Title:

Antibody repertoire development in fetal and neonatal piglets. XXIV. Hypothesis: The ileal Peyer patches (IPP) are the major source of primary, undiversified IgA antibodies in newborn piglets.

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19 **Abstract:**

20 The ileal Peyers patches (IPP) of newborn germfree (GF) piglets were isolated into blind loops and the
21 piglets colonized with a defined probiotic microflora. After 5 weeks, IgA levels in the intestinal lavage (IL)
22 of loop piglets remained at GF levels and IgM comprised ~70% while in controls, IgA levels were elevated
23 5-fold and comprised >70% of total Igs. Loop piglets also had reduced serum IgA levels suggesting the
24 source of serum IgA had been interrupted. The isotype profile for loop contents was intermediate between
25 that in the IL of GF and probiotic controls. Surprisingly, colonization alone did not result in repertoire
26 diversification in the IPP. Rather, colonization promoted pronounced proliferation of fully switched
27 IgA⁺IgM⁻ B cells in the IPP that supply early, non-diversified “natural” SIgA antibodies to the gut lumen
28 and a primary IgA response in serum.

29

30 **Keywords:**

31 germfree animals; ileal Peyers patches; immunoglobulin A; blind ileal loops

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34 1. Introduction

35 The GI tract of mammals is exposed first at birth to environmental antigens in the form of dietary
 36 proteins, normal gut microbiota and enteric pathogens. Experimental studies indicate that development of
 37 adaptive immunity in newborn piglets to defined T-dependent and T-independent antigens, depends on
 38 exposure to colonizing microflora, purified PAMPs or TLR ligands generated by viral infection, while
 39 dietary antigens alone appear to have little effect (Butler et al 2002; 2005; 2013). Potential antigens and
 40 adjuvants are greeted by enterocytes and their associated lymphoid tissues, i.e. Peyer's patches. It has been
 41 known for >40 year that germfree (GF) mice have little or no serum or secretory IgA (Benveniste et al 1971)
 42 as is true for GF piglets (Butler et al 2000). Following colonization of GF mice, there is pronounced
 43 development of the Peyers patches in the upper ileum and jejunum (Pollard and Sharon, 1970). These are
 44 considered the major source of IgA⁺ cells in the GI tract and the source of the SIgA in the gut lumen. The
 45 situation is more complex in swine, sheep and other artiodactyls since they possess two types of Peyers
 46 patches (Binns and License 1985; Pabst et al 1988; Barman et al 1997; Yasuda et al 2006). Those found in
 47 the lower ileum, designated the ileal Peyer patches (IPP), are a continuous array of tightly-packed follicles
 48 extending 1-3 meters that are rich in B cells, whereas those in the jejunum (JPP) appear as isolated follicles
 49 especially in SPF and conventional piglets (Barman et al 1997). In contrast to the IPP, these are rich in T
 50 cells and active in overall lymphocytes trafficking (Binns and License 1985). The JPP are generally
 51 considered the analog and homolog of conventional GALT found in lab rodents and humans and play an
 52 essential role in mucosal immunity (Yasuda et al 2006) and their development is antigen dependent (Pollard
 53 and Sharon 1970; Macpherson et al 2005). By contrast, the IPP appear in fetal life and even in GF piglets
 54 increase in length with age even in germfree (GF) piglets (Pabst et al 1988). While JPP follicles increase 2-
 55 fold in diameter after exposure to gut flora, those in the IPP are not altered (Barman et al 1997). However,
 56 they gradually involute until at six months they are replaced or converted to isolated follicles that resemble
 57 the JPP (Carlens 1928; Barman et al 1997; Reynolds and Morris 1983; Pabst et al 1988; reviewed in Butler
 58 and Sinkora 2013). The concept that hindgut lymphoid tissues in newborns may simply convert to

conventional GALT has also been proposed for rabbits (Weinstein et al 1994). In lambs, the IPP disappear in 18 month (Reynolds and Morris 1983).

The Cebra group indicated that in mice ~90% of intestinal SIgA was “natural” SIgA of unknown specificity (Talham et al 1999) while others showed that SIgA in the gut lumen is bound to bacteria, as demonstrated in fecal studies (van der Waaig et al 19996) and in *in vitro* experiments (Mathias and Corthesy, 2011). Binding of this natural SIgA was shown to be non-Fab dependent, but rather mediated by glycans, largely present on secretory component (SC; Mathias and Corthesy 2011; Perrier et al 2006). SC is the portion of the poly Ig receptor that remains attached to dimeric IgA, and which contains 15-24 % glycan based on molecular mass (Hughes et al 1999). SC alone binds toxin A of *C. difficile* and limits its adherence to brush border membranes (Dallas and Rolfe 1998). While both free SC and SIgA inhibit attachment of bacterial pathogens (Wold et al 1990; Schroter et al 1998), SC fails to bind or block rotavirus, suggesting that the protective effect of natural SIgA, does not extend to viruses (Perrier et al 2006). These studies suggest that the initial value of SIgA seems to be its availability in large amounts and that Fab-dependent specificity can be a later added through events in the JPP.

The IPP in sheep account for 90% of all Peyers patches and can account for 1% of total lamb weight making them the largest lymphoid tissue/organ (Reynolds and Morris, 1983). In sheep, lymphogenesis begins in the fetal liver and B cells appear in spleen at 48 days of gestation (DG; 145 day gestation; Press et al 1993). In swine, B cell lymphogenesis begins in yolk sac, then moves to fetal liver where IgM⁺ cells can be seen at 45DG and finally to bone marrow (BM) at 55DG (110 day gestation; Sinkora et al 2002; 2003; 2005; 2009). In sheep and cattle, the spleen appears to be the major site for B cell development and few cells are found in BM (Miyasaka and Morris, 1988; Yasuda et al 2006) while BM is the major site for B cell lymphogenesis throughout life in swine (Sinkora and Sinkorova, 2014). In sheep, lymphoid structures resembling the JPP appear at 75DG in the jejunum but 110 DG in the ileum (Reynolds and Morris 1983;

82 Press et al 1993). In swine the IPP are evident at 70DG and at 90DG transcribe all isotypes (Butler et al
83 2001).

84 The IPP of sheep have been regarded as primary lymphoid tissues for B cells (Reynolds and Morris
85 1983; Reynolds 1986; Griebel and Hein 1996). Since there can be different views on the definition of
86 primary versus secondary lymphoid tissue, we define here our usage in swine. We ascribe to the definition
87 that *primary* refers to the site of B- or T-cell lymphogenesis and the repertoire that develops at those sites
88 constitutes the *primary repertoire*. Tissues to which these cells emigrate and may further develop their
89 repertoires, are regarded as *secondary* lymphoid tissue. By this definition, the IPP of swine are secondary
90 lymphoid tissue. Destruction of primary lymphoid tissue, e.g. thymectomy, BM irradiation, or bursectomy in
91 chickens, eliminates all T- and B-cells, respectively. Since surgical resection of the IPP at birth in piglets
92 does not affect B cell development or maintenance, it does not qualify as primary lymphoid tissue for swine
93 (Butler et al 2011a; Sinkora et al 2011). Hence Ig⁺ cells in the IPP at 90DG are believed to have emigrated
94 from primary sites in the fetal liver and BM.

95 The work by Reynaud et al (1991; 1995) on the IPP of sheep followed on the heels of their seminal
96 observation that antibody repertoire development in the Bursa of Fabricius in the chicken was the result of
97 templated mutation (gene conversion; Reynaud et al; 1987). The Reynaud team cloned and sequenced a
98 reasonable number of V λ J λ recombinants from the sheep IPP and compared their sequences to 18 known
99 germline V λ sequences. They concluded from this comparison that deviations from the germline sequences
100 were evidence for non-templated SHM and that the IPP of sheep generated a rather enormously diverse
101 repertoire by untemplated SHM. However, their interpretation was flawed because they did not compare
102 their sequences to the >100V λ germline genes that were later identified. Jenne, Reynolds and colleagues
103 went on to show that most all of the sequence variants described by Reynaud et al could be found among the
104 many germline V λ genes of sheep (Jenne et al 2003; 2006). Since their conclusion about SHM was debunked
105 and they provided no independent evidence of AID activation, any conclusion that there is high level AID

activity in the sheep IPP should be viewed with suspicion. Their subsequent article (Reynaud et al 1995) stating the development of the IPP repertoire was antigen independent is not in doubt and totally consistent with what we have observed in swine since the IPP develop during the last third of gestation and there is no antigen trafficking across the placenta in artiodactyls.

While our resection experiments did not support the role of the IPP as primary B cell lymphoid tissue according to our definition, they failed to identify any unique role for the largest secondary lymphoid tissue in fetal and newborn piglets. Compared to conventional Peyer's patches (JPP) they have some peculiar features which no doubt is one of the reasons, together with their early involution, why Reynolds and Morris (1983) considered them primary lymphoid tissue. Reynolds (1986) reported extensive cell death in sheep IPP, reminiscent of what occurs in BM during VDJ recombination yet they presented no evidence that recombination is occurring in the IPP. Similar apoptosis occurs in GC after antigen-driven selection, yet the process in the IPP is antigen independent in sheep and piglets. As shown in this report and elsewhere by others, there is vigorous B cell proliferation in a tissue nearly devoid of T cells; cells generally considered essential for both B cell proliferation and antigen-dependent B cell selection. If apoptosis is due to selection, what is the selector? If B cells can proliferate without T cells, what is the mechanism? Finally, if the IPP is a primary B cell organ, why does it transcribe all three major isotypes in an antigen-free fetal environment while primary B cells in BM express only surface IgM (Butler et al 2001)? Our observations have parallels to the original work of Reynaud et al (1991). They showed that individual follicles were oligo- or monoclonal which is consistent with our microdissection studies on the IPP of newborn piglets. We constructed lineages suggesting that following a series of proliferative steps, in which no mutations occurred, yet some lineages diverged through single mutations (Butler et al 2006). This level of SHM was similar to the very low level seen in many fetal lymphoid tissues (Butler and Wertz, 2012) and also similar to that reported by Jenne et al 2003 in sheep IPP, but inconsistent with the massive SHM claimed by Reynaud et al 1991.

In an effort to obtain more insight into features of the IPP, we isolated the IPP of newborn piglets into blind ileal loops which prevents access to colonizing bacteria and other contents of the gut lumen while still retaining communication via the mesentery. Provided herein are data on IgM, IgG and IgA levels in loop contents, in intestinal lavage (IL), whole saliva (SAL), bronchio-alveolar lavage (BAL) and serum of loop piglets and controls reared in germfree isolators and colonized with a probiotic flora. Furthermore we provide data on IgG3 transcription, heavy chain variable region repertoire diversification and flow cytometric analyses of T- and B cells in the IPP of loop piglets. We propose from these studies that the initial role of the IPP immediately after colonization is to provide broadly-specific “natural” SIgA antibodies to the gut lumen, some of which also enter the blood vascular system. We propose these undiversified SIgA antibodies are best equipped to provide first responder protection against enteric pathogens, either due to their broad specificity or their ability to bind bacteria through glycan interactions and thus to prevent translocation of these and commensal gut bacteria that could result in septicemia.

2. Materials and methods

2.1. Derivation and maintenance of isolator piglets

Piglets were recovered by Caesarian section at 112 days of gestation in the manner previously described (Minatis and Jol 1978). Piglets were distributed in four experimental groups: (A) four were used for preparation of ileal loops, (B) three were sham-operated piglets with a transected lower ileum, (C) four were untreated, colonized controls and (D) two were untreated GF controls. All surgeries were done 48 hours after Caesarian recovery as described below and elsewhere (Santiago Mateo, 2010). Piglets were then transferred to isolator units and were maintained on a diet of Esbilac[®] (Pet Ag, Hamilton, IA). After five days, piglets in groups A-C were then colonized with a defined probiotic gut flora (Harvey et al 2005). One animal in group A underwent unsuccessful surgery and was removed from the study. All animals were maintained under these conditions for five weeks and during this period monitored for the unwanted appearance of pathogenic bacteria.

In a parallel study, non-surgically manipulated isolator piglets were infected with swine influenza virus (SIV) to demonstrate that a viral PAMP could also stimulate development of adaptive immunity (Butler et al 2013). While SIV targets the lung, other tissues can be infected such as the IPP. The infection was resolved in all animals but a few animals had persistent lung lesions suggesting their infection had been more severe and more systemic. For additional comparative purposes, previously published data from fetal, conventional 5 months young and parasite (helminth) infected conventional (PIC) animals were included in some cases (Butler et al 2011b). All samples from mentioned animals were generated from the same origin, collected using identical methods and processed by the same approaches in the same laboratories.

2.2. Formation of isolated ileal loops

The surgical protocol used in this study has never before been performed on GF newborns that were thereafter maintained gnotobiotically in isolator units. The procedure was approved by the Institutional Animal Care and Use Committee of the South Dakota State University and is described in detail elsewhere (Santiago-Mateo et al 2010). Surgery was performed inside an Ohio[®] Care Plus[®] Incubator (OHMEDA Medical, Madison, WI) attached to a sterile isolator unit. Piglets were placed under general anesthesia (95% O₂/5% isoflurane for induction; Baxter Pharmaceutical Products, Inc, Deerfield, IN) and were maintained on to 2% isoflurane/98% O₂ during the surgery. Laparotomy was done through a 2-4 cm left flank incision. Construction of the isolated ileal loops were performed by rejoining 2-5 cm of the distal ileum, in end-to-end anastomosis, to form a one meter isolated loop that included 95% of the IPP. Therefore a small segment (5 cm) of the IPP remained outside of the loop. In sham controls, transection was performed 5 cm proximal to the ileocecal junction. Bowel continuity in both groups was performed by an end-to-end, single layer, jejunoileal or ileal anastomosis. After returning the intestines into the abdominal cavity, the abdominal wall was closed and the piglets were allowed to recover from surgery. Surgical piglets received an intramuscular analgesic, Buprenorphine hydrochloride (0.01 mg/kg body weight; Henry Schein, Inc, Melville, NY), immediately after surgery and at 12-hr intervals for the first 48 hours post-surgery. Piglets were given

Lactated Ringer's solution (LRS; Hospira, Inc., Lake Forest, IL) orally upon recovery post-surgery, and feeding with Esbilac was resumed 4 hrs post-surgery.

2.3. Sample collection

Periodic blood samples were taken from surgically manipulated and control piglets for determination of IgM, IgG and IgA levels. At necropsy, intestinal lavage (IL), loop contents, bronchio-alveolar lavage (BAL) and whole saliva (SAL) were collected for the same purpose. Throughout the report, "loop piglets" refers to those animals with ileal loops while "loop contents" or merely "contents" refers to the lavage of the loop itself. Also at necropsy, segments of the isolated loops and regions of the lower ileum containing the IPP were collected into liquid nitrogen for subsequent molecular biological studies. Various tissues (see Preparation of cell suspensions) were recovered in PBS for flow cytometry analyses (FCM). Histological studies show that the ileum samples collected contained follicles packed with Ig⁺ cells as well as a few Ig⁺ cells outside of the follicle. Therefore ~ 90% the VDJ transcripts analyzed (see below) are believed to be derived from the IPP follicles.

2.4. Recovery of total Ig transcripts

Total RNA and cDNA were prepared from ileal loops, sections of the ileum and other control lymphoid tissue as previously described (Butler et al 2011b). Total IgG transcripts were recovered by PCR as previously described involving a two-stage process first using an FR1 5' primer and an anti-sense C γ 2 primer that anneals to a region common to all swine C γ genes (Butler et al 2009) followed in the second round by a hemi-nested primer regiment in which a FR1 3' primer and the same anti-sense C γ 2 primer were employed (Butler and Wertz 2006). Samples were collected from multiple sites for each animal and on average, used to make two pools per piglet. Therefore, each RID value (see below and Fig. 4) does not represent an individual piglets but a separate pool so there can be two or more points for each piglet. Altogether >1800 clones were

recovered and analyzed. In addition, we include for comparison, RID data on ~320 clone clones recovered from the IPP of neonatal piglets and the JPP from young pigs from previously published studies.

2.5. Relative transcription of IgG3

The cDNA product recovered was cloned into pCR4TOPO and individual insert-containing clones transferred to individual microtiter wells. These clones were propagated overnight, their plasmid DNA recovered by alkaline hydrolysis and transferred to nylon membranes as described previously (Butler and Wertz, 2006). Cloned plasmid DNA was then sequentially hybridized with a 32-P pan IgG or a 32-P IgG3 specific probe as previously described (Butler et al 2009). The hybridization results allowed the proportion of IgG3 transcripts in the total IgG pool to be calculated. On average ~50 clones were analyzed for each sample. Our previous studies have shown that a decline in IgG3 expression is a marker for B cell activation (Butler and Wertz 2006).

2.6. Calculation of variable heavy chain antibody repertoire diversification

A convenient feature of the porcine VH gene system is that only 5-7 VH genes comprise the pre-immune repertoire and diversification thereafter is largely by SHM of the same VH genes (Butler et al 2011b). All of these genes are located at the 3' end of the locus (Eguchi-Ogawa et al 2010). This phenomenon has been studied for nearly two decades and a system devised after monitoring hundreds of sequences (Sun et al 1994; 1995; 1998; Sun and Butler 1996; Butler et al 2006; 2007; 2011b). These have been submitted to GenBank and summarized in a number of articles (Butler et al 2006; Butler and Wertz 2012; Sinkora and Butler 2016; Butler et al 2016). As in any other mammal, these genes appear to have evolved by gene duplication and genomic gene conversion (Butler et al 2011c). Also, like other mammals, somatic mutations almost exclusively accumulate in CDR1 and CDR2 (Butler et al 2006) making it possible to monitor repertoire diversification by monitoring changes in CDR1 and CDR2. This is done using the hybridization procedure described below and the results used to generate a Repertoire Diversification Index

(RDI) which parallels the frequency of SHM (Butler and Wertz, 2012). The RDI equation is based on the principle that as the VH repertoire diversifies: (a) either other minor VH genes are used (other than those indicated in the footnote “c” below) or (b) that the VH genes that dominate the pre-immune repertoire (see footnote “c”) become somatically hypermutated (SHM) and therefore fail to hybridize with the CDR-specific probes. In either case, the proportion of so-called “unknown (UNK)” or partially unknown clones, (see equation below) increases and thus indicates the VH repertoire has diversified.

In this study, the >1800 VDJ-containing cDNA clones recovered from piglets were sequentially hybridized with two VH gene cocktails; one for the CDR1 region and the second for the CDR2 region of the major seven porcine VH genes (Butler et al 2011b). We have previously shown that the use of CDR probe cocktails provides the same result as when the fourteen different CDR1 and CDR2 probed were sequentially used for hybridization (Butler et al 2011b). Hybridization results were used to calculate a repertoire diversification index (RDI) using the equation below:

$$RDI = \frac{UNK^a + [CDR1 + CDR2]^b / 2}{\text{Full hybridization}^c}$$

a/= UNK includes VHN (IGHV15) and other minor non-mutated VH genes

b/= partially hybridizing clones; hybridizing with one CDR cocktail only

c/= complete hybridization with a mixture of probes specific for both CDR1 and CDR2 of VHA (IGHV4), VHA *(IGHV10), VHB (IGHV6), VHB*(IGHV12), VHC, VHE (IGHV8), and VHF (IGHV11). VHC has so far not been mapped. We use both the vernacular and IMGT nomenclature when describing these genes.

2.7. Preparation of cell suspensions

Cell suspensions were prepared essentially as previously described (Sinkora et al., 2014; Potockova et al., 2015). Briefly, heparinized (20 U/ml) blood was obtained by intracardial puncture. Cell suspensions from the mesenteric lymph nodes (MLN) were prepared in cold phosphate buffered saline (PBS) by carefully

teasing apart the tissues using a forceps. Erythrocytes were removed from the blood and MLN using hypotonic lysis. Cells from the bone marrow were directly flushed from the femur and leukocytes were purified using a Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation (Sinkora and Sinkorova, 2014). Cells from IPP, JPP and isolated loops were prepared by incubating diced tissue in digestion media [RPMI-1640, 100 U/ml collagenase type IV (Sigma-Aldrich, St. Louis, MO), 2 % fetal calf serum (FCS)] at 37 °C for 1 hour. Supernatants from incubated tissue were filtered through a 70µm mesh nylon membrane, washed three times with PBS and the lymphocytes recovered using a 40 % - 80 % Percoll gradient (GE Healthcare, Uppsala, Sweden) at 600x G for 20 min. All cell suspensions were finally washed twice in cold PBS containing 0.1% sodium azide and 0.2% gelatin from Cold Water Fish Skin (PBS-GEL), filtered through a 70 µm mesh nylon membranes and cell numbers determined by hemacytometer.

2.8. Immunoreagents

The following mouse anti-pig monoclonal Abs, whose source and specificity were described earlier (Sinkora et al., 2007; Stepanova and Sinkora, 2012, 2013), were used as primary immunoreagents: anti-IgM (M160, IgG1), anti-IgA (M1456, IgG2a), anti-CD2 (MSA4, IgG2a or 1038H-5-37, IgM), anti-CD3 (PPT3, IgG1 or PPT6, IgG2b), anti-CD4 (10.2H2, IgG2b), anti-CD8 (76-2-11, IgG2a), anti-CD21 (IAH-CC51, IgG2b) and anti-TCRγδ (PPT26, IgG1 or PPT16, IgG2b). Goat polyclonal Abs specific for mouse immunoglobulin subclasses labeled with fluorescein isothiocyanate, R phycoerythrin, Phycoerythrin/Cyanin 7 tandem complex or Allophycocyanin were used as secondary immunoreagents (all secondary reagents from Southern Biotechnologies Associates, Inc., Birmingham, AL). Primary isotype-matched mouse anti-rat monoclonal Abs were used as negative controls. Secondary polyclonal Abs were tested for cross-reactivity (no primary monoclonal Abs) and also for cross-reactivity with primary isotype-mismatched mouse anti-pig monoclonal Abs. All immunoreagents were titrated for optimal signal/noise ratios.

2.9. Staining of cells and flow cytometry

Staining of cells for flow cytometry analysis was performed as described previously (Sinkora et al., 2007; Sinkora et al., 2013) by indirect sub-isotype staining. Briefly, multi-color staining was done using cells that had been incubated with a combination of three (three-color staining) or four (four-color staining) primary mouse mAbs of different sub-isotypes. Cells were incubated for 30 minutes and subsequently washed twice in PBS-GEL. Mixtures of goat secondary polyclonal Abs specific for mouse immunoglobulin subclasses that had been labeled with fluorochromes were then added to the cell pellets in appropriate combinations. After 30 minutes, cells were washed three times in PBS-GEL and measured on a standard FACSCalibur flow cytometer (BDIS, Mountain View, CA). Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. The PCLysis software (BDIS, Mountain View, CA) was used for data processing. All cell numbers in this report are stated as relative numbers (proportions).

2.10. Concentration of IgG, IgA, IgM and total protein

The levels of IgM, IgG and IgA in serum and secretions was measured by sandwich ELISA as previously described (Butler et al 2002; 2011a). Data were then treated by ELISANALYSIS as previously described (Peterman and Butler, 1989). Each value entered is the mean from 4-6 points in the linear region of the titration plot for each sample. Because of the inherent difficulty of comparing absolute values among body secretions, in some cases we present data as Ig profiles. In all secretions, total protein was determined by NanoDrop spectrophotometry. In the absence of gut flora, there is a paucity of Igs in the IL, so much of the TP is formula protein which may have skewed the data for this group.

2.11. Bacteria examination

Rectal cultures were obtained periodically and a loop culture was recovered at necropsy, to assess for contamination by pathogens before and after colonization with the probiotic microflora. Specimens were applied to blood agar and cultured aerobically and anaerobically.

2.12. Statistical analysis

Differences among experimental groups were analyzed by one-way ANOVA using GraphPad Prism software. Differences among the median frequency values for lymphocyte populations and their subsets were analyzed by a one-way ANOVA Student–Newman–Keuls test. The level of statistical significance is reported in p values. Data obtained using sandwich assays were determined using ELISANALYSIS. As indicated above, each value is the mean of 4-6 points so “n” is under-represented in the statistical analyses.

3. Results and discussion

Table 1 shows that with the exception of IgM in IL, the concentration of the major Igs in all secretions is < 1 µg/ml in GF controls after rearing for 5 weeks in isolators and IgM comprises ~70% in the IL (Fig. 1). The only significant difference between colonized controls piglets (probiotic) and colonized sham operated controls (sham) was in the mean IgG levels in the IL; i.e. 0.39 µg/ml and 2.83 µg/ml respectively (data not shown). The mean value for the combined groups (1.43 µg/ml; Table 1) was due to sham piglet 101B3; seven of the eight animals in the combined groups had values similar to the non-manipulated controls. Including this animal also influenced the proportional data in Fig. 1 (see below). Since there were no other differences between probiotic and sham piglets, and the focus of the study was not on comparing the effects of surgery, for simplicity we combined the sham and probiotic into a single group (Table 1). The elevated IgG in surgically manipulated piglets, including sham piglets, is discussed below. Overall, colonization results in a 5-10 fold increase in IgA in SAL, BAL and in the IL of probiotic-sham controls compared to GF

piglets while levels in the IL of loop piglets remained the same as in GF piglets. Actually, IgG levels were higher than IgA in the IL of loop piglets primarily because IgA levels did not increase (Table 1; Fig. 1). The low IgA levels in the IL of loop piglets could not be ascribed to having used diluted specimens since total protein levels were the same as in probiotic+sham animals (Table 1).

When the loop content was examined, IgA was 2.5-fold higher and IgM was 7-fold higher than in the IL of colonized piglets (Table I). The higher absolute levels are not unexpected, since the loops are blind. To place this into perspective, we compared the Ig profiles in the loop content with those in the IL of the various treatment groups (Figure 1). The Ig profile of the loop contents was intermediate between that of GF and probiotic-sham controls. As mentioned above, because of the elevated level of IgG in the IL of piglet 101B3, the Ig profile in the IL of the sham piglets differs from that for probiotic controls. However, in seven of the eight piglets in the sham- probiotic group, IgA accounted for ~75% of the total Igs and their profiles were indistinguishable from that of the probiotic group (Figure 1).

The greatly elevated IgG in serum of loop piglets (Table 1) but the relative low level of IgG in the loop content suggests that the serum IgG is not derived from the IPP in blind loops since it comprises < 10% of total Igs recovered from the loop contents which is indistinguishable from that in the IL of GF piglets (~6%; Table 1; Figure 1). The elevated level of serum IgG in loop piglets could result from systemic surgical trauma but not infection since microbial cultures were negative. The degree of elevation of serum IgG appears related to the magnitude of the surgical manipulation since we observed the same phenomenon with the more radical resection of the lower ileum and caecum (Butler et al 2011a). Alternatively, the elevation of IgG may represent antibodies to the intestinal flora that were translocated due to the absence of the secretory IgA barrier as demonstrated by Sait et al (2007). The elevated IgG in the BAL is most likely due to transudation from serum, a phenomenon also seen in rabbits (Butler et al 1983). Collectively data in Fig.1 and Table 1 show that isolation of much of the IPP into blind loops, deprives the remaining gut of IgA suggesting that the IPP is the major source of IgA in the gut lumen of colonized neonatal piglets.

To better characterize GF, probiotic, sham and loop piglets, we also analyzed the proportion of different subpopulations of lymphoid cells by flow cytometry. Figure 2 shows that the proportions of B cells (Fig. 2A) and $\gamma\delta$ T cells (Fig. 2C) was comparable in the IPP of isolated loops and the IPP from sham / probiotic animals while there was no difference in $\alpha\beta$ T cells (Fig. 2B). This indicates that the IPP in isolated sterile loops behave similarly to IPP that had access to bacteria. An increase in B cells and a corresponding decrease in $\alpha\beta$ T cells in IPP is typical following of gut colonization by microbiota, which we described as a shift from a fetal- type to an adult-type lymphocyte distribution (Sinkora et al., 2011). Thus, changes in lymphoid distribution in IPP occur without direct contact with colonizing microbiota. To further demonstrate whether effector subpopulations of lymphocytes were also redistributed, we analyzed antibody-forming B cells and plasma cells having a $CD21^-$ phenotype (Sinkora et al., 2013, 2014); these can be further subdivided into resting $CD2^-CD21^-$ (Fig. 2D) and effector $CD2^+CD21^-$ (Fig. 2E) subpopulations. We also analyzed effector $CD4^+CD8^+$ $\alpha\beta$ T cells (Fig. 2F). The proportions of all three effector subpopulations were comparable in IPP of probiotic, sham and loop animals, and was significantly different from GF animals (Fig. 2D-F).

Figure 3 shows that two weeks after colonization (21D) there is a spike in serum IgA and IgM levels but not IgG in probiotic-sham control piglets; a pattern consistently seen in earlier studies (Butler et al 2002; 2013). After 5 weeks, the serum IgA and IgM in probiotic-sham piglets was 5-fold higher than in GF piglets while serum IgA levels in loop piglets increased < 2-fold (Table 1; Figure 3) suggesting an interruption in its source (Table 1; Figure 3). Figure 3 also shows that **loop piglets** have an aberrant or delayed **serum** response profile in which the IgM and IgA “spike” is delayed beyond 21D. This seems consistent with the intermediate Ig profile in IL of loop piglets (Fig. 1).

The two-week spike in serum IgM is generally attributed to the primary innate antibody response in higher vertebrates but in textbooks this does not extend to IgA. In initial studies using isolator piglets, we observed that colonization resulted in a 3-5 fold increase in serum IgA relative to adult serum levels (Butler

et al 2000). Thus, it seems that the early spike in IgA represents a type of “primary” IgA response similar to that attributed to IgM and could be related to the precocial CSR to IgA in utero. This early appearance of serum IgA is not unlike that reported by Macpherson et al (2000) in mice (see below).

Unpublished studies (Butler, Weber, Francis; see Suppl. Figure 1) show that purified TLR ligands alone given to GF piglets results in the same primary serum IgA and IgM response but not for IgG. These IgA and IgM antibodies especially recognized enterobacteria but also Gram positive *Listeria*, *S. mutants* and *Lactococcus*. The latter are frequently encountered in some mucosal infections. The supplemental figure also shows that colonization with *E. coli* G58-1 and 933D results in a robust and broadly specific response to various bacteria, not just those used for colonization and that colonization also generated an IgG response. It is unlikely that such broad specificity, e.g. to *Listeria*, *Lactococcus*, *Lactobacillus* and *S. mutans*, is due to cross-reactivity. Rather, newborn piglets appear programmed to secrete within two weeks, IgM and IgA antibodies that can recognize pathogens that threaten their survival and prevent translocation of commensal gut bacteria.

There are currently three **recognized** pathways by which IgA from B cells in the IPP might reach the serum. First, IgA-committed B cells from the IPP traffic to the MLN and as plasma cells secrete their products that enter the blood vascular system. Second, IgA-committed cells traffic to the MLN, enter a germinal center reaction and after selection return to the gut lamina propria (Strober et al 2005; Brandtzaeg et al 2005; Mattingly and Waksman 1978). A portion of the IgA produced by these cells enters the lymphatics and proceeds to the blood. It is known that a significant proportion of mucosal IgA cells in another artiodactyl substantially contribute to serum IgA levels (Gorin and Gould 1979) and in pIgR^{-/-} mice that cannot secrete IgA across the epithelial barrier, serum IgA levels rise 100-fold (Sait et al 2007). Assuming the paradigm that an IgA response is a secondary response requiring GC formation, T cell help and B cell selection, one would not expect this IgA to enter blood just two weeks after colonization. A third possibility is that this unexpected primary serum IgA response is directly or indirectly from B cells in the IPP

similar to that from T independent B-1 B cells in mice (Macpherson et al 2000). Except for humans that produce monomeric IgA in bone marrow (Crago et al 1984), 90% of IgA in the serum of non-primate mammals including swine, is polymeric (Vaerman 1973) and is derived from mucosa-associated plasma cells. In our studies with piglets, serum IgA is ~90% polymeric which in secretions appear as SIgA (with bound SC). Considering these possibilities and knowing that the IPP is perhaps the largest lymphoid tissue in newborn piglets, we speculate that the primary serum IgA responses originates from the same IgA⁺ cells in the IPP that accounts for the SIgA in the gut lumen (Table 1; Figure 1). Our analysis of IgA-switched B cells by flow cytometry showed that the majority of fully switched IgA⁺IgM⁻ B cells can found IPP (Fig. 2G). These were resting CD2⁻CD21⁻ and/or effector CD2⁺CD21⁻ antibody-forming B cells and plasma cells (Fig. 2H). While partially switched IgA⁺IgM⁺ B cells can be found in different tissues, with highest occurrence in mesenteric lymph nodes, fully switched IgA⁺IgM⁻ B cells are substantially enriched only in IPP (Fig. 2I). Thus, although a trafficking of partially switched IgA⁺ B cells to the MLN and other site is probable, fully switched IgA⁺ B cells reside mostly in IPP, and may contribute directly to SIgA in the gut lumen as well as blood, which is consistent with the IgA deficiency of loop piglets.

The IgA and IgM in SAL and BAL is most likely produced locally from B-blasts which may be immigrants from the IPP during fetal development. It has been shown that there is preferential migration out of the IPP and these are predominantly B cells (Binns and License 1985). This is also the case in sheep (Reynolds et al 1991). Homing receptors like those associated with the immigration of IgA⁺ blast cells from the gut to the mammary gland also appear to be involved in homing to the respiratory tract (Bourges et al 2008; Salmon 1999). Thus, the IPP may also be a major source of especially IgA⁺ cells that migrate to other mucosal sites such as the salivary glands or bronchial lymph nodes according to the concept of the common mucosal immune system (McDermott and Bienenstock 1979). We did not see a difference between loop and probiotic piglets as regards IgA in BAL and SAL suggesting that IgA precursors in the fetal IPP had already migrated to the bronchi and salivary glands and probably to other mucosal sites. Alternatively, the cells responsible in the bronchia and salivary glands have an independent origin. As discussed above, studies on

the Peyer's patches of mice and humans favor the view that most lymphocyte trafficking goes from the Peyer's patches to the MLN when the antigenic stimulus originates in the GI tract as in the case of colonization (Strober et al 2005; Mattingly and Waksman 1978; Liu and Macpherson 1991; Brandtzaeg et al 2005).

The most surprising observations in this study was that variable heavy chain repertoire diversification in VDJ clones recovered from the IPP of GF newborns, probiotic-sham and those recovered from the IPP of isolated loops did not differ (Fig. 4). However, two of the SIV infected isolator piglets with persistent lesions displayed a 10-fold elevation of RDI in their IPP clearly showing that repertoire diversification can also occur in the IPP during systemic infections (Fig. 4). Double-strand RNA produced by RNA viruses stimulate through TLR 7 (Kawai and Akira 2006) which can substitute for bacterial PAMPs, in activating adaptive immunity (Butler et al 2013). These exceptional piglets indicate that a virulent infection and colonization with a benign microbiota can have different effects on repertoire diversification in the IPP. Figure 4 also shows that at 5 months, conventional piglets have a 10-fold increase in RDI which is temporally consistent with the time when the JPP replaces the IPP (Carlens 1928; Pabst et al 1988; Barman et al 1997). In mice, the JPP equivalent is active in GC formation, SHM and B cell selection so an increase in RDI would be expected. Therefore the degree to which the RDI in gut-associated secretory lymphoid tissue seems to depend on the type of TLRs activated and the age of the animal, i.e. whether or not the transition from IPP to JPP has occurred. The JPP are easily recognized in older piglets as discrete bulges on the ileum just like in mice, rats and rabbits. With 5 week old animals, the distinction is challenging and requires microscopy.

Early events in the IPP appear restricted to CSR and rapid proliferation of IgA⁺ cells (Allen and Porter 1973a; Butler et al 1981; 2002; Pabst et al 1988). However, the CDR3 spectratypic analysis of the IgA transcripts from the IPP of fetal and GF piglets displays a Gaussian distribution (Butler et al 2001) suggesting the absence of B cell selection that normally depends on antigen-dependent GC formation. This observation combined with the low degree of repertoire diversification in the piglet IPP and paucity of SHM in sheep IPP (Jenne et al 2003) (Fig. 4) seems to fit the data of Macpherson et al (2000) indicating the initial

gut SIgA response occurs without cognitive T cell help involving recognition of a peptide, which requires processing by the antigen presenting B cell that recognized the intact antigen using its B cell receptor, followed by display of the peptide on class II. The displayed peptide is then recognized by the T cell receptor on α/β T cells and with the help of CD40/CD 40 ligand interaction, leads to a T-dependent response. Such a process would result in a delayed IgA response by both newborn mice and piglets. The T-independent IgA response that avoids this pathway and delay in mice, is ascribed to B-1 B cells (Macpherson et al, 2000) that are believed to originate in the T cell poor peritoneum (Strober et al 2005). This fits well to a feature of the IPP in swine and sheep, namely that there is a very low proportion of T cells compared to classical JPP (Binns and Liciense 1985; Pabst et al 1988; Barman et al 1997; Griebel et al 1992). Whether these natural antibodies are derived from B-1 cells in swine is a mute point, since two populations of swine B cells are not currently recognized using conventional mouse phenotypic markers (Bertrand and Wortis 2002; Wilson and Wilkie 2007). CD5 and IgD expression are at most quantitative markers so concluding for example, that all rabbit B cells by these criteria is problematic, since rabbits have no gene for IgD (Ros et al; 2004). Whatever one wishes to call the B cells in the IPP of fetal and newborn swine, those making IgA, display an unselected repertoire (Butler et al 2001), a low RDI (Fig. 4) and since IgA is being produced during fetal life in an organ with few T-cells, the phenomenon resembles the T-independent IgA response in mice described by Macpherson et al 2000.

The precocial CSR *in utero* appears to be at least quantitatively special to piglets and perhaps other artiodactyls (Lin et al 2014). While direct switch from IgM to IgA is well-recognized (Strober et al 2005; Janeway et al 2005), fetal CSR has been poorly studied in all species. In swine there is a predominant transcription of IgG3 at mid-gestation in fetal piglets that presumably accounts for the dominance of IgG in fetal and newborn serum (Butler et al 2001; Butler and Wertz 2006). IgG3 is considered the most ancestral IgG, and IGHC3 is located immediately downstream of the IgM-IgD complex suggesting that its appearance in fetal piglets is stochastic (Butler et al 2009; Eguchi-Ogawa 2012) and may not be AID-dependent much like the expression of IgD. The sharp reduction in IgG3 transcription appears to be an early marker for B cell

activation which also coincides with the proliferation of IgA⁺ cells and their predominance of IgA in the IL. Figure 5 shows the disappearance of IgG3 transcripts in loop piglet IPP, the IPP of the probiotic-sham group and in the MLN and spleen. This also coincides with FCM observations showing that after 5 weeks, there is the same proportion of resting CD2[−]CD21[−] (Fig. 2D) and effector CD2⁺CD21[−] (Fig. 3E) (Sinkora et al 2013, 2014). This might also explain why the Ig profile in the IL is intermediate between that of GF and probiotic controls (Fig. 1) and the primary serum IgA response in loop piglets is aberrant or delayed (Fig. 3). Evidence that B cells in the IPP of blind loops begin differentiation without encountering the probiotic bacterial culture suggests the response is either a stochastic response to trauma or is mediated by cytokines generated in the remaining intestine.

Our earlier studies showed that the IPP are not required for B cell development or maintenance of Ig levels (Butler et al 2011a; Sinkora 2011) which by our definition, eliminates their classification as primary lymphoid tissue. However, this left unanswered the question as to the major role of what is arguably the largest secondary lymphoid tissue in fetal and newborn piglets. The lack of repertoire diversification in the piglet IPP in the first weeks after birth indicates that the IgM and IgA are expressing undiversified natural antibodies reminiscent of those described in mice by Macpherson et al (2000) and the low frequency of SHM in the sheep IPP (Jenne et al 2003). Without selection (Suppl. Fig. 2) and with little SHM, one suspects they possess the low affinity and broad specificity of germline-encoded recombinants. Broadly specific IgA secreted into the gut lumen, would be valuable in recognizing a broad spectrum of enteric pathogens and benign microflora and thus preventing their translocation and possible septicemia (Sait et al 2007). Their appearance in serum indicates they could also be useful for protection against systemic pathogens. This could explain why the IPP in swine develop *in utero* and there begin their switch to IgA. If the development of protective mucosal immunity in the gut depended on contact with a specific bacterial epitope processed through the T-dependent pathway reviewed above, it would delay the production of non-diversified SIgA and place the newborn at risk.

One may nevertheless question why the enormous amounts of passive maternal antibodies of all isotypes that are ingested by suckling piglets would not provide similar protection? To begin with, >80% porcine colostral antibodies are IgG and all isotypes are absorbed indiscriminately intact by the “open” neonatal gut to primarily provide systemic protection, equaling what human infants obtain through *in utero* transfer of IgG (Butler, 1974; Butler et al 2015). Concomitant with the transition to mature milk (perhaps triggered by colonization), non-specific absorption by the gut ceases (“closure”) and IgA becomes the major Ig in lacteal secretions, (Bourne and Curtis 1973; Klobasa et al. 1987), some of which become crypt-associated (Butler et al 1981; Allen and Porter 1973b). These lacteal IgA antibodies reflect the sow’s mucosal immunological experience with intestinal pathogens that results from repeated adaptive responses resulting in highly specific IgA⁺ B cells primed in the gut that migrate to the colostrum-forming mammary gland using specific homing receptors and the resulting plasma cells that secrete these specific antibodies into milk and colostrum, i.e. the gut-mammary gland axis (Bohl and Saif 1975; Roux et al 1977, Salmon 1987; reviewed by Butler et al 2015). Thus, colostral IgA theoretically differs from the broadly-specific IgA that appears after colonization or after exposure to TLR-ligands (Suppl. Figure 1). The broadly-specific natural antibodies could enable the newborn to recognize foreign antigens never encountered by the sow. Whether the highly-specific lacteal IgA ingested by the piglet also has the capacity to prevent translocation or absorption of intact dietary allergens, remains to be tested, since the dam has most likely developed oral tolerance to commensal gut bacteria and dietary antigens (Duchmann et al 1995; Karlsson et al 1999). In any case, the scenario of events we have proposed above, may be overshadowed by the glycan-dependent binding of SIgA to bacteria, regardless of its broad Fab specificity (Mathias and Corthesy, 2011; Perrier et al 2006; van der Waaig et al 1996). Binding differs among bacterial strains but can be up to 90% (Mathias and Corthesy, 2011). This would render initial repertoire diversification unimportant. More important would be the need for rapid secretion of SIgA into the gut lumen after exposure to bacteria. Such secretion would require rapid CSR, proliferation of IgA⁺ cells and the secretion of copious amounts of SIgA into the gut lumen. This appears to be precisely what occurs in the IPP of piglets.

What we propose is a hypothesis for an initial role for the IPP of swine which may also apply to other artiodactyls that possess IPP. Briefly, we propose that the IPP provide an immediate wave of broadly-specific SIgA to the gut lumen that either due to its broad specificity or ability to bind directly to bacteria via glucans on SC, provides a first responder mucosal barrier that does not required a diversified repertoire. Although they may possess some autoimmune character, their lectin and low affinity is unlikely to cause intestinal damage. While this natural IgA provides the immediate protection, highly-specific antibodies from colostrum/milk or later generated through T cell dependent, antigen-driven events in the developing JPP, take over the secretory immune response in the gut as the IPP undergo involution. The JPP activity is critical since glycan binding does not protect against viruses (Perrier et al 2006). The T-independent IgA in young mice may serve the same function.

The data reported are from limited but labor-intensive studies that were not pursued due to lab closure and retirement. Confirming our contention that the IPP in newborn piglets are the major source of gut IgA and IgM or IgA⁺ blasts for export, future experiments will require blocking Ig and cell migration from the isolated loops. This can be done surgically at birth but because the IPP develops *in utero*, the results from such studies might be inconclusive since B cell migration could have occurred before birth (see above). One alternative could be to knockout those genes responsible for the development of the IPP architecture which would theoretically prevent the development of the IPP during fetal life. A second GMO alternative could be knocking out the polyIg receptor as was done in mice (Uren et al 2003) which could have a similar effect. Knock-out technology has been used in other swine research (Mendicino et al 2011; Ramsoondar et al 2011; Rodgers et al 2008; Ito et al 2014; Sieren et al 2014). The GMO approach is theoretically possible and should be encouraged.

The study described has a number of weaknesses that might have been corrected had we the opportunity to continue the work. For one thing, serum samples should have been taken at more frequent intervals to pinpoint the serum response profile and enough piglets with loops constructed so some piglets

could have been periodically sacrificed throughout the study and their loop contents analyzed. Kinetic studies could also have benefited from using Thiry-Velly loops so loop contents could be regularly sampled and the probiotic culture administered to the loop of some piglets but not others. In addition, follow-up studies by others should examine the specificity of the various isotypes. First, is the question of whether elevated serum IgG in loop piglets is a systemic immune response to translocated probiotic flora due to a deficiency of the IgA barrier like reported in pIg^{-/-} mice (Sait et al 2007)? The specificity of all isotypes in the IL to the probiotic flora versus the bacterial panel in Suppl Fig. 1 should be measured to determine if binding could be competitively blocked by defined glycans or free SC. These tests might help to explain why the response to the panel of bacteria used is so cross-reactive. It is possible that some of the binding shown in Fig. 1 Suppl., could be glycan-mediated since IgM is the most glycosylated Ig and also complexes with SC during transport. Although not tested in this study, the dimeric IgA in serum presumably lack SC, so recognition must be binding site specific or involve α -chain glycans. Finally, there is a need to determine using appropriate markers, if GCs form in the IPP of newborn piglets. If GC never form in newborns or those colonized with a probiotic flora, it would support the view that proliferation of IgA⁺IgM⁻ B cells is T-independent.

Some may criticize that data were obtained using a contrived model, i.e. the isolator piglet rather than using conventionally-reared piglet. Reviews of this model and its variants emphasize that science proceeds by reducing the number of variable in an experiment; the isolator piglet model eliminates the influence of maternal passive antibodies and other regulatory factors and places the exposure to these and environmental factors under the control of the experimenter (Butler and Sinkora 2007; Saif et al 1996; Sinkora and Butler, 2016). Therefore what we observed in an isolator piglet model may not be seen in conventional piglets because of masking by passive antibodies and changes induced by regulatory factors transmitted through suckling. Identifying those differences are also important in establishing the best hypotheses. Fortunately the piglet model allows this to be tested by experimental administration of colostrum and milk as previously

described (Klobasa et al 1981; 1987). Without further data, we believe the IPP of newborn isolator piglets functions in the same manner as those in newborn conventional piglets.

4. Conclusion

Since are previous work has shown that the IPP in swine are not primary B cell lymphoid tissue or a bursa of Fabricius equivalent, it seemed important to identify a role for this very large lymphoid complex in newborn artiodactyls, that as adults, provide much of the world's food supply. While our observations do not resolve the question of the role of the IPP, they are the first to offer data to formulate a new hypothesis to explain their role for newborn artiodactyls. Briefly, we propose that the IPP provide on demand a broadly-specific, T-independent primary SIgA response in the neonatal gut that may also function through glycan binding to colonizing pathogens and normal gut flora. Both activities of this SIgA eliminate the need for immediate repertoire diversification, but are able to provide without delay, a barrier to bacterial translocation. This could explain why this organ develops *in utero* independent of environmental stimuli and that CSR to IgA begins in utero.

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Figures

Figure 1

The Ig profiles in the ileal lavage (IL) from four treatment groups and for the blind ileal loop contents. The difference in the profile and standard error comparing probiotic and sham controls is attributable to piglet 101B3.

Figure 2

The frequencies of lymphocyte types collected from IPP at necropsy after 5 weeks. Lymphocytes were isolated from IPP of different experimental groups (indicated at top) and analyzed for proportion of B cells (A), $\alpha\beta$ T cells (B) and $\gamma\delta$ T cells (C). The proportions of effector subpopulations of B cells; CD2[−]CD21[−] (D) and CD2⁺CD21[−] (E) as well as CD4⁺CD8⁺ $\alpha\beta$ T cells (F) are also shown. Data are given for the IPP of GF piglets (open bars), IPP of colonized control piglets (dotted bars), IPP of sham-operated controls (light-gray bars), a remaining small segment of the IPP in piglets with loops (dark-gray bars) and from the IPP within the ileal loops (black bars). Error bars represent $\pm$ SEM and statistically significant difference $P < 0.05$ are indicated by an asterisk. Panel G shows representative flow cytometry analysis of IgM and IgA expression on cells isolated from the bone marrow (BM), blood, mesenteric lymph nodes (MLN) and IPP of colonized animals. Further analyses of IgA⁺IgM[−] B cells that are abundant in IPP (R1) showed that vast majority of them are CD21 negative (H). Statistical analyses for IgA⁺IgM[−] and IgA⁺IgM⁺ B cells are also shown (I). Coloring of bars correspond to tissue coloring depicted in G. Error bars represent $\pm$ SEM and statistically significant difference $P < 0.05$ are indicated by an asterisk.

Figure 3

Developmental changes in serum levels for piglets in the same treatment groups indicated in Table 1. LEFT: Kinetic data. RIGHT: Comparison of serum levels at necropsy. Error bars represent $\pm$ SEM and statistically significant difference $P < 0.05$ are indicated by an asterisk. Legend on the figure.

Figure 4

Repertoire diversification in VDJ transcripts recovered from the IPP of 5 week isolator piglets. Data include previously published data for some animals experimentally infected with swine influenza virus (SIV) and also for JPP and other secondary lymphoid tissues (designated Pool) from 5 months conventional animals (see section 2.1.). On average, 50 VDJ clones were examined for each sample reported. Each point is for a mixture of samples, not a single sample from a single animal (see section 2.4.). There are no differences in the IPP among loop, probiotic-sham and GF piglets; $p = 0.95$; The RDI for SIV-infected isolator piglets is significantly elevated as is the RDI in JPP and pooled samples of 5 months conventional animals ($p = 0.006$ to 0.01).

Figure 5

The proportion of IgG3 transcripts relative to total IgG transcripts. Data were generated from the IPP, MLN and spleen of treatment groups from this study after rearing piglets for 5 weeks in isolators (Loop, Probiotic, Germfree), compared to fetal and parasite-infected conventional (PIC) piglets. Data for fetal and PIC piglets were generated in other studies (see section 2.1.). Colonized animals have significantly lower relative levels of IgG3 transcripts at $p < 0.01$. NT = not tested.

Table 1: Immunoglobulin Concentrations (µg/ml) in Serum and Secretions at Necropsy

Specimen	Ig	Piglet Group		
		Loop	Probiotic + Sham	Germfree
BAL	IgA	5.57±0.38 ^a	5.40±0.55 ^a	0.67±0.07
	IgG	62.20±12.89 ^a	3.04±0.64 ^b	0.25±0.02
	IgM	8.53±1.91 ^a	1.91±0.32 ^b	0.27±0.04
	TP	1997± 347	1096 ± 99	1300±71
SAL	IgA	6.67±1.23 ^a	6.80±0.79 ^a	0.45±0.03
	IgG	3.24±0.84 ^a	0.71±0.22 ^b	0.15±0.03
	IgM	0.59±0.08 ^b	0.49±0.04 ^b	0.25±0.03
	TP	2780± 133 ^a	2986±228 ^a	805±3.54
IL	IgA	1.36±0.16 ^b	8.09±2.97 ^a	0.92±0.29
	IgG	2.90±0.18 ^a	1.43±0.27 ^a	0.19±0.04
	IgM	1.90±0.16 ^b	2.96±0.32 ^b	2.05±0.02
	TP	987±102	1050±52	9150±3288 ^c
Serum	IgA	13.23±4.55 ^b	37.81 ±4.60 ^a	7.90 ±0.23
	IgG	1185±275.25 ^a	202 ± 9.98 ^a	53.75±7.91
	IgM	120.33±13.70 ^a	164.43 ±11.87 ^a	26.50±0.22
	TP	NA	NA	NA
Content	IgA	25.6±8.51	NA	NA
	IgG	5.33±1.27	NA	NA
	IgM	20.73±2.97	NA	NA
	TP	5500±3119	NA	NA

a = Higher than GF,

b = Same as GF,

c = Higher than loop and probiotic-sham

All statistical differences are significant at $p < 0.05$

Figure 1

Figure 2:

Figure 3:

Figure 4:

Figure 5: