Expression analysis of LILRB3, LAIR1 and LXRA genes in porcine monocytes stimulated with Salmonella LPS

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ABSTRACT

Salmonella sp. is a zoonotic infection that infects the intestinal tract of domestic animals and humans and causes food poisoning. It grows mainly on macrophages that have differentiated from monocytes. In this study, we examined the changes in gene expression of three receptor genes in swine monocytes induced by lipopolysaccharide (LPS) from S. enterica serovar Typhimurium. The relative expression level of the LILRB3 gene in both the LPS-stimulated and control groups increased after 6 h of culture, and after 24 and 48 h the relative expression level decreased to the same extent as that at the start of the culture. The relative expression level of the LAIR1 gene significantly increased 24 and 48 h after culture in the LPS-stimulated group. In the LXRα-1 variant of the LXRα gene, the relative expression level increased with time in the LPS stimulation group. In the LXRα-2 variant, the gene expression level significantly increased in the LPS-stimulated group after 6 and 48 h of culture, whereas in the control group, the expression level increased with time.

Key words: LAIR1, LILRB3, LXRA, porcine monocytes, Salmonella LPS

INTRODUCTION

Salmonella sp. is a zoonotic disease that infects the intestinal tract of livestock and humans and causes food poisoning. This bacterium often causes bacterial food poisoning similar to Campylobacter sp. A total of 80% of Salmonella food poisoning is caused by Salmonella in livestock and poultry (Cloeckaert 2006; Chlebicz & Śliżewska 2018). Since the emergence of a drug-resistant Salmonella (Threlfall 2002), there has been an increased need for the prevention of Salmonella infection in livestock.

Salmonella is an intracellular parasitic bacterium belonging to the gram-negative bacilli that has been serologically classified into more than 2500 types. The isolation of various serotypes has been reported in pigs. S. enterica serovar Typhimurium has been isolated from a wide range of hosts, including cattle, pigs, horses, and chickens, in addition to its original host, murine, and is important not

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Monocytes circulate in the bloodstream for approximately 3 days and then migrate to tissues in the body and differentiate into macrophages and dendritic cells by the stimulation of chemokines and cytokines secreted by various cells. Recently, it has been reported that high doses of lipopolysaccharide (LPS) promote rapid monocyte differentiation in mice (Kew et al. 2017). Receptors expressed on the surface of monocytes are involved in such chemotaxis and the differentiation of monocytes.

The leukocyte immunoglobulin-like receptor gene (LILR) belongs to the immunoglobulin superfamily and is a receptor gene that is widely expressed on the cell surface of leukocytes. In humans, LILRs are present in the gene region called the leukocyte receptor complex (LRC) on chromosome 19 and 13 genes, including active, suppressed, soluble, and pseudogenes, have been identified to date (Norman et al. 2003). The active LILRA subfamily has arginine in the cell membrane region and a short cytoplasmic region. LILRA transmits activation signals via signaling molecules associated with the immunoreceptor tyrosine-based activation motif structure. The suppressed LILRB subfamily has no arginine in its cell membrane region and has a long cytoplasmic region. The LILRB controls an activation signal by an inhibitory structure called the immunoreceptor tyrosine-based inhibitory motif (ITIM).

Porcine LILR genes are mainly located on chromosome 6 and in the pig genome sequence (Sscrofa 11.1) there are two human LILRA2-like genes, one LILRA3-like gene, 15 LILRA6-like genes, and five LILRB3-like genes that have been identified (Sambrook et al. 2006; Li et al., 2017). Porcine LILR genes are involved in the activation and suppression of immune cells similar to the immune-related receptor in humans; however, details are unknown. The LILRB3 gene was first cloned from a human monocyte cDNA library and RT-PCR analysis showed that the LILRB3 gene is mainly expressed in myeloid monocytes and B cells and lungs (Arm et al. 1997). In addition, the LILRB3 gene has been reported to be weakly expressed in NK and dendritic cells (Borges et al. 1997).

The leukocyte-associated immunoglobulin-like receptor 1 gene (LAIR1) is a collagen-binding receptor that is present on the LRC of human chromosome 19 and inhibits NK cell and other immunity functions (Meyaard et al. 1997). Results from the porcine genome analysis project showed that the LAIR1 gene was present on chromosome 6 and was highly expressed in the spleen (Li et al. 2017). However, there has been no study to date on the relationship between the LAIR1 gene and immune function in pigs.

The liver X receptor (LXR) is a member of the nuclear receptor superfamily and regulates the transcription of genes involved in lipid and glucose metabolism (Repa et al. 2000). Two isoforms have been identified in the LXR gene, called LXRα (NR1H3) and LXRβ (NR1H2). The LXRs gene in humans and mice is strongly expressed in several tissues including the liver, kidneys, intestinal tract, macrophage, lungs, and spleen (Korf et al. 2009). LXRs and their ligands are reported to be negative regulators of macrophage inflammatory gene expression (Joseph et al. 2003). Two variants of porcine LXRα gene have been identified, with LXRs-1 expressed in the liver, kidneys, small intestine, heart, muscle, thymus, spleen, and brain, and LXRs-2 expressed only in the thymus and spleen (Thadtha et al. 2006).

In this study, we investigated gene expression profiles of three receptor genes, LILRB3, LAIR1, and LXRα, which were obtained by stimulating pig monocytes with LPS derived from S. Typhimurium.

MATERIALS AND METHODS
Isolation of porcine peripheral blood mononuclear cells

Blood samples were collected from eight healthy and mature pigs (Duroc female) at the Saga Prefectural Livestock Experiment Station using a 20 mL vacuum blood collection tube containing heparin and the collected blood was diluted 2-fold with cold phosphate-buffered saline (PBS). Blood was divided into 8 equal parts and gently layered on a 50 mL centrifuge tube containing 20 mL...
Gene expression in porcine monocytes

Lymphoprep (Abbott Diagnostics Technologies, USA). After centrifugation at 2000 rpm for 20 min at 20 °C, the peripheral blood mononuclear cells (PBMCs) were carefully collected from each centrifuge tube using a pipette, centrifuged at 1600 rpm for 10 min, and then the supernatant was removed by decantation and stored in a deep freezer.

Antibody cell magnetic separation

The PBMCs stored in the deep freezer were thawed rapidly at 37 °C. A 2 mL sample of a well-cooled MACS Buffer (10% FBS and 0.2% EDTA-PBS) was added to the melted PBMC solution and the mixture was centrifuged at 20 °C and 300 × g for 10 min, after which the supernatant was removed. After adding 90 µL of MACS Buffer and stirring well, 5 µL of the antibody was added, mixed well by tapping, and allowed to stand in a refrigerator for 10 min. Anti-porcine SWC3a-FITC (Novus Biologicals, USA) was used as the antibody. After washing twice with MACS Buffer, the antibody-labeled PBMC was mixed with 90 µL of MACS Buffer, and then 5 µL of Anti-FITC MicroBeads (Miltenyi Biotec, USA) was added and mixed, and the mixture was allowed to stand at 4 °C for 15 min. After washing once with MACS Buffer, the MACS column was set in a magnetic field and the filter was set on the column. A round tube for negative collection was set, the sample was applied on the filter, and washing with MACS Buffer 500 µL was performed three times. When all the buffer was dropped, it was removed from the magnet with the column and filter connected and then set in a round tube for positive recovery. A 1 mL sample of the MACS Buffer was added and the buffer was then completely removed, after which the cells were extruded with a plunger. After centrifugation of both round tubes at 20 °C and 300 × g for 10 min, the supernatant was removed, and the MACS Buffer was suspended in 260 µL and stored at −80 °C. Antibody magnetic separation was repeated three times for LPS stimulation.

LPS stimulation of monocytes

Monocytes were obtained by magnetically separating antibodies suspended in 0.1 mL of PBS in a 60 mm Petri dish and 4.9 mL of RPMI1640 medium (20% FBS and 1% mixed antibiotics [penicillin 5 mg/mL, streptomycin 5 mg/mL, and neomycin 10 mg/mL]) was added. To the LPS stimulation group, 0.05 µg of LPS from S. Typhimurium was added. The cells were cultured in a 5% CO2 incubator at 37 °C for 6, 24, and 48 h. To recover the adherent monocytes, they were gently washed twice with 1 mL of 0.05% trypsin solution and incubated at 37 °C for 10 min. RPMI1640 medium (5 mL) was poured into the Petri dish, the cells were gently shaken to completely remove the cells, and the collected solution was centrifuged in a 15 mL centrifuge tube at 300 × g for 10 min to remove the supernatant. The cells were suspended in 100 µL of 0.2% EDTA-PBS and stored at −80 °C. LPS stimulation of monocytes was repeated three times.

RT-qPCR

Total RNA was extracted from each sample according to the manufacturer’s protocol using a RNeasy Plus Micro Kit (Qiagen, the Netherlands) and the expression levels of LILRB3, LAIR1, and two variants of the LXRα gene were estimated by RT-qPCR. Using a One Step SYBR® PrimeScript™ RT-PCR Kit (Takara Bio Inc., Japan), 5.2

Table 1. Primers used for RT-qPCR

| Gene    | Annealing temperature (℃) | Forward sequence | Reverse sequence | Referenced mRNA sequence |
|---------|---------------------------|------------------|------------------|--------------------------|
| β actin | 63.0                      | ggaacctgaaccgactacctca | gaggtctttctgtgctcctca | U07786 |
| LILRB3  | 64.0                      | caaacgctgacctccgactt XM_005664837 | tcacggcagcatagaggttc | XM_005664837 |
| LAIR1   | 61.0                      | tcaaggagagggaggaggagg | tctggctcctgttgccttg | XM_003127459 |
| LXRα-1  | 60.5                      | gacaggaagacagacagc | tctgcgtgctgctgctg | AB254405 |
| LXRα-2  | 62.0                      | gacaggaagacagacagc | gacagacacagtgcctgtg | AB254406 |

LILRB3: leukocyte immunoglobulin-like receptor B3
LAIR1: Leukocyte-associated immunoglobulin like receptor 1
LXRα-1 and LXRα-2 are variants of porcine Liver X Receptor α gene(LXRA) reported by Thadtha et al.(2006).
μL of purified water, 10 μL of buffer, 0.8 μL of primeScript1stStepEnzymeMix, 1 μL of each primer pair, and 2.0 μL of total RNA were mixed in a PCR tube. After a reverse transcription reaction at 42 °C for 5 min and a stop of the reaction at 95 °C for 10 s, a PCR of 40 cycles was performed using a LightCycler96 Real-Time PCR System (Roche, Switzerland). A melting curve was prepared to confirm that there was no non-specific amplification and then a Cq value was obtained using the regression method.

Table 1 shows the primer sequences of each gene, the annealing temperature, and the mRNA sequences used for preparing the primers. Each primer sequence was prepared from the mRNA sequence using the Primer3 program (Untergasser et al. 2012) to be arranged in two exons across introns. Using β-actin as a reference gene, the relative expression level with respect to the average ΔCq value of the control sample at the beginning of the culture in each repeated test was estimated using the ΔΔCq method. The student’s t-test was used for the significant difference test of the relative expression level and a significant difference was determined at p < 0.05.

Animal ethics
The present study was conducted based on the Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd edition, 2010 (https://www.asas.org/docs/default-source/default-document-library/ag_guide_3rded.pdf).

RESULTS AND DISCUSSION
The average relative expression level of the LILRB3 gene increased after 6 h of culture in both the LPS-stimulated and control groups. However, there was a large variation between repetitions and no significant difference could be detected. After 24 and 48 h, the relative expression levels decreased to the same extent as that at the start of the culture (Fig. 1).

The LILRB3 gene belongs to a suppressive LILR subfamily B that has an ITIM structure and responds to immune stimulation. In the present study, there was no difference in gene expression between the LPS-stimulated and control groups, indicating that the LILRB3 gene did not respond to LPS stimulation from S. Typhimurium. In both groups, the relative expression level increased 6 h after culturing and then decreased to the same level as that at the start of the culture. Therefore, the monocytes that had flowed in the blood responded to the environmental change of culturing in a stationary Petri dish and then adapted to that environment.

The relative expression level of the LAIR1 gene increased significantly at 24 and 48 h of culture in the LPS-stimulated group and the relative expression levels of the other samples were almost the same as those at the start of the culture (Fig. 2). This result indicated that the LAIR1 gene

![Fig.1 Relative gene expression ratio of LILRB3 to the control group at the start of culture in LPS stimulation test.](image1)

![Fig.2 Relative gene expression ratio of LAIR1 to the control group at the start of culture in LPS stimulation test.](image2)
Thus, it has been reported that LPS stimulation increases the expression of suppressive LILR genes such as LILRB3 and LAIR1 in monocytes, and the results of LAIR1 in this study are consistent with these reports. On the other hand, LILRB3 gene expression was not changed by LPS stimulation in this study. This difference in the results may be due to the difference in the animal species of pig and mouse and the difference in the kind of LPS bacteria.

The LILR gene group includes many genes and the sequence homology between them is very high (Li et al. 2017). Because many variant mRNAs are expressed from each gene, it is difficult to select primers and set PCR conditions such as the annealing temperature to accurately separate and estimate the expression level of each gene and variant. Many genes in the LILR gene group have very low expression levels and large numbers of monocytes are required for detection by RT-qPCR. We also tried gene expression analysis of LILRA5, LILRA6, LAIR2 and other LILR genes, but in this study we could perform gene expression analysis only for LILRB3 and LAIR1. If a large amount of blood is available, similar studies should be undertaken to examine other LILR genes.

In the LXRα-1 variant of the LXRα gene, the average relative expression level increased with the passage of culture time in the LPS stimulation group. However, there was a large variation between iterations and no significant difference was observed. In the control group, the relative expression level barely changed from that at the start of the culture (Fig. 3). Therefore, LPS stimulation from S. Typhimurium caused the monocytes to respond and increased the gene expression of the LXRα-1 variants. This increase in expression over time may have some relation to the differentiation of monocytes into macrophages by LPS stimulation.

In the LXRα-2 variant, the average gene expression was increased in the LPS-stimulated group after 6 and 48 h of culture, however there was a large variation between iterations and no significant difference was observed (Fig. 4). It is unknown why the expression level decreased significantly after 24 h of culture in the LPS-stimulated group. However, it was approximately twice the relative gene expression level after 24 h of culture in the control group, indicating that the LXRα-2 variant was more sensitive to LPS derived from S. Typhimurium than the LXRα-1 variant.

In studies using cultured cells and mice, Joseph et al. (2003) showed that LXRs and their ligands are negative regulators of macrophage inflammatory gene expression after LPS stimulation and bacterial infection. The porcine LXRα-2 variant may also be an outlet for the control of excessive inflammatory reactions by gram-negative bacteria such as S. Typhimurium.

The immunity of humans and livestock is complicated, and not only promotes an immune response such as inflammation against the invasion of pathogens and toxins, but also poses a danger to the maintenance of the living body when it is not possible to control excessive inflammatory reactions. In the present study, the expression of three immunosuppressive receptor genes, LILRB3, LAIR1, and LXRα, differed greatly depending on the time elapsed in culture in a Petri dish and LPS stimulation from S. Typhimurium. In particular, the expression of immunosuppressive receptor genes, LAIR1 and LXRα, was increased by LPS stimulation from S. Typhimurium. It is unknown what the significance of these receptors is for the transmission of S. Typhimurium to macrophages; however,
Further research is required on these genes to prevent *Salmonella* infection in livestock and poultry.

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