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Short communication

Feeding a high dosage of zinc oxide affects suppressor of cytokine gene expression in *Salmonella* Typhimurium infected piglets

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ABSTRACT

Suppressor of cytokine signaling (SOCS) proteins play an important role in the regulation of the immune response by inhibiting cytokines. Here we investigated the effects of zinc oxide fed at three different dosages (LZN = 57 ppm, MZN = 167 ppm, HZN = 2425 ppm) to weaned piglets that were or were not orally infected with *Salmonella enterica* serovar Typhimurium DT 104. We detected higher expression of SOCS3 six days after weaning for all analyzed piglets, regardless of the infection or the zinc feeding, suggesting a stress induced immune response. Whereas, SOCS1 showed only higher transcript amounts in *S.* Typhimurium infected piglets, especially the LZN group. This might indicate an infection regulating effect of zinc oxide in the infection model. After 42 days of infection, the expression of SOCS2, SOCS4, and SOCS7 was increased only in animals fed the highest concentrations of zinc oxide, while non-infected piglets at the age of 56 days showed no regulation for these genes. The up-regulation of SOCS genes in the mesenteric lymph nodes of piglets fed a diet with a very high concentration of zinc over 6 weeks suggests that such treatments may impair the immune response.

1. Introduction

Zinc is an essential trace element that plays an important role in the metabolism and also the immune system. It is a co-factor of more than 300 enzymes (Chasapis et al., 2012) some of which are active in immune cells. Zinc deficiency causes immunodeficiency and increased morbidity after infection, while supplementation with zinc can improve the health of children with zinc deficiency by reducing diarrhea (Gupta et al., 2003; Rahman et al., 2001). Zinc is often used as a food additive to improve the health status and performance of weaned piglets. In an earlier study we found higher levels of shedding of *Salmonella* Typhimurium and lower frequencies of T cells in the ileal lymph nodes of piglets after feeding a high dosage of zinc as zinc oxide for six weeks (Janczyk et al., 2013). This suggested an impairment of the immune response that may be mediated through the regulation of SOCS genes. *Salmonella* Typhimurium is responsible for approximately 12% of all salmonellosis outbreaks in humans in the European Union (European Food Safety Authority, 2010) and is a major problem for human health. Salmonellae are found in the gut-associated lymphatic tissue (GALT) from where they invade to regional mesenteric lymph nodes and other lymphatic organs. This triggers an inflammatory immune response, which needs to be tightly regulated to ensure the complete removal of the infectious agent. In this process, suppressor of cytokine signaling (SOCS) proteins act as negative regulators of the cytokine-JAK-STAT and other pathways and are also involved in allergy, tumor-genesis and inflammatory diseases (Yasukawa et al., 2000). Usually they are not highly expressed, but are up-regulated after exposure of cells to cytokines (Matsumoto et al., 1999). Conversely, the SOCS proteins are utilized by pathogens to alter or evade the host's immune response (Delgado-Ortega et al., 2013). Possible alterations have been described by various authors, i.e. *Mycobacterium avium* infected human macrophages showed a reduced response to IFNγ and a decreased phosphorylation of STAT1 accompanied by an over-expression of SOCS1 and SOCS3, which directly correlated with the unresponsiveness of the macrophages to IFNγ (Vazquez et al., 2006). Bruel et al. (2010) demonstrated an induction of SOCS2 mRNA in porcine intestinal cells that were co-cultured with *Entamoeba histolytica*. Thereby the parasite drives the T auxiliary response towards Th2 and Th17 orientation, which diminishes the possibility of a full recovery from the infection (Guo et al., 2008).

Abbreviations: GALT, gut associated lymphatic tissue; JAK, janus kinase; SOCS, suppressor of cytokine signaling; STAT, signal transducers and activators of transcription.

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Table 1
Primer sequences, annealing temperatures of primer sets (°C), expected PCR fragment sizes (bp), accession numbers and references.

Gene	Primer sequence 5′ > 3′	Annealing temperature (°C)	PCR product length (bp)	Refenrence and Accession numbers
RPL-19	*AAC TCC CGT CAG CAG ATC C*	*60*	*147*	Meurens et al. (2009) AF435591
RPL-19	*AGT ACC CTT CCG CTT ACC G*			
SOCS1	*CGC CCT CAG TGT GAA GAT GG*	*62*	*110*	Delgado-Ortega et al. (2013) EW101597
SOCS1	*GCT CGA AGA GGC AGT CGA AG*			
SOCS2	*CTG CGC ATC GAA TAC CAA G*	*58*	*190*	Delgado-Ortega et al. (2013) NM 001097461
SOCS2	*TGT AGA GCG GTT TGG TCA G*			
SOCS3	*CAC TCT CCA GCA TCT CTG TC*	*62*	*105*	Delgado-Ortega et al. (2013) NM 001123196
SOCS3	*TCG TAC TGG TCC AGG AAC TC*			
SOCS4	*TCC TGG GAC AGG CTC TAT G*	*59*	*170*	Delgado-Ortega et al. (2013) ES445034
SOCS4	*GGT ACT TGG GAG GTG TTT C*			
SOCS5	*ACG CTG TGT TTG CAG TCT C*	*58*	*89*	Delgado-Ortega et al. (2013) DB784235
SOCS5	*ACT TTC CAA GCT CCC TGT C*			
SOCS6	*ATC TCT AGC CGG TGA CTT CG*	*62*	*178*	Delgado-Ortega et al. (2013) XM 001926570
SOCS6	*GCC CTT CTG CTT CTG TTT CG*			
SOCS7	*CAC TTG TGG ACG TGG ACA TC*	*62*	*162*	Delgado-Ortega et al. (2013) ENSSSCT00000019652
SOCS7	*GGA AAG ACT GCA GGG AAG AC*			
CIS	*GGG AAT CTG GCT GGT ATT GG*	*62*	*126*	Delgado-Ortega et al. (2013) BE014034
CIS	*CCG ACA GTG TGA ACA GGT AG*			

To our knowledge, there is no data about the role of SOCS in pigs after *Salmonella* Typhimurium infection. Therefore, we investigated the levels of expression of the SOCS family members 1–7 and the cytokine-induced STAT inhibitor (CIS), which belongs to the same protein family. The analyses were done in weaned piglets that had been fed three different levels of dietary zinc and that were or were not challenged with *Salmonella enterica* serovar Typhimurium DT 104 (*S.* Typhimurium).

2. Material and methods

2.1. Tissue samples

Tissues were collected from purebred German Landrace piglets of both sexes. The piglets were weaned at the age of 28 days and randomly assigned to one of three dietary groups, that received different levels of zinc supplementations (group LZN = 57 ppm, group MZN = 167 ppm, and group HZN = 2425 ppm). Four days after weaning piglets were orally infected with *S.* Typhimurium DT 104 (1.0×10^{10} to 1.5×10^{10} CFU/piglet) via an oral tube leading into the throat. The preparation of the bacteria is described elsewhere (Janczyk et al., 2013). Animals from each group were euthanized either two or 42 days after infection (dpi). Ileal lymph nodes (2 dpi/34 days of age: LZN, n = 8; MZN, n = 9; HZN, n = 8; 42 dpi/72 days of age: LZN, n = 12; MZN, n = 11; HZN, n = 10) were collected. N refers to the number of different animals from which the lymph nodes were isolated from. In addition, ileal lymph nodes from healthy, not infected piglets, that received identical diets (LZN, MZN, HZN), were collected at day 34 and 56 days of age (n = 8/feeding group). The experiment was approved by the local animal welfare authority (Landesamt für Gesundheit und Soziales, Berlin) under the ID: G0348/09.

2.2. Expression analysis

The RNA was isolated using the NucleoSpin® RNA II Kit (Macherey-Nagel). The quality and quantity of each sample was determined using the 2100 Bioanalyzer (Agilent Technologies). Only samples with a RIN (RNA integrity number) of 7.5 or higher were used. Afterwards, cDNA synthesis (SuperScript® VILO™ cDNA Synthesis Kit and Master Mix, Life Technologies) was performed with the reactions incubated for: 10 min at 25 °C, then 60 min at 42 °C and finally 5 min at 85 °C. Relative expression analysis was performed on a ViiA™ 7 Real Time PCR System (Life Technologies) using SYBR® Select Master Mix (Applied Biosystems®) and primers (Invitrogen™) specific for each SOCS gene (Table 1). RPL-19 was used as a reference gene as described in previous studies (Kreuzer et al., 2014). The qPCR was performed under the following conditions: 2 min at 50 °C, 10 min at 95 °C (hold stage), then 40 cycles of 15 s at 95 °C, 20 s at 58 °C or 62 °C (according to Table 1), 40 s at 72 °C (PCR stage), then for 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C (melt curve stage). Melting curves were analyzed to assess the specificity of the qPCR reaction and results were normalized internally using the average *Cycle quantification* (Cq) of RPL-19. The ΔΔCt- method (Cycle threshold; Livak and Schmittgen, 2001) was used to determine the relative expression of each SOCS gene. Data are shown as relative expression values compared to the MZN group, sampled 2 dpi, as the amount of zinc in the MZN group is commonly used in starter diets for piglets. Differences between groups were assessed by *t*-tests and were considered significant at $P < 0.05$.

3. Results and discussion

We detected higher relative expression of SOCS3 in age group 1, six days after weaning, than in age group 2 in all dietary groups ($P < 0.05$) in *S.* Typhimurium infected and non-infected piglets (Fig. 1). Therefore, we assume an inflammatory immune response attributable to stress early after weaning, with higher expressions of SOCS3 in all groups.

SOCS1 showed a higher relative expression in age group 1 compared to age group 2 only in *S.* Typhimurium infected piglets, whereas non-infected piglets showed no significant differences of the expression level between age groups (Fig. 1). There was only a tendency of lower transcript amounts in the HZN group at age group 2. Additionally, we detected the highest transcript amounts of SOCS1 and SOCS3 in the low level zinc group at age group 1 in *S.* Typhimurium infected animals (Fig. 1). Both SOCS proteins regulate the differentiation towards T_{H1} or T_{H17} cells and their alteration could prevent the elimination of infections (Delgado-Ortega et al., 2013). The lower expression of SOCS1 and SOCS3 in the zinc supplemented groups MZN and HZN within *S.* Typhimurium infected animals could be an indicator of a better regulation of the immune response. Therefore, a positive influence of high dosages of zinc early after weaning could be assumed in the used infection model. Piglets with no *S.* Typhimurium challenge did not show higher transcript amounts of SOCS1 and SOCS3 for the LZN group. This might indicate that the suboptimal zinc supply in the low zinc feeding group plays a more predominant role within the infection model.

After prolonged feeding of the zinc diets for six weeks (age group 2) we detected in the HZN group higher expression levels of SOCS 2, SOCS 4 and SOCS7 in *S.* Typhimurium infected piglets,