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

François MEURENS[1]*, Mustapha BERRI[1], Gael AURAY[2], Sandrine MELO[1], Benoît LEVAST[1], Isabelle VIRLOGEUX-PAYANT[1], Claire CHEVALEYRE[1], Volker GERDTS[2], Henri SALMON[1]

[1] Institut National de la Recherche Agronomique (INRA), UR1282, Infectiologie Animale et Santé Publique, F-37380 Nouzilly (Tours), France
[2] Vaccine and Infectious Disease Organization and Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon S7N 5E3, Saskatchewan, Canada

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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 3×10^8 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

* Corresponding author: Francois.Meurens@tours.inra.fr

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

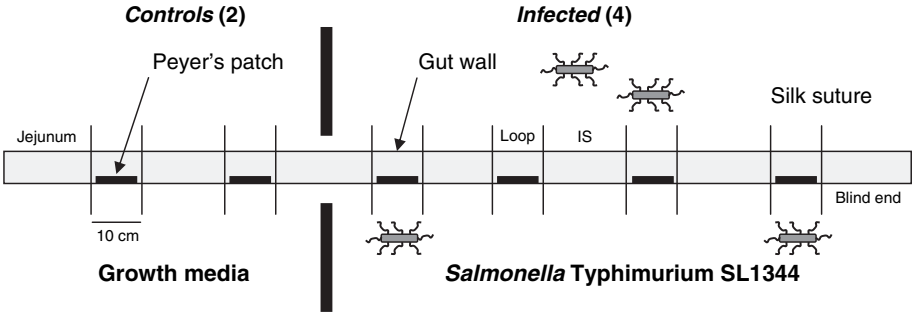

Figure 1. Schematic presentation of the jejunal segments used in the experiment ($N = 3$ piglets). Tissues (Peyer's patch and Gut wall) were collected after 24 h. Two loops were used as control (Growth Media) and four loops were infected with *Salmonella enterica* subspecies *enterica* serovar Typhimurium 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 3×10^8 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 3×3 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 AS: TAAACTCCAGCATCCCAGAC	60	172	**EST** BP170456
BAFF/TNFSF13B	S: GAGAGCAGCTCCATTCAAAG AS: GCATGCCACTGTCTGCAATC	60	103	NM_001097498
CCL20/MIP3 alpha	S: GCTCCTGGCTGCTTTGATGTC AS: CATTGGCGAGCTGCTGTGTG	66	146	NM_001024589
CCL25/TECK	S: GCCTACCACAGCCACATTAAG AS: GCTTCCCGCACACCATCTT	64	136	NM_001025214
CCL28/MEC	S: GCTGCTGCACTGAGGTTTC AS: TGAGGGCTGACACAGATTC	62	144	NM_001024695
CCR9	A: TACGGCTATGACGCCACACC AS: ACGGCACCCACGATGAACAC	69	143	NM_001001624
CCR10	A: GCCCGCAGAGCAGGTTTCC AS: CAAAGAGACACTGGGTTGGAA	66	136	NM_001044563
CCRL1GSP	S: CTGCCTGCCTTCTTCACAGTAGCTTTCATC AS: CCACCCATGAACTGCATTAACTGCCCAGAAG	72	na	**EST** BW955277
CCRL1nGSP	A: CCTTCTATTCACTCTGCCCTTCTGG SS: CCCGCCCTTGTCTTACATCCAGGAG	72	na	**EST** BW955277
CCRL1/CCX-CKR	A: ACAGATACTGGGCAGTAACG AS: CACACCTCGCTTTGTGATTG	61	147	NM_001097430
CD40L	A: TACGCCCAAGTCACCTTCTG AS: AGACTCCGCCCAAGTGAATG	62	172	AF248545
CX3CL1/Fractalkine	S: GCAGCTCCTAGTCCATTAC AS: CACCATTCTGACCCAGAAG	58	167	**EST** CK464144
CX3CR1	A: ACCTTGCCCTTCTGGACTC AS: ACGGTCCGGTTGTTCATGG	60	182	**EST** BX919199
CXCL2/GRO beta	S: TGCTGCTCCTGCTTCTAGTG AS: TGGCTATGACTTCCGTTTGG	60	171	NM_001001861
CXCL10/IP-10	S: CCCACATGTTGAGATCATTGC AS: CATCCTTATCAGTAGTGCCG	60	168	NM_001008691
FOXP3	A: GGTGCAGTCTCTGGAACAAC AS: GGTGCCAGTGGCTACAATAC	65	148	AY669812
GATA3	A: CCCGTCCTACTACGGAAAC AS: GTGGTGGATGGACGTCTTG	60	193	**EST** BW971285
GM-CSF/CSF-2	S: GAAACCGTAGACGTCGTCTG AS: GTGCTGCTCATAGTGCTTGG	62	150	DQ108393
HPRT-1	S: GGACTTGAATCATGTTTGTG AS: CAGATGTTTCCAAACTCAAC	60	91	DQ815175
IL1 beta/LAF	S: AGAAGAGCCCATCGTCCTTG AS: GAGAGCCTTCAGCTCATGTG	62	139	NM_001005149
IL2/TCGF	S: GCCATTGCTGCTGGATTTAC AS: CCCTCCAGAGCTTTGAGTTC	63	159	NM_213861
IL4/BCGF	S: CAACCCTGGTCTGCTTACTG AS: CTTCTCCGTCGTGTTCTCTG	65	173	NM_214123
IL5/EDF	S: TGGAGCTGCCTACGTTAGTG AS: TCGCCTATCAGCAGAGTTCG	64	105	NM_214205
IL6/IFN beta 2	S: ATCAGGAGACCTGCTTGATG AS: TGGTGGCTTTGTCTGGATTC	62	177	NM_214399
IL8/CXCL-8	S: TCCTGCTTTCTGCAGCTCTC AS: GGGTGGAAAGGTGTGGAATG	62	100	NM_213867
IL10/B-TCGF	S: ACCAGATGGGCGACTTGTTG AS: TCTCTGCCTTCGGCATTACG	65	123	NM_214041
IL12 p35	S: GGCCTGCTTACCACTTGAAC 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 AS: AGGAGTGACTGGCTCAGAAC	62	148	NM_214013
IL13	A: TGGCGCTCTGGTTGACTCTG AS: CCATGCTGCCGTTGCATAGG	67	159	NM_213803
IL15/IL-T	S: CTGAGGATGGCATTCATGTC AS: GGGATGAGCATCACTTTCAG	60	164	NM_214390
IL17a/CTLA8	A: CCAGACGGCCCTCAGATTAC AS: CACTTGGCCTCCCAGATCAC	66	103	AB102693
IL18/IGIF	S: ACATCAAGCCGTGTTTGAGG AS: CACTGCACAGAGATGGTTAC	60	129	EU118362
IL21	S: GGCACAGTGGCCCATAAATC AS: GCAGCAATTCAGGGTCCAAG	62	124	NM_214415
IL22/IL-TIF	S: AAGCAGGTCCTGAACTTCAC AS: CACCCTTAATACGGCATTGG	60	133	AY937228
IL23 p19	S: CTCCTTCTCCGCCTCAAGATCC AS: TTGCTGCTCCATGGGCGAAGAC	70	82	**PEDE Blast** 20050322S038657
IL27 p28	S: GCCCGCCACTTTGCTGAATC AS: GGGCGAAGTGTCATGGAGAG	64	152	**EST** BP439244
IL33	S: AGCTTCGCTCTGGCCTTATC AS: GCTGACAGGCAGCAAGTACC	63	126	**EST** BX924734
IFN gamma	A: GCTCTGGGAAACTGAATGAC AS: TCTCTGGCCTTGGAACATAG	60	167	NM_213948
iNOS	S: GAGAGGCAGAGGCTTGAGAC AS: TGGAGGAGCTGATGGAGTAG	62	178	**EST** BI344008
LTA/ TNF beta	A: CTCCTCAGCGCTCAGAAGTC AS: GAGCGAAGGCTCCAAAGAAG	64	172	NM_214453
MAdCAM-1	A: AGCCTGGGCTCCGTAAAGTC AS: TGGTCAGGGAAGGCGAACAC	68	155	NM_001037998
NK-lysin	S: ATGCGACGGAGAGCAGTTC AS: GTGTCCTCGTTGGGTTGTG	60	156	X85431
NOD2	A: GAGCGCATCCTCTTAACTTTC AS: ACGCTCGTGATCCGTGAAC	63	66	NM_001105295
PBD-1	S: ACCGCCTCCTCCTTGTATTC AS: CACAGGTGCCGATCTGTTTC	62	150	NM_213838
PBD-2	S: TTGCTGCTGCTGACTGTCTG AS: CTTGGCCTTGCCACTGTAAC	62	180	NM_214442
PMAP-37/cathelicidin	S: GCAGTCCTCGGAAGCTAATC AS: CCCGTTCTCCTTGAAGTCAC	62	166	L39641
PR-39/cathelicidin	S: TAATCTCTACCGCCTCCTGG AS: CCCGTTCTCCTTGAAGTCAC	62	151	NM_214450
RPL-19	S: AACTCCCGTCAGCAGATCC AS: AGTACCCTTCCGCTTACCG	60	147	AF435591
RORC/ROR gamma	A: TTCAGTACGTGGTGGAGTTC AS: TGTGGTTGTCAGCGTTGTAG	60	141	**EST** BP164723
Secretory component	S: ACTGGTGTCGCTGGGAAGAG AS: GACCGTGAAGGTGCCATTGC	64	131	**EST** CJ025705
SipA	S: CCAACGCAATGGCGAGTCAC AS: GCCGTCTCCGTTTGATGCGT	68	96	NC_003197.1
SMAD2	A: TGAGTGCCTAAGTGACAGTG AS: CCAGAAGAGCAGCAAATTCC	60	143	**EST** DB812041
SMAD3	A: CGCAGAACGTCAACACCAAG AS: AGCTCATGGTGGCTGTGAAG	62	139	**EST** BX926114
STAT3	A: TGCAGCAGAAAGTGAGCTAC AS: CCGGTCTTGATGACTAATGG	60	166	NM_001044580

Table I. Continued.

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

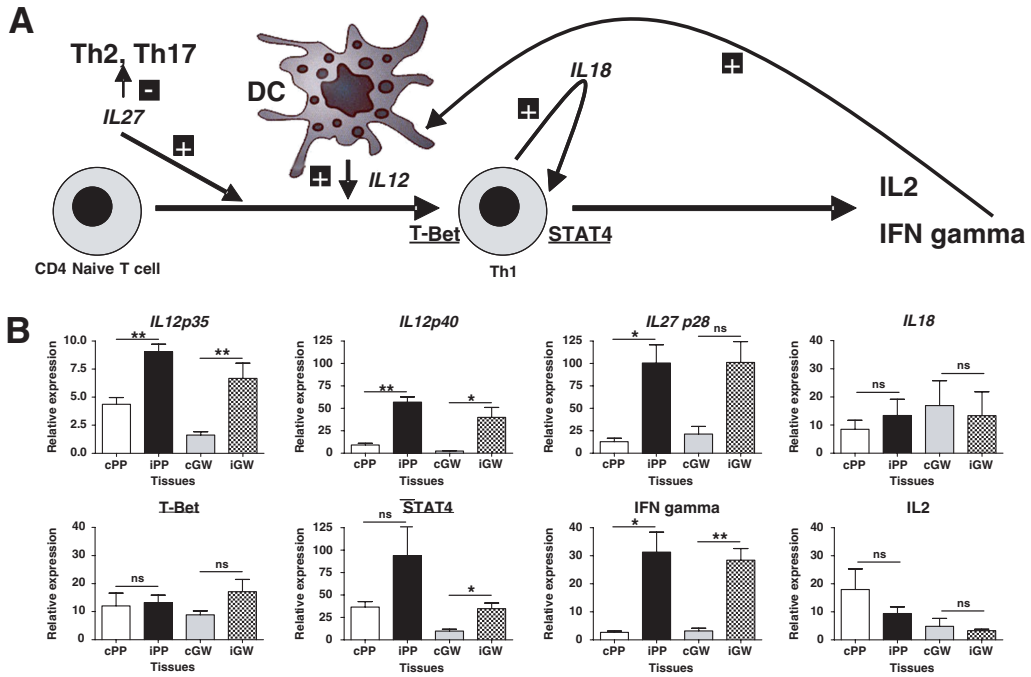

Figure 2. (A) Schematic presentation of the T-helper type-1 (Th1) mediated immune response. (B) Relative mRNA expression of Th1 cytokines and transcription factors. Data were presented as mean ± S.E.M. for a total of 8 control and 12 infected loops. * $P < 0.05$, ** $P < 0.01$, ns: not significant (Student's 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.

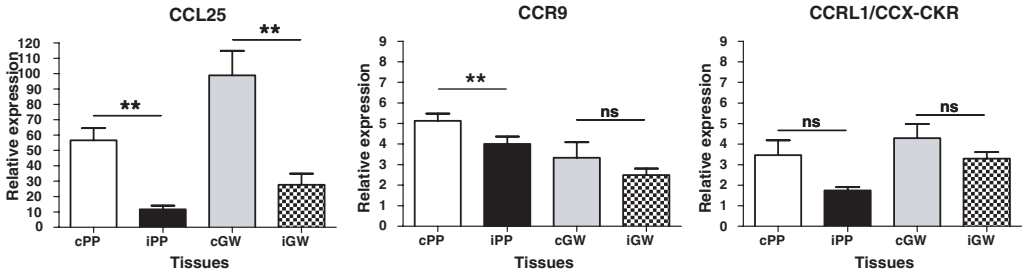

Figure 3. Relative mRNA expression of CCL25 and its two receptors, CCR9 and CCRL1. Data were presented as mean ± S.E.M. for a total of 8 control and 12 infected loops. * $P < 0.05$, ** $P < 0.01$, ns: not significant (Student's Paired t-test or Wilcoxon Signed Rank Test (Exact)).

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

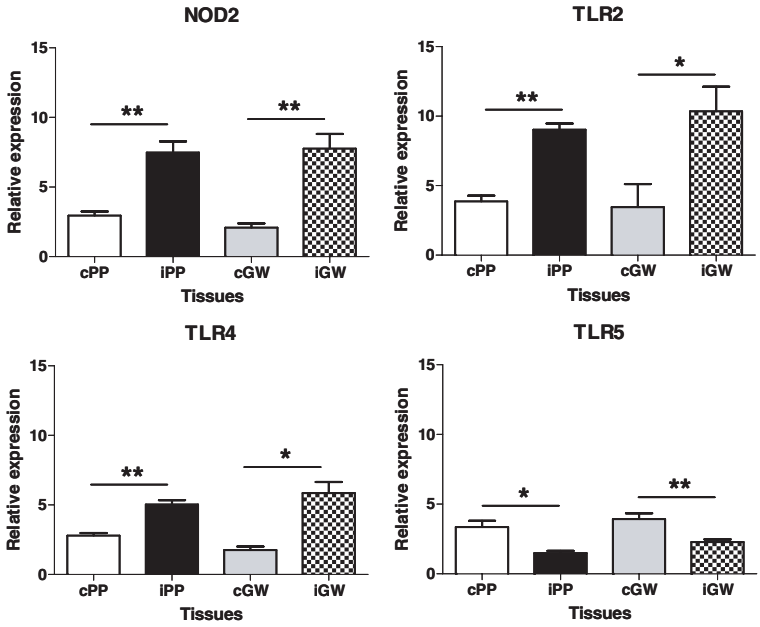

Figure 4. Relative mRNA expression of four pattern recognition receptors (NOD2, TLR2, TLR4 and TLR5) usually associated with *Salmonella*. Data were presented as mean ± S.E.M. for a total of 8 control and 12 infected loops. * $P < 0.05$, ** $P < 0.01$, ns: not significant (Student's Paired t-test or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