Bovine B cells: B lymphopoiesis and the generation of primary immunoglobulin repertoire in fetal cattle

ANNA EKMAN

Department of Veterinary Biosciences
Faculty of Veterinary Medicine
University of Helsinki
Finland

and

Viikki Doctoral Programme in Molecular Biosciences
University of Helsinki

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Supervisors: Prof. Antti Iivanainen, DVM, PhD
University of Helsinki, Finland

Tiina Pessa-Morikawa, PhD
University of Helsinki, Finland

Petteri Arstila, MD, PhD
University of Helsinki, Finland

Reviewers: Prof. Yaofeng Zhao, PhD
China agricultural University, Beijing, China

Prof. Olli Lassila, MD, PhD
University of Turku, Finland

Opponent: Prof. Inga-Lill Mårtensson-Bopp, PhD
University of Gothenburg, Sweden

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Front cover: Images in clockwise order from upper left corner: 1. Heart-shaped group of MGG-stained bovine bone marrow stromal cells, 2. *RAG1* expression on bovine fetal thymus visualized by *in situ* hybridization, 3. OP9-cells growing in a cell culture bottle (picture Eeva Anundi), 4. The animal of interest in this study by Ansa Ekman.

Happy is he who gets to know the reasons for things.
Virgil (70-19 BCE), Roman poet

ABSTRACT

Immunological research is dominated by studies on man and mouse, however, only some aspects of this field are universal among vertebrates. Whilst the production of T cells is universal, B cell production cannot be extrapolated from one species to another. As such, ruminant B cell biology has distinctive features not similar to the conventional man or mouse based models.

Cattle are large long-lived ruminants, of major significance globally. The bovine ileal Peyer's patch, an organ of B cell follicles along the gut, is where V(D)J immunoglobulin gene rearranged B cells proliferate, however it is not known where and how the DNA rearrangements take place before the B cells enter these follicles. Furthermore, it is unclear whether post-recombinatorial modifications, such as somatic hypermutation or gene conversion, facilitate the generation of more antibody specificities of the immunoglobulin genes in these follicles.

Herein, the bovine immunoglobulin light chain genomic locus was characterized and only a moderate number of functional gene segments that cause low combinatorial antibody diversity were found. The lambda locus is the larger of the two light chain loci, containing 25 functional variable gene segments, compared to the kappa locus, which contains only eight. Functional genes comprise less than half of all the variable genes in both loci, the remainder representing unfunctional pseudogenes.

The immunoglobulin genes of the fetal ruminant ileal Peyer's patch can possibly be further modified. Accordingly, the expression of activation-induced cytidine deaminase (AID), a mutator protein, was demonstrated here in fetal cattle ileal Peyer's patch. Sequencing of expressed heavy chain variable genes in these follicles showed ongoing hypermutation. The mutations were concentrated on the complementarity determining regions (CDR) of the variable genes, and on the hotspot target sequences of AID. AID-dependent mutations have usually been ascribed to antigen dependent affinity maturation, but this work demonstrates mutations in fetal immunoglobulin genes before exposure to external antigen.

Bovine B lymphopoiesis is studied here, its localization in the fetal cattle in particular. By analyzing the expression of *RAG1* and *RAG2*, which take part in the rearrangement of immunoglobulin genes, active B lymphopoiesis was demonstrated in fetal bone marrow and lymph node. The expression of surrogate light chain genes *VPREB1* and *IGLL1* was also shown in these same tissues. The expression of these genes implicated that a pre-B cell stage exists in cattle. This was further confirmed by the presence of a phenotypic pre-B cell population in fetal bone marrow and lymph node. *VPREB2* and *VPREB3* were expressed differently from the other surrogate light chain genes, which indicate that their function in cattle might not be related to pre-B cells.

Overall, B lymphopoiesis was shown to take place in fetal, but not in adult, bovine tissues. Pre-B cell related genes *RAG*s, *VPREB1* and *IGLL1* were not expressed in adult tissues. Further, adult bone marrow cells were not able to differentiate into B lineage cells in cell culture. These results suggest that no new immunoglobulin rearrangements are generated during bovine adult life. Thus it is likely that the animal manages its whole life with the peripheral B cell pool produced during the fetal and neonatal period.

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Helsinki, April 2012

CONTENTS

1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by their Roman numerals:

I Ekman, A., Niku, M., Liljavirta, J., Iivanainen, A. (2009). Bos taurus genome sequence reveals the assortment of immunoglobulin and surrogate light chain genes in domestic cattle. BMC Immunol 10, 22.

II Ekman, A., Pessa-Morikawa, T., Liljavirta, J., Niku, M., Iivanainen, A. (2010). B-cell development in bovine fetuses proceeds via a pre-B like cell in bone marrow and lymph nodes. Dev. Comp. Immunol 34, 896-903.

III Ekman, A., Ilves, M., Iivanainen, A. (2012). B lymphopoiesis is characterized by pre-B cell marker gene expression in fetal cattle and declines in adults. Dev. Comp. Immunol. *In press*.

IV Liljavirta, J., Ekman, A., Knight J.S., Pernthaner, A., Iivanainen, A., Niku, M. Activation-induced cytidine deaminase (AID) is strongly expressed in ileal Peyer's patches of bovine fetuses and drives the expansion of the primary antibody repertoire in the absence of exogenous antigens. *Under revision in Mucosal Immunology.*

2. ABBREVIATIONS

AID	activation-induced cytidine deaminase
ATCC	American Type Culture Collection
BCR	B cell receptor
BLAST	basic local alignment search tool
bp	base pair
BSA	bovine serum albumin
C	constant (immunoglobulin gene segment) OR cytosine or cytidine
CD	cluster of differentiation
cDNA	complementary DNA
CDR	complementary determining region
CSR	class-switch recombination
D	diversity (immunoglobulin gene segment)
DNA-PK_{CS}	DNA-dependent protein kinase, catalytic subunit
EBF	early B cell factor
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
Fab	antibody fragment containing the antibody-binding regions
FBS	fetal bovine serum
FC	flow cytometry
FR	framework
GALT	gut-associated lymphoid tissue(s)
GC	gene conversion
HC	heavy chain (of an antibody)
HSC	hematopoietic stem cell
Ig	immunoglobulin (antibody)
ic	intracytoplasmic
IGH	immunoglobulin heavy chain
IGK	immunoglobulin kappa light chain (κ)
IGL	immunoglobulin lambda light chain (λ)
IGLL1	Immunoglobulin lambda-like polypeptide 1
IH	immunohistochemistry
IPP	ileal Peyer's patch
J	joining (immunoglobulin gene segment)
JPPs	jejunal Peyer's patches
kb	kilobase
LC	light chain (of an antibody)
Mb	megabase
µHC	heavy chain of IgM isotype, the product of IGHCµ gene
mIgM	membrane IgM
MPP	multipotent progenitor
NHEJ	non-homologous end-joining
nt	nucleotide
ORF	open reading frame
PBS	phophate-buffered saline (a buffer solution)
PCR	polymerase chain reaction
RAG	recombination activating gene
RT-PCR	reverse transcription PCR
RT-qPCR	reverse-transcription quantitative real-time PCR
RSS	recombination signal sequence
SHM	somatic hypermutation
SJ	signal joint
TdT	terminal deoxynucleotidyl transferase or DNA nucleotidylexotransferase
V	variable (immunoglobulin gene segment)
VPREB	pre-B lymphocyte gene

3. GLOSSARY

AID	Activation-induced cytidine deaminase. An enzyme that deaminate cytidine nucletides in single-stranded DNA. This reaction is essential for initiation of somatic hypermutation, class-switch recombination and gene conversion (see below).
B cell lymphopoiesis	The generation of new B lymphocytes from precursor cells involving rearrangement of immunoglobulin genes.
B cell ontogeny	Development of B cell from the earliest precursor to a mature (but immunologically naïve) cell. Generally ontogeny refers to a development of an organism (Greek *ontos* "being" + *-geneia* "origin").
B cell proliferation	Generation of B cells by cell division, which results in a homogenous population of single developmental stage.
B cell production	A term which can refer to the generation of B cells with lymphopoiesis, B cell proliferation of both.
gene subgroup	A set of V, D, J or C gene segments which share at least 75% identity at the nucleotide level. Sometimes termed "gene family", in which case the nucleotide level identity is at least 80%.
IGLL1	Immunoglobulin lambda like polypeptide-1. *IGLL1* refers to bovine or human gene, the name *lambda5*, *λ5* or *IgII1* is used for the mouse gene. Shows high homology to *IGLC*, but does not recombine in V(D)J recombination. Forms the surrogate LC with VPREB.
NHEJ pathway	Ubiquitous cellular pathway that repairs double-strand breaks in DNA. According to DNA-editing model of AID action, also takes part in AID-dependent DNA-modifying events.
SHM	Somatic hypermutation. A process by which rearranged Ig gene can be further modified by point mutations especially in the CDR regions. Common in secondary lymphoid organs during the so called affinity maturation after antigen challenge, which results in production of antibodies with higher affinities against the given antigen. In some species it might be used also to increase the primary repertoire.
VPREB	The pre-B lymphocyte gene. *VPREB* refers to the bovine or human gene, *VpreB* refers to a mouse gene. Shows high homology to *IGLV*, but is not rearranged in V(D)J recombination. Three different forms are known: *VPREB1* (identified in bovine, human and mouse), *VPREB2* (identified in bovine and mouse) and *VPREB3* (identified in bovine, human, mouse and chicken). Mouse VpreB1 and VpreB2 are known to associate with λ5 to form surrogate LC.

Bovine gene symbols are based on Human Genome Organization's (HUGO) nomenclature guidelines at http://www.genenames.org/guidelines.html. Mouse gene symbols are based on Mouse Genome Informatics nomenclature guidelines at http://www.informatics.jax.org/mgihome/nomen/gene.shtml.

4. INTRODUCTION: Vertebrate immune system protects the host

Cows, and other vertebrates, live in a world full of different micro-organisms, such as bacteria, viruses, fungi and parasites. Some of these are called pathogenic micro-organisms, or *pathogens*, as they can invade the body and cause disease. Pathogens see vertebrates' bodies as sanctuaries where they can reproduce. *Immunology* is the field of research which describes how we defend ourselves against the pathogens, and try to get rid of them (Latin *immunitas*, freedom from).

The body's *immune system* takes care of many important defense functions including pathogen recognition and elimination. It is divided into two different branches, the innate and adaptive immune systems. The *Innate immune system* recognizes and responds to pathogens in a generic way. It is the first of the two to be activated after infection and also the older of the two with respect to evolution. *The adaptive immune system* is responsible for more specific recognition and more delayed responses, and as its name indicates, requires adaptation to the specific infection.

The immune system is composed of white blood cells, which are also called *leukocytes*. They arise from hematopoietic stem cells, as do all blood cells. For innate immunity functions, cells that can engulf and digest pathogens, so called *phagocytic cells*, are of major importance. These include granulocytes, macrophages, and dendritic cells. The *granulocytes,* which constitute 50-70% of all leukocytes in the blood, migrate through blood vessel walls to the site of infection. Monocytes also circulate in the blood, but upon migration into tissues, they differentiate into specialized professional phagocytes, the *macrophages*. The third type of phagocytic cell is the *dendritic cells*, which reside in the tissues. After the phagocytic event, the dendritic cells' function continues, as they display the digested, pathogen-derived, molecules to yet another class of leukocytes, the *lymphocytes*, which are the cellular mediators of the adaptive immune response.

The innate immunity actors described above recognize general features which are common to many pathogens, facilitating an immediate response that is measurable in hours. The subsequent slower, and more specific, response is mediated by lymphocytes. The lymphocytes make up 20-30% of circulating leukocytes in the blood and are composed of *B* and *T lymphocytes*, whose role, although contributing to the same defensive goal, is quite different. T lymphocytes are the cells to which dendritic cells present the pathogens that they have encountered, thus they are activated in a specific manner. Afterward, activated T cells specifically kill the cells which are infected by that same pathogen or, alternatively, trigger pathogen specific antibody production. The cell which produces the antibody is the plasma cell derived from the B cell. B cell is the cell of interest in this study.

Antibody production is one of the hallmarks of an adaptive immune response to a complex extracellular antigen. Activated B cells can produce specific antibodies against an *antigen*, which is, in the course of normal immune response, a recognized, non-self, and pathogen associated molecule. Secreted antibody molecules attach to the surface of the pathogen and tag it for destruction by other immune cells, or prevent its function by other means. This response is not only specific, but it also provides protection against re-infection. Because of this *immunological memory*, the first time a pathogen is encountered, the activation of B cells takes several days, but the response is quite rapid upon re-encounter.

How are the B cells generated in vertebrates' bodies? And how do they produce different antibodies against the pathogens? These questions are explored in this thesis.

5. REVIEW OF THE LITERATURE

5.1. B cell is the antibody producing unit

The vertebrate body has specific *lymphoid organs* that consist mainly of lymphocytes. The thymus and bone marrow are considered to be primary lymphoid organs, as they support the maturation of lymphocytes from immature progenitor cells. The lymph nodes, spleen, and gut-associated lymphoid tissue (*e.g.* ileal Peyer's patches in the wall of small intestine), are examples of secondary lymphoid tissue, where lymphocytes are stored and immunological reactions are started. The "T" in T cells stands for the thymus as T cells are generated there (Miller, 1961; Miller and Mitchell, 1967). The "B" in B cells stands for bursa of Fabricius, a gut-associated lymphoid organ in chicken, as B lymphocytes were first defined in birds (Glick et al., 1956; Cooper et al., 1966). This is the first of many indications that T cell development from progenitor cells, and the production of T cell mass, is uniform throughout vertebrates, but B cell development and production is diverse.

Newly generated *immature B cells* leave the bone marrow and enter the bloodstream having not yet encountered an antigen. B cells migrate between the blood and the secondary lymphoid organs (Nieuwenhuis and Ford, 1976), scanning the bypassing antigens with their cell surface antibodies, called *B cell receptors* (BCR). If a B cell specifically recognizes an antigen, a T cell helps it to become activated (Liu et al., 1991). An activated B cell proliferates vigorously in germinal center situated in the secondary lymphoid tissue, and differentiates into a *plasma cell*. A plasma cell is a small protein factory, which can produce 1500 specific antibody molecules per second (Nossal and Makela, 1962).

An antibody is an immunoglobulin (Ig) protein, which is typically a Y-shaped molecule consisting of two heavy chains (HCs), and two light chains (LCs), linked by disulfide bonds (Fig. 1). The structure of the antibody molecule has been solved to an atomic level (Navia et al., 1979; Harris et al., 1992). The two identical antigen recognition and binding sites in the molecule are formed by both the HC and the LC. These areas of the antibody molecule are called the *variable regions* (Titani et al., 1965).The hypervariable tips of these regions allow billions of slightly different immunoglobulins to exist. As these areas fit to an antigen's shape, they are also called complementary determining regions (CDRs). The other parts of the molecule, called *constant regions*, preserve the structure of the molecule and are not subject to much variation.

5.1.1. Immunoglobulin gene locus is complicated to maximize the antibody repertoire

A human's antibody repertoire is estimated at between 10^7 and 10^9 (Hendershot and Sitia, 2004) or even 10^{11} (Murphy et al., 2007) antibody molecules with different specificities. However, it is estimated that there are only 24500 protein-coding genes in the human genome. If the antibody repertoire would be encoded as such, it would need over three times the size of our genome (Hendershot and Sitia, 2004). Given that the immunoglobulin locus in human is only 2800 – 4120 kB (Lefranc et al., 2009), and the human genome comprises of 3 billion nucleotide base pairs, only about 0.1% of the genome is used to produce the Ig variability. This is possible because of two main mechanisms: 1) Antibody molecules are coded by short gene segments, which rearrange together to build up the complete Ig gene in a process of somatic recombination (Brack et al., 1978), and 2) After the recombination event, these genes can be further modified by mutation in the process of somatic hypermutation (Weigert et al., 1970). The theory of somatic

hypermutation will be discussed more closely when addressing the post-recombinatorial mechanisms of diversity generation.

Ig gene locus rearrangement in B cells was revealed by restriction fragment analysis: the constant region and variable region were separate fragments in non-lymphoid cells, but in the same fragment in the B cells (Hozumi and Tonegawa, 1976). Now we know that a complete LC gene is comprised of variable (V), joining (J) and constant (C) gene segments, and a complete HC gene additionally of the diversity (D) gene segment.

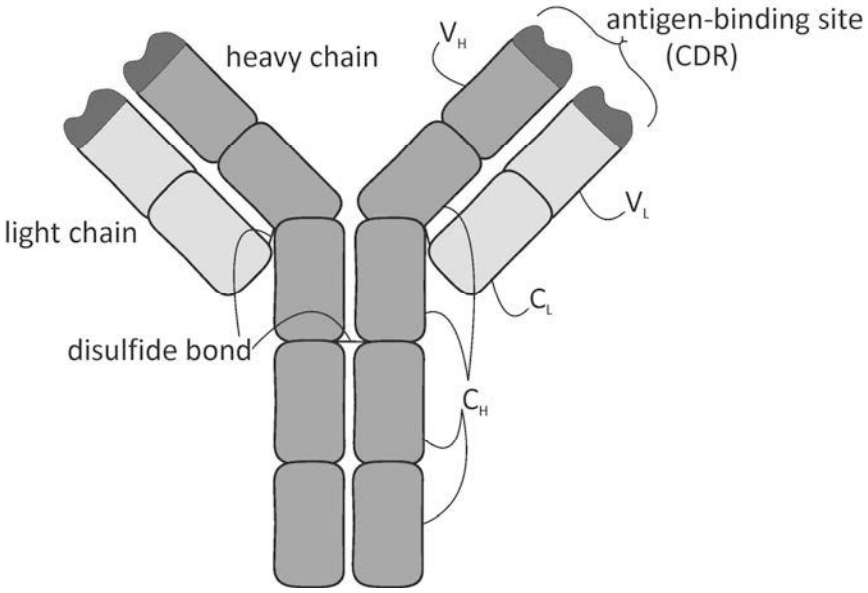

Figure 1. The structure of an antibody molecule. V_H = heavy chain variable region, C_H = heavy chain constant region, V_L = light chain variable region, C_L = light chain constant region, CDR = complementary determining region

Organization of human and mouse immunoglobulin heavy chain locus

The human IGH locus consists of multiple V, D, J and C gene segments. It is situated in chromosome 14, spans 1.3 Mb and comprises 123 *IGHV*, 26 *IGHD*, six *IGHJ*, and 11 *IGHC* genes (Matsuda, 2004). The *IGHV* genes are located successively in the same transcriptional orientation at the 5'end of the locus. Whilst 39 *IGHV*s are functional, 79 are non-functional pseudogenes, and they belong to seven subgroups. Subgroups are defined by the international ImMunoGeneTics information system (IMGT) as a set of V, D, J or C gene segments which share at least 75% identity at the nucleotide level (Giudicelli and Lefranc, 1999; Lefranc et al., 2009). Downstream of the *IGHV* cluster, there are clusters of *IGHD*, *IGHJ* and *IGHC* genes, the transcriptional orientation of all the genes in the IGH locus being similar (Fig. 2). When analyzed further, it has been shown that there have been nine DNA duplication events at the human *IGHV* locus, and they have taken place between 132 and 10 million years ago (Matsuda et al., 1998). Two of the events occurred before the divergence of human and mouse, which was 75 million years ago, and seven duplications after that, which demonstrates a high frequency of recent reorganization in the locus.

The corresponding IGH locus on chromosome 12 of the laboratory mouse (derived from *Mus musculus domesticus*) is two to three times bigger than in human (Riblet, 2004). There are 170 *IGHV*, 13 *IGHD*, four *IGHJ*, and eight *IGHC* genes in the locus. From the *IGHV*s, 101 genes are functional, 69 are pseudogenes,

and they are divided into 15 subgroups. When compared to human, the proportion of functional *IGHV* is larger in the mouse (59% vs. 32%). The *IGHV* pseudogenes in mouse are not evenly spread across the locus. One big subgroup spanning more than half of the locus (J558) has two domains: the 5' end is relatively gene poor and the intergenic distances are long, but the 3' end has tight clustering of genes and pseudogenes (Johnston et al., 2006). As the latter part shows signs of duplications in the area, it seems that there are differences in the evolution between the two parts of this J558 region.

The C region of HC forms the general structure of the molecule and is not in contact with the antigen. There are five different forms of *IGHC*s, with different effector functions of the different isotype antibody molecules: IgM, IgD, IgG, IgA and IgE. The isotype of the antibody can be changed without changing the antigen specificity in the process of class-switch recombination. The B cell receptor (BCR) possesses $IGHC_{\mu}$ (corresponding to isotype IgM), which has no effector function when it is part of the receptor as the C region is inserted in the plasma membrane of the cell.

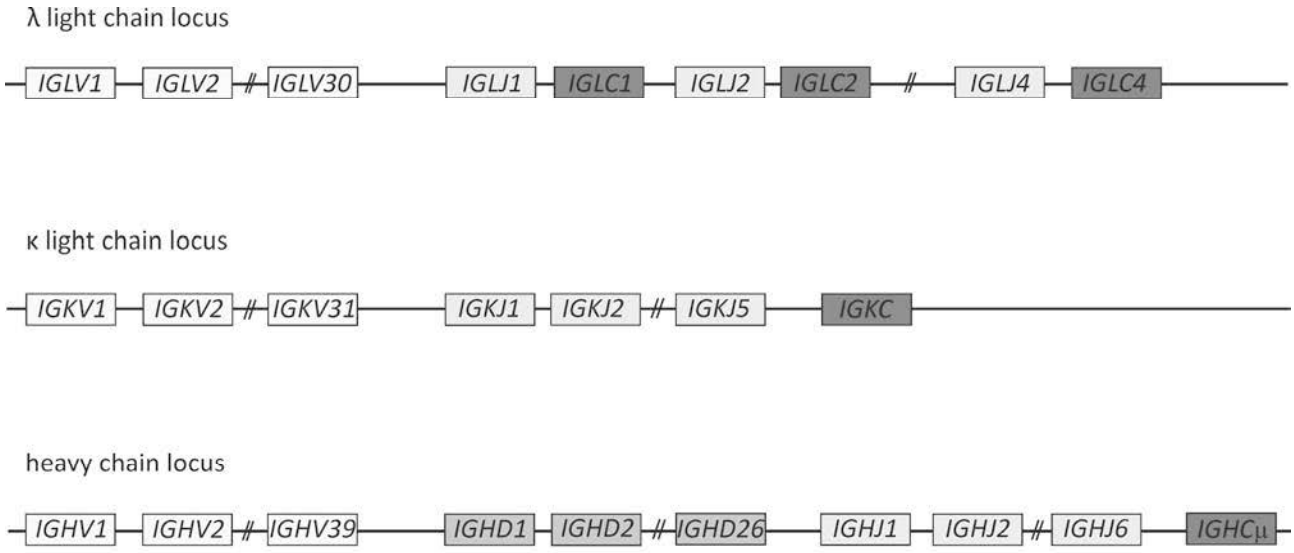

Figure 2. Schematic representation of the germline organization of the immunoglobulin heavy and light chain loci in the human genome, based on Murphy et al. (2007). For simplicity: only functional genes are shown, only single *IGHC* gene is shown, and the figure is not in scale.

Organization of human and mouse immunoglobulin light chain loci

There are two immunoglobulin light chain loci: lambda (λ, IGL) and kappa (κ, IGK). In human antibodies λ and κ are used at a ratio of 40:60, and in mouse antibodies at a ratio of 5:95, for unknown reason (Butler, 1997). The human IGL locus is situated on chromosome 22, spanning 1050 kb and comprising 73-74 *IGLV*, 7-11 *IGLJ*, and 7-11 *IGLC* gene segments (Lefranc and Lefranc, 2004). There are 30 functional *IGLV* genes and 35 pseudogenes, the remainder being open reading frames (ORFs), where there is no evidence of expression, or unclear cases. Whilst 56 to 57 of these genes belong to 11 subgroups, 17 of the pseudogenes are too diverse to fit in to the subgroups. *IGLV* and *IGLC* genes occur downstream of the *IGLV* cluster. They are organized differently than those of the IGH locus, given that there are sets in which each *IGLJ* gene is followed by an *IGLC* gene (Fig. 2), of which only four or five are functional.

The human IGK locus is situated on chromosome 2, spanning 1820 kb and comprising 76 *IGKV*, five *IGKJ*, and a single *IGLC* gene segment (Lefranc, 2001). Of the *IGKV* germline repertoire, 31-35 are functional genes, 32 are pseudogenes, and they are divided into 7 subgroups. The organization of the IGK locus differs dramatically from that of IGL locus. The most noticeable feature is that a major part of the *IGKV* gene region has duplicated. The contig more distal to the *IGKC* contains 36 *IGKV* genes, and the proximal contig contains 40 *IGKV* genes. These two contigs are organized in opposite orientations and the distance between them is 800 kb. All five *IGKJ* genes are functional and they are situated in a cluster between the proximal *IGKV* cluster and the single functional *IGKC* gene. The duplication of the human IGK locus has occurred quite recently, as it is not yet duplicated in the chimpanzee. Based on the divergence of the two copies of *IGKV* clusters, it seems that the duplication has taken place 1 to 2 million years ago (Schäble and Zachau, 1993).

The organization and germline repertoire of LC genes in mouse is distinct to human. The IGL locus on chromosome 16 of the laboratory mouse is much smaller: it spans 240 kb and contains three *IGLV*, five *IGLJ*, and four *IGLC* gene segments (Lefranc and Lefranc, 2004). In each gene segment category, the genes belong to two different subgroups. All *IGLV*s, and three of both *IGLJ*s and *IGLC*s, are functional. The IGL locus is organized so that each *IGLC* gene is preceded by one or two IGLJ genes, and two of these J-C_L sets are preceded by one or two IGLV genes. The organization can be shown as 2 x (V-J-C-J-C_L).

As compared to all of the light chain loci described above, the mouse IGK locus on chromosome 6 is huge, spanning 3.2 Mb. It contains 161 *IGKV*, five *IGKJ*, and a single *IGKC* gene segment (Martinez-Jean et al., 2001). A total of 91 *IGKV*s are functional genes, 60 are pseudogenes, and they belong to 19 subgroups. The individual segments in the *IGKV* cluster comprise both transcriptional orientations, and all *IGKJ* genes can be found downstream of this cluster, four of which are functional, followed by the single *IGKC* gene. There is no large duplication in the mouse κ locus as there was in the human κ locus, but it seems that there have been numerous small duplications (Kirschbaum et al., 1999).

Immunoglobulin gene rearrangements: V(D)J recombination

The purpose of V(D)J recombination is to bring together the separated gene segments of which the complete variable region of the Ig gene is composed of: V and J gene segments for LC, and V, D and J segments for HC. The constant region of the Ig gene is united to the variable region at RNA level, as the J-to-C intron is removed by splicing. The result of these events is V-D-J-C mRNA from which (in this case) HC polypeptide is transcribed. This process is illustrated in Figure 3.

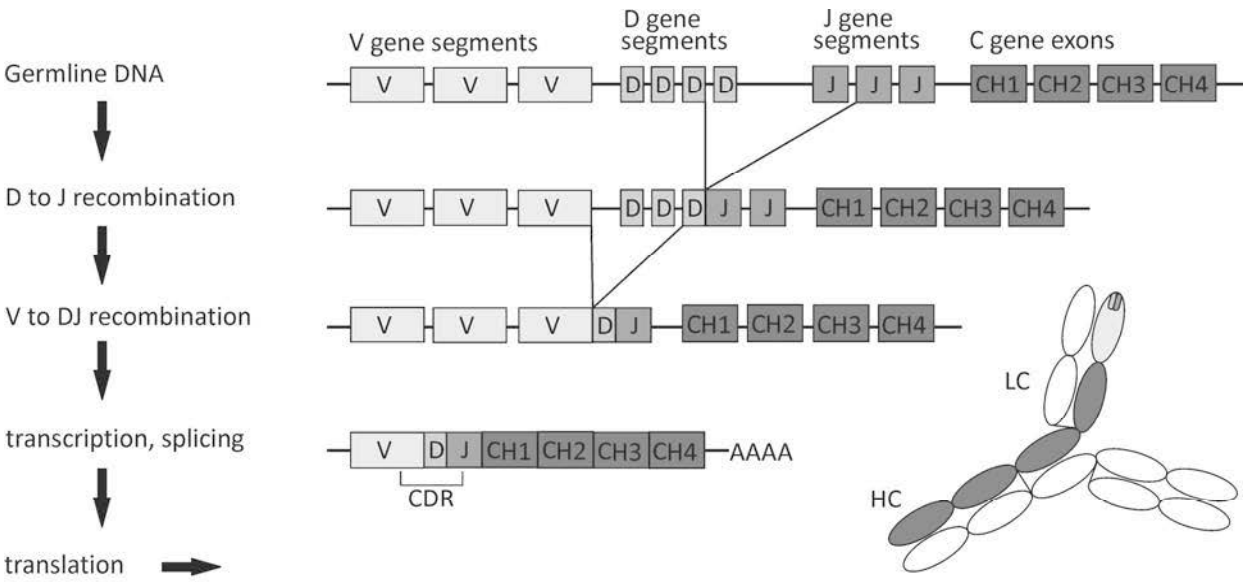

Figure 3. The generation of immunoglobulin HC polypeptide from germline DNA through V(D)J recombination and splicing of RNA, based on Murphy et al. (2007).

To make the cut-and-paste process needed for the recombination, there must be guidance signals in the sequence indicating where to cut and where to paste. Indeed, in 1979 Sakano and co-workers found sequences called recombination signal sequences (RSSs), which flanked J gene segments at the 5' side and V gene segments at the 3' side (Sakano et al., 1979; Tonegawa, 1983). In the recombination, the whole intervening sequence can be excised. An RSS consists of conserved heptamer (5'CACAGTG3'), nonamer (5'ACAAAAACC3') sequences, and a spacer region of conserved length (12 or 23 base pair (bp)). An RSS with a 12-bp spacer (12-RSS) always pairs with an RSS which has a 23-bp spacer (23-RSS). This is called the 12/23-rule and it guarantees that the rearrangement is biologically productive. For example in the kappa light chain, the *IGKV* 3' end 12-RSS pairs with the *IGKJ* 5' end 23-RSS (Fig. 4). In the heavy chain, the 12/23-rule ensures that the diversity segment is included: *IGHV* 3' –23-RSS recombines to *IGHD* 5' –12-RSS and *IGHD* 3' –12-RSS recombines to *IGHJ* 5' –23-RSS. Violations of the 12/23-rule exist, as in the mouse IGK locus there has been noted V-V_K –rearrangements (Vinocur et al., 2009). The frequency of these aberrant rearrangements were calculated to be one in 7500 mature splenic B cells, but is probably greater during lymphocyte maturation.

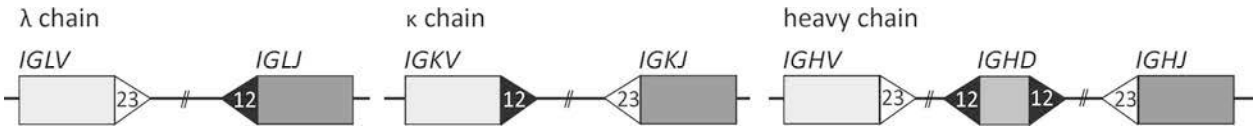

Figure 4. Recombination signal sequences (RSSs) flank V, D and J sequencies and guide V(D)J recombination. "23" RSS consists of conserved heptamer and nonamer sequences separated by 23 bp spacer, "12" RSS consist of conserved heptamer and nonamer sequences separated by 12 bp spacer. 12/23 rule ensures that *IGHD* is included in the recombination. Based on Murphy et al. (2007).

The DNA cutting enzyme V(D)J recombination-activating protein (RAG), composed of RAG1 and RAG2, was found based on the fact that it allowed recombination of RSS flanked sequences when introduced to cells

(Schatz et al., 1989; Oettinger et al., 1990). RAG1 and RAG2 are located adjacent to each other in the genome and in most species both are encoded by a single exon. Between species, their sequences are highly homologous: for example, pufferfish and human RAG1 and RAG2 are 66% and 61% identical at the protein level (Peixoto et al., 2000). RAGs are not identical to each other, and their lack of homology to each other confirms that they have not arisen by gene duplication. In fact, it is thought that RAG1 was a transposon, a sequence that can move itself in the genome, which integrated in the genome of a common deuterostome ancestor (Fugmann, 2010). It integrated proximal to RAG2, which possibly preexisted in the genome, lost its RSSs and could thus no longer move and became permanently locked in place. Consequently, RAG1 and RAG2 evolved a functional interaction leading to V(D)J recombination.

The process of making a double-strand break only requires the presence of both RAG proteins and RSSs (McBlane et al., 1995). There are two steps: first, a nick is introduced at the RSSs adjacent to the coding sequence creating a free 3'-OH. Next, this hydroxyl group attacks the opposite strand creating a hairpin at the coding end and a blunt 5' phosphorylated DNA at the signal end. The catalytic activity of RAG1 relies on three acidic amino acid residues, which in the mouse, for example, are aspartic acids at positions 600 and 708 and a glutamic acid at position 962 (Landree et al., 1999). This catalytic triad also represents the active site in other transposase superfamilies (Kapitonov and Jurka, 2005).

The cleaved ends of double stranded DNA are attached via the non-homologous end-joining (NHEJ) pathway, which compared to RAG proteins, is not lymphoid specific but commonly expressed cellular DNA repair machinery. The cleaved ends are held together by Ku, a heterodimer formed by Ku70 and Ku80, which forms a ring around duplex DNA (Walker et al., 2001). The blunt ends of the signal joint can be readily ligated by DNA ligase IV associated with DNA repair protein XRCC4 (Frank et al., 1998; Grawunder et al., 1998). The coding joint, however, is not ligated precisely, but nucleotides may be added or deleted, which will also contribute to the diversity of the Ig genes. The catalytic subunit of DNA-dependent protein kinase (DNA-PK_{cs}) associated with Artemis nuclease joins the complex formed by Ku. DNA-PK_{cs} activates Artemis by phosphorylation and Artemis opens the hairpin by a single-strand nick at variable places in the coding joint (Ma et al., 2002). The opening of the hairpin can occur at various points along the hairpin leading to hanging single-stranded DNA ends, which may contain nucleotides that were originally complementary and therefore form palindromes. These stretches can be filled and form P-nucleotide additions (P for palindromic) (Lafaille et al., 1989). Lymphoid-specific terminal deoxynucleotidyl transferase (TdT) enzyme also takes part to increase junctional diversity by adding non-templated nucleotides, N-nucleotides, to the ends (Alt and Baltimore, 1982) (Fig. 5). It can add up to 20 extra nucleotides randomly, although it seems to prefer G nucleotide addition (Basu et al., 1983). This happens more commonly in V-D_H and D-J_H junctions during HC rearrangements than in the LC rearrangement (Feeney, 1990; Gu et al., 1990; Bangs et al., 1991; Wasserman et al., 1997). The amount of N-nucleotide insertions also increases after birth with time – they are seldom encountered in perinatal B cells. Nucleotide deletion reactions are catalyzed by exonucleases, one of them in mice is a longer splice variant of TdT (Thai et al., 2002).

Finally the coding end is joined by the DNA ligase IV – XRCC4 -complex. If the rearranging genes (*e.g.* V and J) share the same orientation, the segment between them is excised as B cell recombination excision circle, referred to as deletional recombination (Fujimoto and Yamagishi, 1987). If the genes have the opposite orientation, an inversional recombination takes place: in this case both the signal joint and coding joint are retained in the chromosome. The latter case is relevant in the human κ locus as half of the *IGKV* gene segments are in the opposite orientation compared to J gene segments (Zachau, 1993).

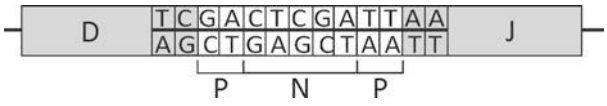

Figure 5. A rearranged D-J_H coding joint containing P-nucleotides (P) and N-nucleotides (N). P-nucleotides originate from the complementary strand and thus form a short palindrome. N-nucleotides are randomly added by TdT. Figure is based on Murphy et al. (2007).

To sum up, there are four mechanisms that contribute to this amount of potentially different antibody molecules: 1) Combinatorial diversity, which means that multiple different copies of Ig gene segments can combine together in different ways. The existence of two separate LC loci provides further possibilities. 2) Junctional diversity, the addition or deletion of nucleotides in gene junctions. 3) Also "combinatorial" as there are many different combinations of HC and LC pairs in single antibody molecule. 4) The repertoire can be widened even after the recombination, as point mutations can be introduced to the rearranged V-region genes in the process of *somatic hypermutation* (SHM). The process takes place in the activated B cells in the secondary lymphoid organs.

5.1.2. From stem cell to an antibody expressing cell

The V(D)J recombinations described above, when completed succesfully both at HC and LC loci, lead to a completely rearranged Ig locus and consequently to the expression of the BCR on the cell surface. The molecular level of the V(D)J recombination has now been described, but what are the stages on a cellular level? The generation of B cells from the progenitor cells, the *B cell lymphopoiesis*, as everything else in cell biology, begins with the totipotent fertilized egg, which is the extreme stem cell.

Stem cells, lymphoid progenitors and other players when it all starts

The hematopoietic stem cell (HSC), a descendant of the totipotent stem cell, can give rise to all blood cells– lymphoid, erythroid, myeloid and megakaryotic cells (Osawa et al., 1996). As it is a stem cell, it carries self-renewal and multilineage differentiation capabilities. Upon embryogenesis, there are three waves of hematopoiesis. The first one in mouse, which is the conventional mammalian model, takes place in extra-embryonic tissue, in the yolk sac on day 7-7.5 of embryonic development (full period 19 -21 days). Cells of hematopoietic lineages other than lymphoid are generated (Cumano et al., 2001). The second wave occurs a few days later (day 8.5-9) in the aorta-gonad-mesonephros region, from where the cells migrate to colonize the liver (Medvinsky and Dzierzak, 1996). At the same time, the placenta has been shown to also function as a hematopoietic organ (Gekas et al., 2005; Ottersbach and Dzierzak, 2005). The first wave of B lymphocyte precursors can be detected In fetal liver at day 13 (Strasser et al., 1989), and B cells are simultaneously generated in the omentum (Solvason et al., 1991) The third and last wave of hematopoiesis starts just before the birth of a mouse (days 17-19) in the bone marrow and continues throughout the life of the animal. During human embryogenesis, the above mentioned points for the hematopoietic waves are days 13-24, days 25-30 and days 70-100, the last one thus beginning long before the birth (Charbord et al., 1996).

The first step for a HSC in the hematopoietic pathway is to lose the capability of self-renewal while retaining the possibility to differentiate to all blood cell lineages. When a HSC divides, some of the daughter cells persist as long-term HSCs (capable of life-long self-renewal)(Jones et al., 1990; Smith et al., 1991) while others have reduced self-renewal potential (short-term HSC) (Smith et al., 1991). Both can give rise to multipotent progenitors (MPPs), which have no remaining self-renewal capacity (Adolfsson et al., 2001).

Two possible models for hematopoiesis thereafter have been presented: The classic model proposes that MPP is the last common progenitor, capable to differentiate to all hematopoietic lineages, after which the cells are committed to either common lymphoid or common myeloid progenitors (Reya et al., 2001). The common lymphoid progenitor would give rise to B-, T-, and NK-cells, while the common myeloid progenitor would differentiate into erythrocytes, monocytes, megakaryocytes, and granulocytes. Dendritic cells could be derived from both lineages (Traver et al., 2000; Manz et al., 2001). According to the other model, called "the alternative model", the cells lose the capability of differentation one by one: first the potential to generate erythroid and megakaryocytic lineages is lost, followed by myeloid potential (Rolink et al., 2000; Adolfsson et al., 2005). The lymphoid potential is retained for the longest time.

The fate of a B cell is guided by the interaction of many transcription factors and cell surface molecules. In mouse, the earliest B cell specific event is the expression of receptor tyrosine kinase Flk2/Flt3 in a subset of MPPs (Adolfsson et al., 2001) (Fig. 6). The expression seems to be under the control of transcription factors PU.1 and Ikaros (Nichogiannopoulou et al., 1999; DeKoter et al., 2002). The activation of Flk2/Flt3 and the straight effect of PU.1 promote the expression of IL-7 receptor (IL-7R) in lymphoid progenitors (Borge et al., 1999). IL-7 is a hematopoietic growth factor which seems to be an essential proliferative stimulus for the B cell progenitors (Lee et al., 1989). Furthermore, mice genetically deficient for the IL-7R have severely impaired early lymphopoieisis (Peschon et al., 1994). In humans, the role of IL-7 in B lymphopoiesis is more controversial. It seems to have a proliferative effect on human B cell precursors (Saeland et al., 1991; Dittel and LeBien, 1995), but it is difficult to exclude the possibility that IL-7 only enhances survival and consequently may not be essential for human B cell development (LeBien, 2000).

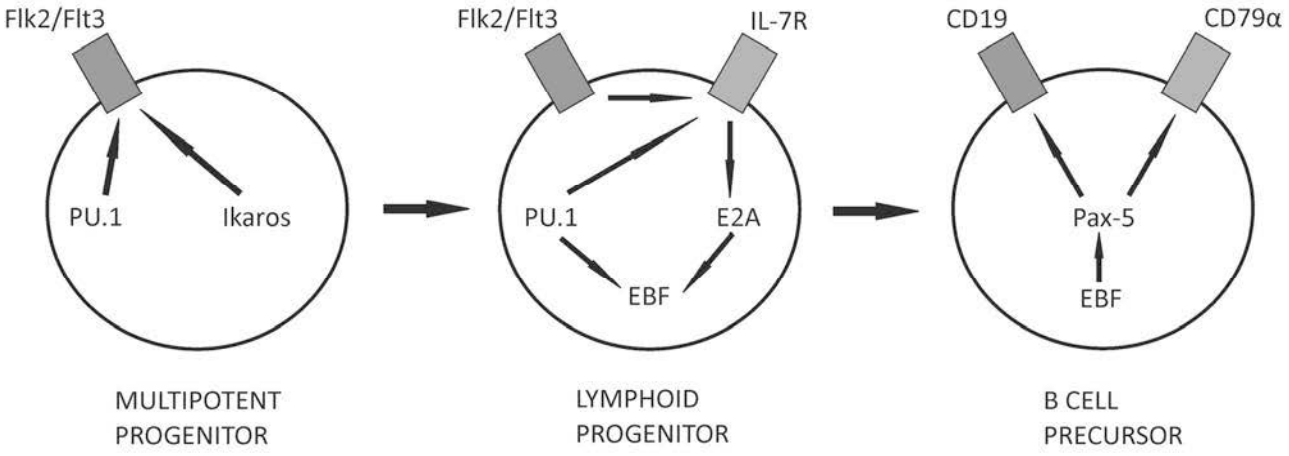

Figure 6. Gene regulatory networks directing the early stages of B cell lymphopoiesis, based on Singh et al. (2005).

The entry to the B lymphoid pathway is controlled by two transcription factors: E2A and the early B cell factor (EBF). Their expression is required for a cell to become a B lineage cell, and that is why they are called primary cell fate determinants (Singh et al., 2005). EBF expression appears to be under the control of E2A, suggested by the finding of a functional binding site for E2A in the EBF promoter (Smith et al., 2002), and by the fact that an E2A deficiency - caused block in B lymphopoiesis can be overcome by expression of EBF (Seet et al., 2004). Similarly PU.1 functions upstream of EBF: EBF restores the early B cell development in PU.1$^{-/-}$ mice (Medina et al., 2004). The control of E2A expression is less clear, but there are suggestions

that signaling through IL-7R would have an effect (Singh et al., 2005). E2A and EBF regulate the expression of RAG1, and B cell specific surrogate LC proteins λ5 and VpreB, which are described in detail below (Bain et al., 1994; Sigvardsson et al., 1997). They also regulate the D-J_H rearrangements in the IGH locus (Bain et al., 1994). Targeted inactivation of these genes results in a block in B lymphopoiesis at the stage where no rearrangements of the Ig locus have yet happened, but has no effect on the T cell, granulocyte, macrophage, or erythroid lineages (Bain et al., 1994; Zhuang et al., 1994).

PAX5, a secondary B cell fate determinant, is also under the control of E2A and EBF. $PAX5^{-/-}$ cells cannot proceed from pro-B cell to pre-B cell stage as the rearrangements stop after D-J_H and proximal V-DJ_H rearrangements (Urbánek et al., 1994). Pro-B cells, as defined by Hardy and colleagues, are cells which have initiated HC rearrangements but not completed them (Hardy et al., 1991). PAX5 expression seems to be needed continuously from the committed B cell progenitor stage until the mature B cell stage to preserve the B cell identity. It regulates the expression of molecules such as CD79α and CD19 (Nutt et al., 1997), and promotes the distal V-DJ_H rearrangements (Hesslein et al., 2003). In addition it represses the expression of myeloid lineage marker genes, such as macrophage colony-stimulating factor receptor (Nutt et al., 1999). Pro-B cells from wild type mice are already committed to the B cell lineage (Allman et al., 1999), but pro-B cells from $PAX5^{-/-}$ mice show a high level of plasticity, as they can differentiate into T cells, NK cells, dendritic cells, granulocytes, macrophages, and erythrocytes (Nutt et al., 1999). Taken together, PAX5 is a factor that developing B cells need continuously in order to stay in the B lineage pathway. Its removal alters the cell into a plastic, stem-cell like phenotype.

Pre-B cell receptor is a precursor receptor that stimulates the proliferation of a V(D)J rearranged cell

During B cell development there are precursor B cells that synthesize intracellular Ig before surface-bound Ig (the B cell receptor) can be detected (Melchers et al., 1975; Raff et al., 1976). This cell stage is called the pre-B cell. A schematic representation of B cell development from pro-B via pre-B to a mature B cell stage is represented by Mårtensson and her colleagues (Mårtensson et al., 2010) (Fig. 7). At the pro-B cell stage, cells start to rearrange the HC locus. When the locus is in the DJ_H rearranged stage, the cells are called pre-BI cells. Subsequently cells, which have successfully reorganized the whole heavy chain locus (VDJ_H stage), form a subpopulation of the previous, called the transitional pre-BI cells. At this point cells start to express the heavy chain μH which pairs with the surrogate LC to form the pre-B cell receptor (pre-BCR) on the cell surface.

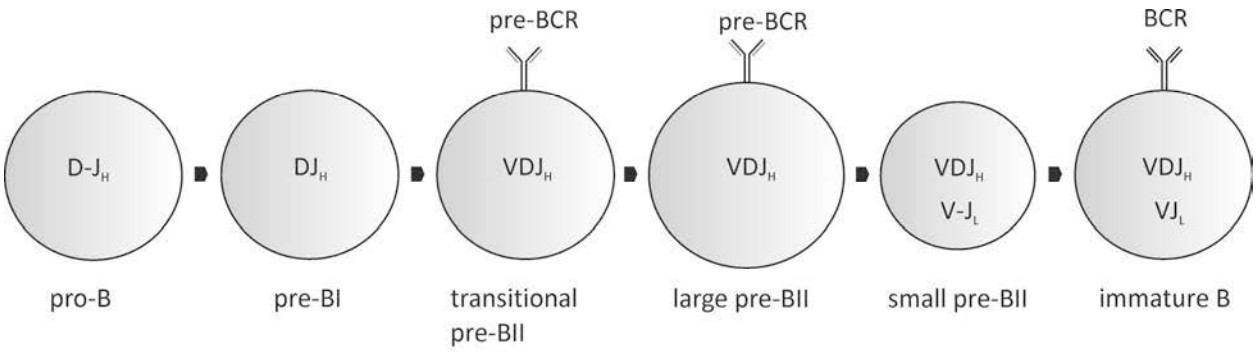

Figure 7. A schematic representation of B cell development, modified from Mårtensson et al. (2010).

Pre-BCR is an Ig-like complex, that consists of a HC pairing with a surrogate LC which resembles LC (Fig. 8). Surrogate LC has two parts: immunoglobulin lambda-like polypeptide 1 (IGLL1, a.k.a. λ5 in mouse) has a homology to the constant part of *bona fide* LC, and VPREB (VpreB in mouse) has a homology to the variable part of *bona fide* LC. The crystal structure of pre-BCR Fab-like fragment has been solved (Bankovich et al., 2007). The N-terminus of IGLL1 and the C-terminus of VPREB have regions which have no counterparts in the LC, so called unique regions. These regions protrude out of the molecule, and replace the CDR3 loop of the LC. The signal transducing part of both receptors, BCR and pre-BCR, is the CD79α/CD79β-heterodimer, which sits at the plasma membrane (Fig. 8). It is unclear how pre-BCR signaling is initiated, however there is evidence of ligand independent signaling. Ohnishi and Melchers suggest that the IGLL1 unique region is responsible for cell autonomous signaling: by using pre-B cell line expressing mutant IGLL1 (unique region deleted) they saw more pre-BCRs on the cell surface and less aggregation and internalization of the receptor (Ohnishi and Melchers, 2003). Electron microscopic visualization of pre-BCR dimers supported also the ligand independent oligomerization (Bankovich et al., 2007). On the other hand, several bone marrow stromal cell-associated ligand candidates have been suggested. Galectin-1 is one of them: it bound to pre-BCR, which led to an immune synapse formation between pre-B cell and stromal cell and initiation of signal transduction from pre-BCR (Gauthier et al., 2002). The IGLL1 unique region was again in charge of the interaction. In another study, the IGLL1 unique region was also responsible for the binding of the pre-B cell to a stromal cell, but for this time the ligand proved to be stromal cell –associated heparan sulfate (Bradl et al., 2003). The third aspect to be taken into consideration is whether or not the cell surface expression is needed for the signaling. Guloglu and Roman showed that pre-BCR signaling driven events, such as proliferation, can also be seen if the pre-BCR is located only intracellularly, in the post-ER membranes (Guloglu and Roman, 2006).

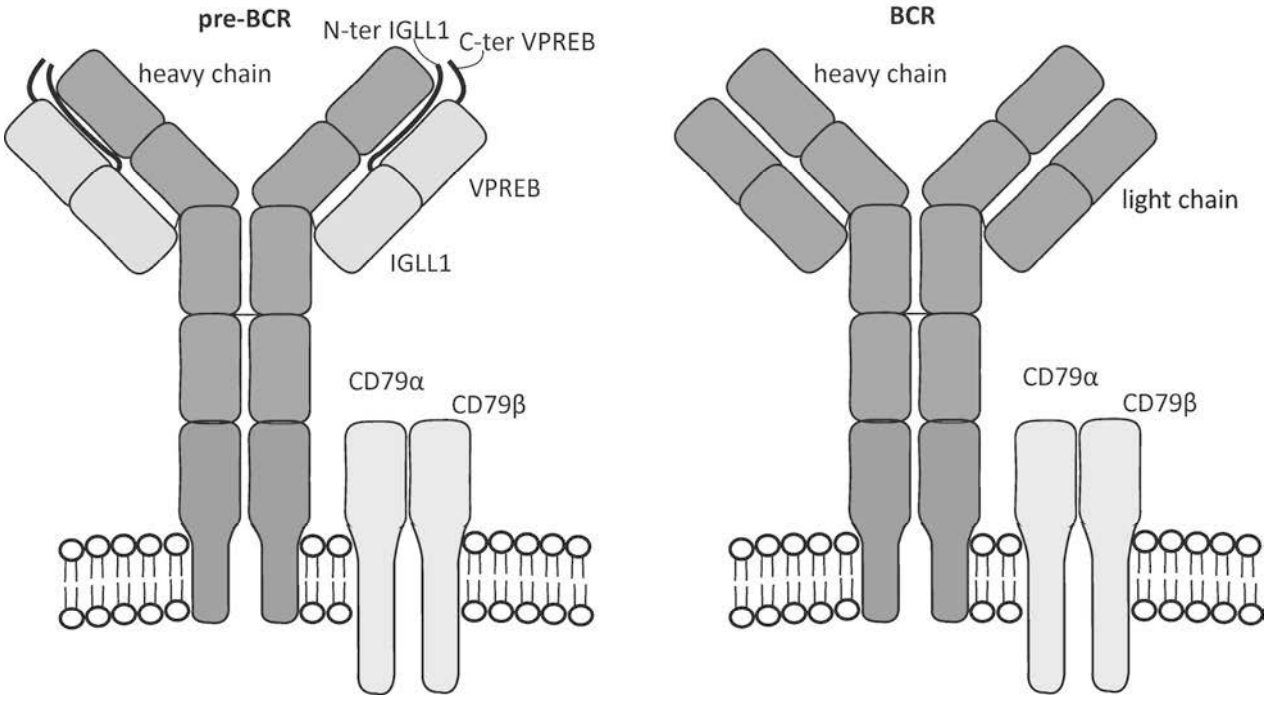

Figure 8. The structure of a pre-BCR and a BCR. Also the signal transducing part CD79α/CD79β is shown.

In mice, there is one gene for λ5 and three genes for VpreB. *λ5* as well as *VpreB1* and *VpreB2*, which are 97% homologues, are situated in the same chromosome as the IGL locus, on chromosome 16 (Kudo and Melchers, 1987; Kudo et al., 1987). *VpreB1* and *λ5* are situated close to each other, separated only by 4 kb, and *VpreB2* is located 1.1 Mb upstream of them (Dul et al., 1996). *VpreB3*, whose homology to *VpreB1* is only 37%, is situated on chromosome 10 (Shirasawa et al., 1993; Ohnishi and Takemori, 1994; Rosnet et al., 1999). In human, there is one gene for IGLL1 and only two genes for VPREB, and they all are situated on the same chromosome as the IGL locus, chromosome 22 (Bauer et al., 1988; Mattei et al., 1991; Rosnet et al., 1999). IGLL5 is also described, but its function is not related to pre-B cells (Evans and Hollis, 1991; Guglielmi and Davi, 1991). Human *VPREB1* and mouse *VpreB1* are 76% homologous, whilst human *VPREB3* (which lies in close proximity to *IGLL1*) and mouse *VpreB3* are 67% homologous, but human *VPREB1* and *VPREB3* are only 40% in agreement.

Mouse *λ5* was found in the 1980s by checking a subtractive cDNA library for genes expressed specially in pre-B cells (Sakaguchi and Melchers, 1986). It was found that part of this gene sequence showed high homology to C and J gene sequences of Ig λ, and it was named *λ5*. A year later, another pre-B cell specific gene was found 4.6 kB upstream of *λ5* (Kudo and Melchers, 1987). It showed homology to V gene regions of IGL, IGK and IGH, and because of that it was designated as *VpreB1*. Another very homologous gene was given the number 2 (*VpreB2*). Both VpreB1 and VpreB2 can assemble with λ5 to form the surrogate LC complex (Dul et al., 1996).

There is some evidence that the role of VPreB3 might differ from VPreB1 and VPreB2. It has been shown that mouse VPreB3 is expressed in pre-B cells together with λ5 and VpreB1, and it associates with μHC (Shirasawa et al., 1993). But furthermore, it seems that interactions between μHC, λ5 and VpreB3 take place only in the cytoplasm and precedes the pre-BCR forming interaction of μHC, λ5 and VPreB1/2 (Ohnishi and Takemori, 1994). This has led to speculation that VpreB3 acts like a kind of chaperone, facilitating the exit of μHC from the endoplasmic reticulum (Mårtensson et al., 2007).

The function of surrogate LC is revealed by mouse knock-out models; *λ5*$^{-/-}$ mice show an incomplete block in B cell development (Kitamura et al., 1992). Pre-BI cells are enriched, but small numbers of B cells are made, and the number increases up to 20% of normal with age. The phenotype of *VpreB1*$^{-/-}$ is less severe; it has an effect, as a small enrichment of pre-BI could be noticed, but the number of B cells is normal (Mårtensson et al., 1999). If a mouse is double-deficient, so *VpreB1*$^{-/-}$ *VpreB2*$^{-/-}$, the outcome is quite similar as in the *λ5* knock-out (Mundt et al., 2001). The impairment of B cell development is noticed again at the pre-BI to preB-II transition, as there are 40-fold less small pre-BII-cells in the bone marrow. A triple knock-out deficient of all three surrogate LC genes (*λ5*, *VpreB1*, *VpreB2*) presents no surprises: the B cell development is severely impaired, similar to mice lacking either *λ5* or *VpreB1* and *VpreB2* (Shimizu et al., 2002). To conclude the situation in the mouse, λ5 and VpreB are needed for normal B cell development. VpreB2 alone is sufficient to support B lymphopoiesis but is not as effective as VpreB1. It has been shown, that VpreB1 is expressed in all cells that express λ5, and that VpreB2 is co-expressed in 30% of these cells (Dul et al., 1996). This kind of data is of course not available from humans. A boy has been described with mutations in both *IGLL1* alleles whose bone marrow harbored no mature B cells (Minegishi et al., 1998). (Minegishi et al., 1998), which appears dissimilar to the mouse model. In humans, the lack of pre-BCR causes a severe, not leaky, block in B cell development.

The role of pre-BCR has been an issue of interest for many decades. It seems that for some of the functions the whole pre-BCR is needed, but for others only μHC without the surrogate LC is sufficient, as recently discussed by Mårtensson and co-workers (Mårtensson et al., 2010). The transition from pre-BI to pre-BII, described above, is incompletely blocked by the lack of surrogate LC. What blocks the transition completely is the lack of signal transducers, CD79α or CD79β (Pelanda et al., 2002), or the lack of the transmembrane domain of the μHC (Kitamura et al., 1991). The conclusion is that signaling-competent μHC, but not pre-BCR, is needed for the B cell differentiation in mice. In humans the surrogate LC appears to be essential for differentiation (Minegishi et al., 1998; Espeli et al., 2006). Another situation, in which the signaling-competent μHC seems to be enough, is the allelic exclusion. In the Ig loci it is of great importance that the Ig gene is expressed only from one allele, so that the cell expresses only antibodies of single specificity. Downregulation of RAG-proteins after the rearrangement of the first IGH allele is coupled to the pre-BCR expression. But, as said, the signaling-competent μHC seems to be enough, because it was shown that downregulation also took place in a mouse devoid of either λ5, VpreB1/2, or the entire surrogate LC (Galler et al., 2004).

There are also situations in which the whole pre-BCR is essential, returning once again to the transition from pre-BI stage to pre-BII stage. Although mouse B cells can pass this point without pre-BCR, the expansion of pre-BII is greatly reduced, which suggests that differentiation and proliferation may occur uncoupled from each other at the pre-BII cells (Espeli et al., 2006; Mårtensson et al., 2007). Pre-BCR also appears to act as a kind of a positive selector for the heavy chains: only those heavy chain molecules that are able to pair with the surrogate LC are accepted further to be a part of the antibody molecule and constitute the repertoire. This is a trimming checkpoint, as more than half of the productively rearranged μHC could not assemble with the surrogate LC (ten Boekel et al., 1997). There are specific examples of pre-BCR selection; the usage of one of the most D-proximal V_H- genes, V_H81X, is varying in the sense that it is common in pre-BI cells but very rare in the pre-BII cells in the bone marrow. The fading of V_H81X from the repertoire is coupled to the expression of pre-BCR, suggesting that it is the selective event. Apart from the above mentioned positive selection, pre-BCR is also coupled to the negative selection. In surrogate LC knock-out mice, there are more autoantibodies directed to nuclear antigens (Keenan et al., 2008). It was stated that these antibodies had more basic residues in their CDR3 region, but the mechanism of selection remains unclear. Similar observations regarding CDR3s harboring multiple basic amino acid residues come from human studies, from the above mentioned case of a boy with mutated *IGLL1* (Minegishi et al., 1998).

The fate of the immature B cell

The status of Ig rearrangements during B cell development in the mouse follows a certain repertoire: 1) in the RAG-1$^+$ VPREB$^+$ preB-I cells both HC alleles are DJ_H rearranged while LC is still in the germline configuration, 2) in the large proliferating VPREB$^{+/-}$ RAG-1$^-$ pre-BII cells the HC is VDJ_H rearranged (LC is still in germline configuration) (Ghia et al., 1996). Half of the pre-BII cells have VDJ_H/DJ_H and another half VDJ_H/VDJ_H configuration (Melchers and Kincade, 2004). According to the allelic exclusion model, functional rearrangement of a H (or L) chain in a particular cell inhibits further H (or L) chain rearrangements in the same cell (Alt et al., 1980). In this case, the existence of VDJ_H/DJ_H configuration proves that allelic exclusion is operative. The existence of the latter VDJ_H/VDJ_H configuration shows that the nonproductive rearrangement in the first allele can not inhibit the rearrangement in the other allele. 3) In the small resting VPREB$^-$ RAG-1$^+$ pre-BII, the LC locus rearrangement is also activated as it is in the VJ_L configuration (Ghia et al., 1996). This rearrangement starts in one IGK allele and continues, if nonproductive, quite likely in the same allele, before trying the rearrangements in the other allele (Yamagami et al., 1999; Melchers and Kincade, 2004). In wild-type mouse, nearly half of the mIgM$^+$ cells have rearranged only one IGK allele the

other being in the germline configuration. In contrast, in small pre-BII compartment it is more infrequent to have only a single VJ_k rearrangement, as nearly two-thirds of the cells harbor secondary rearrangements in the IGK locus. This implies that cells that have not been capable of successful rearrangements in the first place accumulate in this cell population.

Cells that have successively rearranged their whole Ig locus can be roughly divided into two maturity stages, newly formed immature B cells and mature B cells. The increasing expression of mIgD is associated with B cell maturation, so the phenotype of immature B cells is IgM^+ IgD^- changing to IgM^+ IgD^+ in mature B cells (Vitetta et al., 1975). Before the immature B cell can leave the bone marrow, it must have *central tolerance*: it is in order to confirm that the newly formed antibody specificity is not against self-antigens, in other words, the antibody is not self-reactive. If the immature cell possesses an auto-reactive antibody, there are several possible outcomes: they are eliminated by apoptosis, anergized, or their specificity can be altered in a process called *receptor editing*. The latter is frequently used as 25% of antibodies have LCs that have undergone receptor editing (Casellas et al., 2001). After exiting the bone marrow, the peripheral immature B cells should enter the spleen to mature. However, 70% of these peripheral cells do not survive to maturity (Allman et al., 1993). It has been demonstrated that the survival is a selective process instead of a stochastic (Levine et al., 2000). The short-lived immature B cells are selected to the pool of long-lived mature recirculating naïve B cells depending on their Ig specificity. This event seems to shape the mature B cell receptor repertoire.

5.1.3. Post-recombinatorial mechanisms further diversify the antibody repertoire

The essential enzyme: activation-induced cytidine deaminase (AID)

The molecular mechanism of somatic hypermutation (SHM) remained an enigma until 1999 when a cDNA encoding for activation-induced cytidine deaminase (AID) was found in a substraction cDNA library of murine B lymphoma cells (Muramatsu et al., 1999). The library was made with switch-induced and uninduced cells to find genes selectively expressed in B cells during class-switch recombination (CSR). Soon after that, it became evident that AID is also linked to SHM, as $AID^{-/-}$ B cells were defective in both CSR and hypermutation (Muramatsu et al., 2000). AID is known to be expressed almost exclusively in B cells and is the common denominator in the DNA modifying processes of CSR, SHM, and gene conversion (GC) (Muramatsu et al., 2000; Arakawa et al., 2002).

AID mRNA encodes for a protein of 198 amino acids. As soon as it was found, it was noticed to have 34% amino-acid identity to a RNA-editing cytidine deaminase APOBEC-1 (reviewed in (Scott, 1995)). APOBEC-1 converts cytidine to uridine at position 6666 of apolipoprotein B mRNA, and this leads to a stop codon (CAA → UAA). The full-length mRNA is expressed in the liver and the truncated alternative form is expressed tissue-specifically in the intestine, where it is required for dietary lipid absorption. The sequence similarity and the fact that the genes for AID and APOBEC-1 are closely located, possibly because of gene duplication (Muto et al., 2000), led to the proposal that AID could also act as an RNA editing cytidine deaminase (Muramatsu et al., 1999).

In this RNA-editing model (Fig. 9a), AID deaminates cytidine to uridine on mRNA and converts it to an mRNA coding for a yet unknown endonuclease (Honjo et al., 2005). This endonuclease would then attack the Ig gene locus. As this model would necessitate translation of the endonuclease after the AID activation, studies where protein synthesis is inhibited have been performed to find more supporting evidence. An inducible AID protein was constructed, and it was shown that if protein synthesis inhibitor cycloheximide was added before the AID activation, CSR and SHM were blocked (Doi et al., 2003; Nagaoka et al., 2005).

The cellular localization of AID also fits to the RNA-editing model. It has been shown that RNA-editing enzymes shuttle between the nucleus and the cytoplasm; editing takes place in the nucleus and afterwards the enzyme transports the edited mRNA to the cytoplasm (Chester et al., 2003). Both APOBEC-1 and AID have been demonstrated as this type of shuttling protein (Ito et al., 2004).

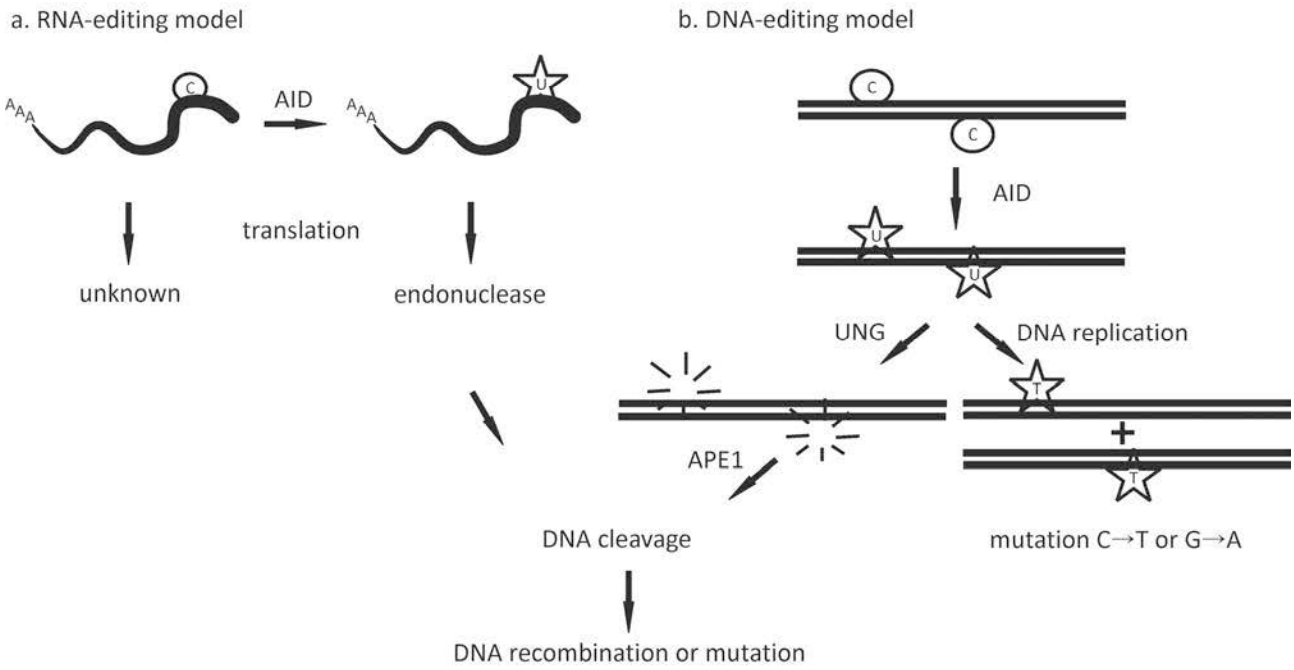

Figure 9. The RNA-editing model (a) and the DNA-editing model (b) of AID function (modified from Honjo et al. (2005)). According to RNA-editing model, AID recognizes a putative mRNA precursor and converts it to mRNA encoding endonuclease. Endonuclease cleaves DNA, which leads to somatic hypermutation, class switch recombination or gene conversion. According to DNA-editing model, AID deaminates deoxycytidine to deoxyuridine, which is recognized by base exision repair enzymes, such as UNG and APE1. Their action leads to DNA cleavage and further to somatic hypermutation, class switch recombination or gene conversion. Additionally, transition mutations can occur without DNA cleavage, as U:G mismatch pair is corrected during DNA replication.

The competing alternative DNA-editing model (Fig. 9b) presents that AID acts directly on DNA to deaminate deoxycytidine to deoxyuridine (Di Noia and Neuberger, 2002; Petersen-Mahrt et al., 2002; Rada et al., 2002). Whether the outcome of the deamination is SHM, CSR, or gene conversion (GC, discussed more closely on chapter 5.2.2.) would depend on how the process continues. SHM occurs by transition mutation (C → T or G → A), when the deoxyuridine produced by AID is recognized as deoxythymidine by DNA polymerases. As the deoxyuridine is substrate for the ubiquitous base-excision repair machinery, however, a uracil-DNA glycosylase (UNG) can remove uracil to form an abasic site, and non-templated replication can generate transition (C → T or G → A) or transversion mutation (C → G or T → A). After UNG action, it is also possible that apurimic/apyrimidinic endonuclease 1 (APE1), also member of the base excision repair machinery, would cleave the phosphodiester backbone. This would lead to GC if DNA replication uses homologous pseudogenes as templates or to CSR if these single-strand nicks are converted to double-strand breaks simultaneously in two different regions flanking the C-region genes. The DNA-editing model is supported by the fact, that ectopic epression of AID induces mutations in the genome of mammalian

cells, yeast and bacteria (Martin and Scharff, 2002; Petersen-Mahrt et al., 2002; Yoshikawa et al., 2002; Mayorov et al., 2005). Further, as the action of UNG and DNA polymerase would conventionally lead to the reinsertion of dC to the empty site, in the *ung* negative phenotype there should have been more mutations, which was the case. $UNG^{-/-}$ lymphocytes also have reduced levels of CSR, which suits the DNA-editing model well (Rada et al., 2002). In conclusion, there is direct evidence of AID-edited DNA, but the evidence of AID-edited mRNA is missing.

It has been noted that mutations are not randomly distributed to the V gene region, but that AID favors cytidines in certain mutational hotspots. AID preferentially targets WRCY/RGYW (W = A/T, R = A/G, Y = C/T) hotspots in the genome (Rogozin et al., 2001; Yu et al., 2004; Longo et al., 2008). It seems that codons in CDR regions are particularly susceptible to replacement mutations and codons in the intervening frame work region are particularly resistant to them (Chang and Casali, 1994). This might not be a consequence of the AID action, but of the selection event afterwards. Evolutionary it is beneficial, as much dissimilarity can be generated while preserving the structural framework.

The SHM and CSR have many things in common in addition to the most apparent, the AID enzyme; their target areas are specific and target transcription is essential, its level correlating to the efficiency of CSR (Lee et al., 2001). Differences between them exist in evolution, as SHM is already observed in bony and cartilaginous fish, but CSR only first appears in amphibians (Litman et al., 1999). Given that two double-stranded breaks are to be joined together in CSR, it is plausible that the molecular mechanisms downstream of AID are different. In summary, AID protein mediates both SHM and CSR, but these mechanisms have evolved separately and are differently regulated.

5.2. Multiple strategies for B cell development

In general, the genome harbors large amounts of Ig gene segments, and antibody variability comes from different combinations of these gene segments. Furthermore, B cells producing these antibodies are generated in the bone marrow of an animal throughout its lifetime. Both of these assertions are true, but only for certain animal species. These species, called the bone marrow species, include man and mouse. For obvious reasons, much of our understanding of immunology is based on these two species. But the more study performed on the immunology of the different species, the more variability has been noticed. It seems more likely that bone marrow species make an exception, as the data from chicken, rabbit, pig, sheep, cattle, dog and horse accumulates. The amount and variation of different Ig gene segments in these species is limited, so they must use other means of generating antibody diversity to compensate for it. The period of new recombinations, B lymphopoiesis, seems also to be restricted to a short time in the beginning of an individual's life. Afterwards the alterations take place in a gut-associated lymphoid tissue (GALT), which gives these species the name GALT species. A summary of B cell development in various mammalian and bird species described in this section is presented in Table 1.

Table 1. B cell development during the fetal period in various mammalian and bird species.

	Known sites of V(D)J rearrangements	Mechanism of post-recombinatorial antibody primary repertoire generation	Site of post-recombinatorial antibody primary repertoire generation
Man	Fetal liver, omentum, bone marrrow	(Combinatorial diversity)	
Cattle	Not known	SHM, GC	IPP, fetal spleen
Sheep	Not known	SHM	IPP
Chicken	Yolk sac, spleen, blood, para-aortic region, thymus, bone marrow, bursa	GC	Bursa
Rabbit	Fetal liver, bone marrow	GC-like, SHM	Appendix, *sacculus rotundus*, IPP
Pig	Yolk sac, fetal liver, bone marrow	(Junctional diversity)	

AGM = aorta-gonad-mesonephros –region, GC = gene conversion, IPP = Ileal Peyer's patch, SHM = somatic hypermutation. Combinatorial and junctional diversity are presented in parentheses as they take place during recombination. For references, see the text.

5.2.1. Ileal Peyer's patch (IPP) is the site for antibody diversification in cattle and sheep

Organization of bovine and sheep heavy chain and light chain loci

The data which is available on the bovine IGH locus is limited, as the locus is largely missing from the current reference genome assembly. The locus is known to be located in chromosome 21 (Tobin-Janzen and Womack, 1992; Chowdhary et al., 1996), but the numbers of different gene segments is, of course, an estimate until the sequence of the whole locus is known. As little as 20 *IGHV* genes belonging to a single subgroup have been reported, although Southern blot data implies the presence of at least three more subgroups (Berens et al., 1997; Saini et al., 1997; Sinclair et al., 1997; Lopez et al., 1998). Seven *IGHD* and six *IGHJ* genes, two of which are functional, have been reported (Zhao et al., 2003; Koti et al., 2008). Cattle specific features include very long CDR3 regions, on average 21 amino acids, which are heavily hypermutated and may compensate for the limited repertoire of *IGHV* genes (Berens et al., 1997). In the constant region, there are7 *IGHC* genes, corresponding to all Ig classes in humans and mouse (Zhao et al., 2002, 2003), and an extra *IGHCμ*-like sequence located in a different chromosome (Hayes and Petit, 1993; Hosseini et al., 2004).

Both LC loci are known to exist in cattle, and the λ:κ-ratio of the produced Ig molecules is 91:9 (Arun et al., 1996). The LC loci are not well characterized. In the IGL locus, 20 germline *IGLV* genes were identified, and 14 of them were pseudogenes (Parng et al., 1996). Two *IGLJ* and at least four *IGLC* genes were also found. There is no information on the IGK locus.

The sheep HC repertoire appears also to be restricted: nine *IGHV* genes have been identified, all belonging to the same gene family (in some species the term "gene family" is used instead of a "subgroup"; in a gene family the identity between segments is > 80%, in a subgroup it is > 75%) (Dufour et al., 1996). Two functional *IGHJ* genes and four pseudogens were identified (Dufour and Nau, 1997). Thus far no *IGHD* genes

have been identified, although they are suspected to exist. Some junctional diversity is found in the form of P- and N-nucleotide additions in both HC and LC (Dufour and Nau, 1997; Hein and Dudler, 1998).

Sheep possess both LC loci, λ and κ. The IGK locus contains probably ten germline *IGKV* gene segments, of which six are expressed (Hein and Dudler, 1998). Of the *IGKJ* genes, two are expressed. Compared to this rather limited IGK locus, the IGL locus holds a much larger number of the genes. Over 70 *IGLV* have been described (Jenne et al., 2003), although this study can be criticized by the use of heterogenic data (alleles included) based on cDNAs (mutations included). Despite the possible large amount of genes, the genetic diversity in those genes is limited (Jenne et al., 2006). Two IGLJ genes can be found (Reynaud, Mackay, et al., 1991; Jeong et al., 2001).

Ileal Peyer's patch a huge continuum of aggregated lymphoid follicles at the wall of the small intestine

Ileal Peyer's patch (IPP) is a group of lymphoid follicles in the wall of the lower small intestine, the ileum. It was originally found by Johan Conrad Peyer in the year 1677, and described by him as intestinal glands (Peyer, 1677). Peyer's patches also exist in the preceding part of the small intestine, the jejunum. In ruminants, pigs, dogs, horses, and humans, Peyer's patches are more concentrated on the ileum, whereas in rabbits, rats, mice, and chicken, they are distributed evenly (Griebel and Hein, 1996). Especially in sheep, pig, and dog, the IPP is a single continuum of aggregated lymphoid follicles (Reynolds and Morris, 1983; HogenEsch et al., 1987; Pabst et al., 1988). Sheep IPP is at its biggest (six weeks after birth) 2.5m long, constituting 100 000 lymphoid follicles and weighing 1.2% of the total body weight (Reynolds and Morris, 1983).

The formation of IPP in sheep and cattle begins at the beginning of the second trimester (full term 150d sheep; 280d cattle). At day 60 in sheep, lymphocytes start to aggregate in the submucosal area (Reynolds and Morris, 1983). By day 75, the cells already form follicle-like structures, and by the last month of the gestation (d120-150), the histology is maintained unchanged. The IPP consists of tens of thousands of densely packed elongated follicles surrounded by a connective tissue capsule. The interfollicular area is sparse. In cattle, the first lymphocytes in the area are detected at day 120, primordial follicles at day 150, and the actual follicles from day 180 onwards (Ishino et al., 1991). The dominant cell population in bovine follicles is IgM^+ B cells (Yasuda et al., 2004). $CD3^+$ T cells are seen mainly in the interfollicular areas. The follicular medulla also contains an extensive network of follicular dendritic cells, at least in the last month of gestation of fetal lamb (Nicander et al., 1991). The IPP starts to involute at the age of three months in the sheep, and at the age of 18 months only a few follicles remain visible (Reynolds and Morris, 1983).

Jejunal Peyer's patches (JPPs) differ for IPP in their histology and ontogeny. The IPP in the sheep is one huge aggregate of follicles, whereas in one JPP (of which there are tens) there are about hundred pear-shaped follicles with a broad interfollicular space (Reynolds and Morris, 1983). The JPPs follicles developed earlier in the fetal period, at day 65 of sheep fetal period versus at day 110 for IPP (Reynolds and Morris, 1983). JPPs remain intact throughout life and do not involute in puberty as the IPP. There are also differences in the cellular composition of the different Peyer's patches after birth, as there are more T cells in JPPs (Miyasaka et al., 1983; Hein et al., 1989). These findings, and others (discussed in (Yasuda et al., 2006)) suggest that the natures of the two kinds of Peyer's patches are different: IPP is responsible for the production of the B cell mass whereas JPPs have more the character of a secondary lymphoid tissue.

As the cells populating the sheep IPP are $mIgM^+$ (Griebel et al., 1992), they are immigrants of relatively advanced state of B cell development. By analyzing the LC rearrangements of the cells in the same follicle, it

seems that very limited number of rearrangements have occurred (Reynaud, Mackay, et al., 1991). The data from cattle leads to the same conclusion; the low amount of Ig gene configurations in a single IPP follicle suggests that it is derived from a small number of B cell clones, in other words that the follicles are oligoclonal (Niku et al., 2002). The oligoclonality suggests also that the entry to the follicles is limited. Indeed, Press and coworkers showed (Press et al., 1996) that a single injection of anti-IgM antibodies to fetal lamb at day 63 prevented the formation of the IPP follicles and caused a depletion of IgM^+ cells. These results reveal that a single wave of IgM^+ cells immigrate the *lamina propria* from the blood circulation, a small number of cells enter each follicle and start to proliferate without further Ig rearrangements.

The evident question raised by these observations is from where do these IgM^+ cells originate? Sheep hematopoiesis is known in detail: it begins in the yolk sac between days 19 and 27 (Al Salami et al., 1985). At the same time, the liver becomes hematopoietic and remains as the main hematopoietic tissue for the rest of the fetal period. Cells positive for leukocyte common antigen CD45 are first seen in the spleen at days 40-45 (Press et al., 1993). By day 48, IgM^+ cells are first being observed in the spleen, and during the next month they proliferate vigorously. The spleen has also been found to be the site of early *IGLV* expression and diversity, both in sheep and in cattle (Lucier et al., 1998; Jeong et al., 2001). The question of whether these splenic B cells could contribute to the colonization of the IPP was addressed by performing a splenectomy, a surgical removal of the spleen, on a 56-day-old fetal sheep (Press et al., 2001). No effect was seen in the composition of the IPP, which indicates that other sites are responsible for the production of Ig gene rearranged immature B cells, which will be the founder cells in the IPP follicles. Sheep fetal bone marrow, when compared to mouse and man, is not a rich source of B lineage cells, as discussed in Reynolds (1997). Approximately 5% of all nucleated bone marrow cells in sheep are B cells, compared to 25-30% in rodents (Osmond and Nossal, 1974). This may be taken as an indication that ovine bone marrow can not bear the responsibility of B lymphopoiesis.

The cell proliferation rate in ovine IPP follicles is extraordinary, even for lymphoid tissue: 5% of the follicular B cells enter metaphase and divided each hour, which is ten times greater than the rate for T cells in the thymus (Reynolds, 1987). If all these cells and their daughter-cells stayed in the follicles and continued dividing, after six days the IPP's weight would be equal to the weight of the whole animal (Reynolds, 1997). It has been shown that less than 5% of the cells emigrate from the tissue (Pabst and Reynolds, 1986). Most cells produced in the IPP die rapidly by apoptosis (Motyka and Reynolds, 1991). The fact that only a small proportion of the produced cells are exported, and the others are doomed to death, implies that some kind of selection event exists. In addition, other actions, such as maturation during the fetal period and age-related involution, are shared between the sheep IPP and the T cell producing thymus. This suggests that the IPP, similar to the thymus, could act as a location for repertoire generation and selection.

Cattle and sheep use somatic hypermutation and gene conversion

The high death rate for follicular B cells in the IPP suggests that a selection event occurs. But what is the basis for the selection, when each follicle is constituted of similar clones of the few founder cells? Reynaud and colleagues (Reynaud, Mackay, et al., 1991) showed by sequencing the sheep IGL locus that Ig genes are still further diversified in the IPP follicles by SHM. Interestingly, this SHM process is independent of foreign antigen, in contrast to the antigen-driven reaction in secondary lymphoid organs (Reynaud et al., 1995). The rate of the mutational process in IPP is comparable to that in the secondary response in the germinal centers in mouse ($0.5 \times 10^{-4} – 1.2 \times 10^{-4}$ / bp per division) (Reynaud, Mackay, et al., 1991). These mutations start at the fetal period, accumulate over time, and concentrate in the CDR regions. A 10-fold clustering of the mutations in CDRs versus framework (FR) regions is detected (Reynaud et al., 1995). Mutations seem to

be addressed to specific codons in CDRs, and replacement mutations seem to be favoured. The SHM generates more diversity to compensate the limited amount of V region gene segments used in the rearrangements. The sheep IGL locus contains many variable region genes, but half of them are pseudogenes, and only two *IGLV* genes contribute to 50% of the rearrangements (Reynaud, Mackay, et al., 1991).

When compared to sheep, there are few studies on the antibody diversity mechanisms in cattle. In one study, sequences from 11-day-old calf show evidence of the GC in the λ locus (Parng et al., 1996). The authors come to the conclusion of GC because of several facts: the λ locus contains a high proportion of pseudogenes, the repertoire in the IPP is composed of a small number of VDJ rearrangements, and the modifications could be traced to the pseudogenes. Another study found evidence of GC but also of SHM in the fetal spleen, prior to the stage of IPP (Lucier et al., 1998).

5.2.2. Chicken is the best studied example of a GALT-species

A continued exploration of different species regarding GALT, by commencing with the chicken model is justifiable, because as mentioned, the avian specific lymphoid organ "bursa of Fabricius" imparted the name for B cells. In fundamental experiments from the 1950's, Glick and colleagues noticed that the surgical removal of bursa after hatching led to a severe inability to produce antibodies after immunization with *Salmonella typhimurium* O antigen (Glick et al., 1956). The avian bursa is a sac-like lymphoid organ located in the dorsal wall of the cloaca. It is composed of longitudinal folds within which there are about 10000 lymphoid follicles. It seems to serve not only as a place for B cell diversification and B cell mass production, but also as a peripheral lymphoid organ.

The chicken LC locus is extremely compact; there is only one locus that spans 25 kb. In that locus, there is a unique functional *IGLV* which rearranges with unique *IGLJ*, so the combinatorial diversity is minimal (Reynaud et al., 1985; Weill and Reynaud, 1987). Upstream of *IGLV* there is a family of approximately 25 pseudogenes, *ΨIGLV*, which are in both transcriptional orientations (Reynaud et al., 1987). The IGH locus follows the same simple pattern although its genomic localization is not yet mapped; there are unique *IGHV* and *IGHJ* gene segments (Reynaud et al., 1989; Reynaud, Anquez, et al., 1991). Between the *IGHV* and *IGHJ* gene segments, there are 16 *IGHD* segments, 15 of which are highly homologous. As with the IGL locus, there is a family of pseudogenes upstream of the *IGHV* gene segment in both orientations, altogether approximately 80 to 100 *ΨIGHV*. A distinctive feature of these pseudogens is that they contain a D gene segment in addition to V gene segment, so they are considered as ΨVD_H (Reynaud et al., 1989). Another speciality is the existence of D-D junctions in a remarkable portion of rearranged B cells. As for the *IGHC* gene segments, there are Cμ, Cα and Cυ in the locus coding for IgM, IgA ans IgY isotypes (Kincade and Cooper, 1973; Magor et al., 1992).

Chicken embryogenesis takes 21 days and ends in hatching. The first B lymphopoietic signs are seen at embryonic day 5 to 6 in the yolk sac, as DJ_H rearrangements are observed (Reynaud et al., 1992).LC rearrangements can be observed at embryonic day 7 (Mansikka et al., 1990). Cells continue to rearrange their Ig loci and a few days later they spread via the circulation. Rearranging cells can first be observed in the spleen and blood, and a few days later in the thymus, para-aortic region, bursa, and bone marrow. The rearrangements at both loci take place nearly simultaneously, in a stochastic manner, and not sequentially in an ordered way, as in mammalian species. No surrogate LC or pre-BCR is described in chickens.

A few million B cell progenitors are generated in a relatively short time window in the first 10-13 embryonic days (Reynaud et al., 1992). Only the cells in the bursa will go on to expand (McCormack, Tjoelker, Barth, et

al., 1989; Reynaud, Anquez, et al., 1991). Each of the 10000 bursal follicles is colonized by approximately 3 B cell progenitors (Pink et al., 1985). A few days later (embryonic day 17), the amount of B cells in bursa has increased from 30000 to 1-2 million, whereas the amount of B cells at other sites, such as in the blood, spleen and bone marrow, has decreased (Reynaud et al., 1992).

The combinatorial repertoire in chicken is, as mentioned, minimal. As chicken only possesses TdT activity in the thymus, it is probably not taking part in the Ig gene modification (Yang et al., 1995). Some junctional diversity is noticed as there are P nucleotides in VJ_L junction (McCormack, Tjoelker, Carlson, et al., 1989), but the main mechanism of antibody diversity generation in chicken is the GC event in the bursa. In the GC, which is initiated by AID, upstream pseudogenes are used as templates and small segments of them are copied to, and replace, the corresponding region in the rearranged VJ_L and VDJ_H regions (Reynaud et al., 1987, 1989; Thompson and Neiman, 1987). Proximally located, highly homologous, and inverted orientation pseudogenes are preferred as templates (McCormack and Thompson, 1990). The 5' end of the converted sequence, which is in total 10-300 nucleotides, displays the highest homology, but the 3' end could occur in regions of nonhomology. As the pseudogenes differ mainly on CDR regions, and a single cell can undergo many rounds of gene conversions, a remarkable variation is generated especially at the CDRs (Fig. 10) (Reynaud et al., 1987).

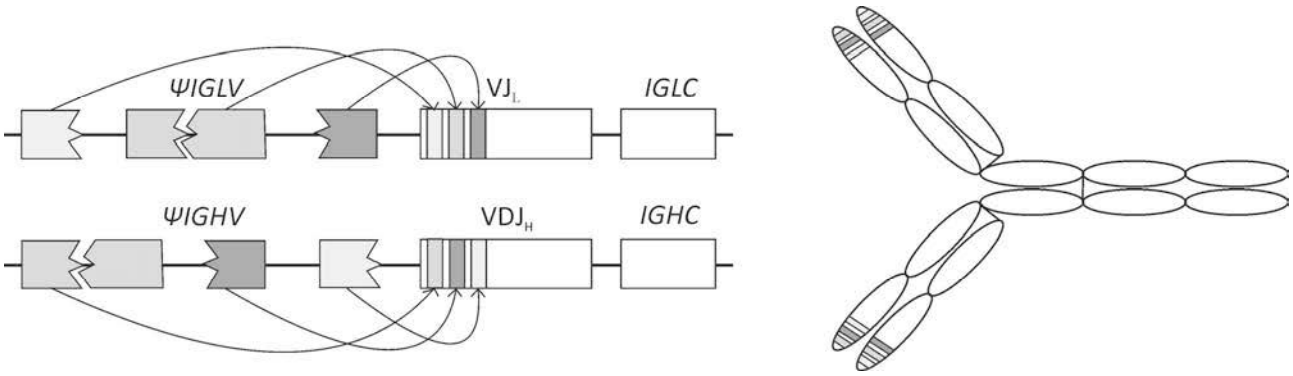

Figure 10. The gene conversion contributes to the variability in the chicken antibody molecule. Originally only single *IGHV* and *IGLV* gene segments recombines in V(D)J recombination. The diversity is achieved through post-recombinatorial gene conversion, in which upstream pseudogenes (*ΨIGLV* and *ΨIGHV*) introduce sequences into the expressed gene thus generating diversified primary repertoire. Based on Murphy et al. (2007).

At the age of two months, each of the 10000 bursal follicle contains approximately 150000 cells. The B cell emigration from bursa starts already before hatching. The estimated emigration rate after hatching corresponds to 1-5% of the daily B cell production (Lassila, 1989). Massive cell death is also seen in the bursa, as the remainder of cells die *in situ* (Motyka and Reynolds, 1991). The whole organ is involuted at the age of sexual maturity, and the only source of B cells in adult birds is the post-bursal cells, which have populated the peripheral lymphoid organs.

5.2.3. The rabbit diversifies the repertoire only after birth

The history of rabbit immune system research rests heavily on allotype studies. In 1956 Jacques Oudin demonstrated the existence of immunoglobulin allotypes, that is, allelic polymorphism of the Ig genes, in

rabbits (Oudin, 1960). Rabbit B cell development has also offered interesting findings, as can be seen by recent research.

The rabbit IGH locus harbors approximately a hundred *IGHV* gene segments (both functional genes and pseudogenes), fourteen *IGHD*, and six *IGHJ* gene segments (Gallarda et al., 1985; Becker et al., 1989; Chen et al., 1996). Rabbits have *IGHC* genes that encode IgM, IgG, IgA and IgE isotypes, and the peculiarity is the presence of 13 nonallelic Cα genes (Burnett et al., 1989). What comes to LCs, κ chains are used in 90% of the cases (Dray et al., 1963). 39 *IGKV* genes, and 2 *IGKC* genes both preceding few *IGKJ* genes have been identified (Benammar and Cazenave, 1982; Sehgal et al., 1999). The data on λ locus is limited, as this locus is hard to study due to its differential organization in different strains, as discussed in Mage et al (2006).

B lymphopoiesis begins in fetal liver at day 14 (full term 30 days), and moves to the bone marrow, demonstrated by the existence of DJ_H and VDJ_H rearrangements (Tunyaplin and Knight, 1995). The most D-proximal *IGHV* gene is used in 80 to 90% of rearrangements (Knight and Becker, 1990). The usage of *IGHD* and *IGHJ* gene segments does not offer much junctional variety, either; J_H4 is used in 90% of the rearrangements and 4 *IGHD* genes are used in nearly 80% of rearrangements (Friedman et al., 1994). In rabbit, V(D)J recombination takes place sequentially, and the presence of surrogate LC together with pro-B and pre-B cells has been shown (Jasper et al., 2003). Between birth and 2-3 weeks of age, the V(D)J rearranged B cells colonize the rabbit GALT; the IPP, *sacculus rotundus* and the appendix. The appendix in particular seems to act as a rabbit bursa-equivalent (Weinstein, Anderson, et al., 1994; Weinstein, Mage, et al., 1994). At birth, rabbits show undiversified V(D)J genes, but they become diversified by two months of age (Crane et al., 1996). The diversification happens in the appendix by a gene conversion–like mechanism and SHM, the latter especially in the D and J regions which do not have the gene conversion templates (Becker and Knight, 1990; Weinstein, Anderson, et al., 1994). This process requires the stimulation of an intestinal antigen. It is not considered to be antigen driven, but it is possibly driven by a superantigen (Rhee et al., 2004). B lymphopoiesis in the bone marrow is reduced more than 99% when rabbits reach the age of 16 weeks (Crane et al., 1996; Jasper et al., 2003). Unlike chicken bursa, and sheep and cattle IPP, the rabbit appendix does not involute but serves as a peripheral lymphoid organ for the rest of the animal's life.

5.2.4. Fetal piglets rely on junctional diversity

Swine also present immunologically interesting and unusual features, which backs up the notion that there are many ways to generate the antibody repertoire and antibody producing cells. Exceptional features include the presence of CSR already in the fetus in the absence of an external antigen, and the existence ofpro-B like cells and mature B cells in the thymus.

Swine IGH locus consists of 15 *IGHV*, 4 *IGHD*, and 5 *IGHJ* gene segments (Eguchi-Ogawa et al., 2010). Swine has the genes for all *IGHC* isotypes: *IGHM* (for IgM), *IGHE* (IgE), *IGHA* (IgA), *IGHD* (IgD) and distinctively 11 genes for *IGHG* (IgG) (Butler et al., 2009). The presence of such a large number of *IGHG*, representing 6 subclasses, is explained at least partly by gene duplication and exon shuffling. Swine has two LC loci, λ and κ, and as in primates, they are expressed in the ratio of 1:1 (Hood et al., 1967; Butler et al., 2005). The κ locus is partially mapped and contains ≈70 *IGKV* and at least six *IGKJ* genes (Butler et al., 2004), on λ locus there is no published data. The usage of different genes in swine is restricted and leads to a limited repertoire. All *IGHV* genes are members of one highly homologous gene family, and seven of them account for more than 90% of the pre-immune repertoire (Butler, Sun, et al., 2011). Of the D and J genes, 2 *IGHD* and one *IGHJ* gene are functional (Eguchi-Ogawa et al., 2010). In the κ locus only 10 *IGKV* genes, and one *IGKJ*, were used (Butler et al., 2004).

Early B lymphopoiesis in fetal piglet, detected by V(D)J rearrangements, begins at day 20 (full term 114 days) in the yolk sac (Sinkora et al., 2003). B lymphopoiesis is first detected in the embryo at day 30 in the fetal liver, where this process predominantly takes place until the bone marrow becomes active at day 45. In swine, TdT is already active from the earliest rearrangements in the yolk sac. Findings in fetal piglets indicate that there might be different rearrangement methods used at different times of fetal B lymphopoiesis; the first wave of progenitors originating from the yolk sac or fetal liver all carry productive in-frame VDJ rearrangement on one allele and no rearrangements on the second allele. In the second wave of progenitors from the bone marrow, 71% carry in-frame rearrangements, this being the predicted value if rearrangements are random and both chromosomes are involved.

Contradictory to other mammalian species, the presence of IgM, IgA and IgG transcripts in fetal tissues demonstrates that CSR already takes place prior to antigenic stimulation (Butler et al., 2001). Interestingly, fetal thymus seems to be the major source of IgA and IgG during fetal life, the reason of which remains unexplained.

The swine IPP resembles that of sheep and the chicken bursa. It develops at about fetal day 70, is dominated by B cells, and involutes several weeks after birth (Barman et al., 1997). Based on the exiguity of V(D)J recombination and low level of SHM, the swine IPP was not regarded as a site of lymphopoiesis or a site where antigen independent repertoire diversification occurs (Butler, Santiago-Mateo, et al., 2011). As there is only low level of SHM, and no GC has been proven in fetal piglets, it seems that the minimal combinatorial repertoire is widened mainly by the TdT, for which onset is extraordirily early (Butler et al., 2000). The resulting junctional diversity in CDR3 explains 95% of the repertoire diversity noticed in fetal piglets.

6. AIMS OF THE STUDY

The aim of the present work was to characterize how cattle B cells develop and how the antibody repertoire is generated in comparison with other species. The increasing detail and variation revealed by immunologic research on different animal species has shown that conventional models do not comprehensively represent the all systems. Knowledge of B cell biology is essential to understand the function of the immune system in cattle, which is intrinsically important as this species is of major global significance in the field of farming and food production.

The specific aims were:

1) To characterize and annotate the bovine immunoglobulin light chain locus including surrogate light chain genes (I, III)

2) To investigate the site and timing of bovine B cell lymphopoiesis (I, II, III)

3) To analyze the role of activation-induced cytidine deaminase (AID) enzyme in bovine fetal B cell development (IV)

4) To compare B lymphopoiesis in fetal and adult cattle (III)

7. MATERIALS & METHODS

This section summarizes the materials and methods used in this thesis work. Detailed descriptions are available in the original publications (I-IV).

7.1. Analysis of tissues

7.1.1. Tissue samples (I – IV)

Bovine tissues were obtained from local slaughter houses. No experiments were performed with live animals. Adult tissue samples were taken from animals aged between one year eight months and nine years. Fetal tissue samples were obtained from slaughtered pregnant cows. The age of the fetuses were determined by the crown-rump length (Evans and Sack, 1973), the ages being between 40 and 290 gestational days. Tissue samples were collected from the thymus, spleen, liver, ileum, lymph nodes (*lymphonodus cervicalis superficialis*, *lymphnodi jejunales*), muscle (*musculus triceps brachii*), and bone marrow (adult samples were taken from the sternum, and fetal samples from the distal epiphysis of the femur).

The time between the death of the animal and the fixation or freezing of the tissue varied. For adult tissues, the delay was maximally 3-4 hours, and for fetal tissues it was maximally up to 8-12 hours. For immunohistochemistry and RNA *in situ* hybridization, samples were fixed in 4% phosphate-buffered paraformaldehyde for 24 h at 22°C, and embedded in paraffin. Four to seven µm sections were cut with a microtome. Bone marrow samples were decalcified with approximately 8% EDTA (pH7) for one to two days before embedding. For RNA extraction, samples were either quickly frozen in liquid nitrogen and stored in at minus 80°C, or stored in RNAlater (Ambion) at 4°C.

7.1.2. Immunohistochemical stainings (II – IV)

Paraffin sections were dewaxed with xylene and rehydrated. Tissue sections were then subjected to heat-induced antigen retrieval in 500 ml of the retrieval solution at 750W for 15 min in a standard microwave oven, followed by a 20 min cooling period. The retrieval solutions were: 10 mM Tris-HCl pH 9.5, 1 mM EDTA (α-CD79α and α-AID); 50 mM glycine-HCl, pH 3 (α-CD21); 2 x SSC, pH 6 (α-IgM). After retrieval, the slides were transferred to the Shandon coverplate system (ThermoShandon), in which the rest of the procedure was carried out. Sections were permeabilized with 0.1% to 1% Tween-20 in PBS, and then blocked for endogenous biotin with avidin (10% egg white powder) and biotin (1 mg/ml D-biotin (Sigma-Aldrich)) incubations. Nonspecific binding sites were blocked by incubation with 1% goat serum. The antibodies used in this thesis work are shown in Table 2. The α-CD79α antibody (clone HM57, Dako) was reported to cross-react with bovine tissues (Mason et al., 1991). The anti-AID antibody was raised against a synthetic peptide corresponding to amino acids 185-198 of human AID, which are identical to the bovine sequence. The interspecies cross-reactivity of it was confirmed by an expected staining pattern in immunohistochemistry The primary antibody was diluted to PBS + 1% BSA, and the incubation took place overnight at 4°C. On the next day, the sections were incubated for two hours at room temperature with the α-mouse Ig secondary antibody (with α-AID the secondary antibody was anti-rat IgG(H+L)). To perform tyramide signal amplification, sections were incubated in avidin D–conjugated peroxidase (Vector), in biotinylated tyramide (Hopman et al., 1998), again in avidin-peroxidase, and finally in the diaminobenzidine substrate. The

sections were counterstained with Mayer's hematoxylin and embedded with Faramount mounting medium (Dako). The immunostained sections were photographed using a Leica DM4000 microscope equipped with an Olympus DP70 camera and Cell^P software (Olympus).

Table 2. The antibodies (or equivalents) used in this thesis work.

Specificity		Name and manufacturer	Application
CD79α	human	HM57, also R-phycoerythrin conjugated form (Dako)	IH, FC (conjugated form)
CD21	bovine	CC21 (Serotec)	IH
IgM	bovine	BM-23 (Sigma), Big73A (Vmrd)	IH (BM-23), FC (Big73A)
Igμ	bovine	Biotinylated (Kirkegaad & Perry Laboratories)	FC
AID	human	EK2-5G9, Ascenion	IH
IgG(H+L)	rat	Biotinylated AffiniPure (Jackson Immunoresearch)	IH
Ig	mouse	Biotinylated (Dako)	IH
Ig	mouse	Alexa Fluor 488 –conjugated (Molecular Probes)	FC
Biotin		Alexa Fluor 647 –conjugated *streptavidin*, used as an antibody (Molecular Probes)	FC
IH = immunohistochemistry, FC = flow cytometry			

7.1.3. RNA *in situ* hybridization (II)

The tissue sections used in RNA *in situ* hybridization were dewaxed and rehydrated similarly to that done for the sections used in immunohistochemistry. They were incubated with 7 μg/ml proteinase K (Sigma) for 15 min, fixed with 4% paraformaldehyde, and to reduce nonspecific binding, they were treated with 0.25% acetic anhydride in 0.1M triethanolamine-HCl. After, the probe was applied to the slides.

The antisense and sense probes to bovine *RAG1* were both 792 nt long, and corresponded to bases 1798-2589 of GenBank accession XM867321. The probes were produced by *in vitro* transcription. For that purpose, the corresponding cDNA was cloned into the pSTBlue-1 vector (Invitrogen). The plasmid was linearized either with PstI (sense) or SalI (antisense) (both from New England BioLabs), and *in vitro* transcribed using SP6 (sense) or T7 (antisense) RNA polymerases (both from Promega) and radioactively labeled [^{35}S]UTP (Amersham). Labeled probes were then precipitated and their amount was determined by measuring the specific activity with a scintillation counter (MicroBeta Trilux 1450, Wallac).

The amount of applied probes to the slides was 15000 counts per min/μl. The slides with applied probes were incubated overnight at 60°C. The next day, the slides were washed twice: the first wash was at 50°C, 5xSSC, 10 mM dithiothreitol; the second wash was at 65°C, 50% foramide, 2xSSC, 20 mM dithiothreitol. After that, the slides were treated with 20 μg/ml ribonuclease A (Roche) and the second wash was repeated. For detection, the slides were dipped in Kodak autoradiography NTB emulsion, which was diluted 1:1 in water, and exposed for 11 days. The slides were developed with a Kodak D-19 developer, fixed with Kodak sodium fixative, counterstained with Delafield's hematoxylin, and embedded with DePex (BDG).

7.2. Analysis of cells

7.2.1. Cells and cell lines (II – III)

For cell culture and flow cytometry, pieces of bovine lymph node and bone marrow were collected and maintained in medium (lymph node: RPMI1640 (Lonza) + 5% FBS (Euroclone); bone marrow: α-MEM (Gibco) + 20% FBS (ATCC) + 1 x Antibiotic Antimycotic solution (penicillin G 100 units/ml, Streptomycin sulfate 0.1 mg/ml, amphotericin B 0.25 μg/ml) (Sigma-Aldrich)) at 4°C, and processed as quickly as possible.

Tissue pieces were mechanically disrupted by cutting with scissors and further tearing with tweezers. With bone marrow pieces, bone forceps were also used. These actions released the cells from the tissue to the media, which was then filtered through a 40 µM or 70 µM cell strainer (Becton Dickinson). Red blood cells were removed with hypotonic lysing buffer (155 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM EDTA). In some cases Ficoll-Paque gradient centrifugation was performed before red blood cell removal. The cells were suspended in the media and calculated with a hemocytometer. After that, they were ready to use in immunofluorescent stainings or in cell culture.

OP9 murine stromal cell line (Nakano et al., 1994) was ordered from ATCC. It was maintained in α-MEM medium (Gibco) supplemented with 20% FBS (ATCC), 1.75 g/l sodium bicarbonate, 1 mM L-glutamine, 50 µM β-mercaptoethanol (Gibco) and 1 x Antibiotic Antimycotic solution (described above).

7.2.2. Cell culture (III)

OP9 murine stromal cells were used to form co-cultures with bovine bone marrow cells. In this co-culture system, the stromal cells provide essential yet uncharacterized contacts and soluble factors for the developing B cell precursors. OP9 cell confluency before the addition of the bone marrow cells was essential, so OP9 cells were grown in advance to form a 50 – 80% confluent monolayer at the bottoms of a six-well cell culture dishes. To each well, 1 x 10^7 bone marrow cells were then added. The formation of lymphoid cell colonies was followed and the cultures were fed once a week by replacing half of the medium. After one week's culture, the OP9 cells were usually overconfluent, so the culture was refreshed: bone marrow cells were collected by vigorous pipetting in the wells, filtrated through the 70 µM cell strainer, centrifuged, resuspended and transferred on to a new lawn of pre-grown OP9 cells. Cultivation was continued yet another week, including the feeding also in the middle of the second week. After two weeks' cultivation, the cells were harvested using the same method, but were then used for analysis in flow cytometry and signal joint quantification.

7.2.3. Flow cytometry (II – III)

Adult and fetal lymph node and bone marrow cells were analyzed similar to bone marrow cells also after the co-cultivation with OP9 murine stromal cells. Three B lineage markers were used, as described in (Jasper et al., 2003): B cells are defined as $CD79\alpha^+$ intracytoplasmic $Ig\mu^+$ (ic-$Ig\mu^+$) membrane Ig^+ (mIg^+), and pre-B cells are defined as $CD79\alpha^+$ ic-$Ig\mu^+$ mIg^-. The antibodies used are described in Table 2. Triple staining was performed by first incubating $1x10^6$ cells/sample with 1 g/ml monoclonal antibody against bovine IgM (Big73A), which would bind to the cell surface IgM. Cells were then washed with 1% BSA-PBS and incubated with the secondary antibody Alexa Fluor 488-anti-Mouse Ig at 2 g/ml, followed by another wash. Because the two other antigens are intracellular, the cells were fixed and permebilized with Intrastain reagent (Dako Cytomation). Next, the cells were stained for the intracellular epitope of CD79α with a 10 g/ml R-phycoerythrin conjugated monoclonal human α-CD79α. Eventually the cells were stained for intracellular Ig with 0.5 g/ml biotin-conjugated α-Ig, followed by a wash and incubation with the Alexa Fluor 647-conjugated streptavidin at 0.5 g/ml. Cells were suspended in PBS and analyzed immediately with LSRII or FACSAriaII flow cytometer (Becton Dickinson).

7.3. Analysis of DNA and RNA

7.3.1. RNA extraction and reverse transcription (I – IV)

RNA was extracted from 50 to 400 mg of frozen or RNAlater preserved tissue. Frozen tissue was first crushed with a mortar, then the tissue was suspended in Eurozol or Trizol RNA extraction reagent (EuroClone, Invitrogen, respectively) and homogenized using a Polytron PT1200 homogenizer (Kinematica

AB) with a 5 mm cutter. The extraction procedure was carried out according to manufacturer's instructions. The further purification was carried out with LiCl precipitation (2.5 M LiCl, Sigma), after which the RNA was dissolved in water. The quality of the RNA was confirmed by assessing the integrity of the RNA (RIN algorithm, RNA Integrity Number) with an Agilent Bioanalyzer (Agilent Technologies). The samples with RIN > 8.0 were used for downstream applications, as that is considered as a limit of good quality RNA (Fleige and Pfaffl, 2006). Reference gene *GAPDH* expression was also analysed with qRT-PCR (see section below) and the upper limit for C_t value was 22. Part of the samples was discarded based on these criteria.

To get rid of possible genomic contamination in the RNA preparation, it was treated with RQ1 DNAse (Promega) or TurboDNase (Ambion) according to manufacturer's instructions. In the reverse transcriptase reaction 20 pmol of 18-mer oligo(dT) primer was added to 1 µg of total RNA, and RevertAid M-MuLV reverse transcriptase (Fermentas) was used according to manufacturer's instructions. RiboLock ribonuclease inhibitor (Fermentas) was added to the reaction. Reactions without the reverse transcriptase were included to control the possible residual genomic DNA contamination. The resultant cDNA preparation was used in RT-PCR or qRT-PCR analysis.

The bovine *IGHV* library from immunized calf (IV) was obtained from Tony Pernthaner, Hopkirk Research Institute, Massey University, New Zealand. Briefly, total RNA was extracted using Trizol (Invitrogen) from a lymph node after vaccinating the animal with an inactivated whole-cell *Streptococcus uberis* vaccine. RNA was DNase treated (DNase I Recombinant, Roche) and first-strand cDNA synthesized using the iScript Select cDNA synthesis kit (BioRad) with equivalent concentrations of the Oligo$(dT)_{20}$ and random primer mixes.

The primary repertoire *IGHV* library was prepared from RNA extracted from IPP of fetus aged 240 gestational days; cDNA were created with target specific primer IgHrev1, and SuperScript III First-Strand Synthesis SuperMix (invitrogen) was used according to manufacturer's instructions. The resultant cDNA was used in amplifying and cloning the library.

7.3.2. Cloning & Sequencing (I – IV)

The cloning primers are shown in Table 3. Full-length clones were prepared when possible. The lengths of the PCR products are available in the publications. The purified PCR fragments were ligated to the pSTBlue-1 vector (Novagen) or pBluescript II SK vector (Fermentas) and sequenced on ABI3130 XL 16-capillary sequencer at the DNA sequencing and Genomics laboratory, Institute of Biotechnology, University of Helsinki, using fluorescently labeled BigDye™ dideoxynucleotides. Cloned sequences have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and the accession numbers are available in the publications.

The PCR reactions amplifying the post-immunization bovine *IGHV* repertoire contained 20 ng cDNA, 0.5 µM of each of 3 reverse primers (Bov VH-R1 – Bov VH-R3, Table 3) in combination with 0.5 µM of each of one of 8 forward primers (Bov VH-F1 – Bov VH-F8), 0.2 µM each dNTP, 2.5 mM $MgCl_2$, 0.05 U Platinum *Taq* DNA Polymerase (Invitrogen) and 1x PCR buffer in a final volume of 20 µl. A 30-cycle programme with annealing temperature of 55°C was carried out. The eight reactions were combined, purified, digested, ligated to vector JSC (GenBank Accession EU109715) and transformed to DH5α-E ElectroMax cells (Invitrogen).

The primary repertoire *IGHV* library (IV) was amplified from the cDNA with Phusion High-Fidelity PCR master mix (Fisher Scientific) using primers spanning the *IGHV* region from leader sequence to *IGHCμ* region (IgHfwd1 and IgHrev1). A 18-cycle PCR program, described in the publication, was carried out. The

purified PCR products were ligated into the pCR 4Blunt-TOPO vector and transformed into TOP10 *E.coli* cells (Invitrogen).

After overnight growth, a total number of 384 clones were picked from both libraries. Plasmid DNA was isolated using alkaline lysis miniprep procedure in 96-well microtiter plates (Birnboim and Doly, 1979), and used as a template in amplification with vector specific T7 and T3 primers (primary repertoire library) and JSCmcsFwd and JSCmcsRev1 (post-immunization library). The PCR products were sequenced as described above.

Table 3. The cloning, expression analysis and sequencing primers, and hydrolysis probe assay primers and probes used during this thesis work.

Primer	Publication	Use	Sequence
VPREB1			
VPREB1-f2	I, III	C, Er	5'-catgtcctgggccctcgt-3'
VPREB1-r1	I, III	C, Er	5'-gcccagcctccttgtccac-3'
QVPreB1_f1	III	Eq	5'-gctccaccgcacaggatgcag-3'
QVPreB1_r1	III	Eq	5'-tctcagcaggaatctcgggcgg-3'
VPREB2			
VPREB2_fw2	III	C	5'-tgtgagcaccatggcctggac-3'
VPREB2_rev2	III	C	5'-tgctggtttgaggaaaatcggttgc-3'
QVPreB2_f2	III	Eq	5'-gcgacactaacgcttacacg-3'
QVPreB2_r2	III	Eq	5'-tttgaggaaaatcggttgct-3'
QVPreB2_f1	III	Eq	5'-tgttcctctctcactacacaggttc-3'
QVPreB2_r1	III	Eq	5'-aggaggtaccggagagggttcc-3'
VPREB3			
VPREB3-fw1	I	C, Er	5'-tgtgtggaggtcccgaag-3'
VPREB3-fw2	I, III	C	5'-cgcagaacagcggactcct-3'
VPREB3-rev1	I, III	C, Er	5'-aggtcaggagtagaagtgg-3'
QVPreB3_f1	III	Eq	5'-ccctcctggcagcctccca-3'
QVPreB3_r1	III	Eq	5'-gctgcctgctcgctgctgat-3'
IGLL1			
L5-f1b	I, II, III	C	5'-ccagcgcgtctgcccaag-3'
L5-f2c	I	Er	5'-tgctggctgggcgtctgg-3'
L5-r3a	I, II, III	C, Er	5'-agaagggacgtaggggaccat-3'
lambda1fw	II	Eq	5'-ccataccagatcagcccaag-3'
lambda1rev	II	Eq	5'-agtcgctgatgagacacacc-3'
L5F21	III	Eq	5'-cccgcatggaagccccatc-3'
L5R21	III	Eq	5'-gacgccacgccagcaccta-3'
RAG1			
Rag3	II, III	C	5'-gagtcttgtgatgggatggg-3'
Rag4	II, III	C	5'-tttggtcatgagcttccggg-3'
QRag1fw	II	Eq	5'-gtcttccattccataaccag-3'
QRag1rev	II	Eq	5'-tagaactcagccgcattg-3'
RAG2			
Rag2fw	II	C	5'-tggtgtatagtcgggggaaa-3'
Rag2rev	II	C	5'-tctggggtttccatctcaag-3'
QRag2fw	II, III	Eq	5'-attcacttgccaataacatcc-3'
QRag2rev	II, III	Eq	5'-ctgatagccaccaacaataac-3'
AID			
Aicda5'	IV	C	5'-aaaaggatccgaactggattccaccatggacagcc-3'

Aicda3'	IV	C	5'-tttgaattcttcttgaaggttggtatcaaagtccc-3'
bAIDfw3	IV	Eq	5'-ggagtccagatcgccatc-3'
bAIDrev2	IV	Eq	5'-gcaagtcatcaacctcgtag-3'
GAPDH			
QGAPDfw	I, II	C, Er, Eq	5'-ctgacctgccgcctggag-3'
QGAPDrev	I, II	C, Er, Eq	5'-aagagtgagtgtcgctgttgaag-3'
PAX5			
Pax5fwc2	III	C	5'-agcatcaagcctggggtaa-3'
Pax5revc1	III	C	5'-aggctatggccttcagtgat-3'
Pax5Qfw2	III	Eq	5'-ttgagaggcagcactactcg-3'
Pax5Qrev2	III	Eq	5'-aggttggccttcatgtcatc-3''
boPAX5gen.for	III	T	5'-tgggtccatccccgact-3'
boPAX5gen	III	T	FAM-tgcgcggcgtctcttaccttcg-BHQ
boPAX5gen.rev	III	T	5'-caccaacaagcgcaagagag-3'
IGLV			
bIGLV_fw1	III	C, Eq	5'-atggccttggcccctctg-3'
bIGLV_rev2	III	C, Eq	5'-gaacagggtgaccgaggg-3'
IGLC			
bLambda qPCR fw1	II	C, Eq	5'-tcctgggtcagcccaagtcc-3'
bLambda qPCR rev1	II	C, Eq	5'-ggtggtcttcacgttgcgg-3'
SIGNAL JOINT			
boVJring.for	III	H	5'-ggtttttgtgccagcctgt-3'
boVJring	III	H	FAM-gtcactgtgcacagtgctccaggcc-BHQ
boVJring.rev	III	H	5'-ggtttttgtctcacttccccc-3'
IGHV-analysis			
JSCmcsFwd	IV	A	5'-gtgtggaattgtgagcggat-3'
JSCmcsRev1	IV	A, S	5'-cgtctttccagacgttagta-3'
IgHfwd1	IV	A	5'-ttgtgctstcagcccccaga-3'
IgHrev1	IV	A	5'-cgcaggacaccaggggggaag-3'
T3	IV	A	5'-attaaccctcactaaaggga-3'
Bov VH-F1	IV	A	5'-gatcggcgcgccadgtgcadctgcgcgagtygg-3'
Bov VH-F2	IV	A	5'-gatcggcgcgccagrtgmagtgyggggartca-3'
Bov VH-F3	IV	A	5'-gatcggcgcgccaggtgsasytgcgggagtca-3'
Bov VH-F4	IV	A	5'-gatcggcgcgccrggtgcwgctgcgsgagtcygg-3'
Bov VH-F5	IV	A	5'-gatcggcgcgccagctggagctacrggagtcg-3'
Bov VH-F6	IV	A	5'-gatcggcgcgccaggtgcrhytgcgggagtcg-3'
Bov VH-F7	IV	A	5'-gatcggcgcgccagstgcagctgcgggamkcg-3'
Bov VH-F8	IV	A	5'-gatcggcgcgccaggtgcagstgmrrgagtcg-3'
Bov VH-R1	IV	A	5'-gatcactagtgacnnggagtccttsncccca-3'
Bov VH-R2	IV	A	5'-gatcactagtgaccavvaghccnnggcccca-3'
Bov VH-R3	IV	A	5'-gatcactagtgaccaggantcbttggcccca-3'
Sequencing primers			
T7	I-IV	S	5'-taatacgactcactataggg-3'
SP6	I-IV	S	5'-catacgatttaggtgacactatag-3'

C = cloning, Er = expression by RT-PCR, Eq = expression by qRT-PCR, H = hydrolysis probe assay, A = IGHV-analysis, S = sequencing primer

7.3.3. Expression analysis by RT-PCR & RT-qPCR (I – IV)

The primers used in reverse transcription PCR (RT-PCR) are presented in Table 3. cDNA from 100 ng of total RNA was added to the reaction mixture. A 40-cycle PCR program was used to amplify the cDNA with the expression analysis primers. The results were analyzed with gel electrophoresis.

The primers used in reverse transcription quantitative real-time PCR (RT-qPCR) are also presented in Table 3. RT-qPCR was performed using SYBR green technology. cDNA from 50 ng of total RNA was added to the reaction mixture, which was either SYBR premix Ex Taq (Takara) or Maxima SYBR Green qPCR Master Mix (Fermentas). All amplifications were carried out using an Mx3005P QPCR system (Stratagene). A 40-cycle program including the last step of dissociation curve analysis was carried out. The specific programs and the product sizes are available in the publications. During all runs, standard curve serial dilutions (3 to 7 points) of the genes quantified were included, both to observe the efficiency of the reaction and to be able quantify the absolute amount of copies in the sample. The produced PCR fragments were sequenced to confirm their identity, and the dissociation curves were also analyzed during all runs for the same purpose. The expression was either normalized in relation to the house-keeping gene control (GAPDH) and in some assays further calibrated to the muscle tissue sample (no significant expression of genes of interest), or normalized in relation to the B lineage transcription factor PAX5.

As there is quite a high sequence homology between bovine *VPREB1*, *VPREB2*, *VPREB3,* and *IGLV* genes, and also between bovine *IGLL1* and *IGLC*, we tested the specificity of *VPREB* and *IGLL1* assays. The particular *VPREB*'s serial dilutions were analyzed alone and with competing templates (separately) of the other *VPREB*s and the *IGLV*. A similar competition assay was carried out with *IGLL1* and *IGLC*. The results of *VPREB* competition assays are presented in supplementary Figure 1 (III): competing templates did not have an effect on the threshold cycles of the subjected *VPREB* at the levels that were comparable to its levels in the tissues. The result of *IGLL1* competition assay (used in (II)) was that the competing template did not have an effect if the amount of *IGLL1* molecules was more than 10^3. In (III), we used an *IGLL1* assay in which the primers were located at the unique region of *IGLL1*, not similar to a constant gene.

Another thing to be aware of in the *VPREB* expression analysis is the possibility that especially *VPREB2* could in principle be expressed in both forms: unrecombined (as a surrogate LC gene) or recombined (as *IGLV*). To differentiate the expression in these cases, two primer pairs were designed. QVPreB2_f1 and QVPreB2_r1 (Table 3) amplified a product from the 5' end of the gene and it could be derived either from unrecombined or recombined DNA, whereas QVPreB2_f2 and QVPreB2_r2 amplified a product from the 3' end of the gene, flanking the RSS, so it could be derived only from unrecombined DNA.

7.3.4. Signal joint quantification by qPCR (III)

To detect ongoing or quite recent V(D)J rearrangement, signal joint (SJ) quantification was performed by quantitative real-time PCR (qPCR) with hydrolysis probe technology. SJs are present in B cell recombination excision circles that are cut from the genome during V(D)J recombination (see III / SFig. 2.). Fetal and adult bone marrow cells were taken before and after co-cultivation with OP9-cells; $1x10^6$ cells were lysed with 100 µl of Lysis Buffer II (from cells-to-cDNA –kit, Ambion). The suspension was incubated briefly, after which it was ready to use as a template in qPCR, using Maxima Probe qPCR Master Mix (Fermentas). Hydrolysis probe assays, including primers and probe, designed to quantify the signal joints between *IGLV* and *IGLJ* genes and to quantify single copy gene *PAX5*. The primers and probes are shown in Table 3. The signal joint primers were designed so that they would detect as many of the putative rearrangements as possible. The bovine specific *PAX5* primers overlapped the exon-intron boundary. The amount of signal

joints was normalized to the amount of *PAX5* using bovine specific primers that overlapped the exon-intron boundary.

7.4. Bioinformatics

7.4.1. Gene identification & annotation (I)

To find and identify bovine IGLV gene segments, cDNAs with matches in the dbEST database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) were used as initial query sequences in a blast search (Altschul et al., 1990) at Ensembl server (http://www.ensembl.org/index.html) against the bovine genomic sequence data. Databases at The Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/) and the Bovine Genome Database (http://genomes.arc.georgetown.edu/drupal/bovine/) were used to gain more evidence for the annotation. Annotations of the genomic sequences were accomplished with Apollo (Lewis et al., 2002) and Otter (Searle et al., 2004) annotation softwares.

7.4.2. Functional & phylogenetic analysis of genes (I, III)

The European Molecular Biology Open software Suite (Rice et al., 2000) was used for sequence analysis. In the *IGLV* gene extraction, several criteria were used to analyze the functionality of the gene, such as the presence of uninterrupted reading frame, consensus splicing sites at exon/intron boundaries, or likely functional signal sequences. Sigcleave, based on (von Heijne, 1986), was used to predict signal peptidase cleavage sites. Multiple sequence alignments were performed with MAFFT (Katoh et al., 2002). Phylogenetic trees were constructed in MAFFT or PHYLIP (Felsenstein, 1989) by using Neighbor Joining algorithm (Saitou and Nei, 1987), and visualized with Archaeopteryx (Han and Zmasek, 2009). Mouse chromosome 16 was examined using the UCSC genome browser (http://genome.ucsc.edu/), and the comparison between mouse and bovine genomic loci was performed using Artemis comparison tool (Carver et al., 2005).

7.4.3. *IGHV* mutational pattern analysis (IV)

The sequence data from fetal ileal Peyer's patch and postimmunization cDNA libraries was analyzed with Geneious software (Drummond et al., 2011). Pairwise identities were compared with BioEdit software (Hall, 1998). High-quality cDNA sequences spanning the FR1-FR3 region, according Kabat boundaries (Kabat and Wu, 1971), were matched to the bovine *IGHV* gene sequences and aligned on the best matching genes using the assembly function of Geneious. Replacement and synonymous mutations in the cDNA alignments and AID target motifs (WRCY and RGYW)(Rogozin et al., 2001; Yu et al., 2004; Longo et al., 2008) in the *IGHV* genes were detected in Geneious. WRCY and RGYW target motifs were detected separately in the forward strand. The numbers of mutations in AID target nucleotides (the third C in motif WRCY and the second G in motif RGYW) and in C/G nucleotides outside the AID motifs, for each individual cDNA sequence, were calculated in Microsoft Excel.

7.5. Statistical analysis (II, III, IV)

The statistical significance of the RT-qPCR data was analyzed by ANOVA with R software (R Development Core Team, 2011)(R Development Core Team, 2011). Normality of the data was confirmed with Shapiro-Wilk's test or with Lilliefors' test, and the homogeneity of variances using Barlett's test. Post-hoc Tukey HSD test was applied for pairwise comparison. In specific cases, non-parametric tests were used.

To statistically assess the role of selection (IV), focused binomial test (Hershberg et al., 2008; Uduman et al., 2011) and global binomial test (Lossos et al., 2000) were used at the web server at

htpp://clip.med.yale.edu/selection. Positive selection is indicated by a greater amount of replacement mutations than expected and negative selection by smaller amount than expected.

The proportions of mutated C/G of all AID target nucleotides and of all C/G nucleotides were compared in PASW Statistics 18, using the nonparametric sign test for related samples.

8. RESULTS

8.1. The diversity of bovine light chain genes is moderate (I, III)

8.1.1. Lambda locus is more extensive than kappa (I)

To characterize the bovine germline repertoire of immunoglobulin LC genes, they were extracted from the *Bos taurus* genome sequence Btau_3.1 (The Bovine Genome Sequencing and Analysis Consortium et al, 2009). The bovine κ locus is located on chromosome 11, and its size is approximately 280 kb (I / Table S3, S for supplementary file). The orientation of variable genes is similar, indicating that recombination takes place by deletion. In contrast, the λ locus is situated on chromosome 17. Its size could not be estimated from Btau_3.1 as there were gaps in the assembled sequence (I / Fig. 4), nor could the inversion recombination be ruled out. Most likely the λ locus is larger than the κ locus, as more variable, joining and constant gene segments are evident.

The λ locus possesses 63 *IGLV* genes, which could be divided into eight subgroups based on sequence similarity and phylogenetic analyses (I / Fig. 1, Table S2). Of these genes, 25 (41%) are apparently functional (I / Table S1). Subgroup 1 is ruminant-specific, and harbors the majority of the functional genes. One subgroup is composed of only 13 pseudogenes (subgroup 5). Variable genes in bovine subgroups 7, 8 and 9 are similar to variable genes in human subgroups 5, 8 and 9, respectively. Of the bovine *IGLV*s, 68% have ovine counterparts. There are 3 genes, *IGLV41*, *IGLV47* and *IGLV53*, which are not closely related to any human, mouse or ovine genes. 3 *IGLJ* and 5 *IGLC* genes could be identified (I / Table S1), but only 2 J-C_L pairs form functional units.

In the κ locus, there are 22 *IGKV*, 3 *IGKJ*, and a single *IGKC* gene segment (I / Table S3). The proportion of functional variable genes compared to all variable genes is similar to the λ locus, as there are 8 (36%) functional *IGKV*s. Variable genes could be divided into 8 subgroups. Subgroup 2 has all but one of the functional *IGKV*s (88%). Of the bovine *IGKV*s, 95% cosegregate with ovine *IGKV*s.

8.1.2. VPREB1, *VPREB2*, *VPREB3* and *IGLL1* exist in the lambda locus (I, III)

Surrogate LC genes *VPREB1*, *VPREB2*, *VPREB3* and *IGLL1* were identified in the bovine genome. The composition of surrogate LCs in cattle, mouse, and human, are presented in Table 4. The cDNA of all cattle surrogate LC genes could be cloned in non-recombined form. *VPREB1*, *VPREB3*, and *IGLL1* contain no RSSs. *VPREB2* contains an atypical RSS, and we first annotated it as a variable gene *IGLV21* (I / Table S1). The discovery of an EST sequence, which was derived from non-recombined *IGLV21*, and the failure to clone the recombined *IGLV21*, showed that the expression of *IGLV21* was not coupled to recombination. This, and the sequence similarity of bovine *IGVL21* to mouse *VpreB2*, promoted its new name *VPREB2*. The protein sequence identity from FR1 to FR3 between cattle and mouse *VPREB1* is 70.8%, *VPREB2* is 63.5%, *VPREB3* is 68.0%, and *IGLL1* is 58.5% (III / Table 2). As can be seen from the phylogenetic analysis in Figure 2 (III), *VPREB1* and *VPREB2* genes form a separate group across species. Indeed, the identity between *VPREB1* and *VPREB2* was 97.1% in mouse, but only 46.0% in cattle however.

Bovine surrogate LC genes were located in the λ locus. *VPREB1* and *IGLL1* were located closely to each other near the constant genes, similar to the mouse (I / Fig. 4, III / Fig. 3). *VPREB3* was also located near the constant genes, and *VPREB2* was located in the middle of variable genes.

Table 4. The surrogate light chain composition in cattle, mouse and human.

Surrogate light chain gene	Cattle	Mouse	Human
VPREB1 (in mouse: *VpreB1*)	•	•	•
VPREB2 (in mouse: *VpreB2*)	•	•	
VPREB3 (in mouse: *VpreB3*)	•	•	•
IGLL1 (in mouse: *λ5*)	•	•	•
IGLL5			•

Cattle genes were identified during this thesis work, mouse genes in (Kudo and Melchers, 1987; Kudo et al., 1987; Shirasawa et al., 1993; Dul et al., 1996), and human genes in (Bauer et al., 1988; Rosnet et al., 1999). *IGLL5* is not related to pre-B cells (Evans and Hollis, 1991; Guglielmi and Davi, 1991).

8.2. Fetal lymph node and bone marrow exhibit signs of ongoing B lymphopoiesis (II, III, this thesis)

8.2.1. Pre-B cells can be found by looking at specific CD markers (II, III)

To characterize where and when bovine B cell lymphopoiesis takes place, the fetus was chosen due to certainty that the process could be detected. *PAX5* expression reflects the amount of B-lineage cells in general, as it is active from early B cell precursors until mature B cell stage (Nera et al., 2006; Cobaleda et al., 2007). To get an overview of where the B cell-rich tissue is located in the fetus, *PAX5* expression was quantitated. This demonstrated that in the fetus, there are high amounts of B-lineage cells in the IPP and bone marrow, reasonable amounts in the lymph node and spleen, and only very small amounts in the thymus and liver (III / Table S3a). Immunohistochemistry was then employed to more closely analyze B cell precursors in specific tissues. As CD79α is an early, but CD21 and IgM are late markers in B cell development, $CD79\alpha^+$ IgM^- $CD21^-$ cells would represent pro-B or pre-B cells (Kearney et al., 1997). When forty bovine fetuses were analyzed by immunohistochemistry, stainings showed that a higher local density of $CD79\alpha^+$ cells in relation to IgM^+ or $CD21^+$cells was seen in the fetal bone marrow and lymph node, implying a $CD79\alpha^+$ IgM^- $CD21^-$ phenotype (II / Fig. 1, Fig. S2). On the other hand, the IPP although full of B cells, accommodated cells of a more developed stage, as they seemed to be IgM^+ and $CD21^+$ already in developing follicles (Fig. 11).

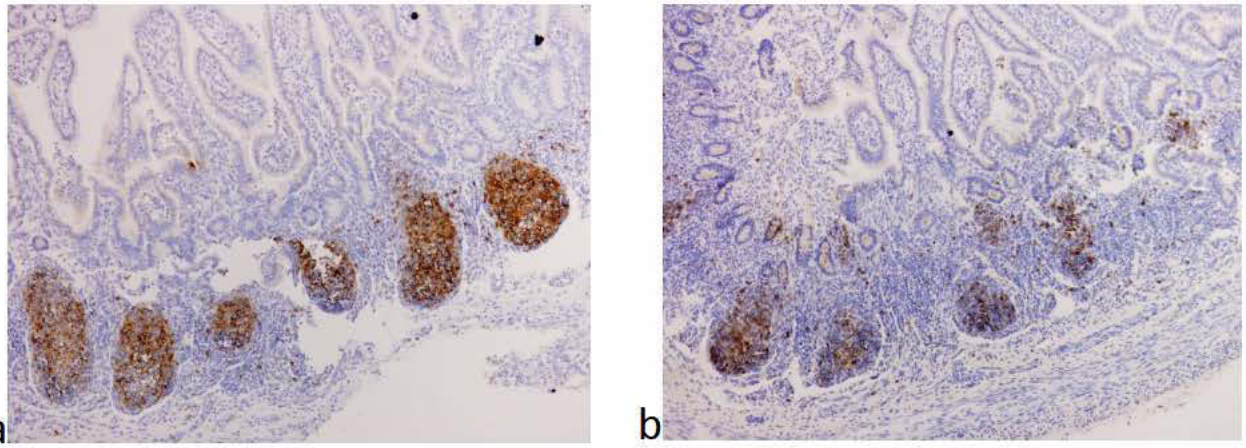

Figure 11. α-CD21 staining (a) and α-IgM staining (b) of bovine fetal IPP, at the stage where the follicles have just been formed (age 205 days). The cells in the IPP follicles are stained with both antibodies. The follicles are situated in the submucosa, between the mucosa facing the gut lumen (at the top of the images), and the muscular layer (at the bottom of the images).

To confirm that a CD79α$^+$ IgM$^-$ cell population existed in the fetal bone marrow and lymph node, cells were extracted from these tissues and a triple staining (α-CD79α, α-ic-Igμ, α-mIgM) was performed to identify pre-B cells (Jasper et al., 2003). Pre-B cells are of the phenotype CD79α$^+$ ic-Igμ$^+$ IgM$^-$, and they comprise 46% and 36% of the lymphocyte gated cells in the bone marrow and lymph node, respectively (II / Fig. 2).

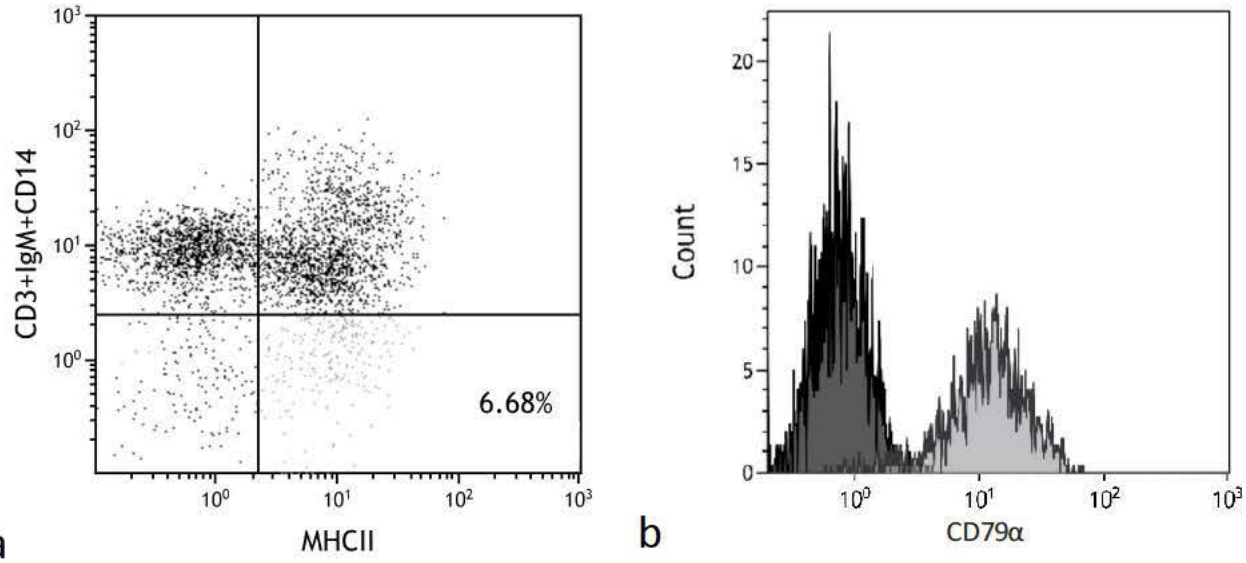

Figure 12. Pre-B cells were isolated from fetal bovine lymph node. a) The sorting strategy was to isolate MHCII single positive cells (see text). b) CD79α-staining of the sorted pre-B cells. The sorted cell population was CD79α$^+$ (light grey) as compared to the control where antibody was omitted (dark grey).

A strategy to isolate the pre-B cells from fetal tissues was then sought. Based on the finding that MHCII is already expressed at the pre-B cell stage in adult mouse bone marrow (Lam and Stall, 1994) and in rabbit bone marrow (Jasper et al., 2003), a sorting strategy based on this surface marker was developed. Lymph node cells were stained with a mixture of antibodies: α-MHCII, α-IgM, α-CD3, and α-CD14. As mature B cells

would be IgM$^+$ MHCII$^+$, T cells CD3$^+$ MHCII$^+$ and monocytes CD14$^+$ MHCII$^+$, cells which were sorted that carried MHCII$^+$ but were negative for all the other markers. These cells comprise 4-13% of lymph node lymphocytes (Fig. 12a). The sorted cells were confirmed to be B-lineage cells, as they were CD79α$^+$ (see Fig 12b).

8.2.2. *RAG* expression reveal the Ig gene rearrangement (II, III)

To obtain more evidence of the ongoing B lymphopoiesis, the expression of the recombination activating genes *RAG1* and *RAG2* was quantitated with RT-qPCR from fetal bovine tissues. The expression of these genes is connected to pro-B and pre-B cells, as the RAG enzymes are essential factors in V(D)J rearrangement, but it is also connected to T cell precursors, as the RAGs are involved in T cell receptor gene rearrangement. Thus, B lymphopoiesis could not be quantified from the thymus, but instead it was used as a positive control. Based on the mRNA expression, both *RAG*s' expression levels are elevated in the fetal bone marrow and lymph node (II / Fig. 4, III / Table 2b, III / Fig. 4).

In order to confirm the localization of RAG1$^+$ cells in the tissue an RNA *in situ* hybridization was performed. The thymus was used as a positive control. The RAG1$^+$ positive tissues bone marrow and lymph node, based on the RT-qPCR, show different staining patterns; there are distinct, single, strongly positive cells in the bone marrow tissue, but in the lymph node, positive cells are more widely spread in the medulla (II / Fig. 5). No other tissue stained positively.

8.2.3. *VPREB1* and *IGLL1* expression profiles are similar to that of *RAG*s (II, III)

As the expression of *VPREB*s and *IGLL1* is restricted to the pro-B and pre-B cell stage, they are good additional markers for B lymphopoietic tissues; thus their expression was quantified with RT-qPCR. The expression of *VPREB1* and *IGLL1* follow a similar tissue distribution as *RAG1* and *RAG2*; high levels of expression are seen in the fetal bone marrow and lymph node, and low levels in other fetal tissues (II / Fig. 3, III / Table 2b, Fig. 4).

Contrary to expectations, *VPREB2* and *VPREB3* are not expressed similarly as the other surrogate LC genes. The expression levels of *VPREB2* are very low in all fetal tissues (III / Table 2b, Fig. 4). High *VPREB3* expression was noticed in the fetal ileum; it was high as such (III / Table 2b), and when normalized to *PAX5* to indicate the cellular expression level, it was moderate (III / Fig. 4).

8.3. Activation-induced cytidine deaminase mutates the *IGHV* genes in fetal cattle Ileal Peyer's patch (IV)

8.3.1. AID is expressed in fetal IPP (IV)

There is evidence of somatic hypermutation of Ig genes in sheep IPP (Reynaud, Mackay, et al., 1991). As it is dependent on AID enzyme (Muramatsu et al., 2000), fetal bovine expression of AID in the IPP was investigated. Bovine AID was cloned recently, but there is no AID expression data in the IPP (Verma et al., 2010). Results consequently showed that AID is expressed in bovine fetal IPP. Of the four lymphoid tissues (bone marrow, lymph node, spleen, ileum) and muscle tissue studied by RT-qPCR, AID expression was strongest in the ileum and spleen (IV / Fig. 1). The mRNA expression results could be confirmed at the protein expression level; immunohistochemistry revealed the localization of AID in the ileum to the IPP follicles (IV / Fig. 2).

8.3.2. *IGHV* genes in fetal cattle IPP harbor mutations, which are focused in AID hotspot sequencies in CDR regions, and are positively selected (IV)

Mutations in an *IGHV* cDNA library from fetal IPP were examined, compared to the lymph node of an immunized calf as a positive control. In both cases, the FR1-FR3 region was examined for 1) the amount of mutations, and their distribution along the region, 2) could a difference be seen in the distribution of replacement and silent mutations between CDR and FR regions and were the mutations in these different regions positively or negatively selected, and 3) if the mutations were focused on the known AID hotspot target sequencies.

The IPP *IGHV* cDNA library from a 240-day-old fetus showed the expression of 10 different *IGHV* gene segments based on 306 reads (IV / Table 1). The mutational load was significant, 14.75 mutations / 1 kb sequence, but about 6 fold lower than in an immunized calf lymph node (IV / Table 2). The CDR regions were nearly three times as mutated as the FR regions, both in the fetal IPP and in immunized calf lymph node (IV / Table 2). The replacement mutations dominated in the CDR regions: they were 3.5 times more usual there than in the FR region, and 2.5 times more usual than silent mutations in the CDR regions (IV / Table 2). Similar, but stronger, preference was also seen in the calf, with corresponding values of 4.6 and 3.6, respectively. Ongoing selection of the mutations could be noted in both cases: mutations at the FR regions were negatively selected and in the CDR regions, they were positively selected (IV / Table 3). The dC (and the complementary dG) at the AID hotspot sequence WRCY/RGYW were more readily mutated in both, fetal IPP and immunized calf, than dCs and dGs outside the motifs (IV / Fig. 3).

8.4. B cell lymphopoiesis is impaired in adult cattle (II, III)

8.4.1. Pre-B cells are sparse in the adult tissues (II, III)

Now that B lymphopoiesis was demonstrated to take place in fetal bovine tissues, the investigation of whether the production of new Ig rearrangements is restricted to the fetal period, or if there is B lymphopoiesis also during the adulthood was pursued.

The amount of B lineage cells present in adult tissues with *PAX5* RT-qPCR. In the adult, B lineage cells are concentrated in the lymph nodes and spleen. These tissues had 107% and 165% of the amount of *PAX5* expression compared to the corresponding fetal tissues, respectively (III / Table 2a). Quite a dramatic loss of B lineage cells compared to fetus took place in the bone marrow and ileum: only 3.1% and 4.7% of the *PAX5* expression could be detected, respectively. In the bone marrow, the low number of B lineage cells could be visualized with the CD79α$^+$ staining (III / Fig. 5).

Pre-B cell populations were specifically sought from the adult bone marrow and lymph node, as these are the tissues in which pre-B cells reside in the fetus. CD79α, ic-Igμ, and mIgM triple staining showed that in both tissues the CD79α$^+$ ic-Igμ$^+$ mIgM$^-$ pre-B population is virtually absent (II / Fig. 2).

8.4.2. *RAG*, *VPREB1* and *IGLL1* gene expression is low in adult tissues (III)

RAG1, *RAG2*, *VPREB1*, and *IGLL1* share a similar expression profile among adult tissues; expression is very low in all tissues (except for the T cell-dependent expression of *RAG*s in the thymus). However, adult bone marrow shows slightly elevated expression of all these genes compared to other adult tissues. Still their expression in the adult bone marrow is low when compared to the fetal bone marrow: 1.4% of the expression of *VPREB1*, 0.5 % of *IGLL1*, 2.9% of *RAG1*, and 1.2% of *RAG2* (III, Table 2b).

The expression profiles and expression levels of *VPREB2* and *VPREB3* differ from those mentioned; they are highly expressed in the adult tissues, especially in the spleen, lymph node, and ileum. Their expression is higher than in the fetal tissues as such (copy numbers; III / Table 2b), and also when normalized to the *PAX5* expression (III / Fig. 4).

8.4.3. Adult bone marrow cells have impaired lymphopoietic capacity (III)

The low amount of B lineage cells and the low expression of *RAG*s, *VPREB1* and *IGLL1* observed in adult tissues both imply that B lymphopoiesis is not active in adult cattle. This was tested *in vitro*; adult and fetal bone marrow cells were cultivated with OP9 murine stromal cells, which support the B lymphopoiesis, and the differentiation of adult versus fetal bone marrow cells towards B lineage cells was estimated. If no signs of B lymphopoiesis in adult culture would be seen, this experimental design would help to determine if the problem *in vivo* is the adult stromal cells not capable of B lymphopoietic support, or the B cell precursors themselves, either missing or with impaired capability to mature to B cells.

Adult and fetal bone marrow cells were co-cultured with OP9 cells for two weeks. During the cultivation, colonies of morphologically lymphoid-like cells coud be seen growing attached to the stromal cells in the fetal co-cultures but not in the adult ones (Fig. 12). After the cultivation, the cultures were analyzed with flow cytometry for scattering characteristics measuring size and granularity. There was a marked difference between populations present in the adult and fetal co-cultures; a population with lymphoid-like scattering characteristics was absent in the adult co-culture, but present at fetal co-culture, constituting 36% of all the cells in the culture (III / Fig. 6). Of this population, 85% were $CD79\alpha^{+}$. To obtain evidence of ongoing λ V-J_L recombination during the cultivation, a signal joint quantification was performed. The amount of signal joints in cells recovered from adult co-cultures compared with the cells freshly isolated from the tissue and before the cultivation was begun was 2.1%, and in the fetal co-cultures it was 530% (III).

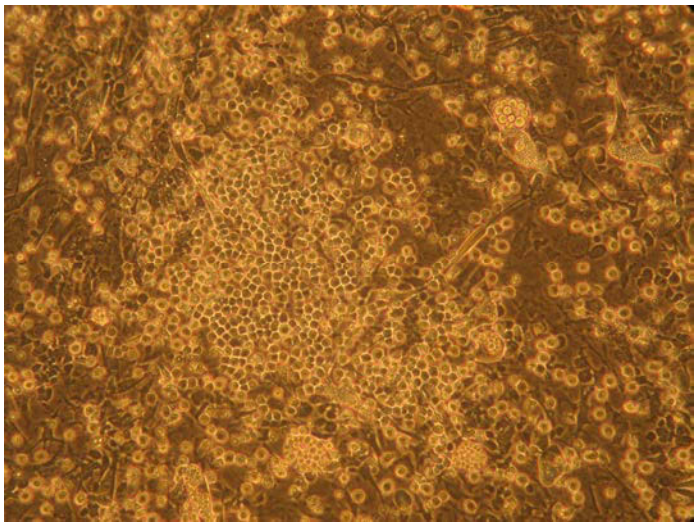

Figure 12. A colony of lymphoid-like cells observed after 7 days of the co-cultivation of OP9 cells with fetal bone marrow cells. These colonies were absent in the adult bone marrow cell co-cultures.

9. DISCUSSION

At the commencement of this project, only a slender knowledge of bovine B lymphopoiesis existed, despite a history of many decades of general B lymphopoiesis research. In the 70s, the pre-B cell stage was recognized in the mouse as an intermediate phase between the Ig HC and LC rearrangements (Raff et al., 1976). In the 80s, the molecular hallmark of this stage, the pre-BCR, was described (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987; Pillai and Baltimore, 1987). Surprisingly, it was discovered that in chicken there is no pre-B stage as the HC and LC rearrange simultaneously (Grossi et al., 1977; Reynaud et al., 1992). The main focus of this work was to characterize Ig rearrangements and the course of B lymphopoiesis in cattle. Here, an evaluation of the materials and the methods used during this study will firstly be presented, followed by a discussion on bovine B cell development based on the results and previous literature, finishing with a proposed model of bovine B lymphopoiesis.

9.1. Evaluation of materials & methods

Post-mortem tissues were used, which might have impacted on the quality of the cells and RNAs used in the analysis; high quality RNA is typically needed for gene expression analysis (Fleige and Pfaffl, 2006). The quality of RNA decreases with time after death, and the degradation process is tissue-dependent (Koppelkamm et al., 2011). For example, bovine skeletal muscle RNA is preserved for much longer than liver RNA (Bahar et al., 2007). To be sure of the quality of the samples, and the reliability of the results, the integrity of the RNA and the expression of the reference gene were examined carefully. When extracting cells from tissues (lymph node and bone marrow), the percentage of dead cells could be quantified by Trypan blue staining. One tenth and one third of the cells were dead in the adult and fetal extractions, respectively. Ficoll-Paque centrifugation was not used to remove dead cells as there are indications that this centrifugation might also remove B cell subsets (Fleisher and Marti, 2001; Bleesing, 2004). The possible cell-type-specific dyeing process cannot be assessed, but light scatter profiles as assessed by flow cytometry indicated that various bone marrow cell populations were preserved as described in the literature (O'Malley, 2002).

In gene expression analysis, the expression of a specific gene must be proportioned to an expression of a reference gene, to allow the comparison of expressions between samples. Usual choices for reference genes are *GAPDH*, β-actin (*ACTB*), or ribosomal RNAs (Bustin, 2000). The ideal reference gene should have a constant expression in different tissues of the organism in all developmental stages. As for *GAPDH*, there are results of suitability (Winer et al., 1999) and unsuitability (Schmittgen and Zakrajsek, 2000; Aerts et al., 2004; Dheda et al., 2004) for particular analyses. In a part of the work, *GAPDH* was used as a reference gene because its expression varied less between samples compared to *ACTB*, which was also tested. PAX5 was used in another part of the work, as its expression can be used to assess the relative amounts of B lineage cells in the tissue samples, and to determine the relative expression of other genes per B cell.

9.2. Drawing a model for bovine B cell development

9.2.1. Restricted germline repertoire causes modest combinatorial diversity

The bovine Ig LC locus was analyzed and annotated as an essential tool for primary repertoire analysis. The analysis was done from the *Bos taurus* genome sequence version Btau_3.1 (The Bovine Genome Sequencing and Analysis Consortium et al, 2009). It is based on whole genome shotgun sequence from a single animal (Hereford L1 Dominette 01449) with a 30% inbreeding coefficient ("Bovine Genome Project,

www.hgsc.bcm.tmc.edu/projects/bovine/,"), which facilitates the analysis by limiting the possibility of polymorphism. Although the data on the more extensive λ locus was quite fragmented, it emcompasses the whole genome, so it is likely that probably most of the functional Ig genes in both loci, λ and κ, were described.

In the bovine λ locus, 25 potentially functional variable genes existed. In human λ locus, there are 30 potentially functional variable genes (Lefranc and Lefranc, 2004). In mouse λ locus, all 3 variable genes are functional. This shows that the rearrangement potential in the bovine and the human λ locus is comparable, and higher than in the mouse λ locus. On the other hand, the bovine κ locus was found to be small containing only 8 functional variable genes. In the human κ locus there are 31-35 functional variable genes and in the mouse 93 (Lefranc and Lefranc, 2004). As can be seen, the bovine germline repertoire encoded by the κ locus is very limited compared to the man and mouse. The differential preferences of the usage of λ and κ are correlated to the sizes of the light chain loci in different animals: the bovine λ:κ is 91:9, and in mouse and man 5:95 and 40:60, respectively (Arun et al., 1996; Butler, 1997).

The variable genes in the bovine λ locus could be divided into 8 subgroups. Four of them are shared with ovine, including subgroup 1 which possesses most of the functional genes (Reynaud, Mackay, et al., 1991; Reynaud et al., 1995, 1997; Hein and Dudler, 1998). 3 subgroups are similar to human counterparts. Subgroup 10 is bovine specific as no closely related genes exist in the ovine, mouse or human genome. This work assessed only the question of bovine LC germline repertoire, not the expressed repertoire which would be analyzed from the cDNA data. In humans, it is known that the λ variable genes expressed are mostly from 3 subgroups (Ignatovich et al., 1997), and in ovine from one subgroup (Reynaud, Mackay, et al., 1991). Half of the repertoire in both species is also contributed by a few genes: 2 in sheep and 3 in humans. Only one study could be sourced on cattle λ LC usage (Parng et al., 1996). The results are based on 20 cDNA sequences, where the authors' conclude that a single family of variable genes is predominantly expressed. In addition, they see only single J-C_L unit in all rearrangements. Based on these results, one could anticipate that the LC usage in both cattle and sheep is restricted.

The bovine HC locus was not analyzed at the same time (2009) as it was mostly missing from Btau_3.1. Afterwards, it has been analyzed in our group (Niku et al., under review) by using mostly UMD_3.1 (Zimin et al., 2009), in addition to raw sequencing data available in trace and high-throughput sequence archives. Of 36 variable genes identified, 9 were classified as functional. The human HC locus harbors 123 variable genes, of which 39 are functional (Matsuda, 2004). In mouse, there are 170 variable genes, of which 101 are functional (Riblet, 2004). Altogether, it seems that the bovine HC repertoire is very restricted. HC together with the LC provides only little material to produce combinatorial diversity to widen the antibody repertoire.

9.2.2. AID mutates *IGHV* genes in fetal cattle IPP, but its contribution to antibody repertoire diversification remains unclear

The post-recombinatorial DNA modifying processes were connected to the diversification of the primary repertoire in the 80s. The limited Ig gene germline repertoire was announced to be compensated by gene conversion in the chicken bursa (Reynaud et al., 1987), or somatic hypermutation in the sheep ileal Peyer's patch (Reynaud, Mackay, et al., 1991; Reynaud et al., 1995). The reported restricted Ig gene germline repertoire connects cattle to the sheep and chicken. AID was shown to be expressed in bovine fetal IPP here. To examine if it is actively mutating Ig genes, an *IGHV* expression library from fetal IPP was sequenced. The amount of mutations indicated an active hypermutation process. In addition, an AID

fingerprint was detected as mutations concentrated in the AID hotspot sequences. The amount of mutations was lower than in the *IGHV* expression library from lymph node of immunized calf, which may be due to the short time from the formation of the IPP, as the fetus was 240 days old. In calves, it has been shown that the amount of mutations accumulates with time (Verma and Aitken, 2011). In the study by Verma and Aitken, 7 mutations/kb were seen in the CDR2 region of a one day old calf. The corresponding number in 240 day fetus described here is 3.1 mutations/kb. It is likely that the accumulating mutations would have also reached similar numbers in this study if the accumulation of mutations had been followed for the next six weeks until the birth of the animal.

The reliability of mutational analysis is dependent on the quality of the cDNA and reference sequences. Consequently, the possible PCR and sequencing mistakes were analyzed by calculating a combined error rate for PCR and sequencing using cloning vector derived sequences in the raw data. The calculated error rate in this assay was 0.22 bp / V gene, thus not affecting the interpretation of the results. These cDNA libraries may include sequences expressed from unknown *IGHV* genes or alleles, which could affect the mutation analyses. These cannot be reliably excluded from cDNA sequence data, which may contain shared mutations or dominating B cell clones. When mutation analyses were repeated with a strictly reduced data set, all cDNA sequences that clustered separately from the best matching *IGHV* gene sequences in phylogenetic trees were removed. A clear trend of overrepresentation of AID target C/G mutations over non-motive C/G mutations was preserved, although the statistical significance was lost due to a very limited total number of mutations.

Evidence is presented here to suggest that AID action and the consequent somatic hypermutation process takes place in cattle fetal IPP before significant exposure to external antigens. The negative selection on the FR regions can be explained by their consequences to the protein structure. At least two possible explanations for the positive selection of the mutations in the CDR regions exist; endogenous antigens drive the selection, or the unmutated Igs do not provide proliferative signals as efficiently as the mutated ones.

Somatic hypermutation is demonstrated here to take place in bovine fetal IPP, but its relative importance to Ig repertoire diversification remains unclear. In sheep, the IPP has been reported to act as a primary lymphoid organ of diversification (Reynaud, Mackay, et al., 1991; Reynaud et al., 1995). These studies have been challenged: the putative variability can arise from previously uncharacterized *IGLV* genes (Jenne et al., 2003). In this criticizing study, however, gene sequences were obtained from probably polymorphic cDNA material, in which the analysis is further distracted by the mutations. Also swine IPP, which is anatomically and developmentally similar to sheep IPP, has been analyzed (Butler, Santiago-Mateo, et al., 2011). The comparison of mutations in the fetal IPP, bone marrow and spleen provide no evidence for a uniquely high frequency of SHM in the IPP. It is concluded that swine IPP is not a primary lymphoid tissue and antigen independent repertoire diversification is not occurring there. Based on this and other findings, the theory of IPP being an artiodactyl “mutant breeding organ”, suggested by several groups (Jerne, 1971; Reynolds and Morris, 1983), is being questioned. Before estimating the importance of the described mutational process in fetal cattle IPP, more studies should be done: 1) mutations should be studied from other fetal tissues to compare their amounts and targeting to those in IPP, and 2) the junctional diversity should be determined. Junctional diversity is important for primary Ig repertoire generation in swine (Butler et al., 2000), and possibly important also in cattle (Koti et al., 2010) and horse (Sun et al., 2010).

9.2.3. B lymphopoiesis is active in fetal cattle and declines in adults

The question which led to this study was: Is it possible that a cow could survive whole of its twenty years' life with only the antibody repertoire generated during fetal and post-natal period? That question was sound, as B cells are produced in the IPP in ruminants, at least in sheep, but this organ involutes at puberty (Griebel and Hein, 1996). Is there *de novo* production of B cells after that? This is the first study to describe fetal cattle B lymphopoiesis, and investigate its presence in adults.

Bovine fetuses were screened for signs of B lymphopoiesis: for the gene expression of recombination enzymes *RAG1* and *RAG*, and pre-B cell connected surrogate LC gene *IGLL1*, and for the presence of population of pre-B cell phenotype $CD79\alpha^{+}IgM^{-}$. Fetal tissues with ongoing B lymphopoiesis are the bone marrow and lymph node. *IGLL1* expression is highest at these tissues, as well as the extrathymic *RAG* expression. The presence of *RAG1* mRNA was confirmed with *in situ* hybridization. Based on immunohistochemical stainings and flow cytometric analyses, pre-B cell populations were present in fetal bone marrow and lymph node. In sheep and cattle the studies of the B lymphopoietic sites have been controversial and inadequate. Spleen has been implied as B lymphopoietic tissue in both species (Press et al., 1993; Lucier et al., 1998; Jeong et al., 2001). There are also results from further studies indicating that spleen could not be responsible for B lymphopoiesis and the production of pre-IPP B cells, as splenectomy did not affect the formation of B cell follicles to IPP (Press et al., 2001). The work presented here is one of the few, in which B lymphopoiesis of an artiodactyl is studied with modern methods. Additionally, a recent report on the pig describes B lymphopoietic capacity of the bone marrow of another artiodactyl species (Butler, Santiago-Mateo, et al., 2011).

These observations were confirmed in the following series of experiments, which showed that the expression of *VPREB,* another part of the surrogate LC, was expressed in fetal bone marrow and lymph node like the *RAG*s and *IGLL1*. We first described *VPREB1* and *IGLL1* based on the sequence similarities. Further investigation confirmed the homologous relationship of these genes in cattle and mouse: in both *VPREB1* and *IGLL1* are located next to each other in the λ locus, in opposite transcriptional orientation to constant genes (Shirasawa et al., 1993). Also flanking genes next to *VPREB1* are conserved in both species. Mouse *VpreB1* and *λ5* (*IGLL1*) assemble to form a surrogate LC complex expressed specifically in pre-B lymphocytes (Sakaguchi and Melchers, 1986; Kudo and Melchers, 1987). Mouse surrogate LC expression is needed for the proliferation of a HC rearranged population (Kitamura et al., 1992; Mundt et al., 2001; Shimizu et al., 2002). Surrogate LC is needed for normal B cell development, and its absence causes an incomplete block and substantially delayed B cell development. As bovine *VPREB1* and *IGLL1* are homologous to mouse counterparts, and their expression profile among fetal tissues is similar to *RAG*s, it can be concluded that they are most likely expressed in pre-B cells in cattle. Furthermore, the expression of *VPREB1* and *IGLL1* in cattle supports the existence of a pre-B cell stage during cattle B lymphopoiesis. Similar findings have been described in rabbits; *VpreB* and *λ5* are expressed selectively in pro-B and/or pre-B cells in rabbit bone marrow (Jasper et al., 2003).

The question of the existence of B lymphopoiesis in adult cattle was then addressed. Cell populations with pre-B cell characteristics, abundant in the fetal bone marrow and lymph node, were practically absent from the corresponding adult tissues. There was no *VPREB1* and *IGLL1* expression, and no extrathymic expression of *RAG*s, in any adult tissues. These results indicate that B lymphopoiesis is not active in adult cattle. Although in mouse and man B lymphopoiesis is active throughout life, for example in chicken, V(D)J rearranged progenitors are generated in only a short time window during embryonic development (Weill

and Reynaud, 1998). Another example is rabbit, in which B lymphopoiesis is reduced over 99% by the age of 16 weeks, measured by the amount of B cell recombination excision circles (Jasper et al., 2003).

The absence of B lymphopoiesis in adults is well suited to the hypothesis of origin of the B cells in rabbits. All rabbit B cells express CD5 (Raman and Knight, 1992), which is the marker for B-1 lineage in mouse (Manohar et al., 1982; Hayakawa et al., 1983, 1984). In mouse, B-1 cells and B-2 (conventional) B cells develop from separate precursors (Hayakawa et al., 1985). B-2 cells develop throughout life from precursors in the bone marrow (Raff et al., 1976), whereas B-1 cells develop early in ontogeny and maintain themselves by a self-renewing process (Hayakawa et al., 1986; Förster and Rajewsky, 1987). The restricted usage of *IGHV* genes is also a common denominator in rabbit B cells and B-1 lineage cells in mouse (Tarlinton et al., 1988; Becker et al., 1990; Carmack et al., 1990; Knight and Becker, 1990). Herzenbergs and co-workers have represented a model of evolutionary layering in the immune system where B-1 cells are of more primitive origin and present in many species, but B-2 cells have evolved in addition as a more adaptable system in some species, including mouse and human (Herzenberg et al., 1992). Rabbit might be a species in which B-2 cells never developed. Cattle B cells have been suggested to also belong to the B-1 lineage (Naessens, 1997). Our results demonstrating the absence of the B lymphopoiesis in adult cattle would support this hypothesis.

To assess the differences of the B lymphopoietic capacity between fetal and adult bone marrow at the cellular level, bone marrow cells from both were cultivated with murine OP9 stromal cells supporting B lymphopoiesis. OP9 stromal cell culture could not support adult bone marrow B cell development. In fetal, but not in adult, bone marrow – OP9 co-culture colonies of proliferating cells appeared in contact with the stromal cells. After two weeks of co-cultivation, a massive population of $CD79\alpha^{+}$ cells was present only in fetal co-culture. In addition, the number of B cell recombination excision circles increased in the fetal co-cultivation, but decreases dramatically in adult co-cultivation. The possible explanations are that precursors for B cells are either absent from adult bone marrow or that their differentitation capacity is lost. The third possibility is that OP9 cells cannot support adult B cell differentiation for an unknown reason. The question of whether B lymphopoiesis can be reactivated in the adult under specific circumstances was not assessed. In rabbits, green fluorescent protein marked adult bone marrow cells differentiated to pro-B and pre-B cells in young recipients (Kalis et al., 2007). B lymphopoiesis could also be re-initiated in adult bone marrow after sub-lethal irradiation. In cattle, B cell depletion by injection with unspecified anti-B cell antigen did not succeed, although T cell depletion by anti-T cell antigen did (Naessens et al., 1998). The authors speculated that a continuous high rate of B cell production prevented the depletion. It remained unclear if the injected amount of the monoclonal was sufficient to deplete B cells from all immune compartments (*e.g.* the IPP of these young animals), in which case the emerging cells would really arise from *de novo* V(D)J rearrangements.

In addition to surrogate LC genes *VPREB1* and *IGLL1*, two other *VPREB*s, *VPREB2* and *VPREB3*, were also described. First *VPREB2* was annotated as λ variable gene *IGLV21*. Its atypical RSS and an unrecombined BLAST hit, together with its similarity to murine *Vpreb2* suggest it is a *VPREB*. To clarify the situation, the level of mRNA derived from non-recombined gene to that from either non-recombined or recombined genes was compared by RT-qPCR. There was no statistically significant difference, which indicates that *VPREB2* does not require recombination for transcription. The data does not exclude the possibility of *VPREB2* recombination under specific circumstances, but however, its cloning in recombined form remained unsuccessful.

The sequence similarity between bovine and murine *VPREB2* is most prominent at the N-terminal region. At the C-terminal region, which is surrogate LC specific, the similarity is low. This C-terminal region in *VpreB1* is probably responsible for the interaction of *VpreB1* with the µHC CDR3, which has been shown with human *VPREB1* (Bankovich et al., 2007). Murine *VpreB1* and *VpreB2* are 97% identical and both can assemble with *λ5* (Dul et al., 1996). The C-terminal part of bovine *VPREB2* being dissimilar to murine counterpart questions bovine *VPREB2*'s ability to assemble to form the surrogate LC. A RT-qPCR assay showed that *VPREB2* was not expressed in the fetal tissues, but it was expressed prominently in adult tissues, namely in the spleen and bone marrow. Similar expression in adult tissues was seen with bovine *VPREB3*: It was expressed in adult spleen and lymph node with exceptionally high levels. *VPREB3* was expressed also in fetal IPP, all of these being tissues where B lymphopoiesis does not take place, but where there are high numbers of more mature B cells. The expression of bovine *VPREB3* is not connected to pre-B cells. The situation is the same in other species, since VPREB3's function appears also to be dissimilar to VPREB1/2's function. In mouse, VPreB3 has been suggested to have a chaperone-function, as its association with µHC precedes µHC's association with λ5 and VpreB1/2 (Ohnishi and Takemori, 1994). Furthermore, VpreB3 was not detected at the cell surface complexes, contrary to VpreB1. VPREB3 has also been described in chicken, which lacks pre-B cells, as the HC and LC rearrange simultaneously (Rosnet et al., 2004). It was expressed in IgM^+ bursal cells and appears to bind free LC molecules preventing their secretion. All known VPREBs, including bovine, lack the surrogate LC specific C-terminal part of the molecule present in VPREB1 (Shirasawa et al., 1993; Rosnet et al., 1999, 2004). In summary, bovine *VPREB2* and *VPREB3* probably have functions unrelated to surrogate LC, shown by their expression uncoupled to *VPREB1*, *IGLL1* and *RAG*s, and supported by the lack or dissimilarity of the surrogate LC specific C-terminal part of the molecule.

The proposed model of bovine B lymphopoiesis based on the studies in this thesis: Bovine B lymphopoiesis begins by Ig gene rearrangements in fetal tissues, specifically in the bone marrow and lymph node, demonstrated by *RAG* expression already during the second trimester of the fetal period. The expression of surrogate LC genes *VPREB1* and *IGLL1*, and the presence of phenotypic population, indicate the existence of pre-B cell intermediate stage. *De novo* production of B lymphocytes can be detected in the bone marrow and lymph node until the end of fetal life. During the last trimester of fetal development, immature B cells immigrate to the follicles in the IPP, where they proliferate and where Ig genes are hypermutated by AID mutator protein. After birth, the B lymphopoietic capacity of the animal is impaired and new B cells are no longer generated through Ig gene rearrangements. Cattle manage the rest of their life with the peripheral B cell compartment produced in the IPP during fetal and neo-natal period.

10. CONCLUSIONS

a) Bovine immunoglobulin κ and λ loci, consisting of variable, joining, and constant genes, and of surrogate light chain genes *VPREB1*, *VPREB2*, *VPREB3,* and *IGLL1*, was characterized. The amount of different genes in the locus is only moderate, and the percentage of unfunctional pseudogenes is high.

b) Bovine B cell lymphopoiesis takes place in the bone marrow and lymph node during fetal development, and proceeds through a pre-B cell stage.

c) The bovine activation-induced cytidine deaminase (AID) is expressed in fetal ileal Peyer's patch, where it mutates *IGHV* genes, but its relative contribution to antibody repertoire diversification remains unclear.

d) B cell lymphopoiesis is not active in adult cattle. Its possible reactivation under specific circumstances needs further investigation.

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