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DISERTAČNÍ PRÁCE

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Vývoj B buněk u prasat a úloha γδ T lymfocytů při imunizaci naivního imunitního systému

The development of swine B cells and the role of γδ T lymphocytes in immunization of naive immune system

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Keywords

B cell lymphogenesis; B lymphocytes; Bone marrow; CD2; CD8; CD21; Cell Differentiation; Germ-free animals; Lymphocyte Subpopulations; Peyer's patches; Porcine immune System; Repertoire Development; T cell development; T cell receptor; γδ T lymphocytes

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Abbreviations

7-AAD	7-aminoactinomycin D
BAL	bronchoalveolar lavage
BCR	B cells receptor
BM	bone marrow
CDR3	third complementary determining region
CFSE	5-(and -6)-carboxyfluorescein diacetate succinimidyl ester
DG	day of gestation
DP	double-positive
FL	fetal liver
GF	germ-free
HC	heavy chain
Ig	immunoglobulin
IL-2, 4, 17	interleukin 2, 4, 17
IFN-α, γ	interferon α, γ
IPP	ileal Peyer's patches
JPP	jejunal Peyer's patches
LC	light chain
mAb	monoclonal antibody
MHC II	major histocompatibility complex class II
MLN	mesenteric lymph nodes
pAb	polyclonal antibody
PBS	phosphate buffered saline
PKC	protein kinase C
PMA	phorbol-12-myristate-13-acetate
pre-BCR	pre B cells receptor
pre-TCR	pre T cells receptor
RAG	recombination-activating gene
SHM	somatic hypermutation
SJC	signal joint circle
SLA DR	swine leukocyte antigen DR
SLC	surrogate light chain
SWC1, **7**	swine workshop cluster 1, 7
TCR	T cells receptor
TCRαβ/γδ	αβ/γδ T cells receptor
TdT	terminal deoxynucleotidyl transferase
YS	yolk sac

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Thesis summary

The process of B cell lymphogenesis in swine remains uncertain. Some reports indicate that pigs belong to a group of animal that use ileal Peyers's patches (IPP) for the generation of B cells while others point to the possibility that the bone marrow is functional throughout life. The functional subpopulations of B cells in swine are also unknown. Together with other ruminants, and also birds, γδ T cells in swine may account for >70% of all T cells which is in apparent contrast with humans and mice. The purpose of this thesis was to address these discrepancies and unresolved issues. The results disprove the existing paradigm that the IPP is primary lymphoid tissue and that B cells develop in IPP in an antigen-independent manner. On the other hand, it shows that bone marrow is fully capable of B cell lymphogenesis and remains active at least for the same period of time as it had been speculated for the IPP. This thesis also identified functionally different subsets of porcine peripheral B cells, and shows that CD21 molecules can be expressed in differential forms. Finally, this thesis identifies two lineages of γδ T cells that differ in many functional and phenotype features. This finding may explain why γδ T cells constitute of minority of lymphocytes in circulation of humans and mice.

Souhrn v češtině

Proces lymfogeneze u prasat zůstává stále do značné míry nejasný. I když některé práce naznačují, že prasata patří do skupiny zvířat, která využívají pro vývoj B buněk ileální Peyrovy pláty (IPP), jiné práce ukazují na možnost, že B lymfopoéza probíhá během života v kostní dřeni. Rozdělení B lymfocytů u prasat do funkčních subpopulací je také stále velmi omezené. Narozdíl od myší nebo lidí může dosahovat podíl γδ T lymfocytů u prasat i více než 70 % z celkového počtu T buněk, stejně je tomu u ostatních přežvýkavců a také například u ptáků. Cílem této práce bylo přispět k porozumění výše zmíněných rozdílností v imunitě prasat a objasnit tato témata. Naše výsledky vyvracejí zavedené paradigma, že IPP u prasat fungují jako primární lymfatický orgán pro vývoj B lymfocytů na antigenech nezávislým způsobem. Naopak se podařilo prokázat, že kostní dřeň plně umožňuje vývoj B lymfocytů a zůstává aktivní minimálně po stejnou dobu, po jakou se předpokládalo, že lymfopoéza probíhá v IPP. Tato práce také umožnila odlišení funkčně odlišných populací prasečích periferních B lymfocytů a ukázala, že molekula CD21 se může na B buňkách vyskytovat v několika různých formách v závislosti na stavu B buněk. Dalším výsledkem této práce je odlišení dvou funkčně odlišných linií γδ T buněk, které mají různé funkce a fenotypové vlastnosti. Tyto nálezy by mohly přispět objasnění minoritního zastoupení γδ T lymfocytů v cirkulaci myší a lidí.

1. General Introduction

1.1. Swine as an experimental model

Swine *(Sus scrofa domesticus*) belongs to the even-toed ungulate order (Artiodactyles). Pigs are nearly the oldest domesticated animals and belong to hoofed mammals that are one of the largest groups on the planet. After fish, pork is the main source of meat in the human diet. For these reasons, swine is one of the most studied veterinary species with substantial progress in understanding its immune system. Better understanding of porcine immunology has clear implications in veterinary medicine, ecology, wildlife management, agriculture, and comparative immunology.

Pigs have been chosen also as a valuable laboratory and experimental model. This is mainly because pigs possess a non-invasive six-layered epitheliochorial placenta that prevents prenatal transfer of maternal immunoglobulins (Igs) to the offspring and even small peptides are not transferred to the fetus (*Sterzl and Silverstein, 1967; Brambell, 1970; Butler, 1974*). This predetermines porcine fetuses and germ-free (GF) piglets as an unique experimental model for the study of completely naive immune system and allows one to distinguish between intrinsic immune factors that develop spontaneously and those arising under the influence of passively acquired antibodies, maternal factors and external antigenic stimuli. Similar closed type of placentation can be found in other ruminants, and also in whales or lower primates, but only swine has numerous offspring and are precocial. Newborn piglets arrive with fur, are sighted, are able to free moving, are ready immediately to accept food and they do not need their mothers for further development. Although some may regard the usage of GF piglets as an artifact that does not reflect conventional conditions, it is still is the best possible animal model in which an effect of any agent on a naive immune system can be studied. In fact, GF piglets are the only animal model known so far in which all major factors that control postnatal development can be controlled by the experimenter *(Butler and Sinkora, 2007; Sinkora and Butler, 2009)*. These factors include (1) diet, (2) maternal regulatory factors, (3) external antigens and pathogens and (4) normal gut flora. Therefore, studies on GF pigs overcome ambiguous studies on laboratory rodents that are altricial and cannot be reared after birth without their mothers, and in which diet is always contaminated to some extent by bacterial components *(Hrncir et al., 2008)*.

Body size, similar nutrition requirements, intestinal/respiratory systems and microflora composition predetermines swine also as medically important species for its usage in xenotransplantation *(Sachs et al., 2001)* and surgery training. Pigs are also often used as an ideal model for human diseases such as inflammation, shock, trauma, wound healing and cardiopulmonary diseases (*Summerfield, 2009*). Recent effort in the generation of gene knock-out pigs also demonstrate that swine is a far better animal model for human medicine than mice (*Rogers et al., 2008*).

Other benefits of using pigs in biomedical research are high cell numbers for experimental work and numerous offspring; thus making the studies more ethnical, cheaper and less time-consuming. Furthermore, all swine genes for Ig heavy chain (HC) *(Sun et al., 1994; Butler et al., 1996)* share a common leader. The most used T cell receptor (TCR) δ family *(Yang et al., 1995)* also share a common leader sequence *(Sun et al., 1994; Butler et al., 1996).* Therefore, repertoire diversification of B and γδ T cell-specific receptors can be studied using a single primer set for each. Last but not least, pig breeds can be easily miniaturized allowing reduced requirements for space and nutrition.

1.2. Lymphocytes mediate adaptive immunity

All eukaryotic organisms initially or exclusively depend on innate immunity for survival against pathogens. However, in higher vertebrates, survival after 96 hours also depends on adaptive immunity. The evolutionary and phylogenetic appearance of adaptive immunity parallels the appearance of lymphocytes. Lymphocytes offer a possibility not available in the innate immune system, i.e. somatic manipulation of genes in which nonfunctional gene segments must be rearranged to encode the productive receptor with unique specificity to antigen. This allowed the functional genetics of an individual to be altered during its lifetime so that survival of the species need not wait for natural selection to act on spontaneous mutations of germ-line genes; hence the term "adaptive". Three types of lymphocyte lineages have been identified in all higher vertebrates studied so far, consisting of B, αβ T and γδ T lymphocytes. Although mechanism of gene rearrangement is similar for all type of lymphocyte receptors, their functions, receptor specificity and lymphocyte development is distinct for each lymphocyte lineage.

1.2.1. Porcine B cells

B cells represent antibody (Ig) producing lymphocytes, with their corresponding B cell receptor (BCR) that recognizes antigens directly. Occurrence of B cells is closely related to hematopoiesis because B cells mostly develop in primary hematopoietic organs. The earliest source of lymphopoietic activity in the porcine embryo is the yolk sac *(Sinkora et al., 2003).* Although very infrequent, the first lymphocytes that can be detected at the 20^{th} day of gestation (DG20) are B lineage cells. However, the number of earliest B cells in yolk sac is extremely low and the contribution of generated cells in yolk sac to overall lymphocyte pool is marginal. The yolk sac in the fetal pig involutes after DG24-27 so termination of its role in lymphogenesis afterward would be expected. Lymphopoietic activity and development of the pre-immune BCR repertoire can be thereafter detected in the fetal liver, which is the major site of B cell lymphogenesis in the porcine embryo from DG30 until at about DG45 when the bone marrow becomes active *(Sinkora et al., 2003).* The onset of B cell lymphogenesis in the bone marrow starting from DG45 is followed by a rapid expansion of B cells in the periphery *(Sinkora et al., 1998b).* Lymphopoietic activity in the liver after this period fades and become insignificant. Lymphopoietic activity in the bone marrow peaks between DG60-DG80 and during this period, most peripheral B cells are generated.

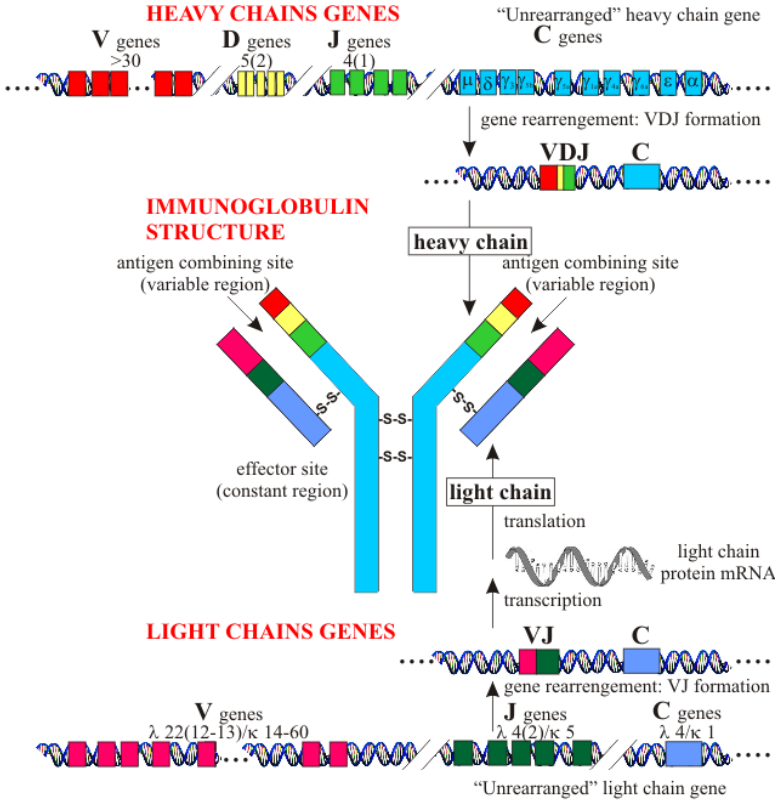
1.2.1.1. Development of porcine B cells at molecular level

Differentiation of B cell precursors from multipotent stem cell to immature B cells can be divided into a series of stages based on the status of Ig gene rearrangement (Fig. 1) and the expression of certain genes (*Hardy et al., 1991; Ehlich et al., 1993; Ghia et al., 1996; Rolink et al., 1999; Hardy and Hayakawa, 2001*). The first step in the generation of the BCR or antibody repertoire involves combinatorial joining of V_H, D_H and J_H gene segments in the Ig HC locus, a process called VDJ rearrangement. A special set of enzymes that selectively marks rearrangement process are active during VDJ rearrangement. These include the recombinase-activating genes (RAG-1 and RAG-2) needed for DNA cleavage at recombination signal sequences, terminal deoxynucleotidyl transferase (TdT) which can facilitate N-nucleotide additions, and various DNA repair enzymes. During VDJ_H rearrangement, the portions of the locus containing unused V_H, D_H or J_H gene segments are excised from genomic DNA in a form of circular extra-nuclear DNA. These so called signal joint circles (SJC) are not duplicated during proliferation of

cells and are often use for detection of active B cell lymphogenesis. Cells that have productively rearranged their VDJ_H down-regulate rearrangement-specific genes including RAG-1 and RAG-2. In the following differentiation, the VDJ_H spliced to the IgM HC (called μHC) associate with surrogate light chain (SLC, consisting of non-polymorphic λ5 plus VpreB chains) and the signaling components CD79α and CD79β to form pre-B cell receptor (pre-BCR), which is expressed on the cell surface (*Melchers et al., 1993*). The functional pre-BCR is thought to deliver signals that stop further rearrangement in the HC locus and thus insures allelic exclusion. Further cellular differentiation involves vigorous proliferation to increase precursor B cell pool, re-expression of rearrangement-specific genes, including RAG-1 and RAG-2, and subsequent Ig light chain (LC) gene segments rearrangement. Rearrangement of LC locus involves combinatorial joining of V_L and J_L gene segments. There are two types of LC, κ and λ, in most mammals that are located in different loci. In mice and humans, LC rearrangement is ordered and λ genes undergo rearrangement only when the κ gene recombination has led to nonfunctional products *(Gorman and Alt, 1998)*. After LC rearrangement is successful, the SLC is replaced with authentic LC (*Ehlich et al., 1993; Ghia et al., 1996*). The authentic BCR is the defining marker of the immature B cell stage, the direct predecessor of transitional stage of T-1 and T-2 B cells (*King and Monroe, 2000*).

All V_H genes in swine are members of a single, highly homologous family sharing sequence homology with the human V_H3 family, and more than 30 variants (genes or alleles) have been identified *(Butler et al., 2006; Butler and Wertz, 2012)*. About 80% of the fetal pre-immune repertoire uses just four V_H genes (V_HA, V_HB, V_HC, V_HE) and by including three additional V_H genes (V_HF, V_HX, V_HY), >95% of the fetal repertoire can be accounted for *(Butler et al., 2000)*. Given: (a) the limited combinatorial diversity in this swine where 7 major V_H genes are used in fetal development, only two functional D_H (D_HA and D_HB) and one functional J_H segments exist (Fig. 1) and (b) the nearly identical framework regions of its V_H3-like genes, >95% of the pre-immune repertoire in swine is determined by junctional diversity in the HC third complementary determining region (CDR3). The LC repertoire is also combinatorially restricted and shows little or no junctional diversity *(Butler et al., 2004)*. Interestingly, both V_H and V_L (and also major histocompatibility complex - MHC) genes exhibit unexpected high sequence similarity with their human counterparts despite differences in phylogeny.

Figure 1: Structure of BCR and scheme of V(D)J recombination



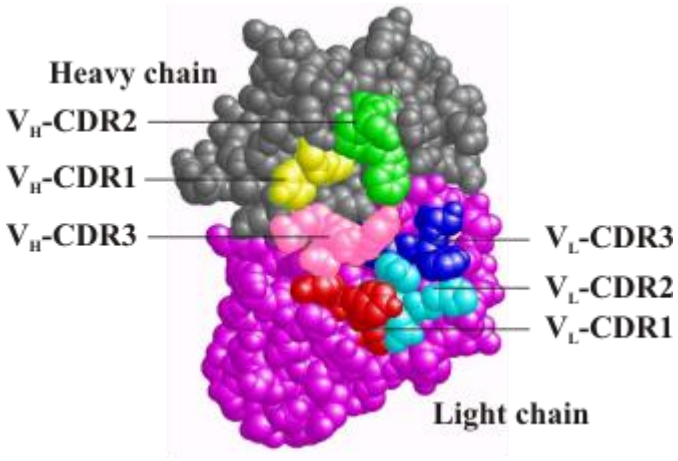
V(D)J rearrangement, also known as somatic recombination, is a mechanism by which BCR (or soluble antibodies) and TCR are generated. This recombination combines Variable (V, in red), Diverse (D, in yellow) and Joining (J, in green) gene segments into one functional gene. In the case of HC rearrangement, V, D and J segments recombinate while in the case of LC (lower part) only V and J segments are used. Constant (C, in blue) gene segments are connected to V(D)J recombination during transcription by splicing. The constant region of HC encodes the same repertoire of isotypes common to other eutherian mammals. There are ~30 V_H, 5 D_H (only 2 functional, indicated within brackets) and 4 J_H (only 1 functional) gene segments. Porcine LC locus consists of 14–60 Vκ, 5 Jκ, 12–13 functional Vλ, and two functional Jλ gene segments. Drawing is based on *Janeway, 2005* and *Butler and Wertz, 2012.*

Repertoire development of B cells (and similarly also T cells) is overwhelmingly determined by the diversity in CDR3. This is because CDR3 region is the most variable part of the molecule due to inclusion of V, D and J segments (Fig. 2). CDR3 also recognize a vast repertoire of antigens and it has been shown that diversity of CDR3 alone can generate full level of repertoire diversification (*Xu and Davis, 2000*). The diversity of the CDR3, CDR3 polymorphism, is often studied by separation of amplified CDR3 regions on polyacrylamide sequencing gels, which provides a clonotypic analysis of B cell receptors, including length. This procedure is called spectratyping. If there is no repertoire diversification, obtained band has typical gaussian or polyclonal distribution. On the other hand, expansion of some B cells with certain specificities result in superimposed bands

corresponding to expanded clones. This leads to non-gaussian or oligoclonal distribution of bands.

B cells development can be monitored by nuclear events such as the extent of rearrangement in the HC and LC locus, the expression of nuclear proteins like RAG-1, RAG-2 or TdT, the presence of SJC or the expression of VpreB transcript as a component of SLC. Although the development of B cell on a molecular level operates by a similar mechanism regardless of the particular species, the monitoring of this development by expression of accessory cell surface markers differs markedly. This inconsistency is apparent namely in species that are extensively studied like humans (key markers are for example CD19, CD10, CD34) and mice (key markers are for example B220, c-kit, CD43, CD19, CD25). The characterization of B cell development by cell surface antigens in other species is not well known due to lack of necessary monoclonal antibodies (mAb) and less progress in molecular knowledge such as unknown sequences for rearrangement-specific genes. In swine, there is no pan-B cell marker other than intracellular CD79α that can be detected by cross-reactive anti-human monoclonal antibody *(Jones et al., 1993)*. Because this mAb is restricted for use in intracellular staining, many studies are done by anti-μHC antibodies that detect all Ig positive cells, thus omitting plasma cells, switched B cells and mainly B cell precursors from detection. Moreover, there is no one study which connects molecular events during B cell development with expression of cell surface markers. For that reason, the exact developmental pathways for B cell in swine are poorly understood.

Figure 2: 3D view of Ig binding site with highlighted CDR regions



Pinnacle view of Ig variable region that constitute antigen binding site. Contributions of individual CDR regions are indicated by different colors and corresponding marks. None CDR regions of HC are in grey while none CDR regions of LC are in purple. Picture is adapted from 3D BLAST structural data.

1.2.1.2. Postnatal B cell development in pigs

The primary B cell lymphogenesis in early fetal ontogeny is thought to be limited to the yolk sac and fetal liver. This was demonstrated in many mammalian species including swine *(Sinkora et al., 2000b)*. However, the final location, course and maintenance of B cell lymphogenesis are very diverse among species. In some species like humans and mice, the B lymphopoietic activity moves to the bone marrow in later stages of embryogenesis and become the ultimate destination of B lymphogenesis throughout life *(Nuñez et al., 1996)*. In species like rabbit, cattle and sheep *(Press et al., 1993; Crane et al., 1996)* in which B cell lymphogenesis in the bone marrow terminates shortly after birth, the bone marrow does not contribute much to the overall B cell poll. Rather the majority of B cells are generated in specialized hindgut follicles where somatic diversification of the pre-generated B cell pool occurs. The anatomical site is not unique because rabbits use the appendix while sheep and cattle utilize the ileal Payer's patches (IPPs). Chickens just do not use the bone marrow at all for B lymphogenesis *(Pink et al., 1985)*. All B cells in chickens are generated in about 3-5 days in the fetal life in yolk sac and thereafter undergo somatic diversification in the bursa of Fabricius *(Reynaud et al., 1992)*. However, hindgut follicles including the bursa are not populated by stem cells. Species that do not use the bone marrow throughout life cannot generate new B cells during life. These species utilize pseudogene templated gene conversion as a supplementary mechanism for diversification of B cell repertoire from a limited number of existing B cells (*Yasuda et al., 2006*). The appearance and utilization of gene conversion parallels the phylogenic limitation in V_H family diversification because the gene conversion can operate only on a highly conservated DNA template *(Yasuda et al., 2006)*. Some authors speculate that gene conversion can be, in some cases, replaced by antigen-independent non-templated somatic hypermutation (SHM) in a similar way as is known for antigen-dependent SHM, the classical secondary immune response (*Maul and Gearhart, 2010*). However, SHM also occurs spontaneously and uses the same mechanism and set of enzymes independently of antigen dependency or independency. Without experiments on GF animals and careful experimental design, it is impossible to conclude whether SHM occurs in antigen dependent or independent manner.

The process of B cell lymphogenesis in swine remains uncertain. Pigs are hoofed mammals like sheep, cattle and horses and they also have IPPs that appears as specialized gut-associated organs *(Butler et al., 1996; Barman et al., 1997; Rothkötter et al., 1999;*

Pabst and Rothkötter, 2006; Yasuda et al., 2006). All porcine V_H genes are members of a single, highly homologous family *(Sun et al., 1994)*. Moreover, gene conversion was predicted to operate in swine because of the occurrence of chimeric V_H rearrangements *(Butler et al., 1996)*. Swine have also limited combinatorial joining diversity with one functional JH, two DH and seven most used VH segments (therefore only 14 possibilities) so there should be other mechanisms of repertoire diversification *(Butler et al., 1996)*. All these findings indicate that gene conversion events might be expected in swine. On the other hand, our previous data indicate that the bone marrow in swine is highly active in B cell lymphogenesis *(Sinkora et al., 2000b)*. It was also shown that chimeric V_H rearrangements are most likely a PCR artifact *(Sinkora et al., 2000b)*. As many as 45% of the clones recovered after PCR amplification of the defined VDJ templates can be V_H gene chimeras resembling somatic gene conversion products observed in the original study *(Butler et al., 1996)*. Moreover, the chimeric V_H rearrangements of swine do not resemble mosaics seen in chicken *(Sinkora et al., 2000b)*. In addition, highly restricted VDJ combinatorial diversity can be compensated by extensive CDR3 junctional diversity *(Butler et al., 2000)* that was particularly demonstrated using transgenic mice with only one V_H *(Xu and Davis, 2000)*. Thus, pigs may represent a species in which B lymphogenesis continues throughout life in the bone marrow but this is a departure from other hoofed animals that share limited V_H repertoires, possess hindgut associated IPPs, do not use bone marrow for B lymphogenesis and may use gene conversion. The uncertainty about the location, course and maintenance of B cell lymphogenesis in swine; the vague information about B cell development; and unclear activation, differentiation and maturation pathways of B cell in circulation lead us to propose the studies in this thesis that should clarify those issues.

1.2.1.3. Phenotype of porcine B cells

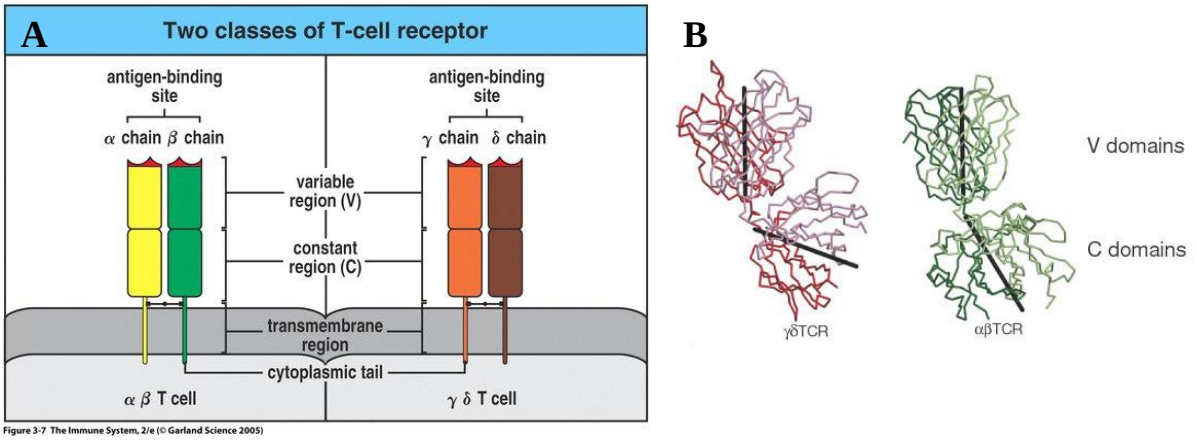
There is no pan-B cell marker for swine other than intracellular CD79α detected by cross-reactive anti-human monoclonal antibodies *(Jones et al., 1993)*. Because identification is restricted to intracellular staining, many studies are done using anti-μHC antibodies that detect all Ig positive cells, thus omitting a part of plasma cells, some switched B cells and B cell precursors from detection. Analysis by this approach showed that all μHC^+ B cells in swine are MHC-II^+, $CD25^{lo}$ and $CD45RC^+$ *(Sinkora et al., 1998b)*. Porcine B cells were shown to express CD2 molecules differentially *(Sinkora et al., 1998a, 1998b, 2005a;*

Sinkora and Butler, 2009), and ontogenetic studies suggested that down-regulation of CD2 occurs as a consequence of B cell activation *(Sinkora et al., 1998b)*. Together with the observation that the expression of CD2 occurs earlier than expression of BCR during B lymphopoiesis *(Sinkora et al., 1998b)*, CD2 may be regarded as a developmental marker of B cell. Although CD2 expression has been considered as a typical T cell-specific marker, expression of CD2 at low density on surface of B cells occurs on all mouse B cells *(Sen et al., 1989, 1990)* and also transiently during early human B cell ontogenesis *(Muraguchi et al., 1992)* and in some cases of neoplasm *(Viciana et al., 1994)*. Other information about subpopulations of porcine B cells is largely missing. The vast majority of studies just identified all B cells using different anti-CD21 mAbs *(Denham et al., 1994, 1998; Takamatsu et al., 1999; Boersma et al., 2001; Makala et al., 2001; Solano-Aguilar et al., 2001; Nielsen et al., 2003)* and anti-IgM or other Ig isotype-specific mAbs *(Sinkora et al., 1998a, 1998b, 2003, Sinkora and Butler 2009; Takamatsu et al., 1999)*. There is some indication that porcine B cells are heterogeneous for CD5 expression but expression of other markers does not correspond with the expression profiles of B1/B2 cells found in mice or humans *(Wilson and Wilkie, 2007)*. There is also indication that some anti-CD21 mAb may recognize porcine B cells differentially (*Sinkora and Butler, 2009)*.

1.2.2. Porcine T cells

Two types of CD3-associated TCR molecules have been identified in all vertebrates studied so far, consisting of either a TCRαβ or TCRγδ heterodimer (Fig. 3). In some species like human, mouse and rat, TCRαβ is expressed on >95% of all T cells. In these so called *γδ low species* γδ T lymphocytes constitute minor subset of all T cells that also are enriched at certain anatomical localizations, such as the epithelia. On the other hand, *γδ high species* such as swine, ruminants, chickens or rabbits have a substantial proportion of γδ T cells in the peripheral blood and lymphoid organs and these may account for more than 70% of the peripheral T cell pool (*Hein and Mackay, 1991; Massari et al., 2012*). The reason for abundance of γδ T cells in these species is unknown.

Figure 3: Structure of TCRαβ and TCRγδ



TCRαβ and TCRγδ have very similar structure (A). Both types of receptors are heterodimers made by two chains, each having variable (V), constant (C), and transmembrane regions *(*modified from *Parham, 2005)*. Protein structure models of human TCRγδ and TCRαβ are also shown (B). The γ and β chains are in lighter colors than δ and α chains. Despite very similar shape, the functions of each type of TCR are different (modified from *Allison et al., 2001*).

While αβ T lymphocytes are focused on foreign peptides presented by self MHC molecules, γδ T lymphocytes are still an enigmatic group of cells with diverse functions. At the effector cell level, they share many features with the αβ T lymphocytes such as potent cytotoxic activity *(Yang and Parkhouse, 1997; Carding and Egan, 2002; Scottet et al., 2008),* regulatory functions including ability to induce dendritic cell maturation and the capacity to produce a variety of cytokines *(Scotet et al., 2008).* γδ T cells also generate and retain immunologic memory (*Blumerman et al., 2007; Morita et al., 2007*) thus clearly possessing the typical characteristic of adaptive immunity. Nonetheless, they respond rapidly to infection *(Xiong and Raulet, 2007)* and their γδ T cell receptor (TCRγδ) recognize unprocessed or non-peptide antigens *(Tanaka et al., 1994; Hayday, 2000),* i.e. fundamentally different ligands from the short peptides that are seen by αβ T cells in the context of MHC molecules. In some cases and species, γδ T cells may also use their TCRγδ as a pattern recognition receptor and recognize directly, for example, some mycobacteria *(Lee et al., 2004)* or some plant extracts *(Summerfield and Saalmuller, 1998).* Due to that nature, γδ T cells are often categorized into unconventional T cells (together with TCRαβ^{+}CD8αβ^{-}CD4^{-} T cells) and hypothesized to have more innate functions acting in specific primary immune response. With recent evidence that γδ T cells can also act as potent antigen-presenting cells (*Takamatsu et al., 2002; Brandes et al.,*

2005) and that they may be involved in management of tumors by recognizing stress induced conserved antigens *(Scotet et al., 2008)*, the γδ T cells probably form a unique link between innate and adaptive immune responses thus belonging to both immune systems and providing an additional mechanism of antigen presentation that could rapidly trigger the αβ T cell immune response. In any case, (a) a very broad spectrum of recognized antigen with no uniform specificities, (b) an extremely different representation in lymphoid tissues in various species with often unique anatomical distribution, (c) an absence of selection for pre-antigen receptors (pre-BCR and pre-TCR) and (d) the unclear differentiation pathways as well as (e) the striking differences in γδ cell activities in different strains and species lead to understandable uncertainty about the exact role of γδ T cells in immunity and any compelling explanation for the evolutionary conservation of γδ T cells at all.

1.2.2.1. Development of porcine T cells at molecular level

The ontogeny and development of T cells in different species are comparable and follow similar pattern. This uniformity is in agreement with the findings of very strong evolutionary conservation of TCR genes that have been conserved since the emergence of jawed vertebrates more than 450 million years ago *(Rast and Litman, 1994)*. Diversity of TCR is generated in a similar fashion as the process of somatic recombination (rearrangement) described for BCR *(Janeway, 2005)*. Similar to the HC and LC of B cells, β or δ chains of the T cell consist of V-J-D-C segments and α or γ chains utilize V-J-C segments, respectively. It is considered that γ and δ chains segments are inserted inside of α/β locus so that rearrangement of TCRαβ excises TCRγδ genes from the genome.

There are at least 19 families of porcine Vβ genes in seven supergroups *(Butler et al., 2005)*. Five Vβ families account for 85% usage in all T cells. Genes for Dβ, Jβ and Cβ are similarly to human, organized in duplicon, each having seven Jβ segments (Jβ1.1 – Jβ1.7 and Jβ2.1 – Jβ2.7). Usage of Jβ1 segments from the 5' D-J-C duplicon is directly correlated with their position in the locus while usage of Jβ2 segments from the 3' D-J-C duplicon is the opposite. In any case, all but one possible Jβ segments are used in T cells. Heterogeneity in CDR3 is too great to allow identification of specific Dβ segments. The considerable number of Vβ and Jβ genes is therefore a reason why porcine Vβ repertoire is much more complex than the porcine V_H repertoire, and resembles the Vβ repertoire in

humans. This suggests a common phylogenetic origin of Vβ families. Sequences of porcine Vα genes and their usage are unknown. There is only information about Jα and Cα *(Uenishi et al., 2003).*

The abundance of γδ T cells in various species correlates with number of Vδ genes since the approximate estimate showed that there are ~16 murine and ~10 human Vδ genes while >30 in chicken *(Kubota et al. 1999),* >23 in cattle *(Hein and Dudler, 1997),* >28 in sheep *(Hein and Dudler, 1993)* and >36 in pigs *(Yang et al., 1995).* Swine utilize at least four different Jδ gene segments (Jδ1 - Jδ4), three putative Dδ segments (Dδ1 - Dδ3) and at least five Vδ families (Vδ1 - Vδ5) with one family (Vδ1) consisting of a large number of members (>31). A significant advantage for a molecular biological study is the observation that all members of the Vδ1 family use essentially the same leader sequences with only one or two nucleotide difference among them (two leader exons are in tandem where the 5' leader is used several-fold more often than the 3' exon). Thus, each member of Vδ family may be amplified using a single set of primers allowing an adequate study of TCRδ repertoire *(Holtmeier et al., 2004; Sinkora et al., 2005b).* Information about porcine Vγ genes is missing. It is only known that pigs use three known Cγ genes *(Saalmuller et al, 1990).*

The rearrangement of Ig HC, TCRβ chain, and TCRδ chain involve V, D and J genes, whereas only V and J genes are involved in Ig LC and the TCRα and γ chains *(Lai et al., 1989).* Among these chains, the TCRδ chain is unique in that it could involve more than two joining steps for the rearrangement. In murine TCRδ chain, three joining events are involved (Vδ to Dδ1, Dδ1 to Dδ2, and Dδ2 to Jδ), and four joining steps are involved in humans (Vδ to Dδ1, Dδ1 to Dδ2, Dδ2 to Dδ3 and Dδ3 to Jδ). This multistep rearrangement provides a mechanism that could potentially generate diversity at the V-D-J junction several orders of magnitude greater than all the Ig and TCR chains. Furthermore, analysis of TCRδ chain CDR3 region revealed length characteristics more similar to Ig HC than TCRβ chain *(Yang et al., 1995).* Therefore, swine extensive Vδ diversity as well as junctional diversity based on three joint events creates a level of diversity that is highest of any lymphocyte antigen receptor studied so far and is in accord with the idea that TCRγδ could recognize antigen directly like Ig *(Rock et al., 1994; Schild et al., 1994).*

1.2.2.2. T cell development at cellular level

T cell progenitors (pro-T cells) of both αβ and γδ T cells are derived from stem cells in primary hematopoietic centers and migrate to the thymus where further T cell differentiation takes place. In early ontogeny, T lymphopoietic activity is directly correlated with the hematopoietic activity of the yolk sac and fetal liver. The first T cells can be detected in thymus at about DG40, i.e. with about 10 days delay in comparison with B cell lyphophogenesis in the fetal liver *(Sinkora et al., 1998b and 2000a)*. The first peripheral T cells appear about 5 days after they are detectable in the thymus, all of which are γδ T cells. This corresponds to other species where γδ cells are the first T cells to develop *(Coltey et al., 1987; Jotereau et al., 1987)*. The first αβ T cells appear in the periphery at about DG55. Therefore, αβ thymocytes require about 15-20 days to express TCRαβ on their surface while γδ thymocytes do so for TCRγδ in less then 3 days *(Sinkora et al., 2000a)*. This is consistent with finding that γδ T cells develop without any $CD3^{lo}$ or $TCR\gamma\delta^{lo}$ transitional stage and do not need strict selection for TCRγδ specificity as for αβ T cells *(Sinkora et al., 2000a)*. The period of lymphopoietic activity from DG20 to DG45 generates the first wave of progenitors because all their lymphocyte progeny originate from the yolk sac and fetal liver. The onset of B cell lymphogenesis in the bone marrow starting from DG45 causes the second wave of thymic colonization and is followed by a rapid expansion of γδ and thereafter αβ T cells in the periphery *(Sinkora et al., 2000a)*. In further development, pro-T cells are continuously derived from stem cells in the bone marrow.

Maturation of porcine αβ T lymphocytes in thymus follows the generally accepted model of intrathymic T cell differentiation derived from studies in other species *(Sinkora et al., 2000a)*. Differentiating thymocytes belonging to the αβ lineage follow a progression from less differentiated, large triple-negative ($CD3^{-}CD4^{-}CD8^{-}$) precursors through a $CD8^{+}CD3^{-}$ immature stage to $CD3^{-}CD4^{+}CD8^{+}$ double-positive (DP) thymocytes. The latter subset proliferates vigorously to form large numbers of small cortical $CD3^{-}$ DP thymocytes that follow a progression through the $CD3^{lo}$ DP stage with different level of CD4 and CD8 expression to final populations of either mature $CD4^{+}CD8^{-}$ T helper or $CD4^{-}CD8^{+}$ T cytotoxic single-positive T cells expressing CD3 at high level.

Developing porcine γδ T cells in the thymus can be subdivided into two main families according to expression of CD4 molecule. The first family of $CD4^{+}$ γδ T cells is

strictly present in the thymus and has no counterpart in the periphery *(Sinkora et al., 2005b)*. These $CD4^+$ γδ T cells bear CD8αβ molecules on their surface, which was not observed on γδ T cells in any other organ than thymus. They are also always $CD1^+$ indicating they do not mature to the terminal stage of T cell development and are not exported from the thymus. Detailed phenotype studies reveal that $CD4^+$ γδ thymocytes represent a transient and independent subpopulation that extinguish their TCRγδ expression, become DP thymocytes and differentiate along the αβ lineage program *(Sinkora et al., 2005a)*. Thus these cells may alter their lineage program by re-entering the cell cycle and re-activation of TCRαβ gene rearrangement. Noteworthy, $CD4^+$ γδ thymocytes are not unique for swine; these cells can be found infrequently in human thymus and also in the fetal liver *(Offner et al., 1997; Aparicio et al., 1989; Wucherpfennig et al., 1993)*, early and transiently during fetal development in mice *(Itohara et al., 1989; Fisher and Ceredig, 1991)* and in chickens *(Bucy et al., 1990)*. The second family of $CD4^-$ γδ thymocytes give rise to all peripheral γδ T cells *(Sinkora et al., 2005b; Sinkora et al., 2007)*. Similarly to the periphery, $CD4^-$ γδ thymocytes can be divided into three subsets according to the expression of CD2/CD8α molecules. They may be further sub-divided also according to expression of CD1 and CD45RC. The common precursors of all γδ T lymphocytes are $CD2^+CD8^-CD1^+CD45RC^-$ γδ T cells. This developmental stage is followed by diversification in the $CD2^-CD8^-$, $CD2^+CD8^-$ and $CD2^+CD8\alpha^+$ subsets, which subsequently mature by loss of CD1 and by eventual gain of CD45RC expression *(Sinkora et al., 2007)*.

1.2.2.3. Phenotype of porcine αβ T cells

Mature αβ T cells exported from thymus are composed of classical homogenous $CD4^-$ $CD8\alpha\beta^+$ cytotoxic and $CD4^+CD8^-$ helper αβ T cells subsets *(Sinkora et al., 2000a)*. However, activation of $CD4^+CD8^-$ helper αβ T cells with various antigens in the periphery leads to expression of CD8α that plays an important role in signal transduction associated with cell effector function. This results in the occurrence of so-called "peripheral DP αβ T cells" that form the effector/memory T cell pool *(Saalmuller et al., 1989; Zuckermann and Husmann, 1996; Yang and Parhouse, 1997)*. A similar effect can be observed in humans or mice where CD8α is also expressed on T helper cells following activation *(Blue et al., 1985; Moebius et al. ,1991; Hori et al., 1991)* or in the case of

malignancies *(Ortolani et al., 1993)* or autoimmunity *(De Maria et al., 1987)*. However, CD8α expression on porcine αβ T helper cells is permanent and is not down-regulated after activation, which results in their increased accumulation during postnatal ontogeny *(Sinkora et al., 1998b)*. In agreement, peripheral $CD4^{+}CD8\alpha\beta^{+}$ αβ T helper cells are absent or very rare before birth and among newborns *(Sinkora et al., 1998b)*. Importantly, existence of these peripheral $CD4^{+}CD8\alpha\beta^{+}$ αβ T cells can be used for easily identification of primed effector/memory T helper cells in pigs (*Zuckermann, 1999*).

1.2.2.4. Phenotype of porcine γδ T cells

Peripheral γδ T cells in swine may be subdivided into $CD2^{-}CD8^{-}$, $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ subsets *(Yang and Parkhouse, 1996; Sinkora et al., 1998b)*, correspondingly to the group of $CD4^{-}$ γδ thymocytes. Individual subsets of peripheral γδ T cells differ in their homing characteristic: $CD2^{+}CD8^{+}$ and $CD2^{+}CD8^{-}$ γδ T cells preferentially accumulate in the spleen while $CD2^{-}CD8^{-}$ are enriched in the circulation *(Saalmuller et al., 1990; Yang and Parkhouse, 1996; Sinkora et al., 1998b*). The same phenotype-dependent pattern of tissue distribution has not been identified in mice or humans although $CD2^{+}CD8^{-}$ γδ T cells as well as γδ T cells expressing CD8 or lacking CD2 molecules have been observed *(Offner et al., 1997; Itohara et al., 1989; Fisher and Ceredig, 1991)*. Moreover, γδ T cells in humans and mice show distinctive TCRγδ gene usage depending on anatomical location *(Deusch et al., 1991)*. Although similar observation was not confirmed in swine, porcine $CD2^{+}CD8^{-}$ γδ T cells express different 46 kDa TCRγ chain than the $CD2^{-}CD8^{-}$ population that carries γ-chains of 37–38 kDa *(Saalmuller et al., 1990; Hirt et al., 1990)*. The porcine $CD2^{+}CD8\alpha\alpha^{+}$ subset has been postulated to be the progeny of peripheral $CD2^{+}CD8^{-}$ γδ T lymphocytes. This is based on observation that: (a) some $TCR\gamma\delta^{+}CD2^{+}$ cells may acquire CD8 upon activation similarly as has been reported for porcine αβ T cells *(Reddehase et al., 1991; Wen et al., 2012)*, (b) $CD2^{+}CD8\alpha\alpha^{+}$ γδ T cells are potentially cytotoxic while other $TCR\gamma\delta^{+}$ cell subsets are not *(Yang and Parkhouse, 1997, De Bruin et al., 1997)* and that (c) $CD2^{+}CD8\alpha\alpha^{+}$ γδ T cell are scarce in porcine fetuses *(Sinkora et al., 1998b)*. In humans and rodents, cells bearing the CD8αα homodimer have been found on $TCR\alpha\beta^{+}$ and $TCR\gamma\delta^{+}$ cells following activation, on a subset of NK cells that are able direct cytotoxicity, on a subset of effector dendritic cells, and on a large proportion of intraepithelial lymphocytes from conventional but not from GF animals *(MacDonald et*

al., 1990; Moebius et al., 1991; Hori et al., 1991; Spetz et al., 1991). These findings support the idea that CD8αα expression may be an indicator of functionally competent cells that have acquired this molecule extrathymically as a result of activation. It follows that some or maybe all peripheral $CD2^{+}CD8\alpha\alpha^{+}$ γδ T cells develop independently of thymus. However, we have recently proved that all $TCR\gamma\delta^{+}$ T cells subsets develop in the thymus and may represent separate cell lineages *(Sinkora et al., 2005b)*. Therefore, it seems that extrathymic maturation of γδ T cells does not involve rearrangement of TCRγδ genes and genesis of γδ T cells *de novo* from common progenitors. More likely, γδ T cells may "only" alter their accessory molecules to change the surface phenotype. It suggests there may be two developmentally distinct subsets of $CD2^{+}CD8\alpha\alpha^{+}$ peripheral γδ T cells: one developing and acquiring CD8αα in the thymus and a second that acquires CD8αα in the periphery as a result of activation of their $CD8^{-}$ thymus-dependent counterparts.

2. Significance, rationale and aims of the thesis

Despite significant progress in porcine immunology over the past two decades, there are several key questions in porcine immunology that need to be addressed:

1. Are Ileal Peyer's patches (IPP) the true site of B cell development in swine?

The process of B cell lymphogenesis in swine remains uncertain. Pigs belong historically to a group of animals that use specialized hindgut-associated IPP for the generation of B cells *(Butler et al., 1996; Barman et al., 1997; Rothkötter et al., 1999; Pabst and Rothkötter, 2006, Yasuda et al., 2006)*. However, our previous data are in conflict with this presumption *(Butler et al., 1996; Sinkora et al., 2000a, 2000b, 2002, 2003; Sinkora and Butler, 2009)*. Thus, pigs may represent another species in which B lymphogenesis continues throughout life in the bone marrow similarly to human and mice while at the same time share the limited V_H repertoire and hindgut associated IPP of cattle and sheep. The issue is unresolved because the role of swine IPP in B lymphogenesis, characterization of B cell subsets and other lymphocyte subsets in this organ has not been studied. All recent conclusions regarding porcine IPP are still at a hypothetical level as they have been extrapolated from work in sheep and the real functional role of porcine IPP is unknown.

2. What is the role of the bone marrow in swine?

Ontogenetic data indicate that the bone marrow in swine is highly active in B cell lymphogenesis during fetal life (*Sinkora et al., 2000a*). The onset of B cell lymphogenesis in the bone marrow starting from the 45^{th} day of gestation is followed by rapid expansion of B cells (*Sinkora et al., 2000a, 2002 and 2003*). The bone marrow appears to remain functional for at least a few weeks after the birth although B cell lineage is masked by the massive occurrence of polymorphonuclear cells (*Sinkora et al., 2003, Sinkora and Butler 2009*). There is no study that would explain the role of the bone marrow in postnatal liver or to show whether B lymphogenesis in the bone marrow is terminated.

3. Are there any functionally different subsets of porcine B cells?

Only known subpopulations of B cells with suggested functional differences can be identified using anti-CD2 mAbs. Ontogenetic studies suggested that down-regulation of

CD2 occurs as a consequence of B cell activation *(Sinkora et al., 1998a)* but these studies did not address functionality directly. We have also speculated that some anti-CD21 mAbs may recognize porcine B cells differentially (*Sinkora and Butler, 2009)*. However, none of studies was performed to directly show whether there are any functionally different subsets of porcine B cells.

4. What is the effect of bacterial colonization and age on phenotype of CD2/CD8 γδ T cell subsets?

Swine, together with ruminants and birds, belong to the group of *γδ high species* in which γδ T cells are not preferentially limited to epithelia and may account for >70% of all T cells *(Hein and Dudler, 1993)*. Traditionally, γδ T cells in swine are subdivided into three subsets based upon their expression of CD2 and CD8, and include $CD2^{-}CD8^{-}$, $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ cells *(Sinkora et al., 1998b, 2005b, 2007; Yang and Parkhouse, 1996, 1997)*. These individual subsets differ in their homing characteristics *(Saalmuller et al., 1990)* and cytotoxic activities *(de Bruin et al., 1997; Yang and Parkhouse, 1997)*. Previous studies revealed the basic distribution of porcine γδ T cells *(Sinkora et al., 1998b)*, their ontogeny *(Sinkora et al., 1998b, 2005a)*, development in the thymus *(Sinkora et al., 2000a, 2005a, 2007)* and the repertoire diversification of their TCR *(Holtmeier et al., 2004)*. However, none of these studies focused on a detailed analysis of peripheral γδ T cells, and no other studies have been performed to explain differences in the phenotypic profile of γδ T cells subsets.

5. Are CD2/CD8 subsets of γδ T cells only one cell lineage with developmental dependency or do they represent independent lineages?

Human and mice γδ T cells are generally considered $CD2^{+}$ *(Jitsukawa et al., 1987; Groh et al., 1989; Rakasz et al., 1997)* thus resembling $CD2^{+}$ γδ T cells in swine ($CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$). This is in sharp contrast with porcine $CD2^{-}$ γδ T cells, which are numerous and preferentially reside in the blood. High occurrence of $CD2^{-}$ γδ T cells is not unique to pigs but also for other members of *γδ high species* such as sheep *(Mackay et al., 1989; Witherden et al., 1995)*, calf *(Clevers et al., 1990)* and birds *(Vainio et al., 1991)*. Unfortunately there is no study that would resolve whether CD2/CD8 subsets represent separate and independent lineages specific for *γδ high species* or if they represent

subsequently developing subsets. Ontogenetic and developmental studies in the thymus indicate separate lineage commitment *(Sinkora et al., 2005a, 2007)*. This would correspond with findings that $CD2^{+}CD8^{+}$ γδ T cells are the only CD2/CD8 subset that occurs with potentially cytotoxic activity *(Reddehase et al., 1991; Yang and Parkhouse, 1997; de Bruin et al., 1997)*. However, there is some evidence that the $CD2^{+}CD8^{-}$ subset can differentiate into $CD2^{+}CD8^{+}$ γδ T lymphocytes upon activation *(Reddehase et al., 1991; Wen et al., 2012)*. There is also evident up-regulation of $CD8^{+}$ γδ T cells during ontology: $CD2^{+}CD8\alpha^{+}$ γδ T cells are scarce in porcine fetuses but their frequency increases with age *(Sinkora et al., 1998b)*. In any case, lineage commitment and/or dependency is not resolved for CD2/CD8 subsets of γδ T cells.

3. Outline of the thesis

The general purpose of this thesis is to address, and at least partially resolve the key questions given in the rationale. To clarify whether resection of IPP leads to B cell immunodeficiency and whether B cell development in IPP is antigen-dependent/independent. In ***publication #1*** we have used GF pigs that were or were not colonized by intestinal bacteria and/or in which IPP were removed by total resection very early after birth. GF animals were chosen because the fetal pattern of antigen-independent development in IPP can be prolonged in these animals or it can be easily stopped and reverted to antigen-dependent development after experimental colonization. The results 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. On the other hand, porcine IPP were shown to be an important, but nonessential mucosal lymphoid tissue for early immune response against colonization and food antigens.

Using semi-quantitative PCR detection of RAG, TdT and VpreB transcripts and the occurrence of SJC and VDJ_H/VJ_L rearrangements in DNA we show in ***publication #2*** that major B lymphopoietic organs during fetal ontogeny of pigs are the yolk sac, fetal liver and bone marrow. In agreement with publication #1, there was little evidence found for B cell lymphogenesis in the IPP. Moreover, bone marrow is shown to be active in B cell lymphogenesis for at least 5 weeks postpartum. Although this particular publication does not address B lymphopoietic activity in the bone marrow after 5 weeks of life, our recent data indicate that B lymphogenetic activity continues much longer. Publication #2 also addresses ordered rearrangement of LCκ and also the LCκ locus known for humans and mice and shows that this textbook paradigm does not apply to swine: VλJλ rearrangement precedes VκJκ rearrangement during B cell lymphogenesis in pigs.

Publication #3 shows that different mAbs against CD21 recognize different amounts of IgM^+ B cells in swine. Cross-reactivity and inhibition studies revealed that mature B cells always express CD21 molecules but probably in two different forms $CD21^a$ and $CD21^b$. We used one of the anti-CD21 mAb (IAH-CC51) together with anti-CD2 to

define four subpopulations of B cells: $CD2^{-}CD21^{b+}$, $CD2^{+}CD21^{b+}$, $CD2^{-}CD21^{b-}$ and $CD2^{+}CD21^{b-}$. We followed these subsets during ontogeny and studied their profiles in the blood and other lymphoid organs. *In vitro* culture studies, analysis of cell size, expression of CD11b and class-switched phenotype together with measurement of proliferation and cell death revealed that these subsets represent distinct populations. Most $CD2^{+}CD21^{b+}$ B cells are naive while $CD2^{-}CD21^{b+}$ B cells appear to be primed. Both these subsets probably mature into $CD2^{+}CD21^{b-}$ B cells that represent the effector, class-switched B cell pool. These effector B lymphocytes may eventually lose CD2 and contribute to $CD2^{-}CD21^{b-}$ cells that perhaps represent antibody-producing and plasma cells. This is the first report showing functionally different subpopulations of porcine peripheral B cells and indicating that end-stage B cells can express differential forms of CD21, which can be significant for their function.

In ***publication #4***, the expression of CD25, CD11b, SWC1 (swine workshop cluster 1), SWC7, MHC class II and the family of CD45 molecules on γδ T cells was studied. These molecules were chosen because of their differential expression on porcine γδ T cells in our preliminary studies. We used GF piglets that have a virgin immune system and compare those with their age-matched conventional mates. This comparison addresses the effect of bacterial colonization and environmental antigens on the development of γδ T cells *in vivo*. In addition, a group of conventional adult pigs was evaluated for effect of age. Analysis was done for all γδ T cells as well as their CD2/CD8 subpopulations. In addition, *in vitro* experiments were performed to explain the effect of different activation stimuli and TCR repertoire studies were conducted to show the extent of diversification. The results generally indicate that CD25 represents an activation molecule that probably marks a functionally distinct subset, expression of CD11b is perhaps connected to early functions of naive γδ T cells in the periphery, SWC1 is lineage specific marker, SWC7 may represent an activation molecule with intrinsic or transient expression, and the expression of CD45RA/RC most likely defines naive and terminally differentiated cells. Evidence that the CD2/CD8 subsets of porcine γδ T cells may represent functionally different cells is given.

Publication #5 is a continuation of publication #4 where peripheral and thymic γδ T cells were studied according to their differential expression of TCRγδ and CD2/CD8 molecules. Sorting and the following *in vitro* cultivation showed that CD2 expression can be used for definition of two lineages of γδ T cells while CD8 molecule can be modulated

on the surface according to actual functional status of $CD2^+$ γδ T cells. In this respect, both $CD2^+$ and $CD2^-$ γδ T cell lineages are equally diversified because CDR3 spectratyping of TCRδ1 genes among CD2/CD8 and $TCR\gamma\delta^{med}$/$TCR\gamma\delta^{hi}$ subsets is comparable. Two levels of TCRγδ were also observed in thymus in addition with CD1 expression which is characteristic for immature cells. Analysis of CD2/CD8 γδ thymocyte subpopulations confirms existence of $CD2^+$ and $CD2^-$ γδ T cell lineages. Because $CD2^-$ γδ cells are missing in the blood of humans and mice but are obvious in other members of *γδ high species* such as ruminants and birds, our findings support the idea that circulating $CD2^-$ γδ T cells are a specific lineage.

4. *(publication #1)*

Ileal Peyer's patches (IPP) 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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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 *Escherichia 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 $CD3ε^+TCRγδ^-$ 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

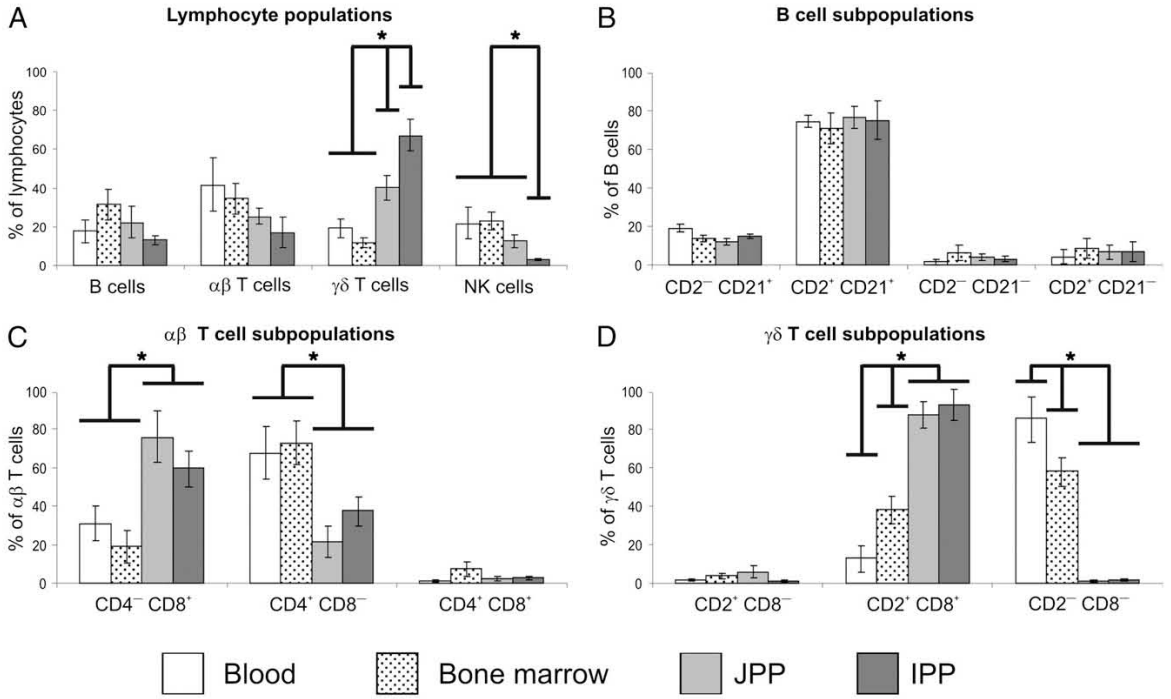

FIGURE 1. The frequencies of lymphocyte types and their subpopulations in different organs of 36-d-old germ-free piglets. Lymphocytes were isolated from blood (open bars), bone marrow (dotted bars), JPP (grey bars), and IPP (black bars) and analyzed for the proportions of B, αβ T, γδ T, and NK cells (*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

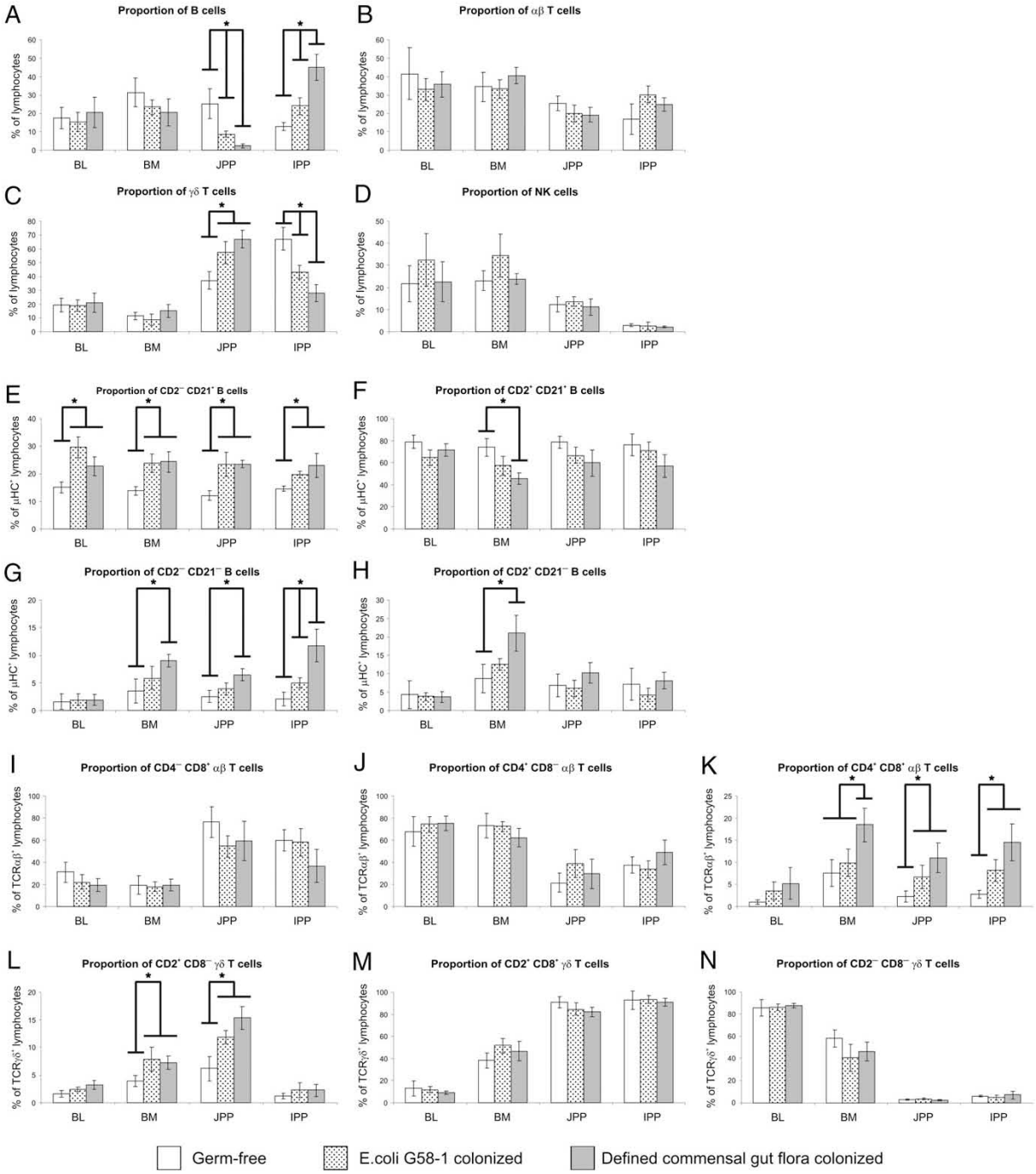

FIGURE 2. The frequencies of lymphocyte types (*A–D*) and their subpopulations (*E–N*) in different organs of 36-d-old germ-free piglets (open bars) and gnotobiotic piglets colonized only by *E. coli* strain G58-1 (dotted bars) or by the defined commensal gut flora (40) (grey bars) at day 5 after birth. 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.

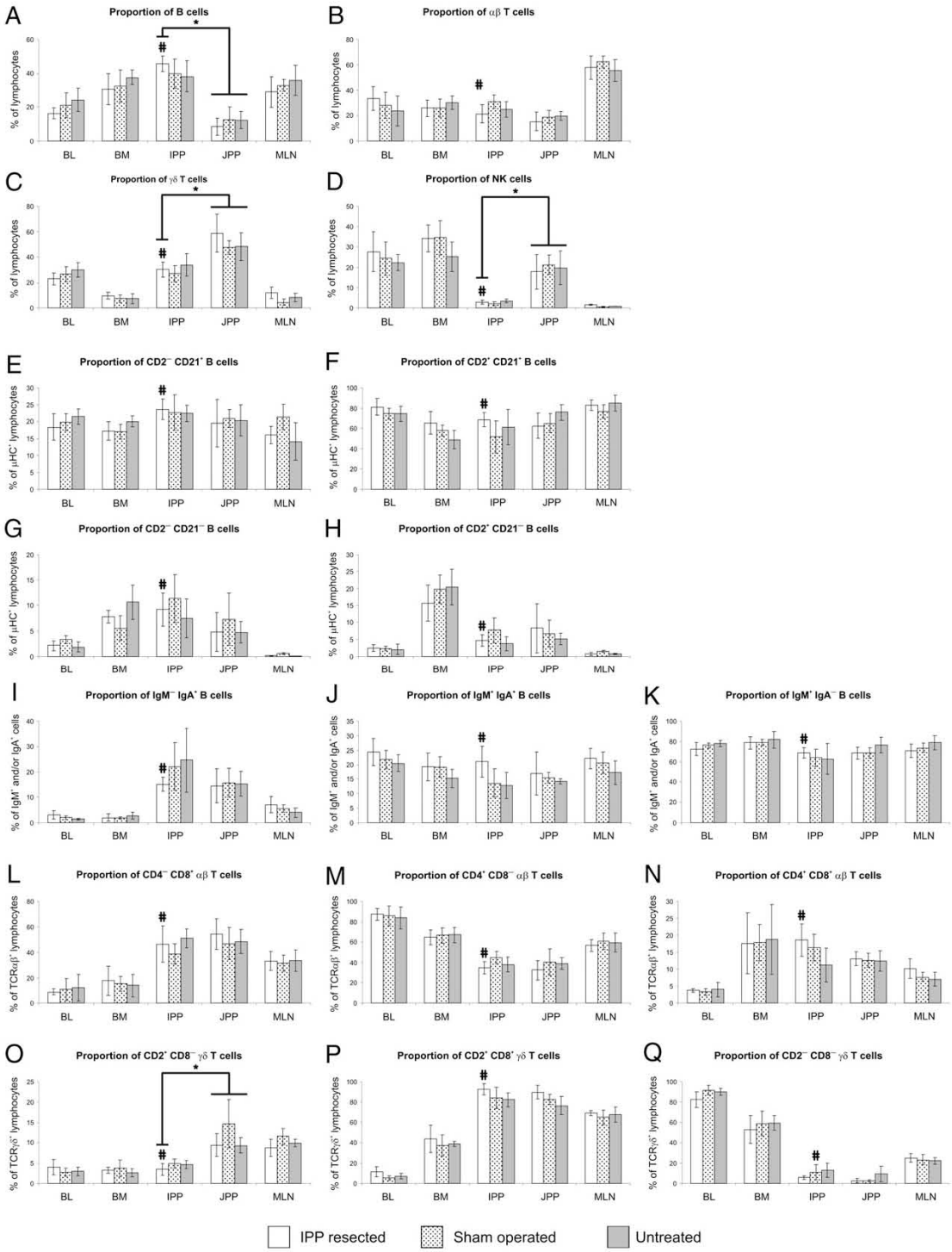

FIGURE 3. The frequencies of lymphocyte types (*A–D*) and their subpopulations (*E–Q*) in different organs of 44-d-old piglets in which IPP were removed by resection (open bars) or were sham operated (dotted bars) at day 2 after birth. Piglets that were not surgically treated are also shown (grey bars). 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

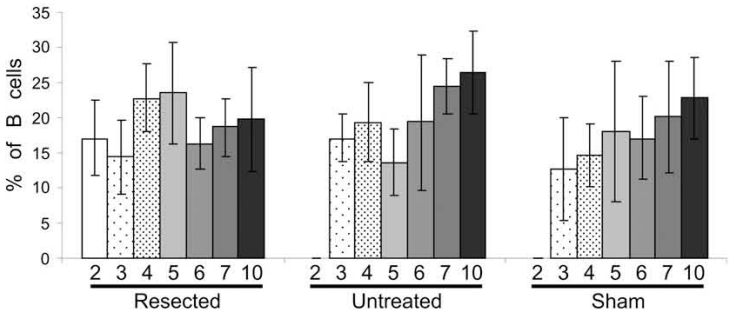

FIGURE 4. The frequencies of B lymphocytes in the blood of IPP-resected, untreated, and sham-operated pigs that were colonized by the defined commensal gut flora (40) at 5 d after birth. Animals were monitored at age 2, 3, 4, 5, 6, 7, and 10 wk (*x*-axis). Numbers of B cells were detected as a proportion 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

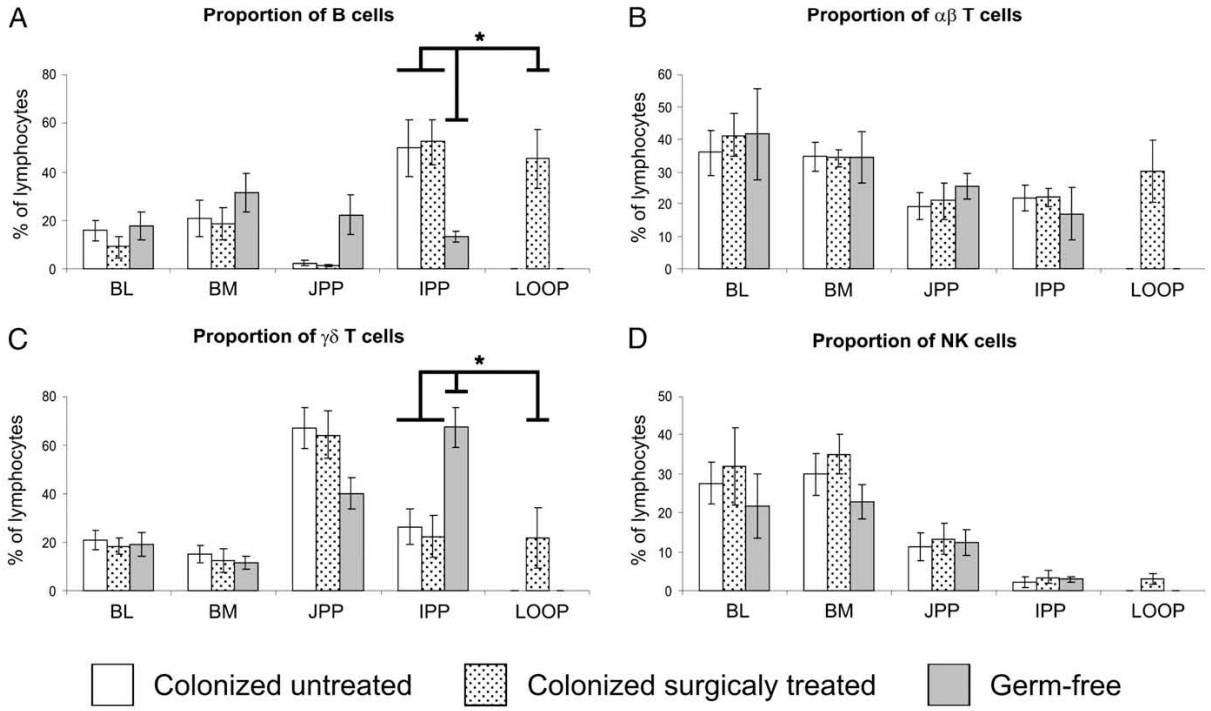

FIGURE 5. Frequencies of lymphocyte types (*A–D*) in different organs of the untreated piglets (open bars) and piglets in which isolated ileal loops were surgically constructed (dotted bars). Both groups were thereafter colonized by the defined commensal gut flora (40) at 5 d after birth. Analysis of 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.

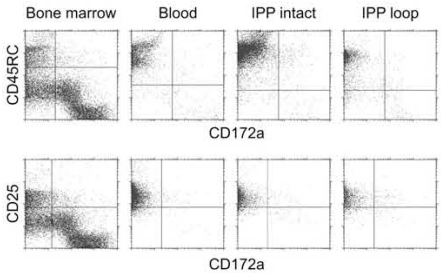

FIGURE 6. Flow cytometry analysis of CD172a, CD45RC, and CD25 expression on MHC-II$^+$ cells. Lymphocytes were prepared from bone marrow (*first column*), blood (*second column*), and IPP (*third column*) of 36-d-old germ-free piglets that were colonized by the defined commensal gut flora (40) at day 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

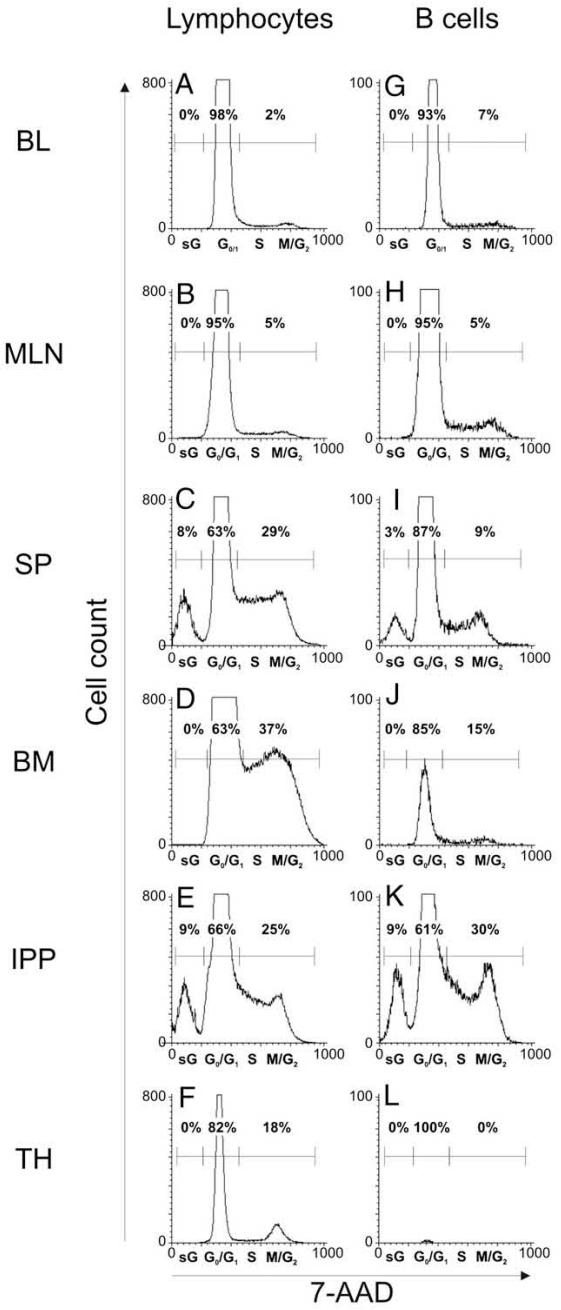

FIGURE 7. Flow cytometry analysis of cell proliferation in different organs. Lymphocytes isolated from the peripheral blood, MLN, spleen, bone marrow, IPP, and thymus of conventional animals were stained with anti-μHC mAb, fixed in 70% ethanol, and the DNA was visualized using 7-AAD. In each staining, 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.

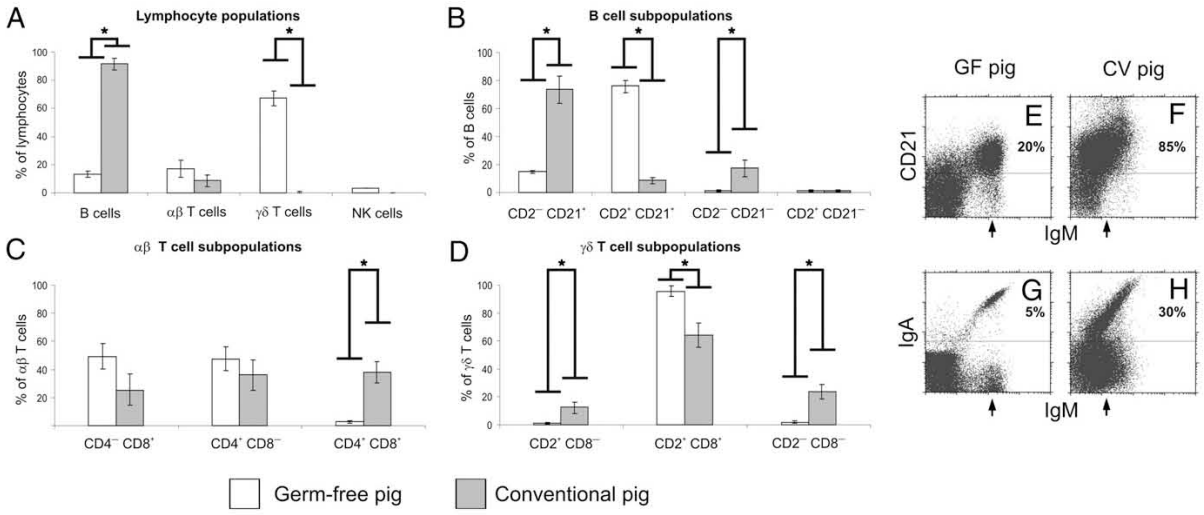

FIGURE 8. The frequencies of lymphocyte types and their subpopulation in IPP of 45-d-old germ-free (open bars) and conventional (grey bars) piglets. The proportions of B, αβ T, γδ T, and NK cells (*A*), CD2/CD21 subpopulations of B cells (*B*), CD4/CD8 subpopulations of αβ T cells (*C*), and CD2/CD8 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.

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5. *(publication #2)*

Antibody repertoire development in fetal and neonatal piglets. XXII. λ rearrangement precedes κ rearrangement during B cell lymphogenesis in swine

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Antibody repertoire development in fetal and neonatal piglets. XXII. λ rearrangement precedes κ rearrangement during B-cell lymphogenesis in swine

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Summary

VDJ and VJ rearrangements, expression of RAG-1, Tdt and VpreB, and the presence of signal joint circles (SJC) were used to identify sites of B-cell lymphogenesis. VDJ, Vλ Jλ but not Vκ Jκ rearrangements or SJC were recovered from yolk sac (YS) at 20 days of gestation (DG) along with strong expression of VpreB and RAG-1 but weak Tdt expression. Vλ Jλ rearrangements but not Vκ Jκ rearrangements were recovered from fetal liver at 30–50 DG. SJC were pronounced in bone marrow at 95 DG where Vκ Jκ rearrangements were first recovered. The Vλ Jλ rearrangements recovered at 20–50 DG used some of the same Vλ and Jλ segments seen in older fetuses and adult animals. Hence the textbook paradigm for the order of light-chain rearrangement does not apply to swine. Consistent with weak Tdt expression in early sites of lymphogenesis, N-region additions in VDJ rearrangements were more frequent at 95 DG. Junctional diversity in Vλ Jλ rearrangement was limited at all stages of development. There was little evidence for B-cell lymphogenesis in the ileal Peyer's patches. The widespread recovery of VpreB transcripts in whole, non-lymphoid tissue was unexpected as was its recovery from bone marrow and peripheral blood monocytes. Based on recovery of SJC, B-cell lymphogenesis continues for at least 5 weeks postpartum.

Keywords: B-cell lymphogenesis; fetal; RAG-1; signal joint circles; Tdt; VpreB.

Introduction

B-cell lymphogenesis take place in the fetal liver (FL) and then in bone marrow (BM) of mice and humans; in both species the process is continuous throughout life.[1,2] In other species the site, duration and features of this process have been less-well studied. In the few cases in which other homeothermic species have been studied, the pattern differs from that in mice and humans. In both hen and rabbit the process is determinant. In rabbit the process essentially terminates after 4 months.[3,4] In the Bovidae the process involves the spleen.[5–7] In some species, full development of the B-cell compartment depends on repertoire diversification in hindgut lymphoid tissues, e.g. the hen bursa, rabbit appendix and the ileal Peyer's patches (IPP) of sheep, leading to the view that higher vertebrates belong to either the BM or 'gut-associated lymphoid tissues' group as regards B-cell development.[8] However, surgical resection of the IPP of piglets does not affect B-cell levels or maintenance or repertoire diversification,[9,10] indicating that swine fit best to the BM group. This report focuses on features at early sites of B-cell lymphogenesis in this BM group mammal.

The use of swine in biomedical research,[11] especially where antibody responses are involved,[12–15] is one method to better understand B-cell lymphogenesis in this species. It was previously shown that VDJ rearrangements first appear in yolk sac (YS) at 20 days of gestation (DG) and later in FL at 30 DG and thereafter in many lymphoid tissues of fetal piglets.[16,17] This criterion for identification of the B-cell lineage is consistent with the detection of putative B-cell precursors in fetal liver and BM.[18] However these studies did not provide convincing evidence that pre-B cells or B cells were actually developed at these sites. The VH gene usage is identical at all of the sites in fetal piglets,[17,19] which could mean that:

(i) B cells are derived from one common site and then disseminated, or (ii) the process follows the same programme at many different sites. In mice, different pathways have been described at both the molecular level[20 22] and the phenotype level.[23] Regarding the latter, two B-cell sub-populations develop at different sites; B-1 cells develop in early fetal life perhaps in the peritoneum whereas B-2 cells develop in late term BM and continuously thereafter in BM. In swine, two populations were only predicted on the basis of the occurrence of in-frame and out-of-frame VDJ rearrangements.[16] B cell lymphogenesis in swine is of interest because this species was originally placed in the "gut-associated lymphoid tissues" category because the continuous IPP of swine are homologous to those in sheep, the latter regarded as primary lymphoid tissue for sheep.[8,24 28]

B-cell lymphogenesis is reviewed in the immunology textbooks. Briefly, the process is recognized by rearrangement of genes coding for the immunoglobulin heavy and light chains that are assembled from germline-encoded V, D and J segments by a series of site-specific recombination events.[29,30] These rearrangements excise circles of intervening DNA that accumulate in the nucleus as signal joint circles (SJC). V(D)J recombination is initiated by the recombination activation gene products, RAG1 and RAG2.[31 34] In the B lymphoid lineage, RAG expression is primarily restricted to developing B cells in the BM.[30,35] There are two distinct waves of RAG expression in BM. The first corresponding to immunoglobulin heavy-chain gene rearrangement at the pro-B-cell stage. The RAG genes are then down-regulated upon expression of a μ heavy chain, and in mice, humans and rabbits these assemble with the surrogate light chain, comprising proteins $\lambda 5$ and VpreB to form the pre-B-cell receptor.[36,37] $\lambda 5$ is homologous to a $J\lambda C\lambda$ product that associates with VpreB to produce a highly charged junction that lacks a conventional CDR3.[38 40] VpreB, which is expressed selectively at the early pro-B and early pre-B cell stages, is also designated CD179, and encodes the immunoglobulin iota chain.[41] Later, the RAG genes are re-expressed in pre-B cells during V-J light-chain rearrangements. In mice and humans, rearrangement in the κ light-chain locus precedes that for λ.[2,42] However, low-level RAG expression is also found in B cells in peripheral lymphoid organs, especially after immunization.[43 46] This re-expression is believed to be the result of receptor editing, which has been most frequently identified with secondary rearrangements in the light-chain loci.[47] In addition, Chun *et al.*[48] reported the detection of low levels of the RAG-1 transcript in the murine central nervous system by PCR, *in situ* hybridization, and Northern blot analyses. However, an authentic RAG-2 transcript could not be reproducibly detected in the central nervous system.

Terminal deoxynucleotidyl transferase (Tdt) is a nuclear enzyme that catalyses the addition of non-templated (N) nucleotides to the free 3′-OH ends of fragmented or nicked DNA.[49] So far, the only known physiological function of Tdt is the random addition of nucleotides to the V(D)J junctions of immunoglobulin heavy-chain and T-cell receptor gene rearrangements[50 53] and rarely at junctions during immunoglobulin light-chain rearrangements.[54 56] The N additions effectively increase diversity of the repertoire of the antigen receptors on B and T cells.

Data presented summarize the expression of RAG, Tdt, VpreB and the presence of SJC in DNA and VJ rearrangements in light-chain loci. Using semi-quantitative PCR, we show that only λ rearrangements are present in YS and FL meaning that $V\lambda J\lambda$ rearrangement precedes that for $V\kappa J\kappa$ in this species by at least 30 days. Furthermore we show that especially VpreB can be widely recovered including from non-lymphoid tissues and monocytes. Robust B-cell lymphogenesis in BM appears to be limited to early postnatal life.

Materials and methods

Experimental animals

Animals used in the study included: (i) pregnant gilts procured from certified suppliers as previously described;[57] (ii) Minnesota miniature/Vietnam-Asian-Malaysian crossbred piglets bred in Novy Hradek;[58] (iii) isolator piglets reared as previously described[59,60] and (iv) conventionally reared young and adult pigs.[19] All pigs were healthy and normal at necropsy. All animal experiments were approved by the National Animal Disease Center Institutional Animal Care and Use Committee (NADC-IACUC) and the Ethical Committee of the Institute of Microbiology, Czech Academy of Science, according to guidelines in the Animal Protection Act and housed according to NADC IACUC Guidelines.

Collection of animal tissues for transcript studies

At 20, 30, 50 and 95 DG, pregnant gilts were killed, and tissues from at least five fetuses were recovered. These included YS at 20 DG, FL at 30 and 50 DG and BM, spleen and IPP at 95 DG. BM was recovered from long bones of fetal, isolator and older conventional pigs after removal of cartilaginous ends, extrusion with saline and preservation in TriZol or DNAZol (Invitrogen, Carlsbad, CA). Placenta and uterus were obtained from gilts as negative control tissues.

Preparation of cell suspensions for flow cytometry

Cell suspensions for flow cytometry were prepared as previously described.[61 63] Briefly, heparinized (20 U/ml) blood was obtained by intracardial puncture. Leucocytes from the BM were isolated by washing femur contents

with PBS. Erythrocytes from all suspensions were removed using hypotonic lysis and washed twice in cold PBS. 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, all chemicals Sigma-Aldrich, St Louis, MO), filtered through a 70-μm mesh nylon membranes and cell numbers were determined by haemacytometer. Recovery of leucocytes sufficient for flow cytometry at 20–50 DG was not possible using current technology.

Flow cytometry and cell sorting

A variety of mouse anti-pig monoclonal antibodies were used (see Supplementary material, Table S1). Goat polyclonal antibodies specific for mouse immunoglobulin sub-classes labelled with fluorescein isothiocyante (FITC), phycoerythrin (PE) or allophycocyanin were used as secondary immunoreagents (Southern Biotechnologies Associates, Inc., Birmingham, AL). Staining of cells for flow cytometry was performed as described previously by indirect sub-isotype staining.[62,63] Briefly, multi-colour staining was performed using cells that had been incubated with a combination of three primary mouse monoclonal antibodies of different sub-isotypes. Cells were incubated for 15 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary polyclonal antibodies specific for mouse immunoglobulin sub-classes that had been labeled with FITC, PE and allophycocyanin conjugates were then added to the cell pellets in appropriate combinations. After 15 min, cells were washed three times in PBS-GEL and analysed by flow cytometry.

Samples were measured or sorted on a FACS Calibur or a FACS AriaIII flow cytometer (BDIS, Mountain View, CA). In each measurement, 300–700 thousand events were collected. Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. The PCLysis software (BDIS, Mountain View, CA) was used for data processing.

Preparation of DNA, cDNA and gene-specific PCR

Tissues that had been stored in liquid nitrogen were pulverized and their DNA was extracted into DNAZol as previously described.[17] RNA was extracted from the same tissues and treated with DNase for two rounds for 30 min at 37° and then converted to cDNA.[17,59] The effectiveness of DNase treatment in removing contaminating DNA from RNA was tested. Briefly, we tested whether VDJ rearrangements could be recovered by PCR from RNA before and after DNase treatment (see Supplementary material, Fig. S1). Recovery of VDJ from DNase-treated RNA would indicate DNA contamination.

Selection of gene-specific PCR primers

Sequences were recovered by PCR amplification using the primers listed in the Supplementary material (Fig. S2). Primers used to recover VDJ, VκJκ and VλJλ rearrangements have been previously described.[64 66] Primers for amplification of RAG-1 were specific for the unique core sequence[67] that were found in nine different species; five are compared in the Supplementary material (Fig. S3a). The region amplified shares 85% homology among these species. Primers used for recovery of porcine VpreB were based on the sequence alignment of porcine VpreB with that of seven other species including mouse and human.[60] The per cent homology referenced to pig is given. Similar criteria were used for the generation of primers for porcine Tdt. Primers for SJC generated in D-J and V-DJ rearrangements were based on recent genomics map data.[68] Primer sequences and the size of the expected product are summarized in the Supplementary material (Fig. S2) and the latter are confirmed by agarose electrophoresis.

Rationale for semi-quantitative PCR

Using the primers described in the Supplementary material (Fig. S2), products of the expected size were recovered (Figs 1, 2 and 3). In the case of SJC, multiple products are possible because swine have two functional DH segments (DHA and DHB) and one functional JH.[68,69] The V-DHA product of 309 bp and the DHB-JH product (370 bp) were most pronounced (Fig. 3). Distinguishing the products is best seen in agarose gels, not by quantitative PCR. Concatemers of VDJ are also commonly seen at higher concentrations (Fig. 1). The doublet produced by VλJλ amplification results from using a mixture of plasmid DNA that represents the two major porcine Vλ families, each of which uses a different leader sequence producing products that differ by 60 nucleotides (Fig. 2). Again we consider it valuable to observe the nature of the products.

The PCR assays were conducted using a five-fold dilution sequence of the target DNA using an intial DNA concentration of 50 ng. Titrations were directly visualized on agarose gels (Figs 1, 2 and 3). We preferred this approach for its direct value, because there are no established quantitative PCR protocols for the genes or transcripts studied. In the case of VDJ and SJC recovered from DNA, titration end-points were normalized to those for VDJ recovery in the same sample based on the premise that the amount of VDJ product reflects the number of B or pre-B cells (Table 1A). In the case of transcripts we provide only titration end-points because differences in copy number cannot be normalized.

$$\text{Normalized end-point} = \frac{\text{SJC end-point} \times 100}{\text{VDJ end-point}}$$

Figure 1. Comparative titration of products after two rounds of PCR for VκJκ and VλJλ in 30 days of gestation (DG) fetal liver (FL), 20 DG yolk sac (YS), 95 DG spleen (SP) and 95 DG bone marrow (BM). Note that there is no product in YS and FL but it is recovered at 95 DG.

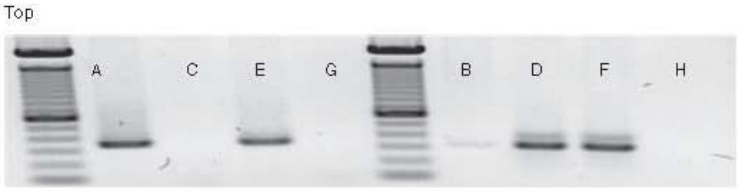
β-actin is a relatively stable cytoskeletal protein generally thought to be present at some level in most cells, although there may be differences among cell types.[70] Our data are derived from tissues comprised of many cell types, so β-actin was used only as an internal control for positive transcriptional activity.[71–73]

Assays for VκJκ and VλJλ rearrangements

To validate the specificity and sensitivity in the recovery of rearrangements in the κ and λ loci, we conducted studies using plasmid DNA that contained VκJκ and VλJλ rearrangements. Assays were performed using equal amounts of κ and λ plasmid DNAs. Results were obtained using the semi-quantitative PCR system described and are given in Figs 1,2 and 3 and in Table 1).

Analysis of junctional diversity in VDJ and VλJλ rearrangements

Junctional diversity for VDJ rearrangements was established by assigning CDR3 to the region between the arginine codon at the 3′ end of VH and the tryptophan (W) codon in JH. The gap determined the length, and changes within were recorded as 5′ and 3′ additions and D_HA and D_HB usage. In the case of VλJλ rearrangement, CDR3 was defined as the region between the ultimate cysteine of Vλ and the phenylalanine of Jλ.[74] Assignment



Plasmid Used	Primer set used	
	kappa	lambda
kappa	A	B
lambda	C	D
Kappa + lambda	E	F
H_2O	G	H

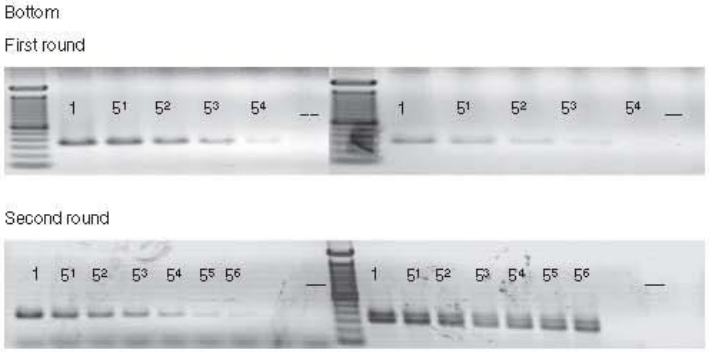


Figure 2. The specificity and sensitivity of the PCR amplification of rearranged VκJκ and VλJλ. Top: Specificity of primers for λ and κ when applied to plasmids containing λ and κ or a mixture (κ + λ). Lanes labelled A, B etc are explained below Fig. 1(a). Bottom: Sensitivity of PCR assays for VκJκ (left) and VλJλ (right) determined by titration. Right lanes on each gel (marked with) are reagent blanks.

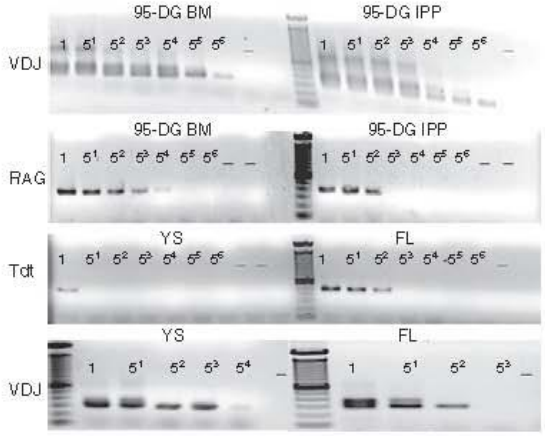


Figure 3. Example of the titration analysis of PCR products from four different lymphoid tissues. The titration of the VDJ product from the same tissue was performed in parallel with that for RAG 1 and TdT. The secondary (larger) PCR product seen when the VDJ product is used at low dilutions is the result of concatemers. YS, yolk sack; FL, fetal liver; BM, bone marrow at 95 days of gestation (DG); IPP, ileal Peyer's patches at 95 DG.

of mutations was based on reference to the consensus sequence of the V_H or $V\lambda$ gene used.

Results

λ locus rearrangement precedes κ in swine

In an earlier report, we presented data showing that λ transcripts were detected before κ transcripts in fetal piglets.[66] At that time we suggested the possibility that recovery of λ represented expression of $\lambda 5$ because in mice and humans rearrangements in the κ locus occur before those in the λ locus.[42] Here we show that in YS and FL, only $V\lambda J\lambda$ rearrangements were recovered whereas $V\kappa J\kappa$ rearrangements were first seen at 95 DG (Fig. 1, Table 1). Periodic samples between 50 and 95 DG were not collected or examined so the actual time when the $V\kappa J\kappa$ rearrangement appears was not pinpointed. This surprising early appearence of λ before κ of course raised a question about the specificity and sensitivity of the PCR assays used to recover $V\lambda J\lambda$ and $V\kappa J\kappa$. We addressed this in an experiment shown in Fig. 2 by showing that both primers sets are specific and that after one or two rounds of PCR, sensitivity is equal. Even the small difference in sensitivity (if it exists) cannot explain the complete absence of $V\kappa J\kappa$ in YS and FL whereas the end-point titre for $V\lambda J\lambda$ is 625 (Table 1B). To suggest this could rest on whether plasmids versus cDNA were used does not explain the ease of recovering $V\kappa J\kappa$ rearrangements in 95-DG BM or IPP (Table 1B). Given these observations and controls, we believe that our data confirm that somatic rearrangements in the λ locus precede those in the κ locus; this is the opposite of what is seen in mice and humans.

Table 1. PCR titration end point. (A) PCR titration end points* and end point ratio for signal joint circles and VDJ rearrangement. (B) PCR titration end points* from transcripts

		End point			End point ratio†	
(A)	Age	VDJ	VD	DJ	VD	DJ
BM	95DG	625	125	125	20	20
5W BM	5 week	625	125	5	20	0.8
IPP	95DG	625	1	0	0.16	0
5W IPP	5 week	625	5	0	0.8	0
Spleen	95DG	3125	5	0	0.8	0
Thymus	95DG	625	5	5	0.8	0.8
FL	50DG	15625	1	25	6.4e ^{3}	0.16
YS	20DG	15625	0	25	0	0.16

(B)	Age	VDJ	RAG 1	Tdt	VpreB	β actin‡	$V_\kappa J_\kappa$	$V_\lambda J_\lambda$
Fetal BM	95DG	15625	625	625	625	15625	625	15625
5 week BM	5 week	625	25	25	625	15625	125	625
Fetal IPP	95DG	15625	25	1	625	625	125	3125
5 week –IPP	5 week	125	625	125	25	125	125	5
Fetal spleen	95DG	625	25	5	15625	15625	25	125
Placenta	112§	125	1	1	3125	625	25	25
Fetal thymus	95DG	625	15625	625	625	3125	25	25
Uterus	1124	25	25	1	25	1	0	0
YS	20DG	3125	25	1	3125	15625	0	625
FL	50DG	3125	5	25	625	625	0	625

*The end point values based on two or three replicates.
†End point ratios compared with VDJ; see Materials and methods.
‡Used as evidence for successful preparation of cDNA, because the β actin segment amplified crosses an intron (see Supplementary material, Fig. S2).
§Collected from sows during Caesarean delivery.

As the primers used to recover the λ product used anti-sense $J\lambda$, the products recovered in YS and FL are authentic rearrangement products from the λ locus, not conventional $\lambda 5$. To confirm this we cloned and sequenced various $V\lambda J\lambda$ rearrangement from early fetuses and found them to be diverse like those in our panel of ~ 300 $V\lambda J\lambda$ rearrangements obtained from various tissues and animals of different age (Fig. 4; J. Vasquez, K.L. Wells, N. Wertz, J. E. Butler, unpublished). In a sample of seven $V\lambda J\lambda$ sequences from 20-DG YS, four different $V\lambda$ genes and two different $J\lambda$ genes were used. We highlighted (using a box) an example recovered from 20-DG YS containing a $V\lambda$ gene frequently found in older fetuses and adults (Fig. 4). Data show that there are no unique features of the $V\lambda J\lambda$ sequences recovered from 20–50-DG samples, such as CDR3 length, additions or deletions. However all early $V\lambda J\lambda$ rearrangements used the $V\lambda 8$ family (IGLV8; J. Vasquez, K.L. Wells, N. Wertz, J.E. Butler, unpublished).

Vλ	FR 1	CDR1	FR 2	CDR2	FR3	CDR3	FR4
				A			
8-2	IQEPAMSVSPGGTVTLTCAFN	SGSVTASNY	PGWYQQTPGQPPRQLIY	DTN	NRPAGVPSRFGGAISANKATLRITGAQAEDEADYYC	ALEKSSA LI	FGGGTHLTVL
8-3	IQEPAMSVSLGGTVTLTCAFS	SGSVTDSNW	PGWFQQTPGQPPRTLIY	QTN	NRPNGVPSRFSGAISGNKATLTITGAQAEDEADYFC	ALWKSCMD VP	FGGGTHLTVL
8-4	IQEPAMSVSLGGTVTLTCAFS	SGSVTTSDY	PSWFQQTPGQPPRLLIY	RTN	NRPTGVPSRFSGSISGNKAALTITGAQADDEADYFC	TLSKSGGN VP	FGGGTHLTVL
8-4	IQEPAMSVSLGGTVTLTCAFS	SGSVTTSDY	PSWFQQTPGQPPRLLIY	RTN	NRPTGVPSRFSGYISGNKVALTITGAQAKDEADYLC	SLYKTVF TI	FGGGTHLTVL
8-4	IQEPAMSVSLGGTVTLTCAFS	SGSVTTTNY	PGWFQQTPGQPPRLLIY	RTN	NRPTGVSSRFSGAISGNKAALTITGARANDEADYFC	SLNKATAN AI	FGGGTHLTVL
8-5	IQEPAMSVSLGGTVTLTCAFS	SGSVTSSNY	PGWFQQTPGQPPRQLIY	STN	SRPTGVPSRFSGAISGNRAALTITGAQAEDEADYFC	VLYKSST NI	FGGGTHLTVL
				B			
8-4	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	LSWFQQTPGQPPRLLIY	RTN	SRPTGVPSRFSGAISGNKAALTITGAQAKDEADYFC	TLYKSS LI	FGGGTHLTVL
8-4	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRLLIY	RTN	SRPTGVPSRFSGAISGNKAALTITGAQAKDEADYFC	TLYKSSA NI	FGGGTHLTVL
8-4	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRLLIY	RTN	NRPTGVPSRFSGAISGNKAALTITGAQANDEADYFC	TLYKSSAK VP	FGGGTHLTVL
8-4	IQEPAMSVSLGGTVTLTCAFS	SGSVTSSNY	PSWFQQTPGQPPRTVIY	RTN	SRPTGVPSRFSGAISGNKATLTITGAQAEDEADYFC	ALEKSGAT VI	FGGGTHLTVL
8-4	IQEPAMSVSLGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRLLIY	RTN	SRPTGVPSRFSGAISGNKAALTITGAQAKDEADYFC	TLYKSSAN VP	FGGGTHLTVL
8-4	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRQLIY	STN	NRPTGVPSRFSGAISGNKAALTITGAQAEDEADYFC	ALYKSSANVVP	FGGGTHLTVL
				C			
8-4	IQEPAMSVSLGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRLLIY	RTN	NRPTGVPSRFSGAISGNKAALTITGAQANDEADYFC	TLYKSSAN VP	FGGGTHLTVL
8-4	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRLLIY	RTN	NRPTGVPSRFSGAISGNKAALTITGAQANDEADYFC	TLYKSSA NI	FGGGTHLTVL
8-5	IQEPAMSVSLGGTVTLTCAFS	SGSVTSSNY	PGWFQQTPGQPPRTVIY	STN	SRPTGVPSRFSGAISGNKATLTITGAQAEDEADYFC	ALYKSC SI	FGGGTHLTVL
8-5	IQEPAMSVSLGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRLLIY	YTN	SRPTGVPSRFSGAISGNKATLTITGAQAEDEADYFC	ALYKSC TN	FGGGTHLTVL
8-5	IQEPAMSVSLGGTVTLTCAFS	SGSVTSSNY	PGWFQQTPGQPPRTVIY	STN	SRPTGVPSRFSGAISGNKATLTITGAQAEDEADYFC	ALYKSCT NI	FGGGTHLTVL
8-5	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRQLIY	STN	NRPTGVPSRFSGAISGNKAALTITGAQAEDEADYFC	ALYKSN NI	FGGGTHLTVL
8-5	IQEPAMSVSPGGTVTLTCAFS	SGSVTTSNY	PSWFQQTPGQPPRQLIY	STN	NRPTGVPSRFSGAISGNKGALTITGAQAEDEADYFC	ALYKSS NI	FGGGTHLTVL
λ5	IQEPAMSVSLGGTVTLTCAFS	SGSVTSSDY	PGWFQQTPGQPPRTVIY	STN	SRPTGVPSRFSGAISGNKATLTITGAQAEDEADYFC	ALRKSGGT VT	FGGGTHVTVL

Figure 4. The sequence of Vλ*J*λ rearrangements recovered from 20-days of gestation (DG) yolk sac (YS), 50-DG fetal liver (FL) and adult peripheral blood mononuclear cells (PMBC). The boxed sequence is an example of a rearrangement that is found in all three groups. Changes in 3′ Vλ in CDR3 from the consensus for each Vλ gene are underlined. There were no deletions from Jλ. The Vλ genes are designated according to those used in a submitted manuscript (J. Vasquez, K.L. Wells, N. Wertz, J. E. Butler, submitted for publication). Also shown is the λ5-like sequence reported in GenBank accession NM_001243319.

High levels of SJC were only recovered from the BM of fetal and young pigs

The SJC are indicators of ongoing B-cell development. These were only recovered in large amounts from the BM of fetuses and young pigs (Table 1A). When normalized to the level of VDJ rearrangements in the same tissue, values of 20 were obtained. VD-SJC were recovered in small amounts from all lymphoid tissues except YS but their relative levels were < 1 (Table 1A). DJ-SJC were also recovered from fetal thymus, YS and FL. Although VDJ rearrangements were readily recovered from YS and FL, recovery of SJC was relatively poor. The failure to recover VD-SJC from YS was notable; SJC could also not be recovered from the BM of adult swine (data not shown).

B-cell lymphogenesis in the IPP is weak or absent during fetal and neonatal life

In spite of the observation that RAG-1, Tdt and VpreB are transcribed in the IPP, when results are normalized to SJC : VDJ ratios, there is little evidence to support the role of this tissue as a site of B-cell lymphogenesis. This is in agreement with data reported elsewhere that involved surgical resection of the IPP of newborn piglets to show that this tissue has no effect on B-cell development or maintenance of B-cell levels.[9,10]

Expression of RAG-1 and VpreB transcripts does not fit expectations

Recovery of transcripts for RAG-1 and VpreB was unexpected from non-lymphoid tissues. RAG-1 was expressed in nearly all tissues including uterus but not placenta (Table 1B). The same was true for VpreB, but high levels were also recovered from placenta. The recovery of RAG-1 transcripts from fetal thymus was expected because it is also required for T-cell development. Recovery of β-actin confirmed successful preparation of cDNA. These unexpected results prompted the sorting of leucocytes followed by testing for VpreB, Tdt and RAG (Fig. 5a,b). The transcripts for VpreB, Tdt and RAG-1 were found in sorted cells that are putative precursors of B cells in the BM (Fig. 5c). These progenitors express low levels of VDJ rearrangements (Fig. 5c) compared with mature BM B cells (Fig. 5d) but no κ light-chain rearrangement (Fig. 5c). Interestingly, we found that VpreB transcripts could be found in sorted monocytes (Fig. 5b). As expected, there was no VDJ or VJ rearrangement in these monocytes (Fig. 5b). Weak expression of VpreB and RAG was also detected in mature BM B cells (Fig. 5d) but polymorphonuclear cells did not express any VDJ or VJ rearrangements (Fig. 5b). These results indicate that in sorted BM sub-populations, VpreB, Tdt and RAG transcripts are present in the developing B-cell lineage and in monocytes.

Because we detected VpreB and RAG transcripts in non-lymphoid tissues like the uterus and placenta (Table 1B), we wondered whether transcripts for these could be recovered from peripheral blood leucocytes. Subpopulation of adult blood cells were sorted by flow cytometry and inspected for the presence of transcripts. Figure 5(e–h) shows that VpreB cannot be detected in sorted mature B cells although there is weak expression of RAG in the cells (Fig. 5f). Like BM, sorted monocytes contain VpreB

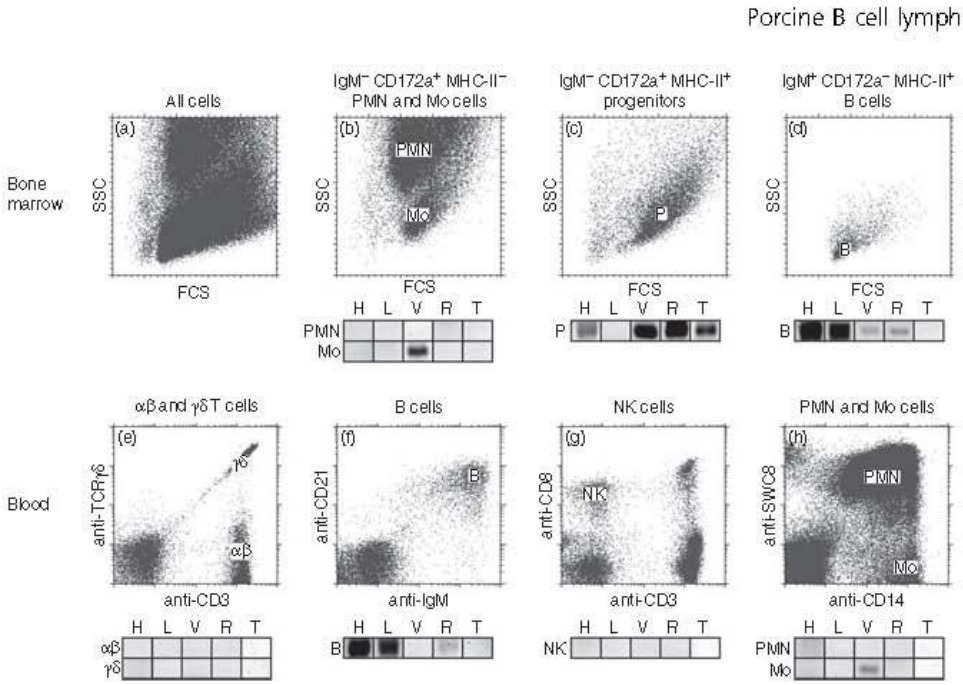


Figure 5. Analysis of transcripts from flow cytometry sorted cells prepared from newborn bone marrow (a–d) and adult blood (e–h). The bone marrow cells (dot plot a) were sorted according to scatter characteristic and expression of IgM, CD172a and MHC II into polymorphonuclear IgM⁻ CD172a⁺ MHC II⁻ cells [polymophonuclear cells (PMN) in dot plot b], monocytes (Mo in dot plot b), IgM⁻ CD172a⁺ MHC II⁺ B cell progenitors (P in dot plot c), and IgM⁺ CD172a⁻ MHC II⁺ mature B cells (B in dot plot d). A similar approach was used for sorting of blood stained by monoclonal antibodies against CD3, TCR$\gamma\delta$, IgM, CD21, CD8, CD14 and SWC8. In this case, $\gamma\delta$ **T** cells ($\gamma\delta$ in dot plot e), $\alpha\beta$ **T** cells ($\alpha\beta$ in dot plot e), mature B cells (B in dot plot f), natural killer cells (NK in dot plot g), polymorphonuclear cells (PMN in dot plot h), and monocytes (Mo in dotplot h) were sorted. All sorted populations were lysed in TRIzol and their RNA/cDNA preparations were inspected by reverse transcription PCR for presence of transcripts (see below each dot plot) for VDJ heavy chain rearrangement (**H**), VJ light chain rearrangement κ (L), VpreB (V), RAG 1 (R) and TdT (**T**). Note that amplification of VJ rearrangement for κ gave the same output as for λ. Results are representative of three independent experiments.

transcript but no detectable VDJ and VJ (Fig. 5h). Other cell populations like $\alpha\beta$ and $\gamma\delta$ T cells (Fig. 5e), natural killer cells (Fig. 5g) or polymorphonuclear cells (Fig. 5h) do not contain any transcripts characteristic of developing or mature B cells.

N-region additions in heavy-chain VDJ rearrangements are more frequent at DG 95

The low expression of Tdt in 20-DG YS, but higher expression in fetal BM (Table 1A), prompted us to ask whether the number of N-region additions in 71 CDR3 sequences from VDJ rearrangements from 20-DG YS, 30- and 50-DG fetal liver was different from that in BM, IPP and spleen from 95-DG fetuses. The 33 sequences from 95-DG animals were treated as one group (called mix) because differences between tissues were not significant. Data show that total N region additions were significantly greater at 95 DG than in FL and YS, especially in terms of 5′ additions (Table 2). However, the usage of DHA and DHB was similar at all time-points.

Table 2. The number of N region additions in 71 CDR3 sequences from 20 days of gestation (DG) yolk sac (YS), 30 DG and 50 DG fetal liver (FL) and from bone marrow, spleen and ileal Peyer's patches from 95 DG piglets (Mix)

Tissue	*n*	5′Add	D_HA/D_HB	3′Add	5′Add + 3′ Add
20 DG YS	11	3.0 ± 2.0	6/5	6.6 ± 5.2	9.6 ± 5.2
30 DG FL	12	4.0 ± 2.0	6/6	9.8 ± 6.8*	9.8 ± 6.01
50 DG FL	15	3.0 ± 3.0	6/9	8.7 ± 6.4	8.7 ± 7.64
95 DG (Mix)	33	7.0 ± 8.0†	19/14	12.0 ± 8.3*	18.6 ± 11.0†

*Significantly higher than 20 DG YS.
†Significantly higher than 20 DG YS, 30 DG FL and 50 DG FL.

CDR3 diversity in VλJλ rearrangements

Figure 4 reveals that CDR3 diversity in VλJλ rearrangements is small and N-region additions are seldom seen. The frequency of N-region additions in VλJλ rearrangements pales in comparison to events in the heavy chain. This same difference between junctional diversity in

heavy- and light-chain rearrangements has been reported by others.[74 77]

Discussion

B-cell lymphogenesis in mice and humans is continuous throughout life in BM,[1,2] which differs from the determinant process described for hen and rabbit; the process ceasing at birth or shortly thereafter.[3,4] Knowing whether the process is continuous or determinant in swine may have implications for animal health because artiodactyls comprise 50% of all mammals and are an important food and agricultural species. This is the first study in which the identification of B-cell lymphogenesis was based on using markers that are standard in mice, humans and rabbits. In addition to factors required for somatic rearrangement, we used a product dilution assay to quantify rearranged VDJ, VκJκ and VλJλ at different times during development. As the study involved tedious technology based on PCR and flow cytometry, we have supplied some of the technical details in the Supplementary material.

Titration results for SJC were expressed relative to the recovery of VDJ, the latter was chosen as a measure of the total B cells or pre-B cells in the sample. In the case of cDNA, such normalization could not be justified because of differences in the rate of transcription of different genes that can result in large differences in copy number. Hence, only PCR titration end-points are of use.

Our findings confirm an earlier report that VDJ rearrangements are present in YS at 20 DG.[16] However, when compared with BM, recovery of VD SJC is conspicuously low and this observation extends to FL. This might suggest that active rearrangement primarily involves DJ at this stage of B-cell lymphogenesis. However this interpretation leaves unexplained why full VDJ rearrangements are recovered from this tissue. Perhaps the rate of rearrangement is very low and as DJ rearrangements have been reported to occur simultaneously on both alleles,[29,78] V-DJ SJC would be present at lower levels than DJ SJC. As all SJC are rapidly degraded, a slow rate of B-cell lymphogenesis in YS might also explain our results and also our inability to recover SJC from adult BM. Therefore, B-cell lymphogenesis in these tissues may not be absent, but occurs at a frequency too low to be detected by the methods we have employed.

Although Tdt is expressed in YS, its expression is considerably lower than in fetal BM at 95 DG. To address whether this might be the result of differences in transcript copy number versus function, we looked for the effect of Tdt on N-region additions. We show a progressive increase in N-region additions in VDJ rearrangements with fetal age (Table 2) consistent with the pattern reported for mice and humans.[79] Hence the ease of recovery of Tdt transcripts from fetal liver may reflect the presence of alternative transcripts that do not have transferase activity. However, we found little evidence for N-region additions in VλJλ rearrangement regardless of age and consistent with other reports.[75 77] The preferential appearance of VλJλ rearrangements as early as 20 DG in YS and its continuance in 30 and 50-DG FL before the appearance of VκJκ rearrangements in BM at DG 95 (Fig. 1) clearly differs from the pattern seen in mice and humans.[42] It should be remembered that in many ungulates like cattle and the horse, > 90% of all immunoglobulins use λ light chains even though they possess a functional κ locus.[80] Early appearence of VλJλ rearrangement in the spleen of fetal sheep has also been reported.[7] Furthermore, vertebrates like hen, use only λ. We show that the VλJλ rearrangements recovered at 20 DG contained known V and J elements, making them authentic rearrangements, and contained the same spectrum of Vλ and Jλ segments that is seen in older animals (Fig. 4). In fact, four different Vλ and two different Jλ segments are represented in just seven sequences. The control studies we conducted eliminated the possibility that our results reflect differences in the specificity of primers used or the sensitivity of the respective PCRs (Fig. 2). Interestingly, when we submitted our sequence data for the VλJλ product recovered from 20-DG YS to a BLAST search, it was identified as a λ5-like sequence in GenBank (http://www.ncbi.nlm.nih.gov/projects/genome/guide/pig/). This reported "λ5" was in fact a VλJλCλ transcript and was identical to a common member of the porcine Vλ genes in our panel of ~300 Vλ genes (Fig. 4; Vasquez *et al.* unpublished). The annotated sequence was simply a rearranged λ light chain and not conventional λ5. No conventional λ5 sequence has yet been reported for swine.

One heretical explanation for our findings is that a conventional surrogate light chain is absent in swine. Rather, swine and perhaps other ungulates that use a predominance of λ light chains, may proceed directly to the use of authentic VλJλ rearrangements. That said, we offer no evidence for the absence of a surrogate light chain in swine. If it exists, it clearly does not involve the λ5-like element annotated in GenBank unless λ5 in swine is different from that in mouse.

Our most recent studies have shown that swine (and sheep) do not belong to the "gut-associated lymphoid tissues" category of higher vertebrates as has been proposed.[8] Hence, the increase in RAG-1 and Tdt expression (Table 1B) in the IPP of 5-week-old piglets may suggest that RAG-1 and Tdt are being re-expressed perhaps as part of receptor editing. We addressed this issue by normalizing titrations for SJC to the titration of rearranged VDJ (Table 1A). DJ-SJC were not recovered in IPP and VD-SJC were recovered 125-fold less frequently than in BM. This finding favours the interpretation that expression of RAG-1 in the IPP of 5-week-old piglets

is associated with receptor editing/revision not B-cell lymphogenesis.

The recovery of VDJ rearrangements in thymus was previously observed.[81] The thymus of newborn calves and piglets contains B cells and synthesizes immunoglobulin.[82 84] Whereas the number of B cells in the medullary and cortex areas is quite small[85] the subcapsular region is rich in cells believed to be of the B-cell lineage. It is therefore likely that some lymphocyte stem cells that populate the thymus start in the direction of B-cell lymphogenesis and these could explain the recovery of thymic SJC.

The expression of RAG-1 and especially VpreB in non-lymphoid tissues was unexpected based on paradigms of B-cell lymphogenesis that were primarily established using laboratory rodents.[2] A careful analysis of the core sequence for RAG-1 that was amplified in the PCR assay we employed was highly conserved among the five species compared (see Supplementary material, Fig. S3A). The sequence amplified for VpreB was not found in any genes submitted to GenBank other than those for VpreB (see Supplementary material, Fig. S3B). Hence, recovery of transcripts for VpreB and RAG-1 should reflect transcripts of authentic RAG-1 and VpreB and not other genes. After two successive rounds of DNase treatment before the preparation of cDNA, data showed that VDJ-IgM or VκJκ could not be amplified, indicating that contaminating DNA was absent (see Supplementary material, Fig. S1). Furthermore we tested whether these markers of active B-cell lymphogenesis were transcribed in isolated leucocytes from BM and peripheral blood. For these reasons we sorted different subpopulations to test whether VpreB, RAG and Tdt are expressed in other than developing B cells. Results indicate that VpreB can be expressed at least in monocytes and probably in their macrophage progenies. Transcripts may also be found in other non-B cells that were not included in analyses. Studies show that macrophages may contain partial DJ rearrangement.[86] This is probably because they share a common lymphoid precursor.[87,88] On the other hand, Tdt and RAG can be expressed not only as a consequence of T-cell and B-cell development in primary lymphoid organs but also because of receptor editing, which can take place anywhere in the periphery.[47,89] This might explain our findings of weak expression of RAG in mature B cells sorted from the blood.

The enigma surrounding VpreB is more complicated. Based on sequence homologies (see Supplementary material, Fig. S3B) it is difficult to explain our results as some spurious PCR artifact. Given the heretical possibility that there is no Pre-B-cell receptor in swine, the tissue-wide expression of VpreB may have more global implications, which suggests that VpreB expression is not restricted to B-cell development. As we have been unable to identify previous studies that have employed PCR to recover transcripts for these genes from solid tissues, we believe that it remains an issue that needs further investigation in all species.

Our results suggest that expression of VpreB, Tdt and RAG transcripts are unreliable indicators of B-cell lymphogenesis in swine. We bypassed criticism of quantitative PCR by providing raw, visual titration data (Figs 1, 2 and 3). Nevertheless we encountered unexpected results for VpreB, RAG-1 or Tdt in many situations. By contrast, data that we report on SJC or light-chain rearrangement is not subject to the same criticism because it comes from measuring actual rearrangement events that occur in the DNA and cannot be skewed by concerns over transcript copy number or whether these transcripts are functional or whether quantitative PCR is reliable.

We conclude that in swine B-cell lymphogenesis begins in the YS, and in agreement with mouse and human studies, heavy VDJ rearrangements show fewer N-region additions in the early stages of development than later in fetal life and that light-chain rearangement shows little junctional diversity. By contrast, light-chain rearrangement begins in the λ locus in this species, continues in FL, meaning 75 days earlier than when κ rearrangements appear in late fetal life. The pattern of B-cell lymphogenesis in late fetal BM, which is associated with pronounced recovery of SJC, continues postnatally at least for 5 weeks after birth but is questionable in older animals. Our findings suggest that swine may resemble the rabbit in the lack of continuous B-cell lymphogenesis, do not belong to the gut-associated lymphoid tissues group[8] but perhaps belong to a group of mammals such as artiodactyls in which λ light-chain rearrangement precedes that for κ.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Effectiveness of double DNase treatment of RNA.

Figure S2. Primer sets used in the PCRs for the study.

Figure S3. Sequence alignment of amplified and flanking sequences of porcine *Rag-1* (A) and for *VpreB* (B) compared with sequences for other species in GenBank.

Table S1. Monoclonal antibodies used for flow cytometry.

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6. *(publication #3)*

Different anti-CD21 antibodies can be used to discriminate developmentally and functionally different subsets of B lymphocytes in circulation of pigs

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Different anti-CD21 antibodies can be used to discriminate developmentally and functionally different subsets of B lymphocytes in circulation of pigs

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ABSTRACT

Monoclonal antibodies IAH-CC51, BB6-11C9.6 and B-Ly4 are routinely used to detect CD21 orthologue on the surface of porcine B lymphocytes. Cross-reactive studies show that IAH-CC51 and B-Ly4 recognize only a portion of B cells that are positive for pan-specific BB6-11C9.6. This indicates that CD21 is always present on all mature B cells but can be expressed in at least two differential forms, and these were assigned as $CD21^{a}$ and $CD21^{b}$. We used IAH-CC51 together with anti-CD2 to define four subpopulations of B cells. Ontogenetic and *in vitro* culture studies, analysis of cell size, expression of CD11b and class-switched phenotype together with measurement of proliferation and cell death, revealed that these subsets represent distinct populations. Phenotypic and functional features collectively suggest that $CD21^{b+}$ B cells are less mature than $CD21^{b-}$. The present work is the first to show that distinct subsets of mature B cells can express differential forms of CD21.

1. Introduction

CD21 is complement receptor type 2, which binds cleavage products of the C3 complement protein and IFN-α, and belongs to the family of the regulators of complement activation (Carroll et al., 1988). It is expressed mainly on B-cells, follicular dendritic cells and also on fractions of other cell types depending upon the species (Zabel and Weis, 2001). CD21 interacts with CD23, CD35, CD19 or CD81. The signal transduction complex CD19/CD21/CD81 enhances BCR signaling in response to complement-coated antigens and therefore is strongly involved in B cell responses to T cell-dependent antigens (Carter and Fearon, 1992; Matsumoto et al., 1993). CD21 also facilitates internalization of immune complexes by B cells to enhance antigen presentation (Zabel and Weis, 2001). Recent findings indicate that engagement of CD21 on B cells promotes the survival of both mature and transitional B cells (Molnar et al., 2008). CD21 functions also as receptor for Epstein–Barr virus (Fingeroth et al., 1984), which indicates its high conservation and significant function. Interspecies conservation may also be evident from observations that some anti-CD21 monoclonal antibodies (mAbs) cross-react between different species as in the case of anti-human B-Ly4 (Boersma et al., 2001) or anti-bovine IAH-CC51 (Boersma et al., 2001; Denham et al., 1994, 1998) with pigs. Broad species-overlapping reactivity of other anti-CD21 mAbs was also documented in other species (Saalmuller and Aasted, 2005).

The expression profile of CD21 on developing B cells in mouse and humans closely matches its role (Zabel and Weis, 2001). CD21 is not present on precursor B cells and begins to express on transitional (T1–T3) immature B cells by different cell surface densities. Developing human B cells acquire CD21 together with IgM already in the bone marrow and are exported to the periphery as T1 cells. Immature T1 cells in mice are exported from the bone marrow to the periphery without CD21 expression and acquire CD21 subsequently in the spleen as they transfer into T2 stage (Loder et al., 1999). All subsequent mature B cells are thereafter CD21 positive until B cells become plasma cells (Zabel and Weis, 2001). However, while all mAbs against human and mice CD21 recognize all mature B cells, there are two populations of mature B cells in sheep: $CD21^{-}$ and $CD21^{+}$ (Liu et al., 2008). These two subpopulations in sheep were shown to have distinct recirculation characteristics, phenotype and tissue distributions (Gupta et al., 1998; Young et al., 1997). Because of these characteristic and their differential expression of CD11b, they could represent B1 and B2 subset homologues but further analyses does not support this suggestion (Chevallier et al., 1998; Gupta et al., 1998). Some reports in pigs also indicate that porcine mature B cells could express CD21 differentially (Sinkora et al., 2011; Takamatsu et al., 1999), and we have speculated that different $CD21^{+/-}$ populations could have

Abbreviations: 7-AAD, 7-aminoactinomycin D; DG, day of gestation; MLN, mesenteric lymph nodes.

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different function in swine immunobiology with special impact in viral or pathogenic immunology (Sinkora et al., 2005a; Sinkora and Butler, 2009). However, the phenomenon of differential CD21 expression was never studied in more details.

Information about different subpopulations of porcine B cells is largely missing. The vast majority of studies just identified all B cells using different anti-CD21 mAbs (Boersma et al., 2001; Denham et al., 1994, 1998; Makala et al., 2001; Nielsen et al., 2003; Solano-Aguilar et al., 2001; Takamatsu et al., 1999), anti-IgM (Sinkora et al., 1998a, 1998b, 2003, 2009, 2011; Takamatsu et al., 1999) or anti-human CD79α mAbs (Lee et al., 2008; Sinkora and Butler, 2009). It is also known that that all IgM^+ B cells in swine are MHC-II^+, $CD25^{lo}$ and $CD45RC^+$ (Sinkora et al., 1998a,b). In addition, porcine B cells were shown to express CD2 molecules differentially (Sinkora et al., 1998a,b, 2005a; Sinkora and Butler, 2009), and ontogenetic studies suggested that down-regulation of CD2 occurs as a consequence of B cell activation (Sinkora et al., 1998b).

This report confirms the existence of two populations of porcine mature B cells differing in expression of CD21. However, we have shown that different mAbs against CD21 recognize different amounts of IgM^+ B cells. Cross-reactivity and inhibition studies revealed that mature B cells always express CD21 molecules but probably in two different forms $CD21^a$ and $CD21^b$. Ontogenetic, phenotypic and functional studies revealed that two $CD21^{b+/-}$ subpopulations represent functionally distinct subsets of porcine peripheral B cells. These findings explain not only differential expression of CD21 in pigs and probably sheep but may have impact also for humans and mice.

2. Materials and methods

2.1. Experimental animals

Animals used in the study were: (1) Czech Large White pigs [35 animals] and (2) Minnesota miniature/Vietnam–Asian–Malaysian crossbred pigs bred in Novy Hradek [78 animals including fetuses and germ-free animals] (Sinkora et al., 2000, 2003). Both types of pigs gave the same results. Fetuses were obtained by hysterectomy. Gestation age (DG) was calculated from the day of mating (gestation in swine is 114 days (Sinkora et al., 2002)). Germ-free piglets were recovered from gilts by sterile hysterectomy at DG112 and were kept in isolator units under germ-free conditions at all times. All animal experiments were approved by the Ethical Committee of the Institute of Microbiology, Czech Academy of Science, according to guidelines in the Animal Protection Act.

2.2. Preparation of cell suspensions and cell cultures

Cell suspensions were prepared essentially as previously described (Sinkora et al., 1998a, 2005b). Briefly, blood was obtained by intracardial puncture and erythrocytes were removed using hypotonic lysis. Cell suspensions from mesenteric lymph nodes (MLN) and spleen were prepared in PBS by teasing apart the tissues using a forceps and then by passage through a 70 μm mesh nylon membrane. Lymphocytes were separated by Histopaque-1077 gradient according to a protocol recommended by the manufacturer (Sigma–Aldrich, St. Louis, MO). Cell suspensions for flow cytometry were thereafter washed twice in PBS containing 0.1% sodium azide and 0.2% gelatin from Cold Water Fish Skin (PBS-GEL) while those for cell cultures were transferred to cultivation medium (see below), filtered through a 70 μm mesh nylon membrane and cell numbers were determined by hemacytometer.

Cell cultures (Stepanova and Sinkora, 2012) were done in RPMI-1640 medium supplemented with L-Glutamine and 25 mM HEPES, 10% fetal bovine serum, 100 U Penicillin and 0.1 mg/ml Streptomycin. Final concentration of cells was always set to 2×10^6 cells per ml and cells were cultivated with 50 ng/ml PMA and 1 μg/ml Ionomycin or 10 μg/ml CpG oligodeoxynucleotides ODN 10103 (Coley Pharmaceutical Group, Wellesley, MA) or 20 μg/ml LPS or 2 μg/ml PWM or without any stimulation (all chemical except specifically stated from Sigma–Aldrich, St. Louis, MO).

2.3. Immunoreagents

Mouse anti-pig mAbs used in this study, whose source and specificity were described earlier (Sinkora et al., 1998a, 2003, 2007), are listed in Table 1. Goat polyclonal antibodies specific for mouse immunoglobulin subclasses labeled with fluorochromes were used as secondary immunoreagents (Southern Biotechnologies, Birmingham, AL). All immunoreagents were titrated for optimal signal/noise ratios. Unlabeled isotype-matched mouse anti-rat mAbs were used as negative controls replacing used mouse anti-pig mAbs. No background staining was observed during any experiments. Rabbit anti-swine IgM polyclonal antibody B27 was used for IgM masking experiments (kindly provided by John E. Butler, University of Iowa, IA) and goat anti-swine Ig polyclonal antibody labeled by FITC was used for detection of all Ig classes (Sevac, Prague, Czech Republic).

2.4. Staining of cells

Staining of cells for flow cytometry analysis was performed as described previously (Sinkora et al., 1998a, 2000, 2007) by indirect sub-isotype staining. Briefly, multi-color staining was done using cells that had been incubated with a combination of primary mouse mAbs of different sub-isotypes. Cells were incubated for 30 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary polyclonal Abs specific for mouse immunoglobulin subclasses that had been labeled with different fluorochromes 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. Detection of CD79α was done by intracellular staining using IntraStain kit according to a protocol recommended by the manufacturer (DakoCytomation, Glostrup, Denmark).

The DNA content of multi-color-stained cells was determined using the DNA intercalating probe 7-aminoactinomycin D (7-AAD) (Sinkora et al., 2000, 2005b). 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 (2000g, 10 min, 4 °C) and washed in cold PBS containing 0.1% sodium azide. The pellets were then incubated with 50 μl of 7-AAD (40 μg/ml) for 20 min at 4 °C in dark until measured using flow cytometry.

Proliferation history of cells was determined by CFSE [5-(and -6)-carboxyfluorescein diacetate succinimidyl ester] in which suspensions of 4×10^7 fresh lymphocytes in 1 ml of PBS were stained by 110 μl of 50 μM CFSE solution (prepared from 5 mM stock of CFSE in DMSO by 100× diluting with PBS) under vigorous mixing for 5 min. Final suspension was 10× diluted by PBS supplemented with 5% fetal calf serum, washed three times in the same diluting solution, resuspended in culture medium and cultivated (see cell cultures). After cultivation, suspensions were stained by indirect sub-isotype staining as described above and analyzed by flow cytometery.

2.5. Flow cytometry

Samples were measured on a standard FACSCalibur or FACSAria III flow cytometer (BDIS, Mountain View, CA). Electronic compensation was used to eliminate residual spectral overlaps between

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Table 1
List of mAbs used in this study.

Specificity	Clones	Isotype	Donor/provider
Anti-IgM	M160	IgG1	K. Nielsen, CFIAB OLF, Ottawa, Canada
	M157	IgG2a	K. Nielsen, CFIAB OLF, Ottawa, Canada
Anti-IgG	K138.4C12	IgM	J.K. Lunney, API USDA, Beltsville, MD
Anti-IgA	M1459	IgG1	K. Nielsen, CFIAB OLF, Ottawa, Canada
	M1456	IgG2a	K. Nielsen, CFIAB OLF, Ottawa, Canada
Anti-CD2	MSA4	IgG2a	C. Hammerberg, UM, Ann Arbor, MI
	1038H-5-37	IgM IgG3	D.H. Sachs/C.A. Huang, MGH, Charlestown, MA
	PG168A		VMRD, Pullman, WA
Anti-CD11b	MIL4	IgG1	K. Haverson/C.R. Stokes, UB, Bristol, UK,
Anti-CD21	IAH-CC51	IgG2b	C.J. Howard, IAH, Compton, UK
	BB6-11C9.6	IgG1	Southern Biotechnologies, Birmingham, AL
	B-Ly4		BD Bioscience Pharmingen, Franklin Lakes, NJ
Anti-CD79α	HM57	IgG1	DakoCytomation, Glostrup, Denmark

individual fluorochromes. Doublet discrimination module was used in DNA content analysis allowing single-cell events to be discriminated from doublets and higher multiplets. PCLysis software (BDIS, Mountain View, CA) was used for data processing.

3. Results

3.1. Different anti-CD21 mAbs antibodies recognize different epitopes

There are several different anti-CD21 mAbs (Boersma et al., 2001; Denham et al., 1994, 1998) that recognize swine CD21 orthologue from which clones IAH-CC51, BB6-11C9.6 and B-Ly4 were used in this report. Since mature porcine IgM$^+$ B cells differ in expression of CD2 (Sinkora et al., 1998a,b, 2005a; Sinkora and Butler, 2009), CD2/CD21 staining was used to study the specified anti-CD21mAbs. All anti-CD21 mAbs give a very similar staining profile on IgM$^+$ B cells in young piglets (Fig. 1A–C). However, when used in adult pigs, the staining profiles are different (Fig. 1D–F): BB6-11C9.6 stains all IgM$^+$ B lymphocytes (Fig. 1D) like in the case of young piglets (Fig. 1A–C) but IAH-CC51 and B-Ly4 stain only a part of IgM$^+$ B cells (Fig. 1E and F, respectively). Apparently, B-Ly4 (Fig. 1F) always recognizes more IgM$^+$ B cells than IAH-CC51 (Fig. 1E) indicating there are three subsets as regards to CD21 expression: BB6-11C9.6$^+$ B-Ly4$^+$ IAH-CC51$^+$, BB6-11C9.6$^+$ B-Ly4$^+$ IAH-CC51$^-$ and BB6-11C9.6$^+$ B-Ly4$^-$ IAH-CC51$^-$. These three populations are evident from Fig. 1G.

To further prove that these mAbs recognize different epitopes, flow cytometry cross-reactivity and inhibition studies were performed (Fig. 1G–I). As expected, IAH-CC51/B-Ly4 staining revealed three subpopulations of IgM$^+$ B lymphocytes (Fig. 1G) while BB6-11C9.6 stained all IgM$^+$ B cells with a substantial number of IAH-CC51$^-$ (Fig. 1H) and B-Ly4$^-$ cells (Fig. 1I). Again, B-Ly4 recognizes more BB6-11C9.6$^+$ B cells (Fig. 1G and I, 47%) than IAH-CC51 (Fig. 1G and H, 28%). Inhibition studies on the binding of each mAb to the target antigen by the other mAb gives the same profiles as shown in Fig. 1G–I indicating that selected anti-CD21 mAbs detect different epitopes (data not shown). These results prompted Western-blot and mass spectrometry studies that would show all three mAbs recognize proteins with the same molecular weight and sequence. However, we were unable to precipitate antigen with IAH-CC51 or B-Ly4 mAbs from cell lysates or purify antigen from cell lysates by affinity chromatography with mAbs bound to UltraLink support or isolate antigen from reversible cross-linked antigen with mAbs by DTSSP directly on cell surface. For that reason we cannot determine whether BB6-11C9.6 recognizes the

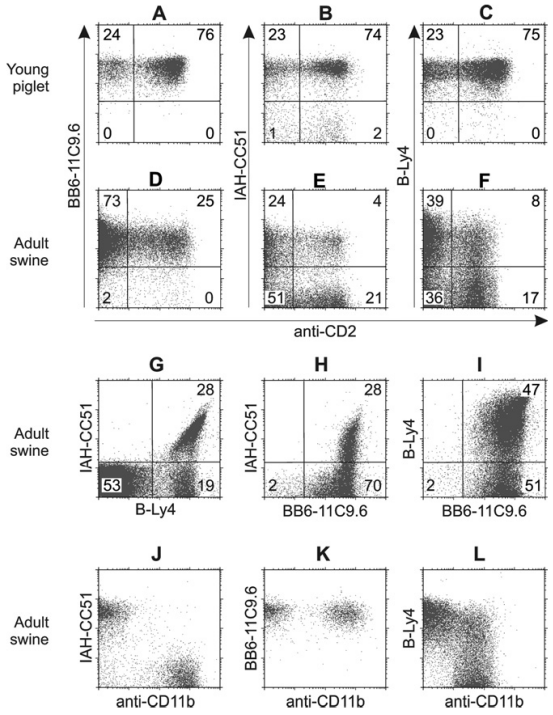

Fig. 1. Anti-CD21 mAbs IAH-CC51, BB6-11C9.6 and B-ly4 probably recognize the different epitopes on the same molecule. The leukocytes isolated from the peripheral blood of 7-days-old (A–C) and 6-months-old (D–J) conventional pigs were triple stained by anti-IgM, anti-CD2 and one of the anti-CD21 mAbs, gated on IgM$^+$ B lymphocytes only and their CD2/CD21 subpopulations were analyzed (A–F). Numbers in dot-plots represent the percentage of IgM$^+$ B cells. Three-color flow cytometry cross-reactive analysis between individual anti-CD21 mAbs is also shown for IgM$^+$ B lymphocytes (G–I). Analysis of CD11b and CD21 expression detected by mAb IAH-CC51 (J), BB6-11C9.6 (K) and B-Ly4 (L) on IgM$^+$ B lymphocytes done by three-color flow cytometry is also shown. All data are representative of at least four independent experiments in which 23 animals were analyzed.

CD21 isoform that is always present on the surface of cells or whether it is pan-specific and recognizes all isoforms in their conservative region. In any case, because IAH-CC51 always clearly recognizes two subsets of mature adult B cells, this particular mAb was used in further studies described in this report and is hereafter referred as CD21^b specific, while molecules recognized by BB6-11C9.6 were assigned to be CD21^a.

3.2. CD21^b and CD11b are expressed on IgM$^+$ B cells in mutually exclusive fashion

Experiments in sheep showed that CD21 negative and positive cells differ also in expression of CD11b (Chevallier et al., 1998; Gupta et al., 1998; Young et al., 1997). While ovine CD21$^-$ B cells are CD11b$^+$, CD21$^+$ B cells are CD11b$^-$. Fig. 1J shows that mutually exclusive expression of CD21^b and CD11b seen in sheep can also be observed in swine. However, pan-specific mAb recognizing CD21^a shows no difference in CD21 expression for CD11b$^+$ or CD11b$^-$ mature B cells (Fig. 1K). Comparison of CD11b and CD21 expression recognized by B-Ly4 shows that a part of B-Ly4$^+$ cells can be either CD11b$^+$ or CD11b$^-$. This finding was also the reason we choose IAH-CC51 and not B-Ly4 for further studies described in this report.

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3.3. B cells differ in expression of CD2 and CD21^b during ontogeny and between individual tissues

Analysis of fetal piglets showed that the vast majority of B lymphocytes in the blood have CD2$^+$CD21^{b+} phenotype (Fig. 2A). Similar results were found in germ-free piglets that have had no access to external antigenic stimuli (Fig. 2B). This is in contrast to conventional piglets of the same age which show substantial number of CD2$^-$ and/or CD21^{b-} B cells (Fig. 2C). Increased frequencies of CD2$^-$CD21^{b+}, CD2$^+$CD21^{b-} and CD2$^-$CD21^{b-} B lymphocytes are even more pronounced in old conventional pigs (Fig. 2D). These findings prompted ontogenetic studies of CD2/CD21^b B cell subpopulations in the blood, spleen and MLN. In these studies, differences between blood and spleen were negligible (data not shown) so only blood (Fig. 2E) and MLN (Fig. 2F) was analyzed further. Ontogenetic analysis proved that fetuses are virtually devoid of CD21^{b-} B cells in both the blood (Fig. 1E) and MLN (Fig. 1F) and the majority of all B cells are CD2$^+$CD21^{b+}. CD21^{b-} B cells never constitute a considerable population in MLN (Fig. 2F) but become prominent postnatally in the blood (Fig. 1E) first as CD2$^+$CD21^{b-} and thereafter also as CD2$^-$CD21^{b-} subsets. Because detection of B cell subpopulations is proportional, the increase in the frequency of CD21^{b-} B cells in older conventional animals is to the detriment of CD2$^+$CD21^{b+} B lymphocytes (Fig. 1E). B cells with CD2$^-$CD21^{b+} phenotype never compose a substantial population during fetal life in any organ but become more frequent after birth when they constitute ~20–30% of all IgM$^+$ B cells independent of age and studied tissue (Fig. 1E and F).

3.4. CD21^{b-} B lymphocytes are prominent after in vitro cultivation

The effect of cultivation on porcine B cells purified from the blood, spleen and MLN was studied using late term fetuses (DG90–DG114), young piglets (1–8 wk old) and adult swine (3–12 mo old). B lymphocytes isolated from spleen were comparable to blood (Fig. 3A) and therefore the data are not shown. Differences between fetuses, young piglets and adult pigs were (1) more accelerated changes in lymphocyte subset composition as the pigs get older (data not shown) and (2) the fact that fetuses are virtually devoid of CD21^{b-} and CD2$^-$ B cells (Fig. 2A, E and F).

The results of cultivation experiments revealed a vigorous decrease in the proportion of CD2$^-$CD21^{b+} B cells in the blood (Fig. 3A) and also MLN (Fig. 3B). There was also a substantial decrease in the frequency of CD2$^+$CD21^{b+} B cells in the blood (Fig. 3A) but only a slight decrease in the MLN (Fig. 3B). On the other hand, the proportion of CD2$^+$CD21^{b-} and CD2$^-$CD21^{b-} B cells always increased (Fig. 3A and B). When comparing different organs, it is notable that the increase in the frequency of CD2$^+$CD21^{b-} B cells during culture is delayed in the MLN (Fig. 2B) in comparison with the blood (Fig. 2A). The similar phenomenon but in less extent can be observed for the proportion of CD2$^-$CD21^{b-} B cells whose increase is also more pronounced in the blood (Fig. 2A) than MLN (Fig. 2B). This is not a true for CD2$^-$CD21^{b+} B cells which decline comparably in all analyzed tissues (Fig. 3A and B). Changes in the proportions of CD2/CD21^b B cell subsets observed during cultivation are in direct accordance with *in vivo* ontogenetic data (Fig. 2E and F), except CD2$^-$CD21^{b+} B cells which increased *in vivo* but decrease *in vitro*. To elucidate a possibility that changes in CD2/CD21^b

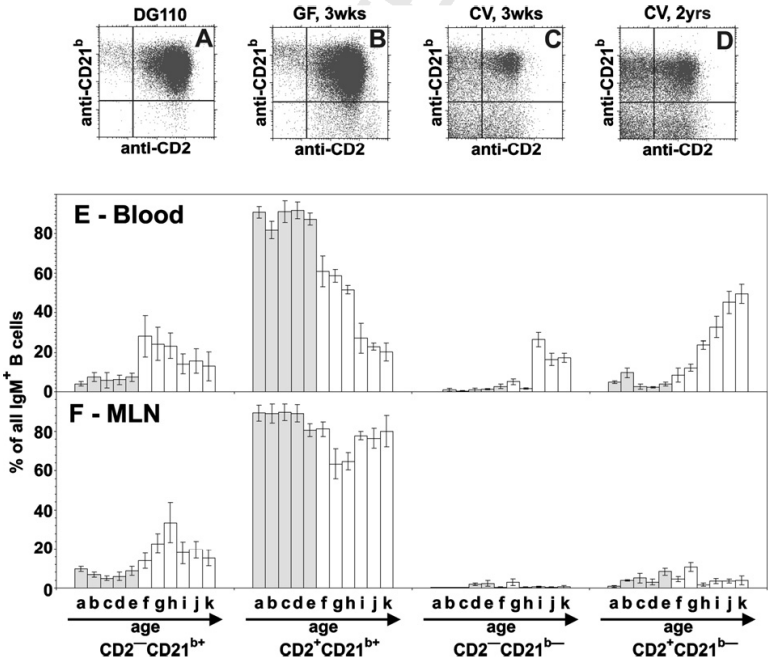

Fig. 2. The frequencies of CD2/CD21^b B cell subpopulations in the blood and MLN during ontogeny. Isolated lymphocytes were triple stained with combinations of anti-IgM, anti-CD2 and anti-CD21^b mAb (IAH-CC51), and IgM$^+$ B lymphocytes were analyzed for CD2 and CD21^b expression. Dotplots A–D show representative analysis for DG110 fetus (A), 3-wk old germ-free (B) or conventional (C) piglets and 2-yr old sow (D). The proportions of individual CD2/CD21^b B cell subpopulations (*x*-axis) expressed as a percentage of all IgM$^+$ B lymphocytes in the blood (E) and MLN (F) during ontogeny are also shown. Age of animals is depicted on the *x*-axis for each individual B cells subset for fetal (grey bars: a = DG70, b = DG80, c = DG90, d = DG100, e = DG110) and postnatal pigs (open bars: f = 1 wk, g = 2 wk, h = 1 mo, i = 6 mo, j = 1 yr, k = 3 year). Bars represent mean values and error bars represent ± SEM obtained from at least four animals. Eighty three animals were analyzed in total.

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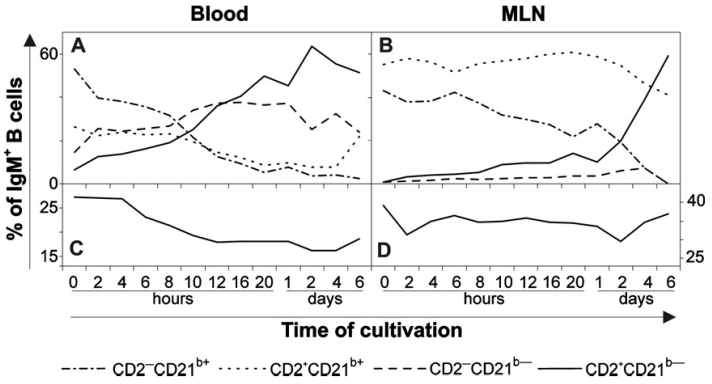

Fig. 3. Analysis of CD2/CD21^b subpopulations of IgM$^+$ B cells during cultivation. Lymphocytes purified from the peripheral blood (A) and MLN (B) of 3 mo old piglet were cultivated (*X*-axis) in medium only, triple stained by anti-IgM, anti-CD2 and anti-CD21^b (IAH-CC51) mAbs, and IgM$^+$ B cells were analyzed by flow cytometry for the proportion of CD2$^-$ CD21^{b+} (dash-dotted line), CD2$^+$CD21^{b+} (dotted line), CD2$^-$ CD21^b (dashed line) and CD2$^+$CD21^b (solid line) subpopulations. The proportion of IgM$^+$ B cells among all cells in the culture is also shown for blood (C) and MLN (D). The results are representative of four independent experiment performed on different pigs (3 fetal animals, 2 newborns, 3 young piglets and 2 adult pigs).

subpopulations are merely due to dying of B cells, analysis of IgM$^+$ B cells among all cells in the culture was done. This analysis showed almost no observable change in the proportion of IgM$^+$ B cells during cultivation of MLN cells (Fig. 3D) while in the blood there was a moderate decrease (Fig. 3C).

To enhance observed changes and to see the effect of activation on development of B cells *in vitro*, we have included different mitogens and activators in cultures that included PWM, PMA + Ionomycin or CpG (Fig. 4). However, the effect of these agents on porcine IgM$^+$ B cells was unexpectedly low. No apparent influence by the selected activators to development of any CD2/CD21 B cell subpopulations could be observed in either blood (Fig. 4A–D) or MLN (Fig. 4E–H) in postnatal animals. The only effect was seen in fetal animals where in both the blood and MLN activators inhibited the decrease of CD2$^+$CD21^{b+} (Fig. 4J and N, respectively) and the increase of CD2$^+$CD21^{b-} B cells (Fig. 4L and P, respectively). In this respect, PWM was the most powerful, and was the only activator that caused an obvious increase of CD2$^-$CD21^{b-} B cells (Fig. 4K and O, respectively). It should be noted that LPS does not have any effect at all and for this reason it is not included in Fig. 4.

3.5. Only CD2$^+$CD21^{b+} and CD2$^+$CD21^{b-} B cells can be generated de novo

To demonstrate whether accumulation of CD2$^+$CD21^{b-} B cells is caused by formation of new B cells in the culture or is merely due to dying of their CD2$^+$CD21^{b-} B cell counterparts, we have performed culture experiment in which the surface expression of IgM was masked at the start of the culture by staining with rabbit anti-swine IgM polyclonal antibody. This experimental approach makes the detection of originally seeded B cells impossible and only newly generated IgM$^+$ B cells can be detected by staining with anti-IgM mAb at the end of the culture. Fig. 5 shows that only IgM$^+$ B cell subsets that can be detected in the cultures are CD2$^+$CD21^{b+} and CD2$^+$CD21^{b-} B cells. The control culture performed with presence of sodium azide and/or at 4 °C did not show any newly generated IgM$^+$ B cells (data not shown).

3.6. Different CD2/CD21^b subsets of B cells have different proliferation rate and susceptibility to apoptosis

To resolve whether the changes in subset composition during cultivation (Figs. 3 and 4) are caused by proliferation and/or apoptosis we have analyzed all B cell subset for their cell cycle phase by staining with 7-AAD (Fig. 6). Using 7-AAD does not allow usage of more than two additional fluorochromes (IgM and CD2 or IgM and CD21^b) and therefore CD2 and CD21^b B cell subsets were analyzed separately (Fig. 6). The inspection of cell cycle phases of individual B cell subpopulations indicates decreasing number of cells in resting $G_{0/1}$ cell cycle phase for CD2$^-$ B cells with no proliferation, but some apoptosis after 1 day of culture (Fig. 6B). The same phenomenon was found in MLN (Fig. 6F). A similar decrease in resting cells was observed for CD21^{b+} B cells except that there was no observable apoptosis and some cells proliferate at the end of culture (Fig. 6C). This tendency is even more pronounced in MLN (Fig. 6G). It is notable that even no apoptosis was detected and cells proliferated there was no elevation in the number of CD21^{b+} resting B cells (Fig. 6C and G). On the other hand, the number of CD21^{b-} resting cells remains approximately on the same level throughout culturing with some cells dying and some dividing (Fig. 6D and H). The difference in the frequency of resting CD21^{b-} B cells in the blood (Fig. 6D) and MLN (Fig. 6H) is caused by their rare occurrence in the MLN. CD2$^+$ B cells (Fig. 6A and E) behave similarly to CD21^{b-} B cells (Fig. 6D and H). The only differences are that they contain more proliferating than apoptotic cells and that the proportion of resting cells in MLN is decreasing with time of culture (Fig. 6A and E).

From these results it is evident that CD2$^-$CD21^{b+} did not proliferate and did not die because no apoptosis was detected in CD21^{b+} B cells (Fig. 6C and G) and no mitosis was detected in CD2$^-$ B cells (Fig. 6B and F). Having characterized CD2$^-$CD21^{b+} B cells, the rest of CD21^{b+} B cells are only mitotic (Fig. 6C and G) and therefore CD2$^+$CD21^{b+} B cells have capacity to divide but do not die. Similar logic can be applied to the rest of CD2$^-$ B cells that are only apoptotic (Fig. 6B and F). Therefore, the CD2$^-$ CD21^{b-} can die by apoptosis but have no capacity to proliferate. It follows that CD2$^+$CD21^{b-} B lymphocytes combine cells being able to both proliferate and also die by apoptosis. This is because CD2$^+$ (Fig. 6A and E) as well as CD21^{b-} (Fig. 6D and H) B cells are also able to divide and die but their counterparts can only die (CD2$^-$ B cells, Fig. 6B and F) or divide (CD21^{b+} B cells, Fig. 6C and G). To prove that our conclusions are correct, CFSE staining was performed to analyze proliferation history in individual CD2/CD21^b subpopulation (Fig. 6I–L). Results confirmed that only CD2$^+$CD21^{b+} (Fig. 6J) and CD2$^+$CD21^{b-} B cells (Fig. 6K) have the capacity to divide.

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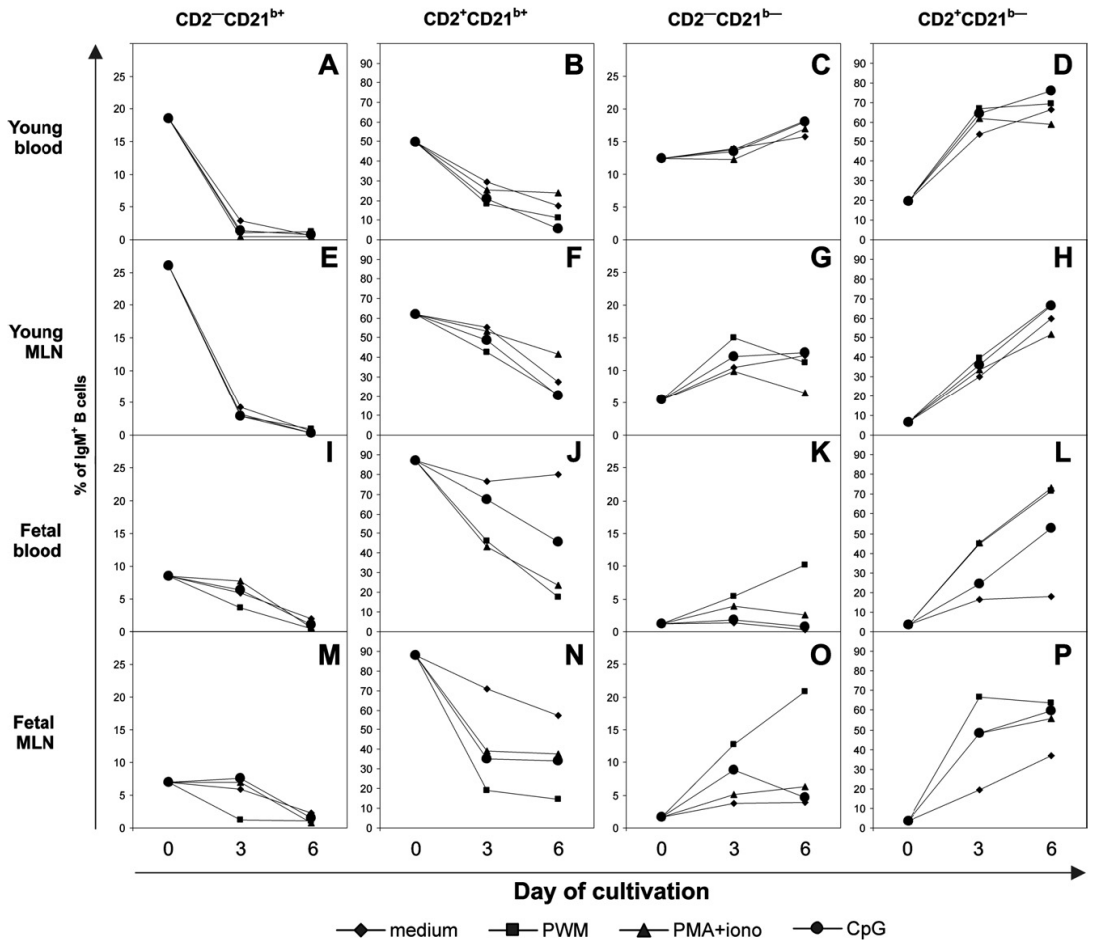

Fig. 4. The frequencies of CD2/CD21^{b} B cell subpopulations in the blood and MLN during cell cultures. Lymphocytes purified from the peripheral blood (A–D and I–L) and MLN (E–H and M–P) of 1 mo old piglets (A–H) and DG100 fetuses (I–P) were cultured with medium only (diamonds) or with addition of PWM (squares) or PMA and ionomycin (triangles) or CpG ODN (circles). At the end of each cultivation (x-axis, 0 stands for fresh cells) cells were triple stained and IgM^{+} B lymphocytes were analyzed by flow cytometry for the proportion of CD2^{-} CD21^{b+} (far left column), CD2^{+}CD21^{b+} (inside left column), CD2^{-} CD21^{b-} (inside right column) and CD2^{+}CD21^{b-} subpopulations (far right column). The results are representative of three independent experiments performed on different pigs (1 newborn, 1 young piglet and 1 adult pig).

3.7. CD21^{b-} B lymphocytes are larger then CD21^{b+}

Analysis of cell size (Fig. 7A) showed that small-sized lymphocytes (cells in R1) are mostly CD2^{+}CD21^{b+} (Fig. 7B). However, a majority of medium-sized and large-sized lymphocytes (Fig. 7A, cells in R2 and R3, respectively) are CD2^{-}CD21^{b-} (Fig. 7C and D, respectively). Thus while small lymphocytes are composed ~80% CD21^{b+} and ~20% CD21^{b-} B cells (Fig 7B), medium to large lymphocytes are composed ~40% CD21^{b+} and 60% CD21^{b-} B cells (Fig. 7C and D).

3.8. CD21^{b-} B lymphocytes are composed of either switched IgG^{+}IgM^{+} or IgG^{+}IgM^{-} cells while CD21^{b+} are uniformly IgG^{-}IgM^{+}

There is no pan-B cell marker for swine other than intracellular CD79α (Igα, Mb1) detected by cross-reactive anti-human mAb HM57 (Lee et al., 2008; Sinkora and Butler, 2009). We have used this mAb together with anti-Ig to detect CD2/CD21^{b} phenotype of class-switched B cells (Fig. 7E–I). Results show that while Ig^{+}-CD79α^{+} B cells (Fig. 7E, R2) are enriched by CD21^{b+} B cells (Fig. 7H), Ig^{-}CD79α^{+} class-switched B cells (Fig. 7E, R1) are consistently CD21^{b-} (Fig. 7G). Interestingly, there is also minor subpopulation of Ig^{+}CD79α^{-} B cells (Fig. 7E, R3) which probably represent memory and plasma cells (Lee et al., 2008), and which had mostly CD21^{b-} phenotype (Fig. 7I). Alternative analysis of class-switched B cells was done using staining against IgM and IgG (Fig. 7J–N) or IgA (data not shown because output was similar as for IgG staining). Results confirmed that pre-class-switched IgM^{+}IgG^{-} B cells (Fig. 7J, R6) are consistently CD21^{b+} (Fig. 7N), class-switching IgM^{+}IgG^{+} B cells (Fig. 7J, R5) are mostly CD21^{b-} (Fig. 7M) and post-class-switched IgM^{-}IgG^{+} B cells (Fig. 7J, R4) are uniformly CD2^{-}CD21^{b-} (Fig. 7L).

4. Discussion

Data reported herein describe populations of porcine B cells differing in expression of CD21^{b}. Such differential expression is in accordance with studies in sheep (Chevallier et al., 1998; Gupta

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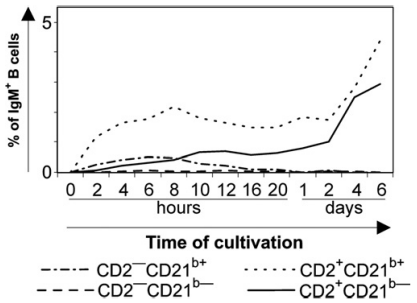

Fig. 5. The proportion of CD2/CD21^{b} subpopulations of IgM^{+} B cells in culture of peripheral blood lymphocytes after masking of the surface expression of IgM on B cells by pre-staining using rabbit anti-swine IgM polyclonal antibody. This approach makes the detection of originally seeded B cells impossible so only the newly generated IgM^{+} B cells can be detected using anti-IgM mAb. Cultivation was performed in medium only and resulting cells were triple stained by anti-IgM, anti-CD2 and anti-CD21^{b} (IAH-CC51) mAbs. Newly formed IgM^{+} B cells were divided into CD2 CD21^{b+} (dash-dotted line), CD2^{+}CD21^{b+} (dotted line), CD2 CD21^{b} (dashed line) and CD2^{+}CD21^{b} (solid line) subsets. The results are representative of three independent experiment performed on different pigs (3 young piglets and 1 adult pig). Similar results were obtained using cells isolated from MLN or spleen.

et al., 1998; Liu et al., 2008; Young et al., 1997) and could indicate that a part of mature B cells in some species do not need CD21 for their functions. This might be possible because CD21 can be shed from B cells upon activation (Masilamani et al., 2003). However, analysis of three different mAbs reveals that CD21^{b-} B cells do not lose CD21 but express its differential form because CD21^{a} molecules detected by BB6-11C9.6 mAb are present on the surface of the same cells. Differential forms of CD21 are known in humans (Carroll et al., 1988; Zabel and Weis, 2001) and mice (Molina et al., 1990) where they are generated by alternative splicing or in sheep where they carry different levels of ubiquitination (Hein et al., 1998; Liu et al., 2008). Thus although different forms of CD21 can be expressed during B cell development and may account for different functional properties, epitope specific mAbs have not been described until this report. Finding that CD21 is always expressed on the surface of mature B cells unifies different findings in swine, sheep, mice and human and confirms the critical function of CD21 in BCR signaling (Carter and Fearon, 1992; Matsumoto et al., 1993). It also corresponds with CD21 shedding experiments because only a partial release of CD21 could be detected (Masilamani et al., 2003). It also agrees with the generally accepted postulation that engagement of CD21 on B cells promotes the survival of both immature transitional and mature B cells (Molnar et al., 2008).

Our studies are unable to resolve what accounts for binding differences of the anti-CD21 antibodies used because we were unable to biochemically characterize a CD21^{b} form. However, this study is rather focused to the fact that different forms of CD21 can be expressed during B cell development. These forms can be discriminated by IAH-CC51 mAb and partially also by B-Ly4 mAbs, although we do not know the functional significance of IAH-CC51^{-} B-Ly4^{+} B cells in this moment. Diverse reactivity of different anti-CD21 mAbs may have several explanations. There can be (1) differently spliced products of CD21, like in humans (Carroll et al., 1988; Zabel and Weis, 2001), (2) different allosteric conformational isoforms of CD21, (3) different post-transcriptional modifications of CD21, like the level of glycosylation or ubiquination known in sheep (Hein et al., 1998; Liu et al., 2008), (4) different configuration of molecular complexes like CD21/CD35 or CD19/CD21/CD81 or (5) different molecules, like CD35 and CD21 in mice (Molina et al., 1990). In any case, results show that the CD21^{b} epitope recognized by IAH-CC51 mAb cannot be detected as B cells mature and proceed from naive to effector and finally to class-switched B cells. This conclusion is supported by analysis of different CD2/CD21b subsets of B cells (Table 2). The findings prove that CD2^{+}CD21^{b+} B cells are almost exclusively the subset during fetal life and most of them therefore represent naive B cells with IgM^{+}IgG^{-}IgA^{-} phenotype. Despite observations that they do not die, some of them have capacity to divide and they can be generated *de novo*, their frequency decreases in cultivation. Since this decrease is compensated by an increase in the frequency of CD2^{+}CD21^{b-} subset, these results indicate that some of the CD2^{+}CD21^{b+} cells can mature into CD2^{+-} CD21^{b-} B cells (Table 2). On the other hand, CD2^{-}CD21^{b+} B cells appears very early after birth and remains stable during further life indicating that they may represent primed B lymphocytes. This is supported by the finding that they are mostly resting B cells with IgM^{+}IgG^{-}IgA^{-} phenotype. Since their numbers in cultivation promptly decrease, these cells most likely also mature into CD2^{+-} CD21^{b-} B cells (Table 2). The CD2^{+}CD21^{b-} B cells appear later after birth and they accumulate as animals get older, which is a sign of an effector, class-switched and memory B cell pool. This is supported by the finding that a substantial number of these cells are large and have either pre-switched IgM^{+}IgG^{+}/IgA^{+} or switched IgM^{-}IgG^{+}/IgA^{+} phenotype. Cultivation experiments indicate that CD2^{+}CD21^{b-} B cells are result of CD2^{-}CD21^{b+} and CD2^{+}CD21^{b+} B cells maturation (Table 2). The CD2^{-}CD21^{b-} B cells appear later in postnatal ontogeny and their frequency remains thereafter stable suggesting they may represent antibody-producing and plasma cells. This is supported by the finding that they have mostly pre-switched IgM^{+}IgG^{+}/IgA^{+} or switched IgM^{-}IgG^{+}/IgA^{+} phenotype. These cells can die by apoptosis but have no capacity to proliferate and cannot be generated *de novo*. However, their proportion slowly increases in cultivation, which indicates that CD2^{+}CD21^{b-} B cells probably further mature, lose CD2 and contribute to the CD2^{-}CD21^{b-} compartment (Table 2).

Similar CD21^{+} and CD21^{-} subsets of B cells were found in sheep (Chevallier et al., 1998; Gupta et al., 1998; Young et al., 1997). Although it was speculated that these may represent B1 and B2 analogues known from mice, further studies negated this conclusion. CD21^{-} mature B cells in sheep are absent in fetuses and early neonatal lambs but become prominent with advancing age (Gupta et al., 1998). They are large in size, they have a higher propensity to apoptosis, a higher proportion of them are cycling and they respond more rapidly to stimulation (Chevallier et al., 1998). They are also mainly located in the blood and spleen, localize preferentially in splenic marginal zone and do not recirculate via lymph nodes and secondary B-cell follicles, including Peyer's patches (Chevallier et al., 1998; Gupta et al., 1998; Young et al., 1997). These findings, together with results of this work, collectively indicate that CD21^{b-} B cells have the phenotype and behavior associated with effector functions. Moreover, most CD21^{-} B cells lack L-selectin (CD62L) which together with lack of recirculation throughout the lymph nodes leads to speculation that they represent a memory B cell pool (Gupta et al., 1998; Young et al., 1997) just like in the case of T cells (Mackay et al., 1990) and in agreement with studies in humans (Kansas et al., 1985) and mice (Kraal et al., 1998). It is notable that the decreasing proportion of CD21^{b+} B cells was observed earlier with stimulation of immune system by African Swine Virus infections (Takamatsu et al., 1999), Porcine CircoVirus type 2 infections (Nielsen et al., 2003) or by the developing mucosal immune system after the birth (Makala et al., 2001; Solano-Aguilar et al., 2001). None of these studies however recognize that CD21^{b-} represented effector stages of mature B cells. They rather concluded that the decreasing number of CD21^{b+} cells was due to a decrease in a total number of B cells.

Our results demonstrate that MLN do not contain CD21^{b-} B cells even in older conventional animals. This indicates that CD21^{b-} B cells are redistributed throughout body but not to MLN. Recirculation studies done in sheep show that CD21^{-} B cells do not home

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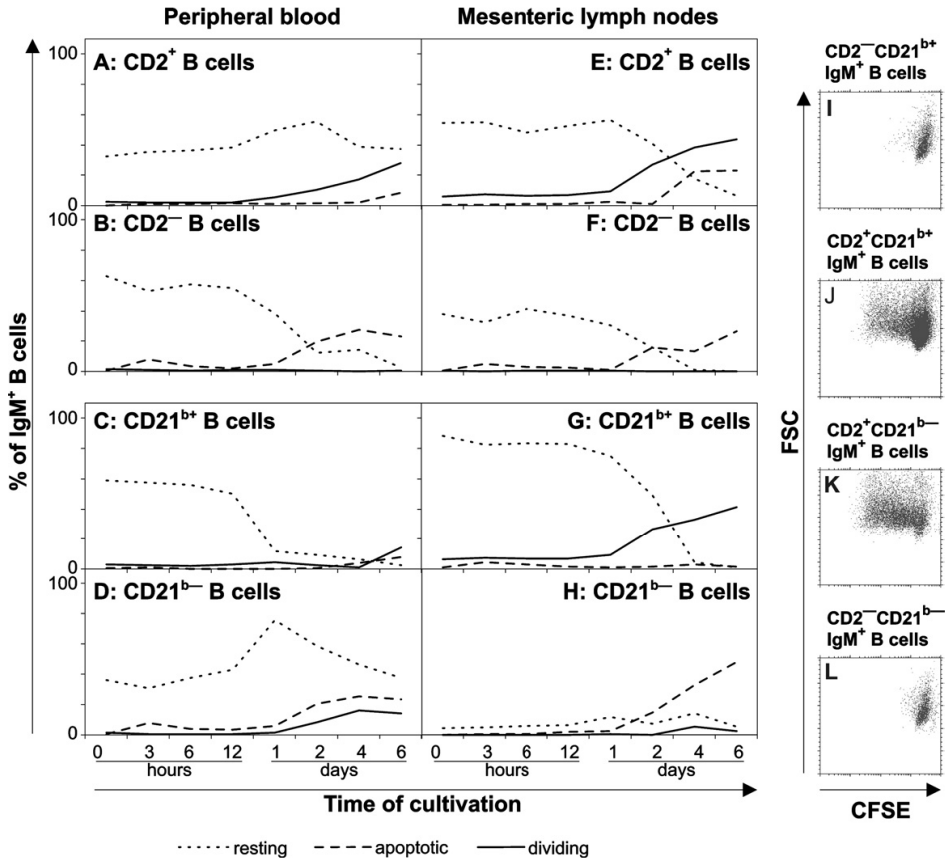

Fig. 6. Flow cytometry analysis of cell cycle phases in IgM$^+$ B cells during cultivation. Lymphocytes purified from the peripheral blood (A–D) and MLN (E–H) were cultivated (*x*-axis) in medium only, double stained on cell surface and their DNA was finally labeled by 7-AAD. The proportion of apoptotic (sub-G cell cycle phase – dashed line), resting ($G_{0/1}$ cell cycle phase – dotted line) and dividing cells ($S + G_2$ cell cycle phase – solid line) for individual CD2 (A, B and E, F) and CD21^b (C, D and G, H) subpopulation of IgM$^+$ B cells are shown. Representative CFSE proliferation analysis of CD2/CD21^b subpopulations of IgM$^+$ B cells that were gated from four-color flow cytometry stained blood lymphocytes cultivated for 4 days is also shown (I–K). All results are representative of at least four independent experiments (3 fetal animals, 2 newborns, 3 young piglets and 2 adult pigs).

into lymph nodes and secondary follicles (Gupta et al., 1998; Young et al., 1997). The results indicate that MLN contains only naive and primed mature B cells (CD21^{b+}CD2$^+$ and CD21$^+$CD2$^-$, respectively) but not effector, fully class-switched and memory B cells (CD21^{b-}) which probably leave MLN and traffic to the blood and periphery including other gut-associated lymphoid tissues (GALT). Interestingly, MLN are the only GALT that do not contain CD21^{b-} B cells. Peyer's patches, intraepithelial lymphocytes and lamina propria contain a significant amount of CD21^{b-} B cells (Sinkora et al., 2011). This could mean effector, class-switched and memory B cells from MLN are redistributed to other GALT as well. These results fully correspond to mucosal studies done earlier by other groups (Makala et al., 2001) and can explain why authors find decreasing number of CD21^{b+} cells in all compartments of gut except MLN.

There is a striking inverse relationship between expression of CD21 and CD11b found in sheep (Gupta et al., 1998) and also swine (this study). Mutually exclusive expression of CD21^b and CD11b on B cells in swine and sheep can help to elucidate the role of naive CD21^{b+}CD11b$^-$ and effector CD21^{b-}CD11b$^+$ B cells and can unify different findings across different species. There is no clear subpopulation of IgM$^+$CD21$^-$ B cells that could be detected in the mature B cells pool of mice or humans. However, there is still the possibility that mouse and human CD21 undergo similar conformation changes in effector B cells but there is a lack of adequate mAbs that could distinguish it. In any case, human and mice B cells express CD11b differentially (Ghosn et al., 2008; Kawai et al., 2005), which is similar to swine and sheep. Expression of CD11b molecules on mouse B cells is usually considered the classical marker of B-1 cells and marginal zone B cells. Nevertheless, human CD11b$^+$ B cells do not fall into the same category as described for mice and they were rather shown to represent the memory B cell pool (Kawai et al., 2005). Moreover, recent findings in mice disrupt the concept of B-1 cells and expression of CD11b as about half of peritoneal B-1 cells were shown to be CD11b$^-$ (Ghosn et al., 2008). Significantly, those CD11b$^-$ B cells appears early in ontogeny and are progenitors of CD11b$^+$ B cells that are more differentiated and cannot reconstitute the CD11b$^-$ B cell pool (Ghosn et al., 2008). Because expression of CD11b in swine and sheep fully correspond to the proposed revised concept, CD21^{b+}CD11$^-$ B cells could generally represent naive B cells subset, while CD21^{b-}CD11b$^+$ B cells could represent experienced effector B lymphocytes.

In summary, this work explains differential finding of CD21 expression in different species. We believe that CD21 is expressed on mature B cells in all homoeothermic animals including sheep,

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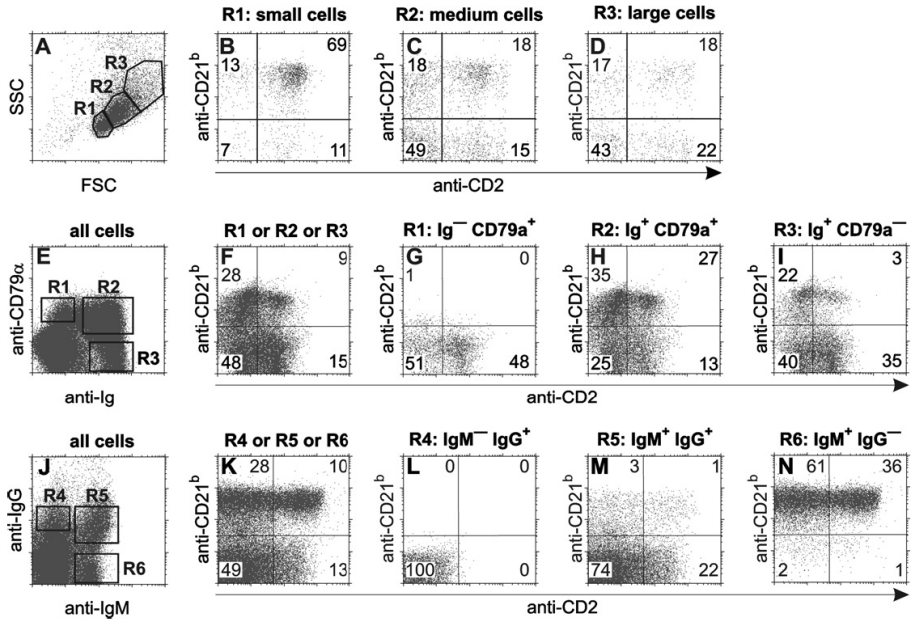

Fig. 7. Characteristics of CD2/CD21 subpopulations of porcine B cells. Upper row (A–D) shows that CD21^{b} B cells are larger than CD21^{b+}. Blood lymphocytes from 6 mo old adult pig were triple stained with anti-IgM, anti-CD2 and anti-CD21^{b} (IAH CC-51) mAb. Based on cell size (A), three subsets of IgM^{+} B cells were distinguished and gated: small-sized (R1), medium-sized (R2) and large-sized (R3). The CD2/CD21 expression on IgM^{+} B lymphocytes in R1, R2 and R3 is also shown (B, C and D, respectively). These results were confirmed in all experiments regardless age and breed of animals (fifteen animals were analyzed in total). Lower two rows (E–N) show the expression of Ig, IgM, IgG, CD79α, CD2 and CD21^{b} molecules on fresh lymphocytes isolated from peripheral blood of 6-months-old swine detected in each row by four-color flow cytometry. The Ig/CD79α subpopulations of B cells were gated from all cells by regions R1, R2 and R3 (E) and expression of CD2 and CD21^{b} (detected by IAH CC-51) on gated B cell subpopulations were analyzed in separate dotplot F (all B cells positive for CD79α and/or Ig), G (Ig^{-} CD79α^{+} B cells), H (Ig^{+}CD79α^{+} B cells) and I (Ig^{+}CD79α^{-} B cells). Note that intracellular staining for CD79α was used after extracellular staining for other molecules. Similar approach was used for analysis of IgM/IgG subpopulations of B cells, which were gated from all cells by regions R4, R5 and R6 (J). The expression of CD2 and CD21^{b} molecules on gated B cell subpopulations were analyzed in separate dotplot K (all B cells positive for IgG and/or IgM), L (IgM^{-} IgG^{+} B cells), M (IgM^{+}IgG^{+} B cells) and N (IgM^{+}IgG^{-} B cells). Note that analysis of IgM/IgA subpopulations revealed comparable results as for IgM/IgG staining and for that reason it is not shown.

Table 2
Characteristics of CD2/CD21^{b} subpopulations of mature B cells in swine.

Characteristics	CD2^{+}CD21^{b+}	CD2^{-} CD21^{b+}	CD2^{+}CD21^{b}	CD2^{-} CD21^{b}
Present before birth:	Yes	No	No	No
Appearance after birth:	Decreasing	Early	Later	Later
IgM/IgG/IgA phenotype:	IgM^{+}IgG^{-} IgA^{-}	IgM^{+}IgG^{-} IgA^{-}	IgM^{+}IgG^{+}/IgA^{+} IgM^{-} IgG^{+}/IgA^{+}	IgM^{+}IgG^{+}/IgA^{+} IgM^{-} IgG^{+}/IgA^{+}
Cell size:	Small	Small to large	Large	Large
Spontaneous apoptosis:	No	No	Yes	Yes
Spontaneous proliferation:	Yes	No	Yes	No
Generation *de novo*:	Yes	No	Yes	No
Cultivation:	Decrease	Decrease	Increase	Increase
Possible maturation to:	CD2^{+}CD21^{b}	CD2^{+}CD21^{b}	CD2^{-} CD21^{b}	End-stage

and also that there are different forms of CD21 or the CD19/CD21/CD81 complex depending on the developmental stage of mature B lymphocytes in humans and mice. Some evidence for this statement can be found in healthy humans (Isnardi et al., 2010). The different CD21 forms should also have different functions and perhaps binding capacity for C3 and/or IFN-α. In any case, differential expression of CD21^{b} detected by IAH-CC51 can be used to monitor two functionally different subpopulations of mature B cells in swine. It remains to be definitively determined whether CD21^{b-} B cells arise from their CD21^{b+} counterparts and therefore both subsets are differential maturation stages within the same lineage or whether they represent distinct subpopulations. The results of this study and our recent unpublished findings with flow cytometry sorted CD21^{b+} B cells that can generate CD21^{b-} B lymphocytes indicate developmentally dependent stages.

5. Conclusions

In conclusion, this work shows that three anti-CD21 antibodies, which are routinely used by many investigators to detect CD21 on a surface of porcine B cells, recognize different amounts of mature B cells. From these studies it is evident that mAb IAH-CC51 together with anti-CD2 can be used to recognize a functionally distinct CD2/CD21^{b} subsets of B cells in swine. This work also shows that CD21 molecule detected by mAb BB6-11C9.6 is always

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present on a surface of mature B cells. By our knowledge, this is the first report indicating that end-stage B cells can express differential forms of CD21, which can be significant for their function.

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7. *(publication #4)*

The expression of CD25, CD11b, SWC1, SWC7, MHC-II, and family of CD45 molecules can be used to characterize different stages of γδ T lymphocytes in pigs

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The expression of CD25, CD11b, SWC1, SWC7, MHC-II, and family of CD45 molecules can be used to characterize different stages of γδ T lymphocytes in pigs

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ABSTRACT

The expression of selected molecules was chosen to study porcine γδ lymphocytes and their CD2/CD8 subsets in different lymphoid organs *in vivo* and *in vitro*. Results indicate that many γδ T cells can constitutively express CD25 and MHC-II and that the frequency of γδ T cells positive for CD25, CD11b, SWC1 and SWC7 can be increased by stimulation. A diversified TCRδ repertoire was found inside $CD25^+$, $CD11b^+$, $SWC1^-$ and $CD45RA^-$ cells. Ontogenetic studies revealed various age and/or colonization dependency for expression of all studied molecules except of SWC7. Findings generally indicate that CD25 represent an activation molecule that probably marks a functionally distinct subsets, expression of CD11b is perhaps connected to early functions of naive γδ T cells in the periphery, SWC1 is lineage specific marker, SWC7 may represent an activation molecule with intrinsic or transient expression, and the expression of CD45RA/RC most likely defines naive and terminally differentiated cells.

1. Introduction

T lymphocytes of γδ lineage are evolutionary conserved cells which develop in the thymus similarly to αβ T cells (Xiong and Raulet, 2007). However, γδ T cells do not need any selection for pre-antigen receptors (like pre-BCR or pre-TCR αβ) and therefore mature faster than αβ T cells, develop without any TCR^{low} transitional stage and are released much earlier to the periphery (Sinkora et al., 2000a, 2005a, 2007; Xiong and Raulet, 2007). At the effector cell level, γδ T lymphocytes share many features with αβ T cells such as potent cytotoxic activity, regulatory functions including ability to induce maturation of dendritic cell, and the capacity to produce a variety of cytokines (Scotet et al., 2008). They also generate and retain immunologic memory. On the other hand, they respond rapidly to infection (Xiong and Raulet, 2007), are probably involved mainly in mucosal immunity (Hiromatsu et al., 1992; King et al., 1999), can act as potent antigen-presenting cells (Brandes et al., 2005; Takamatsu et al., 2002) and their TCR recognizes a broad spectrum of unprocessed or non-peptide antigens without any requirement for MHC co-signalization (Tanaka et al., 1994). They can also be involved in managing of tumors by recognizing stress-induced conserved antigens (Scotet et al., 2008). Due to their nature, γδ T cells are often categorized into unconventional T cells and probably form a unique link between innate and adaptive immune responses.

Swine together with ruminants and birds belongs to the group of γδ high species in which γδ T cells are not preferentially limited to epithelia and may account for >70% of all T cells (Hein and Dudler, 1993). Traditionally, γδ T-cells in swine are subdivided into three subsets based upon their expression of CD2 and CD8 and include $CD2^-CD8^-$, $CD2^+CD8^-$ and $CD2^+CD8^+$ cells (Sinkora et al., 1998, 2005b, 2007; Yang and Parkhouse, 1996, 1997). These individual subsets differ in their homing characteristic (Saalmüller et al., 1990) and cytotoxic activities (de Bruin et al., 1997; Yang and Parkhouse, 1997). Our previous studies revealed basic distribution of porcine γδ T cells (Sinkora et al., 1998), their ontogeny (Sinkora et al., 1998, 2005a), development in the thymus (Sinkora et al., 2000a, 2005a, 2007) and the repertoire diversification of their TCR (Holtmeier et al., 2004). However, none of these studies focused on a detailed analysis of peripheral γδ T cells, and no other studies have been performed to explain differences in the phenotypic profile of γδ T cells subsets.

In this report, the expression of CD25, CD11b, SWC1, SWC7, MHC class II and family of CD45 was studied. These molecules were chosen because of their differential expression on porcine γδ T cells in our preliminary studies. CD25 is α-chain of IL-2 receptor and it is expressed on activated and regulatory T cells

Abbreviations: BAL, bronchoalveolar lavage; BM, bone marrow; GF, germ-free; IL-2, interleukin 2; MHC-II, major histocompatibility complex class II; MLN, mesenteric lymph nodes; PBS, phosphate buffered saline; PMA, phorbol-12-myristate-13-acetate; SWC1, SWC7, swine workshop cluster 1,7.

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software (BDIS, Mountain View, CA) was used for data processing. Lymphocyte gate for analysis was set according to light scatter characteristics (forward versus light scatter).

2.6. Cell cultures and stimulation in vitro

Cell cultures were done in RPMI-1640 medium supplemented with L-glutamine and 25 mM HEPES, 10% fetal bovine serum, 100 U penicillin and 0.1 mg/ml streptomycin (all chemicals PAA, Pasching, Austria) in CERTOMAT CS-20 CO2 incubator (Sartorius Stedim, Aubagne Cedex, France). Final concentration of cells was always set to 10^6 cells/ml. One third of the cells was cultured with 50 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma), one third of cells was supplemented by 100 U/ml porcine recombinant interleukin 2 (IL-2, RayBiotech or Prospec) and one third was cultured without any stimulation. Culturing times were 4, 8, 14, 24 and 72 h.

2.7. PCR amplification and CDR3 spectratyping

The diversity in the TCRδ repertoire is overwhelmingly determined by the diversity in the delta chain third complementary region (CDR3). Thus, separation of CDR3 regions for TCRδ on polyacrylamide sequencing gels provides a clonotypic analysis of porcine γδ T cells (Holtmeier et al., 2004). This procedure is called CDR3 polymorphism or spectratyping and was performed on sorted subpopulations of γδ T cells to show their level of diversification. Total amount of 30–50 thousand γδ T cells or their subpopulations were either (1) sorted into PBS, centrifuged and dissolved in 0.5 ml TriZol or (2) directly sorted into 0.5 ml TriZol. Both methods gave the same output. In a particular analysis, only the same amount of sorted cells was used for preparation of RNA and cDNA, both done as previously described (Holtmeier et al., 2004; Sinkora et al., 2000b, 2007). Gene segments for VDJ regions of TCRδ1 (TRDV1)–TCRδ5 (TRDV5) were PCR amplified using previously described primer pairs (Holtmeier et al., 2004) that have been modified for the purpose of this study (Table 1A). Each sample was amplified by five separate reactions using FR1 specific sense primers (Vδ1–Vδ5) and a common anti-sense primer for constant region of δ-chain (Cδ-1). As controls for determining relative transcript expression and efficiency of amplification, a portion of β-actin was amplified from cDNA (Sinkora et al., 2003). Efficiency of PCR amplification was constantly checked on agarose gels. Amplified segments were next re-amplified for CDR3 regions by five separate reactions using FR3 specific sense primers (Vδ1–Vδ5) and a common 32^P-labeled anti-sense Cδ-2 primer (Table 1B). The re-amplified CDR3s were then separated on sequencing gels using Sequi-Gen GT apparatus (Bio-RAD Laboratories, Hercules, CA). Gels were dried in a 583 gel dryer (Bio-RAD Laboratories, Hercules, CA) and images were obtained either directly by Fluorescent Image Analyser FLA-7000 (Fujifilm corporation, Yokyo, Japan) or indirectly by Kodak X-Omat Blue XB-1 film developed in Medical X-Ray Processor 102 (Kodak, Rochester, NY).

Table 1
Sequences of oligonucleotides used for PCR amplification and CDR3 spectratyping.

A	Sense Vδ1-FR1	AAGTTA(C/T)TCAAGACCAGCCAG
	Sense Vδ2-FR1	ACTCAGCCTCAATGGGAAGT
	Sense Vδ3-FR1	AAGCTCAGACCACAATCACAG
	Sense Vδ4-FR1	TCTACAGATGTGGTGTATGAGG
	Sense Vδ5-FR1	AGACTTCCCTGGAAGAGGTG
	Anti-sense Cδ-1	CAAGACAAGCAACATTTGTTCC
B	Sense Vδ1-FR3	CTCACCATTTCAGCCTTACAG
	Sense Vδ2-FR3	ATCTCAGCCTCCCAGCTTGA
	Sense Vδ3-FR3	CAATCTCTTCCTTACAACTGGC
	Sense Vδ4-FR3	TCGAGTTTGACACTGAGTGACT
	Sense Vδ5-FR3	TTCACTTGGTGATCTCCTCAGT
	Anti-sense Cδ-2	AACGGATGGTTTGGAATTAGGC

2.8. Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences among the median frequency values were analyzed by one way analysis of variance (anova) – Tukey's Multiple Comparison test using GraphPad Prism4™ software (GraphPad Software, San Diego, CA). The level of statistical significance is reported in *P*-values: $P < 0.05$ was considered significant (marked *); $P < 0.01$ was considered strongly significant (marked **); and $P < 0.001$ was considered very strongly significant (marked ***).

3. Results

3.1. Distribution of γδ T cells and their subpopulations

Analysis of γδ T cells isolated from different organs shows that their distribution is tissue-specific (Fig. 1A). While γδ T lymphocytes can constitute up to 50% and 30% of all lymphocytes in the blood and spleen respectively, the MLN and bone marrow are poor sources for γδ T cells. A comparison of germ-free piglets with conventional piglets and adult pigs shows that the proportion of γδ T cells in most tissues studied is comparable. The exception is only the blood where the proportion of γδ T cell decreases with age and the BAL where they increase (Fig. 1A). The proportion of γδ T cell is also higher in MLN from germ-free animals in comparison with adults.

Analysis of the CD2/CD8 subpopulations of γδ T cells also reveals tissue-specific patterns (Fig. 1B–D). For example, $CD2^+CD8^+$ γδ T cells are infrequently found in the blood but can be found in solid tissues (Fig. 1B). $CD2^+CD8^-$ γδ T cells are also infrequently found in the blood and in some solid tissues like lung and tonsil (Fig. 1C). This cell subset is mainly enriched in the spleen and thymus (Fig. 1C). On the other hand, $CD2^-CD8^-$ γδ T cells are very frequent in the blood and constitute almost 90% of all γδ T cells (Fig. 1D). This γδ T cell subset can be also found in solid tissues ranging from 20 to 80% (Fig. 1D).

A comparison of germ-free piglets with conventional piglets and adult pigs shows that the proportions of CD2/CD8 subsets in some tissues changes with age. Age-dependent changes are characterized by a similarity between young germ-free and young conventional animals but this is different from adult animals. For example, such changes can be observed in the spleen where the proportion of $CD2^+CD8^+$ γδ T cells increases with age (Fig. 1B) while $CD2^+CD8^-$ decreases (Fig. 1C) or in the jejunum where the frequency of $CD2^+CD8^+$ γδ T cells decreases (Fig. 1B). On the other hand, some changes in the proportions of CD2/CD8 subsets are dependent on the status of bacterial colonization. Colonization-dependent changes are characterized by similarities between young conventional and adult conventional animals but this is different from germ-free animals. For example, such changes are observed in the lung where the proportion of $CD2^+CD8^+$ γδ T cells increases with colonization (Fig. 1B) while $CD2^-CD8^-$ cells decreases (Fig. 1D) or in the ileum where the frequency of $CD2^+CD8^+$ γδ T cells decreases (Fig. 1B) while $CD2^+CD8^-$ increases (Fig. 1C).

Stimulation by PMA or IL-2 does not lead to any significant changes in the proportional composition of CD2/CD8 subsets in any studied tissue (data not shown).

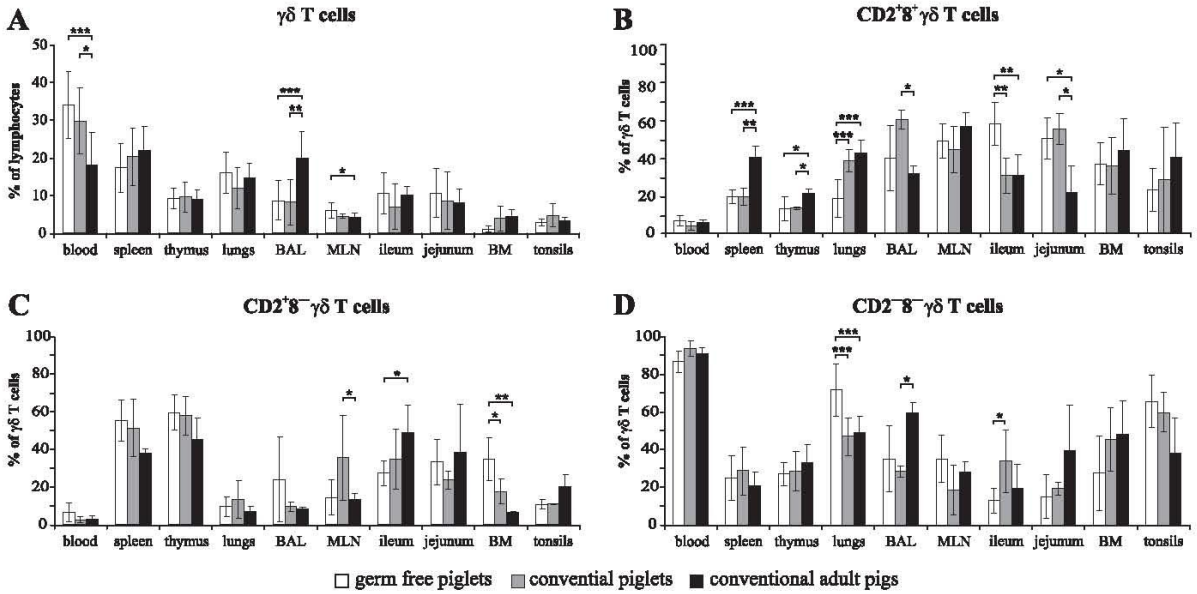

Fig. 1. The frequencies of γδ T cells (A) and their CD2/CD8 subpopulations (B–D) in different organs (*x*-axis) of germ-free piglets (open bars), conventional young piglets (gray bars) and conventional adult pigs (black bars). Error bars represent ±SD and significant differences between tissues are shown and indicated by asterisks according to Section 2.

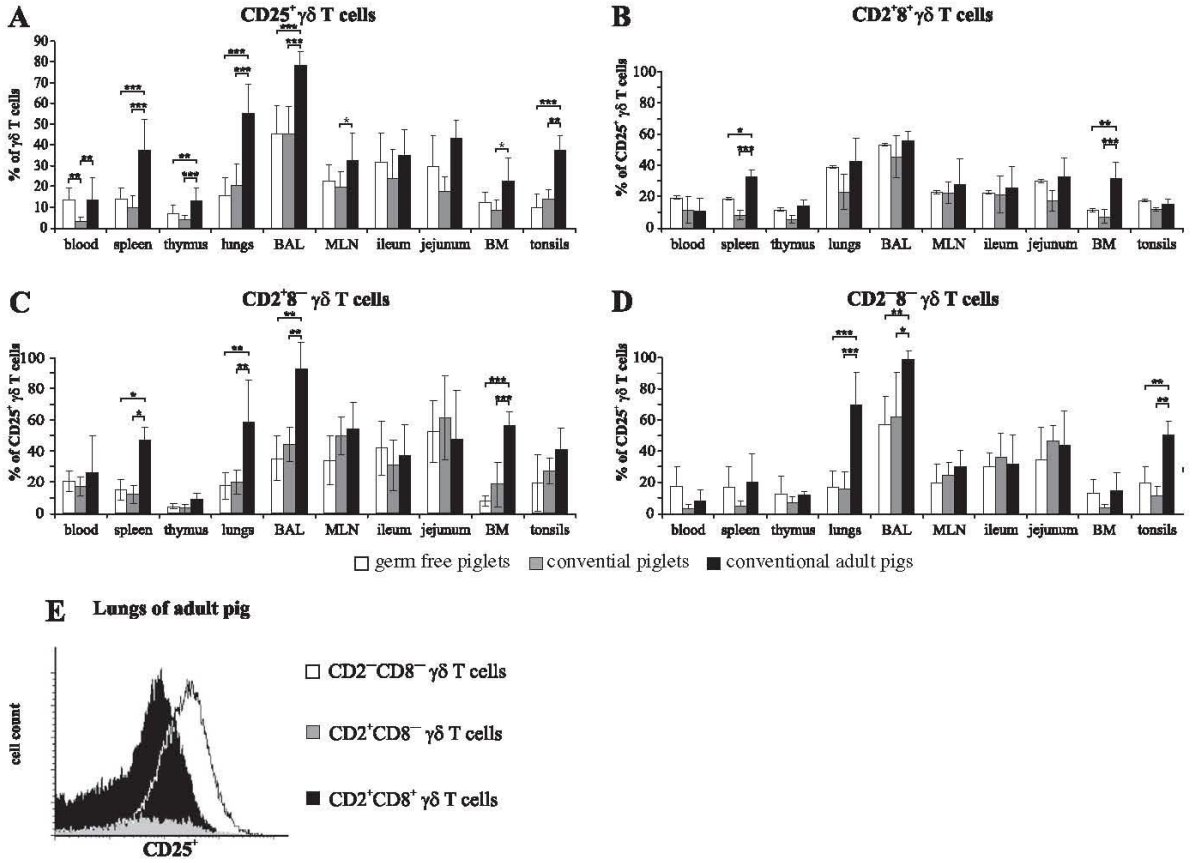

Fig. 2. The frequencies of CD25+ γδ T cells (A) and their CD2/CD8 subpopulations (B–D) in different organs (*x*-axis) of germ-free piglets (open bars), conventional young piglets (gray bars) and conventional adult pigs (black bars). Level of CD25 cell surface expression on the CD2/CD8 γδ T cell subsets isolated from lungs of adult pig is also shown as representative example (E). Error bars represent ±SD and significant differences between tissues are shown and indicated by asterisks according to Section 2.

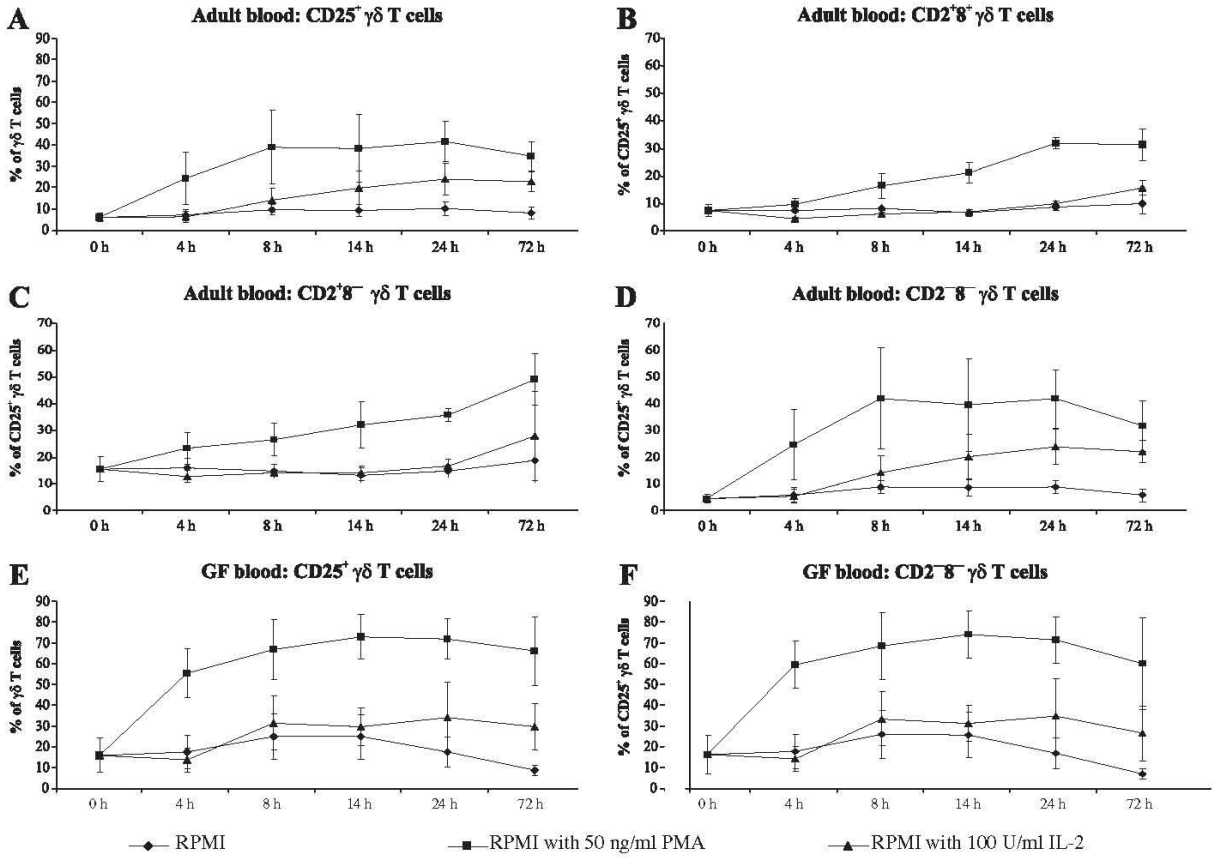

Fig. 3. The frequencies of CD25$^+$ γδ T cells (A) and their CD2/CD8 subpopulations (B–D) during cell cultures with medium only (diamonds) or with addition of PMA (squares) or IL-2 (triangles). Representative analyses of cells isolated from blood of adult conventional pigs are shown because other organs had comparable output. Comparison of CD25$^+$ frequencies among γδ T cells (E) and their CD2 CD8 subset (F) isolated from germ-free pigs is also shown. The results are representative of three independent experiments. Culturing times are indicated on *x*-axis, and 0 h stands for fresh cells. Error bars represent ±SD.

3.2. Native expression of CD25 on γδ T cells

The frequency of freshly isolated γδ T cells that are positive for CD25 is stable and independent of age or colonization status but only in ileum and jejunum (Fig. 2A). Other organs like the spleen, thymus, lung, BAL and tonsils show significantly higher proportion of CD25 positive cells in adult animals in comparison with young germ-free and conventional piglets (Fig. 2A). The increase in the frequency of CD25$^+$ γδ T cells in these tissues is therefore age-dependent but independent of bacterial colonization. The MLN and bone marrow shows differences only between young and adult conventional pigs while blood levels in all groups are less clearly defined (Fig. 2A).

Analysis of the CD2/CD8 subpopulations of γδ T cells for expression of CD25 reveals that the age-dependent increase in CD25$^+$ γδ T cells can be ascribed mainly to CD2$^+$CD8$^-$ γδ T cells (Fig. 2C). There is also an age-dependent increase for CD2$^-$CD8$^-$ subset but only for γδ T lymphocytes isolated from lung, BAL and tonsil (Fig. 2D) and for CD2$^+$CD8$^+$ subset in the spleen and bone marrow (Fig. 2B).

The expression of the CD25 molecule occurs on the surface of γδ T cells in two densities: high and low (Fig. 2E). Notable is the observation that while subset of CD2$^-$CD8$^-$ γδ T cells is almost exclusively CD25^{hi}, CD2$^+$CD8$^-$ and CD2$^+$CD8$^+$ subsets are almost exclusively CD25^{lo}.

3.3. Expression of CD25 on γδ T cells after in vitro stimulation

The proportion of CD25$^+$ γδ T cells can be increased by PMA and IL-2 stimulation. All analyzed organs including the blood, spleen, thymus, lung and MLN yielded similar results so only representative results from the blood are shown (Fig. 3). Stimulation by PMA results in a faster and more vigorous increase in the proportion of CD25$^+$ γδ T cells than stimulation by IL-2 (Fig. 3A). Increased proportion of CD25$^+$ γδ T cells can already be observed after 2 h of culturing with PMA (data not shown).

Detailed analyses reveal that PMA stimulation caused a proportional increase of CD25$^+$ γδ T cells in all CD2/CD8 subpopulations (Fig. 3B–D). However, IL-2 stimulation increases the proportion of CD25$^+$ cells only in CD2$^-$CD8$^-$ γδ T cell subset (Fig. 3D) and has no effect on CD2$^+$CD8$^-$ (Fig. 3C) or CD2$^+$CD8$^+$ subsets (Fig. 3B).

A comparison of adult conventional pigs (Fig. 3A) with germ-free piglets (Fig. 3E) shows that PMA stimulation in adults is slower and less intensive. Four hours after PMA stimulation, only about 25% of adult γδ T cells are CD25$^+$ (Fig. 3A) while 55% in germ-free animals (Fig. 3E). There are no significant differences between germ-free and adult conventional pigs as regards stimulation with IL-2 (compare Fig. 1A with 1E). In both of these groups only the CD2$^-$CD8$^-$ γδ T cell subset responds to IL-2 stimulation and extent of stimulation is comparable (Fig. 3D and 3F).

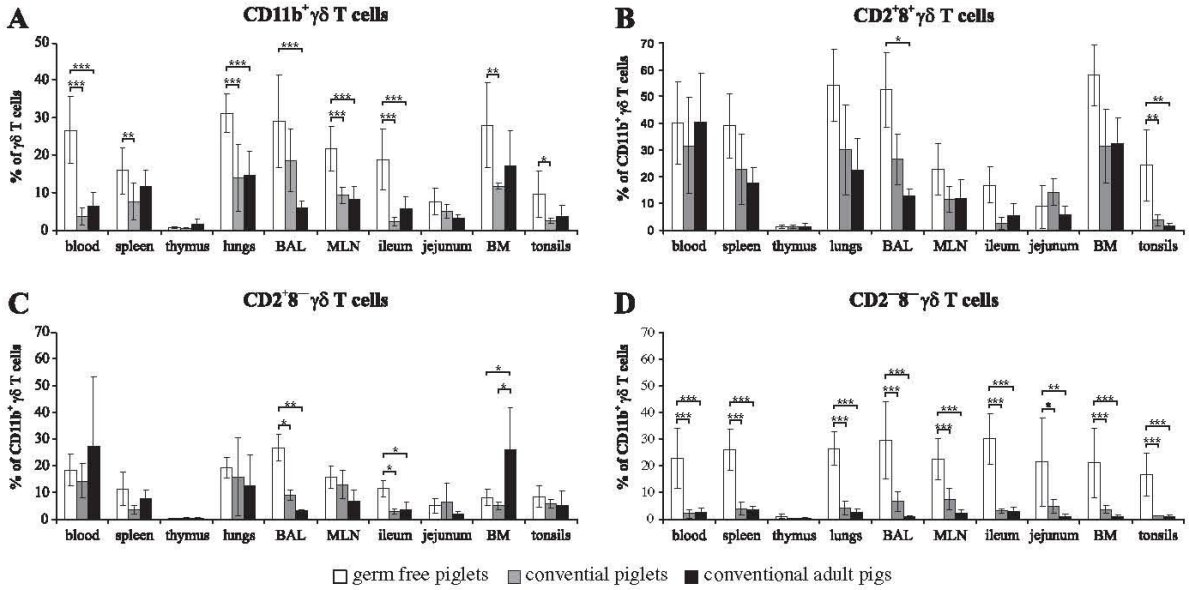

Fig. 4. The frequencies of CD11b$^+$ γδ T cells (A) and their CD2/CD8 subpopulations (B–D) in different organs (*x*-axis) of germ-free piglets (open bars), conventional young piglets (gray bars) and conventional adult pigs (black bars). Error bars represent ±SD and significant differences between tissues are shown and indicated by asterisks according to Section 2.

Young conventional animals resemble germ-free piglets (data not shown) indicating that γδ T cells isolated from young animals are more susceptible to PMA stimulation. This corresponds with the native expression of CD25 on γδ T cells showing an age dependency of CD25$^+$ expression (Fig. 2).

3.4. Native expression of CD11b on γδ T cells

The frequency of freshly isolated γδ T cells that are positive for CD11b is significantly higher in germ-free animals in comparison with age-matched conventional piglets (Fig. 4A). A higher proportion of CD11b$^+$ γδ T cells can be observed in almost all analyzed organs. The only exception is the thymus where CD11b$^+$ γδ T cells always represent a minority of cells irrespective of age and colonization status and the jejunum where differences were insignificant (Fig. 4A). A similar decreases in the proportion of CD11b$^+$ γδ T cells can be observed when germ-free piglets are compared with adult conventional pig but while differences are significant for the blood, lung, BAL, MLN and ileum, they are insignificant in other organs like spleen, bone marrow and tonsils.

Analysis of the CD2/CD8 subpopulations of γδ T cells for expression of CD11b reveals that the colonization-dependent decrease in the proportion of CD11b$^+$ γδ T cells can be mainly ascribed to CD2$^-$CD8$^-$ γδ T cells (Fig. 4D). This subpopulation is almost devoid of CD11b expression in conventional animals, independent of age (Fig. 4D). Some decrease of CD11b can be also found in CD2$^+$CD8$^+$ (Fig. 4B) and CD2$^+$CD8$^-$ (Fig. 4C) γδ T cell subsets but not in all tissues. Unique is the increase of CD11b$^+$ γδ T cells in CD2$^+$CD8$^-$ subset in the bone marrow of adult animals (Fig. 4C).

3.5. Expression of CD11b on γδ T cells after in vitro stimulation

The proportion of CD11b$^+$ γδ T cells can be increased by PMA stimulation but not by stimulation with IL-2 (Fig. 5A). As in the case of CD25 expression, other analyzed organs including the blood, spleen, lung and MLN had comparable output and therefore only representative results from the blood are shown (Fig. 5). The exception is thymus where stimulation by PMA has no effect at all (data not shown).

A detailed analysis of γδ T cells subpopulations reveals that PMA stimulation caused proportional increase of CD11b$^+$ γδ T cells namely in CD2$^-$CD8$^-$ subset (Fig. 5D). A similar effect can be seen in CD2$^+$CD8$^-$ γδ T cells but is less evident and generally slower (Fig. 5C). On the other hand, CD2$^+$CD8$^+$ γδ T cells do not respond to PMA stimulation (Fig. 5B).

A comparison of pigs of different age and colonization status shows that the proportion of CD11b$^+$ γδ T cells can be easily increased by PMA stimulation in germ-free piglets (Fig. 5A), is much slower in young conventional piglets (Fig. 5E) and that adult conventional pigs are almost non-responsive (Fig. 5F). This indicates that the responsiveness of γδ T cells to the expression of CD11b$^+$ after stimulation decreases with both age and colonization. Together with results on native expression of CD11b on γδ T cells (Fig. 2), colonized and older pigs have less CD11b$^+$ γδ T cells and their relative number cannot be increased by stimulation.

3.6. Expression of SWC1 on fresh γδ T cells and after stimulation in vitro

The majority of γδ T cells in adult conventional animals are positive for SWC1 (Fig. 6A). A comparison with germ-free animals reveals that in some organs there is a significantly lower proportion of SWC1$^+$ γδ T cells (like in the thymus and bone marrow, Fig. 6A) while the proportion is higher in the gut (ileum and jejunum, Fig. 6A). Differences in the proportion of SWC1$^+$ γδ T cells are sometimes colonization-dependent, for example in the bone marrow (Fig. 6A). Analysis of the CD2/CD8 subpopulations shows that the majority of SWC1$^+$ γδ T cells are always found in the CD2$^+$CD8$^+$ subset and this is independent of age or colonization status (Fig. 6B and C). In this regard, difference between CD2$^+$CD8$^+$ and CD2$^+$CD8$^-$/CD2$^-$CD8$^-$ in the thymus (Fig. 6B) is more emphasized than in the lungs (Fig. 6C). Analyses of other organs show that they more resemble lungs, except of blood in young animals which resemble more thymus (data not shown). Stimulation by PMA

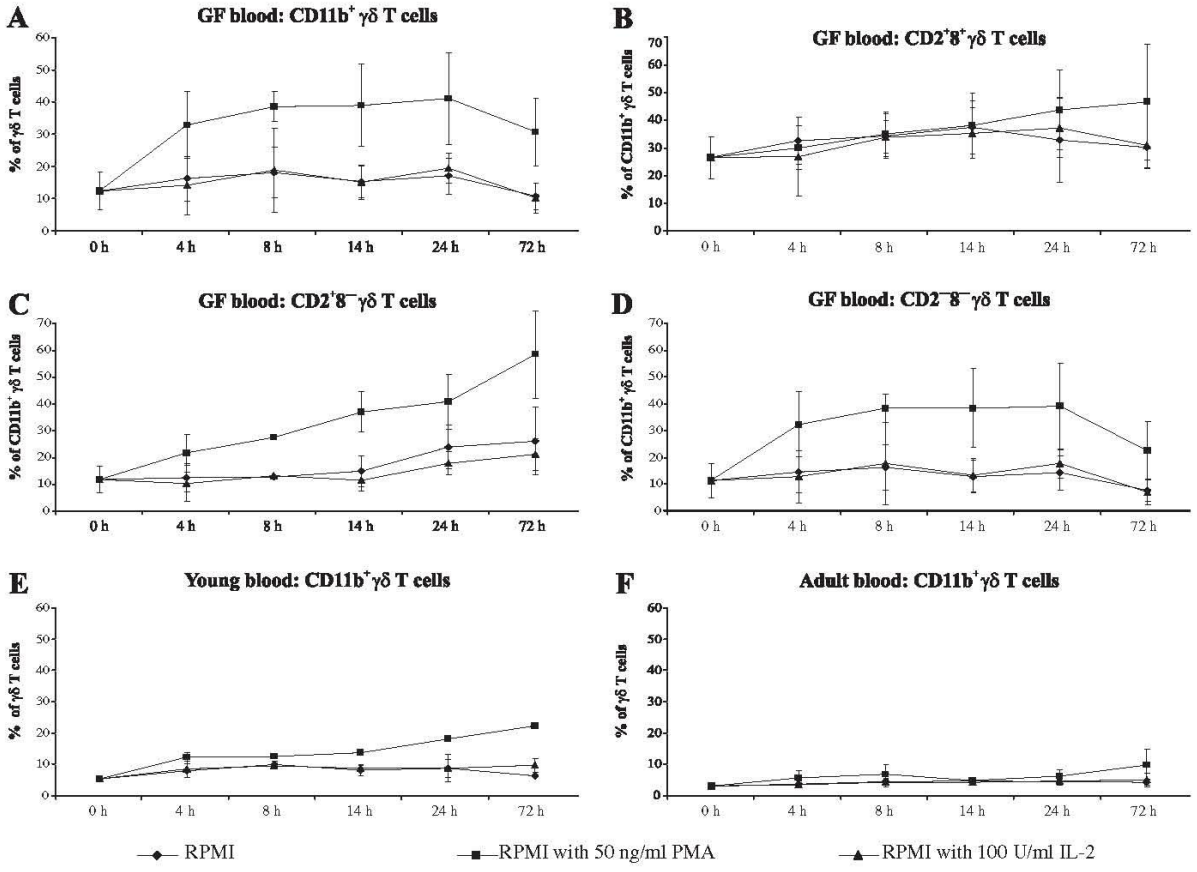

Fig. 5. The frequencies of CD11b$^+$ γδ T cells (A) and their CD2/CD8 subpopulations (B–D) during cell cultures with medium only (diamonds) or with addition of PMA (squares) or IL-2 (triangles). Representative analyses of cells isolated from blood of germ-free piglets are shown because other organs had comparable output. Comparison of CD11b$^+$ frequencies among γδ T cells isolated from young conventional piglets (E) and adult conventional pigs (F) is also shown. The results are representative of three independent experiments. Culturing times are indicated on *x*-axis, and 0 h stands for fresh cells. Error bars represent ±SD.

always leads to a slight increase in the frequency of SWC1$^+$ γδ T cells (Fig. 6D and E), although it is insignificant in some organs like the spleen and lungs of adult pigs (data not shown). In any case, we have never observed a decrease in the proportion of SWC1$^+$ γδ T cells after PMA stimulation. Stimulation with IL-2 does not lead to any significant change in the proportion of SWC1$^+$ γδ T cells in any studied organ (Fig. 6D and E).

3.7. Expression of CD45RA and CD45RC on fresh γδ T cells and after stimulation in vitro

As in the case of SWC1, a majority of γδ T cells are positive for CD45RA (Fig. 6F) and CD45RC (Fig. 6K). The exception is the thymus where they constitute a minority. Generally, CD45RA and CD45RC molecules have very similar expression profiles on porcine γδ T cells in any studied aspect, and therefore are hereafter referred as CD45RA/RC. Ontogenetic studies reveal several differences between adult conventional and germ-free piglets. Like in the case of SWC1, there is significant lower proportion of CD45RA/RC$^+$ γδ T cells in the thymus and bone marrow of germ-free animals while it is higher in other organs like the lung, BAL, ileum or MLN (Fig. 6F and K). The bone marrow differs moreover between young conventional and germ-free piglets, which indicates a colonization-dependent effect (Fig. 6F and K). Unlike SWC1 where the majority of positive cells are always found in the CD2$^+$CD8$^+$ subset, the majority of CD45RA/RC$^+$ γδ T cells in many tissues are found in the CD2$^+$CD8$^+$ and the CD2$^-$CD8$^-$ subset. Therefore they resemble the thymus (Fig. 6G and L), although the difference between CD2$^+$CD8$^+$/CD2$^-$CD8$^-$ and CD2$^+$CD8$^-$ in the thymus is more obvious because CD45RA/RC expression is almost absent on the CD2$^+$CD8$^-$ subset (Fig. 6G and L). The exception is some tissues of adult pigs like lungs, BAL, MLN, jejunum and tonsils where CD45RA/RC$^+$ γδ T cells are found also in CD2$^+$ CD8$^-$ subset (Fig. 6H and M, only lungs shown). *In vitro* experiments revealed a major difference from SWC1 expression profile because stimulation by PMA or IL-2 does not lead to any significant changes in the frequency of CD45RA/RC$^+$ γδ T cells (Fig. 6J and O, only spleen shown). The exception is again thymus where a significant decrease in the proportion of CD45RA/RC$^+$ γδ T cells is observed after PMA stimulation (Fig. 6I and N).

3.8. Expression of MHC-II on fresh γδ T cells and after stimulation in vitro

The proportion of MHC-II$^+$ γδ T cells in germ-free animals is low in the blood, spleen, thymus, lung, BAL, bone marrow and tonsils (Fig. 7A). Higher percentages can be found in the gut associated lymphoid tissues like MLN, ileum or jejunum (Fig. 7A). Ontogenetic studies show a general tendency towards an increasing proportion of MHC-II$^+$ γδ T cells with age or colonization (Fig. 7A). Tissues like

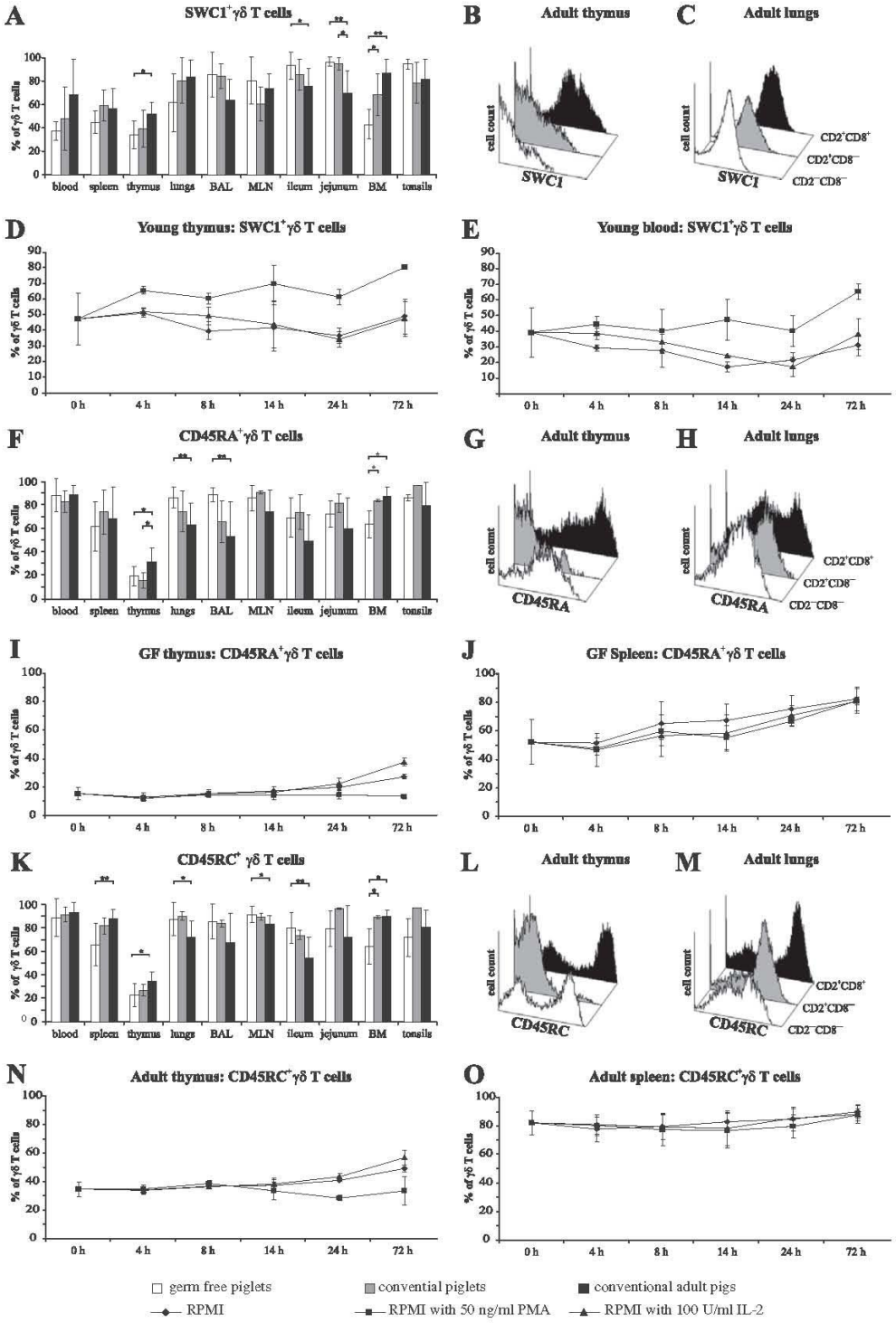

Fig. 6. Summarization analyses of cell frequencies in different organs, level of cell surface expression on the CD2/CD8 γδ T cell subsets, and cell frequencies during cell cultures for SWC1 (A–E), CD45RA (F–J) and CD45RC (K–O). The frequencies of SWC1$^+$ (A), CD45RA$^+$ (F) and CD45RC$^+$ (K) γδ T cells in different organs (*x*-axis) of germ-free piglets (open bars), conventional young piglets (gray bars) and conventional adult pigs (black bars) are shown. Error bars represent ±SD and significant differences between tissues are shown and indicated by asterisks according to Section 2. Representative analyses of cell surface expression level for SWC1 (B–C), CD45RA (G–H) and CD45RC (L–M) on different CD2/CD8 γδ T cells subsets isolated from different organs (depicted above each histogram) is also shown. Lastly, the frequencies of SWC1$^+$ (D–E), CD45RA$^+$ (I–J) and CD45RC$^+$ (N–O) γδ T cells during cell cultures with medium only (diamonds) or with addition of PMA (squares) or IL-2 (triangles) are shown. Representative analyses of cells isolated from different tissues of different pigs (depicted above each graph) are shown for comparison of differences. The results are representative of three independent experiments. Culturing times are indicated on *x*-axis, and 0 h stands for fresh cells. Error bars represent ±SD.

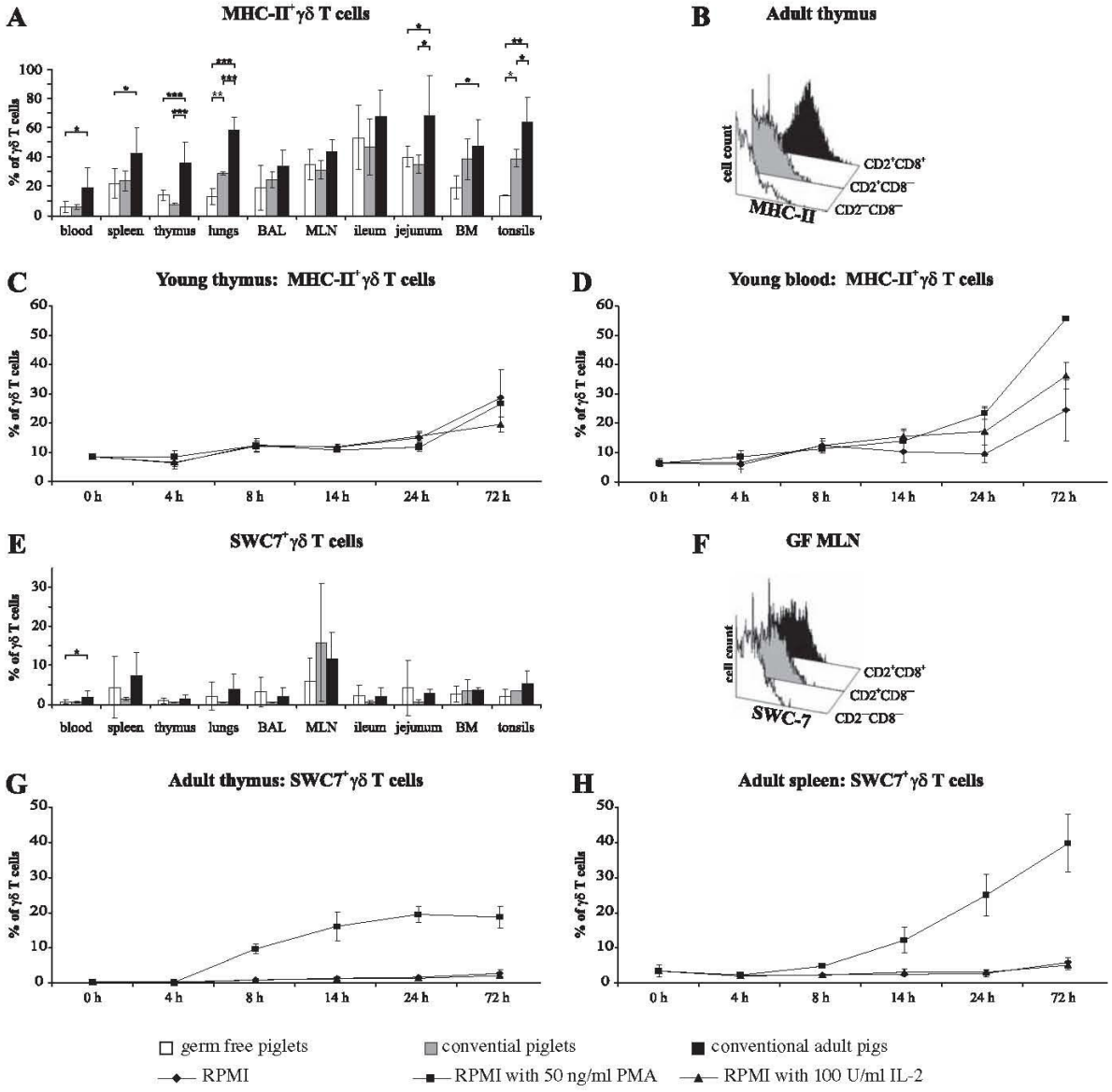

Fig. 7. Summarization analyses of cell frequencies in different organs, level of cell surface expression on the CD2/CD8 γδ T cell subsets, and cell frequencies during cell cultures for MHC-II (A–D), and SWC7 (E–H). The frequencies of MHC-II$^+$ (A), and SWC7$^+$ (E) γδ T cells in different organs (*x*-axis) of germ-free piglets (open bars), conventional young piglets (gray bars) and conventional adult pigs (black bars) are shown. Error bars represent ±SD and significant differences between tissues are shown and indicated by asterisks according to Section 2. Representative analyses of cell surface expression level for MHC-II (B), and SWC7 (F) on different CD2/CD8 γδ T cell subsets isolated from different organs (depicted above each histogram) is also shown. Lastly, the frequencies of MHC-II$^+$ (C–D), and SWC7$^+$ (G–H) γδ T cells during cell cultures with medium only (diamonds) or with addition of PMA (squares) or IL-2 (triangles) are shown. Representative analyses of cells isolated from different tissues of different pigs (depicted above each graph) are shown for comparison of differences. The results are representative of three independent experiments. Culturing times are indicated on *x*-axis, and 0 h stands for fresh cells. Error bars represent ±SD.

blood, spleen, thymus, lung, bone marrow or tonsils, which have a low amount of MHC-II$^+$ cells show a significant increase in the proportion of MHC-II$^+$ γδ T cells but there is not a significant increase in tissues like BAL, MLN or ileum with initially high levels of MHC-II$^+$ (Fig. 7A). Analysis of the CD2/CD8 subpopulations shows that the majority of MHC-II$^+$ γδ T cells in all analyzed tissues can be found in CD2$^+$CD8$^+$ subset, less in CD2$^+$CD8$^-$ subset and the least in CD2$^-$CD8$^-$ subset (Fig. 7B, only thymus shown). Short-term *in vitro* stimulation mostly does not lead to changes in the proportions of MHC-II$^+$ γδ T cells (Fig. 7C). An exception is the higher percentage of MHC-II$^+$ γδ T cells in the blood and spleen after 72 h (Fig. 7D, only blood shown). Because we did not continue to study cells beyond 72 h, we cannot exclude a long-term effect of activation on MHC-II expression.

3.9. Expression of SWC7 on fresh γδ T cells and after stimulation in vitro

The proportion of SWC7$^+$ γδ T cells is generally very low (Fig. 7E). Slightly higher percentages can always be found in the

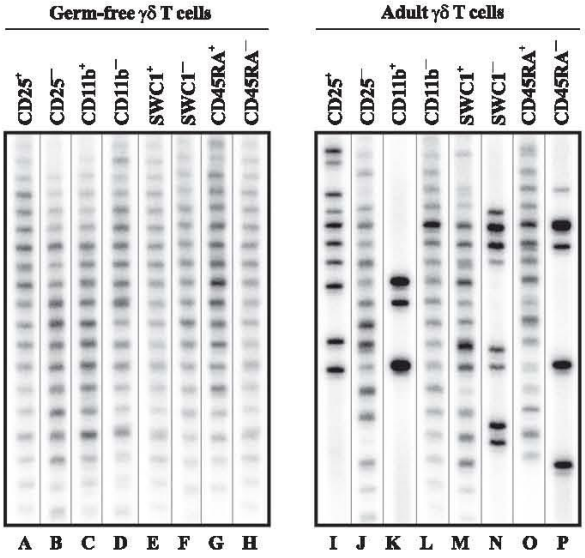

Fig. 8. Representative CDR3 length analysis (spectratyping) of flow cytometry sorted subpopulations of γδ T cells. Splenic γδ T cells from germ-free piglets (left panel A–H) and adult conventional pigs (right panel I–P) were sorted by flow cytometry according to their further phenotype (depicted above each line) and analyzed for Vδ1 CDR3 spectratype. Note that cells from other tissues gave similar output. The results are representative of five independent experiments.

MLN and spleen (Fig. 7E). It is important to emphasize that the proportion of SWC7$^+$ cells considerable vary among individuals. These inter-individual differences are the reason for the large standard deviations in the results obtained (Fig. 7E). In any case, the proportion of SWC7$^+$ γδ T cells is not age or colonization dependent. Analyzes of the CD2/CD8 subpopulations does not show any significant correlation between SWC7$^+$ cells and any particular γδ T cell subset (Fig. 7F). Notable is the increased proportion of SWC7$^+$ γδ T cells after PMA stimulation (Fig. 6G and H). This increase is vigorous, fast, occurs in all tissues and in all CD2/CD8 γδ T cell subsets, and it is not dependent on age or the colonization status.

3.10. Analysis of TCR diversity in sorted subpopulations of γδ T cells

The diversity of TCRδ repertoire in flow cytometry sorted subpopulations of γδ T cells was studied by CDR3 spectratyping (Fig. 8). The results for Vδ1 are shown, as an example, because it is the most varied family (containing more than 30 members while the rest of the Vδ families contains 1–2 members). TCRδ repertoire is diverse (polyclonal) in any sorted subpopulation in germ-free animals (Fig. 8A–H). Sorted subpopulations from adult pigs also show no diversification for CD25$^-$ (Fig. 8J), CD11b$^-$ (Fig. 8L), SWC1$^+$ (Fig. 8M), and CD45RA$^+$ (Fig. 8O) γδ T cells. However, sorted CD25$^+$ (Fig. 8I), CD11b$^+$ (Fig. 8K), SWC1$^-$ (Fig. 8N), and CD45RA$^-$ (Fig. 8P) γδ T cells from adult pigs show highly restricted (oligoclonal) Vδ repertoire. Such oligoclonal pattern is typical for clonally expanded cell clones as a result of previous stimulation (Butler et al., 2007).

4. Discussion

Data reported here show that porcine γδ T cells are mainly enriched in the blood, spleen and lung and their frequency increases also in the BAL of adult animals. Such distribution is different from mice and humans (Carding and Egan, 2002) but is similar to other γδ high species like ruminants (Hein and Mackay, 1991) or birds (Sowder et al., 1988). Thus while γδ T cells in γδ-low-species reside mainly in epithelial layers, they are widespread in secondary lymphoid organs and circulation of γδ-high-species. Notable is the universal occurrence in the lung of both groups which would be consistent with their first line of defense function (Xiong and Raulet, 2007). Unfortunately, a detailed comparison of γδ T cell populations from different species is still lacking, namely because mouse studies use Vγ for identification of subsets, human studies are limited to peripheral blood and studies from other species are rather incomplete. This manuscript adds relevant information on distribution of selected molecules on porcine γδ T lymphocytes and their CD2/CD8 subsets and compares the finding with those from other species. The use of germ-free animals addressed the influence of age and/or colonization on development of γδ T cells.

Our studies demonstrate that CD25 is clearly an activation molecule which is consistent with its function on αβ T cells (Sinkora et al., 2001). The frequency of CD25$^+$ γδ T cells increases quickly with stimulation and it is the only increase inducible by stimulation with IL-2. Our studies cannot exclude whether this increase is a result of new CD25 expression on the surface of existing cells or whether it is result of CD25$^+$ γδ T cell proliferation. CDR3 spectrotyping shows that CD25$^-$ γδ T cells from adult pigs display a non-diversified polyclonal TCR repertoire while CD25$^+$ γδ T cells contains previously expanded clones with diversified oligoclonal TCR repertoire. However, there is major discrepancy between the expression of CD25 on αβ versus γδ T cells. Expression of CD25 on αβ T cells is not permanent and is down-regulated after activation. Thus CD25$^+$ αβ T cells are infrequent in adults (Sinkora et al., 2001). In contrast, the number of CD25$^+$ γδ T cells is naturally high already in germ-free animals and besides increases with age. A similar phenomenon is common among other species (Shibata et al., 2008; Ullrich et al., 1990). The expression of CD25 can distinguish between two functionally different subsets: CD25$^+$CD122$^-$ IL-17-producing and CD25$^-$CD122$^+$ IFN-γ-producing γδ T cells (Shibata et al., 2008). IL-17-producing CD25$^+$ γδ T cells together with Th17 αβ T cells were found to be essential for protection against pulmonary infections (Khader et al., 2009) which is in agreement with our observations of increased frequencies of CD25$^+$ γδ T cells in lung, BAL and tonsils. Therefore CD25 could not only mark activated γδ T cells but functionally distinct subset that is involved in the neutrophil-mediated clearance of the pathogen (Khader et al., 2009; Shibata et al., 2008).

Expression of CD11b is probably connected to the early function of naive γδ T cells that are exported out of the thymus. This is evident from observation that (1) expression of CD11b is negligible in the thymus, (2) germ-free animals contain a majority of CD11b$^+$ γδ T cells and their frequency decreases with age and colonization and (3) the frequency of CD11b$^+$ γδ T cells can be substantially increased by activation in germ-free animals while the same is difficult in conventional piglets and almost impossible in adults. This finding indicates that the expression of CD11b can be induced on peripheral naive γδ T cells and could be critical for their function and further maturation. This would correspond with expression profile of CD11b in mice where it is associated with acquisition of cytotoxic capacity (McFarland et al., 1992), and humans where it is found on the CD8$^+$CD28$^-$ subset of memory T cells (Yamada et al., 1985). However, CD11b is probably down-regulated with maturation of porcine γδ T cells because CD11b$^+$ γδ T cells do not accumulate with age. Such an expression profile and sensitivity to activation was also seen in other γδ-high species (Graff and Jutila, 2007) but not in humans where CD11b is expressed on the vast majority of γδ T cells (Graff and Jutila, 2007). Therefore it is possible that CD11b expression is diminished after activation in some species

similarly to the expression of CD8α and MHC-II on T cells and CD25 on B cells (Sinkora et al., 2002b; Sinkora and Butler, 2009). Evidence that CD11b$^+$ γδ T cells represent a population of maturating and proliferating cells is their oligoclonal TCRδ reperoire. When these cells lose CD11b with maturation, their oligoclonality is diluted into a surplus of CD11b$^-$ polyclonal cells.

SWC1 and SWC7 are porcine molecules with unknown function and human/mice homologe. The expression of SWC7 was studied mainly on porcine B cells, and there is an entirely different profile from T cells (Bullido et al., 1999). There are only few reports, to our knowledge, about expression of SWC7 on porcine T cells, and those are merely summarized reports from Swine CD Workshops (Bullido et al., 1999; Sinkora et al., 2001). In agreement with those reports, we also found that SWC7 is activation molecule because its expression can be substantially increased after activation. However, the frequency of SWC7$^+$ γδ T cells does not show any age or colonization dependency, and distribution on the CD2/CD8 γδ T cells subsets is equal, which is significantly different from expression of CD25 or CD11b. Notable is our finding that the number of SWC7$^+$ γδ T cells differs substantially among individual animals and those with a high proportion of SWC7$^+$ γδ T cells are infrequent. According to these findings we believe that SWC7 represents an activation molecule with transient expression and its occurrence depends on the actual status of analyzed animal, although its expression on γδ T cells can be activated in all animals.

SWC1 is expressed on the majority of γδ T cells and its expression can be further increased by activation. This corresponds to other finding showing increased expression of SWC1 after immunization (Köhler et al., 1997). However, other reports show down-regulation of SWC1 after activation with ConA (Saalmüller et al., 1987; Sinkora et al., 2001) or contact with viral recall antigen (Sinkora et al., 2001). Discrepancies were explained by the possible role of SWC1 in a very early stage of activation (Köhler et al., 1997). In any case, none of these studies was done on γδ T cells, and due to the former limitation in availability of reagents they could not effectively discriminate between even basic lymphocyte populations. Detailed analysis of γδ T cells in this report shows that although expression of SWC1 can be activated, it does not resemble CD25, nor CD11b, because oligoclonal (diversified) TCR repertoire was confined to the SWC1$^-$ γδ T cells. Together with observation that SWC1 expression is relatively infrequent on the thymic γδ T cells, these observations point to the possibility that SWC1 is a lineage specific marker. In that case SWC1$^-$ as well as SWC1$^+$ could develop in the thymus. While lineage of SWC1$^+$ γδ T cells has a broad spectrum of TCR reactivity with no repertoire diversification, the SWC1$^-$ γδ T cell lineage can be activated to SWC1$^+$ whereafter it becomes diversified in response to particular antigens. After stimulation and maturation, diversified survivors return and stay in SWC1$^-$ γδ T cell pools where they represent effector/memory cells. This would correspond also with higher occurrence of SWC1$^-$ γδ T cells in some tissues of adult animals (particularly ileum and jejunum), and also with the observation that SWC1 expression can be induced in thymus of germ-free and young conventional piglets.

Human studies use CD45 isoforms to discriminate between functionally different subsets: the expression of CD45RO defines memory phenotype (CD45RO$^+$CD45RA$^-$) while the expression of CD45RA defines naive and terminally differentiated cells (CD45RO$^-$CD45RA$^+$) (Dieli et al., 2003). However, the same phenotype was not confirmed in mice. According to human studies we expected: (1) a positive response to stimulation for CD45RA$^-$ γδ T cells, (2) a negative response for CD45RA$^+$ γδ T cells, and (3) a concentration of the oligoclonal TCR repertoire in the CD45RA$^-$ γδ T cell subset. This is exactly what we have found. Therefore, the expression of CD45RA/RC in swine probably represents a marker of naive and terminally differentiated γδ T cells. However, CD45RA/RC is substantially reduced in the thymus so that naive CD45RA/RC$^+$ phenotype is acquired in the periphery after export from the thymus. This corresponds with earlier results on the development of γδ thymocytes (Sinkora et al., 2005a, 2007). Unfortunately, due to its current unavailability we could not employ anti-CD45RO mAb which has been used to define memory γδ T cells in humans (Dieli et al., 2003).

γδ T cells are capable of antigen presentation (Brandes et al., 2005; Cheng et al., 2008; Takamatsu et al., 2002) and therefore MHC-II expression should be its natural feature. However, unlike humans (Brandes et al., 2005) and mice (Cheng et al., 2008) where MHC-II expression on γδ T cells is transient and can be observed only after activation, porcine MHC-II$^+$ γδ T cells can be easily detected in germ-free piglets and they occur in thymus as well as in the periphery. Expression of MHC-II increases mainly with age of animals but short-term activation does not lead to clearly enhanced MHC-II expression. Inability of MHC-II stimulation on porcine γδ T cells is in contrast with other reports (Brandes et al., 2005; Cheng et al., 2008) and can be explained by observation that porcine γδ T cells constitutively express MHC-II molecules. Constitutive expression of MHC-II on T cells is a well known peculiarity of the porcine immune system (Saalmüller et al., 1991; Sinkora and Butler, 2009). On the other hand, our studies cannot exclude the effect of long-term activation on MHC-II expression (Rehakova et al., 1998).

Our studies allowed for the first time to compare features of different CD2/CD8 subsets of porcine γδ T cells. It is known that different CD2/CD8 γδ T cell subsets are differentially located in various tissues (Saalmüller et al., 1990; Sinkora et al., 1998; Yang and Parkhouse, 1996). However, except for the indication that CD2$^+$CD8$^-$ can differentiate into CD2$^+$CD8$^+$ γδ T cells (Reddehase et al., 1991), there is no report showing whether the CD2/CD8 subsets represent separate and independent lineages or if they represent subsequently developing subsets. Ontogenetic and developmental studies in the thymus indicate separate lineage commitment (Sinkora et al., 2005a, 2007). Results of this work point to a possibility that the CD2$^-$CD8$^-$ subset is mostly composed of naive cells because the majority of them has the naive CD45RA/RC$^+$ phenotype and there are only few MHC-II$^+$ cells capable of antigen presentation. CD2$^-$CD8$^-$ γδ T cells is the only subset responding to IL-2 stimulation. Albeit CD2$^-$CD8$^-$ γδ T cells can express high levels of surface CD25, CD25$^+$ cells in this subset are generally found infrequently in tissues other than lung and BAL. Notable is a very high incidence of CD2$^-$CD8$^-$ γδ T cells in the blood. On the other hand, CD2$^+$CD8$^-$ may represent an effector/memory subset because the majority of them lack CD45RA/RC and many of them express CD25 throughout different organs. Moreover, this subpopulation has more MHC-II and CD11b expression than the CD2$^-$CD8$^-$ subset. Finally, CD2$^+$CD8$^+$ γδ T cells probably represent a terminally differentiated subset with re-expressed CD45RA/RC, in which most of cells remain largely unresponsive to activation. Subset of CD2$^+$CD8$^+$ γδ T cells has the highest expression of MHC-II and SWC1, many of which express CD11b and in which expression of CD25 is very frequent. This would correspond with other findings that this particular subset is cytotxic (de Bruin et al., 1997; Yang and Parkhouse, 1997). In any case, although this work substantially increases our knowledge of porcine γδ T cells and gives view into possible functional characteristic of their CD2/CD8 subsets, it still cannot resolve their developmental dependency or independency.

5. Conclusions

In conclusion, we have characterized peripheral γδ T cells and their subsets in swine identifying putative lineages, differentiation,

developmental and activation markers. Evidence that the CD2/CD8 subsets of porcine γδ T cells may represent functionally different cells is given. However, our studies can not exclude possibility that they still represent separate cell lineages.

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8. *(publication #5)*

Porcine γδ T lymphocytes can be categorized into two functionally and developmentally distinct subsets according to expression of CD2 and level of TCR

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Porcine γδ T lymphocytes can be categorized into two functionally and developmentally distinct subsets according to expression of CD2 and level of TCR.

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Porcine γδ T cells have two levels of TCRγδ expression. While TCRγδ^{med} cells are mostly CD2$^+$CD8$^-$ and CD2$^+$CD8$^+$, TCRγδ^{hi} cells are highly enriched for CD2$^-$CD8$^-$. This distribution is independent of bacterial colonization and it is already established in the thymus prior to export of γδ cells to the periphery. Sorting and cultivation experiments revealed that CD2$^-$ CD8$^-$ γδ cells are unable to acquire CD2 and CD8, while CD2$^+$ subsets can gain or loose CD8. There is also differential susceptibility for proliferation between CD2$^+$ and CD2$^-$ γδ cells. While CD2$^-$CD8$^-$ almost do not proliferate, proliferation of CD2$^+$CD8$^-$ and CD2$^+$8$^+$ is substantial. Population of CD2$^-$ γδ cells is also absent in CD1$^+$ immature thymocytes. In addition, subpopulations of CD2$^+$ and CD2$^-$ γδ cells in the thymus differ in expression of auxiliary surface molecules such as CD25, CD45RA/RC and MHC-II. Moreover, TCRγδ^{hi} cells can generate TCRγδ^{med} cells but never the opposite. The only exception is the thymus where a few TCRγδ^{med} cells can be induced to TCRγδ^{hi} but only under IL-2 influence. Repertoire of TCRδ is polyclonal in all subsets indicating there is the same extent of diversification and equal capability of immune responses. Results collectively indicate that CD2 expression determines two lineages of γδ cells that differ in many aspects. Because CD2$^-$ γδ cells are missing in the blood of humans and mice but are obvious in other members of γδ high species such as ruminants and birds, our findings support the idea that circulating CD2$^-$ γδ T cells are a specific lineage.

Swine belongs to the group of γδ high species in which γδ T lymphocytes are not preferentially limited to epithelia and may account for >70% of all T cells (1, 2). Traditionally, γδ T-cells in swine are subdivided into three subsets based upon their expression of CD2 and CD8 and include CD2$^-$CD8$^-$, CD2$^+$CD8$^-$ and CD2$^+$CD8$^+$ cells (3-7). These individual subsets differ in their homing characteristic (8) and cytotoxic activities (7, 9). However, except for the indication that CD8$^-$ can differentiate into CD8$^+$ γδ T cells (10), there is no report showing whether the CD2/CD8 subsets represent separate and independent lineages or if they represent subsequently developing subsets. Ontogenetic and developmental studies in the thymus indicate separate lineage commitment (5, 11). On the other hand, results of recent work (12) point to a possibility that the CD2$^-$CD8$^-$ subset is mostly composed of naive cells, while CD2$^+$CD8$^-$ cells may represent an effector/memory subset, and CD2$^+$CD8$^+$ γδ T cells probably represent terminally differentiated cells. This would correspond with the finding that the latest subset is cytotoxic (7, 9) and would indicate that CD2/CD8 subpopulations of γδ T cells represent subsequently developing stages.

It is generally accepted that unlike αβ T cells, γδ T lymphocytes develop in the thymus without any selection for pre-antigen receptors

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Abbreviations used in this paper: SLA-DR, Swine MHC-II leukocyte antigen type DR.

(like pre-TCRαβ), and also without any $CD3^{lo}$ or TCR^{lo} transitional stage (5, 11, 13, 14). However, it is known that TCR can be expressed on γδ T cells in two densities: medium and high (15, 16). Unfortunately, the relevance of $TCR\gamma\delta^{med/hi}$ expression was never studied in more detail. Studies in αβ T cells indicate that differential expression level of TCR may be a result of activation and is regulated by tyrosine kinase Lck (17).

Our previous studies revealed the basic distribution and phenotypic profile of porcine γδ T cells (3, 12, 18), their ontogeny (3, 11), development in the thymus (5, 11, 13) and the repertoire diversification of their TCR (19). In the present study, we used high-speed flow cytometry sorting and cultivation techniques to characterize different subpopulation of γδ T cells defined by TCRγδ, CD2 and CD8 expression. Results show many differential features of $CD2^{-}CD8^{-}$ versus $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ γδ T cells and collectively indicate that CD2 expression determines two lineages of γδ cells in swine.

Materials and methods

Experimental animals

Animals used in the study were Minnesota miniature/Vietnam-Asian-Malaysian crossbred piglets bred in Novy Hradek (3, 20). All pigs were healthy and normal at slaughter. Germ-free piglets were recovered from gilts by hysterectomy at the 112th day of gestation. Gestation age was calculated from the day of mating. After birth, germ-free piglets were kept in isolator units under germ-free conditions at all times and monitored for the unwanted appearance of bacteria. All animal experiments were approved by the Ethical Committee of the Institute of Microbiology, Czech Academy of Science, according to guidelines in the Animal Protection Act.

Preparation of cell suspensions

Cell suspensions were prepared essentially as previously described (3, 21). Briefly, heparinized (20 U/ml; LECIVA-Zentiva) blood was obtained by intracardial puncture. Cell suspensions from the spleen and thymus were prepared in cold phosphate-buffered saline (PBS) by carefully teasing the tissues using forceps and then by passage through a 70 μm mesh nylon membrane. In the case of the blood and spleen, lymphocyte fractions were purified using a Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation. Before staining for flow cytometry, all cell suspensions were washed twice in cold PBS containing 0.1% sodium azide and 0.2% gelatin from Cold Water Fish Skin (PBS-GEL, all chemicals Sigma-Aldrich, St. Louis, MO), filtered through a 70 μm mesh nylon membranes, and cell numbers were determined by hemacytometer.

Immunoreagents

The following mouse anti-pig monoclonal antibodies (mAbs), whose source and specificity were described earlier (3, 4, 21), were used as primary immunoreagents: anti-TCRγδ (PPT26, IgG1 or PPT16, IgG2b or PGBL22A, IgG1), anti-CD1 (76-7-4, IgG2a) anti-CD2 (MSA4, IgG2a or 1038H-5-37, IgM), anti-CD8 (76-2-11, IgG2a or PT36B, IgG1, VMRD- Pullman, WA), anti-CD25 (K231-3B2, IgG1), anti-CD45RA (FG2F9, IgG1), anti-CD45RC (MIL5, IgG1), anti-SLA-DR^{3} (1038H-12-34, IgM). Goat polyclonal antibodies (pAbs) specific for mouse immunoglobulin subclasses labeled with FITC, PE, PE/Cy5 (alternatively PE/Cy7) or APC were used as secondary immunoreagents (Southern Biotechnologies Associates, Inc., Birmingham, AL). All immunoreagents were titrated for optimal signal/noise ratios. In the case of indirect sub-isotype staining, primary isotype-matched mouse anti-rat mAbs were used as negative controls. Secondary pAbs were tested for cross-reactivity (no primary mAbs) and also for cross-reactivity with primary isotype-mismatched mouse anti-pig mAbs. No background or false staining was observed. In some cases, directly labeled anti-TCRγδ/PE-DY747 mAb (PPT16, Exbio, Prague, Czech Republic) and/or different directly labeled mAbs were used. These were labeled with Zenon Labeling Technology (Molecular Probes, Eugene, OR) according to a protocol recommended by the manufacturer.

Staining of cells

Staining of cells for flow cytometry was performed as described previously (3, 11, 21) 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 pAbs conjugated with different fluorochromes were then added to the cell pellets in appropriate combinations. After 15 minutes, cells were washed three times in PBS-GEL and analyzed by flow cytometry. In some experiments, direct staining was used to elucidate the effect of direct versus indirect staining. In that case, the procedure was the same as described above but only one 30 minutes incubation step was used. In the case of intracellular staining, cells that had been indirectly stained for cell surface

molecules were subsequently intracellularly stained using IntraStain Kit according to a protocol recommended by the manufacturer (DakoCytomation, Glostrup, Denmark).

Flow cytometry and cell sorting

Samples were measured or sorted on standard FACSCalibur or FACSAriaIII flow cytometers respectively (BDIS, Mountain View, CA). In each measurement, 300 – 700 thousand events were collected. Sorted cells were collected into 1) inactivated fetal bovine serum (PAA, Pasching, Austria) in the case of cultivation or 2) empty tubes in the case of PCR amplification. Electronic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. FSC-A/FSC-W parameters were used for elimination doublets. The PCLysis or FACSDiva software (BDIS, Mountain View, CA) was used for data processing.

Proliferation assay and CFSE labeling

Proliferation history of cells was determined by CFSE (Sigma-Aldrich, St. Louis, MO; [5-(and -6)-carboxyfluorescein diacetate succinimidyl ester] using techniques described previously (22). Briefly, suspensions of $4x10^7$/ml fresh or $1x10^6$/ml sorted cells in PBS or PBS with 5% fetal bovine serum respectively were stained by 5 μM/ml CFSE solution under vigorous mixing for 5 min. Final suspension was 10x diluted by PBS supplemented with 5% fetal calf serum, washed three times in the same diluting solution, resuspended in culture medium and cultivated. After cultivation, suspensions were stained by indirect sub-isotype staining as described above and analyzed by flow cytometry.

Cell cultures and stimulation in vitro

Cell cultures were done in RPMI-1640 medium supplemented with L-Glutamine and 25 mM HEPES, 10% fetal bovine serum, 100 U Penicillin and 0.1 mg/ml Streptomycin (all chemicals PAA, Pasching, Austria). Final concentration of cells was always set to $2x10^6$ cells per ml and cells were cultivated with one of the following: 50 ng/ml Phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, St. Louis, MO), or 100 U/ml porcine recombinant interleukin 2 (IL-2, RayBiotech, Norcross, GA), or 10 ng/ml porcine recombinant IL-4 (ProSpec, Ness Ziona, Israel), or 5 μg/ml Concanavalin A (ConA, Sigma-Aldrich, St. Louis, MO), or without any stimulation or cells were stored at 4°C. Some combinations of above mentioned activators were also used and PMA was sometimes used with 1 μg/ml ionomycin (Sigma-Aldrich, St. Louis, MO). Culturing times were 3, 4 or 7 days.

Confocal microscopy

Confocal microscopy was done to test the effect of cross-linking with secondary pAbs to patching and capping of stained molecules. Cell suspensions from the spleen were washed in cold PBS, followed by incubation for 30 min at 4°C or 37°C with directly labeled or unlabeled primary anti-TCRγδ, anti-CD2 and anti-CD8 mAbs. Afterwards, cells were washed twice in PBS, and (1) in the case of directly labeled mAbs fixed with 2% paraformaldehyde for 15 min or (2) in the case of unlabeled mAbs incubated with the appropriate fluorescence labeled secondary pAbs for an additional 30 min at 4°C or 37°C, washed twice in PBS and fixed with 2% paraformaldehyde. Resulting cell suspensions were visualized by Olympus IX-81 microscope equipped with SV-1000 confocal system and analyzed by Olympus FV10-ASW 2.0 Viewer software (Olympus Corporation, Tokyo, Japan). Cell suspensions were also analyzed by flow cytometry for number of positive cells and expression level of stained molecules.

PCR amplification and CDR3 spectratyping

The diversity in the TCRδ repertoire is overwhelmingly determined by the diversity in the delta chain third complementary region (CDR3). Thus, separation of CDR3 regions for TCRδ on polyacrylamide sequencing gels provides a clonotypic analysis of porcine γδ T cells showing their level of diversification (19). This procedure for measuring of CDR3 polymorphism is called spectratyping and was performed essentially as described previously (5, 12, 13, 19). Briefly, 50 or 100 thousand sorted cells were immediately after sort dissolved in 0.5 ml TRI Reagent (Sigma-Aldrich, St. Louis, MO). In a particular analysis, only the same amount of sorted cells was used for preparation of RNA and cDNA (by random hexamer primers). Gene segments for VDJ regions of TCRδ1 (TRDV1) were PCR amplified and efficiency of PCR amplification was checked on agarose gels. Amplified segments were next re-amplified only for CDR3 regions using 32^P-labeled primers and the product was separated on sequencing gels. Gels were dried and images were obtained by Fluorescent Image Analyser FLA-7000 (Fujifilm corporation, Yokyo, Japan).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). Differences among the median frequency values for sorted and thereafter cultivated populations at 37°C and 4°C were analyzed by one way analysis of variance (anova) – Dunnett`s Multiple Comparison Test. Difference between populations of cells originating from one sample were analyzed by paired t-test. All other comparisons were analyzed by unpaired t-test. In

*all analyses, GraphPad Prism4™ software (GraphPad Software, San Diego, CA) was used. The level of statistical significance is reported in P-values: P < 0.05 was considered significant (marked *); P < 0.01 was considered strongly significant (marked **); and P < 0.001 was considered very strongly significant (marked ***).*

Results

γδ T cells have two levels of TCRγδ expression

Natural expression of TCR on porcine γδ T lymphocytes occurs in two densities: medium and high (Fig. *1A*). These two subsets are differentially distributed among CD2/CD8 γδ T cells: while TCRγδ^{med} are preferentially CD2^{+} (either CD2^{+}CD8^{+} or CD2^{+}CD8^{-}) TCRγδ^{hi} are mostly CD2^{-}CD8^{-}.

Indirect staining causes patching of molecules on the surface of positive cells but there is no capping, colocalization, induced antigenic modulation, receptor mediated endocytosis or activation.

Indirect sub-isotype staining was mostly used throughout this report. Although we can easily control cross-reactivity and non-specific binding of used secondary pAbs, we cannot rule out potential cross-linking of key signaling molecules on the surface of cells. For this reason, an effect of direct and indirect staining on the patching and capping of stained molecules on the surface of γδ T cells was studied by confocal microscopy (Fig. *1B*). Direct staining always produced uniform circumferential fluorescence of TCRγδ, CD2 and CD8 on γδ T cells independently of cultivation temperature (Fig. *1B*, cells *B1-B6*). On the other hand, indirect staining always produced patching of surface molecules (Fig. *1B*, cells *B7-B12*). Formation of patches after cross-linking with a secondary pAbs at 4°C (Fig. *1B*, cells *B7-B9*) indicate that retention of TCRγδ, CD2 and CD8 does not require a dynamic cellular response and was a result of cross-linking alone. This is in agreement with the observation that there was no induced capping and no clear colocalization of TCRγδ /CD2/CD8 (Fig. *1B*, cells B7-B9 and cells *B10-B12*). Moreover, the possibility of induced antigenic modulation and/or receptor mediated endocytosis by indirect staining was excluded by examination of the same cells by flow cytometry (Fig. *1B*). If any of these effects would have taken place, there should be numerical and/or expression level differences between samples kept at 4°C and at 37°C, which was not observed.

The effect of indirect staining on the responsiveness of γδ T cells was also examined in a culture. Table I shows that behavior of γδ T cells that were cultivated either unstained or pre-stained by indirect staining is comparable. In agreement with confocal microscopy, there are no differences in proliferation activity (Table I, left two columns), modulation of TCRγδ expression level (Table *I*, right two columns) and/or alteration of CD2/CD8 phenotype (data not shown). This type of analysis also allows examination of the effect of various activators and their combinations. The results show that proliferation of γδ T cells occurs spontaneously in medium alone and can be significantly ($P<0.01$) increased by IL-2 and ConA activation (Table *I*, left two columns). There was no synergistic effect of IL-2 with either PMA or ConA. Although ionomycin increased proliferation caused by PMA, the differences were not statistically significant. For this reason and also because low cell viability and peculiar scatter characteristic of γδ T cells after ionomycin treatment, activation by PMA plus ionomycin was not further used in this study. Modulation of TCRγδ expression level (Table I, right two columns) occurs also spontaneously in medium alone and can be significantly ($P<0.01$) increased namely by IL-2 activation. In fact, stimulation by IL-2 was significantly different from any other treatment. Again, addition of IL-2 do not synergize with PMA or ConA.

Sorted TCRγδ^{med} and TCRγδ^{hi} subsets have different features

Spleen was chosen for most sorting experiments because there is approximately the same CD2/CD8 γδ T cell subsets ratio (12). In any case, confirmation experiments done on other tissues had comparable outputs (data not show). Sorted cells were cultivated for 2-7 days without any activators (at 4°C or 37°C) or supplemented with PMA, IL-2, IL-4 or ConA (Fig. *2*). Results show that sorted TCRγδ^{med} cells (Fig. *2A*) are unable to increase TCR expression and never become TCRγδ^{hi} (Fig. *2C*). On the other hand, there is always a part of sorted TCRγδ^{hi} cells (Fig. *2B*) that spontaneously decrease TCR expression (Fig. *2D*). This down-regulation occurs fast and is detectable within hours. Down-regulation of TCRγδ does not occur at 4°C (Fig. *2D*, see data for 4°C) and is independent of anti-TCRγδ mAb, direct or indirect staining procedures and particular fluorochrome used (data not shown).

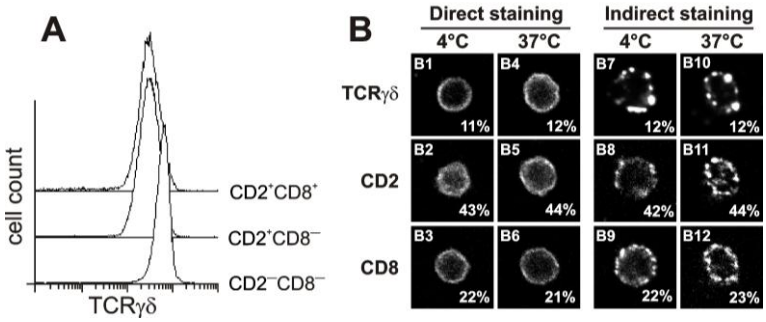

FIGURE 1. γδ T cells have two levels of TCRγδ expression (A). Splenocytes from 2-8 wk old piglets were triple stained by anti-TCRγδ, anti-CD2 and anti-CD8 and expression level of TCRγδ is shown for gated $CD2^{-}CD8^{-}$, $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ γδ T cells. Flow cytometry results are representative of all analyzed animals (12 in total). Indirect staining causes patching but no capping, colocalization or receptor mediated endocytosis (B). Splenocytes from 5 wk old piglets were triple stained by anti-TCRγδ, anti-CD2 and anti-CD8 directly at 4°C (B1-B3) or 37°C (B4-B6) or indirectly at 4°C (B7-B9) or 37°C (B10-B12), and positive γδ T cells were analyzed by confocal microscopy. In each staining, 50 positive γδ T cells were scored and representative results are shown. The same cells were also analyzed by flow cytometry for the frequency of positive γδ T cells (the percentages shown in each examination) and expression level of stained molecules (data not shown).

Supplemented activators can enhance the down-regulations so that PMA had the highest impact, followed by IL-2 and IL-4 (Fig. *2D*).

Analysis of CD2/CD8 subpopulations within $TCR\gamma\delta^{med}$ or $TCR\gamma\delta^{hi}$ sorted cells revealed that sorted $TCR\gamma\delta^{med}$ cells are mostly composed of $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ γδ T cells (Fig. *2E*). On the other hand, sorted $TCR\gamma\delta^{hi}$ cells are highly enriched for $CD2^{-}CD8^{-}$ γδ T cells (Fig. *2F*). This corresponds to analysis of CD2/CD8 γδ T cell subpopulations in unsorted cells (Fig. *1A*). Cultivation of sorted cells without or with selected activators have a small effect on the proportions of individual CD2/CD8 subpopulations for both $TCR\gamma\delta^{med}$ (Fig. *2E*) and $TCR\gamma\delta^{hi}$ (Fig. *2F*) sorted cells. We further analyzed $TCR\gamma\delta^{med}$ (Fig. *2D*, region R1) and $TCR\gamma\delta^{hi}$ (Fig. *2D*, region R2) cells resulting from cultivation of originally sorted $TCR\gamma\delta^{hi}$ cells. The analysis showed that newly generated $TCR\gamma\delta^{med}$ cells (Fig. *2G*) as well as cells that do not change their $TCR\gamma\delta^{hi}$ expression (Fig. *2H*) had similar proportion of CD2/CD8 subpopulations as originally sorted cells (compare Fig. *2G* and *2H* with *2F*). The only exception is a slightly higher representation of $CD2^{+}CD8^{+}$ within newly generated $TCR\gamma\delta^{med}$ cells (Fig. *2G*), which was observed in all experiments.

Sorted CD2/CD8 subpopulations of γδ T cells alter their phenotype in vitro

Cultivation of sorted $CD2^{-}CD8^{-}$, $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ subsets of γδ T cells from spleen with different activators revealed that $CD2^{-}CD8^{-}$ γδ T cells are unable to acquire CD2 and/or CD8 molecules *in vitro* (Fig. *3A-E*). However, $CD2^{+}$ subsets of γδ T cells can change their phenotype by gain (Fig. *3F-J*) or lost (Fig. *3K-O*) of CD8. While a gain of CD8 on $CD2^{+}CD8^{-}$ cells is mainly influenced by IL-2 (Fig. *3I*), the loss CD8 can largely occur spontaneously (Fig. *3L*) but can be increased by activators (Fig. *3M-O*).

CD2/CD8 subsets of γδ T cells have different proliferation activity

Analysis of several pigs (n=20) for spontaneous and induced proliferation of γδ T cells revealed that animals can be divided into two groups. The first group is non-proliferative where there is negligible spontaneous proliferation of γδ T cells in RPMI, and where induced proliferation by PMA or IL-4 is also minimal (Fig. *4A*). Stimulation by IL-2, however, caused apparent proliferation (Fig. *4A*). The second group is proliferative due to the apparent proliferation of γδ T cells in RPMI and under stimulation by PMA or IL-4 (Fig. *4B*). As in the case of non-proliferative animals, stimulation by IL-2 caused substantial increase of proliferation (Fig. *4B*). Analysis of CD2/CD8 γδ T cell subsets for proliferation capacity showed that IL-2 stimulation caused mainly proliferation of $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ γδ T cells while $CD2^{-}CD8^{-}$ proliferation was substantially reduced, and this occurs in both non-proliferative (Fig. *4C*) and proliferative (Fig. *4D*) animals. Notably, the same proportion of cycling cells among CD2/CD8 subsets was also observed during spontaneous proliferation in proliferative animals (data not show).

To further investigate the direct effect of stimulation on individual CD2/CD8

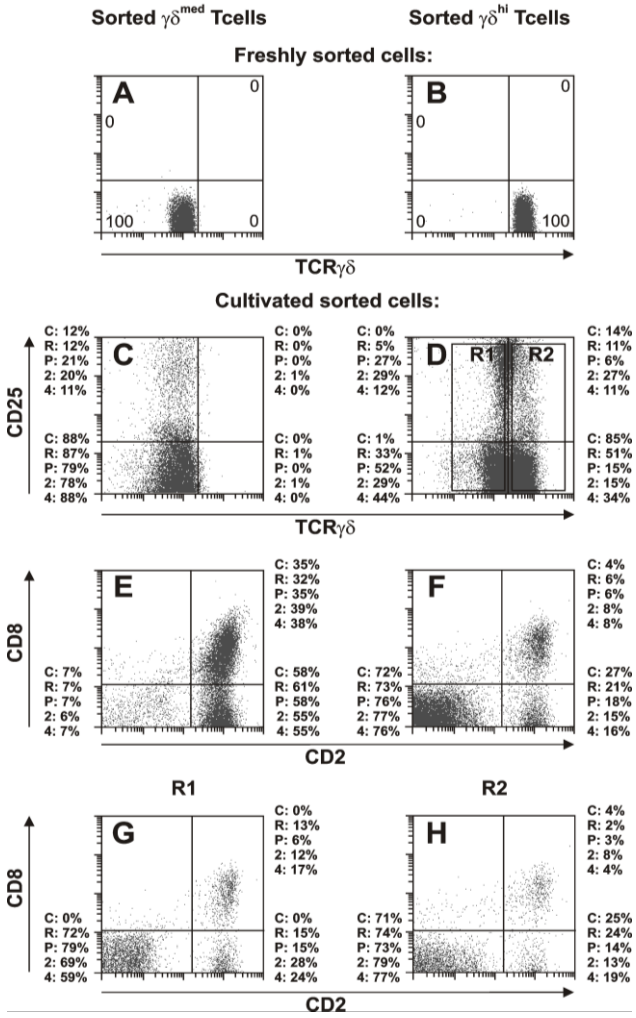

FIGURE 2. Sorted TCRγδ^{med} and TCRγδ^{hi} subsets have different features. Splenocytes from 2-8 wk old piglets were stained for TCRγδ and sorted by flow cytometry according to TCRγδ surface density into TCRγδ^{med} (A) and TCRγδ^{hi} (B) subsets. Sorted cells were cultivated for 3 days under different conditions and reanalyzed by four color flow cytometry after re-staining with anti-TCRγδ and anti-CD2, anti-CD8 and anti-CD25. The dotplots C-H show representative analysis of resulting cells from cultivation with IL-2 and percentages of resulting cells for all culture conditions are stated beside of each quadrant: C= control cultivation at 4°C in medium alone, R= cultivation in RPMI medium only, P= cultivation with PMA, 2= cultivation with IL-2 and 4= cultivation with IL-4. Dotplots C and D show expression of TCRγδ/CD25 on resulting cells from sorted TCRγδ^{med} and TCRγδ^{hi} splenocytes respectively. Dotplots E and F show expression of CD2/CD8 on resulting cells from sorted TCRγδ^{med} and TCRγδ^{hi} splenocytes respectively. Dotplot G and H show expression of CD2/CD8 on resulting cells from sorted TCRγδ^{hi} splenocytes gated by region R1 for TCRγδ^{med} and R2 for TCRγδ^{hi} respectively (region are shown in dotplot D). The results are representative of 9 independent experiments.

subpopulations and to avoid effect of CD2/CD8 phenotype alteration during cultivation we performed flow cytometry sorting (Fig. *4E*). Cultivation of sorted subpopulations confirmed data from mixed cell cultures (Fig. *4C* and *D*) and showed that sorted CD2$^-$CD8$^-$ γδ T cells always have the lowest proliferative activity, followed by sorted CD2$^+$CD8$^-$ and CD2$^+$CD8$^+$ γδ T cells (Fig. *4E*). However, because proliferative and non-proliferative animals were used together, the only significant difference was found for IL-2 stimulation.

Proliferation activity of sorted CD2/CD8 subpopulation as shown in Figure *4E* was always lower than for non-sorted cells where other cell types were present. There was also an apparent lack of activation effect by different activators (namely ConA) on proliferation of

TABLE I. Effect of different activators and indirect pre-staining on proliferative activity and phenotype modulation of γδ T cells *in vitro*[1]

	proliferating γδ unstained	proliferating γδ pre-stained	TCRγδ^{hi} cells unstained	TCRγδ^{hi} cells pre-stained
4°C	N/A	N/A	33 ± 5	28 ± 5
medium alone	7 ± 6	5 ± 4	45 ± 5	40 ± 3
PMA	10 ± 7	7 ± 2	35 ± 3	40 ± 6
PMA + IL-2	22 ± 10	28 ± 7	44 ± 6	48 ± 4
PMA + ionomycin	19 ± 12	19 ± 13	46 ± 5	42 ± 5
IL-2	30 ± 5	33 ± 3	60 ± 6	62 ± 5
IL-4	4 ± 2	3 ± 1	46 ± 8	44 ± 9
ConA	35 ± 13	35 ± 12	44 ± 4	43 ± 5
ConA + IL-2	37 ± 13	32 ± 15	44 ± 5	45 ± 6

[1] The splenocytes isolated from 4 wks old pigs were divided into 2 groups from which cells in one group were left unstained while cells in second group were pre-stained by indirect staining with anti-TCRγδ, anti-CD2 and anti-CD8. Each group was thereafter divided into two subgroups and cells in one subgroup from each group were loaded by CFSE. All four subgroups were thereafter cultivated for 4 days under different conditions (table lines) and cells loaded by CFSE were analyzed by flow cytometry for the frequency of proliferating γδ T cells (table headline) while cells without CFSE were analyzed for the proportions of TCRγδ^{hi} cells (table headline) and also individual CD2/CD8 subpopulations of γδ T cells (data not shown) after final re-staining with the same antibodies. Cells kept at 4°C did not change expression of cell surface molecules and had the same phenotype as fresh splenocytes. Values represent mean ± SD obtained from four animals. There were no statistically significant differences between unstained and pre-stained samples.

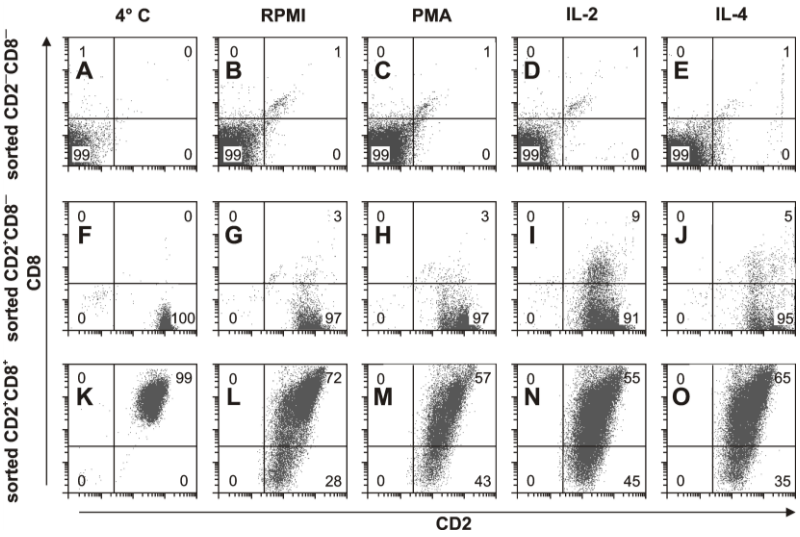

FIGURE 3. Sorted CD2/CD8 subpopulations of γδ T cells alter their phenotype *in vitro*. Splenocytes from 2-8 wk old piglets were stained for TCRγδ, CD2 and CD8 and sorted by flow cytometry into three CD2/CD8 γδ T cell subsets (individual rows). Sorted cells were cultivated for 3 days under different conditions (individual columns) and reanalyzed by three color flow cytometry after re-staining with the same mAbs. Cells kept at 4°C did not change expression of cell surface molecules and had the same phenotype as freshly sorted splenocytes. Stimulation by ConA had a similar effect as cultivation in medium only (data not shown). The results are representative of 13 independent experiments.

sorted γδ T cells when compared with non-sorted cells (compare Fig. *4E* and Table *I*). This observation initiated experiments in which pure γδ T cells and their individual CD2/CD8 subsets were sorted, stained by CFSE and half of them was cultivated alone while the second half was cultivated with an isogeneic mixture of unsorted cells (Fig. *4F-G*). Analysis of spontaneous (Fig. *4F*) and IL-2 induced (Fig. *4G*) proliferation showed that pure sorted γδ T cells always have a lower proliferation capacity than sorted γδ T cells cultivated with isogeneic unsorted cells (Fig. *4F-G*). The same applies to CD2/CD8 subpopulations of γδ T cells (Fig. *4F-G*). However, the capacity of individual CD2/CD8 subpopulations of γδ T cells to proliferate remains unchanged: $CD2^{-}CD8^{-}$ γδ T cells always have the lowest proliferative activity followed by sorted $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ γδ T cells (Fig. *4F-G*).

Thymic γδ T cells can be divided into three subsets according to level of TCRγδ and the expression of CD1 molecule

Expression density of TCRγδ on thymocytes is medium and high (Fig. *5A*), resembling the periphery. However, thymic γδ T cells contain developing precursors which can be identified by expression of CD1 (5, 11, 13). While $CD1^{+}$ γδ thymocytes are strictly $TCR\gamma\delta^{med}$, $CD1^{-}$ γδ thymocytes can be $TCR\gamma\delta^{med}$ or $TCR\gamma\delta^{hi}$ (Fig. *5A*). These three subsets differ in expression of CD2 and CD8 (Fig. *5B*) and also CD25, SLA-DR (MHC-II) and CD45RC/RA (Fig. *5C*). $TCR\gamma\delta^{med}CD1^{+}$ thymocytes are characteristic of the highest frequencies of $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ while $CD2^{-}CD8^{-}$ cells are almost absent (Fig. *5B*). $TCR\gamma\delta^{med}CD1^{+}$ thymocytes also express low amounts of CD45RC, CD25 and SLA-DR molecules (Fig. *5C*). On the other hand, $TCR\gamma\delta^{med}CD1^{-}$ thymocytes contain all CD2/CD8 subpopulations but most abundant are $CD2^{+}CD8^{-}$ cells (Fig. *5B*). $TCR\gamma\delta^{med}CD1^{-}$ thymocytes also contain low frequencies of $CD45RC^{+}$, $CD25^{+}$ or $SLA\text{-}DR^{+}$ cells (Fig. *5C*). Finally, $TCR\gamma\delta^{hi}CD1^{-}$ thymocytes are characteristic by a predominance of $CD2^{-}CD8^{-}$ cells (Fig. *5B*), which is similar to blood. This subset has a much higher amount of CD45RC expression than $TCR\gamma\delta^{med}$ thymocytes and also higher expression of CD25 and SLA-DR (Fig. *5C*).

Sorted γδ thymic subsets can change their phenotype during cultivation

While sorted $TCR\gamma\delta^{med}CD1^{+}$ thymocytes (Fig. *6A*) keep the same TCRγδ/CD1 phenotype during cultivation, even with activators (Fig. *6B*), sorted $TCR\gamma\delta^{med}CD1^{-}$ (Fig. *6C*) and $TCR\gamma\delta^{hi}CD1^{-}$ (Fig. *6E*) thymocytes can change their phenotype *in vitro* (Fig. *6D* and *6F*, respectively). Similarly to their peripheral counterparts, a portion of $TCR\gamma\delta^{hi}CD1^{-}$ thymocytes (Fig. *6E*) becomes $TCR\gamma\delta^{med}CD1^{-}$ (Fig. *6F*). This down-regulation of TCRγδ occurs quickly (it is visible in a day), spontaneously and it can be increased by activators, especially using PMA (data not shown). On the other hand, a small fraction of sorted $TCR\gamma\delta^{med}CD1^{-}$ thymocytes (Fig. 6C) can become $TCR\gamma\delta^{hi}CD1^{-}$ (Fig. *6D*). This up-regulation of TCRγδ occurs very slowly (few cells can be detected after 3 days but a significant amount at the 7th day), only by IL-2 stimulation, and could not be observed in any analyzed tissue except the thymus.

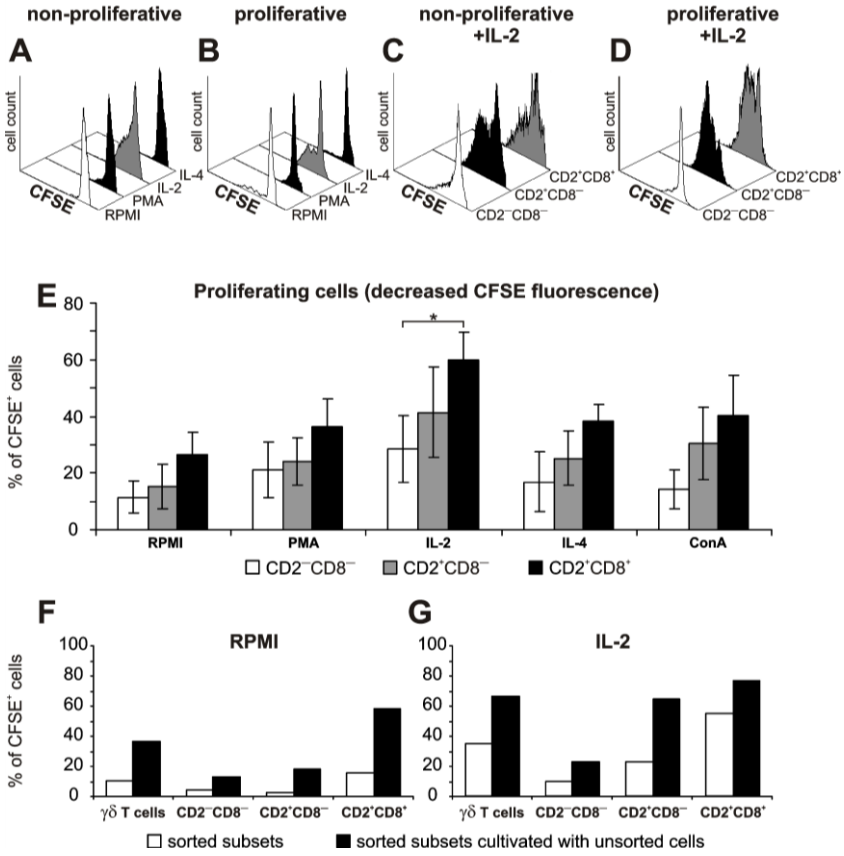

FIGURE 4. CD2/CD8 subsets of γδ T cells have different proliferation activity. Fresh splenocytes were labeled with CFSE, cultivated for 4 days in medium alone (RPMI) or with PMA, IL-2 or IL-4 and finally cell-surface stained for TCRγδ, CD2 and CD8. Representative analysis of CFSE fluorescence of all γδ T cells for groups of animals that showed low (A) or high (B) spontaneous proliferation activity under different cultivation condition (Z-axis) is shown. Further analysis (C and D) of IL-2 stimulated γδ T cells from histogram A and B respectively for proliferation activity of individual CD2/CD8 subsets (Z-axis) is also shown. Graph E shows the results of the second type of experiment which involves the same cell-surface staining, flow cytometry sorting of individual CD2/CD8 γδ T cell subpopulation, labeling with CFSE, cultivation for 4 days under different conditions (x-axis) and cell-surface re-staining. The proportions of proliferating cells (with decreased CFSE fluorescence) for sorted $CD2^{-}CD8^{-}$ (white bars), $CD2^{+}CD8^{-}$ (gray bars) and $CD2^{+}CD8^{+}$ (black bars) γδ T cells are shown. Bars represent mean values and error bars represent ± SD obtained from five animals. Significant difference is shown and indicated by asterisks according to materials and methods. The last experiments (F-G) involved the same type of experiments and analysis as described for graph E but while a half of sorted cells was cultivated alone (white bars), the second half was cultivated with isogeneic mixture of unsorted cells (black bars). Cultivation in medium alone (F) or with IL-2 (G) is shown. The result is representative of two independent experiments with the same outcome.

When $TCR\gamma\delta^{med}CD1^{-}$ thymocytes were sorted (Fig. *6C*) and thereafter cultivated (Fig. *6D*), we have analyzed resulting cells for CD45RC, CD25 and SLA-DR expression (Fig. *6G-I*). The results showed that newly generated $TCR\gamma\delta^{hi}CD1^{-}$ cells appear only in IL-2 conditioned cultures (Fig. *6G-I*, white-dashed bars) with a similar phenotype as freshly isolated $TCR\gamma\delta^{hi}CD1^{-}$ thymocytes (Fig. *6G-I*, gray bar for 4°C) but are significantly different from original $TCR\gamma\delta^{med}CD1^{-}$ cells (Fig. *6G-I*, white bar for 4°C). Newly generated $TCR\gamma\delta^{hi}CD1^{-}$ cells have much higher expression of CD45RC than parental $TCR\gamma\delta^{med}CD1^{-}$ cells and also higher expression of CD25 and SLA-DR. On the other hand, $TCR\gamma\delta^{med}CD1^{-}$ cells that keep their phenotype during cultivation (Fig. *6G-I*, white bars except for 4°C) do not change their CD45RC, CD25 and SLA-DR expression (Fig. *6G-I*, compare the white bars of the other four treatment groups with white bar for 4°C). However, when cultures were prolonged to 7 days, resulting $TCR\gamma\delta^{med}CD1^{-}$ cells increased their CD45RC, CD25 and SLA-DR expression (data not shown). This increase occurs spontaneously but to a lesser extend than for newly generated $TCR\gamma\delta^{hi}CD1^{-}$ cells.

When $TCR\gamma\delta^{hi}CD1^{-}$ thymocytes were sorted (Fig. *6E*) and thereafter cultivated (Fig. *6F*), we have also analyzed resulting cells for

CD45RC, CD25 and SLA-DR expression (Fig. *6G-I*). The results show that newly generated TCRγδ^{med}CD1$^{—}$ cells have comparable expression of CD45RC, CD25 and SLA-DR (Fig. *6G-I*, compare the gray-dashed bars of the other four treatment groups with gray bar for 4°C). The only exception is increased CD25 expression in PMA stimulated cultures. TCRγδ^{hi}CD1$^{—}$ cells that kept their phenotype during cultivation are also mostly comparable in expression of CD45RC, CD25 and SLA-DR (Fig. *6G-I*, compare the gray bars of the other four treatment groups with gray bar for 4°C). Again, the only exception is CD25 expression in PMA and IL-2 stimulated cultures. Differential increase in CD25 expression results in a significant difference of CD25 expression between TCRγδ^{hi}CD1$^{—}$ and TCRγδ^{med}CD1$^{—}$ cells that are the product of sorted and cultivated TCRγδ^{hi}CD1$^{—}$ thymocytes and for all culture conditions except PMA (Fig. *6H*, compare gray and gray-dashed bars).

In agreement with the finding that sorted (Fig. *6A*) and thereafter cultivated (Fig. *6B*) TCRγδ^{med}CD1^{+} thymocytes keep the same phenotype during cultivation, we mostly did not observe significant changes in expression of CD45RC, CD25 and SLA-DR (Fig. *6G-I*, compare the black bars of the other four treatment groups with black bar for 4°C). The only exception was increased expression of CD25 in PMA conditioned cultures.

The last analysis of sorted and cultivated TCRγδ/CD1 γδ thymocyte subsets includes an examination of proportional distribution of CD2 and CD8 expression on the resulting cells (Fig. *6J*). Results show that while TCRγδ^{med}CD1^{+} and TCRγδ^{hi}CD1$^{—}$ thymocytes did not change their proportions of CD2/CD8 subsets, TCRγδ^{med}CD1$^{—}$ thymocytes are enriched for CD2^{+}CD8^{+} subset (compare Fig. *6J* with *5B*). Analysis of newly generated TCRγδ^{med}CD1$^{—}$ cells originating from TCRγδ^{hi}CD1$^{—}$ cells have a decreased proportion of CD2$^{—}$CD8$^{—}$ subset to the detriment of CD2^{+}CD8$^{—}$ and CD2^{+}CD8^{+} subsets (Fig. *6J*). On the other hand newly generated TCRγδ^{hi}CD1$^{—}$ cells originating from TCRγδ^{med}CD1$^{—}$ cells have increased proportion of CD2$^{—}$CD8$^{—}$ subset to the detriment of CD2^{+}CD8$^{—}$ and CD2^{+}CD8^{+} subsets (Fig. *6J*).

Analysis of TCR diversity in sorted subpopulations of peripheral and thymic γδ T cells

The diversity of TCRδ repertoire in flow cytometry sorted subpopulations of γδ T cells was studied by CDR3 spectratyping (Fig. *7*). Only Vδ1 family was studied because it is the most varied family (containing more than 30 members) while the rest of the Vδ families (Vδ2-Vδ5) contains 1-2 members (19). Analyses of sorted TCRγδ^{med} and TCRγδ^{hi} (Fig. *7A-B*) and CD2/CD8 (Fig. *7C-E*) subpopulations of γδ T cells isolated from spleen showed that their Vδ1 repertoire is diverse (polyclonal) in all sorted subpopulations. Note that γδ T cells isolated from the blood gave the same results (data not shown). Similar analysis of CDR3 polymorphism for sorted TCRγδ/CD1 subpopulations of γδ thymocytes (Fig. *7F-H*) showed also polyclonal Vδ1 repertoire.

TCRγδ^{med} cells do not lose TCR completely as evidenced by intracellular staining

In some experiments, TCRγδ down-regulation occurs to the level resembling TCRγδ$^{—}$ cells. To exclude the possibility that TCRγδ is completely lost from the surface but cells maintain TCRγδ in cytoplasm, we have performed intracellular staining for TCRγδ (Fig. *8*). Staining of cells by both mAbs PPT16 and PPT26 (recognizing CD3 molecule expressed specifically on γδ T cells) showed a clear subpopulation of intracellularly positive but extracellularly negative cells for TCRγδ (Fig. *8A* and *B* respectively, region R2). Further analysis of this population indicated that these cells resemble αβ (Fig. *8D*) rather than γδ T lymphocytes (Fig. *8C*). Such an explanation is possible mainly because PPT16 and PPT26 are directed against the CD3 specific form

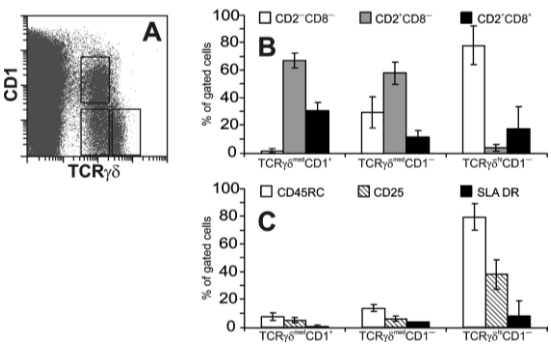

FIGURE 5. Thymic γδ T cells can be divided into three subsets according to level of TCRγδ and the expression of CD1 molecule. Freshly isolated thymocytes were gated by three regions according to their expression of TCRγδ and CD1 (A). Cells inside these three regions were analyzed for proportion of CD2/CD8 subsets (B) or for expression of CD45RC, CD25 or SLA-DR (C). Bars represent mean values and error bars represent ± SD obtained from at least four animals.

exclusively expressed with TCRγδ. This was confirmed by staining for CD3 (Fig. *8E*) where the frequency of $CD3^+$ $TCR\gamma\delta^-$ αβ T cells (20%) corresponds to the proportion of cells in region R2 (Fig. *8A* and *B*).

It is likely that fixation during intracellular staining changes the CD3 molecule to a form which can be recognized by mAbs against the CD3-specific forms for TCRγδ, such as PPT16 and PPT26. When we used TCRγδ-specific mAb PGBL22A that recognize the same cells as PPT16 or PPT26 (Fig. *8F*), the artifact of intracellular staining was eliminated (Fig. *8G*).

Moreover, this staining using PGBL22A mAb (Fig. *8G*) proved that TCRγδ molecules are not completely lost from the surface during down-regulation and that all γδ T cells are included in the analyses. Evidence that PPT16 and PPT26 mAbs recognize the same γδ T cells as PGBL22A is also shown (compare Fig. *8H* with *8F*).

Discussion

Data reported herein describe populations of porcine γδ T cells differing in expression of their TCR. Evidence that TCRγδ is expressed differently can be found in other reports (15, 16) but according to our knowledge this phenomenon was never studied in detail. Porcine $TCR\gamma\delta^{med}$ and $TCR\gamma\delta^{hi}$ cells are distributed

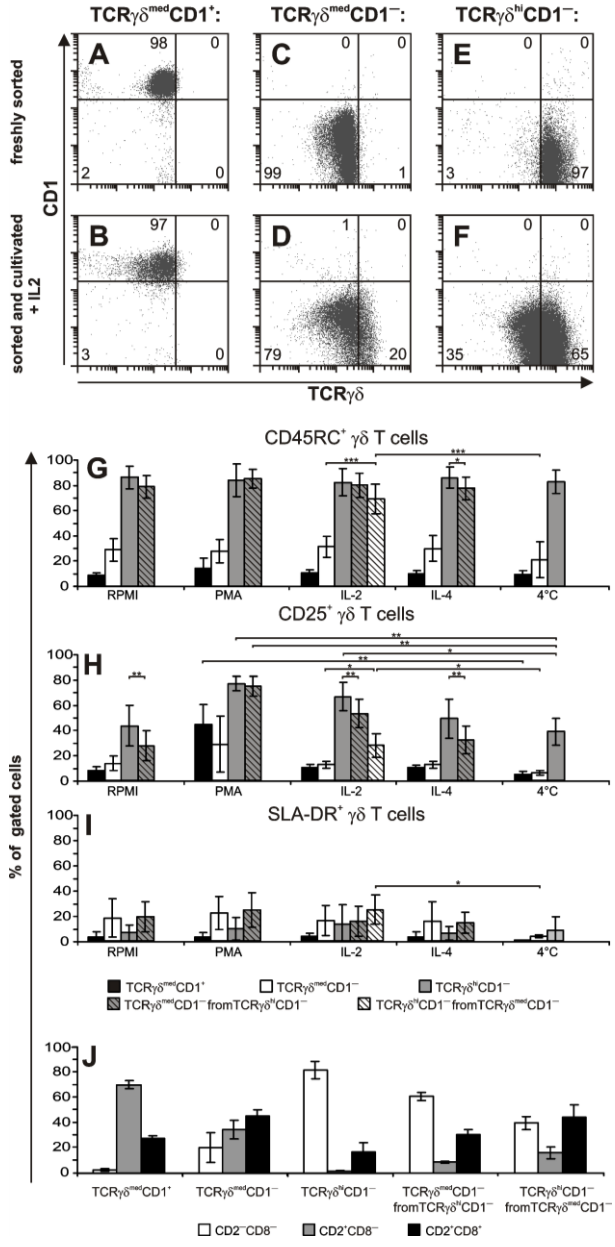

FIGURE 6. Sorted γδ thymic subsets can change their phenotype during cultivation. According to expression of TCRγδ/CD1 as shown in figure 5A, three population of γδ T cells were sorted by flow cytometry as pure subpopulations and analyzed just after sorting (A, C and E) and also after 7-day cultivation and re-staining (B, D and F). Dotplots B, D and F show representative analysis of γδ thymocytes cultivated with IL-2. While up-regulation of TCRγδ, shown in dotplot D, occurs only with IL-2, down-regulation of TCRγδ, shown in dotplot F, occurs with various activators and also spontaneously. Re-staining of resulting cells, shown in dotplots B, D and F, in some cases also involved anti-CD45RC, anti-CD25, anti-SLA-DR and anti-CD2/anti-CD8 mAbs. The proportions of $CD45RC^+$ (G), $CD25^+$ (H), $SLA\text{-}DR^+$ (I) and CD2/CD8 subpopulations (J) cells among particular gated TCRγδ/CD1 subset are also shown for 3-day cultivation. Different cultivation conditions are shown on x-axis for graphs G-I. Note that RPMI stands for cultivation in medium alone and cells kept at 4°C did not change expression of cell surface molecules and had the same phenotype as freshly isolated thymocytes. Bars represent mean values and error bars represent ± SD obtained from four animals. Significant differences between different cultivation conditions are shown and indicated by asterisks according to materials and methods. Analysis for CD2/CD8 subpopulations (J) is shown only for IL-2 conditioned cultivation because it generates $TCR\gamma\delta^{hi}CD1^-$ from $TCR\gamma\delta^{med}CD1^-$ γδ T cells.

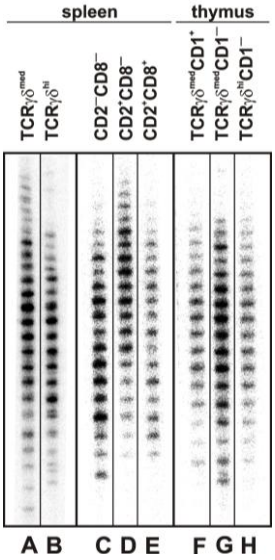

FIGURE 7. Analysis of Vδ1 diversity in sorted subpopulations of γδ T cells. Splenic γδ T cells (A-E) and γδ thymocytes (F-H) were sorted by flow cytometry according to their phenotype (depicted above each line) and their cDNA was analyzed by CDR3 spectratyping for Vδ1. The results are representative of five independent experiments. Note that sorted cells from the blood gave the same output as from the spleen.

differently among CD2/CD8 subsets, which are know to home differentially into various lymphatic tissues: while $CD2^{-}CD8^{-}$ are preferentially $TCR\gamma\delta^{hi}$ and are enriched in the blood, $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ are prevalently $TCR\gamma\delta^{med}$ and accumulate in other tissues (8). Such findings are in accordance with reported findings in cows, where $CD8^{+}$ and $CD8^{-}$ γδ T cells also exhibit a defined tissue tropism. This unequal distribution throughout different tissues is probably connected to their differential expression of L-selectin and E-selectin ligand (23). Moreover, another work in cows showed that $CD8^{+/-}$ γδ T cell subpopulations differ in expression of many other molecules such as galectin-1, prolactin, IgE-dependent histamine-releasing factor, epidermal growth factor, IL-10 or Gro-γ, IL-1, CD44, CD18, MHC class I (15). Interestingly, although authors of these findings did not study differential level of TCRγδ expression, they noted that $CD8^{+}$ γδ T cells express lower levels of TCR. These findings collectively indicate that γδ T cells with different level of TCRγδ and CD2/CD8 phenotype express auxiliary surface molecules and produce soluble factors that influence the immune system and explain tissue-specific accumulation of γδ T cell subsets. Similar tissue tropism and functional difference between γδ T cell subpopulations can be found also in humans and mice, although most of these studies discriminate γδ T cell subpopulations on the base of their TCR usage.

Our studies indicate that $CD2^{+}$ and $CD2^{-}$ γδ T cell subsets represent two independent lineages. This is not only because of differential expression of TCR but mainly because while $CD2^{-}$ γδ T cells cannot change their CD2/CD8 expression, $CD2^{+}$ γδ T can modulate CD8 expression. There is also differential susceptibility for proliferation between $CD2^{+}$ and $CD2^{-}$ γδ T cells. While $CD2^{-}CD8^{-}$ almost do not proliferate, proliferation of $CD2^{+}CD8^{-}$ and $CD2^{+}8^{+}$ is substantial. Lower proliferation of $CD2^{-}$ γδ T cells was also observed in mice (24). A population of $CD2^{-}$ γδ T cells is also absent in $CD1^{+}$ immature thymocytes. Moreover, our earlier finding shows that there is a substantial difference between $CD2^{+}$ and $CD2^{-}$ subsets in expression of CD25, CD11b, SWC1, SWC7, MHC-II and the family of CD45 molecules (12). The conclusion that $CD2^{+}$ and $CD2^{-}$ γδ T cell are two lineages is also supported by the finding that they differ in expression of TCRγ chains (8). Furthermore, findings in cows show that these two lineages differ in expression of non-TCRγδ cell antigens WC1 and GD3.5, in expression of cell adhesion molecules and in ability to infiltrate into inflammatory sites (15, 23). In any case, these two lineages are equally capable of immune responses because analysis of CDR3 length polymorphism showed equal diversification of Vδ1 with no restriction of TCR repertoire. Moreover, existence and behavior of these two lineages are independent of gut colonization because germ-free animals were comparable to conventional ones (data not shown).

Human and mice γδ T cells are generally considered $CD2^{+}$ (25-27) thus resembling $CD2^{+}$ γδ T cell lineage in swine. This is in sharp contrast with porcine $CD2^{-}$ γδ T cells, which are numerous and preferentially reside in the blood. High occurrence of $CD2^{-}$ γδ T cells is not unique to pigs but also for other members of γδ high species such as sheep (28, 29), cattle (30) and birds (31). These findings together support the idea that $CD2^{-}$ γδ T cells are a specific lineage, and this lineage is missing in the blood of humans and mice. This may also be the reason, why γδ T cells in humans and mice constitute a minority of T cells in the circulation. There are only a few examples in which $CD2^{-}$

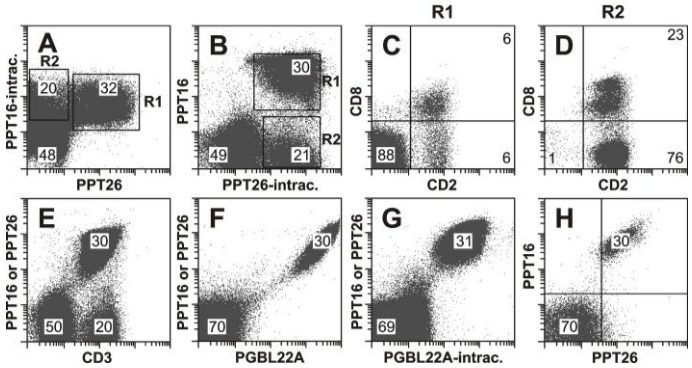

FIGURE 8. TCRγδ^{med} cells do not lose TCR completely as evidenced by intracellular staining. Lymphocytes from the blood were cell-surface stained by anti-CD2, anti-CD8 and either PPT26 (A) or PPT16 (B) mAbs, fixed, intracelullarly stained by either PPT16 (A) or PPT26 (B) mAbs and analyzed for intracellular and extracellular expression of TCRγδ. Two regions, R1 and R2, were set according to TCRγδ expression in dotplot A and B and CD2/CD8 phenotype of gated cells were analyzed further (C for R1, and D for R2). Note that analysis in dotplots C and D are shown for R1 and R2 from dotplot B but results for R1 and R2 from dotplot A were the same. Analyses of the same cells double stained extracellularly by anti-CD3 and PPT16 or PPT26 mAbs (E), extracellularly by PGBL22A and PPT16 or PPT26 mAbs (F), intracelullarly by PGBL22A and extracellularly by PPT16 or PPT26 mAbs (G) and extracellularly by PPT16 and PPT26 mAbs (H) are also shown.

γδ T cells were found in human and mice. Such examples are mouse intestinal (24) or vaginal γδ T cells (27). Other examples in humans are some leukemias (32), autoimmune disorders (33) or some particular individuals (26, 34). Loss of circulating γδ T cells in humans and mice can be connected with non-functional orthologues of WC1 genes known in ruminants and pigs (35). On the other hand, CD2$^+$ γδ T cells in human and mice can be clearly CD8$^+$ or CD8$^-$ (26, 36). We show here that CD8 expression can be modulated on CD2$^+$ γδ T cells in both ways. Moreover, our earlier work indicated that a CD2$^+$CD8$^+$ subpopulation is more mature than CD2$^+$CD8$^-$ (12). Such a conclusion agrees with other studies in humans where CD8$^+$ γδ T cells were shown to have attributes of regulatory cells (37). Also, some studies indicate that CD2$^+$CD8$^-$ γδ T cells can be precursors of CD2$^+$CD8$^+$ γδ T cells (10, 38). These findings collectively indicate that CD8 molecule can be modulated on the surface according to actual functional status of CD2$^+$ γδ T cells.

Sorting experiments show that CD8 expression can be modulated on CD2$^+$ γδ T cells and that TCRγδ can be down-regulated, which is in agreement with studies in mice (16). However, analysis of resulting cells from cultures of TCRγδ^{hi}/TCRγδ^{med} sorted cells does not indicate any significant change in composition of CD2/CD8 subpopulation (Fig. 2). This indicates that down-regulation of TCRγδ can clearly occurs on CD2$^-$CD8$^-$ γδ T cells *in vitro*. On the other hand, when freshly isolated cells were analyzed, CD2$^-$CD8$^-$ γδ T cells are practically absent from the TCRγδ^{med} compartment. Therefore, there has to be some factor(s) that keep expression of TCRγδ^{hi} on the CD2$^-$CD8$^-$ subset *in vivo*. This work is unable to identify those factors although the potential requirement for these factors was identified by addition of unsorted cells, which reconstitute the proliferation activity of pure γδ T cells. Another possibility can be fast turnover of TCRγδ as shown for TCRαβ (17), which can be constitutive or ligand-induced and is dependent on protein kinase C (39), and is maintained *in vivo* but not *in vitro*. Such a conclusion would correspond to our observation that partial loss of TCRγδ expression occurs in hours and this cannot be explained by mRNA modulation and/or proliferation of cells. *In vitro* experiments exclude the possibility that it is caused by early capping and receptor mediated endocytosis due to cross-linking of molecules by indirect staining. In any case, more experiments are needed to show what is responsible for the high expression of TCR on CD2$^-$CD8$^-$ γδ T cell *in vivo*. Our work indicates that distribution of CD2/CD8 subsets among TCRγδ^{hi} and TCRγδ^{med} cells *in vivo* is tightly regulated and is already established in the thymus prior to export of γδ T cells to the periphery. In connection with possible different regulation of the TCR level *in vitro* and *in vivo*, we also cannot exclude the possibility that CD2$^+$ γδ T cells can acquire and/or CD2$^-$ γδ T cells can lose CD2 molecule *in vivo*, which was never observed *in vitro*. This

has to be true at least in thymus where $CD2^{-}$ γδ T cells are generated from $CD2^{+}CD1^{+}$ immature thymocytes (5, 11).

Sorted γδ T lymphocytes are clearly less sensitive to activation of proliferation by different activators than unsorted γδ T cells. Since there is no effect of indirect staining on proliferative response of γδ T cells, decreased proliferation cannot be explained by cross-linking of signaling molecules. More likely, an effect of activators on γδ T cells is indirect and is mediated by other cell types such as sensitive to ConA and IL-2 activation, and these cells are missing in sorted cells. This would agree with studies showing that $CD4^{+}$ αβ T cells are involved (40). In this respect, IL-2 is the best candidate for this indirect function but it is probably not the only factor as evidenced by the inability to fully reconstitute proliferation potential of sorted cells.

Thymic γδ T cells are characterized by the occurrence of $CD1^{+}$ immature cells (13) that are always $TCR\gamma\delta^{med}$. Finding that $CD1^{+}$ γδ thymocytes are composed exclusively of $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{-}$ subsets agrees with previous work showing that the earliest precursors are $CD1^{+}CD2^{+}CD8^{-}$ γδ T cells while $CD1^{+}CD2^{+}CD8^{+}$ γδ T cells are their progeny (5, 11). Apparently, CD8 molecules can be acquired even in immature stages. Also, $CD2^{-}CD8^{-}$ γδ T cells are missing from the $CD1^{+}$ compartment which indicates that they arise after CD1 down-regulation. As regards $CD1^{-}$ γδ thymocytes, distribution of CD2/CD8 subsets among $TCR\gamma\delta^{hi}$ and $TCR\gamma\delta^{med}$ cells resembles the periphery. These $TCR\gamma\delta^{hi}CD1^{-}$ and $TCR\gamma\delta^{med}CD1^{-}$ subsets are probably exported from thymus differently: while $TCR\gamma\delta^{hi}CD1^{-}$ cells ($CD2^{-}$ lineage) are circulating and remain in the blood, $TCR\gamma\delta^{med}CD1^{-}$cells ($CD2^{+}$ lineage) are preferentially homing to tissues. However, there is some difference between CD1 negative $TCR\gamma\delta^{hi}$ and $TCR\gamma\delta^{med}$ thymocytes. It appears that $TCR\gamma\delta^{hi}CD1^{-}$ thymocytes are fully mature with many CD45RA/RC and CD25 expressing cells, which is characteristic of the periphery (12). Similarly to the blood, they can also easily and quickly down-regulate TCRγδ. On the other hand, $TCR\gamma\delta^{med}CD1^{-}$ are still in some transitional stage as evidenced by the low proportion of $CD45RA/RC^{+}$ and $CD25^{+}$ cells. Moreover, there is still a relatively high proportion of $CD2^{-}CD8^{-}$ γδ T cells that probably further mature into $TCR\gamma\delta^{hi}$ cells. Finally, at least a part of these cells is capable of up-regulation of TCRγδ, which can never be detected in peripheral γδ T cells. Significantly, newly generated $TCR\gamma\delta^{hi}$ cells show high expression of CD45RA/RC, CD25 and MHC-II. These findings indicate that $TCR\gamma\delta^{lo}CD1^{-}$ thymocytes may need further maturation and up-regulation of CD45RA/RC, CD25 and MHC-II once they reach their final destination in solid tissues.

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9. General Discussion

9.1. Development of porcine B cells

In the 50's, the work of Glick *et al.* resolved the role of the bursa of Fabricius in the development of B cells in chicken (*Glick et al., 1956*) which caused investigators to search for a mammalian homologue. In the 60's the rabbit appendix was proposed to play such a role (*Archer et al., 1963*), followed in the 70's by the fetal liver and bone marrow in mice (*Owen et al., 1977*) and finally by the IPP of ruminants in the 80's (*Reynolds and Morris, 1983*). The concept that IPP plays a role in development of B cells similar to the bursa of Fabricius or perhaps the rabbit appendix was built mostly on studies in sheep and was supported by findings that (1) surgical removal of IPP resulted in reduction of Ig^+ B cells (*Gerber et al., 1986a, 1986b*), (2) B cells develop in IPP is an antigen-independent manner (*Reynolds and Morris, 1984*), (3) systemic depletion of IgM^+ B cells in fetal lambs causes the failure to develop follicles in IPP (*Press et al. 1996*), (4) IPP have higher proportion of proliferating lymphoid cells than found in thymus (*Reynolds, 1986 and 1987; Motyka and Reynolds, 1991*), (5) the vast majority of the B cells in IPP die by apoptosis *in situ* (*Reynolds, 1986; Motyka and Reynolds, 1991; Andersen et al., 1999*), (6) IPP involute in postnatal ontogeny similarly to the bursa of Fabricius (*Reynolds and Morris, 1983*), (7) activation-induced cytidine deaminase which mediates SHM, gene conversion and Ig isotype switch is present in IPP (*Yasuda et al., 2006*) and (8) diversification of antibody repertoire occurs by SHM or gene conversion like in bursa of Fabricius (*Reynaud et al., 1991; Parng et al., 1996*). However, the observations mentioned in point 3-8 are not directly related to B lymphogenic function of IPP and can be ascribed to the fact that IPP are secondary lymphatic organ where positive selection, proliferation, class-switch and phenotype alteration of B cells occurs due to colonization of gut by bacteria. Occurrence of gene conversion was canceled because the original number of analyzed genes was underestimated *(Jenne et al., 2003).* In any case, the concept of role of the IPP has permeated other scientific reports, reviews and immunology textbooks for more than 20 years and due to the similarity in the organization of IPP, was broadened also to other species including swine, horse and cattle (*Griebel and Hein, 1996; Caro et al., 1998; Rothkötter et al., 1990; Butler et al., 2006; Yasuda et al., 2006; Dobson et al., 2010*).

Results included in this thesis do not support the existing paradigm that the IPP is primary lymphoid tissue (***publication #1***). 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 the 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 marrow and SJC are absent. On the other hand, this thesis shows that the bone marrow is fully capable of B cell lymphogenesis and remains active for at least 3 months whereas no B cell lymphogenesis is present in the IPP (***publication #2***). There is a clear discrepancy of our findings with the original observations in sheep that surgical removal of IPP resulted in reduction of Ig^+ B cells (*Gerber et al., 1986b*) and that B cells develop in IPP in an antigen-independent manner (*Reynolds and Morris, 1984*). 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 (*Gerber et al., 1986b*) that can generate a significant error. Interestingly, the same authors published a conflicting report that B cell deficiency did not alter serum Ig levels (*Gerber et al., 1986b*). It is inconsistent to report that B cell levels are significantly reduced but that serum Ig levels are unaffected. Moreover, the authors never used GF animals to test their statement that B cells develop in IPP in an antigen-independent manner (*Reynolds and Morris, 1984*). We show by the same procedures but using GF animals (***publication #1***) that the development of IPP in isolated ileal loops and anastomosed ileum is comparable only in colonized animals and is clearly different from IPP of GF animals of the same age. Likely, the original work in sheep only observed the effect of antigen and/or lymphocyte relocation throughout the gut. This explanation would be consistent with later findings that agree with our observations. These include studies on distribution of lymphocytes in the IPP and jejunal Payer's patches (*Griebel et al., 1992*) and resection and transposition studies of others (*Rothkötter et al., 1990*). Moreover, some reports in calves (*Ekman et al., 2010*), horses (*Studdert, 1978*) and even sheep (*Pabst et al., 1986*) indicated that bone marrow could have a role in lymphogenesis of B cells, at least for the same period of time as is speculated for the IPP (*Reynolds and Morris, 1983*).

We think that the hypothesis regarding the role of the IPP as a primary B lymphopoietic organ has over time and, because of technology, ease of repetition gone from being a collection of observations to being "institutionalized" into a paradigm. This perpetuation took place during a period in which the focus of basic immunology has

shifted almost entirely to the mouse, so there was little research to challenge paradigms established years ago in non-mouse species. Furthermore, 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. Our current work (***publications #1 a #2***) changes the view of IPP and shows that IPP could be an important but nonessential secondary lymphoid tissue for early immune responses against colonization.

Results showing B lymphopoietic activity of the porcine bone marrow also revealed one interesting feature of B cell development in pigs (***publication #2***). This is the preferential appearance of VλJλ rearrangements prior to the appearance of VκJκ rearrangements, which clearly differs from the pattern seen in mice and humans (*Gorman and Alt, 1998*). It should be remembered that in many Ungulates, like cattle and the horse, >90% of all Igs use LCλ even though they possess a functional LCκ locus (*Butler, 1997*). Furthermore, some vertebrates, like birds, use only LCλ (*Sanders and Travis, 1975*). When early porcine VλJλ rearrangements were submitted to a BLAST search, it was identified as a λ5-like sequence that had been annotated in the porcine genome project (***publication #2***). However, reported "lambda 5" was identical to a common member of the porcine Vλ genes. One heretical explanation for this finding is that there is no λ5 and no preBCR in swine. Swine, and perhaps some other species that use a predominance of LCλ, may proceed directly to the use of authentic VλJλ rearrangements. This would agree with our observation that VpreB expression in pigs is not restricted to B cell development and that VpreB could also have other functions besides the function in pre-BCR. This would also explain why a small percentage of B cells in mice can develop through an alternative pre-BCR-independent pathway in which the LC rearranges independently of the HC (*Kubagawa et al., 1989; Ehlich et al., 1993; Grawunder et al., 1993; Novobrantseva et al., 1999; Shimizu et al., 2002*). Moreover, this could explain why some vertebrates, like birds, can rearrange HC or LC locus competitively (*Benatar et al., 1992*).

All mature B cells in mice and humans express CD21, which is complement receptor type 2. This receptor interacts with CD23, CD35, CD19 or CD81 to form the signal transduction complex CD19/CD21/CD81 enhancing BCR signaling in response to complement-coated antigens (*Carter and Fearon, 1992; Matsumoto et al., 1993*). Engagement of CD21 on B cells also promotes the survival of B cells (*Molnar et al., 2008*). For these reasons, expression of CD21 on mature B cells is probably critical for their function and survival. However, mature B cells in sheep are $CD21^{+}$ or $CD21^{-}$ (*Liu et*

al., 2008) and these two subpopulations were shown to have distinct recirculation characteristics, phenotypes and tissue distributions (*Young et al., 1997; Gupta et al., 1998*). Such findings may imply that a part of mature B cells in some species do not need CD21 for their functions. Some reports in pigs also indicate that porcine mature B cells could express CD21 differentially (*Takamatsu et al., 1999*). We therefore investigated the expression profile of three mAbs that are routinely used to detect CD21 orthologue on the surface of porcine B lymphocytes to explain the phenomenon of mature $CD21^{-}$ B cells (***publication #3***). Cross-reactive studies show that two mAbs recognize only a portion of B cells that are positive for the third pan-specific mAb. Such findings prove that CD21 molecules are always present on all mature B cells but can probably be expressed in at least two differential forms $CD21^{a}$ and $CD21^{b}$. Finding that $CD21^{a}$ is always expressed on the surface of mature B cells unifies different findings in swine, sheep, mice and humans confirms the critical function of CD21 in BCR signaling (*Carter and Fearon, 1992; Matsumoto et al., 1993)*. On the other hand, expression of differential forms of $CD21^{b}$ may account for different functional properties. Ontogenetic and *in vitro* culture studies, analysis of cell size, expression of CD11b and class-switched phenotype together with measurement of proliferation and cell death (***publication #3***) revealed that $CD21^{b+}$ B cells are less mature than $CD21^{b-}$. Studies in sheep showed that most $CD21^{-}$ B cells lack L-selectin (CD62L, *Young et al., 1997*) which together with lack of recirculation throughout the lymph nodes (*Gupta et al., 1998*) leads to speculation that they represent a memory B cell pool just like in the case of T cells (*Mackay et al., 1990*). This is in agreement with studies in humans (*Kansas et al., 1985*) and mice (*Kraal et al., 1998*). There is also a striking inverse relationship between expression of CD21 and CD11b found in sheep (*Gupta et al., 1998*) and also swine (***publication #3***). These particular findings can help to elucidate the role of naive $CD21^{b+}CD11b^{-}$ and effector $CD21^{b-}CD11b^{+}$ B cells and can unify different findings across different species. There is no subpopulation of mature $CD21^{-}$ B cells in mice or humans. However, there is still the possibility that mouse and human CD21 undergo similar conformation changes in effector B cells but there is a lack of adequate mAbs that could distinguish it. Human and mice B cells express CD11b differentially (*Kawai et al., 2005; Ghosn et al., 2008*), which is similar to swine and sheep. Expression of CD11b molecules on mouse B cells is usually considered the classical marker of B-1 cells and marginal zone B cells. Nevertheless, human $CD11b^{+}$ B cells do not fall into the same category as described for mice but rather they were shown to represent

the memory B cell pool (*Kawai et al., 2005*). Moreover, recent findings in mice dispute the concept of B-1 cells and expression of CD11b since about half of peritoneal B-1 cells were shown to be $CD11b^{-}$ (*Ghosn et al., 2008*). Significantly, those $CD11b^{-}$ B cells appear early in ontogeny and are progenitors of $CD11b^{+}$ B cells that are more differentiated and cannot reconstitute the $CD11b^{-}$ B cell pool (*Ghosn et al., 2008*). Because expression of CD11b in swine and sheep fully correspond to the proposed revised concept, $CD11b^{-}$ B cells could generally represent naive B cells subset, while $CD11b^{+}$ B cells could represent experienced effector B lymphocytes. According to these findings we believe that CD21 is expressed on mature B cells in all homoeothermic animals, and also that there are different forms of CD21 or the CD19/CD21/CD81 complex depending on the developmental stage of mature B lymphocytes. Some evidence for this statement can be found in healthy humans (*Isnardi et al., 2010*). If that is true, the different CD21 forms should also have different functions and perhaps binding capacity for C3 and/or IFN-α (interferon α).

9.2. Subpopulations of porcine γδ T cells

Swine, together with ruminants and birds, belongs to the group of *γδ high species* in which γδ T cells are not preferentially limited to epithelia and may account for >70% of all T cells (*Hein and Dudler, 1993*). Traditionally, γδ T cells in swine are subdivided into three subsets based upon their expression of CD2 and CD8 and include $CD2^{-}CD8^{-}$, $CD2^{+}CD8^{-}$ and $CD2^{+}CD8^{+}$ cells (*Yang and Parkhouse, 1996, 1997; Sinkora et al., 1998b, 2005b, 2007*). These individual subsets differ in their homing characteristic (*Saalmuller et al., 1990*) and cytotoxic activities (*de Bruin et al., 1997; Yang and Parkhouse, 1997*). Previous studies revealed the basic distribution of porcine γδ T cells (*Sinkora et al., 1998b*), their ontogeny (*Sinkora et al., 1998b, 2005a*), development in the thymus (*Sinkora et al., 2000a, 2005a, 2007*) and the repertoire diversification of their TCR *(Holtmeier et al., 2004*). However, none of these studies focused on a detailed analysis of peripheral γδ T cells, and no other studies have been performed to explain differences in the phenotypic profile of porcine γδ T cells subsets. We used GF piglets that have a virgin immune system (*Butler et al., 2009),* and compare those with their age-matched conventional mates to show that the expression of CD25, CD11b, SWC1 (swine orthologue of human CD52, *Leitner et al., 2012),* SWC7, MHC class II and family of CD45 may be used for discrimination of functionally and developmentally different subsets of γδ T cells (***publication #4***). The expression of these molecules (except of SWC7) could be also used

for characterization of γδ T cells from other species. Results of this work point to the possibility that the $CD2^{-}CD8^{-}$ subset is mostly composed of naive cells because the majority of them have the naive $CD45RA/RC^{+}$ phenotype like in the case of human (*Dieli et al., 2003*), and there are only a few $MHC\ II^{+}$ cells capable of antigen presentation, as shown for γδ T cells (*Takamatsu et al., 2002; Brandes et al., 2005; Cheng et al., 2008*). $CD2^{-}CD8^{-}$ γδ T cells is the only subset responding to interleukin-2 (IL-2) stimulation by increased CD25 expression (***publication #4***). Albeit $CD2^{-}CD8^{-}$ γδ T cells can express high levels of surface CD25, $CD25^{+}$ cells in this subset are generally found infrequently in tissues other than lung and bronchoalveolar lavage (BAL) (***publication #4***). Notable is a very high incidence of $CD2^{-}CD8^{-}$ γδ T cells in the blood. On the other hand, $CD2^{+}CD8^{-}$ may represent an effector/memory subset because the majority of them lack CD45RA/RC and many of them express CD25 throughout different organs (***publication #4***). Moreover, this subpopulation has more MHC II and CD11b expression than the $CD2^{-}CD8^{-}$ subset (***publication #4***). Finally, $CD2^{+}CD8^{+}$ γδ T cells probably represent a terminally differentiated subset with re-expressed CD45RA/RC (*Dieli et al., 2003*). This subset has the highest expression of MHC II and SWC1, many of which express CD11b and in which expression of CD25 is very frequent (***publication #4***). Moreover, we found that most of $CD2^{+}CD8^{+}$ γδ T cells remain largely unresponsive to activation as measured by the ability to increase CD25 expression (***publication #4***). This would correspond with other findings in pigs that this particular subset is cytotoxic (*de Bruin et al., 1997; Yang and Parkhouse, 1997*). It is also in agreement with findings in mice where increased expression of CD11b is associated with acquisition of cytotoxic capacity (*McFarland et al., 1992*), and humans where CD11b is found on a subset of memory T cells (*Yamada et al., 1985*). The expression of CD25 may also have another function on γδ T cells as it is used to distinguish between two functionally different subsets: $CD25^{+}CD122^{-}$ IL-17-producing and $CD25^{-}CD122^{+}$ IFN-γ-producing γδ T cells (*Shibata et al., 2008*). IL-17-producing $CD25^{+}$ γδ T cells together with Th17 αβ T cells were found to be essential for protection against pulmonary infections (*Khader et al., 2009*) which is in agreement with our observations of increased frequencies of $CD25^{+}$ γδ T cells in lung, BAL and tonsils. Therefore CD25 could not only mark activated γδ T cells but a functionally distinct subset that is involved in the neutrophil-mediated clearance of the pathogen (*Shibata et al., 2008; Khader et al., 2009*). In any case, although ***publication #4*** substantially increases our knowledge of porcine γδ T cells and gives view into possible functional characteristic of

their CD2/CD8 subsets, it cannot resolve their developmental dependency or independency.

To further investigate the subpopulation of porcine γδ T cells we have utilized the finding that they have two levels of TCR expression ***(publication #5)***. While TCRγδ^{med} cells are mostly CD2+CD8^{-} and CD2^{+}CD8^{+}, TCRγδ^{hi} cells are highly enriched for CD2^{-} CD8^{-}. This distribution agrees with tissue tropism in which CD2^{+}CD8^{-} and CD2^{+}CD8^{+} accumulate in solid tissues while CD2^{-}CD8^{-} are preferentially enriched in the blood (*Saalmuller et al., 1990; Yang and Parkhouse, 1996, 1997; Sinkora et al., 1998b, 2005b, 2007*). The distribution is independent of bacterial colonization and it is already established in the thymus prior to export of γδ cells to the periphery ***(publication #5)***. Similar tissue tropism was reported for other members of *γδ high species* such as sheep (*Mackay et al., 1989; Witherden et al., 1995*), calf (*Clevers et al., 1990*) and birds (*Vainio et al., 1991*), and is probably connected to differential expression of L-selectin and E-selectin ligands (*Wilson et al., 1999*). Although distinct tissue tropism of γδ T cell can also be found in members of *γδ low species*, human and mice γδ T cells are generally considered CD2^{+} (*Jitsukawa et al., 1987; Groh et al., 1989; Rakasz et al., 1997*). If CD2^{-} γδ T cells were found in humans and mice, they are preferentially found in solid epithelial tissues like intestine, skin and vagina *(Van Houten et al., 1993; Rakasz et al., 1997)* or in pathological situations like cancers (*Sugimoto et al., 2001*) or autoimmunity (*de Pauli et al., 1990*). These findings together incite the idea that CD2^{-} γδ T cells are a specific lineage, and this lineage is missing in the blood of humans and mice. This may also be the reason why γδ T cells in all *γδ low species* constitute a minority of T cells in the circulation. Loss of circulating CD2^{-} γδ T cells in humans and mice can be connected with non-functional orthologues of WC1 genes known in ruminants and pigs (*Guzman et al., 2012*).

We have shown for the first time that CD2^{+} and CD2^{-} γδ T cell subsets display many differential features and that they represent two independent lineages (***publication #5***). This is not only because of differential expression of TCR but mainly because while CD2^{-} γδ T cells cannot change their CD2/CD8 expression, CD2^{+} γδ T can modulate CD8 expression (***publication #5***). There is also differential susceptibility for proliferation between CD2^{+} and CD2^{-} γδ T cells (***publication #5***). While CD2^{-}CD8^{-} almost do not proliferate, proliferation of CD2^{+}CD8^{-} and CD2^{+}8^{+} is substantial. Lower proliferation of CD2^{-} γδ T cells was also observed in mice (*Faure et al., 1988*). A population of CD2^{-} γδ

T cells is also absent in $CD1^+$ immature thymocytes (***publication #5***). In addition, subpopulations of $CD2^+$ and $CD2^-$ γδ cells in the thymus differ in expression of auxiliary surface molecules such as CD25, CD45RA/RC and MHC II (***publication #5***). ***Publication #4*** supports these findings by showing that there is a substantial difference between $CD2^+$ and $CD2^-$ subsets in expression of CD25, CD11b, SWC1, SWC7, MHC II and also the family of CD45 molecules in the periphery. The conclusion that $CD2^+$ and $CD2^-$ γδ T cell are two lineages is also supported by the finding that they differ in expression of TCRγ chains (*Saalmuller et al., 1990*). Furthermore, findings in cows show that these two lineages differ in expression of non-TCRγδ cell antigens WC1 and GD3.5, in expression of cell adhesion molecules and in the ability to infiltrate into inflammatory sites (*Wilson et al., 1999; Meissner et al., 2003*). In any case, these two lineages are equally capable of immune responses because analysis of CDR3 length polymorphism showed equal diversification of Vδ1 with no restriction of TCR repertoire (***publication #5***).

Modulation of CD8 expression on $CD2^+$ γδ T cell lineage is not unique for *γδ high species* and can also be clearly observed on $CD2^+$ γδ T cells in human and mice (*Jitsukawa et al., 1987; Sato et al., 1993*). We show that CD8 expression can be modulated on $CD2^+$ γδ T cells in both ways (***publication #5***). Moreover, ***publication #4*** indicated that a $CD2^+CD8^+$ subpopulation is more mature than $CD2^+CD8^-$. Such a conclusion agrees with other studies in humans where $CD8^+$ γδ T cells were shown to have attributes of regulatory cells (*Bhagat et al., 2008*). Also, some studies indicate that $CD2^+CD8^-$ γδ T cells can be precursors of $CD2^+CD8^+$ γδ T cells (*Choi and Lillehoj, 2000; Wen et al., 2012*). These findings collectively indicate that CD8 molecule can be modulated on the surface according to actual functional status of the $CD2^+$ γδ T cell lineage.

General conclusion

In conclusion, this thesis disproves the existing paradigm that the IPP is primary lymphoid tissue and that B cells develop in IPP in an antigen-independent manner. On the other hand, this thesis shows that the bone marrow is fully capable of B cell lymphogenesis and remains active at least for the same period of time as it had been speculated for the IPP. This thesis also identified for the first time functionally different subsets of porcine peripheral B cells and shows that CD21 molecules can be expressed in differential forms, which may account for different functional properties of mature B cells. This thesis also identified two lineages of γδ T cells that differ in many functional and phenotype features. This finding may explain why γδ T cells constitute a minority of lymphocytes in circulation of humans and mice.

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Curriculum vitae

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Education and Academic Training:

2003 – 2006	Faculty of Nature Science in Charles University in Prague, department of Cell Biology, biology (obtain a degree B.Sc.)
2006 – 2008	Faculty of Nature Science in Charles University in Prague, department of Cell biology, Immunology (obtain a degree M.S.)
2008 – present	Faculty of Nature Science in Charles University in Prague, Doctoral Educational Programs in Biomedicine, Immunology

Professional Experience:

2004 – 2006	Institute of Animal Physiology and Genetics, AS CR, v.v.i. Liběchov, laboratory of Experimental Oncology (later laboratory of tumor biology) – elaborating of bachelor thesis on the theme „Expression of Heat Shock Proteins in Progreding and Regreding Tumors in MeLiM Pigs“. Supervisor: Dr. Alena Morávková, Ph.D.
2006 – 2008	Department of Microbiology and Genetics in Charles University in Prague, laboratory of Virology – elaborating of master thesis on the theme „Preparation of Antibodies against Large T Antigen (LT) of Mouse Polyomavirus and the Study of Interactions of LT with cellular structures". Supervisor: Dr. Alena Morávková, Ph.D.
2008 – present	Department of Immunology and Gnotobiology, Institute of Microbiology AS CR, v.v.i., Nový Hrádek, Department of Immunology and Gnotobiology, PhD project – "The development of swine B cells and the role of γδ T lymphocytes in immunization of naive immune system". Supervisor: Dr. Marek Šinkora, Ph.D.

Projects:

2010 Grant Agency of Charles University Grant 43-251119. The Phenotype of γδ T lymphocytes in swine. Investigator: Kateřina Štěpánová

List of Publications

Sinkora, M., Stepanova, K., Butler, J.E., Francis, D., Santiago-Mateo, K., Potockova, H., Karova, K., Sinkorova, J., 2011. 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. J Immunol., 187(10), 5150-5161

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