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Tissue-specific mRNA expression profiles of porcine Toll-like receptors at different ages in germ-free and conventional pigs

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ABSTRACT

Toll-like receptors (TLRs), key initiators of innate immune responses, recognize antigens and are essential in linking innate and adaptive immune responses. Misrecognition and over-stimulation/expression of TLRs may contribute to the development of chronic inflammatory diseases and autoimmune diseases. However, appropriate and mature TLR responses are associated with the establishment of resistance against some infectious diseases. In this study, we assessed the mRNA expression profile of TLRs 1–10 in splenic and ileal mononuclear cells (MNCs) and dendritic cells (DCs) of germ-free (GF) and conventional pigs at different ages. We found that the TLR mRNA expression profiles were distinct between GF and conventional pigs. The expression profiles were also significantly different between splenic and ileal MNCs/DCs. Comparison of the TLR expression profiles in GF and conventional newborn and young pigs demonstrated that exposure to commensal microbiota may play a more important role than age in TLR mRNA expression profiles. To our knowledge, this is the first report that systematically assesses porcine TLRs 1–10 mRNA expression profiles in MNCs and DCs from GF and conventional pigs at different ages. These results further highlighted that the commensal microbiota of neonates play a critical role through TLR signaling in the development of systemic and mucosal immune systems.

1. Introduction

Toll-like receptors (TLRs) are a type of pattern recognition receptor (PRR) that interact with microbe-associated molecular patterns (MAMPs) and activate signaling pathways to induce innate immune response and also to initiate specific adaptive immune responses (Kawai and Akira, 2009; Werling and Jungi, 2003). There are 11 TLR family members in humans, among them, TLR11 is a pseudogene (Chuang and Ulevitch, 2001; Ishii et al., 2008). Human TLRs 1–10 are divided into two subpopulations according to their cellular localization (Chuang and Ulevitch, 2001; Kawai and Akira, 2009). TLRs 1, 2, 4, 5, 6 and 10 are localized to the cell surface, while TLRs 3, 7, 8, 9 are primarily localized in intracellular vesicles such as the endosome and the endoplasmic reticulum (Kawai and Akira, 2009; Rich et al., 2012; Takeda and Akira, 2015). To date, MAMPs for all human TLRs, with the exception of TLR 10, have been identified (Lee et al., 2012; Takeda and Akira, 2015). The MAMPs for human TLRs 1–9 are triacyl lipopeptides, peptidoglycan, double-stranded RNA, lipopolysaccharide, flagellin, diacyl lipopeptides, single-stranded RNA (TLR7/8), CpG DNA, respectively (Takeda and Akira, 2015). After recognizing specific MAMPs, signaling pathways downstream of the TLRs are triggered and type I interferon (IFN) and inflammatory cytokines are produced (Kawai and Akira, 2011; Werling and Jungi, 2003). Moreover, TLRs recognize numerous synthetic (imiquimod) and endogenous ligands [danger-associated molecular patterns and products of damaged tissue (heat shock proteins, endoplasmin)] and play a crucial role in shaping intestinal immune function and maintaining gut homeostasis (Abreu, 2010; Frosali et al., 2015).

TLRs are mainly found in tissues and cells involved in immune function, such as mesenteric lymph nodes (MLNs) and the spleen, as well as those exposed to the exterior environment such as mucosa (including epithelial cells and subepithelial components) in the intestine and the lung (Zarember and Godowski, 2002). The expression profiles of TLRs differ among tissues and cell types (Flo et al., 2001; Muzio et al., 2000; Zarember and Godowski, 2002).

Abbreviations: DCs, dendritic cells; GF, germ-free; IFN, interferon; IL-4, interleukin 4; MAMPS, microbe-associated molecular patterns; MLNs, mesenteric lymph nodes; MNCs, mononuclear cells; PRR, pattern recognition receptor; PP, Peyer's patches; SLE, systemic lupus erythematosus; TLRs, Toll-like receptors.

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For example, the TLR4 mRNA expression was higher in murine CD11c+ splenic DCs than in CD11c+ lamina propria DCs, whereas the TLR5 mRNA expression showed the opposite trend (Uematsu et al., 2006). Among these immune cells, dendritic cells (DCs) are critical mediators to achieve TLR signaling functions (Hemmi and Akira, 2005; Reis e Sousa, 2004). Additionally, DCs in different anatomical locations have varying functions, which may be associated with the different TLR mRNA expression profiles (Iwasaki and Kelsall, 1999; Uematsu et al., 2006). For instance, murine naïve CD4+ T cells activated by DCs from the Peyer's patches (PP) produced higher levels of interleukin 4 (IL-4) and IL-10 than those activated by splenic DCs (Iwasaki and Kelsall, 1999); mucosal DCs promoted the differentiation of Th17 cells and contributed to IgA B cell class switching (Denning et al., 2007; Sato et al., 2003). Therefore, it is necessary to assess and compare the TLR expression in DCs from different anatomical sites.

Although TLRs are important for host defense, recognition of self-molecules by TLRs and loss of negative balancing of TLR signals are associated with pathological (chronic) inflammation and autoimmune disease (Kawai and Akira, 2010; Marshak-Rothstein, 2006; Marshak-Rothstein and Rifkin, 2007). This has led to an increase in the study of TLRs as therapeutic targets for immune disorders (Keogh and Parker, 2011; Li et al., 2013). Currently, most models tested are *in vitro* or murine models *in vivo* (Li et al., 2013). However, as demonstrated by the different expression patterns of TLR4 in monocytes and macrophages after LPS treatment, the expression and regulation of TLR function differs between mice and humans (Bryant and Monie, 2012; Rehli, 2002; Vaure and Liu, 2014). This suggests that the murine model may not be adequate for studies of human TLRs and highlights the need for alternative animal models. Pigs are being increasingly recognized and used as a relevant model for studies of infectious disease and human immunity (Fairbairn et al., 2011; Gonzalez et al., 2010; Yang et al., 2014; Yang and Yuan, 2014 Yang et al., 2014, 1996; Zhang et al., 2013); however, knowledge of the porcine TLR expression and function is limited in comparison to mice and humans. Existing evidence suggests that the pig TLR system may be closer to that of humans than the murine system is (Jungi et al., 2011; Vaure and Liu, 2014). Although, porcine, human and murine TLR4 promoter sequences were similar, murine TLR4 promoter exhibited significant differences in the regulation of gene expression; whereas porcine TLR4 promoter shared more common features with the human TLR4 promoter (Roger et al., 2005; Thomas et al., 2006). More studies of porcine TLR expression and function are needed to evaluate if the pig model can effectively mimic and predict human conditions and outcomes.

The immune system of the neonate is less developed than that of the adult and this may extend to TLR expression (Bailey et al., 2005; Lee and Mazmanian, 2010; Pott et al., 2012). There are two important periods during the development of the immune system—immediately after birth and after weaning. In the former period, neonates are exposed to non-sterile environments, and in the later period, the organism undergoes extensive exposure to new antigens due to the introduction of solid food and non-milk based diets (Bailey et al., 2005). Therefore, in addition to adulthood, birth and weaning were chosen as two important time points for examination in this study. Additionally, this study used germ-free (GF) animals to provide a comparative control to define how the microbiota/diet affects the developing immune system (Falk et al., 1998; Lee and Mazmanian, 2010; Macpherson and Harris, 2004).

We assessed the TLR1–10 mRNA expression profiles in mononuclear cells (MNCs) and DCs from spleen, ileum and MLNs in GF and conventional pigs at newborn, weaning and adult stages to compare the difference of TLR mRNA expression in tissue-specific and age-dependent manner.

2. Materials and methods

2.1. Animals and experimental design

In this study, five groups of pigs (newborn GF, newborn conventional, young GF, young conventional, adult conventional) were used. Four pigs were included in each group as replications. GF pigs (Landrace × Yorkshire × Duroc) were hysterectomy-derived, fed with cow milk and maintained in sterile isolation units as described previously (Saif et al., 1996; Yuan and Saif, 2002). Specific pathogen-free conventional pigs (Landrace × Yorkshire × Duroc) were naturally derived from Landrace × Yorkshire sows bred to Duroc boars, nursed on the sows until 3 weeks then switched to plant based solid diet. 1- to 4-day-old and 4-week-old GF and conventional piglets were euthanized and ileum, spleen and MLNs were collected. Additionally ileum, spleen and MLNs were collected from conventional adult pigs (Landrace × Yorkshire) (at the average age of 10–11 months). As no GF adult pigs were available due to facility limitations (inability to maintain adult pigs in GF isolators); there was no GF adult pig group.

2.2. Isolation of MNCs and DCs from spleen, ileum and MLNs

MNCs were isolated from spleen, ileum and MLNs as described previously (Yuan et al., 1996). DCs were isolated from MNCs of different tissue origin of individual pigs. MNC numbers were too low to yield adequate amounts of DCs from newborn pigs for further study. Cell separation buffer (MACS buffer) consisted of PBS, 2 mM EDTA and 0.5% BSA, filtered sterilized and stored at 4 °C. MNCs were counted and centrifuged at 300 × *g* for 10 min at 4 °C. The pellet was resuspended in 1 mL MACS buffer per unit (10^7 MNCs/unit), centrifuged at 300 × *g* for 10 min at 4 °C. The cell pellet was resuspended in 80 μL MACS buffer per unit, followed by the addition of 10 μL of mouse anti-porcine CD3 antibody (Ab) (IgG1) (SouthernBiotec, Birmingham, Alabama, USA), 10 μL of mouse anti-porcine CD21 Ab (IgG1) (SouthernBiotec, Birmingham, Alabama, USA), 2.5 μL of mouse anti-porcine SWC1 Ab (IgG2b) (AbD Serotec, Raleigh, NC, USA), and 2.5 μL of mouse anti-porcine SWC9 Ab (IgG1) (AbD Serotec, Raleigh, NC, USA), that binding with the surface marker of T cell, B cell, granulocyte/monocyte and macrophage respectively, then mixed gently and incubated at 4 °C for 20 min. The cells were then washed with 1 mL MACS buffer per unit of MNCs and centrifuged at 300 × *g* for 10 min at 4 °C. The cell pellet was resuspended in 80 μL of MACS buffer, followed by the addition of 20 μL of anti-mouse IgG MicroBeads (Miltenyi Biotec, San Diego, CA, USA) per unit of MNCs, and gently mixed and incubated at 4 °C for 20 min. The cells were washed with 1 mL MACS buffer per unit of MNCs and centrifuged at 300 × *g* for 10 min at 4 °C, followed by resuspension in 500 μL of MACS buffer. LD columns (Miltenyi Biotec, San Diego, CA, USA) were placed on QuadroMACS™ separator (Miltenyi Biotec, San Diego, CA, USA) followed by adding the MNCs for DC negative selection, following the manufacturer's recommendations.

2.3. Examination of isolated DC purity by flow cytometry

As described previously, 10^5 cells were stained with mouse anti-porcine CD3e-FITC (SouthernBiotec, Birmingham, Alabama, USA), mouse anti-porcine CD21-FITC (SouthernBiotec, Birmingham, Alabama, USA), mouse anti-porcine SWC1-FITC (AbD Serotec, Raleigh, NC, USA) and mouse anti-porcine SWC9-FITC (AbD Serotec, Raleigh, NC, USA) monoclonal antibodies (mAb) to characterize the frequencies of T cell, B cell, granulocyte/monocyte and macrophage and to determine the purity of isolated DCs by flow cytometry (Vlasova et al., 2013). Acquisition of 50,000 events was conducted using MACSQuant® Analyzer flow cytometer (Miltenyi Biotec, San