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B Cell Lymphogenesis in Swine Is Located in the Bone Marrow

Marek Sinkora and Jana Sinkorova

A course and a site of B cell development in swine are not firmly known. In this study, we show that B cell lymphogenesis is located in the bone marrow (BM). According to expression of MHC class II (MHC-II), CD2, CD21, CD25, CD45RC, CD172a, swine workshop cluster (identification number) (SWC) 7, and μHC, porcine BM cells were resolved into seven subsets representing sequential stages of development. Profile of rearrangement-specific products and transcripts from sorted BM cells confirmed the proposed developmental pathway. The same developmental pathway was further proven by analysis of selection for productive rearrangements in Ig H chains and also by cultivation studies. Cultivation also showed that earliest precursors with incomplete DJ rearrangements can still revert their B cell differentiation and develop along myeloid lineage, whereas this is impossible for later developmental stages. Proliferation and the apoptotic potential of individual developmental stages as well as critical checkpoints were also identified. Colocalization experiments showed early colocalization of MHC-II/CD2/CD172a is replaced by colocalization of MHC-II/CD2/CD21/SWC7/μM in immature cells, whereas CD25 and CD45RC did not colocalize with any other studied molecules. In this study, we also finally prove that the BM in pigs is fully functional in adult animals and that B lymphogenesis occurs there throughout life. To our knowledge, this is the first study showing a course and a direct site of B cell lymphogenesis in swine. The Journal of Immunology, 2014, 193: 000–000.

Development of B cells in all known species is characterized by the formation of the BCR or Ig by combinatorial joining of gene segments for H chains (HCs) and L chains (LCs) on the DNA level (1). Studies in humans and mice have shown that during this rearrangement processes, pro-B cells with germine configuration of all V, D, and J gene segments begins with IgM HC (μHC) recombination of D to J segments to form pre-B-I cells (1–3). Further development involves transition to pre-B-II cells that have fully rearranged VDJ. The early pre-B-II cell is a critical developmental stage in which the rearranged HC is tested by a surrogate LC (SLC) (CD179a) (4). Further cellular differentiation to late pre-B-II cell stage involves vigorous proliferation to increase the cell pool and also subsequent LC gene rearrangement. After this step, the SLC is replaced with an authentic LC (4). The authentic BCR is the defining marker of the immature B cell stage, the direct predecessor of transitional cells (5).

During HC and LC rearrangement, intervening DNA between individual V, D, and J segments is excised and ligated to form signal joint circles (SJC). Because these SJC are not further replicated and they are exponentially diluted during subsequent proliferation of B cells (6), presence of SJC is often used as markers for recent B cell lymphogenesis. Rearrangement is also characterized by the expression of rearrangement-specific genes. Such transcripts include RAG-1 and RAG-2 needed for DNA cleavage (7), TdT that facilitates the addition of nontemplated nucleotides (8), and various other DNA regulatory and repair enzymes. The presence of these rearrangement-specific products and transcripts can also be used for monitoring of B cell development. Importantly, although the development of B cells on a molecular level operates by a similar mechanism regardless of species, the monitoring of this development by expression of accessory cell surface markers differs markedly (2, 3). This inconsistency is apparent in species that are extensively studied, such as humans (key markers are, for example, CD19, CD10, CD34) (2) and mice (key markers are, for example, B220, c-Kit, CD43, CD19, CD25, HSA) (3).

Development of a functional B cell requires that rearrangement is productive, that is, that it generates an open reading frame or in-frame (IF) sequence that can lead to a translatable protein or does not contain stop codons (6). Unlike the LC locus, the HC locus can be rearranged only once because of the loss of recombination signal sequences during VDJ rearrangement and the nonrandomness arrangement of VH, Dζ, and JH segments in the HC locus (9). This is particularly demonstrated in swine that have only one functional JH (10). B cell precursors that do not succeed in making a productive rearrangement using the first chromosome have only one more chance to generate a translatable μHC by using the second chromosome. Therefore, status of HC rearrangement in individual cell subsets can provide a useful means of monitoring B cell development.

There are some known deviations from the above-described paradigms, one of which includes species that do not use the BM for generation of B cells throughout life and that use alternative pathways for generation of the B cell repertoire. These species use specialized GALTs located in a hindgut, and include gallinaceous birds that use the bursa of Fabricius, rabbits that use the sacculus rotundus, and ungulates, including swine, that use ileal Peyer’s patches (10, 11). However, our previous work disproved...
B CELL LYMPHOGENESIS IN SWINE

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Materials and Methods

Experimental animals

Animals used in the study were Minnesota miniature/Vietnamese-Asian-Malaysian crossbred pigs from Novy Hradek (15). Fetuses were obtained by hysterectomy (15). Germ-free (GF) piglets were recovered from gilts by sterile hysterectomy at day 112 of gestation and were kept in isolator units under GF conditions on sterile formula as previously described (16). GF piglets were included because of their naive immune system in which the BM contains a minimum of polymorphonuclear cells, which appear in conventional animals as a consequence of colonization and postnatal infections. Moreover, there is an absence of activated, effector, and memory B cells and plasma cells that may recirculate back to the BM. Staining of cells

The following mouse anti-pig mAbs, whose source and specificity were determined animals as a consequence of colonization and postnatal infections. Moreover, there is an absence of activated, effector, and memory B cells and plasma cells that may recirculate back to the BM. Staining of cells

Preparation of cell suspensions and cell cultures

Cell suspensions were prepared essentially as previously described (17, 18). Briefly, blood was obtained by intracardiac puncture. Cell suspensions from the spleen were prepared in PBS by teasing apart the tissues using forceps. Erythrocytes were removed from the spleen and blood using hy-

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Preparation of cell suspensions and cell cultures

Cell suspensions were prepared essentially as previously described (17, 18). Briefly, blood was obtained by intracardiac puncture. Cell suspensions from the spleen were prepared in PBS by teasing apart the tissues using forceps. Erythrocytes were removed from the spleen and blood using hypotonic lysis. BM cells were directly flushed from the tibia and/or femur and leukocytes were purified using Histopaque-1077 (Sigma-Aldrich, St. Louis, MO) gradient centrifugation (19). All cell suspensions were filtered through a 70-μm nylon mesh membrane. Cell suspensions for flow cytometry were finally washed twice in PBS containing 0.1% sodium azide and 0.2% gelatin from cold water fish skin (PBS-GEL), whereas those for cell cultures were transferred to cultivation medium (see below). Cell numbers were determined by hemacytometer. Cell cultures (19) were done in RPMI 1640 medium supplemented with t-glutamine and 25 mM HEPES, 10% FBS, 100 U penicillin, and 0.1 mg/ml streptomycin. Final concentration of cells was always set to 1 × 10⁵ cells/ml.

Immunoreagents

The following mouse anti-pig mAbs, whose source and specificity were described earlier (15, 18-21), were used as primary immunoreagents: anti-IgM (M160, IgG1), anti-swine MHC-II leukocyte Ag type DR (1038H-12-34, IgM; MSA3, IgG2a), anti-CD2 (MSA4, IgG2a; 1038H-5-37, IgM; PG168A, IgG3), anti-CD14 (MIL-2, IgG2b), anti-CD21a (BB6-11C9.6, 34, IgM; MSA3, IgG2a), anti-CD2 (MSA4, IgG2a; 1038H-5-37, IgM; PG168A, IgG3), anti-CD14 (MIL-2, IgG2b), anti-CD21b (BB6-11C9.6, IgG1), anti-CD21b (IAH-CC51, IgG2b), anti-CD25 (K231-3B2, IgG1), anti-CD45Rc (MIL-5, IgG1), anti-CD172a (74-22-15, IgG1 or IgG2b), ––swine workshop cluster (identification number) (SWC) (7A) IAH-CCCS5, IgG1; 2F6/8, IgG2a), and anti-SWC8 (MIL-3, IgM). Goat polyclonal Abs specific for mouse Ig subtypes labeled with FITC, PE, PE/cyanine 7 tandem complex, allophycocyanin, allophycocyanin/cyanine 7 tandem complex, or PE/Texas Red tandem complex were used as secondary immunoreagents (all secondary reagents were from SouthernBioTech, Birmingham, AL). Primary isotype-matched mouse anti-rat mAbs were used as negative controls. Secondary polyclonal Abs were tested for cross-reactivity (no primary mAbs) and also for cross-reactivity with primary isotype-mismatched mouse anti-pig mAbs. All immunoreagents were trated for optimal signal/noise ratios. In some cases, directly labeled mAbs (19) or mAbs labeled with biotin N-hydroxysuccinimide ester (15) were used. These were labeled with either Zenon labeling technology (Molecu-

Staining of cells

Staining of cells for flow cytometry was performed by indirect subtype staining as described previously (15, 21). 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 subtypes. Cells were incubated for 30 min and subsequently washed twice in PBS-GEL. Mixtures of goat secondary polyclonal Abs conjugated with different fluorochromes were then added to the cell pellets in appropriate combinations. After 15 min, 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 this case, the procedure was the same as described above but only one 30-min incubation step was used. Indirect and direct staining gave similar results.

The DNA content of sorted cells for cell-cycle studies was determined after cultivation using the DNA intercalating probe 7-aminoactinomycin D (7-AAD) (15, 21). Cells after cultivation were washed in cold PBS contain-

Flow cytometry and cell sorting

Samples were measured or sorted on standard FACScalibur or FACSaria III flow cytometers, respectively (Becton Dickinson Immunocytometry Systems, Mountain View, CA). In each measurement, 300,000–700,000 events were collected. Sorted cells were collected to 1) inactivated FBS (PBS-A, Laboratories, Pasching, Austria) in the case of cultivation, 2) empty tubes in the case of PCR amplification, or 3) slides for microscopy.

Electronomic compensation was used to eliminate residual spectral overlaps between individual fluorochromes. Forward light scatter (FSC) area/FSC width parameters were used for elimination doublets for live cells, whereas a doublet discrimination module was used for the same purpose in DNA content analyses. The PClysis or FACSDiva software (Becton Dickinson Immunocytometry Systems) was used for data processing.

Microscopy of sorted cells

Bright-field microscopy was used for morphological examination of in-
dividually sorted cell populations. Cells sorted on microscope slides were air-dried and specimens were stained by Dip Quick stain (Medical Products, Pardubice, Czech Republic). Confocal microscopy was used for exami-

PCR amplification and detection of transcripts

Detection of different transcripts and gene segments was done on different populations of 50,000–100 sorted cells that were dissolved in 0.5 ml TRI Reagent. In a particular experiment, only the same sort of sorted cells was used for isolation of total RNA and DNA according to a protocol recommended by the manufacturer (Sigma-Aldrich). Total cDNA was prepared using random hexamer primers. Each cDNA preparation was amplified in four concurrent analyses (TdT, RAG, VpreB, and VDJ) whereas each DNA preparation was amplified in three concurrent analyses (DJ, SICs, VDJ), with each analysis consisting of two rounds of PCR. All primers and PCR conditions used for amplifications are listed in Table I.

CDR3 spectratyping

Separation of CDR3 regions for Ig HCs on polyacrylamide sequencing gels provides a clonotypic analysis of BCR repertoire (22, 23). This procedure is called CDR3 length analysis or spectratyping and was performed on un- sorted or sorted B-lineage populations to show their level of selection for IF rearrangements (18). Technically, the CDR3 segments of the amplified VDJs from DNA were reamplified in the third round of PCR using an FR3 up primer that anneals to all porcine Vh genes and a 3′-P-I-labeled 3′-down primer (22, 23). The products were separated on sequencing gels that were dried and images were obtained by fluorescent image analyzer FLA-7000 (Fujiﬁlm, Tokyo, Japan).

Results

Naïve B cells display a nearly uniform phenotype

Screening of naïve B cells in the periphery of fetal and newborn pigs revealed uniform positivity for CD2 (Fig. 1C), CD21a

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According to the expression of auxiliary molecules on naive B cells (Fig. 1), different populations of BM cells were sorted by flow cytometry and their DNA was investigated by CDR3 spectratyping for the presence of an unselected repertoire (Fig. 2). Cell populations in the BM that passed the selection step should contain IF HC rearrangement, with a spectratype that differs by three nucleotides. This is the case of B cells isolated from the periphery where there are no out-of-frame (OF) bands and distribution is polyclonal (Fig. 2A, 2B). The same applies for BM B-lineage cells that are positive for μHC (Fig. 2C), CD21a (Fig. 2D), CD21b (Fig. 2E), CD2 (Fig. 2F), CD25 (Fig. 2G), or CD45RC (Fig. 2H). In contrast, sorted SWC7+ (Fig. 2I) or CD172a(lo/hi) (Fig. 2J–K) BM cells possess oligoclonal CDR3 spectratyping with an abundance of OF bands. These results suggest that early B cell precursors in BM are present in the SWC7+CD172a+ population.

A developmental pathway for the B cell lineage in swine based on spectratypic analysis

Because naive B cells are negative for CD172a expression (Fig. 1) whereas early B cell precursors are positive (Fig. 2), decreasing CD172a expression may allow screening for expression of other auxiliary molecules on developing B cells (Fig. 3). According to the appearance of individual precursor cell subsets, seven discrete subpopulations were identified with a putative developmental pathway going from subset 0 to subset 6 (Fig. 3C–I). Because SWC7 expression together with expression of CD172a gave the best resolution for unambiguous identification of early B cell precursors in subsets 1 and 2 (Fig. 3C), we included further phenotype studies of SWC7 with other auxiliary molecules (Fig. 3J–O). From all of the studies (Fig. 3C–O), the best markers for screening of B cell lymphogenesis were identified. These included staining for CD172a/SWC7 (Fig. 3C), CD172a/μHC (Fig. 3I), SWC7/CD21a (Fig. 3M), and SWC7/μHC (Fig. 3O).

Staining profiles also revealed that the earliest molecules detected on early B cell precursors in subset 1 are CD45RC (Fig. 3D), CD2 (Fig. 3E), and CD25 (Fig. 3F). Interestingly, the expression of CD2 and CD25 showed similar profiles when plotted against CD172a (Fig. 3E and 3F, respectively) and resembled the expression profile for CD21a (Fig. 3G). In contrast, the expression profile for CD45RC (Fig. 3D) more resembled SWC7 (Fig. 3C). Therefore, we wondered whether there is a coexpression of CD45RC, CD2, CD25, and CD21a in early B cell development. However, further analysis showed that none of these markers displayed completely diagonal expression for subsets 1–3 (Fig. 3F–S). For this reason, these molecules are problematic for unambiguous screening early in B cell development. This is especially pronounced in CD2/CD25 staining (Fig. 3R). The independence of CD2, CD25, and CD45RC expression during early B cell development was also emphasized in colocalization experiments (see below).

The relationship of surface phenotype, Ig rearrangement, and expression of genes needed for rearrangement

According to phenotype studies (Fig. 3), subsets 0–6 were sorted by flow cytometry as individual cell populations. After isolation of DNA and RNA from freshly sorted subsets, we further analyzed these populations for 1) the presence of VDJ rearrangement–related products in the DNA, and 2) transcription of genes associated with VDJ rearrangement (Fig. 4) (Table I). Results show that no rearrangement–specific products and transcripts could be detected in subset 0. Subset 1 contained cells that had rearranged DJ, produced the corresponding SJCs, but contained no complete VDJ rearrangement. There was also expression of TdT, RAG, and VpreB in subset 1. Subset 2 resembles subset 1 but also contained a small amount of VDJ rearrangement in both DNA and cDNA. Subsets 3–5 were the same and contained all rearrangement–specific gene segments and transcripts. Subset 6 contained DJ...
and VDJ rearrangements but SJC were not detected and there was also no expression of TdT and very little of RAG, suggesting that these cells were no longer active in B cell lymphogenesis. This pattern was similar to that seen in the blood of GF piglets.

**Morphology and colocalization of surface molecules during individual stages of B cell development**

The morphology of sorted subsets 1–6 is shown in Fig. 5A–F. In comparison with sorted leukocytes from the blood (Fig. 5G–K),
they showed no similarities with monocytes/macrophages or polymorphonuclear lineage cells. Colocalization experiments showed colocalization of MHC-II, CD2, and CD172A molecules in early B cell development (i.e., subsets 1–3) (Fig. 5L). In late precursors (subsets 4 and 5) and immature B cells (subset 6) colocalization of MHC-II, CD2, CD172, SWC7, and μHC molecules was observed (Fig. 5M). Molecules CD21 and SWC7 did not colocalize with any other studied molecules in any developmental stage. It is not fully clear in which developmental step VDJ rearrangement occurs (Fig. 5). For this reason, detailed analyses of individual subsets 0–6 were performed by sorting and subsequent CDR3 analysis (Fig. 6). In this analysis, MHC-II/SWC7/μHC staining was used for identification and sorting of basic subsets 0–3 and detailed analysis of subset 6 (Fig. 6A), and MHC-II/SWC7/CD25 staining was used for basic subsets 0, 1, and 4–6 and detailed analysis of subsets 2 and 3 (Fig. 6B). Both analyses revealed that 1) subsets 0 and 1 do not contain VDJ rearrangement, 2) subsets 2 and 3 contain unselected VDJ rearrangement with many OF bands present, and 3) subsets 4–6 contain selected VDJ repertoires.

Progressive B cell development is associated with selection for productive VDJ rearrangement

Sorting of individual BM subsets and their subsequent cultivation (Fig. 7) showed that subsets 0 and 1 contain a mixture of common precursors and pro-B cells. When cultivated, these subsets can generate some SWC7+/μHC+ cells after 3 d that resemble subset 2 and later also a few SWC7+/μHC+ cells that resemble immature B cells (Fig. 7B). Generation of immature B cells is also evidenced by analysis of CDR3 spectratyping after cultivation that showed the presence of a selected VDJ repertoire (Fig. 7D). Alternatively, a substantial part of sorted subsets 0 and 1 can generate CD14+ and/or MHC-II+ cells of the myeloid lineage (Fig. 7C). Analysis of cell cycle status revealed that a considerable part of subsets 0 and 1 is mitotic when isolated as fresh cells, and these cells do not die during cultivation (Fig. 7E). Results from cultivation of sorted subset 2 resembled findings for subsets 0 and 1 in the generation of immature B cells (Fig. 7F), capability to generate CD14+ and/or MHC-II+ myeloid cells (Fig. 7G), and high proliferative activity when isolated as fresh cells (Fig. 7I). However, a substantial part of these cells dies when they are cultivated (Fig. 7I), and unselected CDR3 spectratyping does not change (Fig. 7H). Cultivation of subset 3 does not lead to any phenotypic (Fig. 7J) or CDR3 spectratyping (Fig. 7L) changes. Cultivated cells from subset 3 also cannot generate myeloid cells (Fig. 7K). When analyzed fresh, all cells are resting, whereas cultivation causes dying of almost all cells (Fig. 7M). Sorted and cultivated subset 4 can lose μHC expressions in 3-d cultures but later it can also generate a sizable amount of immature B cells (Fig. 7N). In agreement, subset 4 already contains a selected VDJ

**Table 1. List of primers used in this study**

<table>
<thead>
<tr>
<th>Amplification (Round)</th>
<th>Sense Primer (5’→3’)</th>
<th>Antisense Primer (5’→3’)</th>
<th>Temperature (°C)</th>
<th>Template</th>
</tr>
</thead>
<tbody>
<tr>
<td>DJ (first)</td>
<td>TGCCCTTGCCCTTACCA</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>56</td>
<td>DNA</td>
</tr>
<tr>
<td>DJ (second)</td>
<td>CGGCCAGACGACGTTCA</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>56</td>
<td>First PCR</td>
</tr>
<tr>
<td>SJC (first)</td>
<td>GCTTTTTGGCCACCGGGTCT</td>
<td>TGAGGCCCCCTCCAAGGA</td>
<td>58</td>
<td>DNA</td>
</tr>
<tr>
<td>SJC (second)</td>
<td>GCACGCGCTCTTGGCCTG</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>58</td>
<td>First PCR</td>
</tr>
<tr>
<td>VDJ (first)</td>
<td>GAGGAGACGACGAGTCA</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>58</td>
<td>DNA</td>
</tr>
<tr>
<td>VDJ (second)</td>
<td>TCACCCTTTCTTCTTCA</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>58</td>
<td>First PCR</td>
</tr>
<tr>
<td>TdT (first)</td>
<td>GCCCTTCTTCTTCTTCA</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>58</td>
<td>First PCR</td>
</tr>
<tr>
<td>TdT (second)</td>
<td>TCTTTCTTCTTCTTCA</td>
<td>TGAGGACGACGACGCTTCA</td>
<td>58</td>
<td>First PCR</td>
</tr>
<tr>
<td>RAG (first)</td>
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<td>GATGCTCTGGACTTCA</td>
<td>55</td>
<td>cDNA</td>
</tr>
<tr>
<td>RAG (second)</td>
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<td>GATGCTCTGGACTTCA</td>
<td>55</td>
<td>cDNA</td>
</tr>
<tr>
<td>VpreB (first)</td>
<td>AAGGAGAGAGAGGTACAT</td>
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<td>VpreB (second)</td>
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<td>cDNA</td>
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<tr>
<td>VDJ (first)</td>
<td>GAGGAGAGAGAGGTACAT</td>
<td>GATGCTCTGGACTTCA</td>
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<tr>
<td>VDJ (second)</td>
<td>CGAGGAGAGAGAGGTACAT</td>
<td>GATGCTCTGGACTTCA</td>
<td>59</td>
<td>cDNA</td>
</tr>
</tbody>
</table>

**FIGURE 4. Detection of rearrangement-specific products and transcripts from sorted BM cells.** According to phenotype studies depicted in Fig. 3, the phenotypes and cell size characteristics of subsets 0–6 were summarized and individual subsets were sorted by flow cytometry. A similar approach was used for sorting of naive B cells from the blood of GF animals (last column). Each sorted subset was thereafter examined for the presence of rearrangement-specific products (DNA) and transcripts (cDNA) by two-round PCR using primer sets noted in Table I. The results are representative of seven independent experiments that included flow cytometry sorting according to expression of CD172a/SWC7, CD172a/μHC, and SWC7/μHC on MHC-II+ BM cells. In a particular experiment, only the same amount of sorted cells was used for isolation of total RNA and DNA. Note that SJC’s are shown only for D-J rearrangement because amplification of SJC for V-J rearrangement gave the same results as for detection of complete VDJ rearrangement.
repertoire and this does not change after cultivation (Fig. 7P). This is the same also for subsets 5 (Fig. 7T) and 6 (Fig. 7X). Similarly to its predecessor subset 3 (Fig. 7K) and its descendant subsets 5 (Fig. 7S) and 6 (Fig. 7W), subset 4 cannot generate any myeloid cells (Fig. 7O). Interestingly, subset 4 is naturally highly proliferative and almost does not die during cultivation (Fig. 7Q). In contrast, all cells from subset 5 (which represent a small cell counterpart to subset 4) are naturally resting and highly sensitive to apoptosis when cultivated (Fig. 7U). Subset 5 also cannot generate significant amounts of immature B cells (Fig. 7R). Sorted immature B cells in subset 6 represent the final stage of B cell development in the BM, and accordingly they do not change their phenotype during culture (Fig. 7V). However, cell cycle studies showed that they are very sensitive to spontaneous apoptosis (Fig. 7Y).

Some peripheral monocytes contain incomplete DJ rearrangement

Cultivation of individual developmental stages revealed that some cells from subsets 0–2 can generate CD14⁺ and/or MHC-II⁺ cells of the myeloid lineage (Fig. 7C, 7G). We therefore wondered whether peripheral myeloid cells may contain DJ and/or VDJ rearrangements. For this reason, peripheral blood was sorted according to expression of SWC8 and CD14 (24) into SWC8⁺CD14⁻ lymphocytes that include B cells (Fig. 8A), SWC8⁻CD14⁺ monocytes (Fig. 8B), and SWC8⁺CD14⁺ polymorphonuclear cells (Fig. 8C). The scatter characteristic of sorted cells was verified by FSC/side light scatter (SSC) analysis (Fig. 8D–F).

Analysis of DJ and VDJ rearrangement amplified from DNA of these sorted cells revealed that although monocytes may contain partial DJ rearrangement, they lack complete VDJ rearrangements (Fig. 8H). Polymorphonuclear cells did not contain either type of rearrangement (Fig. 8I). As anticipated, lymphocytes that include B cells contain DJ as well as VDJ rearrangement (Fig. 8G).

Development of B cells occurs in the BM throughout life

Comparative flow cytometry analysis of the BM cells (Fig. 9A–L) showed that precursor pre–B-I cells (Fig. 9A–H; arrowed subset 2 for large cells and subset 3 for small cells) can be detected in the BM of young GF animals (Fig. 9A–D) but also in adult sows (Fig. 9E–H). The frequency of pre–B-I cells was lower in adults.
(compare arrowed subsets in Fig. 9A–D with corresponding subsets in Fig. 9E–H), but pre-B-I cells can be detected even in 3-y-old pigs. Sorting of subsets 0 and 1 (pro-B cells) from young and adult pigs showed that these cells can carry partial DJ but never complete VDJ rearrangement (Fig. 9M).

**Discussion**

In this study we described the developmental pathway for B cells in swine (Fig. 4). This pathway closely matches what is known from studies in humans (2) and mice (3). However, pigs, together with all other ungulates, were speculated for >30 y to belong to species that use ileal Peyer’s patches for development of B cells (10, 11, 25). We have previously shown that ileal Peyer’s patches are not required for B cell development and maintenance (12–14), and that this role has probably been filled by the BM (26–30). In the present study we have finally shown that B cell lymphogenesis in pigs clearly occurs in the BM. Moreover, we have also shown that B cell lymphogenesis occurs in the BM throughout life. Uncertainty about the lifetime function of the BM was maintained owing to a massive expansion of polymorphonuclear and myeloid cells after birth that mask ongoing B lymphogenesis, and because no markers for precursor cells were available. Now, using sets of
cells was used for each sorted subset. In a particular experiment, only the same amount of sorted experiments. In a particular experiment, only the same amount of sorted

\[ G \]

G (large MHC-II + CD172ahi SWC7

G (large MHC-II + CD172ahi SWC7

\[ M \]

M and CD25 expression occurs almost si-

\[ B \]

B cells (31). In contrast, all mouse B cells are CD2 + (32), and

\[ H \]

HC). The differences in expression of CD2 and CD25 are

\[ I \]

B cell precursors in low surface density until subset 3 (Fig. 3),

\[ J \]

expression. However, CD172a in swine continues to be expressed

\[ K \]

markers typical for B cell such as CD2, CD25, CD45RC, SWC7,

\[ L \]

expression. This is comparable with porcine CD172a–/hi expression.

\[ M \]

CD2172a is expressed mainly on myeloid cells and it has multiple

\[ N \]

CD25 are not always expressed mutually inclusive and why early

\[ O \]

CD25 are not always expressed mutually inclusive and why early

\[ P \]

CD25 are not always expressed mutually inclusive and why early

\[ Q \]

CD25 are not always expressed mutually inclusive and why early

\[ R \]

CD25 are not always expressed mutually inclusive and why early

\[ S \]

CD25 are not always expressed mutually inclusive and why early

\[ T \]

CD25 are not always expressed mutually inclusive and why early

\[ U \]

CD25 are not always expressed mutually inclusive and why early
and/or CD21a (Fig. 3) and contain complete VDJ rearrangement (Fig. 4). This myeloid/lymphocyte developmental dichotomy is impossible for any further subset in B cell development. Such results imply that only large precursor B cells that retain CD172a expression may revert to myeloid lineage. However, our analysis of peripheral myeloid cells showed that they never contain complete VDJ rearrangement although monocytes may contain incomplete DJ rearrangement (Fig. 8). Therefore, only a part of large CD172a− precursor cells that do not finalize VDJ rearrangement may revert to monocytes, which is in agreement with other finding (38). The possibility that some myeloid cells arise from early B cell lineage cells is documented in mice where some cell lines with monocytic characteristics may contain VDJ rearrangement and lymphoid-to-lymphoid but not myeloid-to-lymphoid conversions are possible (39). Importantly, mature peripheral B cells in conventional pigs can express CD172a in low density (M. Sinkora and J. Sinkorova, unpublished observation). However, such expression is inducible and secondary because this work showed that immature B cells from fetal, newborn, and OF piglets do not express CD172a. Re-expression of CD172a may be related to previous receptor editing after Ag stimulation because this work (M. Sinkora and J. Sinkorova, unpublished observation). However, early B cell lineage cells is documented in mice where some cell may revert to monocytes, which is in agreement with other culture conditions.

ducted especially for cultivation studies to prove whether observed changes imply that only large precursor B cells that retain CD172a expression may revert to myeloid lineage. However, our analysis of peripheral myeloid cells showed that they never contain complete VDJ rearrangement although monocytes may contain incomplete DJ rearrangement (Fig. 8). Therefore, only a part of large CD172a− precursor cells that do not finalize VDJ rearrangement may revert to monocytes, which is in agreement with other finding (38). The possibility that some myeloid cells arise from early B cell lineage cells is documented in mice where some cell lines with monocytic characteristics may contain VDJ rearrangement and lymphoid-to-lymphoid but not myeloid-to-lymphoid conversions are possible (39). Importantly, mature peripheral B cells in conventional pigs can express CD172a in low density (M. Sinkora and J. Sinkorova, unpublished observation). However, such expression is inducible and secondary because this work showed that immature B cells from fetal, newborn, and OF piglets do not express CD172a. Re-expression of CD172a may be related to previous receptor editing after Ag stimulation because this work (M. Sinkora and J. Sinkorova, unpublished observation). However, early B cell lineage cells is documented in mice where some cell may revert to monocytes, which is in agreement with other culture conditions.

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Disclosures

The authors have no financial conflicts of interest.

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