Comparative Aspects of Immunoglobulin Gene Rearrangement Arrays in Different Species

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Studies in humans and mice indicate the critical role of the surrogate light chain in the selection of the productive immunoglobulin repertoire during B cell development. However, subsequent studies using mutant mice have also demonstrated that alternative pathways are allowed. Our recent investigation has shown that some species, such as pig, physiologically use preferential rearrangement of authentic light chains, and become independent of surrogate light chains. Here we summarize the findings from swine and compare them with results in other species. In both groups, allelic and isotypic exclusions remain intact, so the different processes do not alter the paradigm of B-cell monospecificity. Both groups also retained some other essential processes, such as segregated and sequential rearrangement of heavy and light chain loci, preferential rearrangement of light chain kappa before lambda, and functional κ-deleting element recombination. On the other hand, the respective order of heavy and light chains rearrangement may vary, and rearrangement of the light chain kappa and lambda on different chromosomes may occur independently. Studies have also confirmed that the surrogate light chain is not required for the selection of the productive repertoire of heavy chains and can be substituted by authentic light chains. These findings are important for understanding evolutional approaches, redundancy and efficiency of B-cell generation, dependencies on other regulatory factors, and strategies for constructing therapeutic antibodies in unrelated species. The results may also be important for explaining interspecies differences in the proportional use of light chains and for the understanding of divergences in rearrangement processes. Therefore, the division into two groups may not be definitive and there may be more groups of intermediate species.

Keywords: B cell development, B cell receptors, cell differentiation, immunoglobulin heavy and light chains, gene rearrangement
INTRODUCTION

Immunoglobulin (Ig) gene rearrangement has evolved in all jawed vertebrates and involves recombination of variable (V), diversity (D), and joining (J) gene segments at their corresponding loci (reviewed in 1). The number of VDJ segments, their organization, orientation and position within the genome, and their frequencies utilized in B cells are known in many species. This is the result of modern genomic sequencing techniques and available and durable single-cell analyzes. Surprisingly, information on the mechanism by which they rearrange in these different species is sparse. In fact, they are based only on findings in mice and to some extent in humans (2), and it is assumed to be the same at least in mammals. The reason for this is understandable, because while genome sequencing is currently a straightforward task, uncovering the mechanism usually requires inbred animals in sufficient numbers and the technology of genetic modification. However, there are some exceptions such as our studies in swine where we characterized the development of B cells during ontogeny (3–6), their development in bone marrow (7), identified different developmental stages of B cells and the order and status of their IgH and IgL rearrangements (8), analyzed redundant rearrangements in the thymus (9), analyzed the order of IgLκ and IgLλ rearrangements during development (10), and showed the consequences of different rearrangement orders in recovered sequences (11) and IgH and IgL rearrangements configurations in individual peripheral B cells (12). These studies on non-transgenic and outbred animals were possible because of the organization of Ig loci and specific immunological properties. Pigs have a highly simplified IgH gene complex in which all VH genes belong to the ancestral VH3 family sharing the same leader and framework sequences, and only one ΨH segment is functional (8, 13, 14). Porcine IgH loci are also restricted to only two VH families and only two functional JH genes for both IgLκ and IgLλ (8, 15–18). Moreover, pigs possess an epithelialchoriocial placenta that prevents the prenatal transfer of maternal Ig (as well as smaller proteins) to the fetus (19, 20). This type of placenta, combined with prolonged gestation and numerous offspring, provides a favorable opportunity to characterize successive developmental steps during fetal life under naive conditions and without influence of extrinsic factors (4). In addition, pigs are precocious and do not require their mothers for survival. Late fetuses can be born aseptically into sterile isolators to easily produce germ-free piglets (18, 21, 22). Such germ-free animals are devoid of effector, memory, and plasma B cells, including long-lived bone marrow plasma cells that could interfere with developmental studies of naïve B cells (5, 8, 12, 23, 24). Here we summarize our findings and compare them with results from other species to show that alternative pathways of V(D)J rearrangement are used.

REVIEW

Mouse Paradigm of V(D)J Rearrangement

A model of B cell development and generation of B cell receptor (BCR) repertoire by V(D)J rearrangement is derived from mouse studies (reviewed in 1, 25). This sophisticated paradigm describes the rearrangement as a tightly sequential process regulated by a surrogate light chain (SLC) composed of λ5 (CD179b) and VpreB (CD179a). For overview of the process see Figure 1, left part. The first wave of the rearrangement occurs in the IgH locus of proB cells by the combinatorial joining of D1H to J1H segments on both chromosomes. The resulting preB-I cells subsequently rearrange a particular VκH segment to one incomplete DJκH rearrangement on the first chromosome. Complete VDJκH rearrangement for IgH is tested in preB-II cells for its productivity by the ability to form preBCR by association with pre-existing SLC (reviewed in 26). If IgH rearrangement is productive and can associate with invariant SLC, the resulting preBCR are anchored into the plasmatic membrane and associated with the signaling components CD79a and CD79b. This membrane complex delivers stop signals for further IgH rearrangement ensuring the selection for productive rearrangement and allelic exclusion (27). If IgH rearrangement is not productive and/or fails to fold correctly with SLC, the cell has one more chance to rearrange VκH to DJκH using the second chromosome. The large preB-II cells die in case of failure but survive, expand, and consecutively become small preB-II cells in case of success. Up to this stage of development, no IgL rearrangement takes place. IgL locus is rearranged only if productive IgH was successfully tested by SLC and the IgH loci are closed for further rearrangement.

Igλ rearrangement thereafter begins in surviving small preB-II cells initially with IgLκ, which continues to rearrange until it is productive and forms an authentic BCR (28). Multiple IgLκ rearrangements (editing) are possible because IgLκ genes, unlike the IgH locus, do not contain a D segment and do not lose recombination signal sequences between unused VL and Jλ segments. Rearrangement in the IgLκ locus is finished when (1) any IgLκ protein can form an authentic BCR with existing IgH and small preB-II cells become immature B cells or (2) all functional Vk and/or Jκ segments on both chromosomes have been used and/or IgLκ loci have been inactivated. Inactivation of IgLκ occurs by excision of Cκ segments from the genome by recombination of any remaining Vk segment or upstream Cκ recombining element (RE) to downstream recombining sequence in mice or κ deleting element in other species (hereafter referred to as KDE recombination) (28, 29; reviewed in 30). The existence of KDE has been demonstrated in all species studied, and the ablation of Cκ segments by Vk–KDE and RE–KDE recombination before any IgLκ rearrangement is thought to ensure the isotypic exclusion (30).

Swine Deviations From Mouse Paradigm

Porcine B cell development begins with Igλ rearrangement in the absence of IgH rearrangement or components of SLC (VpreB and λ5) (8, 12). For overview of the process see Figure 1, right part. Similar to mice, Igλ rearrangement is a beginning, but it occurs only on the first chromosome and the precursors become Igλκ′IgLλ−IgH+. There are no IgH rearrangements in these precursors yet, and if some occur rarely, if they are productive, and if they match productive IgLκ, the precursors become immature B cells expressing BCR. This is only a small fraction of the final Igλκ′ B cell pool as evidenced by cultivation and

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In the absence of IgH, the vast majority of the remaining cells rapidly replace the initial IgLκ rearrangement with successive IgLλ rearrangement. These precursors thus develop from IgLκ+IgLλ+IgH− to IgLκ−IgLλ+IgH− precursors, which continue to rearrange (and consume) further Vλ genes until IgH rearrangement occurs. As indicated by sorting and sequencing studies (11), most of the initial IgLκ genes are inactivated by KDE recombination. Rearrangement of IgH occurs at the next developmental stage and follows the same rules as known from mice: Incomplete DJH rearrangements are primarily formed on both chromosomes followed by complete VDJH on the first chromosome (7, 9). The IgH product is tested for its productivity and ability for surface expression by pairing with pre-existing authentic IgLλ. In case that both IgH and IgLλ genes are rearranged, the IgH-lambda combination is tested for surface expression, and the IgH product is expressed if it passes this test. The IgLλ+ precursors are also tested for their ability to form functional surface antibodies. If both IgLλ+ and IgLλ− precursors are present, the IgLλ− precursors are the ones that are expressed. As indicated by sorting and sequencing studies (11), most of the initial IgLλ+ genes are inactivated by KDE recombination. Rearrangement of IgLλ− occurs at the next developmental stage and follows the same rules as known from mice: Incomplete DJλ rearrangements are primarily formed on both chromosomes followed by complete VDJλ on the first chromosome (7, 9). The IgLλ product is tested for its productivity and ability for surface expression by pairing with pre-existing authentic IgLκ. In case that both IgLλ− and IgLκ are rearranged, the IgLκ−IgLλ combination is tested for surface expression, and the IgLκ product is expressed if it passes this test. If both IgLλ− and IgLκ are rearranged, the IgLκ−IgLλ combination is tested for surface expression, and the IgLκ product is expressed if it passes this test.
rearrangements are productive, the cells become immature IgL⁺ B cells. The probability of productive IgL rearrangement is very high because early rearrangements are direct VJ joins (31–33). The probability of productive IgH rearrangement follows the 1/3 success rule due to shifted reading frames (3, 7, 9). In the case of defective IgH, there is one more chance to rearrange VH to DJH using the second chromosome. If successful, these early immature B cells survive and expand (8). Since initial IgL rearrangement was replaced by successive IgL rearrangement before IgH rearrangement, the early immature B cells are almost exclusively IgL⁺. This is substantially different from mice, in which IgL⁺ B cells are generated earlier (28). The generation of IgL⁺ B cells in porcine bone marrow occurs during the transition to late immature B cells by rearrangement of germline IgL genes on the second chromosome (10). During this process, which gives rise to the majority of immature IgL⁺ B cells, the existing IgL rearrangement is silenced but remains in the IgL⁺ B cells. This is another principal difference from mice because the vast majority of IgL⁺ B cells carry silenced and mostly productive IgL transcripts (10, 11). This peculiarity can be traced even in peripheral mature B cells (12). There may even be additional IgL editing in a small fraction of late immature cells if the second wave of IgL rearrangement is replaced by secondary IgL rearrangement. In this case, the existing secondary IgL rearrangement is again inactivated by KDE recombination to allow secondary IgL rearrangement (10–12).

What Is Essential and What Optional
Studies in pigs have shown that some steps in the sequential process of V(D)J rearrangement are essential, whereas others are optional. The first essential process is that the IgH and IgL loci rearrange at different developmental stages. Thus, the process is sequential, but the respective order may vary (see below). This may be related to a reduction in the number of IgL isotypes during evolution, resulting in only one or two isotypes in birds and mammals. These can be more comfortably controlled than in skates and sharks, which have multiple IgH and IgL loci rearranged simultaneously (34, 35). In any case, once IgH rearrangement begins, it occurs consecutively on the first chromosome and, if not productive, also on the second chromosome, otherwise the developing B cell dies. Our analyses of single cells have never revealed two productive IgH rearrangements (9, 10). Furthermore, the developmental stage at which IgH rearrangements occur is also a crucial checkpoint of differentiation regulated by intrinsic factors of the bone marrow (7) always followed by several rounds of proliferation to increase a cohort of precursor B cells with identical IgH chain (reviewed in 36). Another essential step is also a preferential rearrangement of IgLx before IgLα. This has been demonstrated in many other species (37) and the positions/organizations of κ-enhancers are highly conserved (30). The function of KDE recombination is also critical. Inhibition of IgLx by Cx excision occurs before the switch to IgLα rearrangement.

On the other hand, an optional process includes the independence of IgH and IgL gene rearrangements so that the respective order may vary. While IgH precedes IgL rearrangement in mice, IgL precedes IgH rearrangement in swine. Interestingly, the independence of IgH and IgL rearrangement was predicted based on results with virus-transformed preB cells (38) and demonstrated in IgH deficient mice, which rearrange IgL at normal frequencies and with normal kinetics (39, 40). It has also been demonstrated that a small fraction of developing B cells can rearrange IgL before IgH even in mice under physiological conditions (41). Swine can do this regularly under physiological conditions (8, 10). It should be emphasized that there may also be an intermediate group of animals that rearrange IgH and IgL competitively. An example of this is birds that do it very early in fetal life (42–44). Another optional process is the independence of IgLx and IgLα rearrangements on different chromosomes. Although IgLx rearrange preferentially as explained above, and both IgLx loci are consumed before any IgLα rearrangement in mice, IgLx and IgLα rearrangements on different chromosomes may occur at different developmental stages in pigs. As also demonstrated in swine, IgL editing can occur much later in immature B cells, which is also true for any other species because BCR editing is used in the establishment of central tolerance in late immature B cells (36). The other optional process is the use of SLC to select productive IgH rearrangements. Pigs use an authentic IgL and do not need SLC.

Certainly, there are still some unresolved issues, such as whether incomplete DJH rearrangements on both chromosomes occur in the earliest precursors in all species. In swine, they do as in mice and humans (7, 8). We have also never observed multiple DJH rearrangements in our sequences. However, VH to DJH rearrangement may precede DJH to IJH rearrangement in rabbits (45). In chickens, multiple DJH to DIH rearrangements have been reported before a subsequent rearrangement to the VH gene (46).

It needs to be emphasized that it is not the intention of this report to discuss the molecular mechanisms of the V(D)J rearrangement machinery. It is apparent that essential components are also critical enzymes, such as the recombinase-activating genes (RAG), which are required for DNA cleavage, or the terminal deoxynucleotidyl transferase (TdT), which can facilitate N-nucleotide additions. As indicated by genome sequencing, this is also true for recombination signal sequences (RSS) and regulatory factors required for rearrangement processes. The encyclopedic information on these aspects can be found in other reviews.

Controversial Function of SLC
Originally, SLC was expected to ensure the allelic exclusion. This was disproved by the construction of knockout mice in which different components of SLC were deleted but the exclusions remained intact (47, 48). Only targeted disruption of the membrane exon of IgH genes results in allelic inclusion (27, 49, 50), confirming that anchoring of IgH in the cell membrane is essential. Experiments with transgenic mice have also demonstrated that SLC is not even required for the selection of productive IgH. The deficiency in SLC does not prevent the initiation of IgL rearrangement or the development of mature B cells (47, 48). Although mice lacking SLC have somehow reduced number of peripheral B cells, they have normal serum IgM levels (47) and immune responses (48). All these experiments suggest
that the SLC play only a role in increased efficiency of B cell generation and faster membrane deposition of successfully rearranged IgH in the absence of IgL. The SLC is not necessary if IgL is already present, which happens in all B cells with both IgH and IgL successfully rearranged. Since IgH and IgL rearrangements are independent (38-41), the SLC is not necessary if the order of IgH and IgL rearrangement is reversed.

There are three VpreB in mice (VpreB1, VpreB2, VpreB3) and two in humans (VpreB1 and VpreB3). While VpreB1 and VpreB2 are co-expressed and serve for IgH selection (1), the role of VpreB3 is different and probably interacts with IgH in the endoplasmatic reticulum (26, 51). However, recent studies in several species indicate that the VpreB and λ5 genes may function in other processes than the formation and testing of IgH. In chickens, no homologues of λ5 have been identified and the function of VpreB3 in these animals is the retention of free IgL inside of cells (51). Cows have all three VpreBs but VpreB2 and VpreB3 have biological functions unrelated to B cells development (52). Marsupials have only maintained VpreB3 and do not have VpreB1, VpreB2, or λ5 in the genome (53). Pigs have VpreB1, VpreB3, and λ5 in the genome (10) but these are not used for IgH selection (8, 10) and they are mainly expressed in non-lymphoid cells (8, 10, 33, 54). It is therefore possible that VpreB probes the fitness of other molecules as well, and that its usage in mice for the selection of productive IgH rearrangements is a highly specialized role adopted by only some species.

Expression of IgL on the Cell Surface Without IgH

Initial IgL rearrangements in the absence of IgH can be expressed on the cell surface of early precursors in swine (8, 10). This is a striking observation, as it is generally assumed that IgL cannot anchor to the cell membrane without IgH. However, free IgL are common in human pathogenesis, and these so-called Bence Jones proteins have been known for >170 years (55). This demonstrates that IgL are able to escape from the endoplasmatic reticulum without being chaperoned by IgH. Free IgL in humans are mostly a product of plasma cells in which IgL are produced in excess to IgH (56). This is understandable because humans use IgH before IgL rearrangement and the IgL produced are likely to be in excess only in plasma cells. On the other hand, mice do not produce Bence Jones proteins under normal conditions, indicating a different kind of regulation for IgL synthesis. Early porcine precursors do not have IgH, so IgL is present in excess until IgH rearrangement occurs. Unfortunately, the mechanism by which free IgL attaches to the surface is not fully known. The vast majority of studies investigate the secreted free IgL. However, surface expression of free IgL has been demonstrated in virus-transformed preB cells, which also showed that free IgL do not associate with other proteins (38). More sophisticated studies showed that free IgL associates with the outer membrane via interaction with phospholipids such as sphingomyelin A (57). Importantly, free IgL are only associated with the surface of cells that produce these IgL (57). Our results also exclude the possibility that free IgL on a surface may be acquired incidentally from other sources (8).

The Role of KDE, IgL Isotypic Exclusion and Distribution of IgL Rearrangements in B Cells

Preferential usage of IgL rearrangements on both chromosomes in mice (37) and the mechanism of IgL inhibition by KDE recombination prior to any IgL rearrangements (30) have four important consequences: (1) IgLκ B cells are generated earlier, (2) IgLκ B cells highly predominate over IgLλ B cells, (3) IgLκ B cells have both IgLα loci in the germline, while (4) IgLλ B cells have rearranged IgLκ loci inactivated by Ck ablation (28, 58). This is true and evident in mice, which have hundreds of Vk genes and generate >90% of IgLκ B cells. Indeed, only a few Vk and Jk genes are required for productive IgLκ rearrangement because the 1/3 chance for out-of-frame rearrangement can be overcome by about three successive rearrangements and only on one chromosome. However, the proportional usage of IgLκ and IgLλ genes is not the same in all species, and some use >90% IgLκ (see below and Table 1), which is not easily explained by preferential IgLκ rearrangement and KDE recombination.

In any case, KDE recombination (leading to Ck ablation and inhibition of IgLκ genes before IgLλ rearrangement) appears to be an effective mechanism to achieve isotypic exclusion. Such an exclusion function would be of particular interest given that KDE recombination is highly conserved in many (if not all) species (30). On the other hand, studies in humans showed the co-expression of multiple IgLκ and IgLλ rearrangements in different combinations in single B cells (65). Studies in mice showed that IgLκ production does not inhibit secondary rearrangements and multiple productive IgLκ can be detected in single cells (66). Studies in pigs extended these findings from the perspective of species which use IgLκ before IgH rearrangement and confirmed that multiple and even productive IgLκ rearrangements are present in IgLκ B cells (8, 10-12). Moreover, multiple IgLκ rearrangements can be effectively transcribed in a single cell (12, 65). Also, not all IgLκ B cells undergo Ck deletion by KDE recombination on the first chromosome before rearranging on the second (10, 66). The coexistence of multiple productive IgLκ rearrangements in a single cell is highlighted in fish (67), where KDE cannot control up to four different IgLκ types encoded by distinct Ci genes (18, 34). Therefore, the function of KDE in isotypic exclusion is implausible. More probably, KDE recombination supports efficient switching from IgLκ to IgLλ rearrangement, but has no control over which IgLκ allele is rearranged, whether it is productive, transcribed, and expressed. Our sorting data suggest that unused IgLκ rearrangements are silenced in translation or in export of IgLκ protein because they have the corresponding mRNA but are not expressed on the surface (10). Silencing of the IgH locus is partially known (68) and is ensured by nonsense-mediated decay (NMD) (69). Whether a similar mechanism operates for IgLκ is unknown, namely because different IgLκ are located on different chromosomes and can be silenced even when they are productive. Some reports indicate that silencing operates after
translation on a “best-fit, best-serve” basis when transcription and translation of unused IgL is at a very low level. This would be consistent with findings in mice (66).

It must be emphasized that the final KDE recombination depends on the available number of Jκ rather than Vk genes, since the number of possible successive rearrangements is limited by the smaller number. If a given species has only one functional Jκ, the further editing is only KDE-mediated Cα ablation. This is not at all uncommon as Table 1 shows. Goats, sheep, and cattle have only one functional Jκ among several other nonfunctional ones (59). Furthermore, the remaining Jκ in cattle and sheep has a noncanonical RSS (59) that may favor immediate KDE-mediated ablation because initial functional rearrangement is inefficient or impossible. This could lead to the elimination of almost all IgLκ rearrangements and be only the final step before the evolutionary loss of all IgLκ usage, as occurred in bats (60, 61).

Can Authentic IgL Be Used for Selection of Productive IgH Repertoire?
SLC is invariant and therefore always “productive”. It can theoretically bind to any productive IgH rearrangement to ensure its selection. This appears as a huge advantage over authentic IgL because the initial IgL rearrangements in precursors may be out of frame or nonproductive for other reasons, such as internal stop codons. However, investigation in mice has shown that as many as 50–70% of productive IgH fail to pair with SLC and developing cells become apoptotic (48; reviewed in 1). On the other hand, experiments in pigs have shown that the initial IgL rearrangements used for selection of subsequent IgH rearrangements are >88% in-frame, have no mutations, and no N-additions (11). Such success rate for authentic IgL is considerably higher than has been reported experimentally for SLC. Authentic IgL use also different Vk and Jκ genes and could allow the generation of B cells whose IgH would not be capable of pairing with always the same but always imperfect SLC (48). Therefore, the IgH repertoire selected by authentic IgL should not be biased by the existence of one type of non-polymorphic peptide chains like SLC, but selection is driven by a germline authentic IgL. In fact, each type of IgL with its specific sequence could serve as a different type of SLC, so that rearrangement is highly inefficient or impossible.

Efficiency of B Cell Generation and Evolution of Ig Rearrangement
Based on current knowledge, it is difficult to estimate whether the use of authentic IgL is less redundant and more efficient than SLC-dependent selection (see section above). The established order of IgH before IgL gene rearrangement seems to be the most ancestral because this strategy is used in amphibians (70). However, amphibians and fish do not use SLC and the number of B cells per gram of body mass is > 10-fold lower than in birds or
mammals (70, 71). A similar effect is seen in marsupials, which also keep IgH before IgL rearrangements and lack SLC (53). These mammals have minimal levels of serum antibodies even several months after birth (72), which is comparable to the kinetics of mice with genetically deficient SLC (48). Thus, one group of successor animals could increase the efficiency of B cell generation by employing the existing components of SLC. Others might reverse the order of IgH and IgL rearrangement and omit the SLC requirement or employ yet other mechanisms such as the gene conversion in chickens (44). The reason why some species do not retain the ancestral IgH before IgL rearrangement may be due to their limited IgH repertoire (62). Chickens have only a single functional V<sub>H</sub> and J<sub>H</sub> segment (44) and pigs are restricted to ten closely related V<sub>H</sub> and one J<sub>H</sub> segment (reviewed in 18, 22). Although it has been demonstrated that CDR3 junctional diversity can compensate for the limited combinatorial repertoire (13, 18), limited or demonstrinated that CDR3 junctional diversity can compensate.

In vivo phenomenon was previously observed in the liver prior to functional bone marrow (73) and pigs are rare and had only a higher number of V<sub>I</sub> segments in the genome (61). The explanation for the different efficiency of B cell generation can be the critical checkpoint at a developmental stage where IgH rearrangements occur. This checkpoint has been characterized in mice (73; reviewed in 36), humans (37), and also in swine (7). In mice and humans, the checkpoint is overcome by expression of functional preBCR (74). The same checkpoint occurs in porcine IgL<sup>+</sup> precursors during IgH rearrangement and is overcome by expression of authentic BCR (7, 8). In all species, the checkpoint is regulated by intrinsic factors of the bone marrow (or appropriate stromal cells), and it is followed by several rounds of proliferation to increase a cohort of precursor B cells with identical IgH chains.

However, the generation of B cells without the bone marrow is still possible but very inefficient (3, 74), as in the case of the variants described in the above paragraph.

Two important observations were made in experiments with porcine bone marrow stromal cells: the first confirms that B cells developing in the absence of stromal cells contain the IgH rearrangement on only one chromosome, while stromal cells support the rearrangement on both chromosomes (7). This phenomenon was previously observed in vivo during early ontogeny, when B cells developing in the yolk sac and fetal liver prior to a functional bone marrow were rare and had only a single productive IgH rearrangement (3). Such an observation cannot be made in mice because maturation of B cells in the fetal liver of mice coincides with maturation in the bone marrow, while in fetal pigs there is a 25-day window in between (4). These results collectively indicate that the opening of the second chromosome for rearrangement does not occur in the absence of the bone marrow. The second observation confirms that the absence of stromal cells leads to the accumulation of IgL<sup>+</sup> IgH<sup>+</sup> precursors and the preferential generation of IgL<sup>+</sup> B cells (8, 10). This is also exactly what happens in vivo during early ontogeny, when IgL<sup>+</sup> transcripts are about 20-times more frequent than IgL<sup>+</sup> (54, 75). The apparent absence of IgL<sup>+</sup> transcripts in the yolk sac and fetal liver led us formerly to the incorrect conclusion that IgL<sup>+</sup> might precede the rearrangement of the IgL<sub>K</sub> genes in pigs (54). Differences in the ability of the bone marrow to support B cell development throughout the checkpoint or its timing can therefore explain interspecies differences in the ration of IgL<sub>K</sub>/IgL<sub>L</sub> usage (see below).

### IgL<sub>K</sub> to IgL<sub>L</sub> Ratio in Different Species

As mentioned earlier, >95% of mouse B cells are IgL<sub>K</sub><sup>+</sup>. However, humans and pigs have about the same amount of IgL<sub>K</sub><sup>+</sup> and IgL<sub>L</sub><sup>+</sup>, while species like cows, sheep, horses, dogs, and cats have >90% of IgL<sub>L</sub><sup>+</sup> B cells (63). The enormous interspecies differences are often explained by the disproportionate number of V<sub>K</sub> and V<sub>L</sub> genes in a genome, which in some cases corresponds to the expressed IgL<sub>K</sub>/IgL<sub>L</sub> ratio (25, 64) but in others does not (61); see Table 1.

However, the preferential IgL<sub>K</sub> rearrangements on both chromosomes and the mechanism of KDE recombination do not allow the generation of substantial numbers of IgL<sub>L</sub><sup>+</sup> B cells (see above), especially in species that have relatively high numbers of functional V<sub>K</sub> genes such as horse (19), pig (10), sheep (8), cat (12), or dog (19) (Table 1; www.imgt.org).

According to the results from swine, the difference in the use of IgL<sub>K</sub> compared to IgL<sub>L</sub> is more likely explained by the sequence of IgL rearrangements on different chromosomes and/or the permissiveness of the microenvironment to support efficient B cell development. Although IgL<sub>K</sub> rearrangement begins with IgL<sub>K</sub> and progresses to IgL<sub>L</sub> probably in all mammals (28, 29), the outcome may be the result of two different processes (1): secondary IgL<sub>K</sub> is not consumed before IgL<sub>L</sub> rearrangement and/or (2) secondary IgL<sub>K</sub> rearrangement is not permitted in given developmental step. In species that use IgL<sub>K</sub> before IgH rearrangement such as swine, these possibilities lead to early genesis of IgL<sub>L</sub><sup>+</sup> B cells, while most IgL<sub>K</sub><sup>+</sup> B cells are generated later and require permissive bone marrow stromal cells (10). Therefore, the dozen-fold prevalence of early IgL<sub>L</sub><sup>+</sup> B cells is compensated to approximately 1:1 ratio of IgL<sub>K</sub><sup>+</sup>:IgL<sub>L</sub><sup>+</sup> B cells (25, 64) but in sheep (8) cat (12) or dog (19) (see above), especially in species that have relatively high numbers of functional V<sub>K</sub> genes such as horse (19), pig (10), sheep (8), cat (12) or dog (19) (Table 1; www.imgt.org).

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Additional evidence that the timing and sequence of each rearrangement control the proportional utilization of IgL genes comes from the proportional usage of the different V\(_{\text{L}}\) and J\(_{\text{L}}\) segments in different species. The majority of mouse IgL\(^{+}\) B cells preferentially use 3’ V\(_{\text{K}}\) and 5’ J\(_{\text{K}}\) genes, while more 5’ V\(_{\text{K}}\) and 3’ J\(_{\text{K}}\) segments are used in IgL\(^{\lambda}\) B cells (77, 78) since a pre-existing V\(_{\text{L}}\) can only be edited by a rearrangement that uses more 5’ V\(_{\text{L}}\) along with more 3’ J\(_{\text{L}}\) genes. However, when SLC is inactivated in transgenic mice, they use more 5’ V\(_{\text{K}}\) genes (78) and resemble more swine, which also do not use SLC. The situation in mice is also different from humans, in which both naive and experienced B cells preferentially use more 3’ V\(_{\text{K}}\) genes (79). However, more 5’ V\(_{\lambda}\) genes are used than even in the naive human repertoire (80), likely reflecting a more independent IgL\(^{\lambda}\) rearrangement (28, 73). In any case, the situation is quite different in the pigs, which have been repeatedly reported to use more 5’ V\(_{\text{L}}\) genes in both IgL\(^{\lambda}\) (16) and IgL\(^{\alpha}\) (15, 32, 33, 81). This discrepancy is mainly caused by the early use of IgL\(^{\lambda}\) rearrangement in precursor cells, which is replaced by IgL\(^{\alpha}\) rearrangement before IgH rearrangement (11). The depletion and inhibition of porcine C\(_{\text{K}}\) genes on the first chromosome during this early development has another fundamental limitation: only IgL\(^{\lambda}\) genes on the second chromosome can be used to generate IgL\(^{\alpha}\) B cells; and pigs have only two functional J\(_{\text{K}}\) segments on one allele (18). Such restriction to only two consecutive IgL\(^{\alpha}\) rearrangements is probably the reason why only two major V\(_{\text{K}}\) genes are preferentially used in swine (11, 32) and why the V\(_{\text{K}}\) repertoire is severely restricted compared to V\(_{\lambda}\) (32, 33). Higher flexibility and diversity of IgL\(^{\lambda}\) than of IgL\(^{\alpha}\) has also been found in other \(\lambda\)-high species (61). All these findings indicate that the disproportionate number of V\(_{\text{K}}\) and V\(_{\lambda}\) genes in different species is not the cause of differential V\(_{\text{K}}\) and V\(_{\lambda}\) usage, but the effect of different rearrangement order and/or developmental dynamics.

Another important factor is the limited number of V\(_{\text{L}}\) and/or J\(_{\text{L}}\) gene segments, which approaches zero. If species can rely on one type of IgL, the second may gradually goes unused or be eliminated. This is the case in birds, which have been able to do so probably because they use gene conversion (42–44), but also in bats, which have expanded the V\(_{\lambda}\) genes arrayed upstream of J\(_{\text{L}}\)–C\(_{\text{L}}\) cassettes (58) instead of multiple V\(_{\lambda}\) genes arrayed upstream of J\(_{\text{L}}\)–C\(_{\text{L}}\) as known from other species (21). In any case, ungulates are also interesting as described earlier. In general, these species have sufficient numbers of putative J\(_{\text{K}}\) and J\(_{\text{A}}\) segments, but many of them are mutated and not useful for functional rearrangements. As a result, sheep and cattle have almost no functional J\(_{\text{K}}\) genes. They are either mutated in the W(F)GxG motif and therefore nonfunctional or have noncanonical RSS, making rearrangement inefficient or impossible (59). In comparison, goats have one J\(_{\text{K}}\) segment that is still fully functional, and this may be a reason why they have more IgL\(^{\lambda}\) B cells than sheep and cattle (59). In this respect, sheep and cattle, followed by goats, might just be other species that follow the bats in the complete loss of IgL\(^{\alpha}\) B cells. It is surprising that many thriving species are able to keep the number of functional V(D)J segments to a minimum. This is especially true for IgH, which is critical for BCR formation. For example, pigs have all five J\(_{\text{H}}\) segments “functional” in terms of the WGxG motif and the absence of stop codons (14). However, three of the five have noncanonical RSS (14), and functional experiments have shown that only one of the remaining can be used for functional rearrangement (82). A similar situation appears to apply to goats (59).

**Conclusion**

In summary, different species appear to have evolved different strategies for the order of rearrangement of Ig genes and for the selection of a productive Ig repertoire. Possibilities for these strategies have even been indicated in mice themselves, which showed that the order of rearrangement is independent (27, 40, 41) and SLC is unnecessary (47, 48). Apparently, all these species have survived with comparable success. On the other hand, differential regulation of rearrangement order and mechanisms of repertoire selection may have evolutionary and practical consequences. In the IgL-before-IgH group, extensive editing of the IgL\(^{\lambda}\) repertoire occurs very early and before IgH rearrangement. On the other hand, IgL\(^{\lambda}\) repertoire is edited in the IgH-before-IgL group only when IgL\(^{\lambda}\) is unsuccessful. These principles could lead to higher diversification of IgL\(^{\lambda}\) loci in the IgL-before-IgH group, while higher diversification of IgL\(^{\alpha}\) loci in the IgH-before-IgL group. Another consequence of the enormous differences between species is the possibility of choosing the uncomplicated experimental systems for practical purposes. All porcine V\(_{\text{H}}\) genes share the same leader and framework sequences and only one J\(_{\text{H}}\) segment is functional. Furthermore, both porcine IgL\(^{\lambda}\) and IgL\(^{\alpha}\) loci contain only two families and two functional J\(_{\text{L}}\) genes. This allows the recovery of all VDJ\(_{\text{H}}\) rearrangements using a single non-degenerate primer set or the generation of deficient pigs for B cells by modifying just a single J\(_{\text{H}}\) segment (82). However, it must always be considered if the regulatory components of Ig rearrangement also need to be copied into the genome. The production of B-cell-deficient pigs may be a simple task, but it may be difficult to generate genetically modified pigs that produce a sufficient amount of human antibodies.

**AUTHOR CONTRIBUTIONS**

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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