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Review article

Development of the neonatal B and T cell repertoire in swine: implications for comparative and veterinary immunology

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Abstract – Birth in all higher vertebrates is at the center of the critical window of development in which newborns transition from dependence on innate immunity to dependence on their own adaptive immunity, with passive maternal immunity bridging this transition. Therefore we have studied immunological development through fetal and early neonatal life. In swine, B cells appear earlier in fetal development than T cells. B cell development begins in the yolk sac at the 20th day of gestation (DG20), progresses to fetal liver at DG30 and after DG45 continues in bone marrow. The first wave of developing T cells is γδ cells expressing a monomorphic Vδ rearrangement. Thereafter, αβ T cells predominate and at birth, at least 19 TRBV subgroups are expressed, 17 of which appear highly homologous with those in humans. In contrast to the T cell repertoire and unlike humans and mice, the porcine pre-immune VH (IGHV-D-J) repertoire is highly restricted, depending primarily on CDR3 for diversity. The V-KAPPA (IGKV-J) repertoire and apparently also the V-LAMBDA (IGLV-J) repertoire, are also restricted. Diversification of the pre-immune B cell repertoire of swine and the ability to respond to both T-dependent and T-independent antigen depends on colonization of the gut after birth in which colonizing bacteria stimulate with Toll-like receptor ligands, especially bacterial DNA. This may explain the link between repertoire diversification and the anatomical location of primary lymphoid tissue like the ileal Peyers patches. Improper development of adaptive immunity can be caused by infectious agents like the porcine reproductive and respiratory syndrome virus that causes immune dysregulation resulting in immunological injury and autoimmunity.

fetal / ontogeny / colonization / repertoires / animal model

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1. INTRODUCTION

All eucaryotic organisms initially or exclusively depend on innate immunity for survival against pathogens. However in higher vertebrates, survival after 96 h also depends on adaptive immunity. The evolutionary and phylogenetic appearance of adaptive immunity parallels the appearance of lymphocytes. Their appearance, which occurred somewhere along the evolutionary pathway between jawless fishes and sharks, is often called the "big bang" of immune system evolution [73]. Lymphocytes offered a possibility not available in the innate immune system, i.e. somatic rearrangements of antigen receptor genes. 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 germline genes; hence the term "adaptive". Adaptive immunity is the result of the evolution of three molecular mechanisms that are unique to lymphocytes. First, somatic gene recombination mediated by relatives of bacterial integrase genes called Recombination Activation Genes (RAG). These may have been acquired from marine bacteria through retroviral transfer. Second, B lymphocytes have acquired mechanisms that allow somatically recombined genes encoding the immunoglobulins (IG) to be somatically mutated at a rate 10^5 faster than that for spontaneous mutation of the eucaryotic genome and 10^4 faster than in bacteria[1] [63]. It is currently believed that this somatic mutation process is dependent on activation-induced cytosine deaminase (AID) working together with various constitutively expressed DNA repair enzymes [80]. While contemporary thinking defines adaptive immunity in terms of these mechanisms, studies on protochordates [25] and jawless fish [85] indicate that other organisms may use different somatic mechanisms for adaptive immunity. Third, B and T lymphocytes have evolved a mechanism for silencing one of the alleles encoding the IG and the T cell receptor (TR) genes, respectively. This goes by the name allelic exclusion and assures that any one lymphocyte expresses only one antigen receptor, i.e. lymphocytes are monospecific.

Many of the elements of the innate immune system are concentrated in the same areas served by mucosal or regional immunity (the focus of this volume). Therefore it is

[1] David Weiss, personal communication.

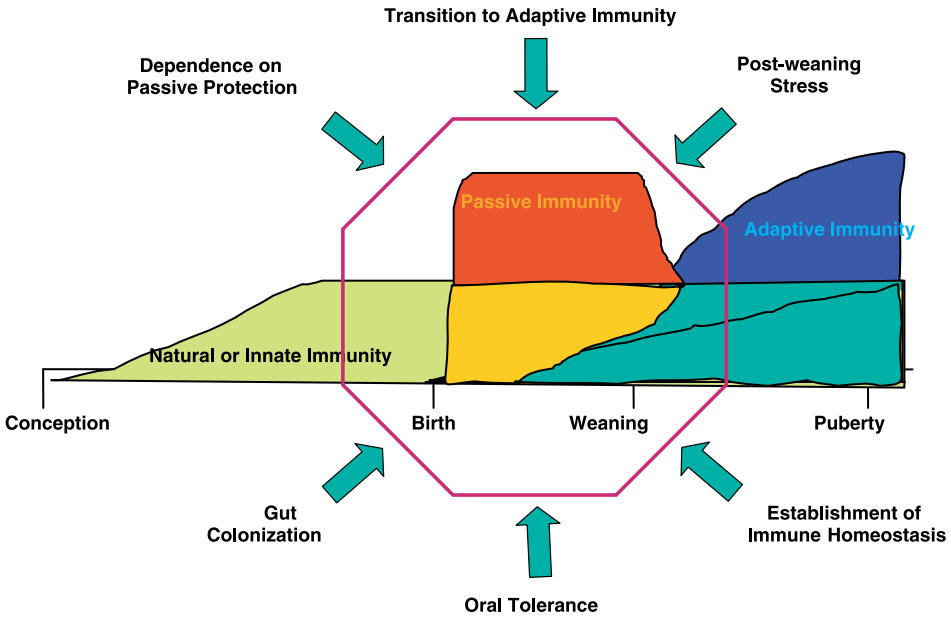

Figure 1. The critical window of immunological development. The super imposed octagon focuses attention to the period during which the many indicated events impact development. (A color version of this figure is available at www.edpsciences.org.)

not unlikely that adaptive immunity first developed in anatomical regions dominated by the mucosal immune system in mammals. This might explain certain phylogenetic and developmental aspects of adaptive immunity. For example, the lymphoid-like tissue of amphioxus [25] is concentrated in the gut and hind gut lymphoid tissues in chickens, sheep and probably swine are developmentally associated with diversification of the B cell repertoire [24, 89, 90] and later with mucosal immunity [121]. This is not per se, an article about mucosal immunity in swine, although certain aspects of T and B cell repertoire development are relevant to regional and/or mucosal immunity.

Development of adaptive immunity in higher vertebrates fits well to the expression that "ontogeny recapitulates phylogeny". The developing mammalian fetus, like invertebrates, depends primarily on innate immunity until late gestation. Thus fetal or neonatal development is characterized by a transition from full dependence on innate immunity to increasing dependence on adaptive immunity (Fig. 1). Immediately after birth in swine, but prenatally in Group I and II mammals in which transplacental IgG transport occurs [9, 20], passive maternal immunity protects the newborn until the adaptive immune system is fully operational[2]. This creates a "critical window" of development for newborn mammals in which innate and passive immunity provide the major protection against pathogens. It is during this critical window that many factors impact the developing neonate and during which time immune homeostasis is believed to normally develop. We believe that maintenance of proper health and well-being of animals depends

[2] Mammals are grouped on the basis of whether maternal IgG antibodies are transferred exclusively in utero (Group I), exclusively post-partum in colostrum and milk (Group III including all large ungulate farm animals) or both before and after birth (Group II, e.g., rodents, carnivores).

on understanding the events that take place during this critical window of development (Fig. 1). Thus, this review focuses on the development of the T and B cell repertoire of piglets and on the role of environmental factors during this critical window.

It is important to emphasize in veterinary immunology that pigs are neither mice nor humans and that certain aspects of the swine immune system are characteristic, if not unique, to the species. An important example of this difference is the manner of providing passive immunity and the precosial nature of its offspring. These features also make swine a valuable model for immunoontogeny that may have implications for all mammals. Information gained using this model can be directly applied in veterinary medicine in such areas as: (1) management practices, (2) development of neonatal vaccines and the time of their application, (3) identification of genetic resistance markers and (4) immunodiagnostics.

2. LYMPHOCYTE DEVELOPMENT AND LYMPHOGENESIS IN FETAL PIGLETS

Swine have a relatively long gestation (114 days) in an environment separated from maternal regulatory antibodies and lymphocytes by an epitheliochorial placenta. IGH V-D-J rearrangements (Fig. 2) and B lymphopoietic activity (Fig. 3A) can be first seen at the 20th day of gestation (DG20) in the yolk sac and thereafter at DG30 in the fetal liver [105, 106]. The yolk sac in the fetal pig involutes after DG24 so termination of its role in lymphogenesis after DG20 would be expected [106]. T lymphopoietic activity can be first detected in thymus at about DG40 (Fig. 3C; [101, 102]). However, hematopoietic activity in the bone marrow does not begin before DG45 (Fig. 3B; [102, 106]). Data suggest that: (a) the yolk sac and fetal liver are the major sites of lymphopoietic activity in the porcine embryo before the bone marrow becomes active,

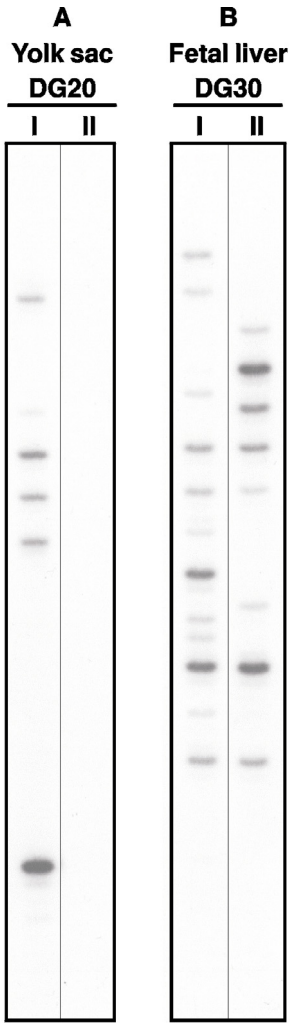

Figure 2. Length analysis (spectratype) of the early CDR3 repertoire. IGH V-D-J rearrangements were first recovered from the yolk sac at DG20 (A) and from fetal liver at DG30 (B). VDJ rearrangements were recovered from DNA (columns I) using a FR1 and J_H primer set (Column I) or from cDNA (Column II) using a FR1 and Cμ primer set for mature transcripts. CDR3 spectratyping was done as described [106]. Briefly, spectratypic analysis is based on the principle that T- and B-cell clones have CDR3 regions that differ in the number of nucleotides. Thus spectratyping is a means for determining the number of clones present. Only in mature transcripts is CDR3 spliced to the heavy chain, e.g. IgM (Cμ).

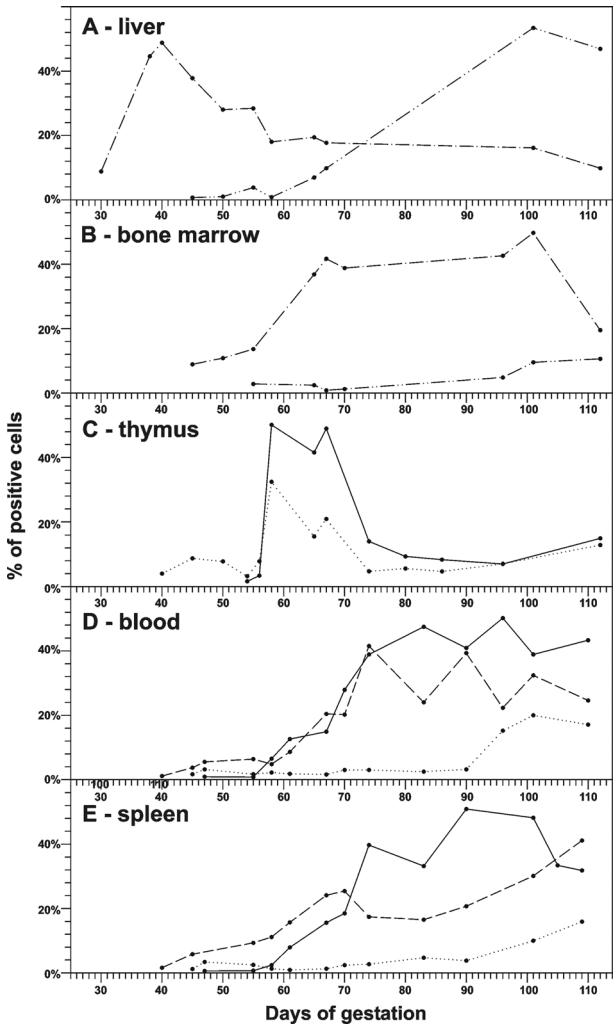

Figure 3. The ontogeny of primary lymphopoietic activity in fetal liver (A) and bone marrow (B) and αβ T cells, γδ T cells and B cells in the thymus (C), fetal blood (D) and spleen (E). The proportion of putative lymphoid progenitors ($CD45^{lo}SWC3a^{-}$ cells, dash-dot line) and mature lymphocytes ($CD45^{hi}SWC3a^{-}$ cells, dash-double-dot line) for fetal liver (A) and bone marrow (B) are presented as a percentage of total $CD45^{+}$ cells. The proportion of αβ T cells (solid line), γδ T cells (dotted line) and B cells (dashed line) for thymus (C), fetal blood (D) and spleen (E) are presented as a percentage of positive cells in lymphoid gate. Data are based on results previously published by Sinkora et al. [101, 105, 107].

(b) these populate the periphery with B cells and (c) the thymus is populated by two waves of T cell progenitors. The first wave of thymocyte progenitors comes from the yolk sac and fetal liver (Fig. 3A) while the second comes from the bone marrow (Fig. 3B).

The first $sIgM^{+}$ B cells appear in fetal blood and spleen on DG40 (Figs. 3D and 3E), i.e. about 10 days after B lymphopoietic activity can be detected in the fetal liver. In the same time period, the first T cells are detected in thymus (Fig. 3C) but not in the

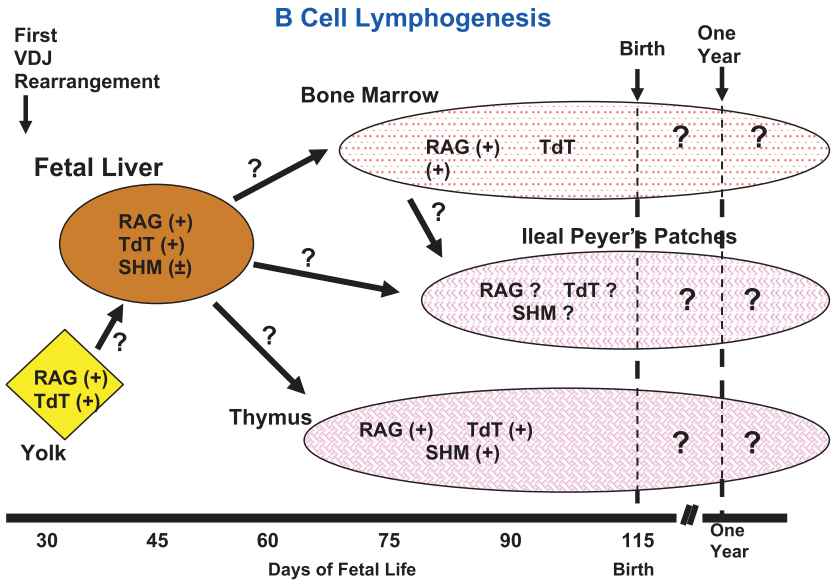

Figure 4. B cell development in piglets. Arrows indicate possible pathways for migration of early B cells from fetal liver to other lymphoid tissue. RAG = recombinase activation gene; TdT = Terminal deoxynucleotide transferase; SHM = somatic hypermutation. It is unknown how long the activities in bone marrow, IPP and thymus continue after birth. (A color version of this figure is available at www.edpsciences.org.)

periphery (Figs. 3D and 3E). The first peripheral T cells are detected in fetal blood and spleen on DG45 (Figs. 3D and 3E). Therefore, B cells are the earliest lymphocytes to appear and 5-18 days before T cells. B cells also remain the major lymphocyte population in the periphery until at least DG55 (Figs. 3D and 3E; [101, 102, 106]).

After the bone marrow becomes the major hematopoietic organ at about DG55-DG65 (Fig. 3B), there is massive expansion of B and T cells in the fetal blood and spleen (Figs. 3D and 3E; [101, 102]). B lymphopoietic activity in the bone marrow peaks between DG65-DG100 (Fig. 3B) and during this period, the majority of peripheral B cells are generated [101, 102, 106]. Fetal bone marrow continues to be active throughout fetal life and also into early neonatal life. Whether it continues in adulthood as in mice and humans [83] has not been established. Figure 4 is a diagrammatic overview of B cell lymphogenesis that incorporates the results described above as well as others that are discussed below.

3. THE B CELL REPERTOIRE

3.1. The genomic potential of the B cell repertoire

Antibodies of the same five isotypes as in mice and humans, i.e. IgM, IgD, IgG, IgE and IgA, are encoded in the swine genome. Similar to the mouse heavy chain genome, there has been no duplication of the IGHG-IGHE-IGHA (Cγ-Cε-Cα) region as seen in humans but there appears to be considerably more diversification of genes encoding IgG subclasses [24]. At least six expressed IgG subclass sequences are known although genomic blots indicate even more may be present ([24, 56]; Fig. 5). Since it is generally recognized that subclass diversity corresponds to diversity of function, swine and

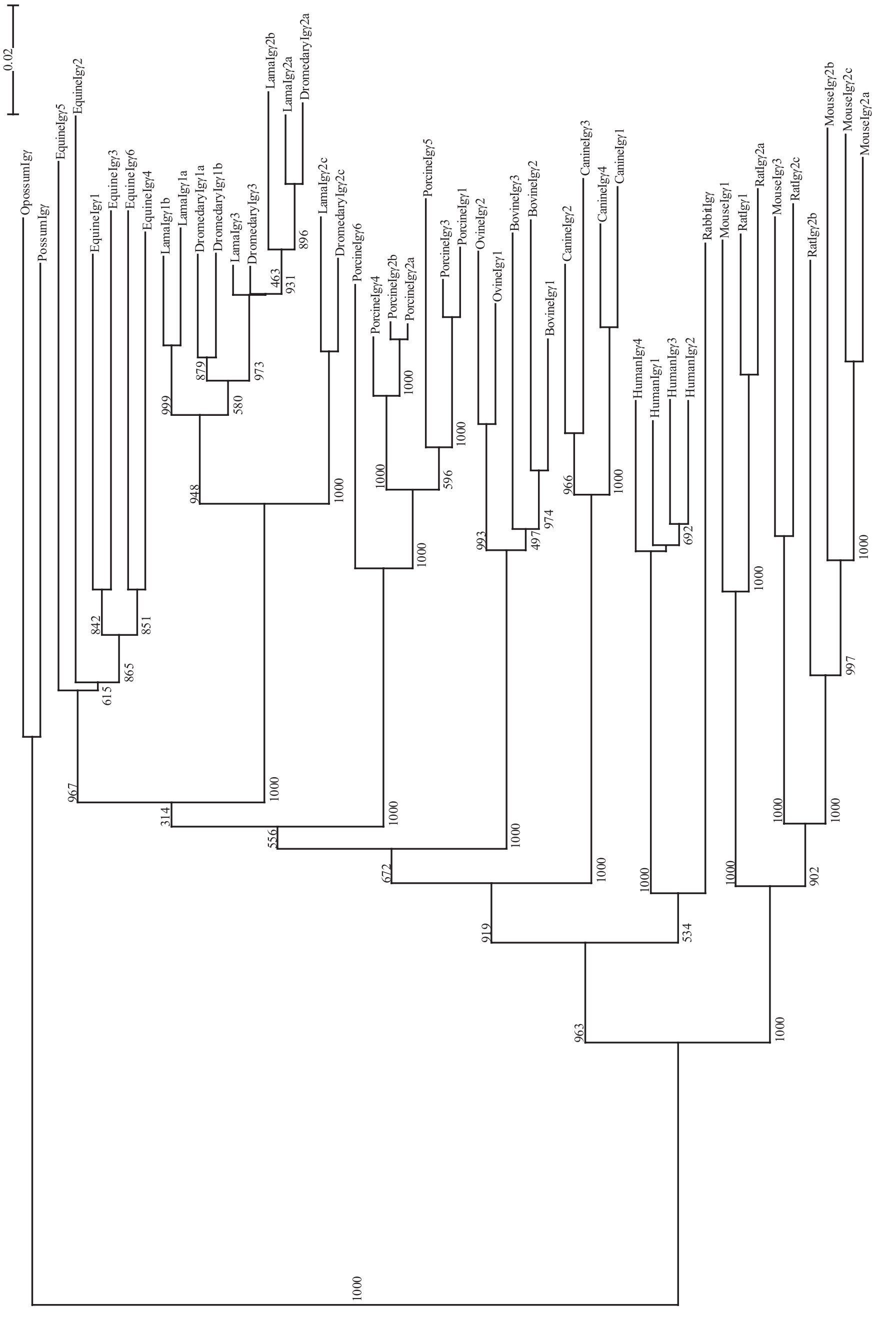

Figure 5. Unrooted nucleotide cladogram comparing the IgG subclass sequences of common mammals. Kindly contributed by Drs Serge Muyldermanns and Nick Deschacht, Free University of Brussels, Belgium.

horses [119] appear to lead the way in this parameter among veterinary species [12, 13, 68]. Since speciation preceded IgG subclass diversification [58, 82] there is no homology among subclasses (except among closely-related species, e.g. cattle-sheep, humans-apes) so that functions ascribed to human and mouse IgG subclasses should not be extrapolated to those subclasses with the same name in other species.

Swine have only one gene for IgA but this occurs in two interesting allelic forms, IgA^a and IgA^b [7, 8]. The latter is unique in missing four amino acids in the lower hinge although there is no evidence to suggest it is associated with any immune deficiency [81].

The IGHD gene in swine has the particularity, as in cattle and sheep, of having a CH1 exon highly similar to the IGHM CH1 exon, that precedes a short switch sequence. The IGHM CH1 exon can be spliced to the IGHD CH1 exon to generate IG delta transcripts with a longer and chimeric constant region [131]. The swine IGHD gene contains two hinge exons, but the second exon is not found in normal cDNAs due to the lack of a normal branchpoint sequence for RNA splicing [131]. Interestingly IgD is nearly absent from peripheral blood B cells and from bone marrow but is prominently expressed in secondary lymphoid tissues [77]. The structural features and expression pattern of porcine IgD therefore differs from IgD in mice and humans.

Swine equally express kappa and lambda light chains in serum Igs [51] and in blood and secondary lymphoid tissues [21, 104]. Thus they differ remarkably from other ungulates like horse, sheep and cattle that use lambda chains > 90% of the time.

The organization of the kappa (IGK), lambda (IGL) and heavy (IGH) loci in swine is similar to most other mammals. The lambda locus contains multiple tandem IGLJ-IGLC genes [12, 24]. The swine kappa locus contains $\simeq$ 80 IGKV genes of which 60 are of a subgroup with homology to the human IGKV2 subgroup [19]. The latter subgroup is preferentially used in the pre-immune repertoire [19, 21]. Expressed porcine IGLV genes comprise predominately two subgroups with homology to human IGLV3 and IGLV8 subgroups[3].

What especially characterizes the porcine variable region repertoire are the IGHV genes. About 30 IGHV gene sequences have been reported although genomic blots indicate only $\simeq$ 20 IGHV genes [24, 113]. Some reported genes may be mutated cDNA sequences, PCR artifacts or allelic variants [103]. Surprisingly all reported IGHV genes of swine belong to a subgroup similar to the human IGHV3 while those of sheep and cattle (also artiodactyls) belong to the *Ovis aries* and *Bos taurus* IGHV1 subgroups, which are similar to the human IGHV4 subgroup [13]. The most striking difference between swine and human/mice is that humans and mice have 7 and 15 subgroups of IGHV genes[4], respectively [66, 68] whereas all swine IGHV genes so far reported are from a single subgroup, similar to the human ancestral IGHV3 subgroup that belongs to clan III[5] [113]. A single subgroup similar to the human IGHV3 subgroup, is not unique to swine. Indeed such a subgroup is exclusively used by chickens, rabbits, camels and monotremes [10, 12]. While the actual number of IGHD diversity genes in swine is unknown, only two are used in > 99% of all VDJ rearrangements. Similar to the chicken, there is only one IGHJ gene [14, 16]. A detailed and comparative description of the porcine Igs and IG genes is available in a recent review [24], and on the Comparative Immunoglobulin Workshop Website[6] and on the IMGT Website[7] [68].

3.2. The pre-immune repertoire

The pre-immune repertoire is defined as the one that develops prior to exposure to

[3] Butler, Wertz, Sun, Wells, unpublished data.
[4] IMGT Repertoire, [on line] http://imgt.cines.fr.
[5] IMGT Index>Clan, [on line] http://imgt.cines.fr.
[6] Comparative Immunoglobulin Workshop, CIgW, [on line] www.medicine.uiowa.edu\CIgW.
[7] IMGT Website, [on line] http://imgt.cines.fr.

environmental antigen or bacteria and potential maternal regulatory factors. In swine it refers to the repertoire that develops during fetal life and that is present at birth in the newborn piglet. Four major IGHV genes, called V_HA, V_HB, V_HC and V_HE dominate the pre-immune VH (IGHV-D-J) repertoire and comprise $\simeq$ 70–80% of all IGHV gene usage [16, 77, 114, 115]. When V_HF, V_HX and V_HY are added, $\simeq$ 95% of the pre-immune repertoire can be accounted for[8]. As indicated above, swine use two IGHD genes 99% of the time and have only a single IGHJ gene so that combinatorial diversity in the pre-immune VH repertoire is highly restricted in comparison to that described for humans and mice [16, 115]. The pre-immune V-KAPPA (IGKV-J) repertoire is dominated by the use of IGKV genes of a subgroup with homology to the human IGKV2 subgroup and a single IGKJ gene, i.e. the pre-immune V-KAPPA repertoire is also highly restricted [19]. The exact degree of restriction in V-LAMBDA (IGLV-J) expression has not yet been established but the same tendency for restricted expression is observed[3]. In sites considered primary lymphoid tissue (Fig. 4), the transcription of lambda to kappa light chains is > 10:1 but in secondary lymphoid tissue it is closer to 1:1 [21]. The predominance of lambda expression in primary lymphoid tissue probably reflects the use of $\lambda 5$ as part of the surrogate light chain complex. Porcine VpreB has been cloned and mAb are being prepared so this hypothesis can be tested[9].

3.3. Why is the pre-immune B cell repertoire restricted?

The basis for antibody gene usage in forming the pre-immune repertoire is not fully understood although there is little evidence that it is random. First, it may be stochastic and dependent on organization of the variable gene locus. It is known that 3' IGHV genes are among the first used [98, 124], that rabbits use their 3' IGHV gene in 90% of early B cells [62] and chickens have only one functional IGHV gene and it is in the 3' position [89]. While position in the locus may play a role, the non-random and restricted usage of V genes in the pre-immune repertoire might also reflect a functional/evolutionary bias. Cohn has described Category I antibodies as those encoded by germline genes that are modified little through recombination because of the inactivity of TdT during early B cell lymphogenesis in mice and humans [32]. Category I heavy chains pair with light chains of a restricted repertoire to generate the specificities attributed to B-1 cells, such as those recognizing bacterial and self antigens (see Sect. 6.1, below). Exactly why this repertoire is restricted and why it is so cross-reactive can only be speculative. It has been called the natural antibody repertoire [26, 84] and its appearance is not stimulated by environmental antigen. Perhaps this natural, pre-immune repertoire is essential for survival of the species since it broadly recognizes the pathogens that can harm the species. Clearly, if an IG does not exist that can recognize the pathogen there is little possibility that an adaptive immune response with refined specificity could ever be generated. It is interesting that the same spectrum of conserved antibodies, including polyreactive autoantibodies [75], is found in both sharks and mammals [74]. Therefore it is not surprising that the pre-immune swine antibody repertoire also recognizes many autoantigens, ubiquitous environmental antigens and their homologs [33]. Further support comes from studies with the porcine reproductive and respiratory syndrome (PRRS) virus in which infection stimulates polyclonal activation of the pre-immune repertoire resulting in destructive autoantibodies [69]. Such a phenomenon also accompanies certain other viral infections [5, 57, 95, 108]. While the retention of autoreactive cells may facilitate anti-tumor immunity, pathogens that can suddenly expand the pre-immune repertoire

[8] Butler, Weber, Wertz, Lemke, unpublished data.
[9] Butler, Sun, Wertz, Muyldermans, unpublished data.

can subvert normal immune function causing harm by: (a) expanding non-tumor autoantibody production and (b) interdict adaptive mechanisms that normally refine the pre-immune repertoire by selecting and expanding clones that are highly specific for pathogens while simultaneously silencing cross-reactive "natural" B cells. Recognition of autoantigens by the pre-immune repertoire is not altogether bad, so long as the eventual effector response is not destruction of vital cells, e.g. beta cells in type 1 diabetes, or molecules that are essential for existence. Rather autoantibody responses could be a means of controlling tumors that generate large amounts of self-antigen, thus stimulating the ever present low affinity autoreactive cells into action.

Since we review many aspects of swine immunology that do not fit the paradigms based on studies in mice, humans or sharks, the concepts discussed above may not universally apply to all mammals or all vertebrates [12, 13]. For example, the Cohn hypothesis does not fit well to the situation in swine in which TdT is active early even in the yolk sac thus generating considerable junctional diversity [16, 106]. Thus, interspecies extrapolations must always be viewed with caution and enterprising student should "think outside the box".

4. THE T CELL REPERTOIRE

4.1. The classification of T cells in swine

Two types of CD3-associated T cell receptors (TR) have been identified in all vertebrate studied so far, consisting of either an αβ or γδ TcR heterodimer. In some species like human, mouse and rat, αβ TcR is expressed on > 95% of all T cells. Unlike rodents and humans, pigs, ruminants and chickens have a higher proportion of γδ T cells in the peripheral blood and lymphoid organs that may account for more than half of the peripheral T cell pool [4, 43].

Maturation of porcine αβ T lymphocytes in thymus follows the generally accepted model of intrathymic T cell differentiation derived from studies in other species. 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 [102]. However, activation with various antigens in the periphery leads to permanent expression of CD8αα on a subset of $CD4^+$ helper αβ T cells leading to so-called double positive peripheral T cell that may selectively mark them as effector/memory T cells [132]. Since occurrence of these peripheral $CD4^+CD8\alpha\alpha^+$ αβ T cells is dependent on external antigenic stimuli, this subset is absent or very rare before birth and among newborns [101, 102].

In comparison with αβ T cells, γδ T cells in swine are traditionally subdivided into three subsets based upon their expression of CD2 and CD8αα and include $CD2^-CD8^-$, $CD2^+CD8^-$ and $CD2^+CD8\alpha\alpha^+$ γδ T lymphocytes [101, 107, 125]. Developmental pathways for γδ T cells have been recently described [107] showing that the maturation of γδ thymocytes occurs after the full expression of γδ TcR. Each of the three γδ thymocyte subsets defined by CD2 and CD8αα expression develop in thymus through separate differentiation pathways originating from $CD1^+CD45RC^-$ to $CD1^-$ $CD45RC^-$ and progressing to $CD1^-$ $CD45RC^+$ cells [107]. In addition to classical γδ T cells that are always $CD4^-$, there is small family of $CD4^+$ γδ T cells in thymus that possess unusual features. This subset co-expresses CD8αβ and CD1, has no counterpart in the periphery, follows a different developmental pathway than other γδ T cells and the majority are actively dividing. Swine γδ T lymphocytes display tissue-dependent phenotypic patterns. $CD2^+CD8\alpha\alpha^+$ and $CD2^+CD8^-$ γδ T cells preferentially accumulate in the spleen while $CD2^-CD8^-$ are enriched in circulation [97, 101, 125]. A subset of the peripheral $CD2^+CD8\alpha\alpha^+$ γδ T lymphocytes has been postulated to be the progeny of peripheral $CD2^+CD8^-$ γδ T lymphocytes upon

stimulation [34, 101, 126] since the change is accompanied by up-regulation of MHC-II expression [107].

4.2. The genomic potential of the T cell repertoire

The TRBV and TRDV[10] repertoire of swine is extremely similar to that in humans. This comes in the wake of a porcine IGHV repertoire that differs substantially from that in humans. This indicates that two highly homologous gene groups (TRBV and IGHV) can diverge greatly from each other in the same species during evolution. We have identified in swine 19 of the 32 known human TRBV subgroups [23, 67] and 17 of these show > 70% sequence similarity to their human homologs. The TRBJ-TRBC region of the locus is identical to that in humans, i.e. TRBJ1-1 to TRBJ1-6 preceding TRBC1 and TRBJ2-1 to TRBJ2-7 preceding TRBC2 [23]. Only one subgroup was highly divergent from any of those expressed in humans and this was designated Vβ100 [2, 23]. TRDV genes belong to five subgroups, three of them with homology to two human TRDV subgroups [127].

4.3. The kinetic of αβ and γδ T cell development

Similar to mice [86] and chickens [27], porcine γδ T cells are the earliest detectable T lymphocytes, developing first in the thymus at DG40 (Fig. 3C) and subsequently populating the periphery at DG45 (Figs. 3D and 3E; [101, 102]). γδ T cells thus require a shorter time period for maturation than αβ T cells and develop without any $CD3^{lo}$ or $TcR\gamma\delta^{lo}$ transitional stage [101]. Mature $CD3\varepsilon^{hi}$ αβ thymocytes are observed at DG55 (Fig. 3C) and their occurrence is preceded by the appearance of $CD3\varepsilon^{lo}$ thymocytes at DG45 [102]. These data therefore suggest that porcine αβ thymocytes require about 15 days to fully differentiate while γδ thymocytes do so in less than 3 days. From findings mentioned in section A (above) it is clear that the earliest T cells recovered from porcine fetuses before DG50 are the progeny of hematopoietic progenitors from yolk sac and/or fetal liver that colonized the embryonic thymus. Prior to this stage of fetal development, the frequency of lymphocytes in the periphery (Figs. 3D and 3E) and the TcR repertoire (see below) is limited. However, the onset of lymphopoietic activity in bone marrow at DG45 (Fig. 3B) is associated with a second wave of hematopoietic progenitors that migrate into the thymus. This is followed by a rapid expansion of T lymphocytes in the fetal blood and peripheral lymphoid organs (Figs. 3D and 3E). This expansion also changes the ratio of αβ/γδ lymphocytes so that αβ T cells predominate in both the thymus and the periphery during the remainder of gestation (Figs. 3D and 3E; [101, 102]).

4.4. The porcine TR V delta (TRDV) repertoire

The TRD genes, encoding the TRδ chain, lies within the TRA locus[10]. The TRD locus is deleted from the genome when the TRA locus is rearranged. The genomic region including the TRAJ genes and TRAC gene, and the TRDJ genes and TRDC gene has been completely sequenced [116]. The TRDC gene consists of three translated exons and a fourth exon that does not have a translated region. Similar to mice and humans, almost all TRDV genes are located on the 5'side of the TRDD diversity genes. One TRDV gene, (TRDV5 according to the nomenclature of [127] and TRDV3 according to [117]) is the inverse orientation of transcription in the locus and located between the TRDC gene and the TRAJ genes. In analogy to humans, four TRDJ genes could be identified. Swine were reported to be phylogenetically more distant from humans than from mice. However, sequencing of the TRDJ and TRDC genes suggested a higher similarity to humans than to mice [117].

[10] The IMGT nomenclature for IG and TR genes has been adopted for the swine. This nomenclature is described in detail by Lefranc and Lefranc [66, 67] and Lefranc et al. [68].

In contrast to the TRDJ and TRDC genes, the precise genomic structure of the TRDV (with the exception of TRDV5, see above) and TRDD genes is not known. However, one group identified multiple TRDV cDNA sequences by anchored PCR of the reverse-transcribed RNA from the thymus of a one month old germfree pig [127]. Thus, the full-length germline sequences of TRDV and TRDD regions are not known and are only estimated based on the sequence similarity of mRNA transcripts. Based on this study, TRDV sequences were placed into five subgroups by the criteria of > 75% nucleotide identity. One TRDV subgroup, TRDV1, is unique in that it consists of a large number of related members. So far, thirty-one distinct sequences have been recovered. All members of the TRDV1 subgroup display highly similar leader sequences in contrast to the diversification of the TRDV1 regions. From Southern blots, the TRDV2 subgroup consists of only two members and TRDV3, TRDV4 and TRDV5 each appear to be represented by a single gene [127]. So far, three putative TRDD genes were described. Thus, the porcine TR δ repertoire offers greater recombinatorial diversity than that described for humans and mice.

In contrast to mice and humans, very little data are available on the rearranged γδ TR repertoire of pigs. An initial study reported 28 CDR3 regions derived from the thymus of a one month old pig [127]. We have analyzed the TR δ repertoire from different mucosal sites including the stomach, duodenum, ileum, Peyers patches, jejunum and colon [48]. Extraintestinal sites like the lung, spleen, thymus and mesenteric lymph nodes were also studied in conventionally reared pigs aged 2 weeks to 5.5 years. TRDV1 to TRDV5 transcripts were amplified by RT-PCR and their CDR3s were spectratyped.

Similar to humans, we observed that the TR δ repertoire of most organs showed increasing restriction with age and was highly oligoclonal in the adult 2 to 5.5 year old pigs. Furthermore, porcine γδ T cells showed a marked compartmentalization not only between different organs like the lung and the intestine, but also within the intestine of old pigs (Fig. 6). We observed a near fingerprint-like CDR3 profile that was typical for each organ and each individual pig. For example, the CDR3 profiles of TR δ transcripts from the left and right lung were very similar by spectratyping and this was confirmed by sequence analysis. Furthermore, the CDR3 profile was identical along the entire duodenum and jejunum, but distinct from that in the colon. Similarities were independent of the TRDV subgroup analyzed.

Together with our previous studies in humans [29, 45–47] and reports of studies on nonhuman primates [71, 87] a general paradigm emerges in which in each organ, different antigens select and maintain the γδ TR repertoire. The homogenous distribution of dominant γδ T cell clones along the small or large intestine is most likely the result of γδ T cells that are selected by ligands in the intestinal tract and undergo expansion and recirculation before lodging throughout the small or large intestine. A local expansion without recirculation would result in a more patchy distribution [44]. This hypothesis is supported by in vivo data demonstrating that proliferating γδ T cells are present in all intestinal compartments [116]. Perhaps these proliferating γδ T cells continuously emigrate via the intestinal lymph and on their migratory route, become distributed along the entire gut. Occasionally we observed identical TR δ transcripts in the intestine and the lungs and similarly, shared clones could be detected along the entire gastrointestinal tract. Thus, subsets of γδ T cells are likely to recirculate and transport immunological information between different compartments of the immune system. This is in line with the observation that intestinal immunization with killed bacteria protects the lung against bacterial infections [36].

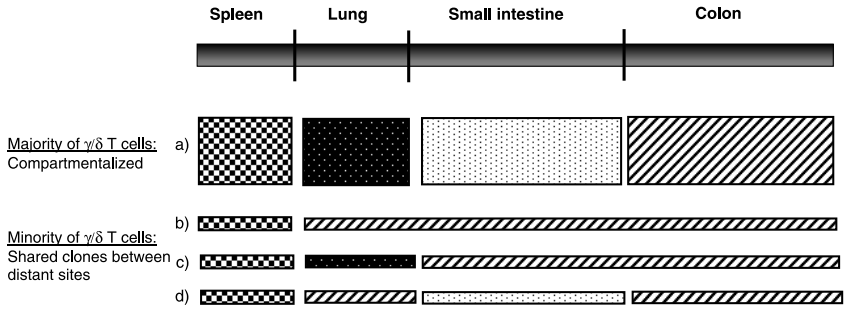

Figure 6. Compartmentalization of the γδ TR repertoire in adult pigs. The various patterns indicate shared (same pattern) or unshared (different pattern) repertoires. a) The vast majority of γδ T cells are compartmentalized as indicated by the distinct patterns. A highly polyclonal repertoire was always present in the spleen. Occasionally shared γδ T cell clones were detected between distant sites (b–d). b) A minority of the same γδ T cells are shared between intestinal tract and in the lung. c) Many are shared along the entire intestinal tract, d) while others are shared between the colon and in the lung.

4.5. Non-polymorphic TRDV during fetal life

Using the same molecular tools described above [48], we analyzed the CDR3 regions of TRDV1 to TRDV5 transcripts from multiple fetal organs from DG38 to DG114 since the first γδ T cells can be detected on DG38 [49] (see above). These studies revealed an invariant TRDV3 transcript in all fetuses from early gestation until birth. No other dominant TR δ transcript could be identified. This invariant TRDV3 transcript was recovered from the earliest γδ T cells suggesting programmed rearrangement as previously proposed for human and murine γδ T-cells [41, 54, 64]. At mid gestation, i.e. DG55, this invariant TRDV3 transcript dominated in all organs analyzed. During further development we observed a gradual loss of predominance. Nevertheless, this invariant transcript was still frequently found in peripheral organs like the intestine and spleen of older fetuses. However, it was absent in the thymus of older piglets, suggesting that this invariant TRDV3 chain was no longer rearranged in late gestation. Thus, the thymic and intestinal γδ TR repertoires partially overlap early in development but diverge in the second half of gestation (Fig. 7). It is likely that invariant TR δ chains are only generated in the thymus early in gestation and γδ T cells expressing this invariant receptor exit the thymus and migrate to distinct peripheral sites where they take up residence and perform specialized functions [42]. Similar data were reported for humans [64, 79].

Invariant TR δ chains have also been described in mice [42]. Murine γδ T cells that populate the epidermis and the reproductive tract during fetal development express two different Vγ chains but the same invariant Vδ chain. This rearrangement exhibits no junctional diversity [1, 41, 53]. This is not surprising since N region additions do not appear in mice until 3 to 5 days after birth when TdT becomes active [6]. In contrast, TdT is active before birth in pigs [16, 106] so the TRDV-D-J rearrangements show marked junctional diversity and N-region additions already during early fetal life. However, the average CDR3 length at mid gestation (< DG70) was much shorter than the average CDR3 length at the end of gestation (DG110), indicating that the activity of TdT increases with time as in humans [46]. Therefore, the persistence of fetal porcine γδ T cells, expressing an invariant TRDV3 chain throughout development, is surprising. It is most likely that

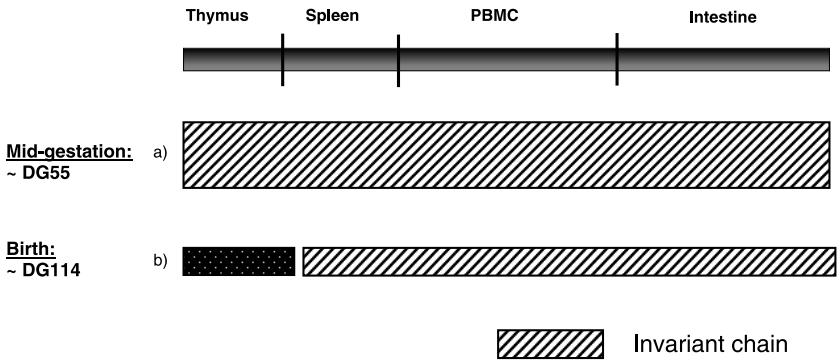

Figure 7. Distribution of the invariant porcine TRDV3 chain. The diagonal pattern depicts the distribution of the invariant receptor. At mid gestation (DG55), the invariant TRDV3 chain predominates in all organs. At birth, the invariant TRDV3 chain is no longer found in the thymus whereas it is still present, albeit at a lower frequency, in the other organs.

the γδ T cell population expressing this invariant TRDV3 rearrangement develops very early in thymus and is then maintained in the periphery by continuous stimulation and proliferation. Both developmental constraints [54] and selective pressure [42] are likely to be important. For example, they might be involved in innate immunological recognition [50] and have therefore remained conserved throughout evolution.

4.6. Clonal diversity of the TRBV repertoire of fetal thymocytes and T cells

Thymocytes and peripheral T-cells from individual fetuses can be fractionated by their co-receptor phenotype. Their TRBV repertoire can be studied by both spectratypic analysis and by determining the frequency of usage of the major porcine TRBV subgroup genes. Studies of this type are ongoing and results will be subsequently published.

5. SPECIAL ISSUES REGARDING B CELL DEVELOPMENT IN PIGS

5.1. Two Bs or not two Bs

The concept of B-1 and B-2 subsets has been well advertised in studies of mice in which flow cytometric (FCM) studies can identify B-1 cells as CD5(+), IgD^{low} whereas B-2 cells are CD5(–), IgD^{high} and also express CD21 and CD23 [3, 95]. Whether these represent two subsets with separate origins or merely transitional stages of B cell development [30, 70, 111] remains controversial. Dogma says that B-1 cells develop early in ontogeny, primarily in the peritoneum, are more easily polyclonally activated [96] and give rise to many antibodies of high cross-reactivity/connectivity that recognize autoantigens and polysaccharide of ubiquitous bacteria. This pattern of recognition also characterizes the pre-immune repertoire [33, 35] (see D-2 and D-3) that is primarily expressed as IgM [37]. Among antibodies of the pre-immune repertoire are autoreactive antibodies, of which a high proportion bind negatively-charged DNA [28] and have arginine residues in their CDR3 regions [65, 99]. This makes them especially suitable for binding bacterial polysaccharides and DNA. In comparison, the mature B-2 repertoire is "refined" and is represented by B cells with more specific receptors and fewer that are autoreactive. In swine, CD5 is expressed on all fetal B cells[11]. However, there are no mAb to IgD

[11] Sinkora J., personal communication.

or CD23 and it remains to be determined if the proportion of CD21(+), and CD23(+) B cells expressing IgD increases during development [31]. Interestingly, expression of CD21 is especially pronounced after birth in conventional lambs suggesting that B-2 cells are either selected or that the B cells that comprise the pre-immune repertoire transition to a B-2 phenotype after exposure to environment stimuli [130].

While B-1 and B-2 cell have not been characterized in swine, B cell V-D-J rearrangements in yolk sac and fetal liver distinguish a subset that differs from that in bone marrow because nearly all those from yolk sac and fetal liver have nearly 100% in-frame rearrangements whereas in bone marrow, 71% are in-frame as predicted if random chance was responsible [106]. Whether B cells differing greatly in the proportion of in-frame rearrangements represent two separate B cell lineages such as the B-1 and B-2 or T-1 and T-2 subsets of mice, remains to be determined. However, the earliest B cell population in swine appears to originate from the first wave of hematopoietic progenitors and these show 100% in-frame IGH V-D-J rearrangements like that reported in chickens and rabbits. In both of the latter species, B cells with 100% in-frame rearrangements are generated during a narrow window in fetal life from yolk sac and fetal liver and are then presumably maintained by mitosis. It may be that B cells generated early in ontogeny in many species are delayed in progressing to V-D-J rearrangement on the second chromosome.

5.2. The role of thymic B cells

The porcine thymus contains three separate populations of B cells. The medullar area contains IG containing cells that primarily express and secrete IgG and IgA [17, 33]. There are also B cells in the cortex and these display a CDR3 spectratype characteristic of the selection of cells with in-frame (productive) rearrangements. However, most B cells in the porcine thymus are found in the interstitial region between the thymic lobules. Surprisingly, these B cells do not display surface IG and display no selection for in-frame rearrangements [16, 106]. These observations raise the question of whether the B cells of the cortex and medulla are derived by in situ lymphogenesis from the pro-like B cells in the interstitium. Are the pro-like B cells of the interstitium merely the result of a defective developmental pathway, e.g. delayed Notch I expression, or a dedicated site of B cell lymphocytes for the mature B cells later found in the cortex and medulla? If the latter, do they have a special role in the thymus? How and why do they switch to transcription and synthesis of IgA and IgG? Is this switch driven by T cells, a special cytokine milieu or a cytokine- and antigen-independent switching effect? Can switch be a stochastic event or must it always be mediated by the above factors? These questions have significance beyond veterinary immunology and may be resolved in mouse models or may have to wait until the necessary tools for swine immunological research becomes available.

5.3. What role is played by the ileal Peyers patches (IPP)?

Gut associated lymphoid follicles of mammals were described more than 150 years ago by Brücke (cited by Griebel and Hein, [40]) but their function has remained elusive. In swine these are found in two separate locations, isolated Peyers patches in the jejunum and as a continuous organ just above the ileal caecal junction; the ileal Peyers patches (IPP). Similar-appearing follicles in the chicken hindgut are known as the bursa of Fabricius and have been shown to be the site of B cell repertoire diversification [78, 90]. Those in sheep appear to play a similar role [91, 92, 128]. Evidence for a similar role of the porcine IPP remains circumstantial. The porcine IPP are detectable on DG70 and at birth (Fig. 4) can express all three major isotypes although IgM predominates prior to exposure to colonizing gut bacteria [18]. Like those of sheep, the

swine IPP undergo early involution or conversion to conventional jejunal Peyers patches. A similar transition may also be true for the rabbit appendix [121]. Unpublished data (Amanda Schoenherr) indicate that B cells in each follicle are derived from 1–3 progenitors, similar to what has been observed in the chicken bursal follicles [89]. Since porcine IgD is not expressed in bone marrow or blood but is abundant in secondary lymphoid tissues [77] it might suggest that developing swine B cells undergo transitional development in some other organ and this could be the IPP.

6. HOW DO MATERNAL AND ENVIRONMENTAL FACTORS IMPACT THE DEVELOPMENT OF ADAPTIVE IMMUNITY?

6.1. The piglet model for studies on immunoontogeny

The epitheliochorial placenta of pigs and the precosial nature of piglets provide several models for immunological studies. Because of the apparent absence of IG transport via the placenta, fetal piglets provide a model for studying the intrinsic development of the immune system without the ambiguity that arises from maternal influences as in rodents. Caesarian-derived piglets can be placed in isolator units that allow investigators to experimentally control the effect of maternal colostrum/milk and intestinal flora on postpartum development of the immune system.

6.2. The role of intestinal flora

Piglets maintained bacteria-free for six weeks have very low serum Ig levels, e.g. 30–50 μg of IgG, but when colonized levels rise 100-fold. Of the major isotypes, IgA is selectively increased [15]. This presumably arises from switched mucosal B cells and not from a unique developmental pathway [72]. Newborn piglets are unable to respond to either T-dependent or T-independent immunogens but monoassociation with a single strain of benign *E. coli* allows immunoresponsiveness to these types of immunogens [18]. We have recently shown that the ability to respond depends on receptors for pathogen-associated molecular patterns (PAMP) such as toll-like receptors (TLR) of which those recognizing bacterial DNA (as CpG-B oligodeoxynucleotides) are very critical [22]. CpG-B appears to primarily expand the abundant IgM B cells of the newborn although the switch to IgG and IgA requires co-exposure to lipopolysaccharide (LPS) [22]. So far, colonization studies have been done with defined flora so its growth and presence can be easily monitored. However studies in mice [52] and rabbits [93] suggest that different organisms have different effects. Single cultures or "monoassociation" may yield different results than with "natural" gut flora. It is noteworthy, and relevant to preliminary data from PRRSV-infected conventional piglets, that the PRRSV-induced immune dysregulation[12] observed in isolator piglets was independent of monoassociation with a single benign *E. coli*.

6.3. The role of colostrum/milk

Colostrum and milk provide the maternal antibodies that provide passive protection to the neonate before its own adaptive immune system has reached proper development (Fig. 1). The role of the mammary gland and its secretions has been extensively reviewed [11, 20]. While the protective role of lacteal secretions by transfer of passive antibodies is well-established, its regulatory role is less well understood. It has been known for some time that maternal IgG has a down-regulatory effect on neonatal IG synthesis [60, 61] perhaps through

[12] Newborn piglets inoculated with wild-type PRRSV develop massive lymphoid hyperplasia, show 10–100 increases in immunoglobulins of all major isotypes, autoantibodies to Golgi, dsDNA and kidney endothelia. The response is polyclonal and not targeted to viral antigens [69].

removal of environmental antigen in the manner of therapeutically administered intravenous immunoglobulin (IVIG) or perhaps by a direct effect on B cells such as cross-linking the B cell receptor (BCR) and the FcγRIIβ. The latter might explain why PRRSV-infected piglets suckling non-immune sows show 10-fold lower IgG levels than isolator piglets and appear to show less immune dysregulation[13]. However, colostrum also contains > 43 enzymes, at least 22 cytokines, chemokines and growth factors [20]. Human milk stimulates growth and maturation of enterocytes [59] perhaps because of the presence of EFF, IGF and CSF [20]. Porcine colostrum contains 1500 ng/mL EGF [55] and up to 250 ng/mL of TGFβ [123]. The latter is generally recognized for its suppression of autoimmune responses and its role in IgA class switch recombination. Thus, non-immune colostrum may provide immune modulators during the critical window of development (Fig. 1) that facilitate the establishment of immune homeostasis.

6.4. The piglet as a model for immune homeostasis

The ability to distinguish "foreign" from "foreign danger" is a lifelong challenge for the adaptive immune system. The host must mount a protective response to the pathogen while avoiding responses that are deleterious, e.g. autoimmunity and allergy. The neonatal period (Fig. 1) is when the "adaptive immune system" develops, matures and apparently also when immune homeostasis develops. It is the time when non-responsiveness to dietary and microbial antigens must be established in the intestinal mucosa [108]. As described above, colonization or exposure to PAMP is required for immunoresponsiveness in newborn piglets [18, 22]. In mice stimulation through TLR is necessary for the induction of oral tolerance and immune homeostasis [88, 120]. It is also within the neonatal window that maternal colostrum or milk is being provided. The meteoric rise in inflammatory bowel disease (IBD) and allergy in the human population in highly developed cultures of Europe, North America, Japan and Korea has been the basis for the so-called hygiene or "dirt" hypothesis [94, 129]. Hence disturbances to normal development within the critical neonatal window (Fig. 1) can disturb development of immune homeostasis. The extreme immune dysregulation caused by the PRRS virus in isolator neonates (deprived of non-immune colostrum and normal gut flora) [69] compared to preliminary data on conventional PRRSV-infected neonates[13], may provide a potential model in which the piglet can be used to yield secretes about immunoontogeny. One may hypothesize that immune dysregulation occurs because the B-1-like pre-immune repertoire is expanded before it transitions to a properly refined B-2 or T-2 repertoire through regulatory events (see Sect. 5.1). It is altogether possible that other neonatal disease problems in swine, especially other viruses, could also result from interference with the proper development of immune homeostasis. The isolator piglet model allow this hypothesis to be tested for nearly any pathogen of interest since piglets can be given sterile colostrum (irradiated) and colonized with various cocktails of bacteria in an effort to mimic the effect of the normal gut flora.

7. IMPLICATIONS FOR VETERINARY PRACTICE AND MEDICAL RESEARCH

The immediate postnatal period is a critical time for the neonate and as research in many mammals has shown, the period when the adaptive immune system matures and when immune homeostasis is established (Fig. 1). Much of the earlier thinking in both human and veterinary medicine has viewed this period "merely" as one when passive antibodies provide temporary protection until

[13] Lemke C.D., Ph.D. thesis, The University of Iowa, December 2005.

the newborn had become fully immunocompetent. While the importance of passive antibodies is not disputed, it has so far not explained the development of immunocompetence. Rather intestinal colonization by bacteria or the PAMP displayed by such bacteria may be the ligands needed for development of immunocompetence [18, 22]. In mice, colonization is also required for the development of oral tolerance [112]. This is not surprising since tolerance develops through an active antigen-driven process [118]. Most likely the same PAMP needed for immunoresponsiveness are also those required for development of oral tolerance [108]. One mechanism proposed for oral tolerance is the generation of immunoregulatory or suppressive T cells (Tregs) [109, 110]. The term Treg is somewhat generic and can include CD4 T cells expressing CD25 and secreting IL-10, others that secrete TGFβ as well as CD8 T cells that suppress via cell-cell interaction [38]. In support of this view, it is known that administration of autoantigens by an oral route can suppress autoimmunity in animal models [76]. If colonization is required for immunoresponsiveness and autoimmune suppression, does the nature of the colonizer matter? While not resolved in regards to oral tolerance, evidence cited above indicates that certain bacteria have a greater effect than others in development of immunocompetence [52, 93] and this might also extend to the development of immune homeostasis during the critical window (Fig. 1).

Another major external influence during the critical neonatal window is maternal colostrum and milk (Fig. 1). As discussed in Section 6.2, conventional piglets reared on non-immune dams suffer much less PRRSV-induced immune dysregulation than colostrum-deprived neonates[13]. This must either be due to the regulatory effect of normal flora discussed above, or because colostrum or milk provides regulatory factors that direct proper development of the adaptive immune system of the newborn. Examples of these regulating effects and the many candidate regulatory factors in colostrum or milk were mentioned in Section 6.3 and are reviewed elsewhere [20].

Recognizing the importance of the neonatal period, the potential effects of maternal factors and normal flora and the differences among mammals, it would seem appropriate for veterinary scientists to begin to "think outside the traditional box". Table I lists a number of potential measures that require experimental testing and might eventually prove therapeutically useful in veterinary medicine.

Accumulating studies also indicate the value of immunodiagnostics. Using neonatal PRRS as an example, simply measuring serum Ig levels and showing they were up to 1000-fold higher in affected piglets than controls, was an indication of polyclonal B cells activation [69]. Using molecular biological methods like spectratyping and V_H gene hybridization, it could also be shown that the PRRS response represented an expansion of the nondiversified, pre-immune repertoire rather than a targeted anti-viral response[13]. Similar methods have revealed that there is selective expansion of certain T cell V gene subgroups[14]. Table II lists a number of molecular/cellular based diagnostic assays that could be valuable in defining and identifying features unique to certain diseases of swine.

Table I. Potential measures that require experimental testing and might eventually prove therapeutically useful in veterinary medicine.

Administration of TLR ligands or other PAMP as pharmaceuticals
Postnatal administration of defined gut floral cultures
Prepartum treatment of sows to stimulate the secretion of important regulatory or growth factors in colostrum or milk
Elimination of antibiotics that destroy valuable commensal gut flora

[14] Butler, Wertz, Lemke, unpublished data.

Table II. Molecular/cellular based diagnostic assays that could be valuable in defining and identifying features unique to certain diseases of swine.

Flow cytometric analyses of leucocytes and T cell subsets
Measurement of total IG levels according to isotype or subisotype by sandwich ELISA
Spectratypic analysis of rearranged antibody variable genes
Spectratypic analysis of expressed T cell receptor genes
Quantitation of IGHV, TRBV and TRDV gene usage
ELISPOT assays for cytokine secretion by blood leucocytes
Cytokine gene expression by real-time PCR

Current research on fetal and neonatal immune development can provide clues as to the nature of required vaccines and the timing and methods of their administration. For killed vaccines, a time or route must be selected when delivery is not compromised by maternal antibodies and when the neonate is sufficiently responsive. Responsiveness may be up-regulated by co-delivery of PAMP like CpG-oligodeoxynucleotides. Of course certain highly effective neonatal vaccines are those which can be given to the prepartum sow and can subsequently result in colostral antibody that can provide protection to the neonate. As indicated in Table I, this procedure might also be extended to stimulation of the secretion of growth or regulatory factors in colostrum and milk. Live viral vaccines should be engineered with the concept of delivering the T and B cell epitopes that can lead to protective immunity and not those as in the example of PRRS, that cause immune dysregulation. Protection versus harm is likely to depend on immune redirection of T cell activity, i.e. establishment of immune homeostasis. Since at least swine are highly outbred, genetic selection may not be as important as in more inbred species, e.g. horses, cattle. However, differences among swine in adhesion receptors for pathogenic *E. coli* are known that can determine health and disease [39]. Although rare, there are well known examples of immune deficiency such as horse SCID [122] and leucocyte adhesin deficiency in cattle, i.e. BLAD [100]. Others, like those in lab animals, are also likely to be revealed.

We believe the health and well-being of young animals is highly dependent on the immediate postnatal events that occur in the critical window of development (Fig. 1) and that appear essential for maturation of the adaptive immune system. We believe more attention should be focused on the neonatal period as regards research, diagnostics and prophylactic therapy.

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