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**Original article**

# Early immune response following *Salmonella enterica* subspecies *enterica* serovar Typhimurium infection in porcine jejunal gut loops

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**Abstract –** *Salmonella enterica* subspecies *enterica* serovar Typhimurium, commonly called *S*. Typhimurium, can cause intestinal infections in humans and various animal species such as swine. To analyze the host response to *Salmonella* infection in the pig we used an in vivo gut loop model, which allows the analysis of multiple immune responses within the same animal. Four jejunal gut-loops were each inoculated with $3 \times 10^8$ cfu of *S*. Typhimurium in 3 one-month-old piglets and mRNA expressions of various cytokines, chemokines, transcription factors, antimicrobial peptides, toll like and chemokine receptors were assessed by quantitative real-time PCR in the Peyer's patch and the gut wall after 24 h. Several genes such as the newly cloned CCRL1/CCX-CKR were assessed for the first time in the pig at the mRNA level. Pro-inflammatory and T-helper type-1 (Th1) cytokine mRNA were expressed at higher levels in infected compared to non-infected control loops. Similarly, some B cell activation genes, NOD2 and toll like receptor 2 and 4 transcripts were more expressed in both tissues while TLR5 mRNA was down-regulated. Interestingly, CCL25 mRNA expression as well as the mRNA expressions of its receptors CCR9 and CCRL1 were decreased both in the Peyer's patch and gut wall suggesting a potential *Salmonella* strategy to reduce lymphocyte homing to the intestine. In conclusion, these results provide insight into the porcine innate mucosal immune response to infection with entero-invasive microorganisms such as *S*. Typhimurium. In the future, this knowledge should help in the development of improved prophylactic and therapeutic approaches against porcine intestinal *S*. Typhimurium infections.

***Salmonella* / Th1 cytokines / pig / CCRL1 / pattern recognition receptor**

## 1. INTRODUCTION

Enteropathogenic *Salmonellae* such as *Salmonella enterica* subspecies *enterica* serovar Typhimurium (commonly called *S*. Typhimurium) and *Salmonella* Typhisuis cause inflammation and necrosis of the small and large intestines of cold and warm blooded animals, resulting in diarrhea that may be accompanied by generalized sepsis. In pigs, all ages are susceptible; however, the disease is most common in weaned and growing-finishing animals. Even though *Salmonella* has been well characterized in terms of genetics, physiology and virulence factors, the understanding of the molecular mechanisms of host pathogen interaction is quite limited. In the pig, few studies have been carried out in the last few years [32, 39, 41–44]. Among

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these studies, some [39, 42] used the original approach of the small intestinal segment perfusion (SISP) [30]. This model was originally described to study the metabolite effects on intestine water absorption capacity [30], but has also proven to be a valuable technique to carry out genomic studies [39]. Markedly different host transcriptional profiles between *Salmonella* serovars Cholerasuis (narrow host range) and Typhimurium (broad host range) have been revealed [39]. Serovar Typhimurium-infected swine presented a transient induction of genes involved in innate and T-helper type-1 (Th1) response early in the infection (24–48 h) followed by a significant repression of Interleukin 12 p35 (IL12 p35), IL12 p40, IL4, IL8 and Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF) [39]. The observed up-regulation of serum IFN gamma and TNF alpha supported the involvement of Th1-mediating cytokines in the porcine response to *Salmonella* infection. The clearance of intracellular pathogens such as *Salmonella* by the host is primarily accomplished by the activation of Th1-mediated immune responses [7, 11, 21, 45]. More recently, a few studies [41–43] have been interested in porcine antimicrobial peptides in the context of *Salmonella* infection. An antimicrobial activity has been demonstrated for porcine beta-defensin 2 (PBD-2) against various bacteria such as *S*. Typhimurium, *Listeria monocytogenes* and *Erysipelothrix rhusiopathiae* and using porcine intestinal cell culture infected with different bacteria, PBD-2 gene expression was shown to increase 10-fold upon infection with *S*. Typhimurium [41]. By contrast, *Arcobacter cryaerophilus* and *Salmonella* Enteritidis, pathogenic bacteria with comparable adhesion and invasion characteristics, failed to increase PBD-2 mRNA expression. Gene expression of PBD-1 was regulated differently since an increase in mRNA expression was only observed upon *Salmonella* Enteritidis infection.

In the current study, we describe the mRNA expression of multiple chemokines, cytokines, pattern recognition receptors, transcription factors and antimicrobial peptides within the Peyer's patch (PP) and the gut wall (GW) of porcine gut loops 24 h after the inoculation of *S*. Typhimurium. This model originally described in sheep [15] is an interesting methodology which allows, similarly to the SISP procedure, the in vivo infection of isolated jejunal segments with a dose of pathogens without removing the blood supply and innervation. Like the SISP procedure, this technique minimizes the effect of individual variation between animals since the control and the infected loops are within the same part of the intestine in the same animal. Moreover, it has been demonstrated that this procedure does not induce any macroscopic or histological alterations in lymph or blood supply with normal cell population and functional mucosal associated lymphoid tissues. This study constitutes a broad and original assessment of the early immune response in the pig gut loop model. In addition, we describe the cloning of ChemoCentryX Chemokine Receptor (CCX-CKR), commonly named CCRL1, the second receptor of CCL25 and we analyzed for the first time its mRNA expression during an infection. As CCRL1, many mRNA expressions were measured to our knowledge for the first time in the pig, particularly the gene coding for recently discovered cytokines such as IL17a, IL22, IL27 p28 and IL33 contributing to a better understanding of porcine immunology.

## 2. MATERIALS AND METHODS

### 2.1. Animals

Landrace piglets between 28 and 32 days of age were used for the experiments. The pigs were healthy and raised in commercial swine herds. Prior to the experiment, the pigs were determined to be free of culturable *Salmonellae* organisms. Twenty-four hours post-surgery, the pigs were euthanized by barbiturate overdose. All experiments were conducted in accordance with the ethical guidelines of the University of Saskatchewan and the Canadian Council for Animal Care.

### 2.2. Bacterial strain, experimental inoculation of intestinal loops and tissue collection

The *Salmonella enterica* subspecies *enterica* serovar Typhimurium strain SL1344 [23] was used

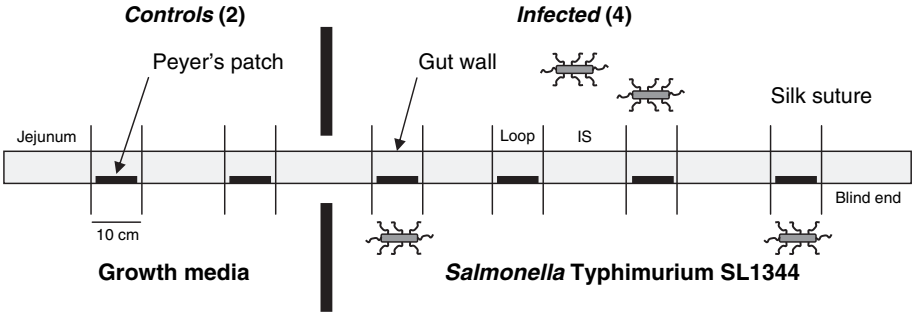


**Figure 1.** Schematic presentation of the jejunal segments used in the experiment ($N = 3$ piglets). Tissues (Peyer's patch and Gut wall) were collected after 24 h. Two loops were used as control (Growth Media) and four loops were infected with *Salmonella enterica* subspecies *enterica* serovar Typhimurium SL1344 (*Salmonella* Typhimurium SL1344). IS: Interspace.

in the current study. Bacteria were prepared freshly for the experiment by cultivation from a frozen stock at 37 °C in Luria Bertani broth (LB: Tryptone 10 g, Yeast extract 5 g, NaCl 10 g/L). Before the experiment, the overnight culture was subcultured 1:100 and incubated for 2 h at 37 °C. Bacteria were then collected in the exponential phase, spun down and resuspended in LB broth. Four jejunal gut-loops were inoculated with $3 \times 10^8$ colony forming units (cfu) of *S*. Typhimurium. As controls, clean bacterial growth media was injected into the two loops. To constitute the loops, a sterile 2–4 m long segment of intestine was surgically prepared in the jejunum, where PP can be individualized, of 3 one-month-old piglets (for a detailed description of the surgical procedure see [15]). This "intestinal-segment" was then subdivided into consecutive segments, designated as "loops" (10–20 cm long, 6 loops), that included a PP, or "interspaces" (20–100 cm long, 7 interspaces), that lacked a visible PP (Fig. 1). All 'loops' were collected 24 h post-surgery before bacteria enumeration. Tissues were cut open in five $3 \times 3$ mm pieces, laid flat, washed with ice cold phosphate-buffered saline, snap-frozen in liquid nitrogen and stored at −80 °C. Additionally few other tissues (duodenum, jejunum, ileum, colon, caecum, mesenteric lymph node, liver, thymus and heart) were collected to check the mRNA expression of CCRL1.

Invasion of *S*. Typhimurium was established, after plating of serial dilution of lumen content and homogenized tissues on SS Agar (Salmonella-Shigella) (Oxoid limited, Basingstoke, UK), by the enumeration in infected and control loops of all the bacteria. The enumeration was performed after an overnight culture at 37 °C. The results showed a massive invasion of the subjacent tissues by the bacteria in infected loops while in control loops only few bacteria, most probably few *Proteus* spp., were detected mainly in the lumen. Moreover a quantitative real-time PCR (qPCR) directed against *sipA* [14] which is involved in the invasion of the bacteria showed a clear up-regulation of *sipA* mRNA expression in all the infected loops versus the control loops confirming the infection in *Salmonella* loops and the absence of bacteria in control loops. Moreover, the level of *sipA* mRNA expression was similar in the different infected loops.

### 2.3. Cloning of the porcine CCRL1 gene

Total RNA was extracted from the pig mesenteric lymph node samples using Trizol reagent (Invitrogen, Cergy-Pontoise, France). The full-length Open Reading Frame (ORF) of porcine CCRL1 was cloned using sequence information of the 3′ end of CCRL1 from a porcine expressed sequence tag (EST) (NCBI accession no. BW955277) and the 5′ end of the porcine CCRL1, which was obtained using a 5′ RACE template switching method [27] with the primer sets CCRL1GSP and CCRL1nGSP (Tab. I). PCR products were cloned using Zero Blunt® TOPO® PCR Cloning Kit for Sequencing from Invitrogen. Inserts were sequenced and their homology to human CCRL1 was determined with Clone Manager 9 (Scientific & Educational Software, Cary, North Carolina, USA). The nucleotide sequence for CCRL1, based on results from five clones,

**Table I.** Primer sequences, annealing temperatures of primer sets, expected PCR fragment sizes and accession numbers.

| Primer name | Primer sequence | Annealing temperature (°C) | PCR product (bp) | Accession number |
|---|---|---|---|---|
| **APRIL/**TNFSF13 | S: TGCTCACCCGTAAACAGAAG<br>AS: TAAACTCCAGCATCCCAGAC | 60 | 172 | **EST** BP170456 |
| **BAFF/**TNFSF13B | S: GAGAGCAGCTCCATTCAAAG<br>AS: GCATGCCACTGTCTGCAATC | 60 | 103 | NM_001097498 |
| **CCL20/**MIP3 alpha | S: GCTCCTGGCTGCTTTGATGTC<br>AS: CATTGGCGAGCTGCTGTGTG | 66 | 146 | NM_001024589 |
| **CCL25/**TECK | S: GCCTACCACAGCCACATTAAG<br>AS: GCTTCCCGCACACCATCTT | 64 | 136 | NM_001025214 |
| **CCL28/**MEC | S: GCTGCTGCACTGAGGTTTC<br>AS: TGAGGGCTGACACAGATTC | 62 | 144 | NM_001024695 |
| **CCR9** | A: TACGGCTATGACGCCACACC<br>AS: ACGGCACCCACGATGAACAC | 69 | 143 | NM_001001624 |
| **CCR10** | A: GCCCGCAGAGCAGGTTTCC<br>AS: CAAAGAGACACTGGGTTGGAA | 66 | 136 | NM_001044563 |
| **CCRL1GSP** | S: CTGCCTGCCTTCTTCACAGTAGCTTTCATC<br>AS: CCACCCATGAACTGCATTAACTGCCCAGAAG | 72 | na | **EST** BW955277 |
| **CCRL1nGSP** | A: CCTTCTATTCACTCTGCCCTTCTGG<br>SS: CCCGCCCTTGTCTTACATCCAGGAG | 72 | na | **EST** BW955277 |
| **CCRL1/**CCX-CKR | A: ACAGATACTGGGCAGTAACG<br>AS: CACACCTCGCTTTGTGATTG | 61 | 147 | NM_001097430 |
| **CD40L** | A: TACGCCCAAGTCACCTTCTG<br>AS: AGACTCCGCCCAAGTGAATG | 62 | 172 | AF248545 |
| **CX3CL1/**Fractalkine | S: GCAGCTCCTAGTCCATTAC<br>AS: CACCATTCTGACCCAGAAG | 58 | 167 | **EST** CK464144 |
| **CX3CR1** | A: ACCTTGCCCTTCTGGACTC<br>AS: ACGGTCCGGTTGTTCATGG | 60 | 182 | **EST** BX919199 |
| **CXCL2/**GRO beta | S: TGCTGCTCCTGCTTCTAGTG<br>AS: TGGCTATGACTTCCGTTTGG | 60 | 171 | NM_001001861 |
| **CXCL10/**IP-10 | S: CCCACATGTTGAGATCATTGC<br>AS: CATCCTTATCAGTAGTGCCG | 60 | 168 | NM_001008691 |
| **FOXP3** | A: GGTGCAGTCTCTGGAACAAC<br>AS: GGTGCCAGTGGCTACAATAC | 65 | 148 | AY669812 |
| **GATA3** | A: CCCGTCCTACTACGGAAAC<br>AS: GTGGTGGATGGACGTCTTG | 60 | 193 | **EST** BW971285 |
| **GM-CSF/**CSF-2 | S: GAAACCGTAGACGTCGTCTG<br>AS: GTGCTGCTCATAGTGCTTGG | 62 | 150 | DQ108393 |
| **HPRT-1** | S: GGACTTGAATCATGTTTGTG<br>AS: CAGATGTTTCCAAACTCAAC | 60 | 91 | DQ815175 |
| **IL1 beta/**LAF | S: AGAAGAGCCCATCGTCCTTG<br>AS: GAGAGCCTTCAGCTCATGTG | 62 | 139 | NM_001005149 |
| **IL2/**TCGF | S: GCCATTGCTGCTGGATTTAC<br>AS: CCCTCCAGAGCTTTGAGTTC | 63 | 159 | NM_213861 |
| **IL4/**BCGF | S: CAACCCTGGTCTGCTTACTG<br>AS: CTTCTCCGTCGTGTTCTCTG | 65 | 173 | NM_214123 |
| **IL5/**EDF | S: TGGAGCTGCCTACGTTAGTG<br>AS: TCGCCTATCAGCAGAGTTCG | 64 | 105 | NM_214205 |
| **IL6/**IFN beta 2 | S: ATCAGGAGACCTGCTTGATG<br>AS: TGGTGGCTTTGTCTGGATTC | 62 | 177 | NM_214399 |
| **IL8/**CXCL-8 | S: TCCTGCTTTCTGCAGCTCTC<br>AS: GGGTGGAAAGGTGTGGAATG | 62 | 100 | NM_213867 |
| **IL10/**B-TCGF | S: ACCAGATGGGCGACTTGTTG<br>AS: TCTCTGCCTTCGGCATTACG | 65 | 123 | NM_214041 |
| **IL12 p35** | S: GGCCTGCTTACCACTTGAAC<br>AS: GCATTCATGGCCTGGAACTC | 64 | 180 | NM_213993 |

**Table I.** Continued.

| Primer name | Primer sequence | Annealing temperature (°C) | PCR product (bp) | Accession number |
|---|---|---|---|---|
| **IL12 p40** | S: CTGAAGAAGACGGCATCACG<br>AS: AGGAGTGACTGGCTCAGAAC | 62 | 148 | NM_214013 |
| **IL13** | A: TGGCGCTCTGGTTGACTCTG<br>AS: CCATGCTGCCGTTGCATAGG | 67 | 159 | NM_213803 |
| **IL15/**IL-T | S: CTGAGGATGGCATTCATGTC<br>AS: GGGATGAGCATCACTTTCAG | 60 | 164 | NM_214390 |
| **IL17a/**CTLA8 | A: CCAGACGGCCCTCAGATTAC<br>AS: CACTTGGCCTCCCAGATCAC | 66 | 103 | AB102693 |
| **IL18/**IGIF | S: ACATCAAGCCGTGTTTGAGG<br>AS: CACTGCACAGAGATGGTTAC | 60 | 129 | EU118362 |
| **IL21** | S: GGCACAGTGGCCCATAAATC<br>AS: GCAGCAATTCAGGGTCCAAG | 62 | 124 | NM_214415 |
| **IL22/**IL-TIF | S: AAGCAGGTCCTGAACTTCAC<br>AS: CACCCTTAATACGGCATTGG | 60 | 133 | AY937228 |
| **IL23 p19** | S: CTCCTTCTCCGCCTCAAGATCC<br>AS: TTGCTGCTCCATGGGCGAAGAC | 70 | 82 | **PEDE Blast**<br>20050322S038657 |
| **IL27 p28** | S: GCCCGCCACTTTGCTGAATC<br>AS: GGGCGAAGTGTCATGGAGAG | 64 | 152 | **EST** BP439244 |
| **IL33** | S: AGCTTCGCTCTGGCCTTATC<br>AS: GCTGACAGGCAGCAAGTACC | 63 | 126 | **EST** BX924734 |
| **IFN gamma** | A: GCTCTGGGAAACTGAATGAC<br>AS: TCTCTGGCCTTGGAACATAG | 60 | 167 | NM_213948 |
| **iNOS** | S: GAGAGGCAGAGGCTTGAGAC<br>AS: TGGAGGAGCTGATGGAGTAG | 62 | 178 | **EST** BI344008 |
| **LTA/** TNF beta | A: CTCCTCAGCGCTCAGAAGTC<br>AS: GAGCGAAGGCTCCAAAGAAG | 64 | 172 | NM_214453 |
| **MAdCAM-1** | A: AGCCTGGGCTCCGTAAAGTC<br>AS: TGGTCAGGGAAGGCGAACAC | 68 | 155 | NM_001037998 |
| **NK-lysin** | S: ATGCGACGGAGAGCAGTTC<br>AS: GTGTCCTCGTTGGGTTGTG | 60 | 156 | X85431 |
| **NOD2** | A: GAGCGCATCCTCTTAACTTTC<br>AS: ACGCTCGTGATCCGTGAAC | 63 | 66 | NM_001105295 |
| **PBD-1** | S: ACCGCCTCCTCCTTGTATTC<br>AS: CACAGGTGCCGATCTGTTTC | 62 | 150 | NM_213838 |
| **PBD-2** | S: TTGCTGCTGCTGACTGTCTG<br>AS: CTTGGCCTTGCCACTGTAAC | 62 | 180 | NM_214442 |
| **PMAP-37/**cathelicidin | S: GCAGTCCTCGGAAGCTAATC<br>AS: CCCGTTCTCCTTGAAGTCAC | 62 | 166 | L39641 |
| **PR-39/**cathelicidin | S: TAATCTCTACCGCCTCCTGG<br>AS: CCCGTTCTCCTTGAAGTCAC | 62 | 151 | NM_214450 |
| **RPL-19** | S: AACTCCCGTCAGCAGATCC<br>AS: AGTACCCTTCCGCTTACCG | 60 | 147 | AF435591 |
| **RORC/**ROR gamma | A: TTCAGTACGTGGTGGAGTTC<br>AS: TGTGGTTGTCAGCGTTGTAG | 60 | 141 | **EST** BP164723 |
| **Secretory component** | S: ACTGGTGTCGCTGGGAAGAG<br>AS: GACCGTGAAGGTGCCATTGC | 64 | 131 | **EST** CJ025705 |
| **SipA** | S: CCAACGCAATGGCGAGTCAC<br>AS: GCCGTCTCCGTTTGATGCGT | 68 | 96 | NC_003197.1 |
| **SMAD2** | A: TGAGTGCCTAAGTGACAGTG<br>AS: CCAGAAGAGCAGCAAATTCC | 60 | 143 | **EST** DB812041 |
| **SMAD3** | A: CGCAGAACGTCAACACCAAG<br>AS: AGCTCATGGTGGCTGTGAAG | 62 | 139 | **EST** BX926114 |
| **STAT3** | A: TGCAGCAGAAAGTGAGCTAC<br>AS: CCGGTCTTGATGACTAATGG | 60 | 166 | NM_001044580 |

**Table I.** Continued.

| Primer name | Primer sequence | Annealing temperature (°C) | PCR product (bp) | Accession number |
|---|---|---|---|---|
| **STAT4** | A: ACCATTCGCTGACATCCTTC<br>AS: TGGGAGCTGTAGTGTTTACC | 60 | 126 | AB20984 |
| **STAT5** | A: CAGCCATCTGGAGGACTAC<br>AS: CATCACGCCATCAAACCAC | 60 | 109 | **EST** CJ011824 |
| **STAT6** | A: TCCCAGCTACGATCAAGATG<br>AS: AGTGAGAGTGTGGTGGATAC | 60 | 171 | **EST** CN155407 |
| **T-Bet** | A: TCAATCCTACTGCCCACTAC<br>AS: TTAGGAGACTCTGGGTGAAC | 60 | 151 | **EST** CJ014895 |
| **TBP-1** | S: AACAGTTCAGTAGTTATGAGCCAGA<br>AS: AGATGTTCTCAAACGCTTCG | 60 | 153 | DQ845178 |
| **TGF beta** | S: GAAGCGCATCGAGGCCATTC<br>AS: GGCTCCGGTTCGACACTTTC | 64 | 162 | NM_214015 |
| **TLR2** | A: ACGGACTGTGGTGCATGAAG<br>AS: GGACACGAAAGCGTCATAGC | 62 | 101 | NM_213761 |
| **TLR4** | A: TGTGCGTGTGAACACCAGAC<br>AS: AGGTGGCGTTCCTGAAACTC | 62 | 136 | NM_001113039 |
| **TLR5** | A: CCTTCCTGCTTCTTTGATGG<br>AS: CTGTGACCGTCCTGATGTAG | 61 | 124 | NM_001123202 |
| **TNF alpha**/TNFSF2 | S: CCAATGGCAGAGTGGGTATG<br>AS: TGAAGAGGACCTGGGAGTAG | 62 | 116 | X54859 |

na: Not available.

was submitted to GenBank (NCBI accession No. NM_001097430).

### 2.4. Messenger RNA expression analysis using real-time PCR

Many mRNA sequences have already been identified in the pig. When genes were not described in this species, tBLASTn searches of the GenBank and PEDEblast EST databases, using known human and murine amino acid sequences, were performed. This methodology enables the identification of porcine expressed sequence tags (EST) corresponding to human and murine sequences (Tab. I). Then, primers (Tab. I) were designed using Clone Manager 9 (Scientific & Educational Software) and were purchased from Eurogentec (Liège, Belgium).

Quantitative real-time PCR (qPCR) was performed using cDNA synthesized as previously described [29]. Diluted cDNA (40×) was combined with primer/probe sets and IQ SYBR Green Supermix (Bio-Rad, Hercules, California, USA) according to the manufacturer's recommendations. The qPCR conditions were 95 °C for 3 min, followed by 45 cycles with denaturation at 95 °C for 15 s, annealing temperature (Tab. I) for 30 s and elongation at 72 °C for 30 s. Real time assays were run on a Bio-Rad iCycler iQ. The specificity of the qPCR reactions was assessed by analyzing the melting curves of the products and size verification of the amplicons. To minimize sample variation, tissue samples of similar size and location and identical quantities of high quality RNA with no signs of degradation were used. Samples were normalized internally using simultaneously the average cycle threshold (*Ct*) of Hypoxanthine PhosphoRibosyl-Transferase 1 (HPRT-1), Ribosomal Protein L 19 (RPL-19) and Tata Box Binding Protein 1 (TBP-1) [33] as references in each sample to avoid any artifact of variation in the target gene. HPRT-1, RPL-19 and TBP-1 genes were selected as the reference genes because of their extremely low variation among samples. A standard curve was generated using diluted cDNA. The correlation coefficients of the standard curves were $> 0.995$ and the concentration of the test samples were calculated from the standard curves, according to the formula $y = -M \times Ct + B$, where $M$ is the slope of the curve, $Ct$ the point during the exponential phase of amplification in which the fluorescent signal is first recorded as being statistically significant above background and $B$ the y-axis intercept. All qPCR displayed efficiency between 90% and 110% according to the equation: qPCR efficiency $= (10^{[-1/M]} - 1) \times 100$. Expression data

are expressed as relative values after Genex macro analysis (Bio-Rad) [40].

### 2.5. Statistical analysis

Data for the comparison of differences in mRNA expression between infected and non infected tissues are expressed as relative values. Most of the data were normally distributed as confirmed by the Shapiro-Wilk normality test (using Statistix 7.0®, Analytical software, Tallahassee, Florida, USA). When the data were paired and normally distributed, group means were compared using Student's Paired *t*-test (using GraphPad Prism® software version 3.00, GraphPad Software Inc., San Diego, California, USA). Paired, non-normally distributed data were analyzed using the Wilcoxon Signed Rank Test (Exact). Differences between groups were considered significant when $P < 0.05$.

## 3. RESULTS

### 3.1. Cloning of porcine CCRL1 gene

The porcine CCRL1 cDNA sequence (GenBank accession number NM_001097430) was found to be 1053 nucleotides in length, encoding a predicted precursor protein of 350 amino acids such as human (GenBank accession number NM_178445) and murine (GenBank accession number NM_145700) CCRL1. At the protein level, 88 and 85% of identity were found to human and murine homologous sequences, respectively. With the cloning of CCRL1, the sequences of the two receptors of CCL25, CCR9 and CCRL1, are now available. CCRL1 mRNA was expressed (between 21 and 28 *Ct*) in various tissues such as the duodenum, jejunum, ileum, colon, caecum, liver, thymus and heart (data not shown).

### 3.2. Cytokine response to *Salmonella* Typhimurium in the gut loop model

To increase our understanding of the early immune response in vivo, we decided to assess the mRNA expression of various Th1, Th2 and newly described Th17 cytokines as well as transcription factors and antimicrobial molecules such as defensins and iNOS (generating Nitric oxide (NO)) after 24 h of infection in the porcine gut loop model. Among cytokines, the mRNA expression inflammation associated cytokines such as IL6, IL8/CXCL8 and TNF alpha were strongly and significantly up-regulated, particularly in the GW (Tab. II). Similarly, mRNA expressions of Th1 cytokines, IL12 p35, IL12 p40, IL27 p28 and IFN gamma, were up-regulated in infected GW and PP (Tab. II and Fig. 2). In contrast, Th2 (IL4, IL5, IL13 and IL33) and Th17 (IL17a, IL21, IL22 and IL23 p19) cytokine mRNA expressions were not up-regulated except for IL33 mRNA expression which was significantly up-regulated in the PP ($P = 0.022$) (Tab. II). Regarding transcription factors associated to Th1 (T-Bet, STAT4), Th2 (GATA3, STAT6) and Th17 (RORC, STAT3) orientations, we did not detect any up-regulation except for STAT4 in the infected GW. For regulatory cytokines, IL10 and TGF beta mRNA expressions were increased in infected GW versus control GW while the forkhead transcription factor FOXP3 was significantly more expressed in the PP than in GW and did not show any increase after infection (Tab. II). Antimicrobial peptide production has been assessed in few studies using intestinal epithelial cell lines and the SISP technique with contradictory results. Consequently, the mRNA expressions of PBD-1, PBD-2, PMAP37, PR39 and NK lysine have been assessed in the gut loop model. Only the expression of PBD-2 mRNA was increased in the infected PP ($P < 0.05$) (Tab. II). Furthermore, iNOS mRNA expression was strongly up-regulated in the infected GW and PP (Tab. II).

GM-CSF, IL1 beta, IL2, IL15, IL18 and LTA mRNA expressions were not up-regulated in the infected tissues (Tab. II).

APRIL and B cell activating factor (BAFF) and their implication in the IgA class-switch recombination (CSR) have been extensively studied in the last few years. In humans and mice, T-cell-dependent IgA CSR is induced by TGF beta and CD40L expressed on activated T cells. On the contrary, APRIL and BAFF directly mediate T-cell-independent IgA CSR. Consequently, we chose to investigate the mRNA expression of these cytokines, which make a link between epithelial cells and

**Table II.** Statistical comparisons between mRNA levels of expression. Levels of expression are shown in the second column (High: Amplification around 17–24 cycle thresholds (*Ct*), very low more than 33 *Ct*). When the data were paired and normally distributed, group means were compared using Student's Paired *t*-test. Paired, non-normally distributed data were analysed using the Wilcoxon Signed Rank Test (Exact).

| Messenger RNA | Level of expression | *P* value | | |
|---|---|---|---|---|
| | | cPP vs. iPP | cGW vs. iGW | cPP vs. cGW |
| **APRIL**/TNFSF13 | High | 0.096 > | 0.127 | 0.069 < |
| **BAFF**/TNFSF13B | High | 0.006 ** < | 0.058 < | 0.009 ** > |
| **CCL20**/MIP3 alpha | High | 0.368 | 0.288 | 0.510 |
| **CCL25**/TECK | High | 0.008 ** > | 0.009 ** > | 0.121 |
| **CCL28**/MEC | Moderate | 0.381 | 0.114 | 0.027 * < |
| **CCR9** | High | 0.004 ** > | 0.559 | 0.227 |
| **CCR10** | Moderate | 0.433 | 0.213 | 0.148 |
| **CCRL1**/CCR11 | High | 0.059 > | 0.318 | 0.573 |
| **CD40L** | Moderate | 0.128 | 0.382 | 0.004 ** > |
| **CX3CL1**/Fractalkine | Moderate | 0.1727 | 0.007 ** < | 0.083 > |
| **CX3CR1** | Low | 0.671 | 0.236 | 0.103 |
| **CXCL2**/GRO beta | Moderate | 0.117 | 0.013 * < | 0.637 |
| **CXCL10**/IP-10 | High | 0.002 ** < | 0.010 * < | 0.565 |
| **FOXP3** | Low | 0.609 | 0.305 | 0.001 ** > |
| **GATA3** | Low | 0.220 | 0.399 | 0.048 * > |
| **GM-CSF**/CSF-2 | Low | 0.452 | 0.156 | 0.227 |
| **IL2**/TCGF | Low | 0.201 | 0.608 | 0.038 * > |
| **IL1 beta**/LAF | Very low | na | na | na |
| **IL4**/BCGF | Very low | 0.616 | 0.061 < | 0.383 |
| **IL5**/EDF | Moderate | 0.086 > | 0.375 | 0.639 |
| **IL6**/IFN beta 2 | Moderate | 0.013 * < | 0.039 * < | 0.592 |
| **IL8**/CXCL-8 | High | 0.149 | 0.001 ** < | 0.843 |
| **IL10**/B-TCGF | Moderate | 0.118 | 0.067 * < | 0.806 |
| **IL12 p35** | Moderate | 0.009 ** < | 0.001 ** < | 0.003 ** > |
| **IL12 p40** | Moderate | 0.003 ** < | 0.027 * < | 0.018 * > |
| **IL13** | Very low | 0.151 | 0.091 < | 0.415 |
| **IL15**/IL-T | Moderate | 0.263 | 0.142 | 0.813 |
| **IL17a**/CTLA8 | Low | 0.541 | 0.062 < | 0.239 |
| **IL18**/IGIF | High | 0.150 | 0.869 | 0.187 |
| **IL21** | Low | 0.319 | 0.553 | 0.238 |
| **IL22**/IL-TIF | Moderate | 0.100 | 0.761 | 0.684 |
| **IL23 p19** | Moderate | 0.325 | 0.922 | 0.036 * < |
| **IL27 p28** | Moderate | 0.019 * < | 0.105 | 0.239 |
| **IL33** | Moderate | 0.022 * < | 0.703 | 0.322 |
| **IFN gamma** | Moderate | 0.010 * < | 0.009 ** < | 0.237 |
| **iNOS** | Moderate | 0.009 ** < | 0.015 * < | 0.831 |
| **LTA**/TNF beta | Low | 0.972 | 0.715 | 0.141 |
| **MAdCAM-1** | High | 0.244 | 0.148 | 0.410 |
| **NK-lysin** | High | 0.164 | 0.354 | 0.181 |
| **NOD2** | Moderate | 0.013 * < | 0.004 ** < | 0.102 |
| **PBD-1** | Moderate | 0.213 | 0.051 > | 0.065 > |
| **PBD-2** | Moderate | 0.017 * < | 0.280 | 0.025 * > |
| **PMAP-37**/cathelicidin | Very low | 0.013 * > | 0.289 | 0.134 |
| **PR-39** | Very low | 0.106 | 0.089 > | 0.027 * > |
| **RORC**/ROR gamma | Moderate | 0.340 | 0.201 | 0.035 * < |

**Table II.** Continued.

| Messenger RNA | Level of expression | *P* value | | |
|---|---|---|---|---|
| | | cPP vs. iPP | cGW vs. iGW | cPP vs. cGW |
| **Secretory component** | High | 0.026 * > | 0.037 * > | 0.122 |
| **SMAD2** | High | 0.648 | 0.613 | 0.052 > |
| **SMAD3** | Moderate | 0.070 > | 0.849 | 0.644 |
| **STAT3** | High | 0.606 | 0.224 | 0.856 |
| **STAT4** | Moderate | 0.363 | 0.015 * < | 0.018 * > |
| **STAT5** | Moderate | 0.828 | 0.846 | 0.629 |
| **STAT6** | Moderate | 0.828 | 0.691 | 0.155 |
| **T-Bet** | Low | 0.710 | 0.207 | 0.127 |
| **TGF beta** | Moderate | 0.253 | 0.002 ** < | 0.005 ** > |
| **TLR2** | Moderate | 0.002 ** < | 0.044 * < | 0.797 |
| **TLR4** | Moderate | 0.001 ** < | 0.020 * < | 0.021 * > |
| **TLR5** | Moderate | 0.020 * > | 0.001 ** > | 0.514 |
| **TNF alpha**/TNFSF2 | Moderate | 0.007 ** < | 0.002 ** < | 0.004 ** > |

cPP: Control Peyer's Patch (PP); iPP: Infected PP; cGW: Control Gut Wall (GW); iGW: Infected GW. * $P < 0.050$, ** $P < 0.010$. na: Not available, expression too low. Higher (>) or lower (<) mRNA expression in the control than in the infected tissues.

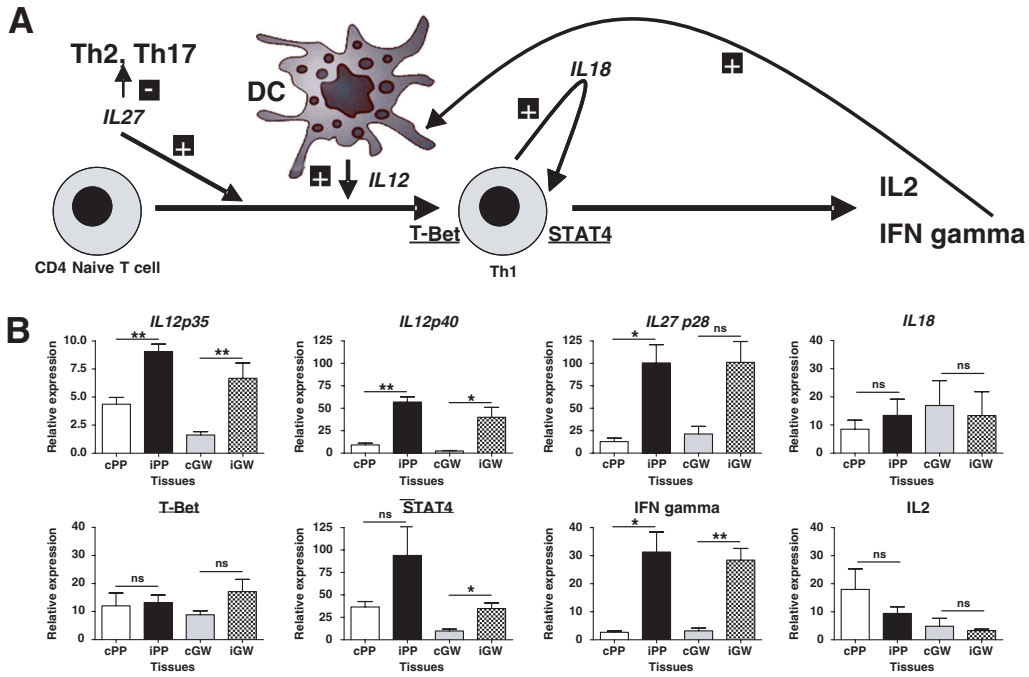


**Figure 2.** (A) Schematic presentation of the T-helper type-1 (Th1) mediated immune response. (B) Relative mRNA expression of Th1 cytokines and transcription factors. Data were presented as mean ± S.E.M. for a total of 8 control and 12 infected loops. * $P < 0.05$, ** $P < 0.01$, ns: not significant (Student's Paired *t*-test or Wilcoxon Signed Rank Test (Exact)). DC: Dendritic Cell; cPP: Control Peyer's Patch (PP); iPP: Infected PP; cGW: Control Gut Wall (GW); iGW: Infected GW.

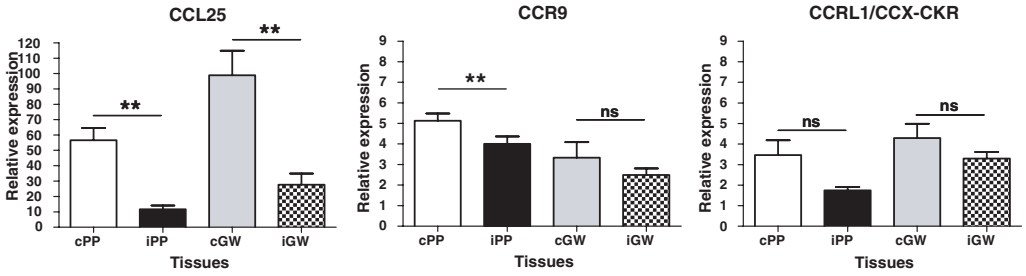


**Figure 3.** Relative mRNA expression of CCL25 and its two receptors, CCR9 and CCRL1. Data were presented as mean ± S.E.M. for a total of 8 control and 12 infected loops. * $P < 0.05$, ** $P < 0.01$, ns: not significant (Student's Paired $t$-test or Wilcoxon Signed Rank Test (Exact)).

IgA secretion by B cells. While the mRNA expression of BAFF was significantly up-regulated in the PP, there was no increase of its expression in the GW after 24 h of infection with *S*. Typhimurium (Tab. II). In contrast to BAFF, APRIL and CD40L were not up-regulated in infected tissues (Tab. II). Regarding TGF beta, which is more expressed as CD40L and BAFF in the PP, the mRNA expression was higher in the infected GW than in the control while there was no significant difference between control and infected PP (Tab. II).

### 3.3. Transcript expression of intestinal chemokines and their receptors and mucosal addressin cellular adhesion molecule 1 in the gut following *Salmonella* Typhimurium inoculation

CCL25 and CCL28 play a crucial role in lymphocyte trafficking to the gut. Since little is known about their expression in the context of an intestinal infection, we decided to assess mRNA expression of these chemokines and their receptors (CCR9, CCR10 and CCRL1) in GW and PP collected 24 h after inoculation of *S*. Typhimurium in the gut loops. Surprisingly, important statistically significant down-regulations ($P < 0.01$) of CCL25 mRNA expression were detected in both PP and GW (Tab. II and Fig. 3). Similarly, but to a lower extent, the mRNA expressions of the two receptors of CCL25, CCR9 and CCRL1 were down-regulated, particularly in the PP which is rich in lymphocytes (Fig. 3). Regarding CCL28 and its receptor, CCR10, we did not observe any down- or up-regulations (Tab. II). Then, we investigated the expression of two chemokines (CX3CL1 and CCL20) involved in dendritic cell recruitment. CX3CL1 was up-regulated at the mRNA level ($P < 0.01$) in the infected GW while CCL20 mRNA expression did not increase in the infected PP nor in the infected GW (Tab. II). The receptor of CX3CL1, CX3CR1, was not up-regulated in infected PP as well as infected GW (Tab. II). Interestingly, CXCL10, a potent chemoattractant of Th1 $CD4^+$ and Natural Killer (NK) cells, was strongly up-regulated at the mRNA level in both infected PP and GW (Tab. II). Moreover, mRNA expression of CXCL2, a neutrophil chemoattractant was up-regulated in the infected GW (Tab. II). Regarding the mucosal addressin cellular adhesion molecule 1 (MAdCAM-1), controlling with its ligand, integrin $\alpha 4\beta 7$, the first steps of B and T cell migration to the gut, we did not observe any up-regulation in infected PP and GW (Tab. II).

### 3.4. Pattern-recognition receptor mRNA expression in the gut following *Salmonella* Typhimurium inoculation

Intestinal epithelial cells and immune cells are able to sense the presence of pathogens via pattern-recognition receptors such as Toll-like receptors (TLR) and intracellular Nod-like receptors. TLR2, 4 and 5 and the nucleotide-binding oligomerization domain-2 (NOD2) are known for their implication in

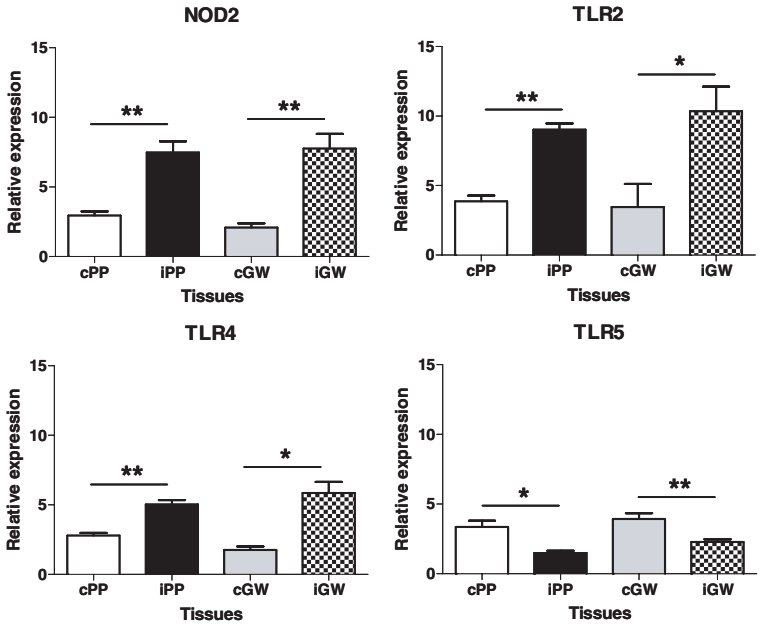


**Figure 4.** Relative mRNA expression of four pattern recognition receptors (NOD2, TLR2, TLR4 and TLR5) usually associated with *Salmonella*. Data were presented as mean ± S.E.M. for a total of 8 control and 12 infected loops. * $P < 0.05$, ** $P < 0.01$, ns: not significant (Student's Paired $t$-test or Wilcoxon Signed Rank Test (Exact)).

the recognition of *Salmonella* spp. Therefore, we assessed their mRNA expression in the gut loop model after inoculation of *S*. Typhimurium. Expressions of NOD2, TLR2 and TLR4 mRNA were significantly up-regulated both in PP and GW in response to *Salmonella* infection while the mRNA expression of TLR5 was significantly down-regulated in both tissues (Fig. 4).

## 4. DISCUSSION

In the current article, the cloning of the porcine CCRL1/CCX-CKR, the second receptor of the intestinal chemokine CCL25 is reported and the mRNA expression of CCL25 and its two receptors is assessed for the first time in the context of *S*. Typhimurium infection. Moreover, a first broad assessment at the mRNA level of the innate immune response and the Th orientation was carried out in response to *S*. Typhimurium in a porcine gut loop model. Several genes involved in the innate response and the establishment of the adaptive response displayed changed expression patterns, demonstrating the complex interactions between the facultative intracellular bacteria *S*. Typhimurium and the intestinal mucosa.

Several papers have focused recently on "scavenger receptors" which are "atypical" receptors playing a role in scavenging or altering the localization of chemoattractant molecules such as chemokines and complement molecules [8, 10, 25]. The "atypical" receptor family comprised the receptors D6, the Duffy Antigen/Receptor for Chemokines and CCRL1/CCX-CKR [8, 10]. CCRL1 was described in mice and humans but there was no data about this receptor in the pig [9, 16, 34, 37]. As previously shown, this receptor of CCL19, CCL21 and CCL25 is expressed in various tissues such as the duodenum, jejunum, ileum, colon, caecum, liver, thymus and heart [20, 34, 37]. At the protein level, the sequence of porcine CCRL1 is very close

to the murine and human sequences with 85–88% identity suggesting similar function in swine. Data from in vitro studies suggest that CCRL1 may be able to act as a chemokine scavenger, at least with one of its chemokine ligands, CCL19 [9]. Further experiments are required to characterize the function of this receptor in the pig and its relation with CCL25. Interestingly, in the current study, parallel decreases were observed for the mRNA expression of CCR9 and CCRL1 receptors and the chemokine CCL25 in the context of an infection with *S*. Typhimurium confirming a previously observed influence of intestinal bacteria on this chemokine mRNA expression [29]. The drastic and significant reduction in the mRNA expression of CCL25 suggests a potential strategy of *S*. Typhimurium to reduce lymphocyte homing to the intestine. Indeed, it is well known that *Salmonella enterica* is able to develop many strategies to escape T cell immunity (for a review see [4]). In contrast to the CCL25/CCR9-CCRL1 axis, the CCL28/CCR10 axis was not induced. The induction of CCL28 is different from that of CCL25 which is atypical with the involvement of caudal-related homeobox 2 [13, 29].

Regarding the cytokines and the Th orientation, a clear up-regulation of the mRNA expression has been observed for IL27 p28, IL12 p35 and p40 and IFN gamma which are Th1 cytokines and for STAT4, a Th1-associated transcription factor. This profile further confirms and completes the Th1 orientation of the immune response to *S*. Typhimurium in the pig [7, 21, 32]. To our knowledge, this is the first broad assessment at the mRNA level of Th orientations in the pig. Indeed, IL27 p28, T-Bet and STAT4 (for Th1 orientation) have not been assessed in this context before. The Th1 response orientation with a strong up-regulation of the mRNA expression of CXCL10 has been previously observed in both PP and GW [39]. The expression of CXCL10, a potent chemoattractant of Th1-type $CD4^+$and NK cells [31], has been shown recently to be controlled by TLR4 in the context of *Salmonella* infection [22] and TLR4 mRNA expression was strongly up-regulated in our conditions. With IL2 and IL18 mRNA expression, we did not observe any up-regulation. This could be explained by the time when the tissues were collected since up-regulation or down-regulation could take place earlier or later. Similarly, it was also probably too early or too late to detect any up-regulation in most of the transcription factor mRNA expressions. Only STAT4 mRNA, in the IL-12 signaling pathway, was more expressed in the infected GW. For genes associated with inflammation, an induction of IL6, IL8 and TNF alpha mRNA was observed at 24 h as previously shown in the pig [39]. However, in our conditions, IL1 beta and GM-CSF mRNA expressions were at very low levels with no increase in the infected tissues. In contrast to Th1 genes, Th2 and Th17 genes did not show any induction except for IL10 in the GW and IL33 in the PP. IL10 mRNA expression increase was associated with TGF beta mRNA expression increase in the same tissue. This double increase could be associated with the induction of T regulatory cells at this location. However, this hypothesis could not be sustained by an increase in FOXP3 mRNA expression which was high in PP and low in GW. IL33 mRNA expression is not documented for the pig in the literature and it is very difficult, so far, to explain this, such as IL33 mRNA expression variation in the context of a *Salmonella* infection. Nevertheless, we can make the hypothesis that this induction, if biologically significant, could be related to the establishment of a humoral response in the PP. BAFF which is involved in the IgA CSR in humans and mice in both PP and *lamina propria* [6, 24, 26] was also up-regulated at the mRNA level in PP. The role of IL33 and BAFF in a humoral response to *Salmonella* in the pig has to be confirmed and further studies are required.

Concerning the recruitment of dendritic cells, we identified a significant up-regulation of CX3CL1 mRNA expression ($P < 0.01$) in infected GW while CCL20 mRNA expression was not altered. These results are a little bit surprising considering reports in mice [18] showing that, at least, two main populations of dendritic cells are located in the gut: One of the sentinels of the intestinal lumen – responding

to CX3CL1 –, sampling and presentating harmless commensal micro-organisms and one of fully competent cells – responding to CCL20 – able to respond quickly and properly to pathogens. This discrepancy could be due to a sequential recruitment of dendritic cells through CCL20 earlier in the infection course.

Regarding the first steps in the recognition of the pathogens by the immune system, a strong up-regulation of NOD2, TLR2 and TLR4 mRNA expression was observed in the PP and the GW. The up-regulation was particularly obvious in the PP where NOD2 is highly expressed in the adult pig [36]. This observation is not surprising since PP M cells constitute a preferential site of entry for the bacteria [17, 19]. Surprisingly, TLR5 mRNA expression was down-regulated in both tissues. Abasht et al. made a similar observation with *Salmonella enterica* serovar Enteritidis in chicken caecum and liver [1]. They hypothesized that the down-regulation of TLR5 RNA expression might be beneficial to protect host cells from over-stimulation by bacterial flagellin [1]. An explanation to this down-regulation could be, as observed with *Borrelia burgdorferi*, that the pathogen lipoprotein-mediated TLR2 stimulation could cause the down-regulation of TLR5 to escape the immune response [5]. TLR2 can act synergistically with NOD2 and TLR4 while there is no evidence of such a synergism with TLR5 [38].

Antimicrobial peptides, NO and NK-lysin are known to be active against *Salmonella* [2, 3, 35, 43, 46]. In our conditions, we detected iNOS up-regulations in both PP and GW ($P < 0.01$) and PBD-2 and PMAP-37 only in PP. The up-regulation of iNOS sustains the anti-*Salmonella* role of NO [2]. In vitro, PBD-2 mRNA expression was increased 10-fold upon infection with *S*. Typhimurium [41] while in vivo PBD-2 was only slightly increased in some infected intestinal segments [42]. In our conditions, PBD-2 mRNA was moderately induced only in the infected PP ($P < 0.05$) in accordance with Veldhuizen et al. observations [42]. However, the in vivo significance of these differences is difficult to appreciate because of the low magnitude of the observed differences and the quite low starting level of expression. Further experiments are needed to definitely elucidate the role of PBD-2 in the protection against *S*. Typhimurium. For PMAP-37 a similar conclusion can be made since levels of expression are low. NK-lysin, PBD-1 and PR-39 genes were not induced in both PP and GW 24 h after the inoculations. This observation for PBD-1 is similar to previous reports [41, 42].

For the current study, one-month-old piglets were used. The mRNA expressions of the different genes were similar to other studies using young piglets [32, 39, 41]. Nevertheless, mRNA expressions could be different in adults as observed previously [12, 28] explaining differences in the response to the infection between young and adult pigs.

In conclusion, the inoculation of *S*. Typhimurium in the gut loop model has enabled a first broad assessment at the mRNA level of the T-helper orientation as well as many newly described genes in response to *Salmonella* in the pig. In the future, all the data collected here should help in the development of improved prophylactic and therapeutic approaches against porcine intestinal *S*. Typhimurium infections.

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