p50). In the present study, the cellular NF-κB p65 and p50 protein production was found to increase in moDCs in response to LPS

stimulation. Notably, NF-κB p65 protein secretion was increased in SFN pre-treated LPS induced moDCs compared to only LPS stimulated cells. Consistent with the role of NF-κB, this implies that pre-treatment with SFN might contribute to the LPS induced inflammatory response. Indeed, SFN pre-treatment suppressed the pro-inflammatory cytokine TNF-α and IL-1ß secretion into the cell culture supernatants, while the cellular TNF-α protein and TNF-α mRNA were increased in this study. It is well known that TNF-α is a proinflammatory cytokine that is rapidly produced following infections, resulting in the initiation of a pro-inflammatory cytokine cascade which can have both beneficial and detrimental effects (Moore et al. 2005). The absence of TNF-α bioactivity correlates with an inability to clear the infectious agent resulting in the significant increase of mortality (Inoue et al. 2012, Wellmer et al. 2001). In contrary, excessive TNF-α production in systemic bacterial infection or sepsis is also resulting in an increased mortality (Apostolaki et al. 2010, Joyee and Yang 2013, Moore et al. 2003). Therefore, the present results indicated that suppression of LPS induced proinflammatory cytokines expression by SFN might have a beneficial effect on bacterial infection.

In conclusion, to the authors' knowledge, the present study firstly identify that the HDAC inhibitor SFN regulates the expression of immune genes critical in porcine moDCs responding to bacterial pathogens. Inhibition of HDACs affects the differentiation from immature to mature moDCs, reduces excessive proinflammatory cytokines expression and increases cellular-resident TNF-α accumulation that might enhance pathogen engulfment and clearance through the activation of NF-κB signalling pathway. Additionally, LPS stimulation was found to alter the expression of genes encoding epigenetic enzymes in porcine moDCs; however, further studies are needed to confirm exactly how SFN affects various HDAC family members and their individual protein targets. Although, the role of epigenetics in the orchestration of the immune response in porcine immune cells is poorly understood, regulation of the inflammatory response by histone modifying enzymes may focus on the host-pathogen interactions and disease susceptibility.

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Author Contributions

Conceived and designed the experiments: XQ, KS, MUC, MJU. Performed the experiments: XQ, MP. Analyzed the data: XQ. Contributed reagents/materials/analysis tools: DT. Wrote the paper: XQ, MJU. Revised manuscript: XQ, KS, DSW, MMH, MJU. Supervised the overall work: MUC, MJU. Contributed to sampling: CN, MP. Contributed to primers: RZ. Statistical analysis: ET, CG-B.

Reference

Andreakos E, Sacre SM, Smith C, Lundberg A, Kiriakidis S, Stonehouse T, Monaco C, Feldmann M, Foxwell BM (2004): Distinct pathways of LPS-induced NF-kappa B activation and cytokine production in human myeloid and nonmyeloid cells defined by selective utilization of MyD88 and Mal/TIRAP. Blood 103, 2229-2237

Apostolaki M, Armaka M, Victoratos P, Kollias G (2010): Cellular mechanisms of TNF function in models of inflammation and autoimmunity. Curr Dir Autoimmun 11, 1-26

Ashburner BP, Westerheide SD, Baldwin AS, Jr. (2001): The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol Cell Biol 21, 7065-7077

Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, Suzuki H, Hayashizaki Y, Hume DA, Sweet MJ, Ravasi T (2006): LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. FASEB J 20, 1315-1327

Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000): Immunobiology of dendritic cells. Annu Rev Immunol 18, 767-811

Banchereau J, Steinman RM (1998): Dendritic cells and the control of immunity. Nature 392, 245-252

Baumann A, Demoulins T, Python S, Summerfield A (2014): Porcine cathelicidins efficiently complex and deliver nucleic acids to plasmacytoid dendritic cells and can thereby mediate bacteria-induced IFN-alpha responses. J Immunol 193, 364-371

Bierne H, Hamon M, Cossart P (2012): Epigenetics and bacterial infections. Cold Spring Harb Perspect Med 2, a010272

Bode KA, Schroder K, Hume DA, Ravasi T, Heeg K, Sweet MJ, Dalpke AH (2007): Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. Immunology 122, 596-606

Calao M, Burny A, Quivy V, Dekoninck A, Van Lint C (2008): A pervasive role of histone acetyltransferases and deacetylases in an NF-kappaB-signaling code. Trends Biochem Sci 33, 339-349

Cao W, Bao C, Padalko E, Lowenstein CJ (2008): Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. J Exp Med 205, 1491-1503

Carrasco CP, Rigden RC, Schaffner R, Gerber H, Neuhaus V, Inumaru S, Takamatsu H, Bertoni G, McCullough KC, Summerfield A (2001): Porcine dendritic cells generated in vitro: morphological, phenotypic and functional properties. Immunology 104, 175-184

Dearman RJ, Cumberbatch M, Maxwell G, Basketter DA, Kimber I (2009): Toll-like receptor ligand activation of murine bone marrow-derived dendritic cells. Immunology 126, 475-484

Do DP, Pai SB, Rizvi SA, D'Souza MJ (2010): Development of sulforaphane-encapsulated microspheres for cancer epigenetic therapy. Int J Pharm 386, 114-121

Facci MR, Auray G, Buchanan R, van Kessel J, Thompson DR, Mackenzie-Dyck S, Babiuk LA, Gerdts V (2006): A comparison between isolated blood dendritic cells and monocyte-derived dendritic cells in pigs. Immunology 129, 396-405

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002): Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci USA 99, 7610-7615

Fan H, Zhang R, Tesfaye D, Tholen E, Looft C, Holker M, Schellander K, Cinar MU (2012): Sulforaphane causes a major epigenetic repression of myostatin in porcine satellite cells. Epigenetics 7, 1379-1390

Ferreira de Oliveira JM, Costa M, Pedrosa T, Pinto P, Remedios C, Oliveira H, Pimentel F, Almeida L, Santos C (2014): Sulforaphane induces oxidative stress and death by p53-independent mechanism: implication of impaired glutathione recycling. PLoS One 9, e92980

Halili MA, Andrews MR, Labzin LI, Schroder K, Matthias G, Cao C, Lovelace E, Reid RC, Le GT, Hume DA, Irvine KM, Matthias P, Fairlie DP, Sweet MJ (2010): Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. J Leukoc Biol 87, 1103-1114

Hu N, Strobl-Mazzulla P, Sauka-Spengler T, Bronner ME (2012): DNA methyltransferase3A as a molecular switch mediating the neural tube-to-neural crest fate transition. Genes Dev 26, 2380-2385

Ingraham CR, Kinoshita A, Kondo S, Yang B, Sajan S, Trout KJ, Malik MI, Dunnwald M, Goudy SL, Lovett M, Murray JC, Schutte BC (2006): Abnormal skin, limb and craniofacial morphogenesis in mice deficient for interferon regulatory factor 6 (Irf6). Nat Genet 38, 1335-1340

Inoue A, Matsumoto I, Tanaka Y, Umeda N, Mihara M, Takahashi S, Sumida T (2012): Murine tumor necrosis factor alpha-induced adipose-related protein (tumor necrosis factor alpha-induced protein 9) deficiency leads to arthritis via interleukin-6 overproduction with enhanced NF-kappaB, STAT-3 signaling, and dysregulated apoptosis of macrophages. Arthritis Rheum 64, 3877-3885

Jones B, Chen J (2006): Inhibition of IFN-gamma transcription by site-specific methylation during T helper cell development. EMBO J 25, 2443-2452

Joyee AG, Yang X (2013): Plasmacytoid Dendritic Cells Mediate the Regulation of Inflammatory Type T Cell Response for Optimal Immunity against Respiratory Chlamydia Pneumoniae Infection. PLoS One 8, e83463

Kallifatidis G, Rausch V, Baumann B, Apel A, Beckermann BM, Groth A, Mattern J, Li Z, Kolb A, Moldenhauer G, Altevogt P, Wirth T, Werner J, Schemmer P, Buchler MW, Salnikov AV, Herr I (2009): Sulforaphane targets pancreatic tumour-initiating cells by NF-kappaB-induced antiapoptotic signalling. Gut 58, 949-963

Kawai T, Akira S (2010): The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. Nat Immunol 11, 373-384

Kim SY, Jeong S, Jung E, Baik KH, Chang MH, Kim SA, Shim JH, Chun E, Lee KY (2012): AMP-activated protein kinase-alpha1 as an activating kinase of TGF-beta-activated kinase 1 has a key role in inflammatory signals. Cell Death Dis 3, e357

Konwinski RR, Haddad R, Chun JA, Klenow S, Larson SC, Haab BB, Furge LL (2004): Oltipraz, 3H-1,2-dithiole-3-thione, and sulforaphane induce overlapping and protective antioxidant responses in murine microglial cells. Toxicol Lett 153, 343-355

Koo JE, Park ZY, Kim ND, Lee JY (2013): Sulforaphane inhibits the engagement of LPS with TLR4/MD2 complex by preferential binding to Cys133 in MD2. Biochem Biophys Res Commun 434, 600-605

Kotian S, Liyanarachchi S, Zelent A, Parvin JD (2011): Histone deacetylases 9 and 10 are required for homologous recombination. J Biol Chem 286, 7722-7726

Kushwah R, Oliver JR, Zhang J, Siminovitch KA, Hu J (2009): Apoptotic dendritic cells induce tolerance in mice through suppression of dendritic cell maturation and induction of antigen-specific regulatory T cells. J Immunol 183, 7104-7118

Lam HC, Cloonan SM, Bhashyam AR, Haspel JA, Singh A, Sathirapongsasuti JF, Cervo M, Yao H, Chung AL, Mizumura K, An CH, Shan B, Franks JM, Haley KJ, Owen CA, Tesfaigzi Y, Washko GR, Quackenbush J, Silverman EK, Rahman I, Kim HP, Mahmood A, Biswal SS, Ryter SW, Choi AM (2013): Histone deacetylase 6-mediated selective autophagy regulates COPD-associated cilia dysfunction. J Clin Invest 123, 5212-5230

Li CJ, Li RW, Elsasser TH, Kahl S (2009): Lipopolysaccharide-induced early response genes in bovine peripheral blood mononuclear cells implicate GLG1/E-selectin as a key ligand-receptor interaction. Funct Integr Genomics 9, 335-349

Liu H, Talalay P (2013): Relevance of anti-inflammatory and antioxidant activities of exemestane and synergism with sulforaphane for disease prevention. Proc Natl Acad Sci U S A 110, 19065-19070

Lunney JK (2007): Advances in swine biomedical model genomics. Int J Biol Sci 3, 179-184

Misaki K, Morinobu A, Saegusa J, Kasagi S, Fujita M, Miyamoto Y, Matsuki F, Kumagai S (2011): Histone deacetylase inhibition alters dendritic cells to assume a

tolerogenic phenotype and ameliorates arthritis in SKG mice. Arthritis Res Ther 13, R77

Moon DO, Kim MO, Kang SH, Choi YH, Kim GY (2009): Sulforaphane suppresses TNF-alpha-mediated activation of NF-kappaB and induces apoptosis through activation of reactive oxygen species-dependent caspase-3. Cancer Lett 274, 132-142

Moore TA, Lau HY, Cogen AL, Standiford TJ (2005): Defective innate antibacterial host responses during murine Klebsiella pneumoniae bacteremia: tumor necrosis factor (TNF) receptor 1 deficiency versus therapy with anti-TNF-alpha. Clin Infect Dis 41 Suppl 3, S213-217

Moore TA, Perry ML, Getsoian AG, Monteleon CL, Cogen AL, Standiford TJ (2003): Increased mortality and dysregulated cytokine production in tumor necrosis factor receptor 1-deficient mice following systemic Klebsiella pneumoniae infection. Infect Immun 71, 4891-4900

Mukherjee R, Choi JW, Choi DK, Oh TS, Liu H, Yun JW (2012): Gender-dependent protein expression in white adipose tissues of lean and obese rats fed a high fat diet. Cell Physiol Biochem 29, 617-634

Mussa T, Ballester M, Silva-Campa E, Baratelli M, Busquets N, Lecours MP, Dominguez J, Amadori M, Fraile L, Hernandez J, Montoya M (2013): Swine, human or avian influenza viruses differentially activates porcine dendritic cells cytokine profile. Vet Immunol Immunopathol 154, 25-35

Mussa T, Rodriguez-Carino C, Pujol M, Cordoba L, Busquets N, Crisci E, Dominguez J, Fraile L, Montoya M (2011): Interaction of porcine conventional dendritic cells with swine influenza virus. Virology 420, 125-134

Myzak MC, Dashwood WM, Orner GA, Ho E, Dashwood RH (2006): Sulforaphane inhibits histone deacetylase in vivo and suppresses tumorigenesis in Apc-minus mice. FASEB J 20, 506-508

Myzak MC, Karplus PA, Chung FL, Dashwood RH (2004): A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer Res 64, 5767-5774

Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E (2007): Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med (Maywood) 232, 227-234

Ngkelo A, Meja K, Yeadon M, Adcock I, Kirkham PA (2012): LPS induced inflammatory responses in human peripheral blood mononuclear cells is mediated through NOX4 and Gialpha dependent PI-3kinase signalling. J Inflamm (Lond) 9, 1

Park JH, Kim SH, Choi MC, Lee J, Oh DY, Im SA, Bang YJ, Kim TY (2008): Class II histone deacetylases play pivotal roles in heat shock protein 90-mediated proteasomal degradation of vascular endothelial growth factor receptors. Biochem Biophys Res Commun 368, 318-322

Pham NA, Jacobberger JW, Schimmer AD, Cao P, Gronda M, Hedley DW (2004): The dietary isothiocyanate sulforaphane targets pathways of apoptosis, cell cycle arrest, and oxidative stress in human pancreatic cancer cells and inhibits tumor growth in severe combined immunodeficient mice. Mol Cancer Ther 3, 1239-1248

Popadin K, Gutierrez-Arcelus M, Dermitzakis ET, Antonarakis SE (2013): Genetic and epigenetic regulation of human lincRNA gene expression. Am J Hum Genet 93, 1015-1026

Provenzano MJ, Domann FE (2007): A role for epigenetics in hearing: Establishment and maintenance of auditory specific gene expression patterns. Hear Res 233, 1-13

Qu X, Cinar MU, Fan H, Proll M, Tesfaye D, Tholen E, Looft C, Holker M, Schellander K, Uddin MJ (2014): Comparison of the innate immune responses of porcine monocyte-derived dendritic cells and splenic dendritic cells stimulated with LPS. Innate Immun (in process) doi: 10.1177/1753425914526266

Raymond CR, Wilkie BN (2005): Toll-like receptor, MHC II, B7 and cytokine expression by porcine monocytes and monocyte-derived dendritic cells in response to microbial pathogen-associated molecular patterns. Vet Immunol Immunopathol 107, 235-247

Ren Y, Xie Y, Jiang G, Fan J, Yeung J, Li W, Tam PK, Savill J (2008): Apoptotic cells protect mice against lipopolysaccharide-induced shock. J Immunol 180, 4978-4985

Roger T, Lugrin J, Le Roy D, Goy G, Mombelli M, Koessler T, Ding XC, Chanson AL, Reymond MK, Miconnet I, Schrenzel J, Francois P, Calandra T (2011): Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. Blood 117, 1205-1217

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386

Ruud TE, Gundersen Y, Wang JE, Foster SJ, Thiemermann C, Aasen AO (2007): Activation of cytokine synthesis by systemic infusions of lipopolysaccharide and peptidoglycan in a porcine model in vivo and in vitro. Surg Infect (Larchmt) 8, 495-503

Sahlin S, Hed J, Rundquist I (1983): Differentiation between attached and ingested immune complexes by a fluorescence quenching cytofluorometric assay. J Immunol Methods 60, 115-124

Savina A, Amigorena S (2007): Phagocytosis and antigen presentation in dendritic cells. Immunol Rev 219, 143-156

Schwab M, Reynders V, Loitsch S, Steinhilber D, Schroder O, Stein J (2008): The dietary histone deacetylase inhibitor sulforaphane induces human beta-defensin-2 in intestinal epithelial cells. Immunology 125, 241-251

Seki T, Yuasa S, Fukuda K (2012): Generation of induced pluripotent stem cells from a small amount of human peripheral blood using a combination of activated T cells and Sendai virus. Nat Protoc 7, 718-728

Serrador JM, Cabrero JR, Sancho D, Mittelbrunn M, Urzainqui A, Sanchez-Madrid F (2004): HDAC6 deacetylase activity links the tubulin cytoskeleton with immune synapse organization. Immunity 20, 417-428

Shakespear MR, Halili MA, Irvine KM, Fairlie DP, Sweet MJ (2011): Histone deacetylases as regulators of inflammation and immunity. Trends Immunol 32, 335-343

Silva-Campa E, Cordoba L, Fraile L, Flores-Mendoza L, Montoya M, Hernandez J (2010): European genotype of porcine reproductive and respiratory syndrome (PRRSV) infects monocyte-derived dendritic cells but does not induce Treg cells. Virology 396, 264-271

Uddin MJ, Nuro-Gyina PK, Islam MA, Tesfaye D, Tholen E, Looft C, Schellander K, Cinar MU (2012): Expression dynamics of Toll-like receptors mRNA and cytokines in porcine peripheral blood mononuclear cells stimulated by bacterial lipopolysaccharide. Vet Immunol Immunopathol 147, 211-222

Vanden Berghe W, Ndlovu MN, Hoya-Arias R, Dijsselbloem N, Gerlo S, Haegeman G (2006): Keeping up NF-kappaB appearances: epigenetic control of immunity or inflammation-triggered epigenetics. Biochem Pharmacol 72, 1114-1131

Vincent IE, Carrasco CP, Herrmann B, Meehan BM, Allan GM, Summerfield A, McCullough KC (2003): Dendritic cells harbor infectious porcine circovirus type 2 in

the absence of apparent cell modulation or replication of the virus. J Virol 77, 13288-13300

Wade PA (2001): Transcriptional control at regulatory checkpoints by histone deacetylases: molecular connections between cancer and chromatin. Hum Mol Genet 10, 693-698

Wellmer A, Gerber J, Ragheb J, Zysk G, Kunst T, Smirnov A, Bruck W, Nau R (2001): Effect of deficiency of tumor necrosis factor alpha or both of its receptors on Streptococcus pneumoniae central nervous system infection and peritonitis. Infect Immun 69, 6881-6886

Yamaguchi Y, Tsumura H, Miwa M, Inaba K (1997): Contrasting effects of TGF-beta 1 and TNF-alpha on the development of dendritic cells from progenitors in mouse bone marrow. Stem Cells 15, 144-153

Youn HS, Kim YS, Park ZY, Kim SY, Choi NY, Joung SM, Seo JA, Lim KM, Kwak MK, Hwang DH, Lee JY (2010): Sulforaphane suppresses oligomerization of TLR4 in a thiol-dependent manner. J Immunol 184, 411-419

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion e.V., Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn

Zhang Y, Zhang YY, Ogata M, Chen P, Harada A, Hashimoto S, Matsushima K (1999): Transforming growth factor-beta1 polarizes murine hematopoietic progenitor cells to generate Langerhans cell-like dendritic cells through a monocyte/macrophage differentiation pathway. Blood 93, 1208-1220

Zhu H, Shan L, Schiller PW, Mai A, Peng T (2010): Histone deacetylase-3 activation promotes tumor necrosis factor-alpha (TNF-alpha) expression in cardiomyocytes during lipopolysaccharide stimulation. J Biol Chem 285, 9429-9436

Table 1. List of primer sequences used in this study

Gene	Primer set	Anneal TM (°C)	Amp. size (bp)	GenBank accession nr.
HDAC1	F: GGAAATCTATCGCCCTCACA	60	157	XM_003356305.2
	R: AAACACCGGACAGTCCTCAC			
HDAC2	F: AACCTGCTGCTTGGAGAAAA	60	201	XM_001925318.3
	R: ACCATCAGGATGCAAAGCTC			
HDAC3	F: CAACCAGGTGGTGGACTTCT	60	152	NM_001243827.1
	R: GCAGAGGGATGTTGAAGCTC			
HDAC4	F: GGTCCTCGCCTACCTTATCC	60	189	XM_003359701.2
	R: GACGCCTGGTAGTTCCTCAG			
HDAC5	F: AGATGCACTCCTCCAGTGCT	60	102	XR_135351.1
	R: GGATGATGGCAAATCCATTC			
HDAC6	F: ATGGACGGGTATTGCATGTT	60	168	XM_003360315.2
	R: GCGGTGGATGGAGAAATAGA			
HDAC7	F: CGTCCCCTACAGAACTCTCG	60	146	XM_003355640.2
	R: TCAGGTTGGGCTCAGAGACT			
HDAC8	F: GGTGACGTGTCTGATGTTGG	60	165	XM_003360365.2
	R: AGCTCCCAGCTGTAAGACCA			165
HDAC9	F: AACTGAAGCAACCAGGCAGT	60	149	XM_003122063.2
	R: CCCAACTTGTCCCAGTGAGT			
HDAC10	F: TCCATCCGAGTACCTTCCAC	60	179	XM_003362070.1
	R: GGCTGCTATGGCCACACTAT			
HDAC11	F: GACAAGCGCGTGTACATCAT	60	143	XM_003483230.1
	R: AGGTTCCTCTCCACCTTCGT			
DNMT1	F: GCGGGACCTACCAAACAT	60	133	DQ060156
	R: TTCCACGCAGGAGCAGAC			
DNMT3a	F: CTGAGAAGCCCAAGGTCAAG	60	238	NM_001097437
	R: CAGCAGATGGTGCAGTAGGA			
CD40	F: TGAGAGCCCTGGTGGTTATC	60	235	NM_214194.1
	R: CTCTCTTTGCCATCCTCCTG			
CD80	F: TCAGACACCCAGGTACACCA	60	189	NM_214087.1

	R: GACACATGGCTTCTGCTTGA			
CD86	F: TTTGGCAGGACCAGGATAAC	60	152	NM_214222.1
	R: GCCCTTGTCCTTGATTTGAA			
TLR4	F: ATCATCCAGGAAGGTTTCCAC	58	235	NM_001097444.1
	R: TGTCCTCCCACTCCAGGTAG			
MD2	F: TGCAATTCCTCTGATGCAAG	60	226	NM_001104956.1
	R: CCACCATATTCTCGGCAAAT			
NF-κB1	F: TGGGAAAGTCACAGAAACCA	60	187	NM_001048232.1
	R: CCAGCAGCATCTTCACATCT			
TBP	F: GATGGACGTTCGGTTTAGG	60	124	DQ845178.1
	R: AGCAGCACAGTACGAGCAA			
IFN-γ	F:AGCTCCCAGAAACTGAACGA	60	225	NM_213948.1
	R:AGGGTTCAAAGCATGAATGG			
TNF-α	F: CCACCAACGTTTTCCTCACT	60	247	NM_214022.1
	R: CCAAAATAGACCTGCCCAGA			
IL-1β	F: GTACATGGTTGCTGCCTGAA	59	137	NM_001005149.1
	R: CTAGTGTGCCATGGTTTCCA			
IL-8	F:TAGGACCAGAGCCAGGAAGA	60	174	NM_213997.1
	R:CAGTGGGGTCCACTCTCAAT			
HPRT1	F: AACCTTGCTTTCCTTGGTCA	60	150	NM_001032376.2
	R: TCAAGGGCATAGCCTACCAC			

F: Forward primer; R: Reverse primer; bp: base pair.

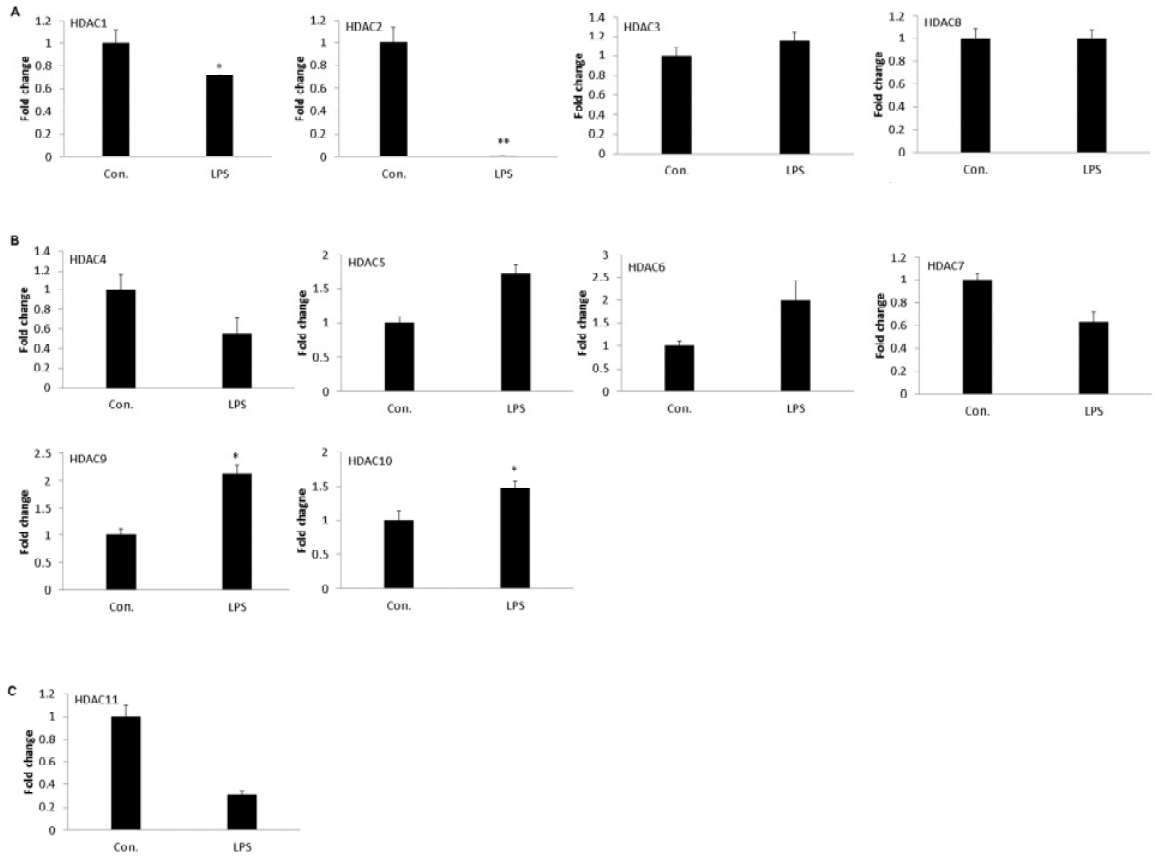

Figure 1. Expression of class I, class II, and class IV HDAC genes in porcine moDCs stimulated with LPS. The expression of HDAC family genes in moDCs were influenced with LPS (1μg/ml) stimulation for 24 h. moDCs were generated from adherent monocytes at day 7 *in vitro*, which were treated with or without LPS. The class I (A), class II (B), and class IV (C) of HDACs mRNA were quantified by qRT-PCR and normalized with the housekeeping gene HRPT1. The results were combined from three independent experiments and each experiment performed in triplicate. The data were represented as the mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$).

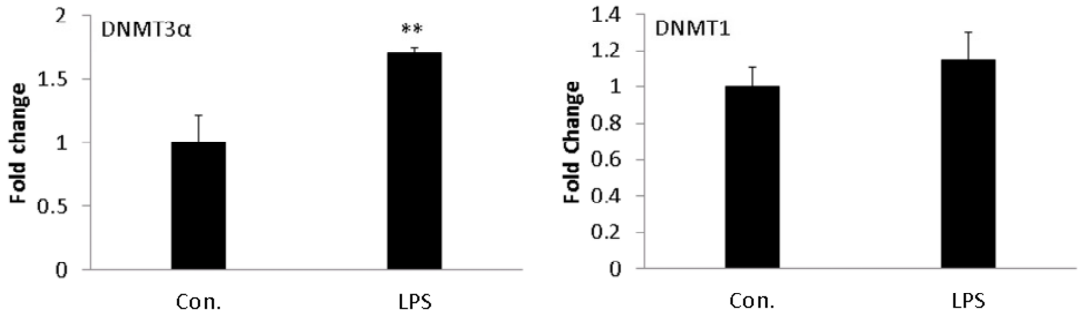

Figure 2. The effects of LPS on DNMT gene expression. Expression of genes that encode the enzymes responsible for methylating CpG sites of DNA were quantified by qRT-PCR including the maintenance methyltransferase DNMT1 and the *de novo* methyltransferase DNMT3a in moDCs in response to LPS exposure (24 h) compared with control. The results were combined from three independent experiments and each experiment was performed in triplicate. The data were represented as the mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$).

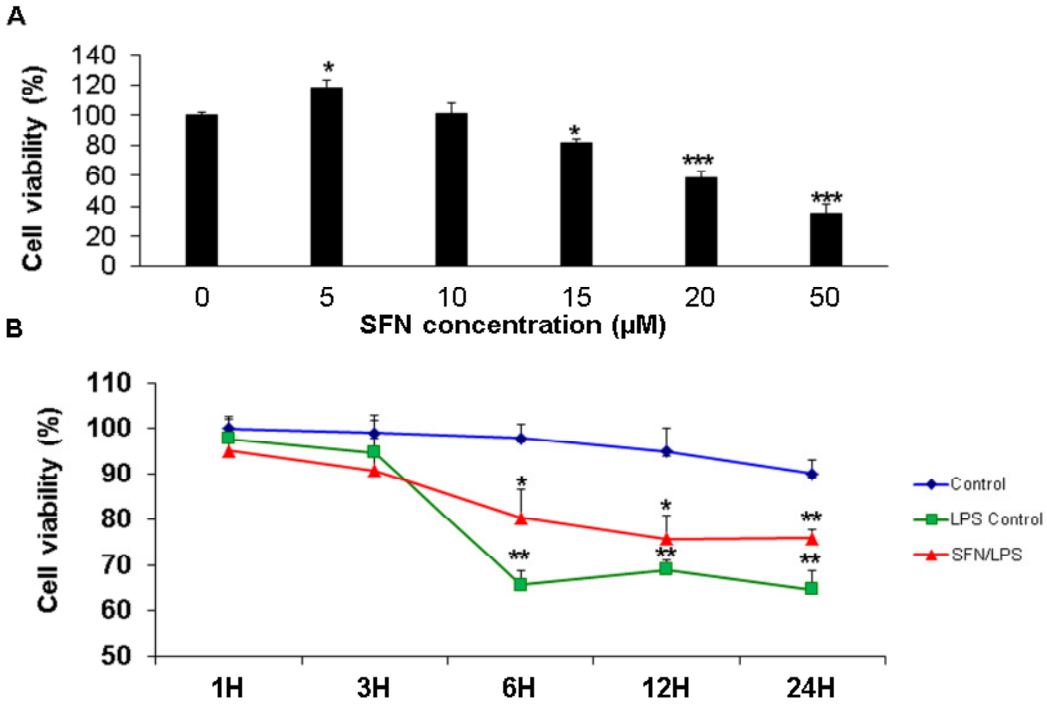

Figure 3. SFN pre-incubation inhibited LPS induce cell death in a time denpendent manner. moDCs at day 7 were used for cell viability assay by WST-1 kit. A SFN dose-dependent assay was used to confirm cell viability of moDCs after stimulating with different concentration of SFN (Control, 5 µM, 10 µM, 15 µM, 20 µM, and 50 µM) for 24h (A). For the effects of SFN on LPS induced cell death, moDCs were pre-incubated 24 h with or without SFN (10 µM) before exposed to LPS (1 µg/ml) for 1, 3, 6, 12, and 24 h (B). The results were combined from three independent experiments and each experiment was performed in triplicate. The data were represented as the mean ± standard deviations (SD) (* $P < 0.05$; **P<0,01; ***P<0,001).

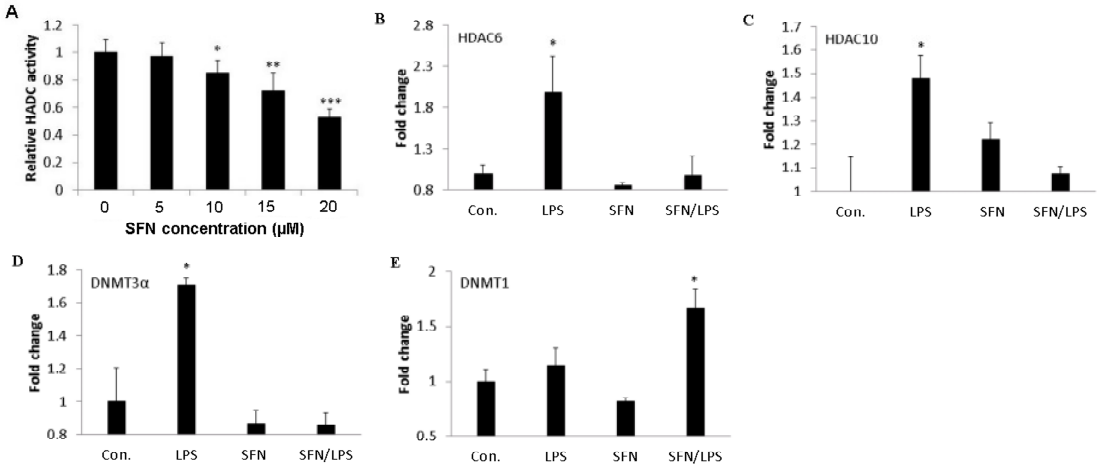

Figure 4. SFN inhibits HDAC activity and regulates genes which encode epigenetic enzymes. moDCs at day 7 in cell culture were used for this experiment. Relative HDAC activity assay was measured using the Color-de-Lys HDAC colorimetric activity assay kit. To confirm the global HDAC deacetylation of moDCs, cells were stimulated with different concentration of SFN (Control, 5 μM, 10 μM, 15 μM, and 20 μM) for 24 h (A). Equal amounts of isolated nuclear protein were subjected to HDAC activity analysis. The effects of SFN (10 μM) on gene expression of epigenetic encoding enzymes in porcine moDCs stimulated with LPS were examined. The HDAC6 (B), HDAC10 (C), DNMT3a (D) and DNMT1 (E) mRNA expression was quantified using qRT-PCR. The moDCs were pre-treated for 24 h with or without SFN before stimulating with LPS (1 μg/ml) for additional 24 h. The results (A, B, C, D, and E) were represented as the mean ± standard deviation (SD) of three independent experiments and each experiment was performed in duplicate (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

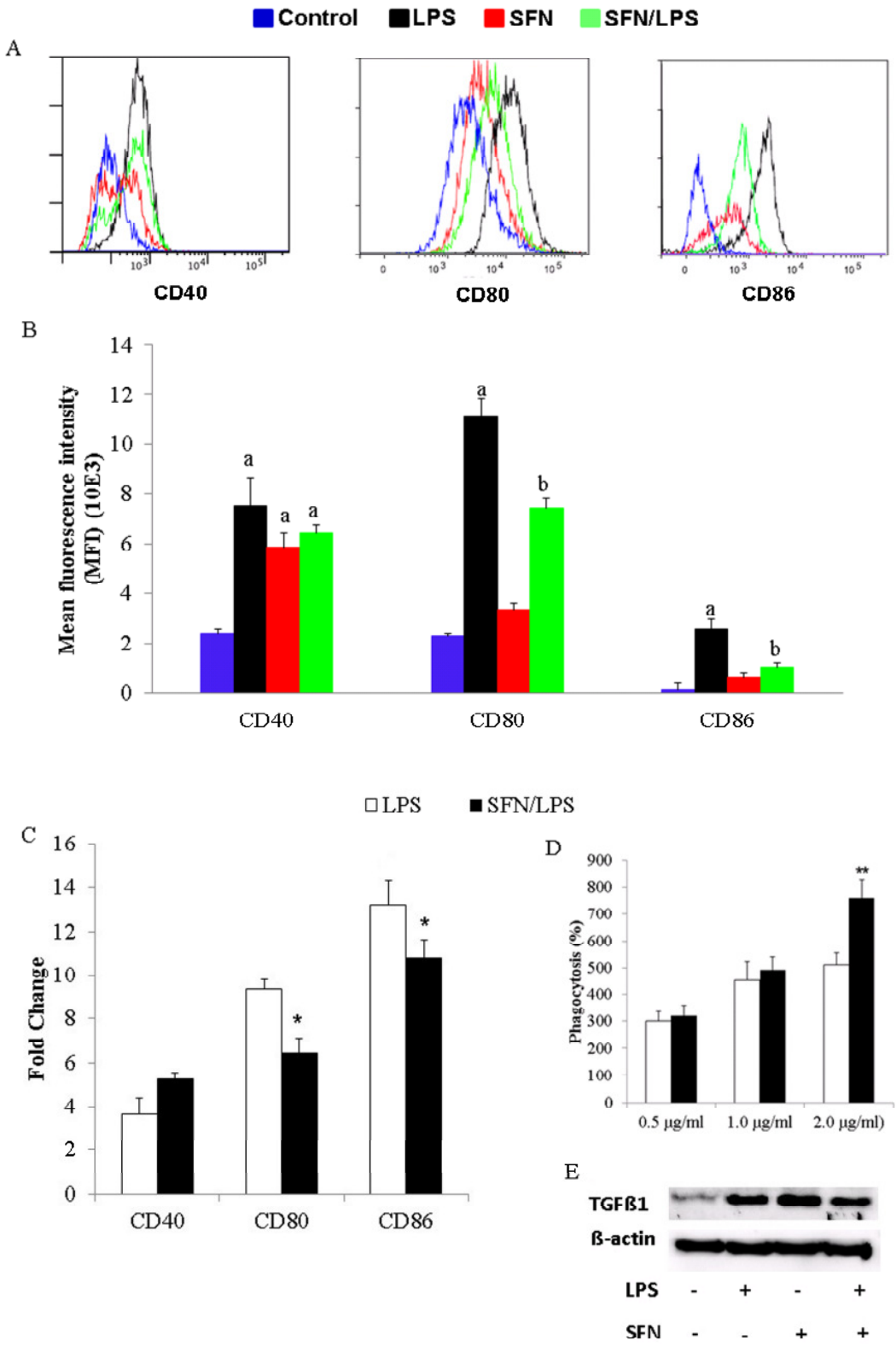

Figure 5. SFN inhibits LPS induced moDC maturation and enhances the phagocytic activity. moDCs at day 7 in culture were used for cell phagocytosis and cell differentiation status analysis. moDCs were pre-incubated for 1 h with or without SFN

(10 µM) before stimulation for 24 h LPS (1.0 µg/ml) or to the indicated concentrations. CD40, CD80, and CD86 cellular surface markers expression were analyzed by flow cytometry (A). The flow cytometry results shown were from one experiment of two independent experiments. CD40, CD80 and CD86 mean fluorescence intensity (MFI) determined by flow cytometry (B). The flow cytometry results were combined from two independent experiments and each experiment was performed from triplications. Data are mean ± standard deviations (SD) (the letters a and b $P<0.01$). The phagocytic activity of moDCs was examined after stimulating with different concentration of LPS (0.5 µg/ml, 1.0 µg/ml , and 2.0 µg/ml) with or without 24 h pre-treatment with SFN (C). The mRNA expression of DCs surface markers CD40, CD80 and CD86 were quantified using qRT-PCR (D). The mRNA expression and phagocytosis results were combined from three independent experiments and each experiment was performed in four replications. The data represented as the mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

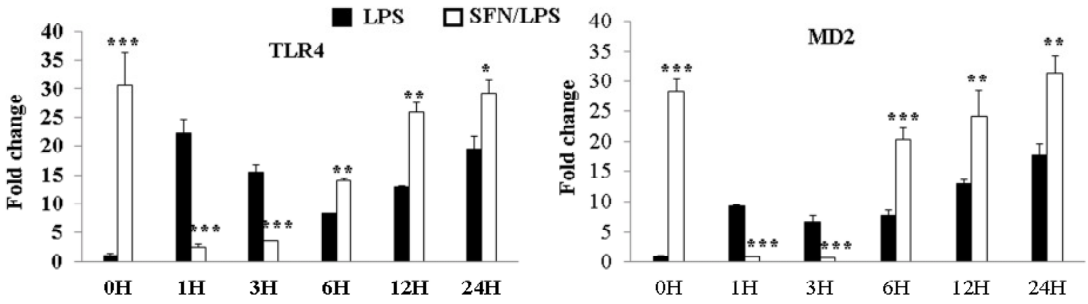

Figure 6. The effects of SFN on LPS induced up-regulation of TLR4 and MD2 gene expression. moDCs were pre-incubated for 1 h with or without SFN (10 µM) before stimulation for 0, 1, 3, 6, 12, 24 h with LPS (1.0 µg/ml). The TLR4 and MD2 mRNA expression were quantified by qRT-PCR. The results were combined from three independent experiments and each experiment was performed in four replications. The data represented as the mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

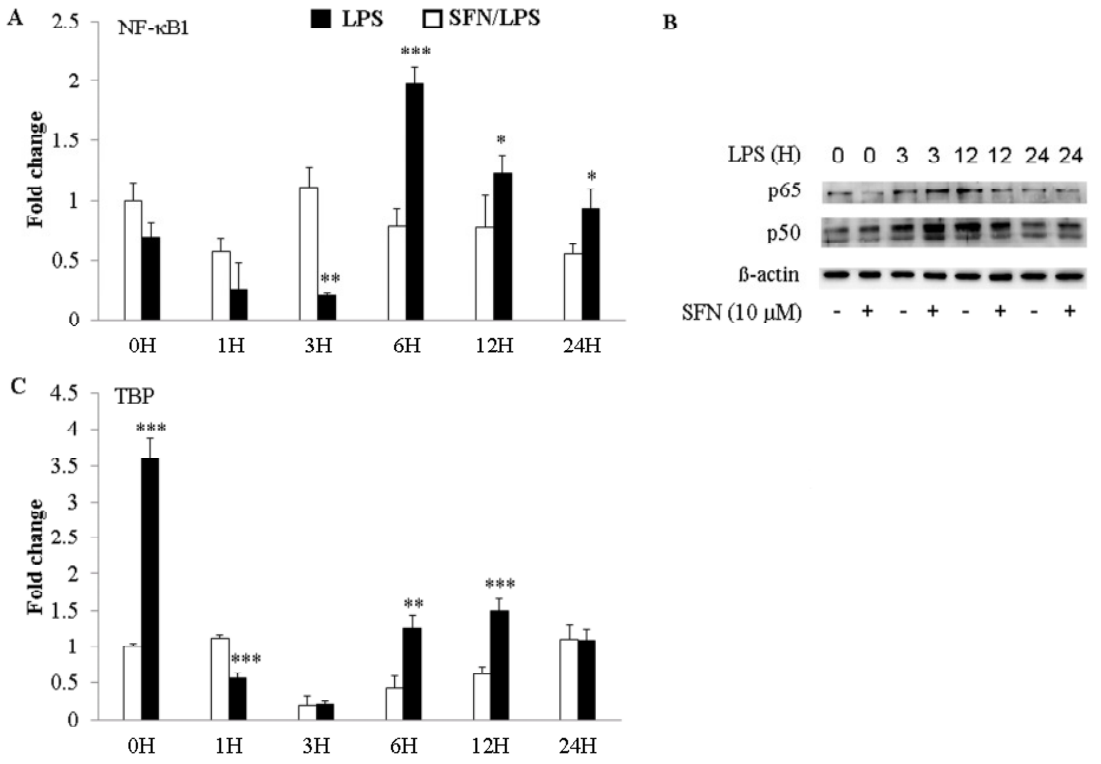

Figure 7. The effects of SFN on LPS induced NF-κB and TBP expression. moDCs were pre-incubated for 1 h with or without SFN (10 μM) before exposure to LPS (1.0 μg/ml) for 0, 1, 3, 6, 12, 24 h or to the indicated time. The transcription factor NF-κB and TBP mRNA expression were quantified by qRT-PCR (A and C). Data are mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) of triplication samples from three independent experiments. NF-κB and TBP protein expression were examined by western blotting. The results were determined from one experiment representative of two experiments. The p50 and p65 protein of NF-κB family were analyzed by western blotting by the selected time points (0, 3, 12, and 24 h) (B). The western blotting result was from one experiment of three independent experiments.

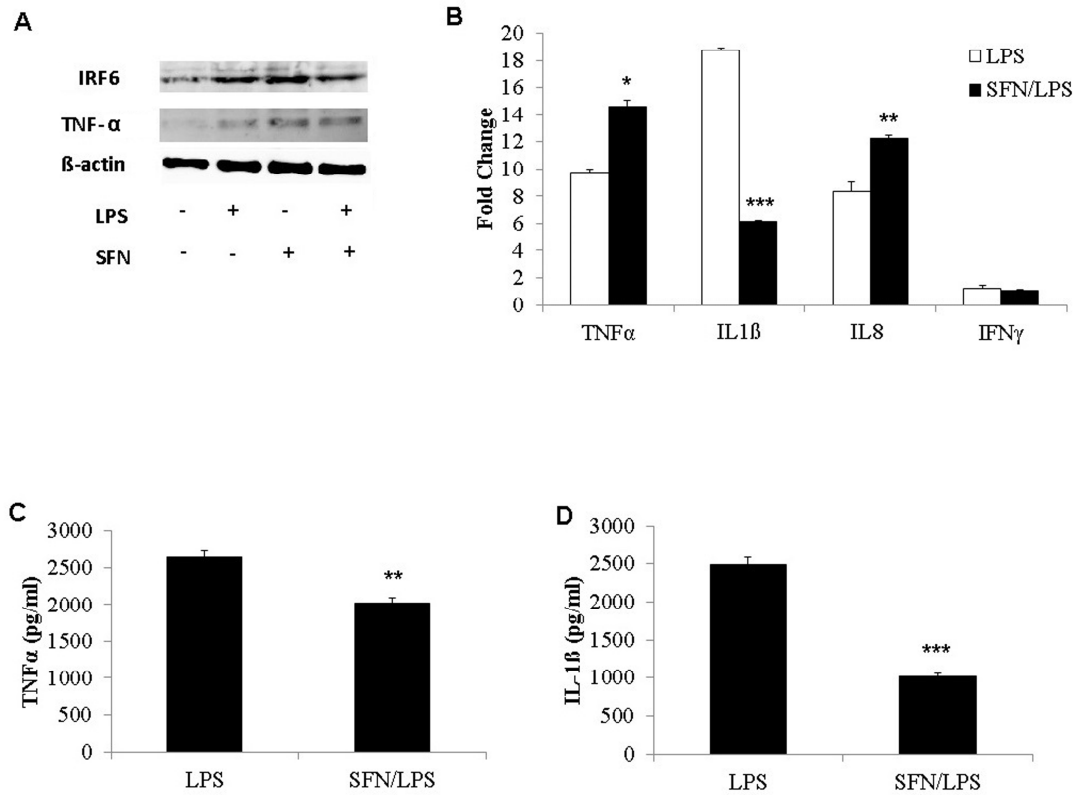

Figure 8. SFN affects gene expressions and protein productions of cytokine. moDCs were pre-incubated for 24 h with or without SFN (10 µM) before stimulation with LPS (1 µg/ml) for additional 24 h. The cell lysate proteins of IRF6 and TNF-α were analyzed using western blotting (A). The effects of SNF on gene expression of pro-inflammatory cytokines TNF-α, IL-1ß, IL-8, and IFN-γ were quantified by qRT-PCR (B). The pro-inflammatory cytokines TNF-α (C) and IL-1ß (D) secreted in cell culture supernatant were determined by ELISA. The results were combined from three independent experiments and each experiment was performed in triplicates. The data were represented as the mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

Chapter 4

Sulforaphane epigenetically regulates the LPS-induced kinetics of innate immune response in porcine monocyte-derived dendritic cells

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Key words: epigenetic; NF-κB; pig; anti-inflammatory response; HDAC inhibitors;

Abstract

Dendritic cells (DCs) are antigen-presenting cells that are specialized in initiating innate immune responses during inflammation. Inflammation is believed to be regulated by epigenetic modification including DNA methylation and histone acetylation. The histone deacetylase (HDAC) inhibitor sulforaphane (SFN), a natural isothiocyanate found in crucifers, exhibits potent ability of immuno-modulatory properties. However its anti-inflammatory actions through epigenetic modifications remain unclear. Therefore, this study aimed to investigate whether SFN epigenetically acts maintaining the DC-mediated immunomodulatory homeostasis of innate immunity during the acute inflammation. To achieve this, the monocyte-derived DCs (moDCs) were generated from porcine peripheral monocytes and exposed to LPS with or without SFN pre-incubation. Following this, we analyzed the SFN induced DNA methylation, HDAC inhibition and expression levels of immune related genes after simulation with LPS. The results unveiled that both SFN and LPS inhibited HDAC activity and caused histone H3 and H4 acetylation. In addition, treatment of moDC either with SFN or LPS induced DNA methylation in the exon 2 of TLR4 and promotor region of MHC-SLA gene. Moreover, pre-incubation of SFN suppressed the TLR4 signal pathway gene and induced inflammatory cytokines (TNF-α, IL-1ß, IL-6 and IL-8) within 3 and 6 hours (h) of LPS stimulation. Surprisingly, unlike to only LPS treatment, SFN treatment induced DNA demethylation in the promoter region of MHC-SLA gene resulting in up-regulation of TLR4, MHC-SLA genes and other immune associated gene expressions after 6 h of LPS challenge. In addition, the protein level of the TNF-α, IL-1ß and IL-8 in cell culture supernatants was significantly inhibited by SFN treatment followed by LPS stimulation in a time dependent manner suggesting that the inhibition of HDAC activity and DNA methylation in restriction of excessive inflammatory cytokines in the fluid. These data demonstrate that SFN causes major epigenetic modulations in regulating the expression of immune genes which are essential in sensing immune responses in porcine moDCs. We might suspect that SFN exerts the protective action and epigenetically influences signalling pathways in experimental conditions employing porcine moDCs in response to stimuli.

Introduction

The innate immune system is the first line of defence against the invading pathogens. In order to detect the microbial attack, the hosts relay on the sentinel cell, such as dendritic cells (DCs) and macrophages. DCs are specialized and professional antigen-presenting cells (APCs) and they regulate adaptive immune response (Fujii et al. 2004). The recognition and presenting of invasive pathogens by DCs are triggered by microbe-specific motifs known as microbial/pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) (Ray et al. 2013, Takeuchi and Akira 2010). Indeed, this process involves the coordinated actions of soluble and cellular molecules comprising complement system, acute phase proteins and membrane-associated/intracellular pattern-recognition molecules (Brown et al. 2011, Ishii et al. 2008, Roger and Calandra 2009). Many PAMPs have been found to be interacting with particular Toll-like receptors (TLRs). For example, LPS, which is one of the best studied immunostimulatory components of bacteria, induces systemic inflammation and sepsis (Jha et al. 2014, Roger and Calandra 2009) and, TLR4 is believed to be an important sensor for LPS (Crosas-Molist et al. 2014). Moreover, LPS-activated TLR4 triggers the mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB) and interferon-related factor (IRF) signal transduction pathways resulting in induction of transcription of immune genes, including cytokines which are critical for the activation of innate and adaptive immunity (Brown et al. 2011, Chen 2005, Doyle et al. 2002).

The recent progress in DCs research has opened new avenues of disease modelling, immunotherapy and transplantation of patient-specific tissues, which attracts immunologists' attention in host defence system. Progress with respect to the epigenetic mechanisms associated with the development and differentiation of the immune system, has advanced considerably in recent years (Rodriguez-Cortez et al. 2011, Suarez-Alvarez et al. 2012). The mechanisms underlying the immunomodulation mainly depend on the epigenetic regulation of genes related to the immune system. Epigenetic mechanisms alter the gene expression by either by hindering accessibility of chromatin at CpG dinucleotide or by modification of nucleosome of the DNA (Bayarsaihan 2011). Generally, methylation and acetylation of histones, DNA methylation are among the epigenetic modification that can control gene transcription and those non coding RNAs including miRNA and siRNA are considered to be posttranscriptional epigenetic regulators of gene action (Bayarsaihan 2011). Histones acetylation is an essential

epigenetic modification controlling chromatin structure, DNA accessibility for transcription factors and gene expression (Bayarsaihan 2011). Steady-state levels of acetylation of core histones result from the balance between the opposing activities of histone acetyltransferases and histone deacetylases (HDACs) (de Ruijter et al. 2003, Roger et al. 2011). The most important signal involved in initiation process of repression is situated in the DNA itself. Although HDAC could be solely responsible for the repression of gene transcription via recruitment to methylated CpG, this is not the case for most genes (Zhang et al. 2005). When HDAC action is inhibited, the transcription of the gene under study is not completely restored. So inhibition of HDACs can result in a general hyperacetylation of histiones, which is followed by the transcription activation of certain genes through relaxation of the chromatin structure. Over the years, many different types of HDAC inhibitors have been developed and they are capable of inhibition HDACs with varying efficiency (at nanomolar to millimolar concentrations) (Mogal and Abdulkadir 2006). For instance, the agents trichostatin A (TSA), vorinostat (SAHA) and suberoylanilide hydromamic acid are typical HDAC inhibitors and have been reported to be able to alter expression of less than two percent of expressed genes in tumor cells based on the study of large-scale gene expression spectrum (Nencioni et al. 2007, Roger et al. 2011).

In recent years, the isothiocyanate sulforaphan (SFN) that naturally occurs in cruciferous vegetable such as broccoli, cauliflower, Brussels sprouts, cabbage and kale is becoming an important group of natural substances with inhibition of HDAC activity. SFN has been reported to exhibit antioxidative, antimicrobial, anti-inflammatory and antitumoral properties (Cheung and Kong 2010, Mrowietz and Asadullah 2005). Numerous mechanisms have been suspected to explain the beneficial effects of SFN on chronic inflammation prevention. SFN enhances bacterial clearance by increasing the phagocytic activity of alveolar macrophages and is beneficial against Gram-negative bacteria infection (Fahey et al. 2002, Johnson et al. 2010). A recent study described that SFN has been suggested to inhibit T cell-mediated autoimmune disease in human DCs through impairment TLR4-induced IL-23 and IL-12 expression and results in up-regulation of the stress response protein heme oxygenase-1 (Geisel et al. 2014). However, these mechanisms include epigenetic changes resulting from the inhibition of HDAC activity (Dashwood and Ho 2008, Myzak et al. 2007).

Although it was shown that SFN may have anti-inflammatory effects, the epigenetic mechanisms of anti-inflammation are less understood. In this study, we used the porcine monocyte-derived DC (moDC) in vitro, which were stimulated with Gram-negative bacterial component LPS, to mimic a state of either infection or inflammatory sepsis. Here, we provided the evidence that SFN protects hyper-activation induced cells death, apoptosis and inflammatory sepsis caused by microbial stimuli through modulating both DNA methylation and histone modification to regulate the HDAC activity and the level of inflammatory cytokine inductions. To the best of our knowledge, the present study provides the first laboratory evidence that SFN recruits the essential epigenetic modifications of transcription and protein synthesis process, thereby resetting the elevated cytokines distribution at the infection site during acute inflammatory condition.

Materials & Methods

Ethics statement

A total of six 35-day-old healthy piglets (Pietrain) with no clinical symptoms or serological evidence of influenza and other respiratory or systemic diseases were used for the study. Animals were housed in the accredited barrier-type animal facilities at teaching and research station of Frankenforst farm, Universty of Bonn, Germany. The feeding, housing and husbandry practices of the animals were carried out in accordance with the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes and in accordance with the German performance testing guidelines, observing the animal protection law (ZDS 2003).

Generation of moDCs

moDCs were derived from cultured porcine monocytes from peripheral blood monocular cells (PBMCs) according the previous description (Qu et al. 2014). Briefly, PBMCs were isolated from porcine peripheral blood using Ficoll-Histopaque (cat. 10771; Sigma, Germany). PBMCs were cultured in Dulbecco's modified Eagle medium (DMEM) (cat. 41966-029; Invitrogen, Germany) supplemented with 2% fetal bovine serum (FBS) (cat. 10270; Invitrogen, Germany) and 500 IU/ml Penicillin-Streptomycin (cat. 15140; Invitrogen, Germany) and 0.5% fungizone (cat. 15290-026; Invitrogen, Germany) for 4 hours (h). The non-adherent cells were removed by vacuum aspiration

and the adherent monocytes were washed two times using pre-warmed (37 °C) DPBS (cat. 14190-094; Invitrogen, Germany). The cleaned monocytes were cultured in RPMI-1640 medium (cat. 21875; Invitrogen, Germany) supplemented with 10% FBS, 1000 UI/ml Penicillin-Streptomycin, 1% fungizone, 20 ng/ml recombinant porcine (rp) granulocyte-macrophage colony-stimulating-factor (GM-CSF) (cat. 711-PG-010; R&D System, UK) and 20 ng/ml recombinant porcine (rp) interleukin-4 (IL-4) (cat. 654-P4-025; R&D System, UK) for 7 days at 37 °C with 5% CO_2. Half of the medium was replaced on the 3rd day with the fresh medium supplemented the full recombinant porcine (rp) GM-CSF (20 ng/ml) and rp IL-4 (20 ng/ml) concentration. After 7 days of incubation, the adherent moDCs were counted and re-cultured in a new plate for the subsequent assays.

Cell treatment conditions

moDCs were seeded in the 6-well plate with the 2×10^6 cells/well concentration and cultured in CO_2 incubator at 37 °C for 24-48 h. Cells were first exposed to 10 µM SFN for 24 h and the untreated cells were used as a control or activated with 1 µg/ml LPS. Afterwards, medium was replaced and 1 µg/ml LPS (cat. # tlrl-3pelps; InvivoGen) added for 1, 3, 6, 12 and 24 h. The cells were then collected for genomic DNA, total RNA and protein extraction or staining for flow cytometry (FAC) or phagocytic activity assay. Likewise the cell culture supernatants were also collected at different time points of treatment to be used for protein investigation using enzyme linked immunosorbent assay (ELISA).

mRNA quantitation using quantitative real time PCR.

Total mRNA from cells was extracted with miRNeasy Mini Kit (cat. 217004, Qiagen) and complementary DNA (cDNA) was synthesized using miScript II RT kit (cat. 218161, Qiagen) as described previously (Qu et al. 2014). Quantitative real time PCR (qRT-PCR) was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems). Gene specific primers (Table 1) were designed using the online Primer3 Program (version 0.4.0) (Rozen and Skaletsky 2000). At the end of PCR reaction, melting curve analysis was performed to detect the specificity of the PCR reaction. Each experiment was performed in triplicates and each sample was quantified in duplicate (technical replication). Primer sequences of specify gene amplifications a given in Table (1). Relative mRNA expression was normalized to the house keeping

genes hypoxanthine phosphoribosyltransferase 1 (HPRT1) and glyceraldehyd-3-Phosphat-Dehydrogenase (GAPDH). Gene expression was statistically analysed using the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001).

Cytokines and chemokine measurement

For cytokines and chemokine investigation, moDCs cell culture supernatants were collected at different time points after LPS-treatment. We used the commercially available enzyme linked immunosorbent assay (ELISA) kits for the quantification of cytokine tumor necrosis factor alpha (TNF-α) (cat. PRA00; R&D systems; Abingdon, UK), interleukin 1ß (IL-1ß) (cat. PLB00B; R&D systems), interferon gamma (IFN-γ) (cat. PIF00; R&D Systems) and chemokine IL-8 (cat. P8000; R&D Systems), as per instructions of the manufacturer.

Western blotting

Cells were harvested and lysed using the commercial AllPrep® DNA/RNA/Protein Mini kit (cat. 80004; Qiagen). Equal amount of cell lysates were loaded and electrophoresed through Precast Gels and transferred onto the nitrocellulose membranes and confirmed with ponceau S staining (Qu et al. 2014). Membranes were incubated with antibodies specific for anti-acetylated histone H3 (H3-Ac) (cat. 06-599; Millipore; MA; USA) and H4 (H4-Ac) (cat. 06-866; Millipore; MA; USA) and ß-actin (cat. Sc-47778; SANTA CRUZ Biotechnology; Heidelberg; Germany) and then identified with horseradish peroxidase (HRP)-conjugated with the secondary antibodies (such as donkey anti-goat, sc-2020, Santa Cruz BIotechnology; goat anti-rabbit, sc-2004, SANTA CRUZ Biotechnology) and SuperSignal West Pico Chemiluminescent Substrate (cat. 34077, Thermo Scientific).

Apoptosis assay

The moDCs were treated with LPS and SFN as previously indicated. Caspase 3 and 9 activities were determined from the cell lysates using the Caspase-3/CPP32 Colorimetric Assay Kit (cat. #K106-25; BioVision; CA; USA) and Caspase-9 Colorimetric Assay Kit (cat. #K119-25; BioVision; CA; USA) according to the manufacturer´s protocol. 100 µg proteins were used for each assay. The samples were measured at 405 nm in a microtiter reader (Thermo max; Germany).

Methylation analysis

Genomic DNA was isolated from each treatment groups of moDCs using an AllPrep® DNA/RNA/Protein Mini Kit (cat. 80004, QIAGEN) according to the manufacturer´s instruction. To analyse methylation of CpG motifs, 300 ng of genomic DNA was bisulfite treated using EZ DNA Methylation-Direct^{TM} Kit (cat. D5020, Zymo Research) following the manufacturer´s protocol. The exon region of TLR4 and promoter region of MHC-SLA gene were applied to the online program Methprimer to appraise the CpG island (Li and Dahiya 2002). The primer pairs, containing the predicted CpG island, were designed by PerlPrimer and Methyl Primer express Software v1.0 (applied Biosystems Inc.) (Table 1) (Marshall 2004). The promoter region of candidate genes was amplified by PCR primer pairs and the PCR amplifications were purified by QIAquick PCR purification kit (cat. 28104, QIAGEN). Following this, the purified PCR products were subcloned into the pGEM T-easy vector (cat. A1360, Promega). A total of 4-8 positive clones from each sample were sequenced by CEQ8000 sequencer system (Beckman Coulter) using the M13 primers.

Statistical analysis

The data were subjected to analysis of variance (ANOVA) procedures using the SAS software package v. 9.2 (SAS institute, Cary, NC, USA). Pairwise comparisons were performed between the treatment groups, using Student's t test and Tukey's multiple comparisons. The data were expressed as means ± standard deviations (SD) and *(*) $P <$* 0.05 were set as statistically significant.

Results

SFN induced histone acetylation and inhibited HDAC activity

In this study, we investigated the influences of pre-treatment of SFN on HDAC activity in either physiological or pathological (LPS challenge) moDCs. The result showed that both SFN and LPS significantly inhibited HDAC activity in porcine moDCs (Fig. 1A). On the other hand, pre-incubation of moDCs with SFN remarkably supressed LPS induced inhibition of HDAC activity. The protein analysis also indicated that both a SFN and LPS treatment was found to induce the histone acetylation and SFN enhanced LPS induced histone H3 but not H4 acetylation (Fig. 1B).

SFN or LPS treatment enhanced while pre-treatment with SFN followed by LPS reduced DNA methylation in the exonic region of TLR4 gene

SFN, acting as a potent functional phytochemical, can induce the anti-inflammatory target in TLR4 signalling (Koo et al. 2013, Youn et al. 2010). In order to further understand the epigenetic modulations of SFN that occurred in the LPS induced TLR4 gene region, we assessed epigenetic-related DNA methylation modification in the exonic region of TLR4 gene (Fig. 2C). The analysis of DNA methylation patterns was generated previously from LPS induced inflammatory moDCs with or without pre-incubation of SFN. First, we quantified the LPS-induced TLR4 gene expression at 24 h and the LPS-induced the TLR4 corresponding gene MyD88 expression with or without SFN pre-treatment in a time dependent manner using qPCR (Fig. 2A and 2B). Unexpectedly, TLR4 was significantly up-regulated by all treatments and the pre-incubation of SFN remarkably increased LPS-induced TLR4 expression (Fig. 2A). SFN pre-incubation significantly inhibited the LPS-induced MyD88 gene expression within 3 h of LPS stimulation. Surprisingly, SFN significantly enhanced the LPS-induced MyD88 gene expression after 6 h of challenge (Fig. 2B). Additionally, the MyD88 gene was significantly increased in SFN treated group (Fig. 2B).

In order to address the whether the alterations of TLR4 and MyD88 gene expressions interfered by epigenetic modification, we further determined the DNA methylation status followed by SFN and LPS treatment. Regarding to the previous study of the alterations of LPS-induced DNMT1 and DNMT3α (data not shown) by SFN, we plotted 10 CpG motifs in the CpG island (Fig. 2C) next to the exon of porcine TLR4 regulation region and examined the DNA methylation status using bisulfite sequencing under the indicated treatments. The result showed that the number of methylated motifs was distinctly higher in the SFN and LPS with SFN pre-treated groups compared to those treated with only LPS and untreated control groups. Moreover, LPS partly demethylated the SFN-induced DNA methylation in SFN+LPS group (Fig. 2D). More interestingly LPS also induced DNA methylation of TLR4 gene in porcine moDCs (Fig. 2D). However, there seems no direct correlation between the DNA methylation and gene expression of TLR4 in porcine moDCs.

SFN or LPS treatment enhanced while pre-treatment with SFN followed by LPS demethylated DNA methylation in the promoter region of MHC-SLA gene

Although the DNA methylation occurred in the TLR4 gene region, the results didn´t explain the relation of DNA methylation of TLR4 and related TLR4 and MyD88 gene expression in porcine moDCs. To further validate the results, the other essential immune mediator gene MHC-SLA with identified promoter region and CpG rich-regions were analyzed for gene expression and DNA methylation levels (Fig. 3B). The qRT-PCR result showed that MHC-SLA1 gene was significantly upregulated in the SFN group (Fig. 3A). Interestingly, the pre-treatment of SFN significantly inhibited the LPS induced MHC-SLA gene expression within 3 h of LPS stimulation, whereas LPS induced gene expression of MHC-SLA1 were remarkably reversed in the SFN pre-treated group at 6 and 12 h post treatment (Fig. 3A). The DNA methylation pattern of 21 CpG motifs in MHC-SLA gene promoter was analyzed after treatment for 24 h with SFN and LPS using was the bisulfite sequencing. (Fig. 3B) As is shown in Fig. 3C, all the treatment groups were found be frequently methylated compared to the control group (Fig. 3C). Notably, the limited results of the samples showed that the pre-incubation of SFN resulted in demethylation of the LPS-caused DNA methylation of MHC-SLA promoter region in porcine moDCs (Fig. 3C).

SFN induced caspase-3 and caspase-9 activity and pre-treatment of SFN inhibited the inflammation induced cell apoptosis

The caspase-9 and caspase-3 are the cell death caspases initiator and executioner of apoptosis respectively (Nadiri et al. 2006). To investigate whether caspase-9 and caspase-3 are involved in LPS induced mature porcine moDCs apoptosis, the SFN induced cell death and the effects of SFN pre-incubation on LPS-induced inflammatory cell apoptosis was studied. The result of this analysis has shown that SFN significantly induced the caspase-3 and caspase-9 Fig. 4A and B. In addition, LPS also significantly increased caspase-9 activity (Fig. 4B). Importantly, the pre-incubation of SFN significantly inhibited caspase-3 and caspase-9 in LPS-induced inflammatory moDCs in pig (Fig. 4A and 4B). On the other hand, the LPS-treatment showed no different caspase-3 activity compared with control group (Fig. 4A).

The SFN dynamically regulated LPS-induced Nrf2 and STAT3 gene expression

The transcription factor expressions were quantified by qRT-PCR in moDCs challenged in a time-dependent manner either with or without SFN pre-treatment. The SFN significantly inhibited LPS-induced up-regulation of Nrf2 gene expression 3 h post

treatment, whereas the effect of the SFN on gene expression of Nrf2 was rapidly reversed 6 h post LPS exposure (Fig. 5A). On the other hand, SFN significantly inhibited LPS-induced STAT3 gene expression 1 h post treatment, but the expression of STAT3 was significantly up-regulated 6 h post treatment (Fig. 5B).

SFN significantly inhibited the LPS-induced pro-inflammatory cytokine secretions

We also determined the effect of SFN treatment on the pro-inflammatory cytokine expressions in LPS-induced inflammatory moDCs. For this, we analysed LPS-induced productions of pro-inflammatory cytokine in cells pre-incubated with or without SFN using qRT-PCR and ELISA. The results from this analysis indicated that SFN pre-treatment significantly down-regulated the expression level of the inflammatory cytokines namely, TNF-α, IL-1ß, IL-8 and IL-6 3 h after LPS stimulation (Fig 6A, C, D, E, G). However, the expression level of these genes was upregulated within 6 and 24 h of post LPS stimulation. Additionally, the protein level of the TNF-α (Fig. 6B), IL-1ß (Fig. 6D) and IL-8 (Fig. 6E) showed that HDAC inhibitor SFN significantly inhibited the inflammatory cytokine productions of LPS stimulation in a time dependent manner.

SFN dynamically regulated the LPS-induced CXCL2 and CCL4 expression level

In order to further clarify the effect of SFN treatment on LPS-induced inflammatory phenotypic plasticity of moDCs, we determined the chemokine gene expressions of CXCL2 and CCL4 in a time dependent manner. The results revealed that SFN pre-treatment significantly inhibited the expressions level of CXCL2 and CCL4 from 1 to 3 h of LPS stimulation (Fig. 7A and 7B), whereas the pre-incubation of SFN rapidly reversed the significant up-regulation of LPS-induced CXCL2 and CCL4 mRNA expressions within 6 to 24 h post stimulation (Fig. 7A and 7B).

Discussion

The present study showed that HDAC inhibitor SFN has an essential role for acetylation of histone and non-histone proteins, in the regulation of inflammatory and innate immune gene expressions and in the cell defensive responses against LPS in porcine moDCs. The global HDAC activity analysis revealed that SFN inhibits HDAC activity and induce the cell apoptosis in absence of LPS challenge. SFN as HDAC inhibitor exerts the DNA methylation induction in both TLR4 and MHC-SLA genes. The TLR4 and MHC-SLA gene transcription are epigenetically altered and further supressed the

secretion of inflammatory cytokines in cell culture supernatants but not in cellular-resident cytokines, in porcine moDC to prevent excessive inflammatory responses. We also reported that SFN and LPS induce DNA methylation of TLR4 and MHC-SLA gene. Notably, in this study, we first report that SFN shows the functional demethylation of inflammatory-induced hypermethylation of MHC-SLA in porcine moDCs. Unexpectedly, we found that SFN sustained the LPS-induced inflammatory and innate immune gene expressions in the prolongation of LPS presence in porcine moDCs (within 6 and 24 h of treatment). Nevertheless, the expression of numerous TLR4/MyD88-dependent genes (such as MD2, MyD88, TBP, STAT3) was strongly inhibited by SFN in the early stage of LPS challenge.

SFN, is a naturally occurring constituent, was first reported to process potent anti-inflammatory properties in response to LPS through inhibition of NF-κb-DNA binding in murine macrophages (Heiss et al. 2001). SFN impaired the production of inflammatory cytokines in response to LPS and increased the anti-inflammatory activity through the inhibition of LPS with TLR4/MD2 complex by preferential binding to MD2 in murine bone marrow derived macrophages (Koo et al. 2013). Indeed, our results consistently showed that the inflammatory cytokine productions induced by LPS were attenuated by pre-incubation of SFN. But surprisingly, the LPS-induced TLR4 gene expression was reversed by SFN of the delaying inflammation process. Therefore, we suspected that the inhibition of LPS-induced inflammatory cytokines in TLR4 signalling pathway by SFN may not through the published mechanism that SFN inhibits the engagement of LPS with TLR4/MD2 complex (Koo et al. 2013, Youn et al. 2010). Therefore, we propose that the anti-inflammatory effects of SFN may involve different mechanisms. SFN could affect the expression of genes potentially involved in immune modulation through the inhibition of HDAC activity via histone hyperacetylation acting as HDAC inhibitor (Dashwood et al. 2006).

Although SFN inhibited HDAC activity has been well studied providing the acting evidences of increase in both global and local histone acetylation status in human cancer cells and peripheral blood mononuclear cells (PBMCs) (Myzak et al. 2004, Myzak et al. 2007), the anti-inflammatory response mechanism via the HDAC function remains unknown. In agreement with the reports, we found not only SFN but also LPS can inhibit the global HDAC activity. Interestingly, in the present study, the relative HDAC

activity levels varied between physiological and pathological status of porcine moDCs based on the fluorescent values. For instance, SFN treatment inhibited the HDAC activity in absence of LPS stimulation, whereas SFN pre-incubation didn´t show changes of HDAC activity on the presence of LPS stimulation compared with normal porcine moDCs group. Reasonably, the inhibition of HDAC activity is accompanied by a global increase in histone H3 and H4 acetylation. Our study revealed that SFN induced a difference in the extent of accumulation of acetylated histones in the absence or presence LPS stimulation. In fact, HDACs act as both positive and negative regulators of innate immune response via TLR signaling. For example, the HDACs deacetylated mitogen-activated protein kinase phosphatase (MKP)-1 which could contribute to sustain p38 activation and trigger TLR-inducible inflammatory response (Cao et al. 2008), whereas the inhibition of HDACs by HDAC inhibitor TSA act as potent and selective negative regulators of LPS-induced pro-inflammatory gene expression and act to prevent excessive inflammatory responses in human macrophages (Aung et al. 2006). Therefore, in this study, the different effects of SFN on the HDAC activity indicate that LPS-induced inflammatory moDCs may have a greater ability to resist the downstream effects of HDAC inhibition, thereby accumulating acetylated histones, than the physiological moDCs in pig. Moreover, the inhibition of LPS-induced HDAC activity may target epigenetic alteration in early stage of acute inflammatory process, which probably prevents the excessive inflammatory cytokines expression.

We next focused upon the epigenetic modifications of SFN on LPS-triggered TLR4 signal transduction and TLR4-induced cell surface molecule expression of MHC-SLA. SFN inhibited the LPS-induced TLR4 expression via the blockade of oligomerization (Youn et al. 2010). In contrast, we found that with the prior administration of SFN, the LPS-induced TLR4 gene expression was not always inhibited within 24 h of stimulationn (data not shown). So far, we know that epigenetic modulation of SFN is not only included the inhibition of HDAC enzymes but also involved in alteration of DNA methylation (Hsu et al. 2011). Therefore, in the present study, we aimed to investigate the effects of prior administration of SFN on DNA methylation status of TLR4 and MHC-SLA on either promoter or exon regions and how alteration in DNA methylation impacts the relative gene expressions in porcine moDCs.

In order to appraise the capacity of epigenetic alteration which mediates the effect by SFN on TLR4 expression, we determined the methylation status of CpG island in TLR4 exon region in response to LPS in moDCs (pathological DCs) as well as control physiological moDCs. In pig, TLR4 doesn´t contain the repeat CpG sequence dinucleotide and doesn´t present a typical CpG island (very scarce CpG sites) neither in the promoter region nor in the first exon. In the present study, the methylated CpG motifs are found around to the second exon region of the TLR4 which occur in both LPS and SFN treatment groups and the SFN causes a more frequent methylation of CpG motifs compared with LPS stimulation. Surprisingly, the prior treatment of SFN could alter the effect of LPS induced DNA methylation of TLR4 in CpG island. The similar finding in previous studies reveal that high-dose (1 µg/ml) LPS-induced TLR4 promoter methylation in human epithelial cells contributes to maintain the homeostasis in intestinal commensal system to resident bacteria and regulating mucosal inflammation in gut (Takahashi et al. 2009). The effects of the hyper-inflammation induced DNA methylation are variable regarding to the different cell types, tissues, species and stimuli. In the LPS-tolerance cell model of macrophages, it has been reported that LPS tolerance caused the DNA methylation to further restrict the expression of the pro-inflammatory cytokines (El Gazzar et al. 2008). The DNA methylation of LPS tolerance may implicate for pro-inflammatory gene silencing associated with severe systemic inflammation. In addition, the effects of SFN on the common epigenetic mark of DNA methylation have been reported in the previous studies that SFN could cause a reduction of DNA methylation to induce a site-specific demethylation in cancer cells (Hsu et al. 2011, Meeran et al. 2010). We found the obviously alteration of DNA methylation in exon region of TLR4 by SFN pre-treatment after 24 h LPS stimulation, although less DNA demethylation show up in this study. It is necessary to point out that the CpG island of TLR4 is far away from the functional promoter region and the CpG island doesn´t exist in the exons. Thus, the SFN or LPS induced DNA methylation of TLR4 may not tightly influence the downstream immune gene expression.

In order to further confirm the epigenetic modulation of SFN on innate immune response in porcine moDCs, we also have investigated another crucial cell surface molecule, the MHC-SLA gene expression. In contrast to TLR4 gene, the gene promoter

of MHC-SLA contains the repeat sequence of CpG motifs and the typical CpG Island containing two critical transcriptional sites (NF-κB and TBP (TATA box binding sites). It has been well studied that the NF-κB plays a pivotal role in the regulation of immune gene including inflammatory cytokines and chemokines in innate immune system (Ruland 2011, Sun et al. 2012). As well, TBP has a majority task to initiate the transcription by the RNA polymerase from the promoter (Pugh 2000). Therefore, it is very important to measure the effect of SFN on the DNA methylation of the HC-SLA gene in the promoter region. Similar to the TLR4 gene, both LPS and SFN could induce DNA methylation of MHC-SLA in the promoter region. It is possible that LPS- and SFN-mediated both HDAC inhibition and DNA methylation which may selectively allow remodelling of chromatin structure to suppress the affinity of functional transcription factors for their binding sites. In other words, the LPS or SFN caused DNA methylation indirectly inhibited the NF-κB and TBP expression (data not shown), leading to the protection of excessive inflammatory cytokine induced sepsis (Kominsky et al. 2011). Notably, in this study, the up-regulation of MHC-SLA in the SFN pre-treatment group seems to be the cause, at least in part, by the DNA demethylation in the promoter region. As we know that MHC-SLA are highly polymorphic in porcine and have been reported to greatly influence the immunological traits (Mallard et al. 1989). The up-regulation of SLA (MHC) in the inflammatory immune cells allows to recognize T cell and to increase the cytotoxic activity (Lecours et al. 2011, Lumsden et al. 1993). Therefore, pre-treatment of SFN in porcine moDC may play a role in the development of the innate and adaptive immunity during a challenge with LPS (or microbes).

Our previous study (data not shown) along with others has reported that HDAC inhibitor could interfere with the activation of NF-κB signal transduction pathway (Horion et al. 2007, Kramer et al. 2006). It is known that LPS-induced TLR4 activation promotes the activation of NF-κB and regulates the expression of inflammatory cytokines, chemokines and transcription factors (Brown et al. 2011). Although quite good numbers of studies have been documented indicating that the HDAC inhibitors negatively regulate the LPS induced pro-inflammatory cytokine productions in TLR4 signal pathway, we report here that SFN acting as a HDAC inhibitor increases phased in the pro-inflammatory cytokine expressions within 24 h LPS stimulation. For example,

consistent with the previous reports, SFN inhibited the TNF-α mRNA expression to LPS for the duration of 3 h. TNF-α is well-known to trigger the mobilization of DCs to peripheral lymph nodes and to promote DCs maturation and to slow the growth of many pathogens (Angeli and Randolph 2006, Randolph et al. 2005). But the amount production of the TNF-α is the double-edged sword. The excessive TNF-α, inducing other inflammatory cytokines such as IL-1ß, IL-8 and IL-6, leads to a chronic inflammatory state and changes the properties of several other immune cell types, which can increase cell apoptosis and implicates the cancer initiation and promotion (Kundu and Surh 2008, Sethi et al. 2008). Therefore, SFN restricting the early production of TNF-α may contribute to the protection of moDC from the excessive TNF-α induced cell apoptosis and hyper inflammatory response in pig. Indeed, the inhibition of caspase 3 and caspase 9, involved in the cell apoptosis (Aggarwal et al. 2004, Mak and Yeh 2002, Manabe et al. 2004), consistent with the TNF-α reduction showed that SFN inhibited LPS induced moDCs apoptosis in pig. In addition, among the pro-inflammatory cytokines, LPS-induced IL-1ß, IL-8 and IL-6, as the principle pro-inflammatory cytokines, function similarly to TNF-α that they mediate acute inflammatory response. Expectedly, likewise the expression of TNF-α, IL-1ß, IL-8 and IL-6 also were inhibited by the SFN to LPS for the duration of 3 h. This result along with the previous studies reasoned that HDAC inhibitors may prove to be beneficial as adjunctive therapy for the infectious-induced septic shock (Bode et al. 2007, Doherty et al. 2013, Roger et al. 2011). Beside the effects of SFN on the production of pro-inflammatory cytokine with LPS stimulation, we also selectively determined the expression chemoattractant of CXCL2 and CCL4, which are strongly induced by LPS stimulation and recruited by professional antigen-presenting cells to promote the cell migration (Le et al. 2004, Wiesner et al. 2010). In the chemokines similarly to the pro-inflammatory cytokines, SFN impaired chemokine expression in porcine moDCs when stimulated by LPS for 3 h. Additionally, it is widely accepted that SFN impairs the inflammatory activity mainly through the activation of transcription factors of Nrf2 and STAT3 (Gonzalez-Guerrero et al. 2013, Lee et al. 2013, Lin et al. 2008). Consistently, in the present study showed the tightly correlation between the inhibition of pro-inflammatory cytokine expression and reduction of Nrf2 and STAT3 of SFN with stimulated LPS. These results indicated that the anti-inflammatory activity of SFN is modulated by multi-signal mechanisms.

Notably, the main finding of the study is the fact that the HDAC inhibitor SFN doesn´t impaire the inflammatory responses of moDCs overall the whole defences procedure against LPS. Yet, we found that the prior administration of SFN increased the phagocytosis of moDCs to LPS challenge converting an acute inflammation into a beneficial immune defence and markedly increasing cell viability (data not shown). In the time-dependent manner with LPS stimulation, SFN dynamically administrated the inflammatory responses. For instance, SFN early (no more than 6 h LPS challenge) inhibited the immune gene expressions including transcription factors, cytokines and chemokines to LPS exposure in antigen specific (TLR4 pathway) and non-specific pathway (MHC pathway), while converse the expression pattern of immune genes after prolongation of LPS challenge (more than 6 h duration of LPS challenge). However, the protein productions of pro-inflammatory cytokine TNF-α, IL-1ß and IL-8 in cell culture supernatants were markedly suppressed at 24 h of LPS stimulation. The SFN up-regulation of the immune genes in the porcine moDCs to LPS may be a reflection of the influence of epigenetic modulation. The HDAC inhibitor SFN induced histone acetylation and DNA demethylation at either the exon or promoter region together leads to up-regulation of the expression of important immune genes. But, interestingly, the LPS induced (for 24 h) protein production of TNF-α from the cell lysates was obviously up-regulated by the SFN administration (data not shown). The inflammation induced TNF-α in fact has cytotoxic functions on immune cells and stimulates numerous inflammatory mediators including IL-1ß, IL-8 and IL-6, which are critical for inflammation and tissue damage (Beutler and Cerami 1988). The relative amount of TNF-α induction during the acute inflammatory infection can survey and evaluate whether the cytokine is protective or destructive for the SFN treatment. The in vivo experiment has reported that the excessive secretion of TNF-α results in severe inflammation in organs and early death (Bekker et al. 2000). Clinically, the classical HDAC inhibitors (such as TSA, SAHA) have been used as the therapeutic reagents in the inflammatory disease via suppression of inflammatory cytokines (Imre et al. 2006, Leoni et al. 2002). In addition, the similar results were found in the NF-κB subunits of p50 and p65 (data not shown). Therefore, in the present study, the SFN guides the induction of pro-inflammatory cytokine distribution in the different inflammatory stages to LPS, either in culture medium or in the cell lysates, which may provide the beneficial function in porcine moDCs.

Taken all together, the present data demonstrated that SFN induced not only histone acetylation but also changed the DNA methylation pattern as major mechanism in regulating the expression immune genes which are essential in sensing immune responses in moDCs. Inhibition of HDACs activity due to histone acetylation and the changes of the DNA methylation pattern together resulted in selectively dominant biologic functions of moDCs in the different development processes of the infection. These modified moDC-mediated immune responses could help to build a capacity of anti-inflammatory responses, to engulf and killing pathogen and to maintain the innate immune response in restricting the inflammation spreading in pig. Additionally, it is important to point out that SFN induced anti-inflammatory response not only undergoes epigenetic mechanisms such as DNA methylation and histone modification but also involves the activation Nrf2 dependent pathway. However, the broad anti-inflammatory and immune-adjustment properties of SFN as HDAC inhibitor was found to be beneficial in limiting the diffusion of inflammation induced tissue damage and increasing the immune responses in the infection site. Thus, this study may suggest that SNF, as a HDAC inhibitor, epigenetically targets the development of acute inflammation during the infection through modulating related immune responses, which may be a better strategy than using HDAC inhibitor as therapeutic agents in a more advanced stage of inflammation.

Conflict of interest

The authors do not have any potential conflicts of interest to declare.

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Reference

Aggarwal BB, Bhardwaj U, Takada Y (2004): Regulation of TRAIL-induced apoptosis by ectopic expression of antiapoptotic factors. Vitam Horm 67, 453-483

Angeli V, Randolph GJ (2006): Inflammation, lymphatic function, and dendritic cell migration. Lymphat Res Biol 4, 217-228

Aung HT, Schroder K, Himes SR, Brion K, van Zuylen W, Trieu A, Suzuki H, Hayashizaki Y, Hume DA, Sweet MJ, Ravasi T (2006): LPS regulates proinflammatory gene expression in macrophages by altering histone deacetylase expression. FASEB J 20, 1315-1327

Bayarsaihan D (2011): Epigenetic mechanisms in inflammation. J Dent Res 90, 9-17

Bekker LG, Moreira AL, Bergtold A, Freeman S, Ryffel B, Kaplan G (2000): Immunopathologic effects of tumor necrosis factor alpha in murine mycobacterial infection are dose dependent. Infect Immun 68, 6954-6961

Beutler B, Cerami A (1988): Tumor necrosis, cachexia, shock, and inflammation: a common mediator. Annu Rev Biochem 57, 505-518

Bode KA, Schroder K, Hume DA, Ravasi T, Heeg K, Sweet MJ, Dalpke AH (2007): Histone deacetylase inhibitors decrease Toll-like receptor-mediated activation of proinflammatory gene expression by impairing transcription factor recruitment. Immunology 122, 596-606

Brown J, Wang H, Hajishengallis GN, Martin M (2011): TLR-signaling networks: an integration of adaptor molecules, kinases, and cross-talk. J Dent Res 90, 417-427

Cao W, Bao C, Padalko E, Lowenstein CJ (2008): Acetylation of mitogen-activated protein kinase phosphatase-1 inhibits Toll-like receptor signaling. J Exp Med 205, 1491-1503

Chen ZJ (2005): Ubiquitin signalling in the NF-kappaB pathway. Nat Cell Biol 7, 758-765

Cheung KL, Kong AN (2010): Molecular targets of dietary phenethyl isothiocyanate and sulforaphane for cancer chemoprevention. AAPS J 12, 87-97

Crosas-Molist E, Bertran E, Sancho P, Lopez-Luque J, Fernando J, Sanchez A, Fernandez M, Navarro E, Fabregat I (2014): The NADPH oxidase NOX4 inhibits hepatocyte proliferation and liver cancer progression. Free Radic Biol Med

Dashwood RH, Ho E (2008): Dietary agents as histone deacetylase inhibitors: sulforaphane and structurally related isothiocyanates. Nutr Rev 66 Suppl 1, S36-38

Dashwood RH, Myzak MC, Ho E (2006): Dietary HDAC inhibitors: time to rethink weak ligands in cancer chemoprevention? Carcinogenesis 27, 344-349

de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB (2003): Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J 370, 737-749

Doherty R, O'Farrelly C, Meade KG (2013): Epigenetic regulation of the innate immune response to LPS in bovine peripheral blood mononuclear cells (PBMC). Vet Immunol Immunopathol 154, 102-110

Doyle S, Vaidya S, O'Connell R, Dadgostar H, Dempsey P, Wu T, Rao G, Sun R, Haberland M, Modlin R, Cheng G (2002): IRF3 mediates a TLR3/TLR4-specific antiviral gene program. Immunity 17, 251-263

El Gazzar M, Yoza BK, Chen X, Hu J, Hawkins GA, McCall CE (2008): G9a and HP1 couple histone and DNA methylation to TNFalpha transcription silencing during endotoxin tolerance. J Biol Chem 283, 32198-32208

Fahey JW, Haristoy X, Dolan PM, Kensler TW, Scholtus I, Stephenson KK, Talalay P, Lozniewski A (2002): Sulforaphane inhibits extracellular, intracellular, and antibiotic-resistant strains of Helicobacter pylori and prevents benzo[a]pyrene-induced stomach tumors. Proc Natl Acad Sci U S A 99, 7610-7615

Fujii S, Liu K, Smith C, Bonito AJ, Steinman RM (2004): The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. J Exp Med 199, 1607-1618

Geisel J, Bruck J, Glocova I, Dengler K, Sinnberg T, Rothfuss O, Walter M, Schulze-Osthoff K, Rocken M, Ghoreschi K (2014): Sulforaphane Protects from T Cell-Mediated Autoimmune Disease by Inhibition of IL-23 and IL-12 in Dendritic Cells. J Immunol 192, 3530-3539

Gonzalez-Guerrero C, Ocana-Salceda C, Berzal S, Carrasco S, Fernandez-Fernandez B, Cannata-Ortiz P, Egido J, Ortiz A, Ramos AM (2013): Calcineurin inhibitors recruit protein kinases JAK2 and JNK, TLR signaling and the UPR to activate NF-kappaB-mediated inflammatory responses in kidney tubular cells. Toxicol Appl Pharmacol 272, 825-841

Heiss E, Herhaus C, Klimo K, Bartsch H, Gerhauser C (2001): Nuclear factor kappa B is a molecular target for sulforaphane-mediated anti-inflammatory mechanisms. J Biol Chem 276, 32008-32015

Horion J, Gloire G, El Mjiyad N, Quivy V, Vermeulen L, Vanden Berghe W, Haegeman G, Van Lint C, Piette J, Habraken Y (2007): Histone deacetylase inhibitor

trichostatin A sustains sodium pervanadate-induced NF-kappaB activation by delaying IkappaBalpha mRNA resynthesis: comparison with tumor necrosis factor alpha. J Biol Chem 282, 15383-15393

Hsu A, Wong CP, Yu Z, Williams DE, Dashwood RH, Ho E (2011): Promoter de-methylation of cyclin D2 by sulforaphane in prostate cancer cells. Clin Epigenetics 3, 3

Imre G, Gekeler V, Leja A, Beckers T, Boehm M (2006): Histone deacetylase inhibitors suppress the inducibility of nuclear factor-kappaB by tumor necrosis factor-alpha receptor-1 down-regulation. Cancer Res 66, 5409-5418

Ishii KJ, Koyama S, Nakagawa A, Coban C, Akira S (2008): Host innate immune receptors and beyond: making sense of microbial infections. Cell Host Microbe 3, 352-363

Jha JC, Gray SP, Barit D, Okabe J, El-Osta A, Namikoshi T, Thallas-Bonke V, Wingler K, Szyndralewiez C, Heitz F, Touyz RM, Cooper ME, Schmidt HH, Jandeleit-Dahm KA (2014): Genetic Targeting or Pharmacologic Inhibition of NADPH Oxidase Nox4 Provides Renoprotection in Long-Term Diabetic Nephropathy. J Am Soc Nephrol 25, 1237-1254

Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA (2010): The absence of the pro-antioxidant transcription factor Nrf2 exacerbates experimental autoimmune encephalomyelitis. Toxicol Sci 114, 237-246

Kominsky DJ, Keely S, MacManus CF, Glover LE, Scully M, Collins CB, Bowers BE, Campbell EL, Colgan SP (2011): An endogenously anti-inflammatory role for methylation in mucosal inflammation identified through metabolite profiling. J Immunol 186, 6505-6514

Koo JE, Park ZY, Kim ND, Lee JY (2013): Sulforaphane inhibits the engagement of LPS with TLR4/MD2 complex by preferential binding to Cys133 in MD2. Biochem Biophys Res Commun 434, 600-605

Kramer OH, Baus D, Knauer SK, Stein S, Jager E, Stauber RH, Grez M, Pfitzner E, Heinzel T (2006): Acetylation of Stat1 modulates NF-kappaB activity. Genes Dev 20, 473-485

Kundu JK, Surh YJ (2008): Inflammation: gearing the journey to cancer. Mutat Res 659, 15-30

Le Y, Zhou Y, Iribarren P, Wang J (2004): Chemokines and chemokine receptors: their manifold roles in homeostasis and disease. Cell Mol Immunol 1, 95-104

Lecours MP, Segura M, Lachance C, Mussa T, Surprenant C, Montoya M, Gottschalk M (2011): Characterization of porcine dendritic cell response to Streptococcus suis. Vet Res 42, 72

Lee JH, Khor TO, Shu L, Su ZY, Fuentes F, Kong AN (2013): Dietary phytochemicals and cancer prevention: Nrf2 signaling, epigenetics, and cell death mechanisms in blocking cancer initiation and progression. Pharmacol Ther 137, 153-171

Leoni F, Zaliani A, Bertolini G, Porro G, Pagani P, Pozzi P, Dona G, Fossati G, Sozzani S, Azam T, Bufler P, Fantuzzi G, Goncharov I, Kim SH, Pomerantz BJ, Reznikov LL, Siegmund B, Dinarello CA, Mascagni P (2002): The antitumor histone deacetylase inhibitor suberoylanilide hydroxamic acid exhibits antiinflammatory properties via suppression of cytokines. Proc Natl Acad Sci USA 99, 2995-3000

Li LC, Dahiya R (2002): MethPrimer: designing primers for methylation PCRs. Bioinformatics 18, 1427-1431

Lin W, Wu RT, Wu T, Khor TO, Wang H, Kong AN (2008): Sulforaphane suppressed LPS-induced inflammation in mouse peritoneal macrophages through Nrf2 dependent pathway. Biochem Pharmacol 76, 967-973

Livak KJ, Schmittgen TD (2001): Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402-408

Lumsden JS, Kennedy BW, Mallard BA, Wilkie BN (1993): The influence of the swine major histocompatibility genes on antibody and cell-mediated immune responses to immunization with an aromatic-dependent mutant of Salmonella typhimurium. Can J Vet Res 57, 14-18

Mak TW, Yeh WC (2002): Signaling for survival and apoptosis in the immune system. Arthritis Res 4 Suppl 3, S243-252

Mallard BA, Wilkie BN, Kennedy BW (1989): The influence of the swine major histocompatibility genes (SLA) on variation in serum immunoglobulin (Ig) concentration. Vet Immunol Immunopathol 21, 139-151

Manabe N, Goto Y, Matsuda-Minehata F, Inoue N, Maeda A, Sakamaki K, Miyano T (2004): Regulation mechanism of selective atresia in porcine follicles: regulation of granulosa cell apoptosis during atresia. J Reprod Dev 50, 493-514

Marshall OJ (2004): PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. Bioinformatics 20, 2471-2472

Meeran SM, Patel SN, Tollefsbol TO (2010): Sulforaphane causes epigenetic repression of hTERT expression in human breast cancer cell lines. PLoS One 5, e11457

Mogal A, Abdulkadir SA (2006): Effects of Histone Deacetylase Inhibitor (HDACi); Trichostatin-A (TSA) on the expression of housekeeping genes. Mol Cell Probes 20, 81-86

Mrowietz U, Asadullah K (2005): Dimethylfumarate for psoriasis: more than a dietary curiosity. Trends Mol Med 11, 43-48

Myzak MC, Karplus PA, Chung FL, Dashwood RH (2004): A novel mechanism of chemoprotection by sulforaphane: inhibition of histone deacetylase. Cancer Res 64, 5767-5774

Myzak MC, Tong P, Dashwood WM, Dashwood RH, Ho E (2007): Sulforaphane retards the growth of human PC-3 xenografts and inhibits HDAC activity in human subjects. Exp Biol Med (Maywood) 232, 227-234

Nadiri A, Wolinski MK, Saleh M (2006): The inflammatory caspases: key players in the host response to pathogenic invasion and sepsis. J Immunol 177, 4239-4245

Nencioni A, Beck J, Werth D, Grunebach F, Patrone F, Ballestrero A, Brossart P (2007): Histone deacetylase inhibitors affect dendritic cell differentiation and immunogenicity. Clin Cancer Res 13, 3933-3941

Pugh BF (2000): Control of gene expression through regulation of the TATA-binding protein. Gene 255, 1-14

Qu X, Cinar MU, Fan H, Proll M, Tesfaye D, Tholen E, Looft C, Holker M, Schellander K, Uddin MJ (2014): Comparison of the innate immune responses of porcine monocyte-derived dendritic cells and splenic dendritic cells stimulated with LPS. Innate Immun

Randolph GJ, Angeli V, Swartz MA (2005): Dendritic-cell trafficking to lymph nodes through lymphatic vessels. Nat Rev Immunol 5, 617-628

Ray A, Cot M, Puzo G, Gilleron M, Nigou J (2013): Bacterial cell wall macroamphiphiles: pathogen-/microbe-associated molecular patterns detected by mammalian innate immune system. Biochimie 95, 33-42

Rodriguez-Cortez VC, Hernando H, de la Rica L, Vento R, Ballestar E (2011): Epigenomic deregulation in the immune system. Epigenomics 3, 697-713

Roger T, Calandra T (2009): TLR2-mediated neutrophil depletion exacerbates bacterial sepsis. Proc Natl Acad Sci U S A 106, 6889-6890

Roger T, Lugrin J, Le Roy D, Goy G, Mombelli M, Koessler T, Ding XC, Chanson AL, Reymond MK, Miconnet I, Schrenzel J, Francois P, Calandra T (2011): Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. Blood 117, 1205-1217

Rozen S, Skaletsky H (2000): Primer3 on the WWW for general users and for biologist programmers. Methods Mol Biol 132, 365-386

Ruland J (2011): Return to homeostasis: downregulation of NF-kappaB responses. Nat Immunol 12, 709-714

Sethi G, Sung B, Aggarwal BB (2008): TNF: a master switch for inflammation to cancer. Front Biosci 13, 5094-5107

Suarez-Alvarez B, Rodriguez RM, Fraga MF, Lopez-Larrea C (2012): DNA methylation: a promising landscape for immune system-related diseases. Trends Genet 28, 506-514

Sun F, Peatman E, Li C, Liu S, Jiang Y, Zhou Z, Liu Z (2012): Transcriptomic signatures of attachment, NF-kappaB suppression and IFN stimulation in the catfish gill following columnaris bacterial infection. Dev Comp Immunol 38, 169-180

Takahashi K, Sugi Y, Hosono A, Kaminogawa S (2009): Epigenetic regulation of TLR4 gene expression in intestinal epithelial cells for the maintenance of intestinal homeostasis. J Immunol 183, 6522-6529

Takeuchi O, Akira S (2010): Pattern recognition receptors and inflammation. Cell 140, 805-820

Wiesner P, Choi SH, Almazan F, Benner C, Huang W, Diehl CJ, Gonen A, Butler S, Witztum JL, Glass CK, Miller YI (2010): Low doses of lipopolysaccharide and minimally oxidized low-density lipoprotein cooperatively activate macrophages via nuclear factor kappa B and activator protein-1: possible mechanism for acceleration of atherosclerosis by subclinical endotoxemia. Circ Res 107, 56-65

Youn HS, Kim YS, Park ZY, Kim SY, Choi NY, Joung SM, Seo JA, Lim KM, Kwak MK, Hwang DH, Lee JY (2010): Sulforaphane suppresses oligomerization of TLR4 in a thiol-dependent manner. J Immunol 184, 411-419

ZDS (2003): Richtlinie Fuer die Stationspruefung auf Mastleistung, Schlachtkoerperwert und Fleischbeschaffenheit Beim Schwein. Zentralverband der Deutschen Schweineproduktion e.V., Ausschussfuer Leistungspruefung und Zuchtwertschaetzung, Bonn

Zhang Y, Fatima N, Dufau ML (2005): Coordinated changes in DNA methylation and histone modifications regulate silencing/derepression of luteinizing hormone receptor gene transcription. Mol Cell Biol 25, 7929-7939

Table 1. List of primer sequences used in this study

Gene	Primer set	Anneal TM (°C)	Amplicon size (bp)	GenBank accession nr.
TLR4	F:ATCATCCAGGAAGGTTTCCAC	58	235	NM_001097444.1
	R:TGTCCTCCCACTCCAGGTAG			
MyD88	F:CCAGTTTGTGCAGGAGATGA	60	185	NM_001099923.1
	R:TCACATTCCTTGCTTTCGAG			
MHC-SLA1	F:AGAAGGAGGGGCAGGACTAT	60	199	NM_001097431.1
	R:TCGTAGGCGTCCTGTCTGTA			
Nrf2	F:GTGCCTATAAGTCCCGGTCA	60	108	XM_003483682.1
	R:ATGCAGAGCTTTTGCCCTTA			
STAT3	F:ATGCTGGAGGAGAGAATCGT	60	159	XM_005668829.1
	R:AGGGAATTTGACCAGCAATC			
TNF-α	F:CCACCAACGTTTTCCTCACT	60	247	NM_214022.1
	R:CCAAAATAGACCTGCCCAGA			
IL-1β	F:GTACATGGTTGCTGCCTGAA	59	137	NM_001005149.1
	R:CTAGTGTGCCATGGTTTCCA			
IL-6	F:GGCAGAAAACAACCTGAACC	58	125	NM_214399.1
	R:GTGGTGGCTTTGTCTGGATT			
IL-8	F:TAGGACCAGAGCCAGGAAGA	60	174	NM_213997.1
	R:CAGTGGGGTCCACTCTCAAT			
CXCL2	F:ATCCAGGACCTGAAGGTGAC	60	152	NM_001001861.2
	R:ATCAGTTGGCACTGCTCTTG			
CCL4	F:CTCTCCTCCAGCAAGACCAT	60	191	NM_213779.1
	R:CAGAGGCTGCTGGTCTCATA			
HPRT1	F:AACCTTGCTTTCCTTGGTCA	60	150	NM_001032376.2
	R:TCAAGGGCATAGCCTACCAC			
GAPDH	F:ACCCAGAAGACTGTGGATGG	60	247	AF017079
	R:ACGCCTGCTTCACCACCTTC			
TLR4-met-nest	F:GTATATGGAGGTTTTTAGGTTAGGG	55	153	AY753179
	R:TCCCTACCCTTACTCAATAAATTAAC			
MHC-SLA-met-nest	F:GTTTGGGGAGAAGTTGAGTAGAGT	58	293	AJ251829.1
	R:AAAAAACAAAAACAAAACAAAATCC			

F: Forward primer; R: Reverse primer; bp: base pair.

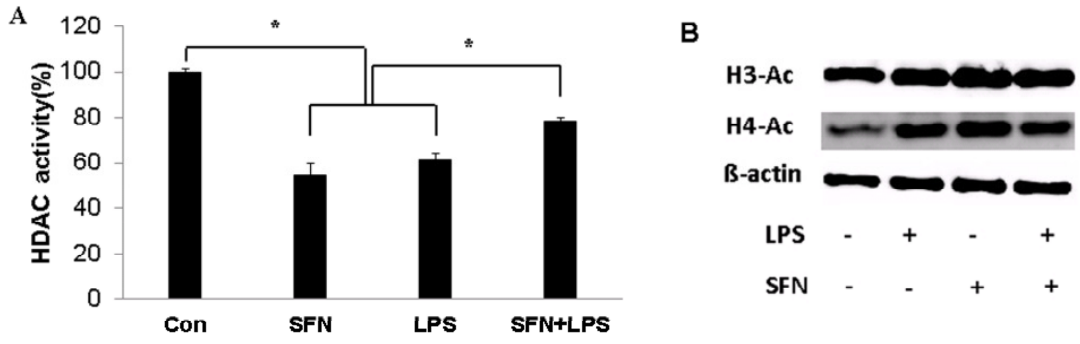


Figure 1. SFN induced histone acetylation and inhibited HDAC activity. moDCs, cultured for 7 days, were pre-incubated for 24 h with or without SFN (10 µM) before exposure to LPS (1 µg/ml). The relative HDAC activity was determined using the Color-de-Lys HDAC colorimetric activity assay kit (A). The result was represented as the mean ± standard deviation (SD) of three independent experiments and each experiment was performed in duplicate *(*p < 0.05*). The histone acetylation of acetylated H3 and acetylated H4 were measured by western blotting (B). The western blotting results were from one experiment of three independent experiments.

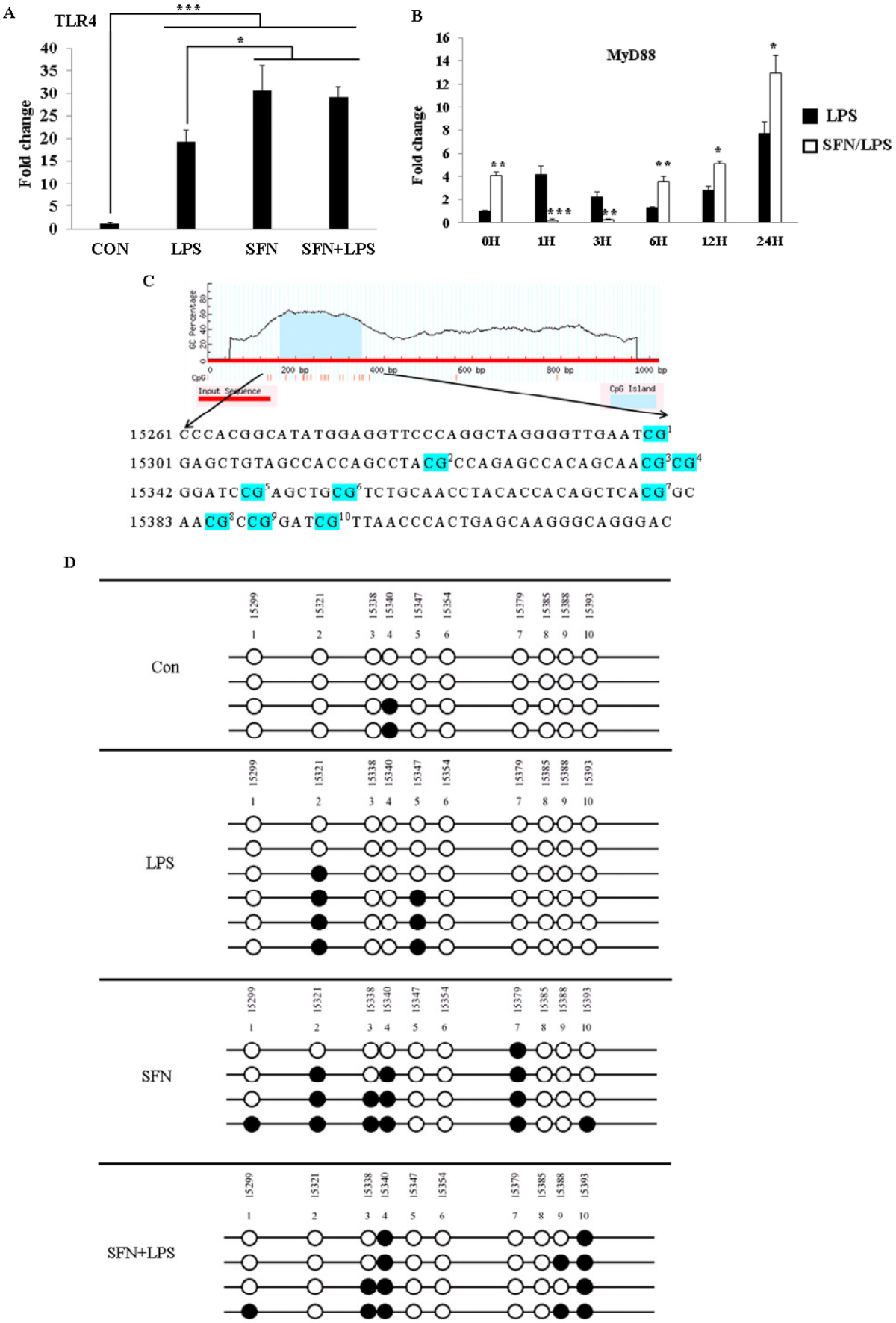
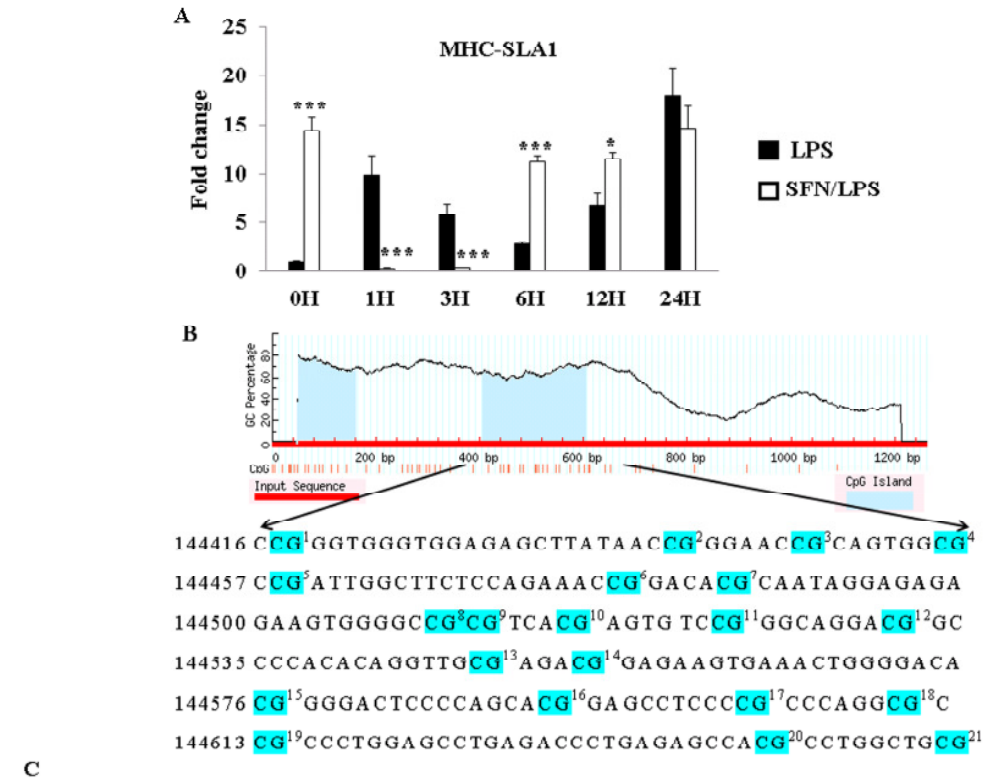

A
TLR4
Fold change
CON
LPS
SFN
SFN+LPS
B
MyD88
Fold change
0H
1H
3H
6H
12H
24H
LPS
SFN/LPS
C
GC Percentage
CpG
Input Sequence
CpG Island
15261 CCCACGGCATATGGAGGTTCCCAGGCTAGGGGTTGAATCG
15301 GAGCTGTAGCCACCAGCCTACGCCAGAGCCACAGCAACGCG
15342 GGATCCGAGCTGCGTCTGCAACCTACACCACAGCTCACGGC
15383 AACGCCGGATCGTTAACCCACTGAGCAAGGGCAGGGAC
D
Con
LPS
SFN
SFN+LPS

Figure 2. SFN or LPS treatment enhanced, while pre-treatment with SFN followed by LPS reduced DNA methylation in the exonic region of TLR4 gene. The effects of SFN 10 (μM) on TLR4 and MyD88 gene expression in response to LPS (1 μg/ml) were quantified by real-time qRT-PCR at indicated times in moDC. moDCs were pre-incubated with or without SFN in response to LPS for 0, 1, 3, 6, 12, 24 h. The TLR4 gene expression was measured at 24h LPS stimulation with or without SFN pre-incubation (A). MyD88 mRNA expression was quantified at 0, 1, 3, 6, 12, 24 h (B). The results were combined from three independent experiments and each experiment performed in triplicate. The data represent the mean ± standard deviations (SD) *(* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)*.10 CpG motifs around exon region of TLR4 were predicted by MethPrimer online (C). DNA methylation status covered the CpG island were quantified by bisulfite sequencing PCR (D). A minimum of four positive clones were randomly picked for sequencing with M13 primers. The sequencing results were visualized by QUMA software. White plots correspond to unmethylated CpGs and black plots correspond to methylated CpGs.


A
MHC-SLA1
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6H
12H
24H
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GC Percentage
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200 bp
400 bp
600 bp
800 bp
1000 bp
1200 bp
CpG
Input Sequence
CpG Island
144416 CCG1GGTGGGTGGAGAGCTTATAACCG2GGAACCG3CAGTGGCG4
144457 CCG5ATTGGCTTCTCCAGAAACCG6GACACG7CAATAGGAGAGA
144500 GAAGTGGGGCCG8CG9TCACG10AGTG TCCG11GGCAGGACG12GC
144535 CCCACACAGGTTGCG13AGACG14GAGAAGTGAAACTGGGGACA
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C

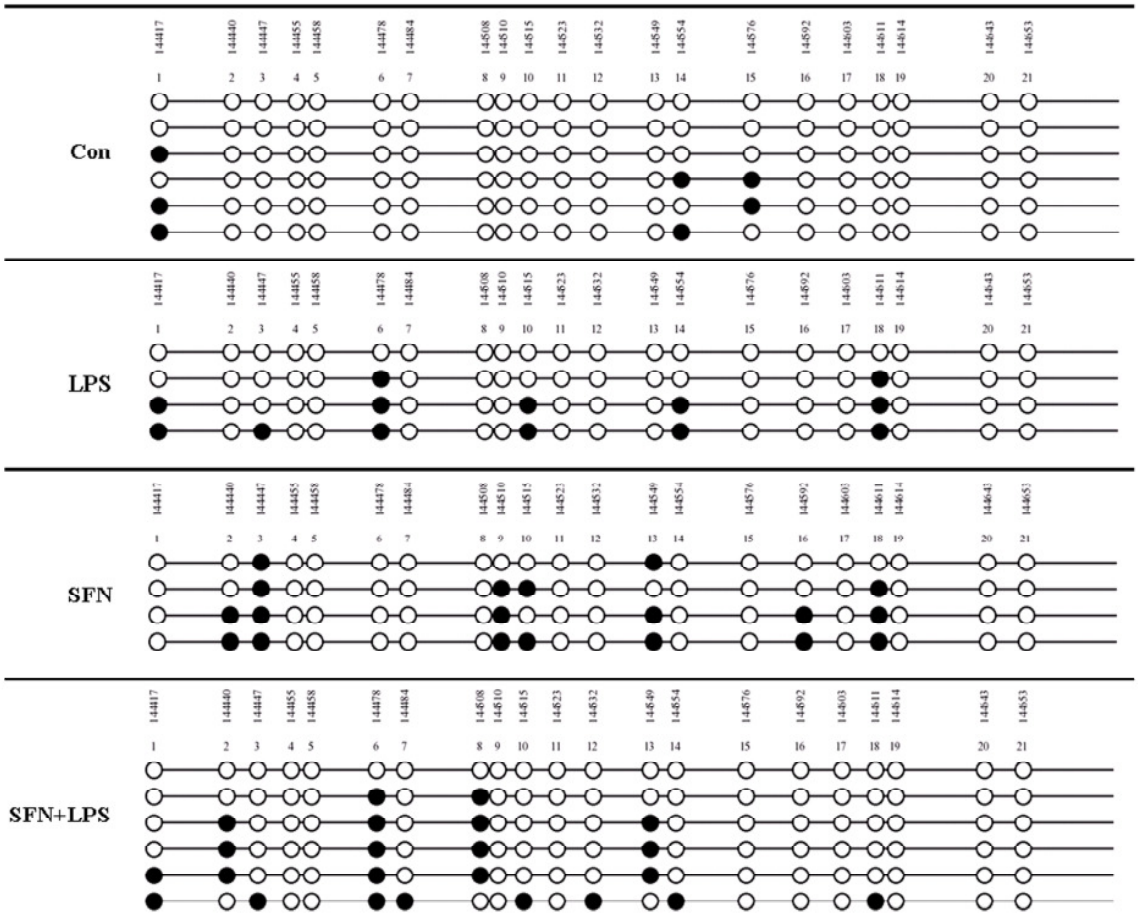
Con
LPS
SFN
SFN+LPS

Figure 3. SFN or LPS treatment enhanced, while pre-treatment with SFN followed by LPS demethylated DNA methylation in the promoter region of the MHC-SLA gene. The effect of SFN on MHC-SLA mRNA expression in response to LPS for the duration of 24 h was measured by qRT-PCR (A). The results were combined from three independent experiments and each experiment performed in triplicate. The data represent the mean ± standard deviations (SD) *(* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$)*. 21 CpG motifs around exon region of TLR4 were predicted by MethPrimer online (B). DNA methylation status within the CpG island were quantified by bisulfite sequencing PCR (C). A minimum of four positive clones were randomly picked for sequencing with M13 primers. The sequencing results were visualized by QUMA software. White plots correspond to unmethylated CpGs and black plots correspond to methylated CpGs.

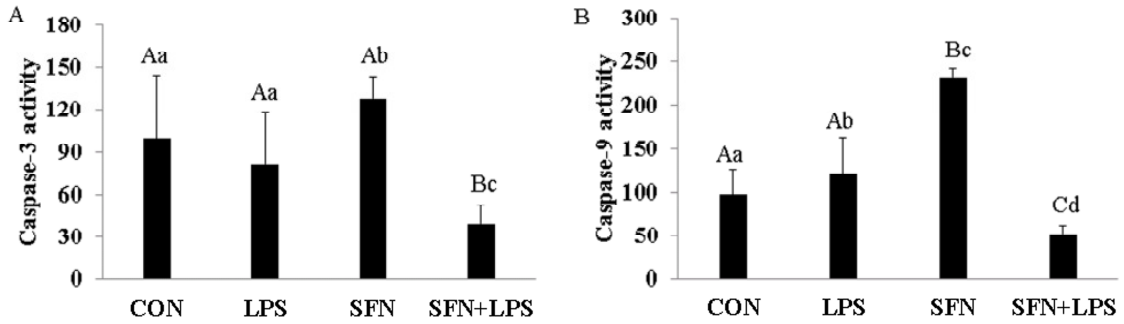

Figure 4. SFN induced caspase-3 and caspase-9 activity, pre-treatment of SFN reduced the inflammation induced cell apoptosis. Cell lysates were isolated from the moDCs, which were stimulated with LPS (1 μg/ml) for 24 h with or without pre-incubation of SFN (10 μM) for 24 h. Caspase-3 (A) and caspase-9 (B) enzyme activities were measured using Caspase-9 Colorimetric Assay Kit and Caspase-3/CPP32 Colorimetric Assay Kit respectively. The results were combined from three independent experiments and each experiment performed in triplicate. The data represent the mean ± standard deviations (SD) (small letters: * $P < 0.05$; capital letters: ** $P < 0.01$).

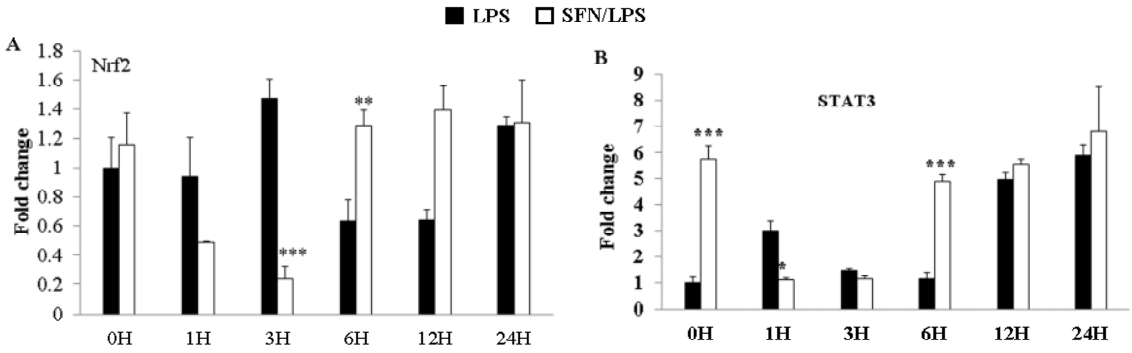

Figure 5. SFN dynamically regulated LPS-induced the gene expressions Nrf2 and STAT3 expression. The effects of SFN on mRNA expressions of Nrf2 (A) and STAT3 (B) in response to LPS for 0, 1, 3, 6, 12 and 24 h in moDCs were measured by qRT-PCR. The results were combined from three independent experiments and each experiment performed in triplicate. The data represent the mean ± standard deviations (SD) (** $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$*).

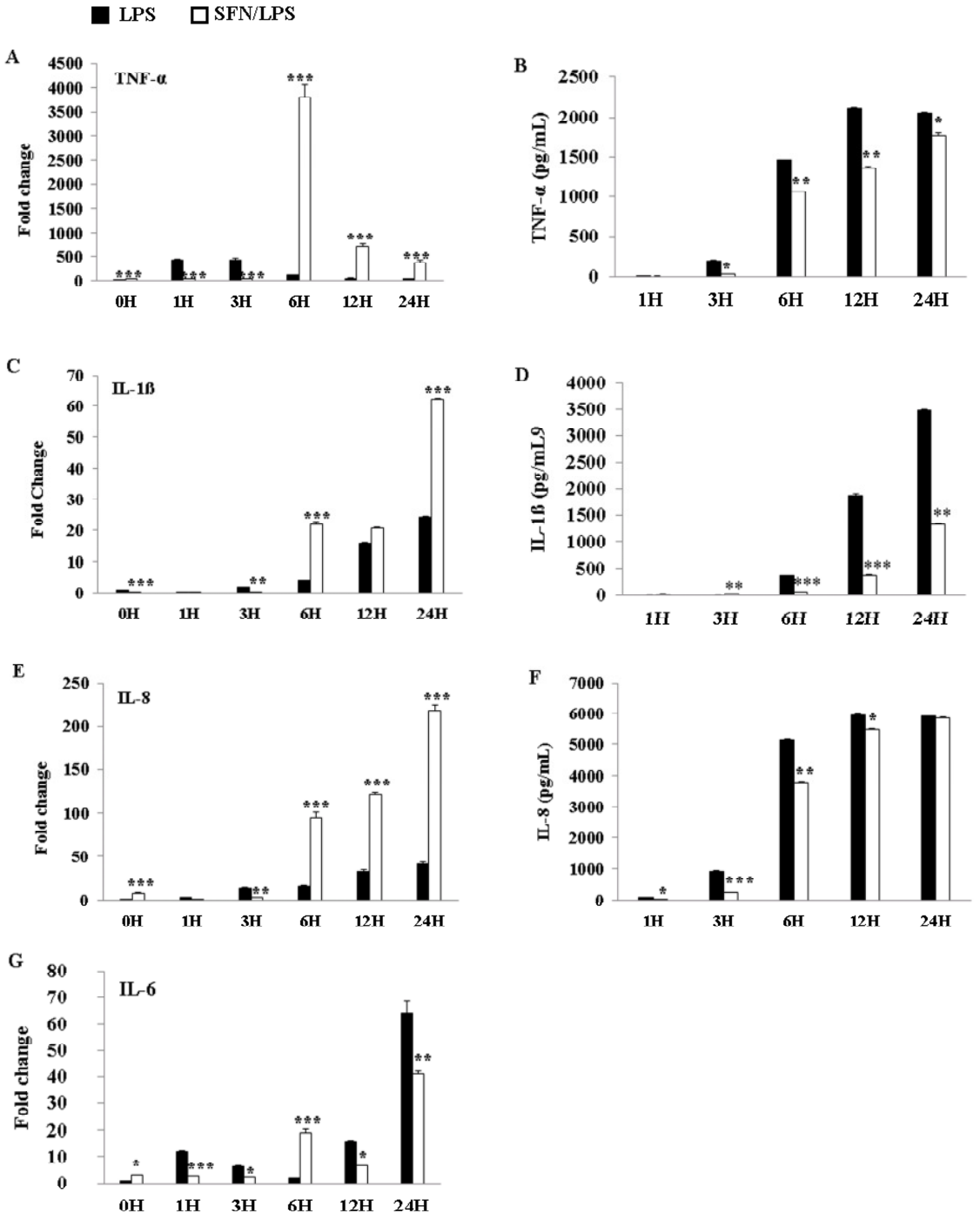

Figure 6. SFN significantly inhibited the LPS-induced pro-inflammatory cytokine secretions. The inflammatory cytokine expressions of TNF-α, IL-1ß, IL8 and IL-6 were quantified using qRT-PCR. moDCs were stimulated with LPS (1 µg/ml) for 0, 1, 3, 6, 12 and 24 h with or without pre-incubation of SFN (10 M) for 24 h. Total RNA and cell culture supernatants were collected at 0, 1, 3, 6, 12 and 24 h for mRNA and protein measurements respectively. TNF-α (A), IL-1ß (C), IL-8 (E) and IL-6 (G) mRNA expressions were quantified by qRT-PCR. TNF-α (B), IL-1ß (D) and IL-8 (F) protein

productions were measured using ELISA in cell culture supernatants. The ELISA data from productions of protein was combined from two independent experiments and each experiment performed in triplicate. The qRT-PCR data of gene expressions was combined from three independent experiments and each experiment performed in triplicate. The data represent the mean ± standard deviations (SD) (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

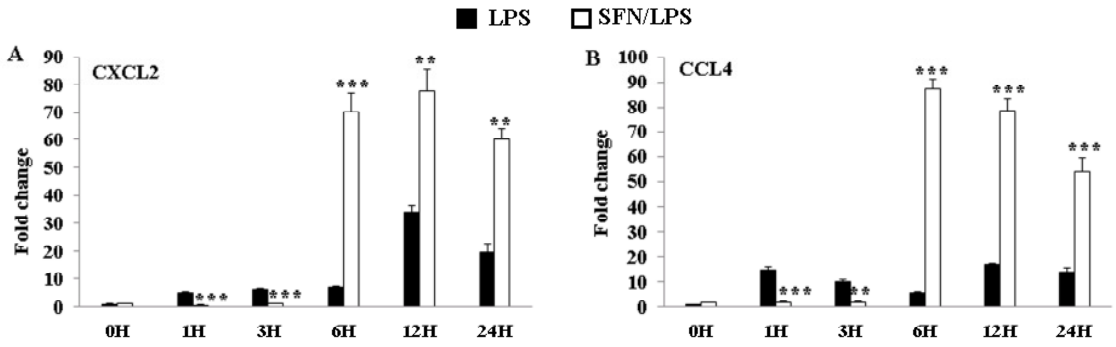

Figure 7. SFN dynamically regulated the LPS-inducedf CXCL2 and CCL4 expression level. The effects of SFN on mRNA expressions of CXCL2 (A) and CCL4 (B) in response to LPS for 0, 1, 3, 6, 12 and 24 h in moDCs were measured by qRT-PCR. The results were combined from three independent experiments and each experiment performed in triplicate. The data represent the mean ± standard deviations (SD) (** $P < 0.01$; *** $P < 0.001$).

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The completion of my thesis and subsequent Ph.D. has been a long journey. I do feel that “all kinds of feelings well-up in one´s heart” when I am completing my dissertation. Grandma Moses said that “Life is what we make it, always has been, always will be”. Indeed, I cannot tightly sit and affectionately express my emotions if I had blockaded to accept the offer of abroad education 4 years ago. Many had questioned whether I should go abroad, as have doubted my tolerance to the Ph.D., as my beloved parents and some of my dear friends referred to it “the challenge”. I am very fortunate that I have received “the challenge” and have been committing the victory, despite I had loosen confidence, got writers block, failed in depression so many times.

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Curriculum Vitae

Education

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09/2008-07/2011	MSc	College of Animal Science and Veterinary Medicine (Department of Clinical Veterinary Medicine), Jilin University, Jilin, China **Masters dissertation:** swine protective immune responses to porcine reproductive and respiratory syndrome virus infection - changes of CD4, CD8 T lymphocytes subsets in different immune status
09/2004-07/2008	B.Sc.	College of Veterinary Medicine, Anhui Science and Technology University, Anhui, China **Bachelor dissertation:** Effect of Se-enriched yeast cultured in Chinese medicine serum antioxidant index on Min Zhong-ma chicken

Project involvement

01/2009-12/2013	"The study of key immune molecules of PRRS after pigs vaccinated with classical swine fever virus vaccine", National Natural Science Foundation Project (grant number: 30972193)
01/2010-12/2012	The project of "Eleventh-Five Year Research" from Ministry of Education of Jilin Province (grant number: (2010)534)
01/2006-12/2008	The Project of Anhui College Discipline Talents from the Ministry of Education of Anhui Province (grant number: 2006KJ197B)

Languages

Mandarin	Mother tongue
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Awards

09/2009-07/2010 Win the Prize of Excellent graduate Scholarship of Jilin University

09/2004-07/2008 Win the Prize of Excellent undergraduate Scholarship four times, Outstanding student leader two times, and Graduates of high academic achievers in Anhui Science and Technology University

Presentations/Abstract

05/09/2013 **Qu X**, Cinar M U, Fan H, Pröll M et al. Comparison of the porcine monocyte-derived dendritic cells and spleenic dendritic cells immune responses to lipopolysaccharide stimulation. *DGfZ/GfT-Gemeinschaftstagung*, Göttingen, Deutschland (Proc)

05/09/2013 Pröll M, Große-Brinkhaus C, Sahadevan S, **Qu X** et al. RNA-Seq Analyse von porcinen dendritischen Zellen nach experimenteller Infektion mit PRRSV. *DGfZ/GfT-Gemeinschaftstagung*, Göttingen, Deutschland (Proc)

19/09/2014 **X. Qu**, M. Pröll, C. Neuhoff, R. Zhang et a. Sulforaphane epigenetically regulates innate immune responses of porcine monocyte-derived dendritic cells induced with Lipopolysaccharide. 44th Annual Meeting German Society for Immunology, Bonn, Germany (Abstr.)

11/10/2014 **X. Qu**, C. Neuhoff, M. Pröll, M. U. Cinar et al. Sulforaphane epigenetically regulates the LPS-induced kinetics of innate immune response in porcine monocyte-derived dendritic cells (moDCs). 2014 Innate Immune Summit, London, UK (Proc)

Publications

19/03/2014 **Qu X,** Cinar MU, Fan H et al. "Comparison of the innate immune responses of porcine monocyte-derived dendritic cells and splenic dendritic cells stimulated with LPS." *Innate Immun.* doi: 10.1177/1753425914526266a

05/2014 **Qu X,** Pröll M, Neuhoff C et al. Sulforaphane epigenetically regulates innate immune responses of porcine monocyte-derived dendritic cells induced with Lipopolysaccharide. *PLoS One.* 2015 Mar 20;10(3):e0121574. doi: 10.1371/journal.pone.0121574

08/2011 **Qu X,** Bai X, Wang Y et al. 2. The changes of peripheral blood T lymphocyte subpopulations and

	the protection for PRRS of pigs vaccinated with CSFV vaccine. *Chinese Journal of Veterinary Science.* 08 CN: 22-1234/R ISSN: 1005-4545
04/2011	**Qu X,** Zhao J, Liang Z et al. The Effects of T Lymphocyte Subset Detection for Flow Cytometry under Different Experimental Conditions. *China Animal Husbandry & Veterinary Medicine.* 04 CN: 11-4843/S ISSN: 1671-7236
08/2010	**Qu X,** Zhao J, Liang Z et al. Progress in Immunology of Porcine Reproductive and Respiratory Syndrome (PRRS). *China Animal Husbandry & Veterinary Medicine.* 37(8): 194-198
07/2012	Fu Y, Shan Y… **Qu X** et al. 4. Immune response and the changes of lymphocyte subpopulations in swine inoculated by porcine reproductive and respiratory syndrome Nsp2Δ1882-2241 attenuated vaccine after CSF vaccination. *Chinese Journal of Veterinary Science.* 07 CN: 22-1234/R ISSN: 1005-4545
01/2012	Fang Q, **Qu X,** Li Y et al. The Observation of Healing Effect of Rats with Acute Lung Injury Treated with Shenmai Injection. *China Animal Husbandry & Veterinary Medicine.* 01 CN: 11-4843/S ISSN: 1671-7236
06/2011	Bai X, Zhao J, Liang Z, **Qu X** et al. Advance in immunology of porcine reproductive and respiratory syndrome virus. *China Animal Husbandry & Veterinary Medicine.* 06 CN: 62-1192/S ISSN: 1673-4696
12/2010	Fang Q, **Qu X,** Gao Y et al. Progress in Research on Therapy of Acute Lung Injury. *Chinese Journal of Biologicals.* 12 CN 1397-1402