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Ileal Peyer's Patches Are Not Necessary for Systemic B Cell Development and Maintenance and Do Not Contribute Significantly to the Overall B Cell Pool in Swine

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Based on studies of sheep, ileal Peyer's patches (IPP) have been regarded as a type of primary lymphoid tissue similar to the bursa of Fabricius in chicken. Because bursectomy results in B cell deficiency, we wondered whether resection of the IPP of piglets would have a similar effect. Comparison of IPP-resected, surgical shams and untreated germ-free piglets, all of which were later colonized with a defined commensal flora, demonstrated that resection of the IPP did not alter the level and phenotype of B and T cells in lymphoid tissues and the blood 10 wk after surgery. Additionally, colonization of IPP caused a shift from the fetal type of lymphocyte distribution to the adult type that is characterized by prevalence of B cells, with many of them representing IgA^+ switched B cells or displaying a more mature $CD2^-CD21^+$ and $CD2^-CD21^-$ phenotype. Moreover, colonization leads to appearance of effector $CD4^+$ $CD8^+$ αβ T helper and $CD2^+CD8^-$ γδ T cells. Comparison of germ-free with colonized pigs and experiments utilizing surgical transposition of jejunal Peyer's patch into terminal ileum or construction of isolated ileal loops indicated that lymphocyte development in IPP is dependent on colonization. Although our studies confirmed higher mitotic and apoptotic rates in IPP, they failed to identify any cell populations that resemble developing B lineage cells in the bone marrow. These results indicate that porcine IPP are not required for systemic B cell generation or maintenance, but they are secondary lymphoid tissue that appears important in immune responses to colonizing bacteria. ***The Journal of Immunology*****, 2011, 187: 5150–5161.**

The organization of lymphoid tissue among mammals is generally similar with several notable deviations. Among artiodactyls, and apparently in whales and probably all ungulates, Peyer's patches (PP) occur in two forms: 1) the ileal Peyer's patches (IPP) that are continuous and occupy 50–200 cm proximal to the ileocecal junction depending on the age of animals, and 2) the jejunal Peyer's patches (JPP) that are isolated or discrete and occupy the upper ileum and jejunum (1–5).

The IPP differ from JPP and classical PP of rodents and humans in B and T cell distribution and in ontogeny. The IPP appear in late gestation, remain dominant during early neonatal life, and involute several weeks to months after birth (3–9). In contrast, JPP develop in middle gestation. Their further development depends mainly on colonization of the gut, and they survive throughout life. Moreover, in conventional animals, IPP are composed mainly of B cells with a minority of T cells present whereas there are more T cells and less B cells in JPP (4, 10).

The exact role and function of the IPP are unknown, but a concept grew out of studies in lambs indicating that the IPP play a role in development of B cells as well as the B cell repertoire in a manner similar to the bursa of Fabricius (5, 11–13) or perhaps the rabbit appendix or sacculus rotundus (14). This concept has permeated other scientific reports, reviews, and immunology textbooks for >20 y, and due to similarity in the organization of IPP it was broadened also to other species (2, 9, 15, 16). It was assumed that there is massive Ag-independent B cell repertoire development in the IPP and that diversification occurs by somatic hypermutation (SHM) and/or gene conversion. This concept was supported by findings that 1) surgical removal of IPP resulted in reduction of Ig^+ B cells (17); 2) B cells develop in IPP in an Ag-independent manner (18); 3) systemic depletion of IgM^+ B cells in fetal lambs causes the failure to develop follicles in IPP (19); 4) IPP have a higher proportion of proliferating lymphoid cells than found in thymus (20, 21); 5) the vast majority of the B cells in IPP die by apoptosis in situ (20, 22, 23); 6) diversification of the Ab repertoire occurs by SHM or gene conversion similar to the bursa of Fabricius (24, 25); 7) IPP involute in postnatal ontogeny similarly to the bursa of Fabricius (5); and 8) activation-induced cytidine deaminase, which mediates SHM, gene conversion, and Ig isotype switching, is present in IPP (2). However, the observations in points 3–8 are not directly related to B lymphogenic function of IPP and can be also ascribed to the fact that 1) B cells are generated at different sites such as the bone marrow or spleen and emigrate to various tissues including IPP where secondary positive selection and proliferation occur (26); 2) changes in B cell phenotype and development are due to colonization of gut by bacteria that include proliferation, selection, class switching, and phenotype alteration after Ag stimuli similarly to germinal reaction (27);

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Abbreviations used in this article: 7-AAD, 7-aminoactinomycin D; IPP, ileal Peyer's patch; JPP, jejunal Peyer's patch; MHC-II, MHC class II; MLN, mesenteric lymph node; PBS-GEL, PBS containing 0.1% sodium azide and 0.2% gelatin from cold water fish skin; PP, Peyer's patch; SHM, somatic hypermutation.

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and 3) gene conversion and/or SHM are not involved in diversification of Ab repertoire because the original number of analyzed genes was underestimated (28) and other gene conversion-like products could be ascribed to the PCR artifacts (29).

To clarify whether resection of IPP leads to B cell immunodeficiency and whether B cell development in IPP is Ag-independent (points 1 and 2 above), we have used germ-free pigs that were or were not colonized by intestinal bacteria and/or in which IPP were removed by total resection very early after birth. Germ-free animals were chosen because the fetal pattern of Ag-independent development in IPP can be prolonged in these animals or can be easily stopped and reverted to Ag-dependent development after experimental colonization and production of gnotobiotic pigs (3, 30, 31). Sheep studies performed under conventional conditions could not effectively discriminate the influence of microbial colonization on the development of B cells in IPP.

In this study we used flow cytometry analysis of lymphocytes in porcine IPP. Swine lymphocytes can be subdivided into four basic populations of B cells according to expression of CD2 and CD21 (31), three populations of αβ T cells according to expression of CD4 and CD8 (32), and three populations of γδ T cells according to expression of CD2 and CD8 (32). Ontogenetic and functional studies showed that $CD2^+CD21^+$ B cells are mostly composed of naive cells, $CD2^-CD21^+$ represent primed and/or activated stages, whereas $CD2^+CD21^-$ and $CD2^-CD21^-$ are Ab-forming and/or memory B cells (31). Swine αβ T lymphocytes contain, except classical $CD4^+$ T helper and $CD8^+$ cytotoxic cells, also effector $CD4^+CD8^+$ T helper cells that express the CD8α molecule as a consequence of previous activation (33). Porcine γδ T cells have phenotype-dependent tissue distribution so that $CD2^+CD8^+$ and $CD2^+CD8^-$ are enriched in lymphoid tissues, whereas $CD2^-CD8^-$ preferentially accumulate in the blood (34). It has been shown that $CD2^+CD8^-$ γδ T cells are infrequent in the periphery during fetal life and may represent an experienced subpopulation (34).

The results in this study disprove the concept that porcine IPP are a significant source of B cells, are required for maintenance of the systemic B cell pool, and/or are a site of B cell lymphogenesis in swine because 1) removal of IPP does not lead to a drop in B cell numbers in blood, 2) resection does not lead to any changes in frequencies of B and T cell subpopulations and their subsets in other lymphoid organs, and 3) B cell lineage populations in IPP do not resemble developing B cell lineage cells in bone marrow and have a phenotype similar to secondary lymphoid organs. Additionally, we show that porcine IPP are an important but nonessential mucosal lymphoid tissue for early immune response against colonization and food Ags, as we have suggested earlier (35), because 1) the fetal type of lymphocyte distribution can be prolonged under germ-free conditions and IPP do not develop an adult type of lymphocyte distribution without external antigenic stimulation; 2) resection of IPP does not lead to an immunodeficiency or even changes in frequencies of B and T cells and their subpopulations, which indicates that IPP are replaceable tissue; and 3) after colonization, IPP contain a high proportion of IgM^-IgA^+ switched B cells and effector stages of lymphocytes, including $CD2^-CD21^-$ B cells, $CD4^+CD8^+$ αβ T helper cells, and $CD2^+CD8^-$ γδ T cells.

Materials and Methods

Experimental animals and surgical procedures

Animals used in this study were as follows: 1) Large White/Landrace crossbred gilts obtained at South Dakota State University, and 2) conventional Minnesota miniature/Vietnamese–Asian–Malaysian crossbred piglets bred in Nový Hrádek (34, 36). Germ-free piglets were recovered from gilts by cesarian section at day 112 of gestation in the manner previously described (37). In this study, all ages of animals are stated as days or weeks after birth, which means the day of recovery. After birth, piglets were kept in isolator units under germ-free conditions at all times and were maintained on a diet of Esbilac (PetAg, Hamilton, IA), which was adjusted daily to meet their daily nutrient requirements and to maintain adequate caloric intake. Piglets designed for surgery treatment were operated ~48 h after birth (total of 16 animals) as described elsewhere (38, 39), whereas other piglets were left untreated (total of 11 animals). Surgical treatment involved 1) a group of piglets with surgically removed IPP (total of eight animals), 2) a group of piglets with surgically constructed isolated ileal loop and simultaneously anastomosed (rejoined) the rest of ileum (total of three animals), or 3) group of sham operated piglets with a transected and thereafter anastomosed lower ileum (total of five animals). Five days after birth, two animals from surgically untreated group were left germ-free (germ-free controls), two animals from surgically untreated group were colonized with benign *Eschericia coli* strain G58-1 (35), and the remaining animals were colonized with a defined commensal gut flora (provided by Dr. Roger Harvey, U.S. Department of Agriculture/Agricultural Research Service, Southern Plains Research Center, College Station, TX) (40). All animals were thereafter maintained in isolator units on the same diet for an additional 4–10 wk and monitored for the unwanted appearance of pathogenic bacteria. At regular intervals, blood samples were recovered and processed, and various lymphoid organs were processed at time of necropsy (see below). All animal experiments and surgical protocols/procedures were approved by the Institutional Animal Care and Use Committee of South Dakota State University and by the Ethical Committee of the Institute of Microbiology, v.v.i., Academy of Sciences of the Czech Republic, according to guidelines in the Animal Protection Act.

Preparation of cell suspensions

Cell suspensions were prepared essentially as previously described (34, 36, 41). Briefly, heparinized (20 U/ml) blood was obtained by intracardial puncture and erythrocytes were removed using hypotonic lysis. Cell suspensions from mesenteric lymph nodes (MLN) were prepared in cold PBS by carefully teasing apart the tissues using a forceps and then by passage through a 70-μm mesh nylon membrane. Cells from IPP, JPP, and isolated loops were prepared by cutting out patch regions that were further cut into pieces and incubated in digestion media (RPMI 1640, 100 U/ml collagenase type IV [Sigma-Aldrich, St. Louis, MO], 2% FCS) at 37°C for 1 h. Supernatants from incubation were filtered through a 70-μm mesh nylon membrane, washed three times with PBS, and lymphocytes were separated with a 40–80% Percoll gradient (GE Healthcare, Uppsala, Sweden) at 600 × *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 70-μm mesh nylon membranes, and cell numbers were determined by a hemacytometer.

Immunoreagents

The following mouse anti-pig mAbs, whose source and specificity were described earlier (34, 36, 41, 42), were used as primary immunoreagents: anti-CD1 (76-7-4, IgG2a), anti-CD2 (MSA4, IgG2a or 1038H-5-37, IgM), anti-CD3ε (PPT3, IgG1 or PPT6, IgG2b), anti-TCRγδ (PPT17, IgG1 or PPT16, IgG2b), anti-CD4 (10.2H2, IgG2b), anti-CD8 (76-2-11, IgG2a), anti-CD11b (MIL-4, IgG1), anti-CD21 (IAH-CC51, IgG2b), anti-CD25 (K231-3B2, IgG1), anti-CD45RC (MIL5, IgG1), anti-CD172a or anti-SWC3 (74-22-15A, IgG2b), anti-SWC7 (IAH-CC55, IgG1 or 2F6/8, IgG2a), anti-μHC (M160, IgG1), anti-IgA (1456, IgG2a), and anti-MHC class II (MHC-II; MSA3, IgG2a or 1038H-12-34, IgM). In some cases, mAbs were also labeled with NHS-LC-biotin (Pierce, Rockford, IL) according to a protocol recommended by the manufacturer. Due to the lack of anti-porcine TCRαβ-specific Ab, αβ T cells in this work were detected as $CD3\varepsilon^+TCR\gamma\delta^-$ cells (36, 42).

Goat polyclonal Abs specific for mouse Ig subclasses labeled with FITC, PE, PE/Cy7, or allophycocyanin were used as secondary immunoreagents (SouthernBiotech, Birmingham, AL). Biotinylated primary Abs were detected by a streptavidin-PE/Cy7 tandem conjugate (SouthernBiotech).

All immunoreagents were titrated for optimal signal/noise ratios and isotype-matched mouse anti-rat mAbs were used as negative controls. No background staining was observed during any experiments.

Staining of cells

Staining of cells for flow cytometry analysis was performed as described previously (34, 36, 41, 42) by indirect subisotype staining. Briefly, multicolor 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 subisotypes. Cells were incubated for 30 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary polyclonal Abs specific for mouse Ig subclasses that had been labeled with FITC, PE, PE/Cy7, and allophycocyanin conjugate were then added to the cell pellets in appropriate combinations. After 30 min, cells were washed three times in PBS-GEL and analyzed by flow cytometry. In the case of subisotype-matched mAbs, staining involved cells stained with mAbs of different subisotypes that were detected by secondary polyclonal Abs labeled with FITC, PE, and allophycocyanin conjugate in appropriate combinations. These cells were later incubated for 10 min with PBS-GEL containing 10% heat-inactivated normal mouse serum to block the free binding sites of the previously bound secondary polyclonal Abs. After washing in PBS-GEL, the cells were incubated for 30 min with a biotinylated subisotype-matched primary mAb for 30 min and subsequently washed twice. Finally, streptavidin-PE/Cy7 was added for 30 min and the cells were then washed three times in PBS-GEL prior to flow cytometry analysis.

The DNA content of multicolor-stained cells was determined using the DNA intercalating probe 7-aminoactinomycin D (7-AAD). Surface-stained cells were washed in cold PBS containing 0.1% sodium azide, centrifuged and fixed with cold (−20°C) 70% ethanol for 1 h at 4°C, centrifuged again (2000 × *g*, 10 min, 4°C), and washed in cold PBS containing 0.1% sodium azide. The pellets were then incubated with 50 μl 7-AAD in cold PBS containing 0.1% sodium azide (40 μg/ml) for 20 min at 4°C in dark until measured using flow cytometry.

Flow cytometry

Samples were measured on a standard FACSCalibur or FACSAriaIII flow cytometer (BD Immunocytometry Systems, Mountain View, CA) and 300,000–700,000 events were collected in each measurement. Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. A doublet discrimination module was used in DNA content analysis that allowed single-cell events to be discriminated from doublets and higher multiplets. The PC-Lysis or FACSDiva software (BD Immunocytometry Systems) was used for data processing. Lymphocyte gate for analysis was set according to light scatter characteristics (forward versus side scatter). Numbers for lymphocyte population (B, αβ T, γδ T, and NK cells) were recalculated to the sum of μHC^+, $CD3^+$, and $CD3^-CD8^+$ NK cells that represented 100% because there were various amounts of debris in the lymphocyte gate. Numbers for subpopulations were not recalculated and represent percentage from a particular lymphocyte population that was 100%. All cell numbers in this report are stated as relative numbers (proportions).

Statistical analysis

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. The strength of association between individual experimental groups was measured using Pearson product moment correlation, and the level of statistical significance (*p* value) for particular correlation coefficients is reported.

Results

IPP in germ-free animals resemble fetal type lymphocyte distribution

Different lymphoid organs of 36-d-old germ-free piglets were analyzed for the proportion of B, αβ T, γδ T, and NK lymphocytes and their subpopulations (Fig. 1). The results show that IPP in germ-free animals resemble more the fetal type of lymphocyte distribution: they are comparable with JPP and have most lymphocytes being B and T cells (Fig. 1*A*). Comparison with other lymphoid tissues shows that IPP and JPP contain considerable amounts of γδ T cells and that IPP are almost devoid of NK cells (Fig. 1*A*). B cell subpopulations subdivided according to CD2 and CD21 expression do not show any significant differences between individual tissues (Fig. 1*B*). The vast majority of B lymphocytes in germ-free animals, independent of tissue, are composed of $CD2^+$ $CD21^+$ cells that were shown to be naive mature B cells (31). Analyses of αβ T lymphocyte subsets show that IPP and JPP contain a significantly higher proportion of cytotoxic T cells and

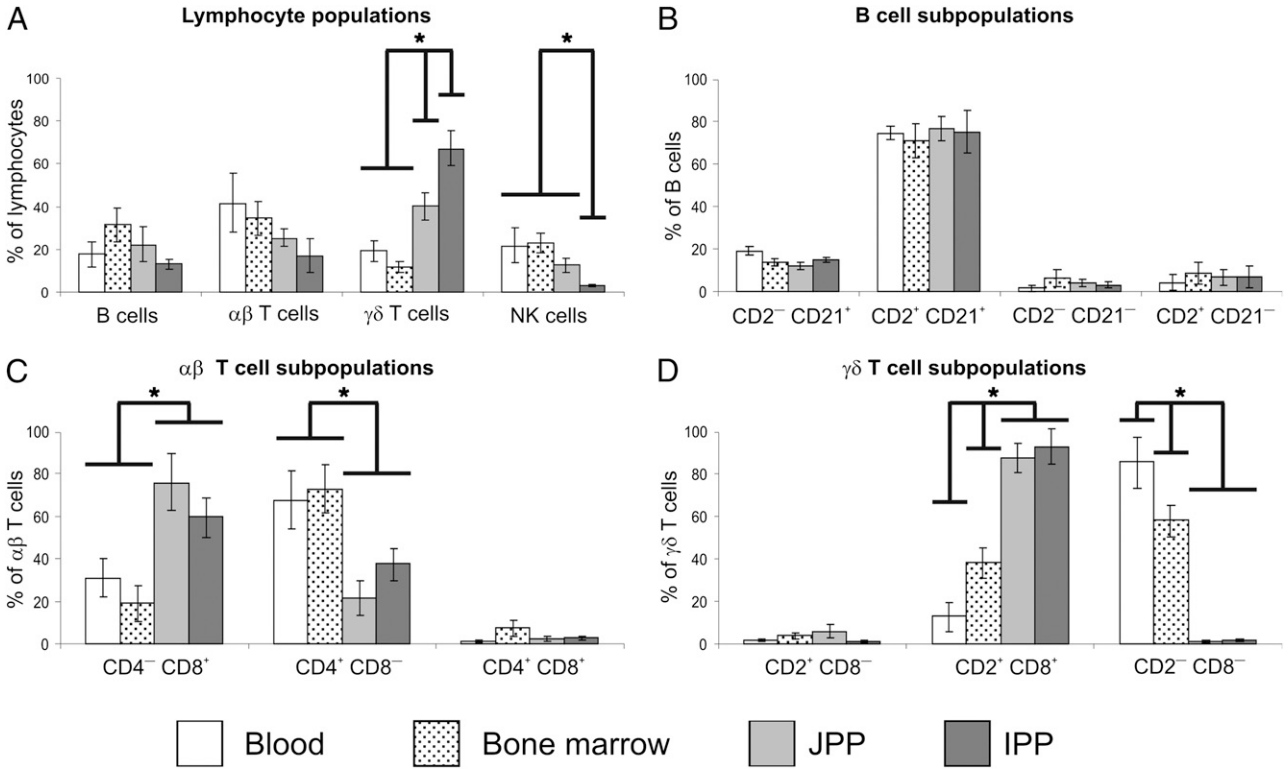

FIGURE 1. The frequencies of lymphocyte types and their subpopulations in different organs of 36-d-old germ-free piglets. Lymphocytes were isolated from blood (open bars), bone marrow (dotted bars), JPP (grey bars), and IPP (black bars) and analyzed for the proportions of B, αβ T, γδ T, and NK cells (*A*). CD2/CD21 subpopulations of B lymphocytes (*B*), CD4/CD8 subpopulations of αβ T lymphocytes (*C*), and CD2/CD8 subpopulations of γδ T lymphocytes (*D*) are also shown. Error bars represent ± SEM. $*p < 0.01$ between tissues. Data are based on analysis of at least four animals from each group.

lower amounts of T helper cells than can be found in blood and bone marrow (Fig. 1*C*). In all organs analyzed, the proportion of effector $CD4^+CD8^+$ T helper cells (33, 34) is negligible (Fig. 1*C*). Lymphocytes of the γδ T cell lineage in IPP and JPP of germ-free animals are almost exclusively $CD2^+CD8^+$ (Fig. 1*D*). The proportion of these cells is significantly lower in the blood and bone marrow where $CD2^-CD8^-$ γδ T cells dominate. The subpopulation of $CD2^+CD8^-$ γδ T cells is negligible in all analyzed organs (Fig. 1*D*).

Colonization results in significant changes in the proportion and phenotype of lymphocytes in the gut

Different lymphoid organs of 36-d-old germ-free and gnotobiotic piglets colonized by only *E. coli* strain G58-1, or by the defined

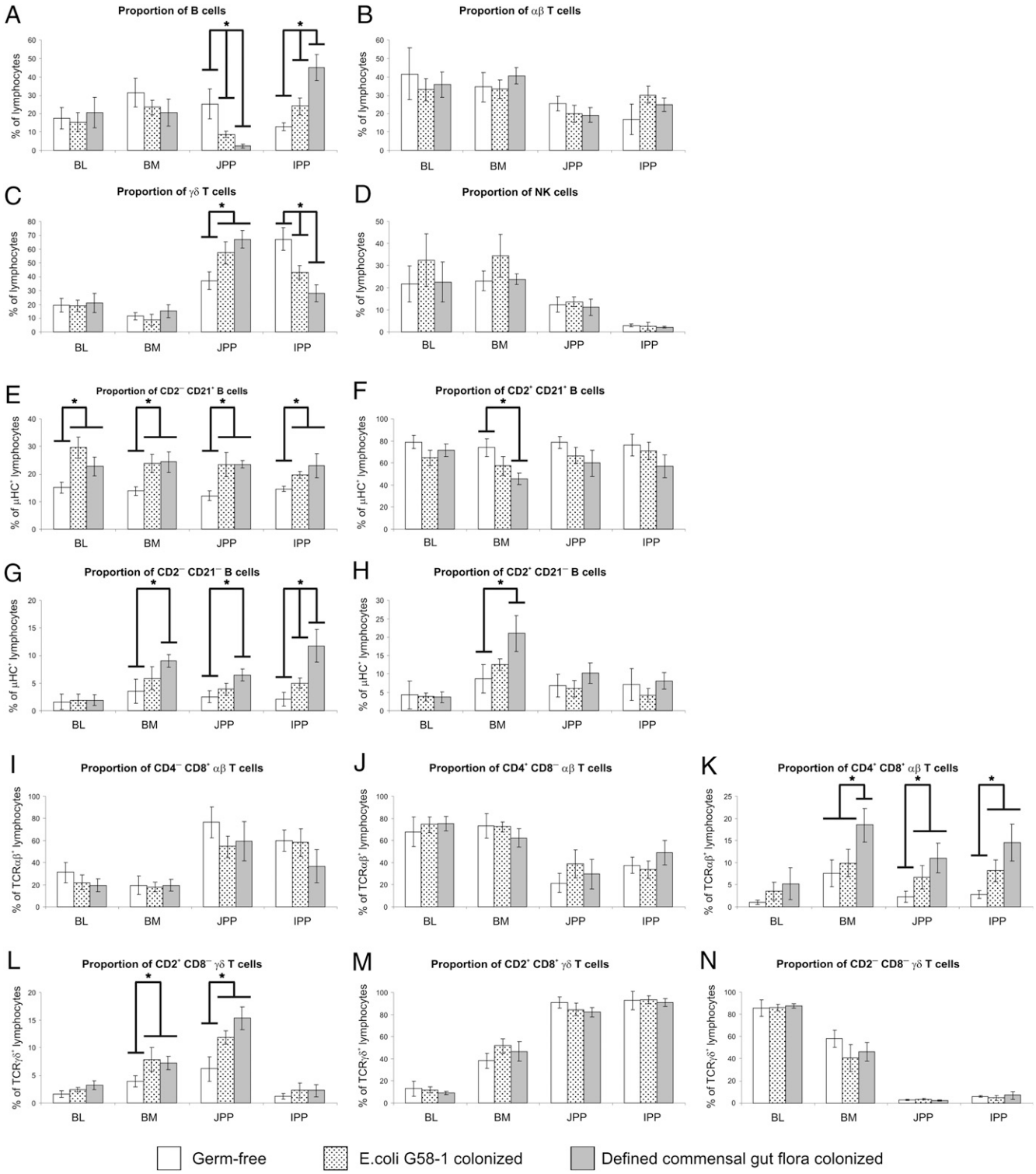

FIGURE 2. The frequencies of lymphocyte types (*A–D*) and their subpopulations (*E–N*) in different organs of 36-d-old germ-free piglets (open bars) and gnotobiotic piglets colonized only by *E. coli* strain G58-1 (dotted bars) or by the defined commensal gut flora (40) (grey bars) at day 5 after birth. Lymphocytes were isolated from blood, bone marrow, JPP, and IPP and analyzed for proportions of B, αβ T, γδ T, and NK cells and their subpopulations. Error bars represent ± SEM. $*p < 0.01$ between experimental groups. Data are based on analysis of at least four animals from each group. BL, blood; BM, bone marrow.

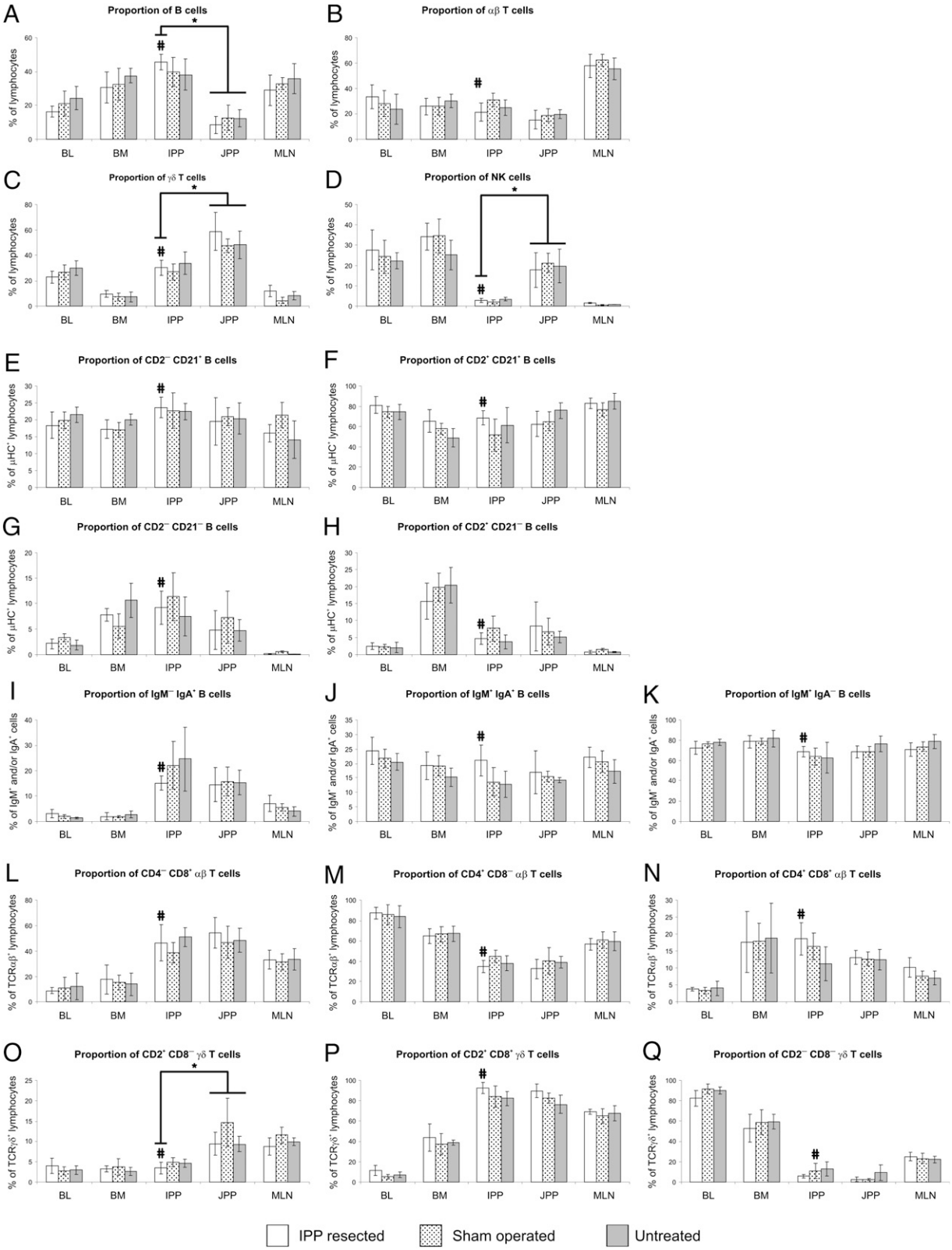

FIGURE 3. The frequencies of lymphocyte types (*A–D*) and their subpopulations (*E–Q*) in different organs of 44-d-old piglets in which IPP were removed by resection (open bars) or were sham operated (dotted bars) at day 2 after birth. Piglets that were not surgically treated are also shown (grey bars). All piglets were subsequently colonized by the defined commensal gut flora (40) at day 5 after birth. Lymphocytes were isolated from blood, bone marrow, IPP, JPP, and MLN and analyzed for proportions of B, αβ T, γδ T, and NK cells and their subpopulations. Error bars represent ± SEM. Individual bars represent average values obtained from five to seven animals done in three independent experiments. Note that there were no significant differences between experimental groups. IPP-resected animals (open bars) lost the lower ileum with IPP during surgical removal and this was replaced by upper ileum during anastomosis. Analysis of relocated upper ileum is represented as IPP and is indicated by # above individual bars. *$p < 0.01$ between relocated upper ileum and JPP. BL, blood; BM, bone marrow.

commensal gut flora at day 5 after birth, were also analyzed for the proportion of B, αβ T, γδ T, and NK lymphocytes and their subpopulations (Fig. 2). The results show that the proportion of B cells does not change in the blood and bone marrow, but it decreases in JPP and conversely increases in IPP after colonization with *E. coli* strain G58-1 and even more after colonization with the defined commensal flora (Fig. 2*A*). These changes are compensated by reverse changes in the proportion of γδ T cells (Fig. 2*C*), whereas frequencies of αβ T cells (Fig. 2*B*) and NK cells (Fig. 2*D*) remain stable. Analysis of B cell subpopulations show that in all analyzed organs, the proportions of $CD2^-CD21^+$ B cells significantly increased with colonization (Fig. 2*E*). This B cell subset was shown to represent activated B cells (31, 43). Colonization effects are most manifested in the bone marrow where the frequency of $CD2^-CD21^-$ (Fig. 2*G*) and $CD2^+CD21^-$ (Fig. 2*H*) subsets also increase by colonization, which is to the detriment of $CD2^+CD21^+$ B cells (Fig. 2*F*). Moreover, an increase in the frequency of the $CD2^-CD21^-$ B cell subpopulation is also significant in JPP and IPP (Fig. 2*G*). B cells lacking the CD21 molecule ($CD2^+CD21^-$ and $CD2^-CD21^-$) were shown to represent effector/memory B cells (31). Colonization of the gut also affects the number of T cells in subpopulations so that there is an apparent increase in number of $CD4^+CD8^+$ αβ T cells in the bone marrow, JPP and IPP (Fig. 2*K*), and $CD2^+CD8^-$ γδ T cells in the bone marrow and JPP (Fig. 2*L*). These subpopulations of T cells were also shown to be effector/memory (31, 33) and are practically absent before birth (34) and in germ-free animals. The proportions of other subpopulations of αβ T cells (Fig. 2*I*, 2*J*) as well as γδ T cells (Fig. 2*M*, 2*N*) do not change with colonization.

Resection of IPP does not lead to changes in the levels of T and B cells and their subpopulations

Germ-free pigs in which IPP 1) were removed by resection, 2) were not surgically treated, or 3) were sham operated at day 2 after birth, and all groups were subsequently colonized by the defined commensal gut flora at day 5 after birth were analyzed for proportions of lymphoid cells in different tissues by flow cytometry at day 44 after birth (Fig. 3). The results demonstrate that the resection of IPP does not cause any significant changes in the proportion of B and T cells and their subpopulations in comparison with sham-operated or untreated animals in any studied tissue (Fig. 3). Analysis of MHC-II and CD25 activation molecules on the surface of either αβ or γδ T cells also did not reveal any differences (data not shown). In accordance with colonization experiments described above, the results proved that IPP generally contains more B cells (Fig. 3*A*) and less γδ T cells (Fig. 3*C*) in comparison with JPP and that there are almost no NK cells in IPP, similar to MLN (Fig. 3*D*). There are accumulations of $CD2^-CD21^-$ B cells in the IPP, JPP, and bone marrow (Fig. 3*G*), and also $CD2^+CD21^-$ B cells in the bone marrow (Fig. 3*H*). Analysis of IgA/IgM subpopulations of B cells disclosed a high proportion of IgM^-IgA^+ switched B cells in IPP and JPP (Fig. 3*I*). IPP also contains cytotoxic αβ T cells (Fig. 3*L*) and both subsets of helper αβ T cells, that is, $CD4^+CD8^-$ (Fig. 3*M*) and $CD4^+CD8^+$ (Fig. 3*N*). IPP and JPP are also enriched for $CD2^+CD8^+$ γδ T cells (Fig. 3*P*), and JPP alone also is enriched for $CD2^+CD8^-$ γδ T cells (Fig. 3*O*) whereas $CD2^-CD8^-$ γδ T cells are infrequent in both tissues (Fig. 3*Q*).

Transposition of JPP into terminal ileum leads to a change in lymphocyte distribution in relocated JPP to resemble IPP

Resection of IPP included surgical removal of ~60 cm lower ileum. During anastomosis, the lower part of the jejunum was artificially connected to the place where IPP originally occur, thus replacing the original lower ileum. Our expectation was that the relocated part of the lower jejunum would more closely resemble the JPP from untreated animals. However, our analysis shows that the relocated lower jejunum in IPP resected animals (Fig. 3, bars marked #) significantly differs from JPP and more closely resembles the IPP in numbers of B cells (Fig. 3*A*), γδ T cells (Fig. 3*C*), NK cells (Fig. 3*D*), and $CD2^+CD8^-$ γδ T cells (Fig. 3*O*). Such results are in agreement with differences in lymphocyte distribution between IPP and JPP for colonized germ-free animals (compare Fig. 3*A* with Fig. 2*A* for B cells, Fig. 3*C* with Fig. 2*C* for γδ T cells, Fig. 3*D* with Fig. 2*D* for NK cells, and Fig. 3*O* with Fig. 2*L* for γδ T cells).

The proportion of B lymphocytes in circulation is stable during 10 wk after IPP resection

Germ-free pigs in which IPP 1) were removed by resection, 2) were not surgically treated, or 3) were sham operated at day 2 after birth, and all groups were subsequently colonized by the defined commensal gut flora at day 5 after birth were analyzed for the proportion of B lymphocytes in the blood by flow cytometry. Periodically sampled blood at 2, 3, 4, 5, 6, 7, and 10 wk does not show any significant changes in the proportion of μHC^+ cells between experimental groups (Fig. 4).

IPP that do not have access to colonizing bacteria or food Ags have the same lymphocyte composition in colonized animals as do IPP that have such access

Results of this work demonstrate that IPP development is Ag-dependent because no IPP development occurs in germ-free animals in comparison with colonized ones (Fig. 2). To investigate whether development of IPP occurs in colonized animals also by indirect contact with gut-associated Ags we surgically constructed isolated ileal loops containing the IPP in the same animals with a rejoined ileum that also contained IPP. Construction of the isolated ileal loops was performed in germ-free piglets at day 2 after birth by separating 20 cm distal ileum, which was then rejoined with an end-to-end anastomosis (44). Because the loops

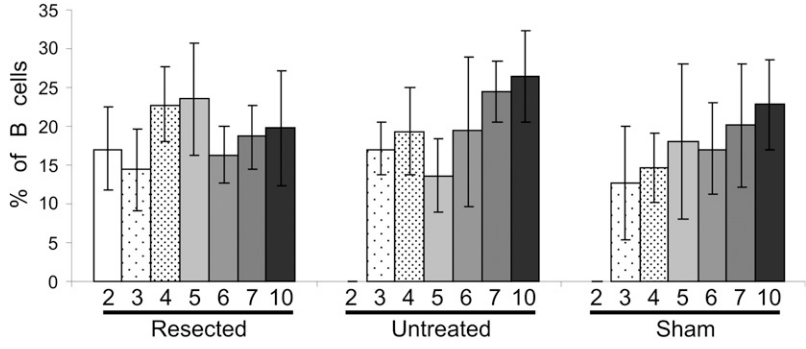

FIGURE 4. The frequencies of B lymphocytes in the blood of IPP-resected, untreated, and sham-operated pigs that were colonized by the defined commensal gut flora (40) at 5 d after birth. Animals were monitored at age 2, 3, 4, 5, 6, 7, and 10 wk (*x*-axis). Numbers of B cells were detected as a proportion of μHC^+ cells among all lymphocytes (100%). Error bars represent ± SEM from at least five animals used in four independent experiments. Note that blood at 2 wk for untreated and sham-operated animals was not sampled and values are therefore not shown.

were constructed in germ-free animals that were only colonized after surgery (at day 5 after birth by the defined commensal gut flora), the loops were not directly exposed to either colonizing bacteria or food Ags whereas the rejoined ileum of the same piglet was exposed to both. The results were compared with animals that were colonized but not surgically treated and also with germ-free animals. Flow cytometry analyses of lymphoid cells at day 36 after birth in all described animals are shown in Fig. 5. The results demonstrate that the distribution of lymphocyte subsets is comparable in IPP isolated from surgically untreated animals, IPP isolated from anastomosed ileum that had access to bacteria, and also IPP isolated from ileal loops that were not exposed to external Ags (Fig. 5). All IPP isolated from different sources resemble the adult type of lymphocyte distribution after colonization, having more B cells (Fig. 5*A*) and less γδ T cells (Fig. 5*C*). This is in sharp contrast to germ-free animals that resemble the fetal type of lymhocyte distribution (Fig. 5, grey bars).

B cell lineage subpopulations in IPP do not resemble bone marrow and have a phenotype similar to secondary lymphoid organs

Some reports (31, 43, 45–48) and our recent unpublished observations indicate that the development of porcine B cell lineage cells can be monitored by stable MHC-II expression, decreasing expression of CD172a (SWC3), and increasing expression of CD2, CD25, and CD45RC. To characterize whether cells in IPP have the same or a different phenotype from bone marrow B cell lineage cells, we analyzed these tissues together with blood in 36-d-old germ-free animals and also in animals of the same age in which surgically constructed isolated ileal loops were made (Fig. 6). The results show that whereas putative precursors of B cells with an MHC-II$^+$CD172a$^+$CD45RC$^-$ and MHC-II$^+$CD172a$^+$CD25$^-$ phenotype can be easily found in bone marrow (Fig. 6), none of these cells can be detected in blood, in IPP from untreated animals, or in IPP in isolated ileal loops that have no direct access to colonizing bacteria. The phenotype of MHC-II$^+$ cells in blood or in IPP is comparable, being MHC-II$^+$CD172a$^-$CD25$^+$CD45RC$^+$ (Fig. 6), which is characteristic of mature B cells.

IPP have high mitotic and apoptotic rates

Comparison of actual mitotic activity of lymphocytes (S+M/G_2 region in Fig. 7) in different lymphoid tissue of conventional piglets shows insignificant proliferative activity of lymphocytes in the blood (Fig. 7*A*) and MLN (Fig. 7*B*), whereas the spleen (Fig. 7*C*), bone marrow (Fig. 7*D*), IPP (Fig. 7*E*), and thymus (Fig. 7*F*) contain considerable amounts of cycling cells. The most active organ in proliferation seems to be the bone marrow, with more than a third of cells cycling (Fig. 7*D*), whereas the spleen (Fig. 7*C*) and IPP (Fig. 7*E*) are comparable with a fourth of cells cycling. Thymus is less active, with approximately a fifth of cells cycling (Fig. 7*F*). When individual organs were inspected for the number of proliferating B cells (Fig. 7, *right column*), the most active organs was the IPP (Fig. 7*K*), followed by the bone marrow (Fig. 7*J*) and spleen (Fig. 7*I*). Proliferation of B cells in the MLN

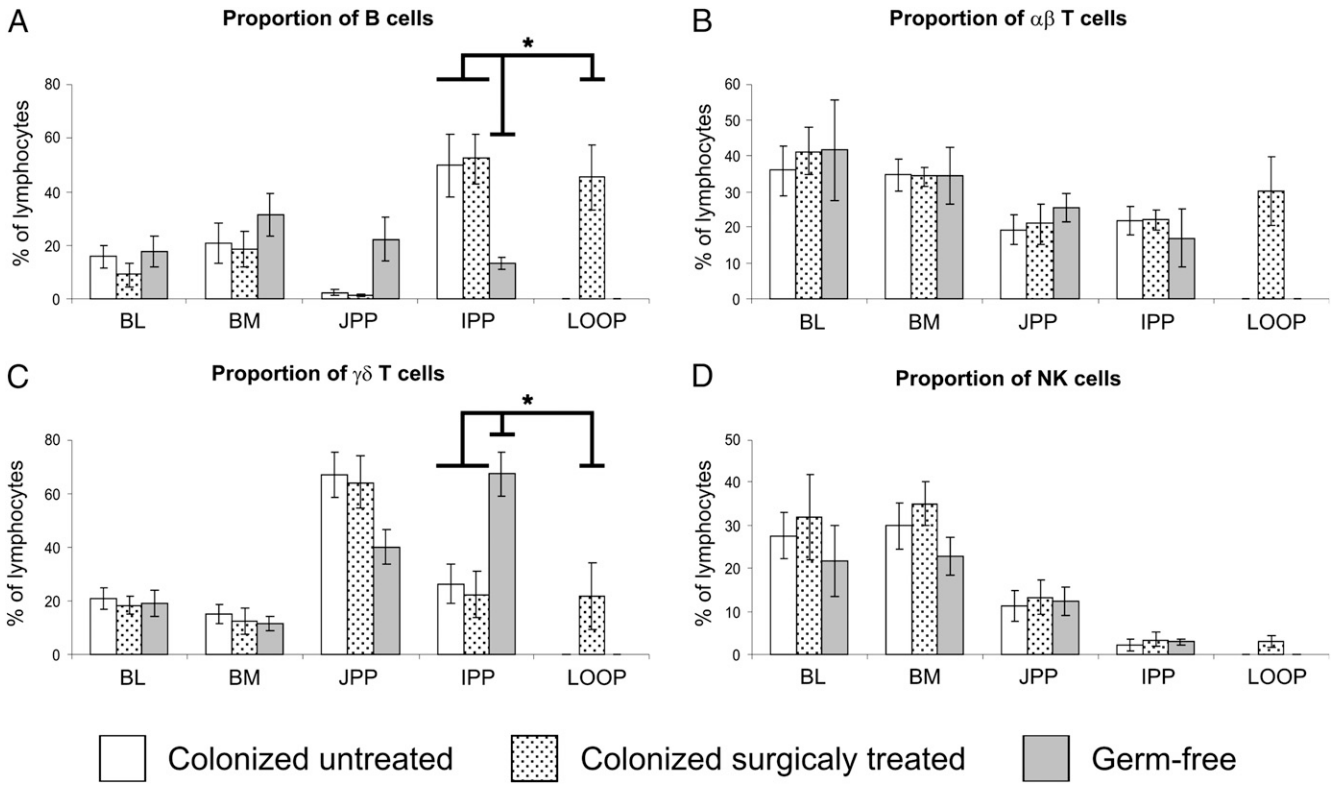

FIGURE 5. Frequencies of lymphocyte types (*A–D*) in different organs of the untreated piglets (open bars) and piglets in which isolated ileal loops were surgically constructed (dotted bars). Both groups were thereafter colonized by the defined commensal gut flora (40) at 5 d after birth. Analysis of germ-free piglets is also shown for comparison (grey bars). Lymphocyte types isolated from the blood, bone marrow, JPP, IPP, and isolated ileal loops were analyzed 36 d after birth. Error bars represent ± SEM. Individual bars represent average values obtained from at least 3 animals. IPP in individual graphs indicates original IPP of lower ileum in untreated and germ-free animals (open and grey bars, respectively); anastomosed IPP of middle ileum in surgically treated animals is depicted by dotted bars. There are no isolated loops in untreated and germ-free animals and values are therefore not shown (LOOP, open, and grey bars). There were no significant differences between IPP isolated from colonized untreated animals (IPP, open bars), anastomosed IPP (IPP, dotted bars), and IPP in isolated ileal loops (LOOP, dotted bars). *$p < 0.01$ in comparison with germ-free animals. BL, blood; BM, bone marrow; LOOP, isolated ileal loops.

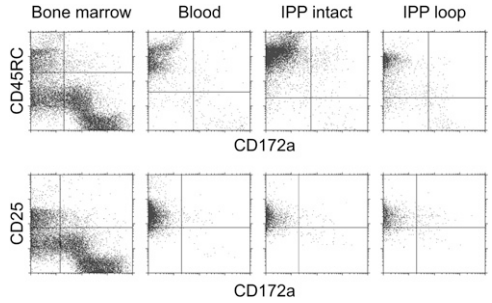

FIGURE 6. Flow cytometry analysis of CD172a, CD45RC, and CD25 expression on MHC-II$^+$ cells. Lymphocytes were prepared from bone marrow (*first column*), blood (*second column*), and IPP (*third column*) of 36-d-old germ-free piglets that were colonized by the defined commensal gut flora (40) at day 7 after birth. Lymphocytes prepared from isolated ileal loops of animals of the same age and colonization are also shown (*fourth column*). Only MHC-II$^+$ cells were analyzed and are shown. Although bone marrow contains putative precursors of B cells with MHC-II$^+$ CD172a$^+$CD45RC$^-$ (*first row*) and MHC-II$^+$CD172a$^+$CD25$^-$ (*second row*) phenotype, none of these cells can be detected in blood, intact IPP, or IPP loops. The results are representative of three independent experiments.

(Fig. 7*H*), blood (Fig. 7*G*), and thymus (Fig. 7*L*) was negligible. Note that the proportion of μHC$^+$ B cells in the bone marrow (Fig. 7*J*) and thymus (Fig. 7*L*) is very low because sIgM$^+$ B cells are rapidly exported out of bone marrow, and the thymus is a site of T lymphogenesis where B cells are scattered (31). For this reason, most B cells that are able to proliferate are concentrated in IPP and spleen.

Actual apoptotic activity (sub-G region in Fig. 7) cannot be detected in freshly isolated cells from the blood (Fig. 7*A*), MLN (Fig. 7*B*), bone marrow (Fig. 7*D*), and thymus (Fig. 7*F*). However, apoptotic cells can be detected in the spleen (Fig. 7*C*) and also in IPP (Fig. 7*E*), and the extent of apoptosis is comparable. When these organs were inspected for the number of apoptotic B cells (Fig. 7, *right column*), these were more frequent in IPP than spleen (compare Fig. 7*K* with Fig. 7*I*). Taken together with proliferation results, these findings shows that the IPP and spleen have higher apoptotic and mitotic rates of B cells than found in other lymphoid tissues.

Physiological colonization in conventional animals causes pronounced prevalence of B cells in IPP

IPP isolated from 45-d-old germ-free and conventional piglets of the same age were analyzed for the proportion of B, αβ T, γδ T, and NK lymphocytes and their subpopulations (Fig. 8). The comparison shows a remarkable difference between experimental groups. In contrast to germ-free animals, the most frequent populations of lymphocytes in conventional animals are B cells, which compose ~90% of all lymphocytes (Fig. 8*A*). The prevalent phenotype of these B cells is CD2$^-$CD21$^+$ (Fig. 8*B*), whereas the prevalent B cell subpopulation in germ-free animals is CD2$^+$ CD21$^+$ B cells (Fig. 8*B*). Conventional animals also have a higher proportion of CD2$^-$CD21$^-$ B cells, which are virtually missing in germ-free animals (Fig. 8*B*). A high proportion of B lymphocytes in conventional animals is at the expense of γδ T cells (Fig. 8*A*). Although γδ T cells are very rare in conventional animals (Fig. 8*A*), analysis of their subpopulation (Fig. 8*D*) showed that conventional animals contain more CD2$^+$CD8$^-$ and CD2$^-$CD8$^-$ and less CD2$^+$CD8$^+$ γδ T lymphocytes than do germ-free pigs. The proportion of αβ T cells is comparable in germ-free and conventional piglets (Fig. 8*A*), and comparable also are frequencies of their CD8$^+$ cytotoxic and CD4$^+$CD8$^-$ helper subpopulations (Fig. 8*C*). However, effector CD4$^+$CD8$^+$ αβ T helper cells have significantly higher representation in conventional than in germ-free animals (Fig. 8*C*). Because both naive CD4$^+$CD8$^-$ and effector

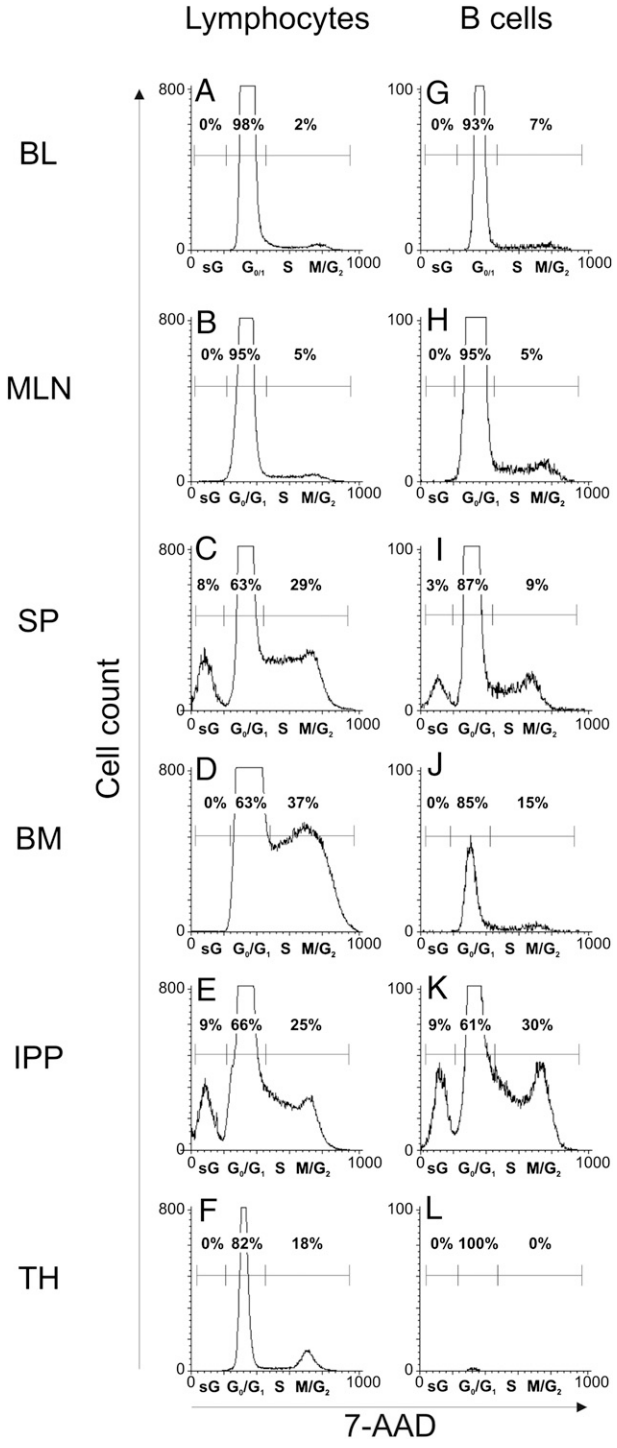

FIGURE 7. Flow cytometry analysis of cell proliferation in different organs. Lymphocytes isolated from the peripheral blood, MLN, spleen, bone marrow, IPP, and thymus of conventional animals were stained with anti-μHC mAb, fixed in 70% ethanol, and the DNA was visualized using 7-AAD. In each staining, all lymphocytes (*A–F*) and μHC$^+$ B cells (*G–L*) were gated and analyzed for DNA content (individual histograms). Positions of cells in apoptotic (sG), resting/gap 1 (G_0/G_1), synthesis (S), and mitosis/gap 2 (M/G_2) cell cycle phase according to 7-AAD fluorescence are indicated on the *x*-axis of each histogram, and percentages of cells are indicated above each histogram. The results are representative of four independent experiments. BL, peripheral blood; BM, bone marrow; SP, spleen; TH, thymus.

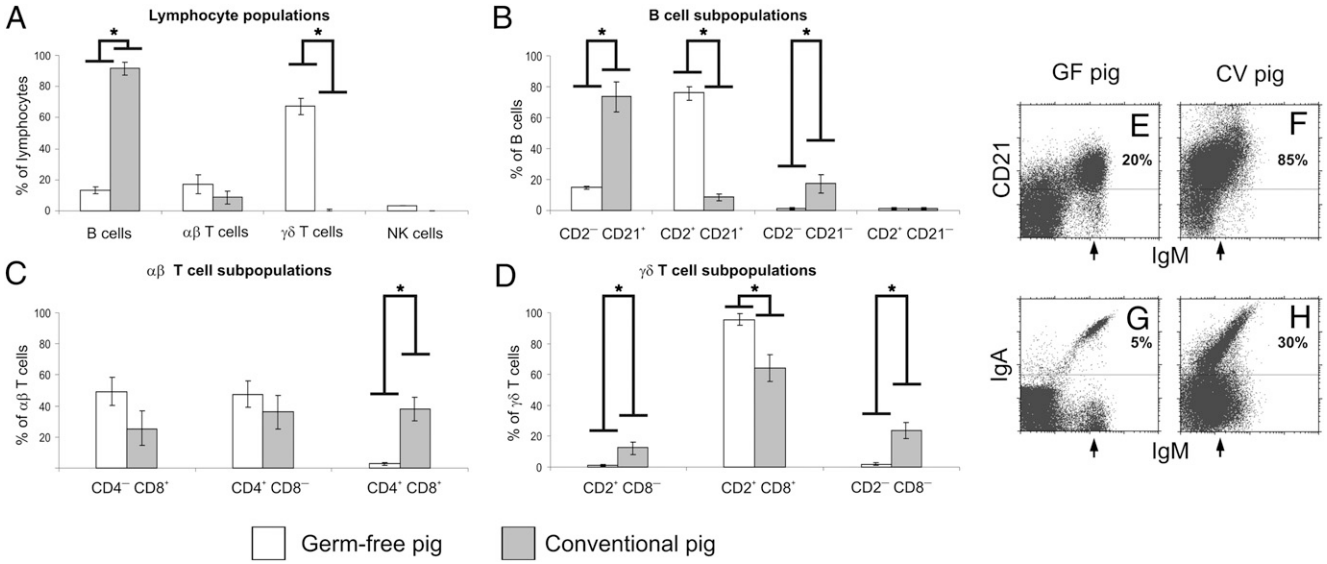

FIGURE 8. The frequencies of lymphocyte types and their subpopulation in IPP of 45-d-old germ-free (open bars) and conventional (grey bars) piglets. The proportions of B, αβ T, γδ T, and NK cells (*A*), CD2/CD21 subpopulations of B cells (*B*), CD4/CD8 subpopulations of αβ T cells (*C*), and CD2/CD8 subpopulations of γδ T lymphocytes (*D*) are shown. Error bars represent ± SEM. $*p < 0.01$ between experimental groups. Individual bars represent average values obtained from at least three animals. Representative analyses of CD21/IgM (*E*, *F*) and IgA/IgM (*G*, *H*) expression on all IPP lymphocytes from germ-free (*E*, *G*) and conventional (*F*, *H*) piglets are also shown. Arrows on *x*-axis of each dot plot indicate expression level of IgM on B cells in germ-free animals (*E–G*), which is significantly higher than in conventional piglets (*F–H*). Similar results were obtained in all analyzed animals.

$CD4^+CD8^+$ αβ T cells (Fig. 8*C*) represent T helper cells in swine, T helper cells in conventional animals are 2- to 4-fold more frequent than are αβ T cytotoxic cells ($CD4^-CD8^+$ cells in Fig. 8*C*). NK cells (Fig. 8*A*) and $CD2^+CD21^-$ B cells (Fig. 8*B*) are infrequent in both germ-free and conventional animals.

Note that the level of IgM expression in germ-free animals (Fig. 8*E*, 8*G*) is significantly higher than that found in conventional piglets (Fig. 8*F*, 8*H*). These results were generated independently of Ab concentrations used for staining and therefore are not related to the number of stained cells. Expression of IgM on B cells isolated from IPP of conventional animals was so low that it was often not distinguishable from negative cells (Fig. 8*F*, 8*H*). For this reason, staining of B cells involved also anti-CD21 (Fig. 8*F*) and anti-IgA (Fig. 8*H*) Abs to effectively discriminate B cells in IPP. In any case, staining for CD21 and IgA clearly demonstrated that B cells are more frequent in conventional animals (Fig. 8*F*) and many of them bear IgA on the surface (Fig. 8*H*) in comparison with germ-free piglets (Fig. 8*E* and 8*G*, respectively).

Discussion

Data reported in this study and in a companion article (39) do not support the existing paradigm, based on studies in sheep and lambs, that the IPP is primary lymphoid tissue. We found that the IPP 1) are not a significant source of B cells because their removal did not lead to an immunodeficiency or a change in frequency of B and T cells; 2) are not required for maintenance of the B cell pool because resection does not lead to a drop in B cell numbers in blood; and 3) are not a site of B cell lymphogenesis because B cell lineage populations in the IPP do not resemble developing B cell lineage cells in bone, and signal joint circles are absent. In particular, our data are discrepant with the work of Gerber et al. (17). These investigators found that resection of the sheep IPP resulted in a prolonged B cell deficiency, although this deficiency did not alter serum Ig levels (49). The latter is consistent with our findings (39). It is possible that this discrepancy could be methodological since evidence for B cell deficiency in lambs with resected IPP was based on manual counting of limited numbers of Ig^+ B cells using a slide smear method (17) that can generate a significant error. These studies conflict internally because it seems incongruent that B cell levels are significantly reduced but that serum Ig levels are unaffected.

Resection and transposition studies reported by others in pigs (50) are in agreement with our own studies. These studies showed that resection of the IPP or the transposition of IPP into the upper jejunum had no effect on the size of the patches or the composition of lymphocyte subsets in the JPP or on the number of B lymphocytes as measured in blood in the subsequent 2 mo. Surprisingly these authors did not challenge the IPP paradigm but retained it in their explanation that the IPP are a primary B cell organ (3, 50). Elements of our data are consistent with certain other observations made in sheep, calves, goats, deer, and swine. For example, the distribution of lymphocytes in the IPP and JPP of germ-free piglets is similar to that reported by Rothkoetter and Pabst (3) and resembles that found in fetal lambs (10). This fetal type of lymphocyte distribution is characterized by a higher proportion of T cells so that IPP and JPP are comparable. Careful inspection of cell phenotypes demonstrates that the T and B cell pools are naive, as evidenced by a vast prevalence of naive $CD2^+$ $CD21^+$ B cells and an absence of effector $CD4^+CD8^+$ αβ T helper cells (Fig. 1). We found that when germ-free piglets are colonized, the fetal type of lymphocyte distribution shifts to the adult type so that the proportion of B cells increases in IPP but decreases in JPP, similar to what is found in sheep (8, 10), calves (51), goats (52), deer (53), and swine (3). The switch from the fetal to the adult type of lymphocyte distribution in the IPP and JPP can be induced by monoassociation with *E. coli* strain G58-1 as well as with the defined commensal gut floral mixture (Fig. 2). We observed that the average proportion of B cells in IPP is ~10% in germ-free piglets, ~20% in *E. coli*-colonized piglets, ~50% in isolator piglets colonized by the defined commensal gut flora (Fig. 2), and >90% in conventional piglets (Fig. 8). The proportional increase in B cells in the IPP after different degrees of colonization clarify so far unexplained dissimilarities in different reports, which indicated variance in the frequency of T cells in porcine IPP ranging from 5 to 70% depending on colonization (3, 23, 54). It also

explains findings in sheep showing absolute prevalence of B cells (8, 10) because all experiments were done on conventional animals. The shift from the fetal to the adult type of lymphocyte distribution and its dependence on degree of colonization indicate that events in the IPP proceed in an Ag-dependent manner, as in secondary lymphoid tissues. This conclusion is supported by findings that the shift is also accompanied by an increase in Ab repertoire diversification (39). It is further supported by the observation that changes are associated with an increase in IgM^+ IgA^+ and IgM^-IgA^+ switched B cells (Figs. 3, 8) (35). There is also a high proportion of activated and effector stage lymphocytes such as $CD2^-CD21^+$ and $CD2^-CD21^-$ B cells and $CD4^+CD8^+$ αβ T cells (Fig. 2), which also characterize secondary lymphoid tissues (31, 33, 34).

Because the purpose of our study focused on the role of the IPP in B cell lymphogenesis or diversification, we studied the phenotype of B cells recovered from the IPP. Studies in sheep (10, 55) and pigs (23) indicated the prevalence of naive B cells in IPP of conventional animals, which were characterized as those with low expression of IgM together with expression of other markers such as BAQ44A in sheep or CD 172a (SWC3) in pigs. It was speculated that low expression of IgM is indicative of an immature stage of B cell development, so the findings were interpreted as evidence for B cell lymphogenesis in IPP. We do not think that these IgM^{low} B cells are involved in B cell lymphogenesis since signal joint circles are virtually absent (39). Instead, we think that they represent experienced B cells that are undergoing class switch recombination, based on the work of others (56). This contention is supported by the observation that they are activated (bear a $CD2^-$ $CD21^+$ phenotype) and many express high levels of IgA (Fig. 8). Previous investigators focused on these cells as primary B cells and did not test for the expression of IgA or other isotypes. Previous investigators also failed to compare the phenotype of these cells with and without the influence of extrinsic Ags. Although naive B cells may initially prevail ($CD2^+CD21^+$), colonization causes a significance increase in numbers of activated $CD2^-$ $CD21^+$ B cells.

Another aspect of B cell biology in the IPP is the high apoptotic and mitotic rate in swine (23) and in sheep (20–22). This finding was also interpreted as evidence of B lymphopoietic activity, since a similar high rate is expected in primary lymphoid organs such as the thymus and the bursa of Fabricius (21). We made a similar finding (Fig. 7) but suggest that it results from immigration of naive B cells, which are autoreactive and undergo positive selection as in any other secondary lymphoid tissues, including the spleen (57). This is because we found the mitotic rate in the IPP to be comparable with that in the spleen, but lower than in the bone marrow and higher than in the thymus. Unfortunately, initial studies by Reynolds (20, 21) did not study the bone marrow or spleen, and they only compared the thymus, which had lesser mitotic and apoptotic activity, corresponding to our results.

Resection experiments also disclose one interesting feature of gut relocation, because the region of the lower jejunum was artificially moved during surgery to the position of the ileum where IPP originally occurred. The results demonstrate that the relocated lower jejunum has a significantly different lymphocyte distribution than JPP and is, on the contrary, comparable to IPP in untreated animals (Fig. 3). This lymphocyte distribution includes a higher proportion of B cells, a lower proportion of γδ T cells, a negligible amount of NK cells, and a lower proportion of $CD2^+CD8^-$ γδ T cells. This indicates that composition of lymphoid cells in the gut is dependent on bacterial load since the ileum has been shown to be colonized by a much higher amount of bacteria than for the jejunum (58).

Moreover, experiments with isolated ileal loops also explain some aspects of sheep studies that used closed ileal loops to demonstrate Ag-independent development of IPP (18). This would correspond to our results showing comparable IPP development in loops and anastomosed ileum. However, the development of IPP is comparable only in colonized animals and is clearly different from IPP of germ-free animals of the same age, which indicate that IPP development is Ag-dependent. Unfortunately, sheep studies were done exclusively in conventional animals and could not demonstrate Ag dependency. We have two explanations for loop- and gut-associated IPP equivalency in colonized animals: 1) gut Ags/derived molecules can be relocated to loops and stimulate IPP development, and/or 2) lymphocytes from gut-associated IPP can be effectively redistributed to loop IPP. Interestingly, a sheep study (18) implied such a possibility by showing that proliferating cells in response to Ag do not stay in IPP but move to other places, including adjacent lymphoid nodes. Furthermore, these studies allowed long-term examination (3–4 mo), demonstrating that IPP involute without direct external antigenic stimulation (18), which further supports the view of IPP as a secondary mucosal tissue in which maturation of B cells is dependent on colonization.

In the 1950s, the role of the bursa of Fabricius in the development of B cells in chicken stems from the work of Glick et al. (59), which caused investigators to search for a mammalian homolog. In the 1960s the rabbit appendix was proposed to play such a role (60), followed in the 1970s by the fetal liver and bone marrow in mice (61), and finally by the IPP of ruminants in the 1980s (5). Studies in mice showed that the PP (equivalent to the JPP in sheep and swine) could not account for the rapid postnatal rise in the number of B cells in circulation (62). The observation that sheep and swine possess two types of PP, IPP and JPP, and that the former developed in fetal life without gut colonization or the influence of maternal IgG, contributed to the idea that the IPP might have a separate role. Thus, the IPP attracted attention and led to the proposal by Reynolds and Morris (5) that it was the artiodactyl equivalent of the bursa of Fabricius. The Reynolds–Morris hypothesis was supported by 1) high mitotic and apoptotic rates (20–22), 2) B cell deficiency resulting from resection of IPP (49), and 3) by the work of Reynaud and colleagues (13, 24) showing Ag-independent repertoire diversification. Because of the existence of IPP homologs among artiodactyls, it came to be regarded in the literature as a primary lymphoid organ for swine, horse, and cattle. This paradigm survived despite subsequent evidence showing that the Ag-independent SHM reported by Reynaud and colleagues was due to an underestimation of the number of initially analyzed Vλ genes (28).

Major factors in maintaining the paradigm regarding the role of the IPP have been time and technology so that a collection of observations became "institutionalized" into a paradigm that continues to be perpetuated in other articles and reviews (2, 13, 15, 16, 50, 52, 53). This perpetuation took place during a period in which the focus of basic immunology shifted almost entirely to the mouse, so there was little research to challenge paradigms established years ago in nonmurine species. Furthermore, those who made the original observation moved on to lucrative studies in mice and humans. It is also surprising that studies to examine the role of bone marrow in the development of B cells and B lymphogenesis in artiodactyls were never undertaken using modern techniques. This is in stark contrast to the abundance of references to the IPP (reviewed, e.g., by Yasuda et al. in Ref. 2). Some reports in calves (63), horses (64), and even sheep (65) indicated that bone marrow could be active in lymphogenesis of B cells, at least for the same period of time as is speculated for the IPP (5). As we show in our companion article (39) and in ongoing studies

(M. Sinkora, manuscript in preparation), the bone marrow is fully capable of B cell lymphogenesis and remains active for at least 3 mo whereas no B cell lymphogenesis is present in the IPP.

In conclusion, data are presented in this study on the distribution of lymphocyte subsets in germ-free piglets, colonized isolator piglets, conventional piglets, and isolator piglets with resected IPP that suggest that the IPP of swine are not a primary lymphoid organ. The porcine IPP can clearly contribute to proliferation of B cells and may be important in the "natural" mucosal immune response to bacteria as discussed elsewhere (39, 66, 67). Data presented in this study and in a companion article (39) are consistent in showing that the IPP of swine are secondary lymphoid tissue. We think that given the similar anatomical homology and developmental behavior of the IPP of swine and sheep, those in sheep play a similar role. It is difficult to imagine that this organ has different functions in each different artiodactyl.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Carlens, O. 1928. Studien über das lymphatische gewebe des darmkanals bei einigen haustieren, mit besonderer berüksichtigung der embryonalen entwicklung, der mengenverhältnisse und der altersinvolution dieses gewebes im dünndarm des rindes. *Z. Anat. Entwicklungsgesch.* 86: 393–493.
2. Yasuda, M., C. N. Jenne, L. J. Kennedy, and J. D. Reynolds. 2006. The sheep and cattle Peyer's patch as a site of B-cell development. *Vet. Res.* 37: 401–415.
3. Rothkoetter, H. J., and R. Pabst. 1989. Lymphocyte subsets in jejunal and ileal Peyer's patches of normal and gnotobiotic minipigs. *Immunology* 67: 103–108.
4. Barman, N. N., A. T. J. Bianchi, R. J. Zwart, R. Pabst, and H. J. Rothkoetter. 1997. Jejunal and ileal Peyer's patches in pigs differ in their postnatal development. *Anat. Embryol. (Berl.)* 195: 41–50.
5. Reynolds, J. D., and B. Morris. 1983. The evolution and involution of Peyer's patches in fetal and postnatal sheep. *Eur. J. Immunol.* 13: 627–635.
6. Binns, R. M. 1982. Organisation of the lymphoreticular system and lymphocyte markers in the pig. *Vet. Immunol. Immunopathol.* 3: 95–146.
7. Pabst, R., M. Geist, H. J. Rothkoetter, and F. J. Fritz. 1988. Postnatal development and lymphocyte production of jejunal and ileal Peyer's patches in normal and gnotobiotic pigs. *Immunology* 64: 539–544.
8. Hein, W. R., L. Dudler, and C. R. Mackay. 1989. Surface expression of differential antigens on lymphocytes in the ileal and jejunal Peyer's patches of lambs. *Immunology* 68: 365–370.
9. Reynolds, J. D. 1987. Peyer's patches and the early development of B lymphocytes. *Curr. Top. Microbiol. Immunol.* 135: 43–56.
10. Griebel, P. J., L. Kennedy, T. Graham, W. C. Davis, and J. D. Reynolds. 1992. Characterization of B-cell phenotypic changes during ileal and jejunal Peyer's patch development in sheep. *Immunology* 77: 564–570.
11. Reynolds, J. D., L. Kennedy, J. Peppard, and R. Pabst. 1991. Ileal Peyer's patch emigrants are predominately B cells and travel to all lymphoid tissues in sheep. *Eur. J. Immunol.* 21: 283–289.
12. Griebel, P. J., W. C. Davis, and J. D. Reynolds. 1991. Negative signaling by surface IgM on B cells isolated from ileal Peyer's patch follicles of sheep. *Eur. J. Immunol.* 21: 2281–2284.
13. Reynaud, C. A., C. Garcia, W. R. Hein, and J. C. Weill. 1995. Hypermutation generating the sheep immunoglobulin repertoire is an antigen-independent process. *Cell* 80: 115–125.
14. Pospisil, R., and R. G. Mage. 1998. Rabbit appendix: a site of development and selection of the B cell repertoire. *Curr. Top. Microbiol. Immunol.* 229: 59–70.
15. Griebel, P. J., and W. R. Hein. 1996. Expanding the role of Peyer's patches in B-cell ontogeny. *Immunol. Today* 17: 30–39.
16. Butler, J. E., J. Sun, N. Wertz, and M. Sinkora. 2006. Antibody repertoire development in swine. *Dev. Comp. Immunol.* 30: 199–221.
17. Gerber, H. A., B. Morris, and W. Trevella. 1986. The role of gut-associated lymphoid tissues in the generation of immunoglobulin-bearing lymphocytes in sheep. *Aust. J. Exp. Biol. Med. Sci.* 64: 201–213.
18. Reynolds, J. D., and B. Morris. 1984. The effect of antigen on the development of Peyer's patches in sheep. *Eur. J. Immunol.* 14: 1–6.
19. Press, C. M., J. D. Reynolds, S. J. McClure, M. W. Simpson-Morgan, and T. Landsverk. 1996. Fetal lambs are depleted of IgM^+ cells following a single injection of an anti-IgM antibody early in gestation. *Immunology* 88: 28–34.
20. Reynolds, J. D. 1986. Evidence of extensive lymphocyte death in sheep Peyer's patches, I: A comparison of lymphocyte proliferation and export. *J. Immunol.* 136: 2005–2010.
21. Reynolds, J. D. 1987. Mitotic rate maturation in the Peyer's patches of fetal sheep and in the bursa of Fabricius of the chick embryo. *Eur. J. Immunol.* 17: 503–507.
22. Motyka, B., and J. D. Reynolds. 1991. Apoptosis is associated with the extensive B cell death in the sheep ileal Peyer's patch and the chicken bursa of Fabricius: a possible role in B cell selection. *Eur. J. Immunol.* 21: 1951–1958.
23. Andersen, J. K., H. Takamatsu, C. A. L. Oura, S. M. Brookes, L. Pullen, and R. M. E. Parkhouse. 1999. Systematic characterization of porcine ileal Peyer's patches, I: Apoptosis-sensitive immature B cells are the predominant cell type. *Immunology* 98: 612–621.
24. Reynaud, C. A., C. R. Mackay, R. G. Muller, and J. C. Weill. 1991. Somatic generation of diversity in mammalian primary lymphoid organ: the sheep ileal Peyer's patches. *Cell* 64: 995–1005.
25. Parng, C. L., S. Hansal, R. A. Goldsby, and B. A. Osborne. 1996. Gene conversion contributes to Ig light chain diversity in cattle. *J. Immunol.* 157: 5478–5486.
26. Meffre, E., and H. Wardemann. 2008. B-cell tolerance checkpoints in health and autoimmunity. *Curr. Opin. Immunol.* 20: 632–638.
27. Yamanaka, T., L. Helgeland, I. N. Farstad, H. Fukushima, T. Midtvedt, and P. Brandtzaeg. 2003. Microbial colonization drives lymphocyte accumulation and differentiation in the follicle-associated epithelium of Peyer's patches. *J. Immunol.* 170: 816–822.
28. Jenne, C. N., L. J. Kennedy, P. McCullagh, and J. D. Reynolds. 2003. A new model of sheep Ig diversification: shift in the emphasis toward combinatorial mechanisms and away from hypermutation. *J. Immunol.* 170: 3739–3750.
29. Sinkora, M., J. Sun, and J. E. Butler. 2000. Antibody repertoire development in fetal and neonatal piglets, V: VDJ gene chimeras resembling gene conversion products are generated at high frequency by PCR in vitro. *Mol. Immunol.* 37: 1025–1034.
30. Butler, J. E., J. Sun, P. Weber, P. Navarro, and D. Francis. 2000. Antibody repertoire development in fetal and newborn piglets, III: Colonization of the gastrointestinal tract selectively diversifies the preimmune repertoire in mucosal lymphoid tissues. *Immunology* 100: 119–130.
31. Sinkora, M., and J. E. Butler. 2009. The ontogeny of the porcine immune system. *Dev. Comp. Immunol.* 33: 273–283.
32. Yang, H., and R. M. Parkhouse. 1996. Phenotypic classification of porcine lymphocyte subpopulations in blood and lymphoid tissues. *Immunology* 89: 76–83.
33. Zuckermann, F. A., and R. J. Husmann. 1996. Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. *Immunology* 87: 500–512.
34. Sinkora, M., J. Sinkora, Z. Rehakova, I. Splichal, H. Yang, R. M. E. Parkhouse, and I. Trebichavsky. 1998. Prenatal ontogeny of lymphocyte subpopulations in pigs. *Immunology* 95: 595–603.
35. Butler, J. E., P. Weber, M. Sinkora, D. Baker, A. Schoenherr, B. Mayer, and D. Francis. 2002. Antibody repertoire development in fetal and neonatal piglets, VIII: Colonization is required for newborn piglets to make serum antibodies to T-dependent and type 2 T-independent antigens. *J. Immunol.* 169: 6822–6830.
36. Sinkora, M., J. Sinkora, Z. Rehakova, and J. E. Butler. 2000. Early ontogeny of thymoctes in pigs: Sequential colonization of the thymus by T cell progenitors. *J. Immunol.* 165: 1832–1839.
37. Minatis, O. P., and D. Jol. 1978. Gnotobiotic pigs: derivation and rearing. *Can. J. Comp. Med.* 42: 428–437.
38. Mateo, K. S., J. Ayres, M. Zhao, J. E. Butler, and D. H. Francis. 2011. Intestinal resection and anastomosis in neonatal gnotobiotic piglets. *J. Am. Assoc. Lab. Anim. Sci.* 50: 361–364.
39. Butler, J. E., K. Santiago-Mateo, X.-Z. Sun, N. Wertz, M. Sinkora, and D. H. Francis. 2011. Antibody repertoire development in fetal and neonatal piglets. XX. B cell lymphogenesis is absent in the ileal Peyer's patches, their repertoire development is antigen dependent, and they are not required for B cell maintenance. *J. Immunol.* 187: 5141–5149.
40. Genovese, K. J., R. C. Anderson, R. B. Harvey, and D. J. Nisbet. 2000. Competitive exclusion treatment reduces the mortality and fecal shedding associated with enterotoxigenic *Escherichia coli* infection in nursery-raised neonatal pigs. *Can. J. Vet. Res.* 64: 204–207.
41. Sinkora, M., J. Sinkorova, and W. Holtmeier. 2005. Development of γδ thymocyte subsets during prenatal and postnatal ontogeny. *Immunology* 115: 544–555.
42. Sinkora, M., J. Sinkorova, Z. Cimburek, and W. Holtmeier. 2007. Two groups of porcine $TCR\gamma\delta^+$ thymocytes behave and diverge differently. *J. Immunol.* 178: 711–719.

43. Sinkora, J., Z. Rehakova, M. Sinkora, B. Cukrowska, H. Tlaskalova-Hogenova, A. T. Bianchi, and B. De Geus. 1998. Expression of CD2 on porcine B lymphocytes. *Immunology* 95: 443–449.
44. Gerdts, V., R. R. Uwiera, G. K. Mutwiri, D. J. Wilson, T. Bowersock, A. Kidane, L. A. Babiuk, and P. J. Griebel. 2001. Multiple intestinal "loops" provide an in vivo model to analyse multiple mucosal immune responses. *J. Immunol. Methods* 256: 19–33.
45. Sinkora, M., J. Sun, J. Sinkorova, R. K. Christenson, S. P. Ford, and J. E. Butler. 2003. Antibody repertoire development in fetal and neonatal piglets, VI: B-cell lymphogenesis occurs at multiple sites with differences in the frequency of in-frame rearrangements. *J. Immunol.* 170: 1781–1788.
46. Perez, C., S. Moreno, A. Summerfield, N. Domenech, B. Alvarez, C. Correa, F. Alonso, A. Ezquerra, J. Dominguez, and C. Revilla. 2007. Characterisation of porcine bone marrow progenitor cells identified by the anti-c-kit (CD117) monoclonal antibody 2B8/BM. *J. Immunol. Methods* 321: 70–79.
47. Layton, D. S., A. D. Strom, T. E. O'Neil, M. M. Broadway, G. L. Stephenson, K. R. Morris, M. Muralitharan, M. S. Sandrin, F. L. Ierino, and A. G. Bean. 2007. Development of an anti-porcine CD34 monoclonal antibody that identifies hematopoietic stem cells. *Exp. Hematol.* 35: 171–178.
48. Sinkora, M., J. Sinkorova, and J. E. Butler. 2002. B cell development and VDJ rearrangements in the pig. *Vet. Immunol. Immunopathol.* 87: 341–346.
49. Gerber, H. A., B. Morris, and W. Trevella. 1986. Humoral immunity in B cell depleted sheep. In *Immunology of the sheep.* B. Morris, and M. Miyasaka, eds. Roche Institute of Immunology, Basel, p. 187–215.
50. Rothkötter, H. J., H. J. Zimmermann, and R. Pabst. 1990. Size of jejunal Peyer's patches and migration of lymphocyte subsets in pigs after resection or transposition of the continuous ileal Peyer's patch. *Scand. J. Immunol.* 31: 191–197.
51. Yasuda, M., M. Fujino, T. Nasu, and T. Murakami. 2004. Histological studies on the ontogeny of bovine gut-associated lymphoid tissue: appearance of T cells and development of IgG^+ and IgA^+ cells in lymphoid follicles. *Dev. Comp. Immunol.* 28: 357–369.
52. Caro, M. R., M. C. Gallego, A. J. Buendia, E. Navarro, and J. A. Navarro. 1998. Postnatal evolution of lymphocyte subpopulations in peripheral blood and lymphoid organs in the goat. *Res. Vet. Sci.* 65: 145–148.
53. Dobson, B. J., C. G. Mackintosh, and J. F. Griffin. 2010. Development of the thymus and Peyer's patches in the first year of life in red deer (*Cervus elaphus*). *Vet. Immunol. Immunopathol.* 137: 93–98.
54. Bianchi, A. T., R. J. Zwart, S. H. Jeurissen, and H. W. Moonene-Leusen. 1992. Development of the B- and T-cell compartments in porcine lymphoid organs from birth to adult life: an immunohistological approach. *Vet. Immunol. Immunopathol.* 33: 201–221.
55. Miyasaka, M., L. Dudler, G. Bordmann, W. M. Leiserson, H. A. Gerber, J. Reynolds, and Z. Trnka. 1984. Differentiation of B lymphocytes in sheep, I: Phenotypic analysis of ileal Peyer's patch cells and the demonstration of a precursor population for sIg^+ cells in the ileal Peyer's patches. *Immunology* 53: 515–523.
56. Fagarasan, S., K. Kinoshita, M. Muramatsu, K. Ikuta, and T. Honjo. 2001. In situ class switching and differentiation to IgA-producing cells in the gut lamina propria. *Nature* 413: 639–643.
57. Pillai, S., A. Cariappa, and S. T. Moran. 2004. Positive selection and lineage commitment during peripheral B-lymphocyte development. *Immunol. Rev.* 197: 206–218.
58. Hao, W. L., and Y. K. Lee. 2004. Microflora of the gastrointestinal tract: a review. *Methods Mol. Biol.* 268: 491–502.
59. Glick, B., T. S. Chang, and R. G. A. Jaap. 1956. The bursa of Fabricius and antibody production on the domestic fowl. *Poult. Sci.* 35: 224–225.
60. Archer, O. K., D. E. Sutherland, and R. A. Good. 1963. Appendix of the rabbit: a homologue of the bursa in the chicken? *Nature* 200: 337–339.
61. Owen, J. J., D. E. Wright, S. Habu, M. C. Raff, and M. D. Cooper. 1977. Studies on the generation of B lymphocytes in fetal liver and bone marrow. *J. Immunol.* 118: 2067–2072.
62. Friedberg, S. H., and I. L. Weissman. 1974. Lymphoid tissue architecture, II: Ontogeny of peripheral T and B cells in mice: evidence against Peyer's patches as the site of generation of B cells. *J. Immunol.* 113: 1477–1492.
63. Ekman, A., T. Pessa-Morikawa, J. Liljavirta, M. Niku, and A. Iivanainen. 2010. B-cell development in bovine fetuses proceeds via a pre-B like cell in bone marrow and lymph nodes. *Dev. Comp. Immunol.* 34: 896–903.
64. Studdert, M. J. 1978. Primary, severe, combined immunodeficiency disease of Arabian foals. *Aust. Vet. J.* 54: 411–417.
65. Pabst, R., M. Miyasaka, and L. Dudler. 1986. Numbers and phenotype of lymphocytes emigrating from sheep bone marrow after in situ labelling with fluorescein isothiocyanate. *Immunology* 59: 217–222.
66. Butler, J. E., Y. Zhao, M. Sinkora, N. Wertz, and I. Kacskovics. 2009. Immunoglobulins, antibody repertoire and B cell development. *Dev. Comp. Immunol.* 33: 321–333.
67. Butler, J. E., and M. Sinkora. 2007. The isolator piglet: a model for studying the development of adaptive immunity. *Immunol. Res.* 39: 33–51.