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RESEARCH

T-independent B-cell effect of agents associated with swine grower-finisher diarrhea

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Abstract

Swine dysentery, spirochetal colitis, and salmonellosis are production-limiting enteric diseases of global importance to the swine industry. Despite decades of efforts, mitigation of these diseases still relies on antibiotic therapy. A common knowledge gap among the 3 agents is the early B-cell response to infection in pigs. Thus, this study aimed to characterize the porcine B-cell response to *Brachyspira hyodysenteriae, Brachyspira hampsonii* (virulent and avirulent strains), *Brachyspira pilosicoli*, and *Salmonella* Typhimurium, the agents of the syndromes mentioned above. Immortalized porcine B-cell line derived from a crossbred pig with lymphoma were co-incubated for 8 h with each pathogen, as well as *E. coli* lipopolysaccharide (LPS) and a sham-inoculum (n = 3/treatment). B-cell viability following treatments was evaluated using trypan blue, and the expression levels of B-cell activation-related genes was profiled using reverse transcription quantitative PCR. Only *S.* Typhimurium and LPS led to increased B-cell mortality. *B. pilosicoli* downregulated B-lymphocyte antigen (CD19), spleen associated tyrosine Kinase (syk), tyrosine-protein kinase (lyn), and Tumour Necrosis Factor alpha (TNF-α), and elicited no change in immunoglobulin-associated beta (CD79b) and swine leukocyte antigen class II (SLA-DRA) expression levels, when compared to the sham-inoculated group. In contrast, all other treatments significantly upregulated CD79b and stimulated responses in other B-cell downstream genes. These findings suggest that *B. pilosicoli* does not elicit an immediate T-independent B-cell response, nor does it trigger antigen-presenting mechanisms. All other agents activated at least one trigger within the T-independent pathways, as well as peptide antigen presenting mechanisms. Future research is warranted to verify these findings in vivo.

Keywords Swine dysentery · Colitis · Salmonellosis · Humoral immunomodulation · Gene pathways

Introduction

Swine dysentery (SD), spirochetal colitis (SC) and swine salmonellosis (SS) are diarrheic diseases affecting swine in the grower-finisher stage, and are associated with decreased growth performance and increased production costs (Funk and Gebreyes 2004; Alvarez-Ordóñez et al. 2013; VanderWaal and Deen 2018). SD is characterized by mucohaemorrhagic diarrhea and colitis. It is caused by *Brachyspira hyodysenteriae* (Harris et al. 1972), B. *suanatina* (Råsbäck et al. 2007) or *B. hampsonii* (Rubin et al. 2013). *Brachyspira pilosicoli* is the causative agent of SC, clinically described as mucoid, watery diarrhea linked to mild colitis when compared to SD (Taylor et al. 1980). *Salmonella enterica* subsp. *enterica* serovar Typhimurium causes watery diarrhea and enterocolitis in growing pigs (Levine et al. 1945). In practice, these three diseases are often controlled and treated using antimicrobial therapy in commercial operations.

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Several different vaccine development strategies have been explored for SD (Song et al. 2009; Mahu et al. 2017; La et al. 2019), and SC (Casas et al. 2017). Despite these efforts, only partial protection has been induced and no effective vaccine for SD or SC is commercially available in the major pork producing countries. In contrast, commercial *Salmonella* vaccines are available in many countries, targeting sows (Denagamage et al. 2007; Smith et al. 2018; Peeters et al. 2020; van der Wolf et al. 2021), piglets (Husa et al. 2009; Farzan and Friendship 2010; Schmidt et al. 2021) or market-agepigs (Denagamage et al. 2007; Peeters et al. 2020), focusing not only in preventing clinical signs but in decreasing shedding and contamination of carcasses at slaughter. However, cross-protection between serovars is questionable, impacting vaccine uptake in commercial farms (Husa et al. 2009; Farzan and Friendship 2010; Moura et al. 2021). Thus, antimicrobials are still used for disease mitigation. Consequently, the emergence of antibiotic resistant strains is a concern given that salmonellosis is linked to animal welfare, food safety, and security (Lekagul et al. 2019; Pholwat et al. 2020).

B-cells express multiple intra and extracellular receptors capable of recognizing antigens, including bacterial, that trigger signals to modulate the innate and adaptive immune responses (Rawlings et al. 2012). T-cell independent B-cell activation takes part in the early response against pathogens through the production of IgM and possible IgD, and serves as a gateway to immunotolerance or immune activation (Boes et al. 2000). The B-cell receptor (BCR) is an important player in this mechanism. It is formed by a membrane-bound immunoglobulin (Ig) and a heterodimeric signaling subunit (CD79a/CD79b) (Reth 1989). Upon BCR crosslinking by antigens, the proximal kinase lyn initiates the signaling cascade phosphorylating tyrosines in the CD79a/CD79b BCR subunits, which results in recruitment and activation of the spleen tyrosine kinase (syk) (Yamanashi et al. 1991; Xu et al. 2005; Geahlen 2009). Syk leads to the phosphorylation and activation of downstream molecular pathways that lead to B-cell activation, proliferation and differentiation or quiescence (Niiro and Clark 2002; Werner et al. 2010).

We hypothesized that B-cell exposure to *Brachyspira hyodysenteriae, Brachyspira pilosicoli, B. hampsonii* and *Salmonella* Typhimurium activates different triggers within the B-cell intrinsic activation pathways. The goal of this study was to investigate the initial mRNA B-cell response to swine enteric pathogens, independently of T-cells.

Materials and methods

B-cell culture

An immortalized porcine B-cell line was established by isolating cells from a 6–7 months old, cross-bred commercial pig, clinically healthy but with splenomegaly identified at slaughter, linked to multicentric lymphoma(Rahe et al. 2020). Cells were cultured at 37 °C with 5% CO_2 in a standard bench-top CO_2 incubator (Thermo Fisher Scientific, Waltham, MA,USA) using high quality polystyrene flasks (Sarstedt, Numbest, Germany). Complete RPMI 1640 media with L-glutamine (Gibco Life Technologies, Co., Grand Island, NY, USA), supplemented with 10 mM HEPES buffer (Gibco Life Technologies, Co., Grand Island, NY, USA), 1X non-essential amino acids (Gibco Life Technologies, Co., Grand Island, NY, USA), 1 mM sodium pyruvate (Gibco Life Technologies, Co., Grand Island, NY, USA), 50 µg/mL gentamycin (Gibco Life Technologies, Co., Grand Island, NY, USA), 5,000 U/mL penicillin-streptomycin (Gibco Life Technologies, Co., Grand Island, NY, USA), and 5% fetal bovine serum (Gibco Life Technologies, Co., Grand Island, NY, USA) (Rahe et al. 2020). The confluence of the B-cell suspended cells was checked every day for the presence of cluster proliferation. Media was changed four times every 5 days. For passaging, the cells and media were pipetted in a 50 mL conical tube (VWR International, Radnor, PA, USA), and centrifuged at 500xg for 5 min at room temperature. The supernatant was decanted and cells resuspended in 10 mL of cRPMI. Then, 2 mL of a cell mixture were added into a flask with 12 ml of cRPMI. Once cells reached 90–100% confluency, they were passaged at a concentration of 1×10^5 cells/mL/flask for inoculation.

Bacterial inocula culture

Salmonella enterica serovar Typhimurium strain X4232 was cultured at 37 °C in Luria-Bertani broth (LB, BD Canada, Oakville, ON, Canada,Costa et al. 2020). *Brachyspira hyodysenteriae* strain G44 (*B. hyo*), the virulent *Brachyspira hampsonii* clade II strain 30,446 (*B. hampsonii*), the non-pathogenic *Brachyspira hampsonii* clade 2 strain KL-180 (*B.* KL180), and *Brachyspira pilosicoli* strain ATCC 51,139 *(B. pilosicoli)* were cultured in brain heart infusion (BHI) broth (Becton and Dickinson Company, Sparks, MD, USA) supplemented with 5% (v/v) of fetal bovine serum, 5% (v/v) of sheep's blood and 1% (w/v) of glucose. and incubated under anaerobiosis (Anaerogen, Oxoid Limited, Basingstoke, United Kingdom) at 39ºCA *B. pilosicoli* aliquot was sonicated (Vibracell Sonicator, Sonics & Materials Inc., Danbury, Connecticut, USA) for 2 min at 20 kHz to inactivate the bacteria (*B. pilo* dead) (Azuonwu et al. 2015).

Inoculation procedure25 mL flasks containing B-cells at 1×10^5 cells/mL were exposed to one of the following inocula: negative control (sham inoculated, n = 6); positive control (100 µg/flask of *E. coli* O111:B4 lipopolysaccharide, LPS, n = 6); *B. hyo* (1.69×10^7 genome equivalents (GE)/mL, n = 6); *B. hampsonii* (1.49×10^9 GE/mL, n = 3), *B. pilosicoli* (3.35×10^{10} GE/mL, n = 6), *B.* KL180 (4.79×10^9 GE/mL, n = 3), *B. pilo* dead (1.26×10^{11} GE/mL, n = 3), and *S.* Typhimurium (4.32×10^9 CFU/mL, n = 6). Inocula were prepared by centrifuging bacterial culture broth at 10,000 rpm (21,385 x g) for 10 min. Next, cell pellets were resuspended in 6 mL of cRPMI and inoculated into the flasks containing B-cells. Co-incubation followed for 8 h at 37 °C in 5% CO_2.

B-cell viability assay

Following the exposure period, B-cell viability was measured using trypan blue (Lonza, Walkersville, MD, USA). Briefly, 0.1 mL of 0.4% trypan blue was added to a 0.4 mL aliquot from each flask, incubated for 2 min at room temperature and counted using a hemocytometer chamber (Hausser Scientific, Horsham, PA, USA) and a light microscope at 40x magnification. Results are reported as total dead cells/total cell count x 100.

Bacterial viability

Before the co-incubation period, 100 µL of each *Brachyspira* inocula were plated on blood agar plates and incubated anaerobically using a commercial system (Anaerogen, Oxoid Limited, Basingstoke, United Kingdom) at 42 °C for 48 h. Similarly, 100 µL of *Salmonella* Typhimurium were plated on LB agar plates (Bectron, Dickinson and Company BD, Sparks, MD, USA) and incubated at 39ºC for 24 h. After the co-incubation period, 100 µL of cRPMI containing any of the *Brachyspira* inocula or cRPMI inoculated with *Salmonella* Typhimurium were plated on their respective medium plates and environmental conditions described above.

Relative mRNA expression levels

Expression of CD19 (B-lymphocyte antigen), CD79b (immunoglobulin-associated beta), lyn (tyrosine-protein kinase), syk (spleen associated tyrosine Kinase), SLA-DRA (swine leukocyte antigen class II), tumor necrosis factor alpha (TNF-α), interferon alpha (IFN-α), interferon beta (IFN-β), and interleukin 10 (IL-10) was evaluated by reverse transcriptase, reverse transcription quantitative PCR. Primers used for amplification are described in Table 1.

The cytokines primers were previously published and validated (Alex Pasternak et al. 2020). All other primers were validated initially *in silico* by verifying primer nucleotide homology with the target template, followed by amplicon size verification and melt-curve analysis using the PCR conditions described below. Following the co-incubation period, flasks containing B-cells and a given inocula were centrifuged at 500 g for 5 min. The supernatant was discarded and 1 mL of RNAlater (Sigma-Aldrich Co., St. Louis, MO, USA) was added to the pellet and vortexed. Samples were stored at -80 °C until processing. RNA extraction was performed using a commercial kit (Qiagen RNeasy, Qiagen, Hilden, Germany) and cDNA was synthesized using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. PCR reactions were conducted in a Bio-Rad CFX instrument (Bio-Rad Laboratories Ltd., Mississauga, ON). Each 25 µL reaction contained 12.5 mL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd, Hercules, CA, USA), forward and reverse primers (20 µM each), and 2 mL of cDNA template. Reactions were incubated at 94◦C for 3 min, followed by 40 cycles of 10 s at 95ºC, 10 s at 59ºC for SLA-DRA and IFN-β; 63.3ºC for IL-10 and CD19; and 65ºC for IFN-α, TNF-α, SYK, LYN and CD79b, and 30 s at 72ºC. Negative and no-template controls were included in each plate ran. All reactions were run in duplicates. Reaction duplicates that differed by more than 1 Ct were repeated.

Table 1 Primer pairs used in this study

Primer	Sequences		
	Forward	Reverse	Efficiency
CD19	5′- GAAATTGCTGAGCCTGAACC-3′	5′- AGCAACAGAACAGCCTTTCC-3′	96%
CD79b	5′- TGATTTGGAGGAGGGAGTTC-3′	5′- CATGGGAGAATGGGTTTGAG-3′	99%
LYN	5′-TTGTTGACAAGAGGCTGTGC-3′	5′ TGGGAAAGACACCAAAGCTC-3′	105%
SYK	5′- CACTTGCCCTTCTTCTTTGG-3′	5′- CGGTTGAAAGGGTTCTTGAG-3′	95%
SLA-DRA	5′- ATCTCCCCTTCATGCCCTCA-3′	5′- AGCTTCAAACTCCCAGTGCT-3′	107%
TNF-α	5′- CCAATGGCAGAGTGGGTATG-3′	5′- TGAAGAGGACCTGGGAGTAG-3′	99%
IFN-α	5′-GGCTCTGGTGCATGAGATGC-3′	5′-CAGCCAGGATGGAGTCCTCC-3′	105%
IFN-β	5′-TGCAACCACCACAATTCCAGAAGG-3′	5′-TCTGCCCATCAAGTTCCACAAGGA-3′	101%
IL-10	5′-GGTTGCCAAGCCTTGTCAG-3′	5′-AGGCACTCTTCACCTCCTC-3′	98%

Statistical analysis

Shapiro-Wilk test was used to evaluate the normality of data. Differences in B-cell mortality levels among the groups were analyzed using one-way ANOVA followed by post-hoc Tukey test. Real-time PCR data were analyzed using generalized linear mixed models based on lognormal-Poisson error distribution, fitted using Marcov chain Monte Carlo sampling (Matz et al. 2013)(mcmc.qPCR package on R version 4.2.0, RStudio, Boston, MA, USA).

Results

B-cell viability

B-cell exposure to LPS ($P < 0.001$, 8.8% ± 0.4%) or *S.* Typhimurium ($P = 0.001$, 11.3% ± 0.5%) significantly increased mortality when compared to the negative control group (5.3% ± 0.2%) for all pairwise comparison. None of the other treatments led to a significant impact on B-cell viability. A summary of the data is presented in Fig. 1.

Relative mRNA expression levels

B-cell exposure to *B. pilosicoli* led to no change in the expression of the BCR signaling component CD79b. In contrast, all other treatments significantly increased CD79b mRNA levels. Other components of the BCR activation pathway (CD19, syk, and lyn) were significantly downregulated only following *B. pilosicoli* exposure (-2.8 fold, $P = 0.0001$; -2.1 fold, $P < 0.0001$; and −1.5 fold, $P = 0.03$, respectively). In contrast, syk mRNA levels was only increased when B-cells were exposed to *B. hampsonii* (2.2 fold, $P = 0.02$) or *B. hyo* (1.7 fold, $P = 0.02$), relative to the negative control group. None of the other treatments significantly altered the expression of lyn.

B. pilosicoli decreased B-cell expression of TNF-α (-2.4 fold, $P = 0.03$), but increased IFN-β (5.5 fold, $P = 0.01$) mRNA production relative to the negative control group. *B. hyo* exposure increased the mRNA levels of IFN-α (2.0 fold, $P = 0.02$) and IFN-β (5.9, $P = 0.01$). *S.* Typhimurium upregulated the expression of IFN-α (1.9 fold, $P = 0.03$) and IL-10 (2.1 fold, $P = 0.03$), in relation to the negative control samples. SLA-DRA was upregulated following *B. hampsonii (*2.5 fold, $P = 0.01$), *B. pilo* dead (2.4 fold, $P = 0.01$), *B. hyo* (1.61 fold, $P = 0.05$), *B. KL180* (1.90 fold, $P = 0.05$), and *S.* Typhimurium (1.7 fold increased compared to control, $P = 0.03$), but remained unaffected in the presence of *B. pilosicoli*. A summary of the RT-PCR data is presented in Fig. 2.

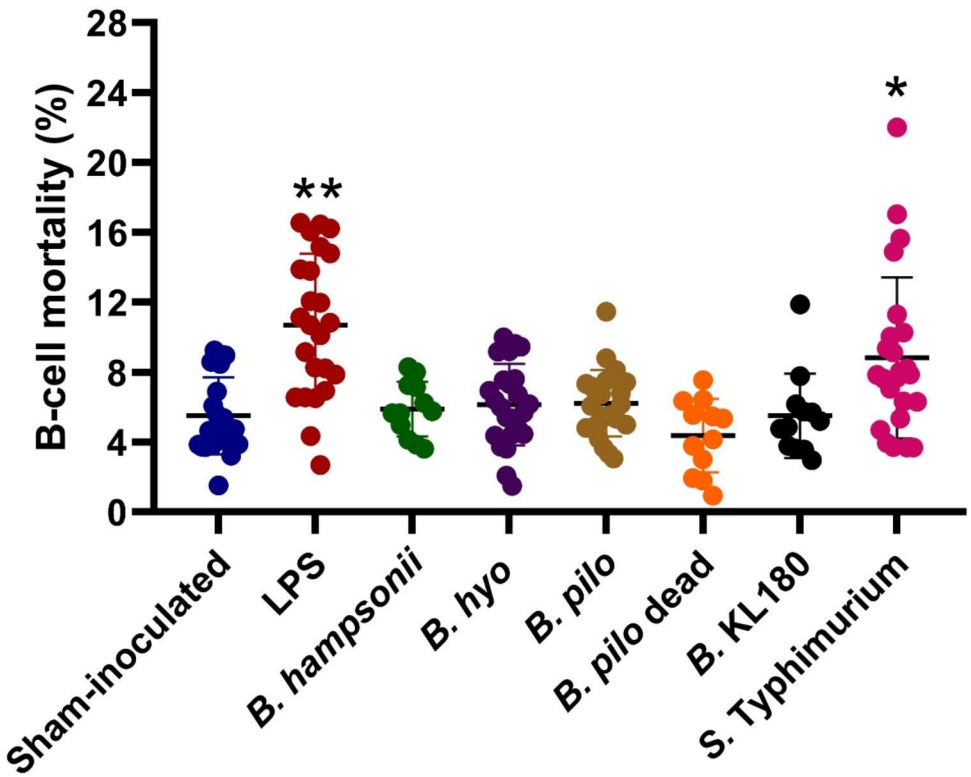

Fig. 1 B-cell mortality after 8 h of exposure to sham-inoculated control (n = 6), LPS (n = 6), *B. hampsonii* clade II 30,466 (*B. hampsonii*, n = 3), *B. hyodysenteriae* G44 strain (*B. hyo*, n = 6), *B. pilosicoli* (*B. pilo*, n = 6), *B. pilosicoli* sonified dead (*B. pilo* dead, n = 3), non-pathogenic *B. hampsonii* clade 2 KL180 (*B.* KL180, n = 3), and *Salmonella* Typhimurium (*S.* Typhimurium, n = 6). *-Denotes statistical significance between *S.* Typhimurium and all groups, except LPS ($P = 0.001$). **-Denotes statistical significance between LPS and all groups, except *S.* Typhimurium ($P < 0.001$)

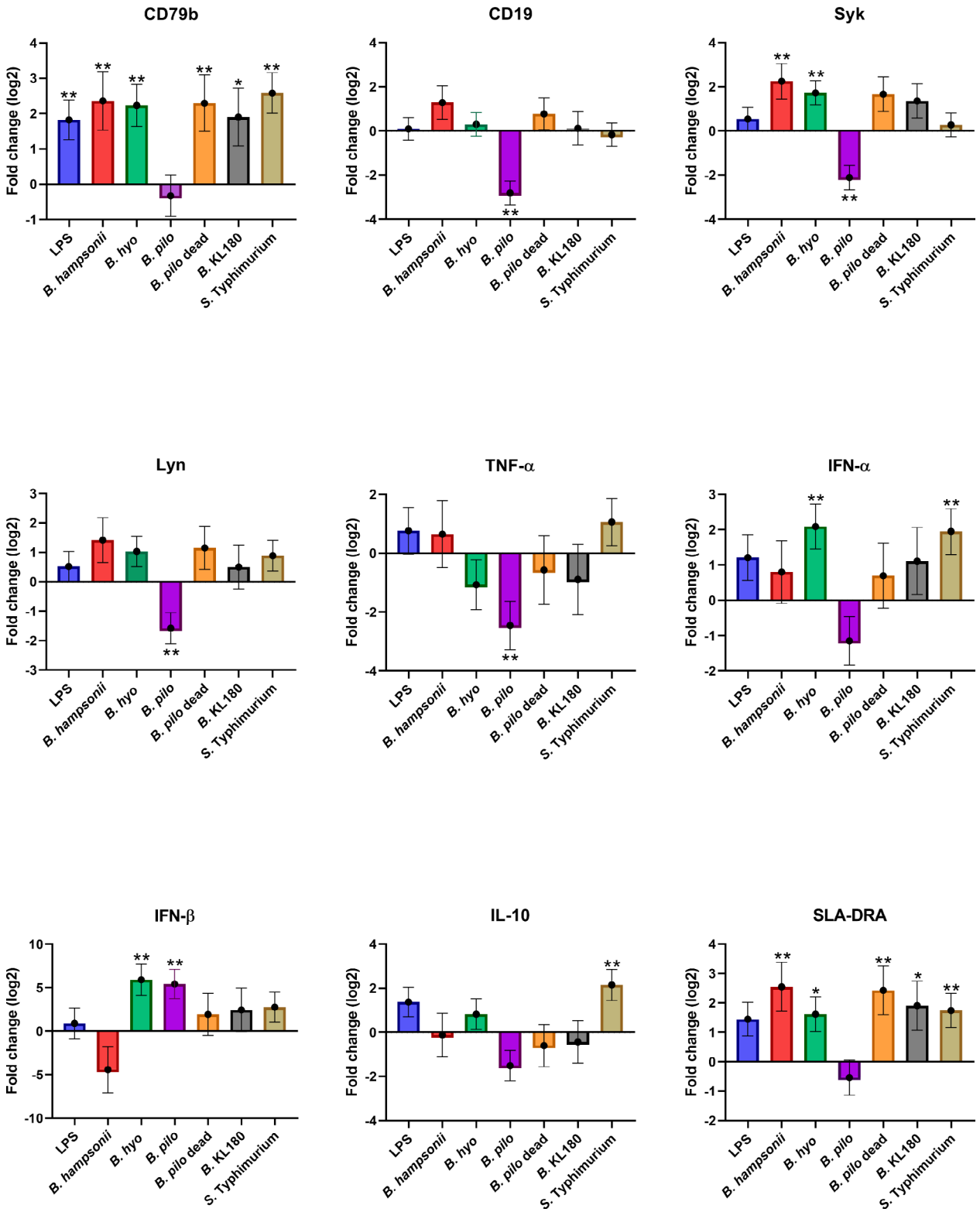

Fig. 2 Expression of B-cell activation and proliferation marker genes after 8 h of exposure to sham-inoculated control (n = 6), LPS (n = 6), *B. hampsonii* clade II 30,466 (*B.hampsonii*, n = 3), *B. hyodysenteriae* G44 strain (*B. hyo*, n = 6), *B. pilosicoli* (*B. pilo*, n = 6), *B. pilosicoli* sonified dead (*B. pilo* dead, n = 3), non-pathogenic *B. hampsonii* clade 2 KL180 (*B.* KL180, n = 3), and *Salmonella* Typhimurium (*S.* Typhimurium, n = 6). measured by quantitative real-time RT-PCR. Bars depict mean fold change (log2) values from eight treatments, relative to the negative control group, and error bars represent 95% confidence intervals. **- Denotes statistical significance ($P < 0.05$). *- Denotes statistical significance ($P = 0.05$)

Bacterial viability

Post-inoculation evaluation of the viability of bacterial inocula resulted in no growth of the *Brachyspira* spp. and *S.* Typhimurium in their respective culture medium.

Discussion

Here we investigated the T-cell independent B-cell response to enteric pathogens associated with grower-finisher diarrhea in pigs. Surprisingly, B. *pilosicoli* downregulated genes involved in B-cell activation and differentiation, and did not trigger the expression of the major histocompatibility complex type II (MHC-II, SLA-DRA gene). *B. hyodysenteriae*, different strains of *B. hampsonii*, *S.* Typhimurium and killed *B. pilosicoli* triggered activating responses by the host cells. Grower-finisher infectious diarrhea directly impacts profit in commercial swine operations (Patterson et al. 2016; Burrough 2017). Understanding B-cell response to pathogens to which antibiotics are largely used in pigs may aid in the development of preventative tools.

Our data showed that B-cell exposure to all treatments other than *B. pilosicoli* upregulated CD79b expression. After antigen binding to BCR, CD79b is the initial signaling trigger involved in B-cell maturation and activation (Koyama et al. 1997; Kraus et al. 2004; Williams et al. 1994). Phosphorylation of the tyrosine-based activation motif (ITAM) on CD79b by Src-family kinases activates syk, followed by downstream signaling molecules, such as phospholipase C gamma 2 (PLC-γ2) and phosphoinositide 3-kinase (PI3K) (Marshall et al. 2000; Niiro and Clark 2002). These molecules form the main BCR signaling cascade involved in B lymphocyte cell-cycle progression and survival pathways (Fruman et al. 1999; Hikida et al. 2003). CD79b expression is up-regulated in mice kidneys infected with *Staphylococcus aureus* (Ziegler et al. 2011), and in sheep mammary tissue infected with *Mycoplasma agalactiae* (Chopra-Dewasthaly et al. 2017). We hypothesize that *B. pilosicoli* likely did not lead to crosslinking of BCR, as no changes in CD79b expression were identified. CD19 is a co-receptor of the B-cell cell-surface signal-transduction complex (including CD21, CD81 and CD225) that plays an important role on B-cell activation by reducing the BCR activation threshold, and by promoting BCR-independent B-cell expansion through c-MYC protein stability (Fearon and Carroll 2003; Scheuermann and Racila 2009; Chung et al. 2012). CD19 deficient mice and humans respond poorly to transmembrane signals, leading to impaired humoral response (Engel et al. 1995; Fujimoto et al. 2000; van Zelm et al. 2006). In contrast, overexpression of CD19 leads to increased humoral response and disruption of tolerance mechanisms (Sato et al. 1996, 2000; Inaoki et al. 1997). Through Akt kinase signaling and tyrosines phosphorylation, CD19 is required for MHC-II-mediated downstream signaling (Mills et al. 2007), and play a role in immunoglobulin-induced activation of B-cell or their antigen-independent development (Otero et al. 2001; Wang et al. 2012). CD19 also plays a role in TLR9 signaling pathways in human B cells (Morbach et al. 2016), which is activated by bacterial DNA (Dalpke et al. 2006). We found that *B. pilosicoli* exposure to B-cells downregulated CD19 expression. Although other molecules and receptors from CD19-activated pathways were not evaluated in the present studywe hypothesize that *B. pilosicoli* may increase the BCR activation threshold, repress B-cell expansion and impair pathogen recognition via MHC-II or TLR-9, thus crippling the early B-cell response to infection and potentially inducing tolerance to *B. pilosicoli* antigens – which would aid in its host-attached lifestyle.

One of the earliest events following BCR activation is phosphorylation of lyn and syk protein kinases (Stepanek et al. 2013). Lyn plays a crucial role in activating or inhibiting BCR signaling (Yamanashi et al. 1991). It can enhance B-cell downstream signaling, phosphorylating ITAMs on B-cell receptor Igα/Igβ (CD79a/CD79b) chains triggering the activation of the spleen tyrosine kinase (syk) (Kurosaki et al. 1994; Johnson et al. 1995). Lyn also phosphorylates tyrosine-based inhibitory motifs (ITIMs) on inhibitory receptors (CD22 and FcγRIIB) that suppress BCR signaling (Cornall et al. 1998; Nishizumi et al. 1998). Syk binds to the BCR (Rolli et al. 2002), phosphorylating not only ITAM tyrosines at CD79a/CD79b but also other proteins, including CD19 and BCAP, activating the PI3K pathway and the SH2 domain-containing leukocyte protein of 65 kDa (SLP-65) (Mócsai et al. 2010; Heizmann et al. 2010). These signals support further development of B-cells from pro-B to pre-B-cell (Turner et al. 1997; Saijo et al. 2003) Here we showed that the expression of lyn and syk were downregulated after B-cell exposure to live *B. pilosicoli.* Lyn-deficient mice have shown reduced numbers of mature follicular B-cells, absence of marginal zone and higher proportion of immature B-cells (Nishizumi et al. 1995; Shahaf et al. 2012). Lyn deficiency is also involved in decreased phagocytosis and autophagy upon *Pseudomonas aeruginosa* infection of mice alveolar macrophages (Li et al. 2016). Syk deficiency also impaired the differentiation and maturation of B-lineage cells (Cheng et al. 1995; Turner et al. 1997; Cornall et al. 2000). Taken together, the decrease in CD19, lyn, and syk expression following *B. pilosicoli* suggest that the B-cell response to this pathogen is weakened from a BCR-dependent or independent activation perspective, potentially leading to tolerance.

SLA-DRA are expressed mainly in antigen presenting cells, and it is a key player in extracellular peptide antigen

processing and presentation, T-cell dependent response and vaccine efficacy (López Fuertes et al. 1999; Lunney et al. 2009). In our study, SLA-DRA was upregulated by B-cell treatment with all inocula, except for live *B. pilosicoli* and LPS. Replication of porcine epidemic diarrhea virus (PEDV) in bone marrow-derived dendritic cells inhibited expression of SLA-DRA, showing PEDV has mechanisms to evade the host immune response (Wang et al. 2021). Our results suggest that the cell line used recognized all the treatments as foreign antigens, except for live *B. pilosicoli*. The mechanism through which *B. pilosicoli* escapes antigen processing and presentation may be a key feature to enable vaccine development in the future.

We found increased expression of IFN-α upon B-cell exposure to *B. hyodysenteriae, S.* Typhimurium, and IFN-β following *B. hyodysenteriae* and *B. pilosicoli* exposure. Type I interferons (IFN-α/IFN-β, T1IFN) are early innate immunity cytokines and have pleiotropic effects on the immune response modulation, with direct and indirect effects on B-cells (Kiefer et al. 2012). Multiple studies have demonstrated the role of IFN-α/IFN-β as immunoregulatory B-cell stimulators during viral infections (Coro et al. 2006; Fink et al. 2006; Swanson et al. 2010; Kiefer et al. 2012). T1IFN were found to enhance B-cell response and activation during the inflammatory process, increasing BCR sensitivity, which is suggested as a link between the innate and acquired immune responses (Morikawa et al. 1987; Braun et al. 2002). In contrast, exacerbated exposure to T1IFN has been shown to be harmful to the host, promoting proliferation of self-reactive B-cells in autoimmune diseases in humans (Theofilopoulos et al. 2004). Thus, the role of T1IFN in response to bacterial infection remains to be clarified (Boxx and Cheng 2016). Here we found that *B. hyodysenteriae* and *S.* Typhimurium led to increased levels of IFN-α, when compared to the control group. Exogenous or endogenous IFN-α was found to modulate B-cell proliferation and their differentiation into antibody-secreting cells (Gujer et al. 2011). Interestingly, Domeier et al. (2018) found evidence that intrinsic B-cell T1IFN signaling causes loss of tolerance in germinal center cells. Also, IFN-α amplifies naïve B-cell activation and immunoglobulin production through TLR-9/MyD88-dependent signaling after stimulation with CpG motifs of bacterial DNA (Giordani et al. 2009). In parallel, IFN-β was upregulated by *B. hyodysenteriae* and *B. pilosicoli*. IFN-β exposure reduces B-cell capacity to respond to antigen mediated signals, focusing its response on immediate innate system measures (Khsheibun et al. 2014). We postulate that *S.* Typhimurium and *B. hyodysenteriae* triggered the observed B-cell responses due to, in part, the increased production of IFN-α. Oppositely, *B. pilosicoli* effect on IFN-β only may explain the lack of antigen-based B-cell response.

TNF-α is one of the earliest responses by B-cells following crosslinking of surface immunoglobulins (Goldfeld et al. 1992). This molecule is a required autocrine factor for B-cell growth, promoting cell differentiation (Boussiotis et al. 1994). Our results revealed that TNF-α was significantly downregulated following B-cell exposure to *B. pilosicoli*, but not killed *B. pilosicoli* or any other treatment. In contrast, a previous study using Caco-2 cells found that inactivated *B. pilosicoli* led to the upregulation of TNF-α, while live *B. pilosicoli* did not significantly change its expression levels (Naresh et al. 2009). Caco-2 are epithelial cells derived from human samples, not pigs. This may explain the differences observed here. Multiple bacterial pathogens have evolved to directly or indirectly supress the production of TNF-α, thus facilitating parasitism (Rahman and McFadden 2006; Luo et al. 2018). It is plausible that *B. pilosicoli* suppresses lymphocyte TNF-α production to support its periplasmatic lifestyle through a mechanism that remains to be clarified.

IL-10 plays a role enhancing B-cell proliferation and differentiation, and regulates MHC-II antigen presentation (Go et al. 1990; Burdin et al. 1997; Vazquez et al. 2015). We found that *S.* Typhimurium was the only pathogen evaluated in this study to increase IL-10 expression after co-exposure with B-cells. Mice infected with *Salmonella* showed rapid differentiation of IL-10-expressing B cells in the spleen by a mechanism involving the myeloid differentiation primary response gene 88 (MyD88) and TLR2 and/or TLR4 (Neves et al. 2010). Although we did not investigate those pathways genes, our results corroborate previous findings that *S.* Typhimurium may hijack IL-10-signalling to favour its intracellular lifestyle.

S. Typhimurium significantly increased B-cell mortality when compared to the negative control group. Previous research in vivo and in vitro indicated that *Salmonella* is able to infect and survive in B-cell endosomal-lysosomal compartments (Rosales-Reyes et al. 2005; Castro-Eguiluz et al. 2009). These cells act as a reservoir for persistence, dissemination and evasion of $CD8^+$ T-cell-mediated responses (Lopez-Medina et al. 2014). This mechanism is linked to a negative regulation in NLRC4, inhibiting the secretion of IL-1β and its cytotoxic effects, preventing B-cell death by pyroptosis (Rosales-Reyes et al. 2012; Perez-Lopez et al. 2013). A second study showed that *Salmonella* could also inhibit B-cell autophagy by activating mTORC1 by secreting its virulence protein SopB (Luis et al. 2022). This may be linked to the overwhelmingly high amount of bacteria to which B-cells were exposed to in our study.

We recognize that there are multiple steps involved in T-independent B-cell activation, and the work presented here focused only on a few key players of these complex mechanisms. Further work dissecting the downstream

effects of the pathways found affected in this study is warranted, especially regarding *B. pilosicoli* interaction with the host. In addition, protein quantification or data on downstream steps other than mRNA expression will help validate the findings presented here.Our findings revealed that *B. pilosicoli* has a profound impact on B-cell activation, both in T-dependent and T-independent manners. An antigenicity spectrum among the other *Brachyspira* tested was also identified, helping explain their varied virulence. *S.* Typhimurium was the only agent to induce B-cell death, among those tested. Further studies on the consequences of the pathogen-B-cell interactions identified here are suggested to help clarify pathogenesis mechanisms, and may fill in gaps leading to vaccine development.

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Data availability The data that support the findings of the current study are available from the corresponding author on reasonable request.

Declarations

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