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The distribution of lymphoid cells in the small intestine of germ-free and conventional piglets

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ABSTRACT

Porcine ileum is populated with a high proportion of B cells but previous studies have shown that they are not developed there. While B cells prevail in the ileum even in germ-free animals, microbial colonization is a major factor that causes even a greater prevalence of B cells in the ileum and further differential representation of lymphoid cells throughout small intestine. Analysis of lymphoid subpopulations showed that the effector cells appear only after colonization. These include class-switched IgM^+IgA^+ B cells, primed $CD2^-CD21^+$ B cells, antibody-producing/memory $CD2^+CD21^-$ B cells, and effector/memory $CD4^+CD8^+$ αβ Th cells. While colonization resulted in a uniform distribution of effector cells throughout the gut, it caused a decrease in the frequency of cytotoxic αβ and $CD2^+CD8^+$ γδ T cells. These results suggest that the ileum is a site where naive B cells expand presumably to increase antibody repertoire but the entire small intestine is immunofunctionally comparable.

1. Introduction

The small intestine is a part of the gastrointestinal tract which is specialized in digestion and resorption of nutrients (Borgström et al., 1957). Since its lumen contains a vast number of exogenous antigens, the immune system of the gut faces a difficult task to protect the tissue from pathogens while tolerating commensal microbes and food antigens (MacDonald and Monteleone, 2005). In contrast to the systemic immune system, there is an anatomic division between inductive and effector sites of the immunity in the gut (Suzuki et al., 2010). In the small intestine, organized gut-associated immune tissue (GALT) such as Peyer's patches or isolated lymphoid follicles represent an inductive site of the immunity, whereas diffuse GALT (intraepithelial lymphocytes and *lamina propria* cells) houses the effector activities (Ramiro-Puig et al., 2008).

It has been shown that gut microbiota is required for normal development of GALT in mice (Kamada et al., 2013) and other species including humans and swine (Grönlund et al., 2000; Haverson et al., 2007; Pabst and Rothkötter, 1999; Rhee et al., 2004). This includes the capacity to make adaptive antibody responses (Butler et al., 2002). In general, the GALT of germ-free (GF) animals is less developed. Isolated lymphoid follicles do not mature, the number of Peyer's patches is lower, they are smaller and lack germinal centers (Bouskra et al., 2008; MacDonald and Monteleone, 2005; Round and Mazmanian, 2014). There are also changes in lymphocyte populations in the small intestine of GF animals. Some effector cell populations, such as secretory IgA plasma cells (Moreau et al., 1978; Yanagibashi et al., 2013), intraepithelial lymphocytes (Suzuki et al., 2002) or $CD4^+$ T lymphocytes in *lamina propria* (Bauer et al., 2006), are profoundly reduced. The overall phenotype of lymphocyte populations is different and shifted toward more naive phenotypes (Sinkora et al., 2011). The differences stated earlier ultimately result in an impaired function of GALT.

In this study, we provide a comparison of lymphocyte populations in the small intestine of GF and conventional (CV) piglets. Even though there have been numerous studies conducted on a gut immune system of swine (Rothkötter and Pabst, 1989; Sinkora et al., 2011), none of these have assessed the lymphocyte populations in the entire small intestine. Our study reveals further insight into the influence of intestinal microbiota on the development of the immune system of swine, perhaps helping to strengthen the swine immunological model as an alternative to the widely used rodent model.

2. Materials and methods

2.1. Experimental animals

Animals used in this study were Minnesota miniature/Vietnamese–Asian–Malaysian crossbred piglets (Sinkora et al., 2000). The total number of 35 animals (both males and females) was divided into four experimental groups based on their age and

Abbreviations: CV, conventional; GALT, gut-associated immune tissue; GF, germ-free; IPP, ileal Peyer's patches; JPP, jejunal Peyer's patches.

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microbial colonization: (1) CV piglets under 2 weeks (6 animals), (2) GF piglets under 2 weeks (5 animals), (3) CV piglets 3–6 weeks old (13 animals), and (4) GF piglets 3–7 weeks old (11 animals). The piglets were bred either under CV conditions at the Institute of Animal Science, v.v.i. (Kostelec nad Orlici, Czech Republic) or under GF conditions at the Institute of Microbiology AS CR, v.v.i. (Novy Hradek, Czech Republic). GF piglets were recovered from gilts by sterile hysterectomy at the 112th day of gestation as described previously (Butler and Sinkora, 2007; Butler et al., 2009; Sinkora and Butler, 2009). The age of GF animals is stated as weeks after the day of recovery. After the recovery, GF piglets were kept in sterile isolator units under GF conditions and were hand-fed by γ-irradiated sterile 6%-fat cow milk reconstituted from evaporated 9%-fat concentrate by dilution with water (Hlinsko Dairy, Czech Republic). GF conditions were monitored regularly for contaminating aerobic and anaerobic bacteria and only animals showing no bacterial contamination were used for further experiments. Piglets were euthanized at selected times by intracardial puncture under general isoflurane anaesthesia, their small intestine was removed and used for preparation of cell suspensions. All animal experiments were approved by the Ethical Committee of the Institute of Microbiology AS CR, v.v.i. according to guidelines in the Animal Protection Act.

2.2. Preparation of cell suspensions

The resected small intestine was cut into 15 sections. The first section contained duodenum, sections 2–12 contained jejunum with jejunal Peyer's patches (JPP) and sections 13–15 contained ileum with ileal Peyer's patches (IPP). We established the border between JPP and IPP as the region where continuous IPP transition into discrete JPP. For this reason, the length of all jejunal sections was the same and proportional to the whole length of the small intestine while the ileum constituted three equal sections of IPP region (approximately 30% of JPP length). The same short section was done for the duodenum. A representative anatomical photography with depicted section strategy is shown in Fig. 1. The cell suspensions were prepared as described previously (Sinkora et al., 2011). Briefly, individual intestine sections were incubated in digestion media (RPMI 1640, 100 U/ml collagenase from *Clostridium histolyticum* type V [Sigma-Aldrich, St. Louis, MO], 2% FCS) at 37 °C for 1 h. Supernatants were filtered through a 70 μm nylon membrane, washed three times with PBS and lymphocytes were separated with a 40 and 80% Percoll gradient centrifugation. All cell suspensions were finally washed twice in cold PBS containing 0.1% sodium azide and 0.2% gelatine from cold water fish skin (washing solution) and filtered through a 70 μm nylon membrane. Cell numbers were determined by hemacytometer.

2.3. Immunoreagents

The following mouse anti-pig mAbs, whose source and specificity had been described earlier (Sinkora et al., 2005a, 2013), were used as primary immunoreagents: anti-CD2 (1038H-5-37, IgM), anti-CD3 (PPT3, IgG1), anti-CD4 (10.2H2, IgG2b), anti-CD8α (76–211, IgG2a), anti-CD21 (IAH-CC51, IgG2b), anti-μHC (M-160, IgG1), anti-TCRγδ (PPT16, IgG2b) and anti-IgA (M1456, IgG2a). Due to the lack of some porcine-specific mAbs and markers, αβ T lymphocytes were detected as $CD3\varepsilon^{+}TCR\gamma\delta^{-}$ cells (Sinkora et al., 2007) and B lymphocytes were detected as IgM^{+} cells (Sinkora et al., 2013). Note that CD21 can be expressed on the surface of mature B lymphocytes in two differential forms and IAH-CC51 mAb can discriminate between functionally distinct populations of B cells (Sinkora and Sinkorova, 2014; Sinkora et al., 2013, 2014). Goat polyclonal Abs specific for mouse Ig subclasses labeled with FITC, phycoerythrin, phycoerythrin/cyanine-7 or allophycocyanin were used as secondary immunoreagents (SouthernBiotech, Birmingham, USA). All immunoreagents were titrated for optimal signal/noise ratios. Primary isotype-matched mouse anti-rat mAbs were used as negative controls. Secondary polyclonal Abs were tested for cross-reactivity (no primary mAbs) and also for cross-reactivity with primary isotype-mismatched mouse anti-pig mAbs.

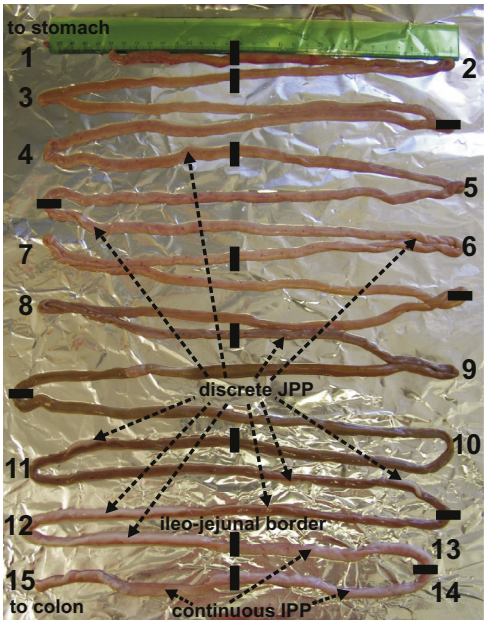

Fig. 1. A representative anatomical photograph of porcine small intestine with depicted section strategy. Black solid lines indicate position of cuts and numbers indicate the position of sections in small intestine (1 = duodenum, 2–12 = jejunum and 13–15 = ileum). Some discrete JPP in the jejunum and continuous IPP in the ileum are pointed to using dashed lines. The border between jejunum and ileum (between sections 12 and 13) was established as the region where continuous IPP transition into discrete JPP.

2.4. Staining of cells

Staining of cells for flow cytometry was performed by indirect subisotype staining as described previously (Stepanova and Sinkora, 2012, 2013). Briefly, cells were incubated with different combinations of primary mouse mAb of different subisotypes for 15 minutes and subsequently washed twice in washing solution. Mixtures of goat secondary polyclonal Ab conjugated with different fluorochromes were then added to the cell pellets in appropriate subisotype combinations. After 15 min, cells were washed three times in washing solution and analyzed by flow cytometry.

2.5. Flow cytometry

Samples were measured on standard FACSAriaIII or FACSCalibur flow cytometers (BD Immunocytometry Systems, Mountain View, USA) and if possible, 300,000–700,000 events were collected for each measurement. The electronic compensation was used to eliminate spectral overlaps between individual fluorochromes. Data were processed with PC-Lysis or FACSDiva software (BD Immunocytometry Systems, Mountain View, USA) employing a gating strategy described earlier (Sinkora et al., 2014). Briefly, lymphocyte gate was set according to light scatter characteristics (forward vs. side scatter). Numbers for main lymphocyte populations (B, αβ T, γδ T and NK cells) were recalculated so the sum of μHC^{+}, $CD3^{+}$ and $CD3^{-}CD8\alpha^{+}$ NK cells equaled 100%, correcting for differing proportions of cell debris in the lymphocyte gate. Numbers for subpopulations were not recalculated and represent a percentage from a particular lymphocyte population that was always 100%. Cell numbers in this report