Tracing the editing history of a single B lymphocyte

Dissertation in Biology

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1. Summary

As part of the humoral immune system, the B cell receptor (BCR) is expressed on B lymphocytes and later, after modification, secreted as an antibody. It is synthesized from a rearranged immunoglobulin (Ig) heavy (H)-chain gene and a rearranged κ or λ light (L)-chain gene. This thesis investigates B cell development as a function of H- and L-chain gene rearrangement in the so called nuclear transfer mouse.

At the beginning of this work the exact germline configuration of the Igλ locus was unknown even in normal mice. Hence, a physical map of the mouse λ L chain and related loci was created: The λ locus was found to stretch over three sections (Vλ2-VλX; Jλ2-Cλ2-Jλ4-Cλ4 and Vλ1-Jλ3-Cλ3-Jλ1-Cλ1), spanning 179,346 bases on chromosome 16. Furthermore, the surrogate L-chain gene $V_{preB}2$ was located 1,077,001 bases downstream of the λ locus; $V_{preB}1$ is 2,180,618 bases 3` of the λ locus and the λ5 gene is located 4,667 bases downstream of the $V_{preB}1$ locus.

Even though the diploid B cells have two H-chain alleles and two alleles for each L-chain locus, κ and λ, antibodies are only expressed from one H-chain allele and one L-chain allele. This phenomenon is called allelic exclusion. Seemingly contradicting allelic exclusion, a distinctive feature of the nuclear transfer mouse is the Ig gene configuration with one H and two κ gene rearrangements. In this work, it was determined that both κ alleles of the mouse are productive, i.e., in-frame. For a functional analysis, mice with the H chain transgene in combination with one or both κ chain genes were generated, some of them on a RAG deficient background. In the absence of RAG, endogenous gene rearrangement is not possible, nor is editing of preformed Ig transgenes. In the absence of RAG, the H chain combined with either κ chain led to a functional BCR on mature B cells. The antibodies containing either κ chain were also detected in the serum of mice, and one of the two HxL combinations was found to be self-reactive.

In general, B cells destroy receptor autoreactivity by editing, i.e., by replacing the self-reactive L-chain allele while preserving allelic exclusion. In RAG sufficient nuclear transfer mice with the autoreactive BCR, however, editing failed to destroy self-reactivity. Instead, a second κ allele was recombined, in addition to the pre-existing one. Breaking allelic exclusion, the surviving B cells recreated dual receptor expression, as

presumably was the case in the original B cell that gave rise to the nuclear transfer mouse. These results indicate that receptor editing does not necessarily destroy the self-reactive allele; and that in normal mice autoimmune antibodies may be fellow travelers in B cells contravening allelic exclusion.

RAG mediates recombination and editing in normal mice, but recombination activity is unnecessary in mice with transgenic Ig genes. Hence, expression of RAG in Ig transgenic mice before Ig genes synthesis may give rise to an artifact that just looks like editing. To address this possibility, RAG expression was analyzed in mice with the H and different L chains using the green fluorescent protein (GFP) as a substitute marker. In mice with the non-autoreactive BCR, very few GFP^+ B cells were found. However, in Ig wild-type B cells and cells with the autoreactive BCR, GFP was widely expressed. These results suggest that in most cells the transgenic Ig loci are expressed before RAG is synthesized; furthermore, that the autoreactive receptor induces BCR editing by re-expressing RAG.

2. Introduction

2.1 The dogma of clonal selection

The central dogma in immunology is Burnet's theory of the clonal selection (Burnet, 1959). It postulates that a single B lymphocyte produces only one out of the vast repertoire of antibodies, present as antigen receptors on the cell surface. If the antigen receptor is capable of reacting with the antigen, the lymphocyte is activated to proliferate (clonal expansion). This theory also easily explains how the immune system tolerates itself. For B cells tolerance is established early in development when the cells are exposed to autoantigens. Upon binding to cognate antigen at this maturation stage, B cells are eliminated, rather than stimulated to proliferate. Even 50 years later this basic concept prevails.

2.2 Central B-cell development

2.2.1 H-chain rearrangement

In mammals the B cell receptor (BCR) is encoded by the immunoglobulin (Ig) heavy- (H) and light- (L) chain genes, both of which are assembled from pools of different gene segments by combinatorial recombination (Fig. 2.1, top line, Fig. 2.3). During B-lymphocyte development in the murine bone marrow, hematopoetic stem cells go through the early lymphoid progenitor, the pre-progenitor (pre-pro B cells), the progenitor (pro-B cells) and the precursor (pre-B cells) cell stage before becoming immature B cells (ten Boekel et al., 1995; Tonegawa, 1983). This so-called "central development" is dependent on the presence of the stromal microenvironment of the bone marrow and its growth factors (Carsetti, 2004). It is an ordered process, in which the H-chain locus is rearranged and expressed before the L-chain loci (Alt et al., 1984; Lennon and Perry, 1990). Starting at the H-chain locus, variable (V), diversity (D) and joining (J) gene segments are recombined to generate an H-chain gene (Fig. 2.1) (Tonegawa, 1983). The recombination is mediated by the recombination-activating gene 1 (RAG1) and RAG2 (Fig. 2.2), which together encode the endonuclease RAG (Oettinger et al., 1990; Schatz et al., 1989). For the active RAG enzyme both RAG1 and RAG2 are required (Mombaerts et al., 1992; Shinkai et al., 1992). RAG introduces double strand breaks at short conserved

recombination signals that flank the Ig gene segments. Subsequently ubiquitous DNA repair proteins of the nonhomologous DNA end-joining pathway ligate these breaks forming contiguous V(D)J segments (Fig. 2.1) (reviewed by Bassing et al., 2002).

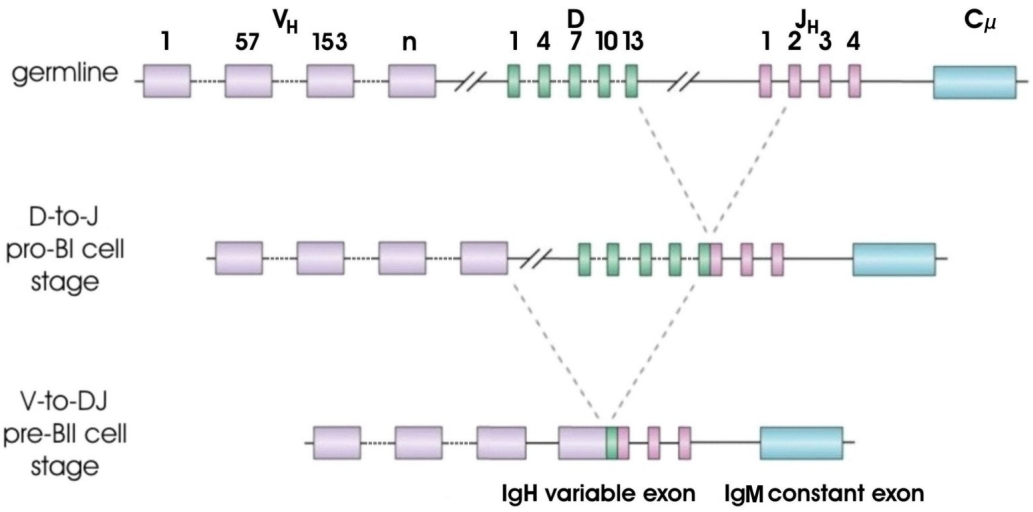

Figure 2.1. **The mouse Ig H-chain locus (IgH) and its recombination steps.**
The top line represents the germline configuration; the second line is a partial rearranged allele DJ_H, and the third line a complete, functional VDJ rearrangement encoding the variable H-chain exon. V_H, variable, D, diversity and J_H, joining gene segments; C_μ, IgM constant gene segment.

At the pre-pro-B cell stage, the H-chain gene segments are in their germ-line configuration (Rolink et al., 1995). Beginning with the transition to the pro-BI cell stage one D and one J_H segments are being recombined on both alleles to form DJ_H segments (Fig. 2.1, second line) (Alt et al., 1984). During the transition of pro-BI cells into large pre-BII cells, a V segment is joined to the DJ_H segment on one allele to generate a contiguous VDJ segment (Fig. 2.1, third line) (Alt et al., 1984). No further canonical rearrangements are possible on this allele, because all D regions are excised. Once transcribed, the gene segment is spliced to the constant gene segment of the μ chain (Cμ), and translated in the cytoplasm as μH chain. For translation into a functional H chain, the rearrangement of the coding segment must be "productive" (denoted VDJ^+), i.e. be in-frame with no premature stop codons. Combined with the surrogate L-chain proteins λ5 and either $V_{preB}1$ or $V_{preB}2$, the μH chain is presented as membrane bound pre-B cell receptor (pre-BCR) on the cell surface (Fig. 2.2). The surrogate L chain genes are expressed from the pro-B cell stage through the large pre-BII cell stage (Dul et al., 1996; Karasuyama et al., 1994; Kudo and Melchers, 1987; Kudo et al., 1992; Sakaguchi et al.,

1986; Sakaguchi and Melchers, 1986). $V_{preB}1$ is expressed in all B cells expressing the λ5 gene, whereas $V_{preB}2$ mRNA is co-expressed in approximatly 30% of the same cells (Dul et al., 1996). On the cell surface, the pre-BCR is associated with the Igα/Igβ heterodimer (Fig, 2.5) which functions as signal transduction unit (Reth, 1994; Wienands et al., 1996).

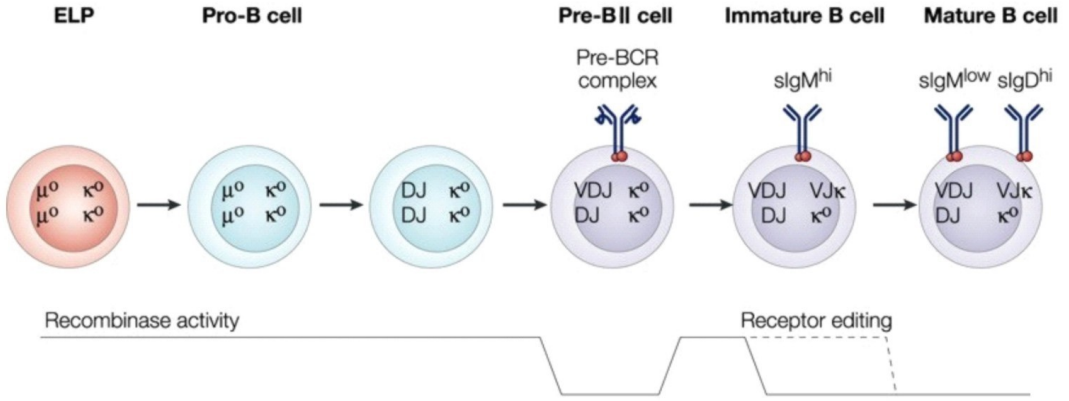

Figure 2.2. **Regulation of V(D)J recombinase activity during lymphocyte development.**
Successive stages of B-cell development and the relative levels of recombination activation gene (RAG) expression at each stage are indicated. BCR, B cell receptor; pre-BCR, precursor-BCR; ELP, early lymphoid progenitor; V, variable, D diversity and J, joining gene segment; κ^o, Igκ in germline configuration; μ^o, IgH in germline configuration; sIgM, surface Ig isotype M; sIgD, surface Ig isotype D; hi, high expression levels; low, low expression levels . (Adapted from Schlissel, 2003)

Not all V(D)J recombination events lead to productive genes. For example the rearrangement on one allele can be out-of frame and/or contains a premature stop codon. In this case a rearrangement at the other allele may rescue the cell (ten Boekel et al., 1995; ten Boekel et al., 1998; Wasserman et al., 1998). From the pre-BII cell stage onward about 60% of all B-lymphoid cells have one productively rearranged H chain allele, with the second allele in DJ_H configuration, while the remaining 40% have two rearranged alleles, one of which is unproductive (denoted VDJ^-) (Coleclough et al., 1981). The presence of a functional pre-BCR mediates clonal expansion, during which there is no gene recombination. Eventually the large pre-BII cells become small resting pre-BII cells (Grawunder et al., 1995; Melchers et al., 1999; Rolink et al., 1994).

Analogous to the BCR in B cells, T lymphocytes carry an antigen receptor on their cell surface, called T cell receptor (TCR). The genes encoding the TCR are also assembled somatically through a recombination process of a different set of V, (D) and J segments mediated by RAG.

2.2.2 L-chain rearrangement

After H-chain synthesis and pre-BCR assembly to signal cellular proliferation, the cells proceed to recombine one of the two L-chain loci, κ or λ. While both L-chain loci contain a number of variable (V) and joining (J) gene segments, they are organized differently.

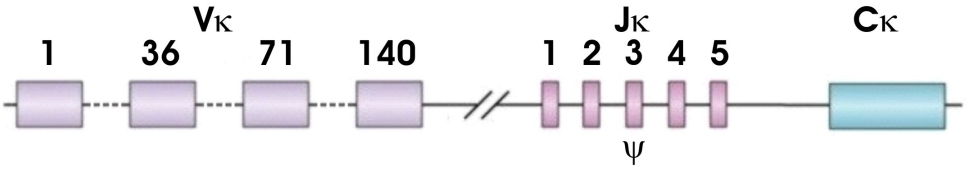

Figure 2.3. **Organization of the mouse Ig κ L-chain (Igκ) locus.** It displays the germline configuration of the Vκ (~140) and Jκ (5) gene segments. The Vκ segments are in different transcriptional orientation. Vκ, variable, Jκ, joining and Cκ, constant region segment; Ψ pseudo.

The mouse Igκ locus is 3.21 Mb in length and comprises 140 Vκ genes in different transcriptional orientation (Fig. 2.3). Seventy-five of the Vκ are functional, 21 potentially functional, and 44 are pseudo genes (Brekke and Garrard, 2004). During recombination, one of these Vκ genes is joined to one of 4 functional Jκ gene segments, Jκ1 , Jκ2 , Jκ4 and Jκ5; Jκ3 is a pseudo gene (Fig. 2.3). Located downstream of the Vκ and Jκ segments is a single κ constant region gene (Cκ) (Roschenthaler et al., 2000). A schematic diagram of the genomic organization of the κ L-chain locus is shown in Fig. 2.3.

The murine λ L chain is located on chromosome 16 and contains three Vλ gene segments (Vλ2, VλX and Vλ1) and four Cλ gene segments, three of which are functional. Each of the Cλ segments is preceded by a Jλ segment. The gene segments are arranged in two clusters: Jλ2Cλ2–Jλ4Cλ4 (Blomberg et al., 1981; Selsing et al., 1982) and Jλ3Cλ3–Jλ1Cλ1 (Blomberg et al., 1981; Miller et al., 1982). Jλ4 and Cλ4 are pseudogenes (Blomberg and Tonegawa, 1982; Miller et al., 1982). In the first complete map of the λ locus, based on pulsed-field gel electrophoresis of large DNA fragments, approximate distances between the gene segments were determined (Storb et al., 1989). However, a precise physical map of the λ locus had not yet been published at the start of the thesis presented here.

Opening the L-chain locus to rearrangement at the resting pro-BII cell stage, RAG expression is increased (Fig. 2.2) (Ehrlich et al., 1993). However, during this time, no H chain V-to-DJ joining occurs. In contrast to the H chain, only one of the two Igκ alleles seems to be accessible for V-to-J rearrangement at one time. This is reflected by different epigenetic states such as methylation, acetylation and chromatin association (Goldmit et al., 2005; Goldmit et al., 2002). Similar studies on the Igλ locus have not been done. Guided by different recombination signal sequences, RAG recombines the various V and J gene segments of the κ L-chain locus far more frequently than the λ locus (Ramsden and Wu, 1991). After an in-frame VJ rearrangement, the L chain is expressed immediately and further rearrangement may cease (Fig. 2.2) (Grawunder et al., 1995). In normal mouse serum, 95% of the L chains are κ and 5% are λ (Cotner and Eisen, 1978; McIntire and Rouse, 1970). Once a BCR, composed of one H chain and one L chain, is presented on the cell surface, the B cell reaches the immature B cell stage. This concludes the central B-cell development (Fig. 2.2).

2.2.3 Allelic and isotypic exclusion

Because B cells are diploid, more than one functionally rearranged allele could in principle be produced at the H-chain locus and each of the L-chain loci, κ and λ. Consistent with the concept of clonal selection, it was found that only one H-chain and one L-chain allele contributes to a functional BCR (Pernis et al., 1965; Weiler, 1965). This phenomenon is called allelic exclusion. It ensures monospecificity at the H-chain locus. At the L-chain loci not only allelic exclusion ensures monospecificity, but also isotypic exclusion, i.e. that an individual B cell expresses only either κ or λ. Because of the lack of a natural allelic marker for the κ or λ locus, allelic exclusion at the L chain has been measured only in mice, in which the κ constant region (Cκ) of one allele had been replaced by the human Cκ sequence. In these mice, 1.5% allelically included cells were found (Casellas et al., 2001). For the H chain however, studies in mice heterozygous for allotypically distinguishable alleles show 99.99% of B cells express the H chain from one allele only (Barreto and Cumano, 2000). This has been confirmed in various H-chain transgenic mice (Chai et al., 1994; Meffre et al., 2000; Rolink et al., 2001). Reports on L-chain transgenic mice on the other hand revealed that not all L chains lead to exclusion of

polypeptide encoded by the other allele (Ritchie et al., 1984). Thus, B cells may contain more than one in-frame rearranged endogenous L allele – these alleles can even be translated into polypeptides (Diaw et al., 2001; Ritchie et al., 1984; Schwartz et al., 1981; Yamagami et al., 1999). In these cases, it is thought that one of the L chains does not pair with the H chain, or the HxL combination does not reach the cell surface. This L chain is therefore not functional and does not violate the allelic exclusion rule.

There are various hypotheses about the establishment of allelic exclusion, but the mechanism is yet unresolved. Functionally, the molecular mechanism that enforces H chain allelic exclusion may be distinct from the one on the Igκ and Igλ loci, as V(D)J recombination must be actively repressed during L-chain gene rearrangement (Bassing et al., 2002). At least for the L-chain locus, a widely accepted model for allelic exclusion is the genetic regulation theory. It proposes a genetic feedback mechanism that turns off RAG after a functional surface BCR has been assembled, thus preventing further recombination (Alt et al., 1982; Coleclough et al., 1981; Wabl and Steinberg, 1982). This will also be the basic model for L-chain allelic exclusion for the scope of this work.

2.3 Peripheral B-cell development

2.3.1 Transitional B cells

With reaching the immature B cell stage, the lymphocytes have completed the central B-cell development. In the next developmental step, called the "peripheral B-cell development", the IgM positive (IgM^+) B cells leave the bone marrow (Carsetti et al., 1995). They travel passively via the bloodstream, exiting in the spleen (Fig. 2.4). At this stage immature B cells are referred to as "transitional" cells as they are "in transit" from the bone marrow to the spleen and also "in transition" from immature to mature B cells (Loder et al., 1999; Su et al., 2004). Essential for progression in the peripheral B-cell development is the supporting microenvironment of the spleen (Carsetti, 2004; Carsetti et al., 2004). The spleen consists of two tissues, the red pulp and the white pulp. In the red pulp, red blood cells are stored, and aged blood cells are destroyed. The white pulp is part of the lymphoid tissue (Fig. 2.4). Here, T cells are arranged around a central arteriola and make up the periarterial lymphatic sheath (PALS) (Chai et al., 1994; Timens, 1991;

Witmer and Steinman, 1984). B cells accumulate in the primary follicles surrounding the PALS and in the marginal zones (Fig. 2.4) (Timens, 1991; Witmer and Steinman, 1984).

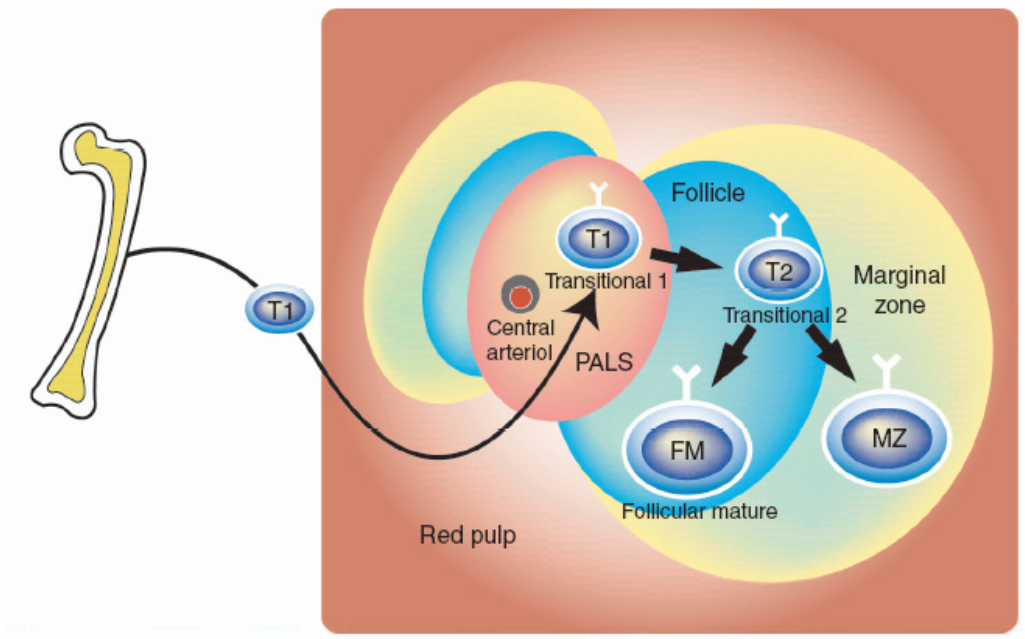

Figure 2.4. **Peripheral B-cell development.**
Immature B cells leave the bone marrow as short-lived, transitional B cells. Transitional 1 (T1) cells travel through the bloodstream and enter the spleen where they reside within the periarteriolar lymphoid sheath (PALS). T1 cells differentiate into transitional 2 (T2) B cells that reside within the splenic follicle. The T2 stage appears to represent a critical step in peripheral B-cell development. Activation of specific signaling events in T2 B cells leads to their further differentiation into either follicular mature (FM) B cells or marginal zone (MZ) B cells. (Adapted from Su et al., 2004)

In addition to IgM transitional B cell express the complement receptor 2 (denoted CD21) on the cell surface (Carroll and Prodeus, 1998). Two major subsets of transitional B cells can be distinguished, transitional type 1 (T1) and transitional type 2 cells (T2). Determined by the expression levels of these markers T1 cells are characterized as $IgM^{+}/CD21^{neg}$. They give rise to the T2 cells, which are determined as $IgM^{+}/CD21^{hi}$ (Carsetti et al., 1995; Loder et al., 1999). Only T2 cells express IgD (δ) on the cell surface. Outside of the bone marrow and the blood, T1 cells are only found in the outer splenic PALS. T2 cells are confined to the primary follicles of the spleen. Only after differentiation into mature B cells are they able to re-circulate among all lymphoid tissues (Cyster, 2003).

The two transitional B cell populations have different responses to BCR engagement. BCR cross-linking in the T2 cells leads to rapid proliferation and the expression of key cell cycle regulators. In addition, T2 cell stimulation also lead to further cell differentiation towards the mature B cell stage (Su and Rawlings, 2002; Petro et al., 2002). None of these responses are observed in the more immature T1 cells (Lam et al., 1997; Su and Rawlings, 2002). On the contrary, in vivo engagement of the T1 BCR results in reactivation of the recombination machinery (Gay et al., 1993; Tiegs et al., 1993). This puts them developmentally closer to early B cells in the bone marrow. T2 cells, like mature B cells, rapidly die in the absence of cell survival signals triggered by a constitutive, tonic BCR stimulation in vivo (Lam et al., 1997). However, in ex vivo experiments on isolated T1 cell populations, such survival signals were not activated. Unlike in T2 cells, cross-linking here promoted cell death (Su and Rawlings, 2002). With their distinct differences in BCR signalling, the transitional B cell stages in the spleen are a critical site for B-cell selection. They mark the last stage before the mature B cells are released to roam the lymphoid tissue (Carsetti et al., 1995; Levine et al., 2000; Nossal, 1994).

During B-cell development, of the approximately 2 x 10^7 IgM^+ B cells which are generated in the bone marrow daily, 10% survive to enter the transitional B cell stage in the spleen and only 1 - 3% enter the mature B cell pool (Melchers et al., 1995; Osmond, 1991; Rolink et al., 2001). This advocates a series of selection steps occurring during peripheral B-cell development (Su and Rawlings, 2002). As reviewed by Su et al., a model is proposed whereby T1 cells are deleted in the spleen by negative selection. T2 cells, in contrast become selected to enter the mature B cell pool (Fig. 2.4) (Su et al., 2004). In this model, T1 cells travelling to the spleen with antigen receptors to soluble self-antigens in the blood are likely to die from antigen-induced apoptosis (Su and Rawlings, 2002). Upon entry into the spleen, T1 cells remain in the outer PALS. Here, additional blood-borne self-antigens trapped by the spleen may further mediate negative selection (Fig. 2.4). The remaining T1 cells enter the primary follicle and become T2 cells. In the microenvironment of the splenic follicles, T2 cells are shielded from soluble antigens exposed to T1 cells. Instead, T2 cells likely encounter a unique set of antigens, possibly by follicular dendritic cells. Here, only T2 cells with BCR affinities giving a

positive response – perhaps to a tonic signal given by the BCR through Igα/β – will be able to escape death and will be selected into the mature B cell pool (Lam et al., 1997; Su et al., 2004).

2.3.2 Autoreactivity

The stochastic nature of the V(D)J recombination process gives rise to an enormously diverse repertoire of as many as 10^{11} different specificities (Janeway et al., 2001). However, a great disadvantage of this huge diversity is the potential to generate autoantibodies, i.e. antibodies that react to self (Fig. 2.5). Following the concept of acquired immunity, self-reactive B cells have to be selected against (Burnet, 1959). This selection is BCR mediated and based on the concept that a single B cell expresses a very limited set of receptors only. If a B cell were to express all receptors, there would be nothing to select from (Burnet, 1959). But how much restricted in its specificity does a given B cell have to be? From a theoretical point of view, the larger the fraction of self-antigens in the antigenic universe, the more the B lymphocytes will have to be restricted. Otherwise, selection against autoreactivity may end up with no repertoire at all. It has long been proposed that most of the generated B cell specificities must be autoreactive (Nemazee, 1993).

Despite the high levels of allelic exclusion, autoreactive antibodies exist in the serum of normal mice and humans (Chai et al., 1994; Coutinho et al., 1995; Dighiero et al., 1985; Hayakawa et al., 1999; Imai et al., 1994; Lacroix-Desmazes et al., 1998). Their existence has been explained by a variety of mechanisms: polyreactivity, antibody networks and positive selection. In general, antibodies are generated to target random antigens mono-specifically. However, some autoantibodies bind a variety of different self and non-self antigens, i.e. they are polyreactive (Diaw et al., 1997; Ichiyoshi and Casali, 1994). Another theory about autoreactivity proposes antibody networks. In the antibody network, one type of antibody binds foreign antigens while another kind reacts to self, more specifically to the antigen receptor (idiotype) of the first antibody kind. These anti-idiotype autoantibodies are thought to control immune responses through the interaction of the antigen receptors - stimulating or suppressing responses and retaining memory (Jerne, 1974; Varela and Coutinho, 1991). In the positive selection model, autoreactive B

cells are recruited into “innate like” B cell populations. These B cells do not need T cell help and reside either in the marginal zone of the spleen as MZ B cells or in the peritoneal cavity as B-1 B cells (Martin and Kearney, 2000; Martin et al., 2001).

2.3.3 Editing

During B-cell development the BCR is generated by a recombination process that is “blind” to autoreactivity (Fig. 2.5a-e). Thus a self-reactive BCR can be created anytime. To analyse the fate of such B cells, mice transgenic for autoreactive BCRs have been generated. In the presence of self antigen in such mice, autoreactive B cells are blocked from entering into the mature naïve B cell repertoire. This is accomplished by three different mechanisms: editing, apoptosis and anergy (unresponsiveness to BCR engagement) (Goodnow et al., 1988; Nemazee, 1996; Nemazee and Burki, 1989). Engagement of an autoreactive T1 BCR results in a reactivation of the recombination machinery, i.e. editing. This leads to the generation of a new, presumably non-autoreactive antibody (Fig. 2.5f-h) (Gay et al., 1993; Tiegs et al., 1993). Were it not for receptor editing, these B cells would either be eliminated from the B cell pool by apoptosis, or made anergic (Goodnow et al., 1988; Nemazee, 1996; Nemazee and Burki, 1989). Recombination at the H-chain locus is strict in its D-to-J and subsequent V-to-DJ rearrangement. Therefore recombination is only possible as long as no VDJ segment is complete. With the elimination of all but one D region, no further canonical recombination is possible at this H-chain allele. The L-chain loci on the other hand are joined by V-to-J recombination. These loci, particularly Igκ, provide multiple opportunities for secondary rearrangement to modify L-chain specificity or destroy autoreactive L-chain expression (Harada and Yamagishi, 1991; Nemazee and Weigert, 2000). For example, given a first rearrangement of a Vκ region with Jκ2 (Fig. 2.5e), this recombination can be edited by further recombination of an upstream Vκ region to one of the downstream Jκ regions 4 or 5 (Fig. 2.5f) (Kouskoff and Nemazee, 2001). Alternatively, the second L-chain allele can be recombined if all Jκ regions on the first allele are exhausted (Fig. 2.5h).

Analyses in normal B-cell development showed multiple V-to-J rearrangements on the same Igκ allele in more than 60% of all pre-BII cells, in more than 40% of all immature B cells and in more than 30% of all mature B cells (Yamagami et al., 1999). More than 80% of these pre-BII cells and more than 50% of these mature B cells used both κ alleles for recombination. B cells carrying multiple Igκ gene rearrangements also have an increased frequency of Vλ-to-Jλ rearrangements in their Igλ loci (Yamagami et al., 1999). Even though some of these multiple rearrangements could be the result of an unproductive recombination or a productive L chain, unable to pair with the H chain, these findings have been interpreted as evidence for multiple Ig rearrangements, i.e. editing. Editing takes place at the pre-BII cell in the bone marrow, and/or the transitional B cell stage in the spleen (Gay et al., 1993; Tiegs et al., 1993). More direct proof for L chain editing was shown by Nussenzweig and colleagues in mice heterozygous for a human and a mouse Cκ allele. Here, 25% of the antibodies in this mouse were created by L-chain editing (Casellas et al., 2001). This receptor editing process is RAG dependent, but elimination of autoreactive B cells occurs equally well in the presence and absence of RAG (Spanopoulou et al., 1994; Xu et al., 1998). Although it is not clear what percentage of L-gene replacements are exclusively induced by autoreactivity, the range given above provides an estimate for what the fraction of self-antigens in an individual mouse, or person, might represent in the antigenic world.

Looking at editing from a mechanistic point of view, the earlier introduced model for L chain allelic exclusion – after a functional surface BCR has been assembled, the enzyme RAG is turned off – has to be expanded (Alt et al., 1982; Coleclough et al., 1981; Wabl and Steinberg, 1982). To incorporate editing in this model, it is stated that RAG activity is turned off after a functional, non-autoreactive Ig has been assembled (Nemazee et al., 2002). While receptor editing and the re- or continued expression of RAG seems a logical measure to replace autoreactive rearrangements, it places a restriction onto allelic and isotypic exclusion. The reactivated recombination machinery poses the threat of rearranging a second L-chain allele and thus breaching allelic exclusion by expressing two L chains.

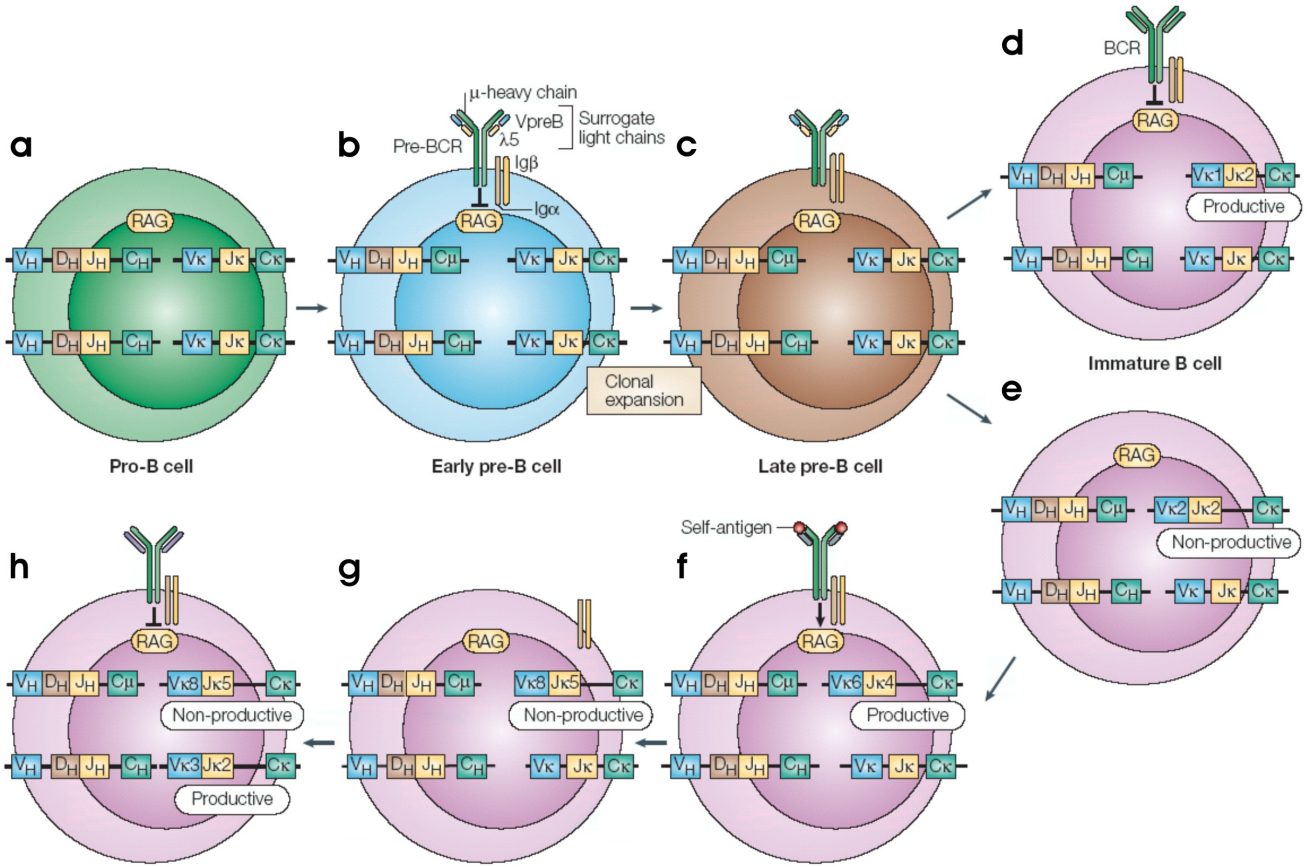

Figure 2.5.

A model for receptor editing and feedback inhibition.

(a) In pro-B cells, κ-alleles are in a germline configuration, RAG is expressed, both H-chain alleles undergo D-to-J recombination. (b) At the pre-B-cell stage, one H-chain allele recombines a V-to-DJ gene segments. The μH-chain pairs with surrogate L chains λ5 and V_{preB} to form the pre-BCR combined with Igα and Igβ. The cells proliferate and RAG is down regulated. (c) RAG is re-expressed after clonal expansion, to rearrange a κ-chain gene, generating the cell-surface BCR. (d) If the initial rearrangement is productive the cell goes on in B-cell maturation. (e) An unproductive rearrangement mediates secondary κ-gene rearrangements in the initially opened allele. (f) If the productive rearrangement is autoreactive, further Vκ-to-Jκ recombination is initiated to destroy the receptor genes in the same allele. (g) If the allele exhausts its recombination options, the second allele can become accessible for rearrangement (h). V, variable, D, diversity and J, joining gene segment; C, constant gene segment; RAG, recombination activation gene; BCR, B cell receptor; pre-BCR, pre-B cell receptor; Igα, BCR associated protein α-chain, Igβ, BCR associated protein β-chain ; λ5, V_{preB}, surrogate L chain genes. (Adapted from Bergman and Cedar, 2004)

2.3.4 Mature B cells

There are three major subsets of mature B cells, the mature follicular (MF) or naïve B cells, the marginal zone (MZ) B cells, and B-1 B cells (Martin and Kearney, 2000). FM B cells reside primarily in the follicles of the splenic white pulp. The MZ B cells are located in the marginal zone of the spleen, a region between the red and the white pulp (Fig. 2.4). B-1 cells represent only a small fraction of the total B cell population; they comprise a population of cells within the peritoneal and pleural cavities. They require the presence of an intact spleen (Wardemann et al., 2002). FM B cells circulate the bloodstream and the lymphatic tissues. They respond to specific antigen stimulation. Co-activated by T cells, they proliferate and differentiate into highly specific effector cells, including plasma and memory B cells. They are the key B-lineage cells to generate the adaptive immune response characterized by the generation of antigen specific antibodies (Carsetti et al., 2004; Su et al., 2004). In contrast to FM cells, MZ and B-1 cells are independent of T cell co-stimulation. They are responsible for the rapid production of secreted IgM made in response to blood-borne antigens and pathogens (Martin and Kearney, 2000; Martin et al., 2001). MZ and B-1 cells are the major source of physiological levels of circulation low-affinity autoantibodies (Hayakawa et al., 1999; Martin and Kearney, 2000).

2.4 Autoreactivity, editing and allelic inclusion

Most B cells produce monoclonal antibodies generated from one H and one L chain gene only. Following the concept of clonal selection, in B cells autoreactive receptors are recognized during B-cell development and altered by receptor editing to prevent autoreactivity. Despite this, autoreactivity is still found in the serum of normal mice and humans. Their existence has been explained by polyreactivity, antibody networks or positive selection (Chai et al., 1994; Coutinho et al., 1995; Dighiero et al., 1985; Hayakawa et al., 1999; Imai et al., 1994; Lacroix-Desmazes et al., 1998). As discussed in chapter 2.2.3, allelic exclusion on the H-chain locus is 99.99% stringent, while on the L-chain locus, 1.5% allelically included cells are found (Barreto and Cumano, 2000; Casellas et al., 2001). Allelic exclusion is thus much less strict on the L -chain locus than on the H-chain locus. A similar phenomenon was observed in T cells. Analogous to the BCR in B cells, the T cell receptor (TCR) in T cells is recombined and expressed from the

TCRα and TCRβ genes. Like the H-chain locus, the TCRβ is assembled by V(D)J recombination, while the TCRα is joined by VJ recombination, similar to the L-chain locus. Both TCR loci also show allelic exclusion. In T cells however, frequent allelic inclusion of the TCRα genes poses an autoimmune 'hazard' due to low level expression of auto-specific receptors (Sarukhan et al., 1998). Transferring this concept onto B cells in the context of receptor editing, there might the possibility that in the editing process, the autoreactive L-chain gene is not destroyed. During editing an L chain encoded by another allele or locus could rescue the cell. As a result, such a B cell would express more than one functional antigen receptor, one of which is autoreactive. Here, this is proposed as a new model to explain autoreactive antibodies in normal serum.

2.5 The nuclear transfer mouse

The nuclear transfer mouse analyzed in this thesis represents the "third generation" of H- and L-chain transgenic mice created to study the involvement of antibodies in the generation and function of B lymphocytes. In "first generation", or conventional Ig transgenic mice Ig genes were expressed under proper promoters but integrated into heterologous chromosomal sites (Grosschedl et al., 1984; Rusconi and Kohler, 1985; Storb et al., 1986). The "second generation" was generated by knocking-in a functional V(D)J rearrangement into the H- and L-chain loci, replacing the germline J-region sequence (Cascalho et al., 1996; Chen et al., 1995; Sonoda et al., 1997). In the most recent generation, these mice were generated by use of the nuclear transfer technology. To generate this mouse, Hochedlinger and Jaenisch harvested mature B lymphocytes from the lymph nodes of an immunologically unchallenged mouse (C57BL/6 x DBA/2 F1) (Fig. 2.6a). The nuclei were extracted and transferred into enucleated eggs, from which the female pro-nucleus had been removed (Fig. 2.6b, c). Subsequently, the eggs were cultured to the blastocyst stage (Fig. 2.6d), from which the embryonic stem (ES) cells were generated. The ES cells were then aggregated with tetraploid embryos, generated by electrofusion of two-cell embryos, and transferred into a foster mother (Fig. 2.6e). The resulting offspring is entirely ES-cell derived, while placental structures were provided by tetraploid cells.

What sets the nuclear transfer mouse apart from other transgenic mouse models is that it starts out with the same gene complement present in the donor B cell (Hochedlinger and Jaenisch, 2002; Rossant, 2002). Another important difference to the "second generation" Ig knock-in mice is that the nuclear transfer mouse has no additional remaining germ line D regions, in the rearranged H-chain allele. Practically, the genotype of the donor lymphocyte was perpetuated in the germline of the mouse. Such mice can, in principle, also be created by cloning all rearranged genes – not only the active alleles, but this has not been done to date.

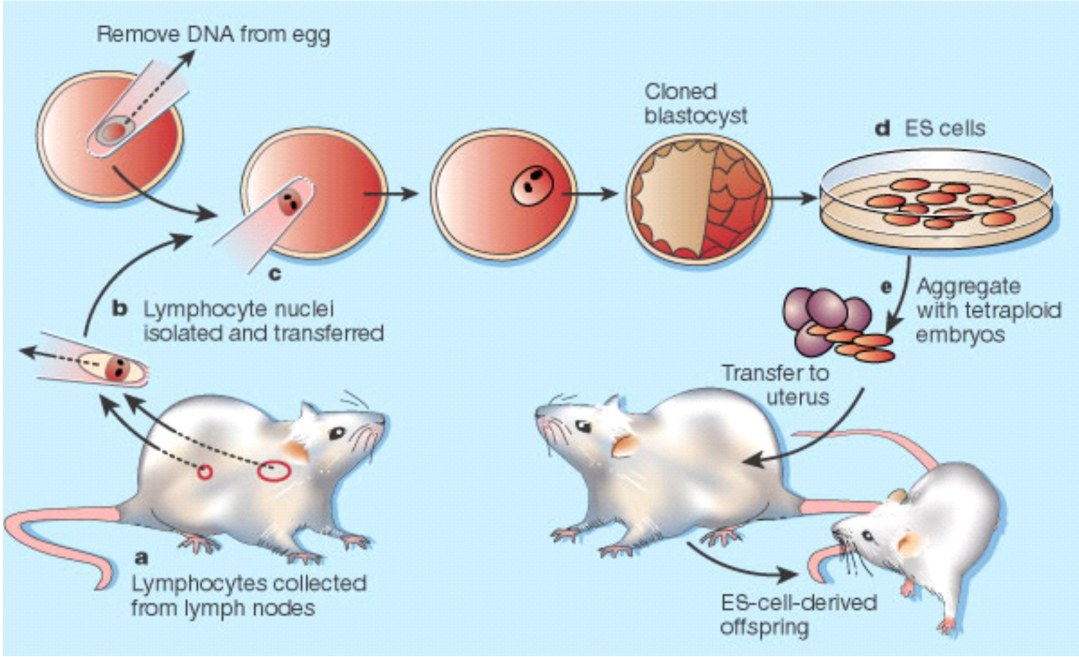

Figure 2.6. **Cloning technique used to generate the nuclear transfer mouse.**
(a) Lymphocytes were isolated from lymph nodes. (b) Nuclei were extracted from B lymphocytes and (c) transferred into enucleated mouse eggs and cultured to the blastocyst stage of development. (d) Embryonic stem (ES) cells were then cultivated from the cloned blastocysts and aggregated to tetraploid embryos (e), generated by electrofusion of two-cell embryos. In the productive chimeras the entire fetus was derived from ES cell, while placental structures were provided by tetraploid cells. The offspring is entirely ES cell derived. (Adapted from Hochedlinger and Jaenisch, 2002; Rossant, 2002)

In the naturally developed B cell that gave rise to the nuclear transfer mouse, the active H-chain allele had the variable gene segment VH22.1 joined to DFL16.2 and JH3 (denoted H^+) (Hochedlinger and Jaenisch, 2002). The second H-chain allele had an irregular D-to-J rearrangement, excluding it from further canonical recombination (Koralov et al., 2005). In the mice we received from Hochedlinger and Jaenisch for our

studies, the second H-chain allele was in germline configuration (denoted H^o). Two κ L-chain segments were described in the original publication (Hochedlinger and Jaenisch, 2002). One allele was characterized to comprise a "variable element of the Vκ 4/5 group, subgroup IV, with similarity to the kk4 gene", in combination with Jκ 1. The other allele had a "variable element of the Vκ 1 group, subgroup II, with similarity to the cu2 gene", in combination with Jκ 2 (Hochedlinger and Jaenisch, 2002).

3. Specific aims

To further the knowledge on Ig genes and their role in B cell development the goal of this work was to study the organization of the Igλ locus and to analyze autoreactivity and receptor editing during B cell development in the nuclear transfer mouse and its offspring.

Despite the fact that B cells are diploid, lymphocytes typically express only one functional antigen receptor, encoded by one H-chain allele and one L-chain allele, a phenomenon termed allelic exclusion (Pernis et al., 1965; Weiler, 1965). Mediated by RAG, the receptor genes are assembled by recombination of different Ig gene segments in a way that is blind to autoreactivity (Schatz et al., 1989; Tonegawa, 1983). To prevent receptor autoreactivity in B cells, a self-reactive BCR induces editing by re-expressing RAG, replacing the L-chain gene while maintaining allelic exclusion (Gay et al., 1993; Nemazee and Weigert, 2000; Tiegs et al., 1993). Once a nonself-reactive BCR is made, editing is stopped.

Generated by cloning, in the nuclear transfer mouse all Ig genes are inherited as present in the donor B cell. However, in addition to its H-chain transgene, the mouse contains two rather than one L-chain rearrangement, which challenges allelic exclusion. It was the aim of this thesis to reconcile the apparent contradiction with classical clonal selection, or to modify current lines of thought.

Specific aim 1:

At the start of this work, it was found that the exact genomic organization of the λ L-chain locus and the location of the VpreB2 gene were unknown. Thus in aim 1, the chromosomal location and distances between the λ L-chain gene segments and the related loci λ5, VpreB1 and VpreB2 were to be determined.

Specific aim 2:

The two in-frame rearranged Igκ genes of the nuclear transfer mouse raised the question of whether or not both L-chain genes direct the synthesis of L chain. Furthermore, if so, do B cells express both L chains in combination with the H chain? These questions are important as they relate to monospecificity of B cells and clonal selection. Under the assumption that both L-chain genes are functional and combine with the H chain, what is the fate of either BCR during B-cell development?

Specific aim 3:

Common to all Ig transgenic mouse models is the question of temporal expression of the preformed Ig genes. This question is important as receptor editing is mostly studied in Ig transgenic mice. Here, this question was addressed in the nuclear transfer mouse, to specify whether RAG or the H (and/or L) chain is expressed first in B cell development. If RAG is re-expressed before the BCR presented, the results would mimic receptor editing but may be artefactual.

4. Materials and Methods

4.1 Materials

4.1.1 Abbreviations

Ab	antibody
APC	allophycocyanine
BCR	B cell receptor
C	constant
Cy5	cyanine dye 5
D	diversity
ELISA	enzyme linked immunosorbent assay
ES cell	embryonic stem cell
FACS	fluorescence activated cell sorting, flow cytometry
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
GFP	green-fluorescent protein
H	heavy
HRP	horseradish peroxidase
Ig	immunoglobulin
Igα/β	BCR associated protein α/β
J	joining
L	light
MF	mature follicular
NCBI	national center for biotechnology information
PALS	periarterial lymphoid sheath
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PBST	PBS with 0.1% Tween-20
PCR	polymerase chain reaction
PE	phycoerithrin
pre-B cell	precursor B cell, pre-B cell
pre-BCR	pre-B cell receptor
pro-B cell	progenitor B cell
RAG1/2	recombination-activation gene 1/2
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
T1/2	transitional stage 1/2
TDT	terminal desoxynucleotidyl transferase
TE	Tris-Cl/EDTA buffer
TMD	3,3’5,5’-tetramethylbenzidine
Tween-20	polyoxyethylen-sorbitan-monolaurat

V	variable
VDJ^- / VDJ^+	unproductive / productive VDJ rearrangement
MZ	marginal zone
Ψ	pseudo
κ	kappa
λ	lambda

4.1.2 Mouse strains

Nuclear transfer mouse: Generated from a single B lymphocyte by nuclear transfer offspring of the nuclear transfer B cell mouse is characterized by one rearranged H-chain gene (H^+) and two rearranged κ L-chain genes (L^{kb4} and L^{cr1}) denoted kb4 and cr1 (Hochedlinger and Jaenisch, 2002). Its offspring was intercrossed to generate the various allelic combinations analyzed here. The mice were studied at 4- to 6-weeks of age.

RAG1 knock-out mouse: The RAG1 gene was rendered non-functional in this mouse by gene targeted insertion of a neomycin cassette into the germline sequence (Mombaerts et al., 1992). The nuclear transfer mouse was crossed with $RAG1^{-/-}$ mice and the F1 mice were intercrossed. The mice were analyzed at 4- to 6-weeks of age.

Human Cκ mouse: The Igκ constant region of this mouse was replaced with the orthologous human sequence creating an Igκ allelic polymorphism (Casellas et al., 2001). To test for allelic exclusion human C_κ mice were crossed with $H^{+/+}$ $L^{kb4/kb4}$ mice and the offspring was analyzed at 3-months of age.

RAG1-GFP mouse	In RAG1-GFP mice part of the RAG1 gene was replaced by the GFP sequence, the original promoter region remained unaltered (Kuwata et al., 1999). To study the RAG expression levels, RAG1-GFP mice were crossed to nuclear transfer mice with different H- and L-chains. The mice were analyzed at 4- to 6-weeks of age.
AIRE knock-out mouse	The sequence of the AIRE gene was disrupted by targeted insertion of a neomycin cassette (Anderson et al., 2002). Spleen and bone marrow cells of such mice were used to test for autoreactive antibodies. The fact that they are AIRE deficient was not relevant here. The organs were harvested at 4- to 6-weeks of age.
C57BL/6	C57BL/6 is the most widely used inbred strain. It is well characterized and a standard reference in immunological and genetic research. Here, spleen and bone marrow cells of C57BL/6 mice were screened for binding of Hxkb4 antibodies. The samples were harvested at 4- to 6-weeks of age.

All mice were kept in a barrier facility under specified pathogen free conditions.

4.1.3 Chemicals

All chemicals were purchased from the distributor with the most competitive pricing. The manufacturer is mentioned in the text if a certain product is preferred.

4.1.4 Buffers and solutions

All buffers and solutions were prepared with double distilled water (ddH_2O), unless stated otherwise. They were either autoclaved prior to use, or made from autoclaved stock solutions.

4.1.5 Kits

QIAprep Spin Miniprep kit	Qiagen, Valencia, CA
TOPO TA-cloning kit	Invitrogen, Carlsbad, CA
QIAquick Gel Extraction kit	Qiagen, Valencia, CA

4.1.6 Oligonucleotides

T_A is the annealing temperature used in the PCR reaction.
The size of the PCR product is given in base pairs (bp).

Genotyping primes for the nuclear transfer mouse:

H-chain locus in germline configuration (H^o): T_A = 60ºC, 750 bp

JH2 (fwd)	5'-CCAGAGATTTATAGGGATCCTGGCCA-3'
JH4 (rev)	5'-GAGGAGACGGTGACTGAGGTTCCTTG-3'

rearranged H-chain locus (H^+): T_A = 60ºC, 800 bp

VH (fwd)	5'-CAGCTTAAGGGCTGAAGACACTGGAAT-3'
JH4 (rev)	5'-GAGGAGACGGTGACTGAGGTTCCTTG-3'

κ L-chain locus in germline configuration (L^o): T_A = 55ºC, 520 bp

5'Jκ1 (fwd)	5'-GGTTAAGCTTTCGCAGCTACCC-3'
3'Jκ2 (rev)	5'-GGTTAGACTTAGTGAACAAGAGTTGAG-3'

rearranged L-chain locus cr1 (L^{cr1}): T_A = 62ºC, 1050 bp

Cr1 (fwd)	5'-CTGGTTTCAAGGTTCACATGTTCC-3'
Jκ5 (rev)	5'-CTCCTAACATGAAAACCTGTGTCTTACACA-3'

rearranged L-chain locus kb4 (L^{kb4}): T_A = 62ºC, 1400 bp

Kb4 (fwd)	5'-GTGGAATTATCCGTGGACGTTCG-3'
Jκ5 (rev)	5'-CTCCTAACATGAAAACCTGTGTCTTACACA-3'

Primers for amplification the two κ L-chain alleles for sequencing:

Primers to amplify the L^{kb4} rearrangement: T_A = 64ºC, 1700 bp

5'kb4 (fwd) 5'-GGGATTTTGAGCTCAGAATGCAACC-3'

Jκ5 (rev) 5'-CTCCTAACATGAAAACCTGTGTCTTACACA-3'

Primes to amplify the L^{cr1} rearrangement: T_A = 64ºC, 1200 bp

κ-generic (fwd) 5'-GGCTGCAG(G/C)TTCAGTGGCAGTGG(A/G)TC(T/A)G G(A/G)AC-3'

Jκ5 (rev) 5'-CTCCTAACATGAAAACCTGTGTCTTACACA-3'

Genotyping primers for the RAG1 knock-out mouse: (Mombaerts et al., 1992)

RAG1 knock-out allele (RAG^{-}): T_A = 57ºC, 1500 bp

Neo Direct (fwd) 5'-GTCACGACGAGATCCTCGCCGTCG-3'

RAG 1-2 (rev) 5'-TTCAAGAGTGACGGGCACAG-3'

RAG1 wild-type allele (RAG^{+}): T_A = 57ºC, 800 bp

RAG1-1 (fwd) 5'-GTGAAGGGACCATTCAGGTAG-3'

RAG 1-2 (rev) 5'-TTCAAGAGTGACGGGCACAG-3'

Genotyping primers for the RAG1-GFP mouse: (Kuwata et al., 1999)

RAG1-GFP allele (RAG^{GFP}): T_A = 55ºC, 1000 bp

RAG 1-5-2 (fwd) 5'-AGGTAGCTTAGCCAACATGG-3'

GFP 3' (rev) 5'-GCTCAGGTAGTGGTTGTCGG-3'

RAG1 wild-type allele ($RAG1^{wt}$) T_A = 55ºC, 1000bp

RAG 1-5-2 (fwd) 5'-AGGTAGCTTAGCCAACATGG-3'

R6 (rev) 5'-CAACATCTGCCTTCACGTCGATCC-3'

Genotyping primers for the AIRE knock-out mouse: (Anderson et al., 2002)

AIRE knock-out allele ($AIRE^-$): T_A=67ºC, 690 bp

AIRE ko 1 (fwd) 5'-AGACTGAGGTGTTCCCTCCCAACCTCAG-3'

AIRE ko 2 (rev) 5'-GTCATGTTGACGGATCCAGGGTAGAAAGT-3'

AIRE wild-type allele ($AIRE^+$) T_A = 66ºC, 500 bp

AIRE wt 1 (fwd) 5'-ATAGCACCACGAACACCCAAG-3'

AIRE wt 2 (rev) 5'-ATATCATTCTCCAACTCCTGCCTCTTT-3'

Sequencing primer for vector pCR 2.1:

M15 (fwd) 5'-GTTGTAAAACGACGGCCAGT-3'

All oligonucleotides were synthesized by Integrated DNA Technologies (IDT, Davis, CA), and dissolved in TE buffer at a 10 µM concentration.

4.1.7 Antibodies and secondary reagents

Antibody	Specificity	Origin; Conjugation	Vendor, Cat. #
B220	Anti-mouse B220	Rat, monoclonal; APC-coupled	BD PharMingen; 553092
IgM^a (μ^a)	Anti-mouse IgM^a	Mouse, monoclonal; biotin-conjugated	BD PharMingen; 553515
IgM^a (μ^a)	Anti-mouse IgM^a	Mouse, monoclonal; PE-conjugated	BD PharMingen; 553517
CD21	Anti-mouse complement-receptor type 2 (CR2)	Rat, monoclonal; FITC-coupled	BD PharMingen; 553818
IgD^a (δ^a)	Anti-mouse IgD^a	Mouse, monoclonal; PE-coupled	BD PharMingen; 553507
Igκ	Anti-mouse Ig kappa	Goat, polyclonal; PE-conjugated	Southern Biotech; 1055-09
Human Igκ	Anti-human Ig kappa	Goat, polyclonal; PE-conjugated	Fischer Scientific; OB1237-21
Igλ	Anti-mouse Ig lambda	Goat, polyclonal; PE-conjugated	Southern Biotech; 1065-09

Secondary reagents for FACS:
Streptavidin conjugated with PE-Cyanine, BD PharMingen, Cat # 554062

Secondary reagents for ELISA:
Horseradish peroxidase (HRP)-coupled goat anti-mouse Ig total antibody (H- plus L-chain), Southern Biotech, Cat # 1010-05

4.2 Methods

4.2.1 Instruments

Centrifuge:	Sorval RC, rotor GSA, SS34 (Sorval, Wilmington, CA) Eppendorf 5415D (Eppendorf, Westbury, NY)
PCR machines:	PTC-100 Peltier Thermal Cycler (MJ Research, Watertown, CA) PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, CA)
Gel electrophoresis:	Hoefer Scientific Instruments (Hoefer Sci., San Francisco, CA)
Gel documentation:	Gel Doc 1000 (Bio-Rad, Hercules, CA)
pH meter:	Oaktron 510 (Fischer Scientific, Pittsburgh, PA)
Cell cytometry:	FACScalibur (BD Bioscience, San Jose, CA)
Incubator:	Incubator 310 (Robbins Scientific, Sunnyvale, CA)
ELISA reader:	SpectraMax plus (Molecular Devices, Sunnyvale, CA)
Heating block	VWR 13250 (VWR Scientific, Hayward, CA)
Cell counter	Coulter Counter Z1 (Beckman Coulter, Fullerton, CA)

4.2.2 Working with nucleic acids

4.2.2.1 Isolation of genomic DNA from tail tissue

Mouse tail biopsy specimens of no longer than 6 mm were cut and immediately transferred into 750 µl of tail-lysis buffer (100 mM Tris-HCl, pH 8.5, 0.5 mM EDTA,

0.2% SDS, 200 mM NaCl, 10 mg/ml proteinase K). They were moved to a rotating tube rack in an incubator (Robbins Scientific, Sunnyvale, CA) and digested at 55ºC for at least 4 hours or overnight. The agitation is important to accomplish complete lysis. Following complete lysis, 750 µl of isopropanol was added. The solutions were shaken until the DNA precipitated. The samples were then centrifuged for 5 min at a relative centrifugal force of 1,600 × *g*. Subsequently, the supernatants were removed and the precipitated DNA was resuspended in 200 µl of TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). To dissolve the DNA, the solution was put in a heating block (VWR Scientific, Hayward, CA) at 60ºC for 20 min. This procedure is a modification of the DNA isolation protocol described by Berns and Jaenisch (Laird et al., 1991).

4.2.2.2 Polymerase chain reaction (PCR)

To genotype the different mice used in this study and to sequence the two L-chain rearrangements of the nuclear transfer mouse, specific DNA segments were amplified by polymerase chain reaction (PCR). This in vitro reaction was originally conceived by Mullis (Mullis et al., 1986). Using specific primers flanking a DNA segment on the 5′ end and on the opposite strand on the 3′ end, the intervening fragment is amplified by a DNA polymerase. A standard reaction consists of 0.01-10 ng of DNA template, 1-4 mM $MgCl_2$, 0.2 mM dNTPs, 0.5 µM forward primer and reverse primer, and 0.5 U of DNA polymerase enzyme. One unit of *Taq* polymerase incorporates 10 nM dNTPs into acid-precipitable material in 30 min at 72ºC.

The consecutive steps of a PCR are carried out at characteristic temperatures. First, the DNA is denatured (94ºC, 4 min). To amplify the template DNA, a sequence of three steps is repeated 25-40 times: (1) denaturing (94ºC, 30 s), in which the two DNA strands dissociate; (2) annealing (depending on the primers, 50-70ºC, 30 s), in which the primers anneal with the single-stranded DNA; and (3) extension (72ºC, 60 s per 1-kb product), in which the polymerase amplifies the primer-flanked DNA fragment. After finishing this cycle, a final amplification step (72ºC, 10 min) ensures full-length synthesis of the fragments.

The annealing temperature (T_A) of a PCR depends directly on the composition of the primers. It is calculated from the melting temperature (T_M) of a primer/DNA duplex, which increases both with its length and with increasing G+C content.

The T_M for every primer was calculated from the empiric formula:

$$T_M = (4(G+C) + 2(A+T))^{\circ}C$$

The T_A was then determined correspondingly:

$$T_A = T_M - 5^{\circ}C$$

This formula was first described by Wallace (Wallace et al., 1979). An online oligonucleotide property calculator was used to determine the T_M based on this formula (http://www.basic.northwestern.edu/biotools/oligocalc.html). Furthermore, the optimal T_A for every primer pair was confirmed by gradient PCR. The annealing temperatures from all primer pairs are given together with the primer sequences (see 4.1.6).

4.2.2.3 Genotyping PCR

To determine the genotype of the different mice, DNA extracted from tail biopsy specimens was amplified using *Taq* DNA polymerase (Invitrogen, Carlsbad, CA). PCR was performed by following the standard protocol in a 25-µl reaction volume at a final $MgCl_2$ concentration of 15 mM. The annealing temperature and the extension time for the different reactions were adjusted according to primer specification and product size (see 4.1.6). The amplification cycle was repeated 40 times.

To distinguish homozygous from heterozygous animals, two PCR reactions were performed for each locus analyzed. Specific to the wild-type and the altered allele, respectively, the combination of the two reactions clearly determined the genotype of each animal.

4.2.2.4 High fidelity PCR

To sequence the two L-chain rearrangements of the nuclear transfer mouse, the respective DNA fragments were amplified using *Pfu* DNA polymerase (PfuTurbo; Stratagene, La Jolla, CA). PfuTurbo has a very low error rate due to its proofreading activity (i.e., 3′-5′ exonuclease). Following the standard protocol, the reaction was performed in a 50-µl volume with 1.5 mM $MgCl_2$. The annealing temperature and the extension time were adjusted according to primer specification and expected product size (see 4.1.6). The amplification cycle was repeated 30 times.

In a second step, 2 U of *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) was added to the reaction mix, which was then incubated for 10 min at 72ºC. This created the 3′-A overhang necessary to ligate the DNA fragment into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA).

4.2.2.5 Cloning of PCR products

PCR products were cloned using the TA cloning kit, the vector pCR2.1 and the included *E. coli* Top10 cells (Invitrogen, Carlsbad, CA). The transformations were done according to the user's manual.

4.2.2.6 Purification of plasmid DNA

Following the manufacturer's protocol, plasmid DNA was purified from *E. coli* Top10 cells of a 5-ml overnight culture using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA).

4.2.2.7 Restriction digest

All DNA restriction digests were performed using New England Biolabs (Ipswich, MA) buffers and enzymes. Following the manufacturer's guidelines, the volumes of the digests were selected so that the DNA concentration was below 300 µg/µl. The added restriction enzyme was not to exceed 10% of the total volume. All digests were performed using 2-3 U of enzyme per 1 µg of DNA. At the appropriate temperature, 1 U of restriction enzyme will digest 1 µg of substrate DNA per hour; incubation times were selected accordingly.

4.2.2.8 DNA gel electrophoresis

Separation analysis of DNA fragments from PCR or restriction digests was performed in 0.8% to 2% agarose gels. The agarose gels were prepared with TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA, pH 8.0) and 0.5 µg/µl ethidium bromide. DNA samples were mixed with 6× DNA loading buffer (glycerol 50% [v/v], bromphenol blue 0.4% [w/v], xylene cyanol 0.4% [w/v]). As a molecular weight marker, 0.4 µg of a 1-kb DNA ladder (Invitrogen, Carlsbad, CA) was included in the electrophoresis. The fragments were separated at no more than 5 V/cm (cm is the distance between the two electrodes). DNA bands were visualized on a UV transilluminator (Gel Doc 1000; Bio-Rad, Hercules, CA) at a wavelength of 312 nm. For extraction of DNA fragments, the desired fragments were cut from the agarose gel after electrophoresis and purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

4.2.2.9 DNA Sequencing

For DNA sequencing, purified plasmid DNA was sent to Davis Sequencing (Davis, CA). They performed all necessary reactions and delivered the nucleotide sequence as a chromatogram file for further analysis.

4.2.2.10 DNA Sequence analysis

All sequence analyses were done with the DNA sequence software Sequencher, version 4.1.2 (Gene Codes, Ann Arbor, MI). Various public genome databases were used as online references: All Ig gene sequences were obtained from or compared in the IgBLAST tool (http://www.ncbi.nlm.nih.gov/igblast) of the National Center for Biotechnology Information (NCBI). The location of different nucleotide sequences within the mouse genome was determined using the UCSC (University of California, Santa Cruz) Genome Informatics tool (http://genome.ucsc.edu/). The physical map of the λ L chain and the related loci was created using the formerly commercial mouse genome database tool created by Celera (http://www.celera.com).

4.2.3 Enzyme-linked immunosorbent assay (ELISA)

An indirect capture ELISA was performed to determine the IgM concentrations for the different sera used in the autoreactivity test. The serum of mice of the following genotypes was analyzed: $H^{o/o}$ $L^{o/o}$ (wt), $H^{+/o}$ $L^{cr1/o}$, $H^{+/o}$ $L^{cr1/o}$ $Rag1^{-/-}$, $H^{+/o}$ $L^{kb4/o}$, $H^{+/o}$ $L^{kb4/o}$ $Rag1^{-/-}$, and $H^{+/o}$ $L^{kb4/cr1}$. The starting dilution for all RAG-sufficient mice was 1:100,000. All RAG-deficient mouse sera were pre-diluted 1:5,000. All dilutions were done in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.3).

To capture the serum IgM, a 96-well ELISA plate (Corning Costar, Fisher Scientific, Pittsburgh, PA) was coated overnight with goat anti-mouse IgM antibodies (4 ng/ml) (Fisher Scientific, Pittsburgh, PA). The wells were rinsed with PBS-T (PBS, Tween-20 0.1% [v/v]) and blocked for 1 hour with 1% (v/v) bovine serum albumin (Fisher Scientific, Pittsburgh, PA). Afterward, the plate was washed three times with PBS. The pre-diluted mouse sera and control antibodies from a mouse standard panel (Southern Biotech, Birmingham, AL) were added in serial dilutions; both were incubated for 2 hours. Three PBS-T washes followed. To detect the amount of captured serum IgM, a

horseradish peroxidase (HRP)-coupled goat antibody to mouse Ig (Southern Biotech, Birmingham, AL) was added for 1 hour, followed by three PBS-T washes. The assay was then developed with HRP-substrate tetramethylbenzidine (BD PharMingen, San Diego, CA). The reaction was stopped after 30 min by adding 3 N NaOH.

After the stop, the color of the solution turned yellow. Its intensity is directly proportional to the amount of substrate processed, i.e., indirectly proportional to the amount of IgM captured. Light absorption in the reaction solution was measured at 450 nm. Plates were read with the SpectraMax plus (Molecular Devices, Sunnyvale, CA). The IgM concentrations were calculated with the help of the mouse standard panel of known concentrations.

4.2.4 Flow cytometry

4.2.4.1 Tissue preparation

In this study, lymphocytes from the peripheral blood, bone marrow and spleen of different mice were collected and analyzed. To determine the characteristics of different cells by flow cytometry, the harvested tissues had to be prepared as single-cell suspensions. Different organs have to be prepared in a tissue-specific manner before antibody staining.

Blood

Whole blood (~200 µl) was drawn by retro-orbital bleeding from mice under inhalation anesthesia with 1.5% methoxyflurane (Metaphane; Mallinckrodt, Mundelein, IL). It was collected in PBS/FCS (2% FCS [v/v], 0.1% NaN_3 [w/v]) with heparin (2 U/ml) to prevent blood coagulation and kept at 4ºC. Red blood cell lysis was performed before antibody staining (see 4.2.4.2).

Spleen

The spleens of euthanized mice were harvested and teased through a 70-µm nylon cell strainer (BD Falcon; BD Biosciences, Rockville, MD) using the plunger of a syringe. The cells were resuspended in PBS/FCS with heparin and kept at 4ºC. The erythrocytes were lysed before antibody staining (see 4.2.4.2).

Bone marrow

The hind leg of a euthanized mouse was harvested, and the femur was extracted. Residual external tissue was removed from the bone, and the hip joint was cut off. The 25-gauge needle of a PBS/FCS-filled syringe (BD Biosciences, Rockville, MD) was inserted into the bone marrow from the knee joint, and the bone marrow was flushed out. The collected cells were then teased through a 70-µm nylon cell strainer (BD Falcon; BD Biosciences, Rockville, MD) using the syringe plunger. Subsequently, the cells were centrifuged (300 × *g*, 5 min, 4ºC) and washed three times with PBS/FCS. For bone marrow samples, no red blood cell lysis was necessary before the antibody staining.

4.2.4.2 Red blood cell lysis

To lyse the erythrocytes in the blood and spleen cell suspensions, the cells were centrifuged (300 × *g*, 5 min, 4ºC), and the supernatant was discarded. Red blood cell lysis buffer (150 mM NH_4Cl, 10 mM $NaHCO_3$, 0.4% EDTA [v/v]) was added and incubated for 10 min at 4ºC. Subsequently, the cells were centrifuged (300 × *g*, 5 min, 4ºC) and resuspended in PBS/FCS. The washing step was repeated three times.

4.2.4.3 Antibody staining

Prior to antibody staining, the number of lymphocytes in the different cell suspensions was determined using a cell counter (Coulter Counter Z1; Beckman Coulter, Fullerton, CA). For antibody staining, $1 – 2 \times 10^6$ cells were centrifuged (300 × *g*, 5 min, 4ºC), resuspended in 50 µl of antibody staining solution (fluorochrome- or biotin-coupled antibodies in PBS) and incubated for 20 min on ice in the dark. Afterward, the cells were centrifuged (300 × *g*, 5 min, 4ºC). If a biotin-coupled first antibody was used, the cells were resuspended in 50 µl of a streptavidin solution (fluorochrome-coupled streptavidin in PBS) and incubated for 20 min on ice in the dark. The cells were then washed two times before analysis.

4.2.4.4 FACS analysis

In flow cytometric analyses, individual cells are held in a thin stream of fluid and passed through a laser beam that causes light to scatter and fluorescence dyes to emit light at specific frequencies. Cells are primarily identified in the forward and side scatter reflecting the approximate cell size and the cell complexity (granularity). In peripheral blood samples, these scatters were used to distinguish lymphocytes, monocytes and

granulocytes. They were also used to exclude dead cells and debris. Fluorescence labeling with one or more markers allows the investigation of cell structures, functions or the developmental stages. For this study the characteristics of 10,000-40,000 cells per sample were acquired using a FACScalibur (BD Bioscience, San Jose, CA). Simultaneously, the intensity of up to four fluorescence dyes was recorded. After acquisition, the data were analyzed using the program CellQuest (BD Bioscience, San Jose, CA). In this study the main focus was on the characteristics of the live lymphocyte population in blood, bone marrow and spleen, as distinguished by the forward and side scatter. The data were depicted in dot plots; the position of each dot reflects the fluorescence intensities measured for one cell. Additionally, a third characteristic can be reflected using different dot colors.

4.2.4.5 Test for autoreactivity

The serum of progeny of the nuclear transfer mouse was analyzed for autoreactive antibodies. The blood of different mice was collected under inhalation anesthesia (Metaphane, Mallinckrodt, Mundelein, IL). From this the serum was harvested after the blood coagulated. The serum of mice of the following genotypes was analyzed: $H^{o/o}$ $L^{o/o}$ (wt), $H^{+/o}$ $L^{cr1/o}$, $H^{+/o}$ $L^{cr1/o}$ $Rag1^{-/-}$, $H^{+/o}$ $L^{kb4/o}$, $H^{+/o}$ $L^{kb4/o}$ $Rag1^{-/-}$, and $H^{+/o}$ $L^{kb4/cr1}$. All sera were of Ig^{a} allotype. Before the analysis, the IgM titers of the different sera were determined by ELISA (see 4.2.3) and adjusted to equal antibody concentration for use in flow cytometry. As a substrate for the test, spleen and bone marrow cells from euthanized C57BL/6, $Aire^{-/-}$, $Aire^{+/-}$ and $H^{o/o}$ $L^{kb4/o}$ $RAG1^{-/-}$ mice were harvested and prepared as single-cell suspensions (see 4.2.4.1). All tested tissue was either of Ig^{b} allotype or from RAG1-deficient mice. The spleen and bone marrow cells were incubated for 30 min with one of the normalized Ig^{a} sera. To detect antibody binding (i.e., autoreactivity), a PE-coupled anti-IgM^{a} antibody (BD PharMingen, San Diego, CA) was added after washing. APC-coupled anti-B220 antibodies (BD PharMingen, San Diego, CA) were used to counterstain the cells. The samples were measured using a FACScalibur (BD Bioscience, San Jose, CA).

4. Results

4.1 The nuclear transfer mouse

Having taken a vital interest in B lymphocytes and B lymphocyte development, the nuclear transfer mouse was very remarkable when it was published. It was generated from a single B cell by nuclear transfer, presenting the next generation in Ig transgenic mice. The most interesting feature of this mouse is that it has one H- and two L-chain gene rearrangements. It is postulated in the clonal selection theory that every single lymphocyte produces only one out of the fast repertoire of antibodies present in the system (Burnet, 1959). Established by allelic exclusion, each antibody is produced from one productive H-chain allele and one productive L-chain allele. Therefore, the H- and two L-chain gene combination found in the nuclear transfer mouse are very unusual. To explore this phenomenon, the objective was to analyze B-cell development in this mouse. As an initial step, the two L-chain gene rearrangements of this mouse were to be evaluated.

4.1.1 The L-chain genes are rearranged in frame

The productivity of the different L-chain alleles was not determined in the original publication (Hochedlinger and Jaenisch, 2002). To find out if both L-chain genes could direct L-chain synthesis (i.e. are productive), it had to be determined whether the gene rearrangements were in-frame. If only one rearrangement was in-frame, the nuclear transfer mouse, and therefore the donor B cell, did not violate the basic allelic exclusion rule.

Using the published sequence fragment of the first L-chain allele referred to as "Vκ region similar to the kk4 gene" by Hochedlinger and Jaenisch, the DNA stretch comprising the first Vκ region was identified using the Celera mouse genome database (Hochedlinger and Jaenisch, 2002). Creating a set of primers annealing upstream of the Vκ gene segment and downstream in the Jκ5 region, the respective variable region gene recombinations were amplified from genomic template DNA by polymerase chain reaction (PCR). The DNA fragment was sequenced and the sequence compared to the Igκ

segments in the NCBI (National Centre for Biotechnology Information) database (www.ncbi.nlm.nih.gov/igblast).

Figure 4.1. **Sequence of the kb4 L-chain allele.**

Tail DNA of a $H^{o/o}$ $L^{kb4/o}$ mouse was isolated by isopropanol precipitation and amplified by PCR using primers annealing upstream of the Vκ region and downstream of Jκ5. The PCR product was sequenced and the sequence was compared to the NCBI database. Dashes, matching bases for V_{kb4} and Jκ1 genes respectively; underneath, amino acid sequence. The grey box indicates the Jκ1 gene sequence. The kb4 allele and NCBI sequences for V_{kb4} (AJ231228) and Jκ1 (V00777). The red box, Cystein residue (Cys 88); blue boxes, Glycins (Gly 98/100), important motifs characteristic for functional κ chains.

The first Vκ region was identified as V_{kb4} combined with Jκ1; denoted the kb4 allele, which encodes L^{kb4} (Fig. 4.1). The sequence alignment gave a 100% match with the corresponding NCBI database entry. The next best alignment had a similarity of 91.2%. No stop codons were found in the analysis of the nucleotide sequence indicating that the rearrangement was in frame. Examination of the amino acid sequence indicated the presence of two Glycine residues in the frame work region 4 of the L chain as well as a Cystein residue in the beginning of frame work region 3. These two Glycines (Gly 98, 100) represent a highly conserved motif in vertebrate L chains, while the Cystein residue

(Cys 88) is mandatory for the L chain to loop via a disulfide bond with a second Cysteine (Cys 22) (Kabat et al., 1983; Xu et al., 1998). These characteristic motifs are important for functional Igκ L-chain proteins. Furthermore, a recent analysis of the κ L-chain locus classifies V_{kb4} as a functional V region segment (Brekke and Garrard, 2004).

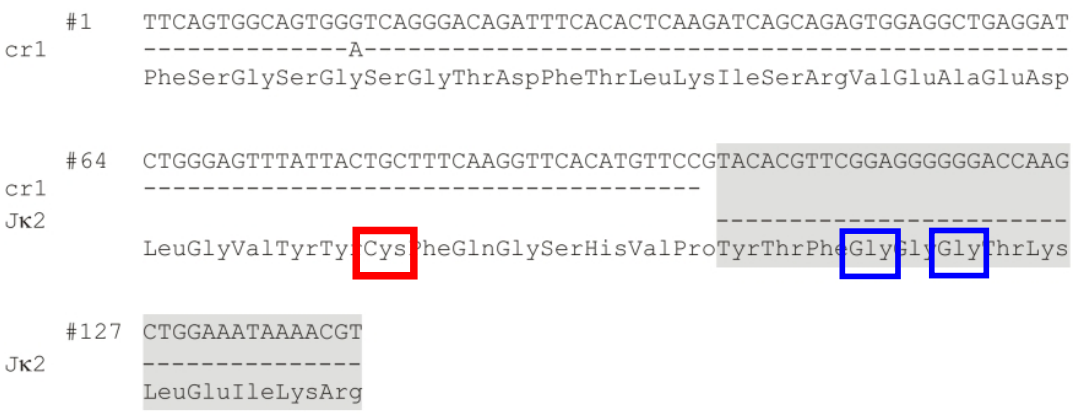

Figure 4.2. **Sequence of the cr1 L-chain allele.**
Tail DNA of a $H^{o/o}$ $L^{cr1/o}$ mouse was isolated by isopropanol precipitation and amplified by PCR using a generic Vκ primer annealing within the Vκ region and a primer annealing downstream of Jκ5. The PCR product was sequenced and the sequence was compared to the NCBI database. Dashes, matching bases for V_{cr1} and Jκ2 genes respectively; underneath, amino acid sequence. The grey box indicates the Jκ1 gene sequence. The cr1 allele compared to NCBI sequences for V_{cr1} (AJ231205) and Jκ2 (V00777). The red box, Cystein residue (Cys 88); blue boxes, Glycins (Gly 98/100), important motifs characteristic for functional κ chains.

The second Igκ allele was published as "Vκ1 segment similar to the cu2 gene" (Hochedlinger and Jaenisch, 2002). An Igκ generic primer (annealing with most within most Vκ regions), in combination with the Jκ5 primer annealing downstream of Jκ5, was used to amplify this allele by PCR. Sequence analysis identified the DNA fragment as V_{cr1} recombined with Jκ2; denoted the cr1 allele, which encodes L^{cr1} (Fig. 4.2). This V region sequence gave a 99.1% match when compared to sequences in the NCBI database. The next best hit had a 95.1% similarity. Analyses showed the recombination was in-frame, with no premature stop codon. Both the Cystein (Cys 88) as well as the Glycine residues (Gly 89, 100) were present indicating a theoretically productive VJκ assembly. Like V_{kb4}, the V_{cr1} segment is also annotated as functional in a recent κ L-chain analysis (Brekke and Garrard, 2004).

In conclusion, the two L-chain alleles, kb4 and cr1, are both in-frame and have joined their Vκ segments to Jκ1 and Jκ2, respectively. Both rearrangements show conserved sequence element motifs important for the synthesis for functional Igκ light chain genes.

4.2 The lambda L-chain and related loci.

While analyzing the two Igκ rearrangements of the nuclear transfer mouse, and studying the available online resourses, it became clear that the Igκ locus was well characterized and mapped by extensive work of H. G. Zachau (Kirschbaum et al., 1998; Roschenthaler et al., 2000; Schable et al., 1999; Thiebe et al., 1999). However, a precise map of the λ L-chain locus had not yet been published and the location of the surrogate L-chain gene $V_{preB}2$ was unknown. It was therefore necessary to create the physical map of the λ L-chain locus and the related loci $V_{preB}1$, $V_{preB}2$ and λ5.

4.2.1 The lambda L-chain locus

A physical map of the λ L-chain locus was compiled using the Celera mouse genome database (www.celera.com) and the DNA sequence of the different λ L-chain genes from the NCBI database. In this map the published order of the λ genes was confirmed (Miller et al., 1988; Storb et al., 1989). All Vλ, Jλ and Cλ segment alignments were found in the contiguous GA_x5J8B7W55B7 scaffold on chromosome 16 (Celera database). The scaffold is 3,625,067 bases long and spans from base 12,061,199 to base 15,868,166. The distances between the exons, their chromosomal location, and their lengths are given in Table 4.1.

Gene	Position on chromosome 16 from (bp)	Position on chromosome 16 to (bp)	Length (bp)	Distance to next gene segment (bp)
Vλ2 exon I	15,596,729	15,596,684	46	93
Vλ2 exon II	15,596,590	15,596,287	304	18,531
VλX exon I	15,577,755	15,577,710	46	103
VλX exon II	15,577,606	15,577,284	323	40,442
Jλ2	15,536,841	15,536,805	37	1,345
Cλ2	15,535,459	15,535,143	317	2,003
Jλ4	15,533,139	15,533,102	38	1,240
Cλ4	15,531,861	15,531,548	314	93,981
Vλ1 exon I	15,437,566	15,437,521	46	93
Vλ1 exon II	15,437,427	15,437,124	304	14,414
Jλ3	15,422,709	15,422 673	37	1,359
Cλ3	15,421 313	15,420,997	317	2 ,103
Jλ1	15,418,893	15,418,857	37	1,153
Cλ1	15,417,703	15,417,384	320	1,077,001[a]

[a] Distance to $V_{preB}2$ exon II

Table 4.1. **The mouse λ L-chain locus.**

Chromosomal positions, lengths and distances of the gene segments. The sequences of the respective λ L-chain genes were retrieved from the NCBI database and compared to the Celera mouse genome database to annotate their genomic location.

The murine λ L chain has three Vλ gene segments – Vλ2, VλX, and Vλ1. As in the IgH and Igκ locus, the Vλ gene segments consist of two exons, one encoding most of the signal peptide sequence and the other encoding the rest of the leader and the variable segment. In the Igλ locus, they are separated by a 93- to 103-base intron. Unlike the Igκ locus, with its clear structure of Vκ and Jκ gene segments followed by a single Cκ constant region, the λ locus contains four Cλ constant region genes; each of these segments is preceded by a Jλ segment (Blomberg and Tonegawa, 1982; Miller et al., 1982). The Jλ and Cλ region gene segments are arranged in two clusters: Jλ2Cλ2–Jλ4Cλ4 (Blomberg et al., 1981; Selsing et al., 1982) and Jλ3Cλ3–Jλ1Cλ1 (Blomberg et al., 1981; Miller et al., 1982). Because of a non-functional RNA donor splice site, Jλ4 and Cλ4 are pseudogenes (Blomberg et al., 1981; Selsing et al., 1982).

The λ locus is divided into three parts that are separated by 40.4 kb and 93.9 kb, respectively (Fig. 4.3). Aligning the sequences of Vλ2 and VλX with their respective leader sequences, these V regions were found in what was defined as the first part of the λ locus, which is 19,446 bases long (Table 4.1) (Sanchez et al., 1990; Tonegawa et al., 1978; Weiss and Wu, 1987). The leader segment of Vλ2 contains a 93-base intron followed by the second part of the gene encoding the rest of the variable gene segment. The leader sequence of the VλX gene contains a 103-base intron, and the rest of the gene segment is contiguous.

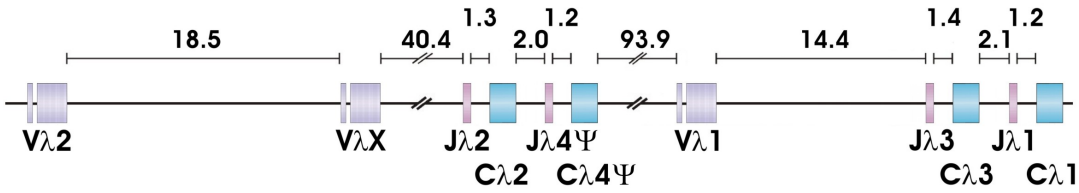

Figure 4.3. **Physical map of the mouse λ L-chain locus**.

Depicted is the genomic organization of the various gene segments according to their genomic location Celera database as presented in Tab. 1. The distances are given in kilobases (kb). Transcriptional orientation is from left to right. Vλ, variable, Jλ, joining and Cλ, constant gene segment, Ψ, denotes pseudo gene segments Jλ4 and Cλ4.

The second part of the locus begins 40,442 bases downstream of VλX; the distance between Vλ2 and Cλ2 is 60.8 kb, and thus somewhat shorter than the 74 kb reported by Storb and co-workers (Storb et al., 1989). This part of the locus contains Jλ2 and Cλ2 and the pseudogenes Jλ4 and Cλ4; it is 5,294 bases long. Downstream of the λ2-λ4 locus are 93,981 bases which do not encode any known gene segment.

The third part of the λ locus is 20,183 bases long; it begins with the Vλ1 and its leader sequence exon, which are separated by a 93-bp intron (Arp et al., 1982; Bernard et al., 1978). Next to Vλ1 and 14,414 bases downstream, are Jλ3 and Cλ3, which are separated by 1,359 bases. The 15,810-base distance between the Vλ1 and Cλ3 is thus smaller than the 19 kb previously published (Storb et al., 1989). Finally, the remaining gene segments of the locus, Jλ1 and Cλ1, are located 2,103 bases 3′ of Cλ3. They are separated by 1,153 bases.

4.2.2 The surrogate L-chain loci $V_{preB}1$, $V_{preB}2$ and λ5.

In addition to the λ L-chain locus, the homologous sequences of the λ L-chain-related surrogate L chain genes λ5, $V_{preB}1$ and $V_{preB}2$ were localized. The genes are expressed in pro-B/pre-BI and large pre-BII cells. They build the pre-B cell receptor (pre-BCR) combined with the μH chain (Dul et al., 1996; Karasuyama et al., 1994; Kudo and Melchers, 1987; Kudo et al., 1992; Sakaguchi et al., 1986; Sakaguchi and Melchers, 1986). The two V_{preB} genes encoded on opposite strands are 97% identical on the nucleotide and amino acid level. $V_{preB}1$ is expressed in all cells expressing the λ5 gene, whereas $V_{preB}2$ mRNA is co-expressed in approximatly 30% of the same cells (Dul et al., 1996). Both genes have sequence homologies to Vλ segments, whereas the λ5 gene has homology to Jλ- and Cλ-gene segments. The physical map of the λ5, $V_{preB}1$ and the $V_{preB}2$ loci is shown in Fig. 4.4.

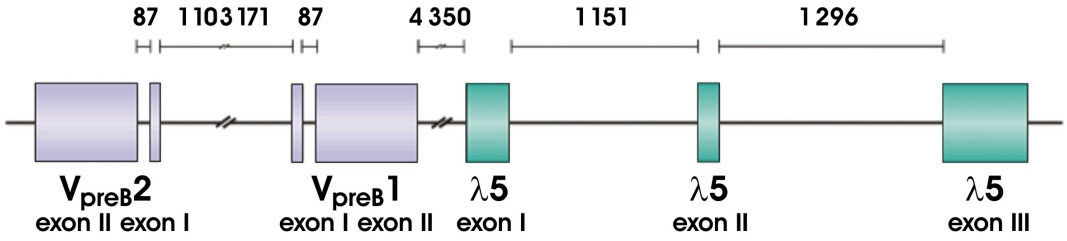

Figure 4.4. **Physical map of the surrogate-L chain genes VpreB1, VpreB2 and λ5.**
Depicted is the organization of the various exons according to their genomic location in the Celera database as shown in Tab. 2. The distances are given in bases. The general transcriptional orientation is from left to right, except for $V_{preB}2$.

The λ5 gene is encoded by three exons that are not rearranged during B-cell development (Sakaguchi and Melchers, 1986). Splicing of the primary transcript yields a 1.2-kb mRNA (Kudo and Melchers, 1987). Exon I contains the sequence encoding the signal peptide (Kudo and Melchers, 1987). The λ5 sequence is in the scaffold GA_x5J8B7W55B7 on chromosome 16. The exons span a 3,076-base-long DNA segment with the same transcriptional orientation as the λ L-chain genes. Thus the distance between the λ5 exons is somewhat smaller than the 3.75 kb published by Kudo and co-workers (Kudo and Melchers, 1987) (Table 4.2). Exon I of λ5 is located 2,185,801 bases downstream of Cλ1. Exon II starts 1,151 bases 3´ of the first exon. Exon III is located 1,295 bases downstream of the second exon; this distance differs slightly from the 1.35 kb published by Kudo et

al., (Kudo and Melchers, 1987). The published exon sequences and the Celera database are 100% identical. The distances between the exons, their positions on chromosome 16, and their lengths are given in Table 2. $V_{preB}1$ is in the same transcriptional orientation as λ5 and the λ L-chain genes. It is composed of two exons that do not rearrange during B-cell development (Kudo and Melchers, 1987). Exon I encodes most of the leader peptide, and exon II starts 87 bases downstream of the first exon. After splicing, they yield a 0.85-kb mRNA. Starting with the first exon, 2,180 kb downstream of Cλ1, the $V_{preB}1$ locus is located between the λ L chain and the λ5 gene. The first $V_{preB}1$ exon is 100% identical in the two data bases. The whole locus spans 516 bases, from base 13,236,765 to base 13,236,250. The distance between the second $V_{preB}1$ exon and the first λ5 exon comprises 4,667 base pairs. This is in consistency with the 4.6 kb distance between VpreB1 and λ5 published by Kudo and Melchers (Kudo and Melchers, 1987). The sequence homology between the $V_{preB}1$ exons II in the two databases is 99%; at position 234 there is either an A or a G. The exact distances of the exons and their respective positions are given in Table 2. Out of all gene loci analysed, only the $V_{preB}1$ locus was annotated in the Celera database. In their Biomolecule Report, this locus spans 849 bases—from base 13,236,904 to base 13,236,056. The $V_{preB}1$ locus was found to stretch 516 bases, from base 13,236,765 to base 13,236,250. The difference in length is due to the untranslated regions, which were included by Celera, but disregarded in this analysis. The distances between the exons, their chromosomal location, and their lengths are given in Table 4.2.

Gene	Position on chromosome 16 from (bp)	Position on chromosome 16 to (bp)	Length (bp)	Distance to next exon (bp)
VpreB2 exon II [a]	14,340,382	14,340,069	314 [b]	87
VpreB2 exon I [a]	14,339,981	14,339,936	46	1,103,170
VpreB1 exon I	13,236,765	13,236,720	46	87
VpreB1 exon II	13,236,632	13,236,250	381	4,667
λ5 exon I	13,231,582	13,231,389	194	1,151
λ5 exon II	13,230,237	13,230,122	116	1,295
λ5 exon III	13,228,826	13,228,507	320	

[a] opposite transcriptional orientation from the λ L-chain and λ5 – VpreB1 loci
[b] length smaller than original gene sequence (see text for details)

Table 4.2. **The mouse surrogate L chain genes $V_{preB}1$, $V_{preB}2$ and λ5.**
Chromosomal positions, lengths, and distances of the gene segments. The sequences of the respective surrogate-L chain genes were retrieved from the NCBI database and compared to the Celera mouse genome database to annotate their genomic location.

Like $V_{preB}1$, the $V_{preB}2$ gene consists of two exons (Kudo and Melchers, 1987). The first exon encodes the leader peptide and is 46 bases long. The second exon encodes the main protein, spanning 381 bases. The whole sequence is 516 bases long, including a 87-base intron. $V_{preB}2$ had been located on chromosome 16 (Kudo and Melchers, 1987), but its precise position had not yet been determined. Searching the Celera database for sequence homologies, a contiguous alignment of 347 bases was found for the first part of the gene. It showed a 100% homology. The sequence included the first exon, the whole intron, and 214 bases of the second exon. A second alignment, being 223 bases in length, was found 57 bases downstream of the first. This homologous sequence represents the second part of exon II, and also shows 100% homology. However, in the Celera database, the 57 bases between the two homologous alignments of $V_{preB}2$ are given as unknown nucleotides (Ns), whereas in our annotation there are 127 bases filling this space. Inspecting this difference it can be concluded that the Ns given in the Celera database do not reflect the correct number of nucleotides. If this is also true for other N regions given in the Celera database, the distances given here for some loci might also change.

The $V_{preB}2$ locus was identified in the Celera database on chromosome 16 starting from base 14,339,936 going to base 14,340,382. It is in opposite transcriptional orientation to the λ L chain, λ5 and $V_{preB}1$ loci. The Celera nucleotide sequence was found 1,077,001 bases downstream of Cλ1 and 1,103,170 bases upstream of $V_{preB}1$.

Taken together it was possible to determine that the λ locus stretches over three parts spanning 179,346 bases on chromosome 16. The exact distances between the exons were defined and confirmed the order of the gene segments as follows: Vλ2-VλX, Jλ2-Cλ2-Jλ4-Cλ4 and Vλ1-Jλ3-Cλ3-Jλ1-Cλ1. All genes segments are in the same transcriptional orientation. Furthermore, the $V_{preB}2$ locus was located 1,077,001 bases downstream 3´ to the λ locus, the $V_{preB}1$ locus is 2,180,618 bases downstream of the λ locus and the λ5 gene is located 4,667 bases downstream of the $V_{preB}1$ locus. Except for the $V_{preB}2$ locus all other loci are in the same transcriptional orientation.

4.2 Ig genes in the nuclear transfer mouse

4.3.1 Designation of the different Ig genotypes

With a better understanding of the genomic organization of the different L-chain genes and related loci, the focus returned to B-cell development in the nuclear transfer mouse. To study the characteristics of the different antibodies of this mouse, the H- and two L-chain alleles of the original mouse were separated and recombined by selectively crossing its offspring. In this study the influence of the H-chain allele derived from the nuclear transfer mouse in combination with either L-chain allele kb4 or cr1 was analyzed. The nuclear transfer derived H-chain allele, denoted H^+, was rearranged by combining V_H region VH22.1, D region DFL16.2 and J_H region J_H3 (Fig. 4.5, first line). The second allele is unrearranged, i.e. in germline configuration, denoted H^o (Fig. 4.5, second line). For the L chain, the two κ L-chain alleles of the nuclear transfer mouse, denoted kb4 and cr1, were analyzed as described in 4.1.1. The kb4 allele has the Vκ region V_{kb4} recombined to Jκ1, the allele is denoted L^{kb4} (Fig. 4.5, third line). The cr1 allele has the Vκ region V_{cr1} recombined to Jκ2, the allele is denoted L^{cr1} (Fig. 4.5, fourth line). An unrearranged κ allele in germline configuration, denoted L^o, was also included. It represents the wild-type allele before recombination. The λ L-chain locus in all mice analyzed was in germline configuration. According to these designations, the genotype of

a wild-type mouse with two unrearranged H-chain alleles and two unrearranged L-chain alleles is $H^{o/o}$ $L^{o/o}$. The genotype of the original B cell mouse is denoted $H^{+/o}$ $L^{kb4/cr1}$ as summarized in Fig 4.5.

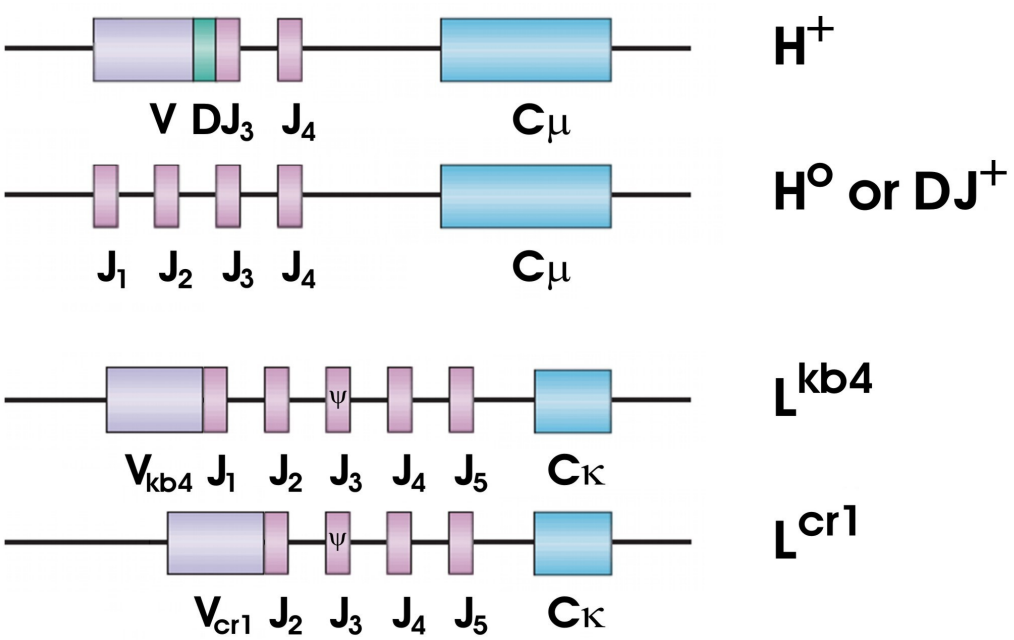

Figure 4.5. **The Ig alleles of the B cell nuclear transfer mouse.**
Schematic representation of the H- and L-chain rearrangements of the original donor lymphocyte. The sequence of the H chain was analyzed by Hochedlinger (personal communication). The sequences of the different L chains were amplified by PCR, sequenced and compared to the NCBI database. Productive H-chain allele (H^+), and silent H-chain allele (H^o, unrearranged; or DJ^+); at the L-chain locus, there are two in-frame alleles, L^{kb4} and L^{cr1}. ψ denotes pseudogene segment Jκ3. V, variable; D, diversity; J, joining gene segment. Cμ, IgM constant region, Ck, Igκ constant region.

4.3.2 Two productive L-chain gene rearrangements

The Ig-gene combination of the donor B lymphocytes with one H- and two L-chain genes was unexpected. It seems to challenge the rule of allelic exclusion, but similar Ig gene combinations have been analyzed before. Other studies showed that B cells may contain more than one in-frame rearranged endogenous L-chain allele (Yamagami et al., 1999). Both alleles might even be translated into polypeptides (Diaw et al., 2001; Schwartz et al., 1981). To not violate allelic exclusion, it is thought that one of the L chains does not pair with the H chain, or the HxL combination does not reach the cell surface; thus one L chain would not be functional. Here, the ability of the respective L chains to pair with this particular H chain was tested in vivo.

To find out which one of the two alleles was the "productive" and the "non-productive" one, they were combined separately with the H^+ allele by breeding. To exclude receptor editing, the mice were put on a RAG1-deficient background. When H- and L-chain genes are introduced into RAG1-deficient mice, complete reconstruction of the B-cell lineage and the emergence of functionally mature B cells is achieved (Spanopoulou et al., 1994; Xu et al., 1998). To detect the presence of a productive BCR on the cell surface of peripheral B lymphocytes by flow cytometry (fluorescence associated cell sorting, FACS), cells were marked with monoclonal antibodies to IgM of allotype a (μ^a) and polyclonal antibodies to Igκ (κ). Since both the H^+ and the H^o alleles were of Ig^a allotype the allotype specificity of the antibody was irrelevant here. It has previously been described that in such mice elimination of autoreactive cells occurs equally well as in the presence of RAG1 (Spanopoulou et al., 1994; Xu et al., 1998). Therefore, B cell stage specific markers were used to track B-cell development. B220 is a member of the protein thyrosine phosphatase family (Johnson et al., 1997). Its expression level correlates directly with the stage of B cell maturity – the higher the B220 expression the more mature the B cell. Here, monoclonal antibodies to B220 were used as an indicator for continuous B-cell maturation. Using monoclonal antibodies to CD21, three expression levels can be distinguished in immature B cells, characteristic for different maturation stages (Kinoshita et al., 1988; Kinoshita et al., 1990). Mature B cells are CD21 low ($CD21^{low}$). Transitional B cells are either CD21 negative ($CD21^{neg}$) in transitional stage 1 (T1) or CD21 high ($CD21^{hi}$) in transitional stage 2 (T2) (Carsetti et al., 1995; Loder et al., 1995).

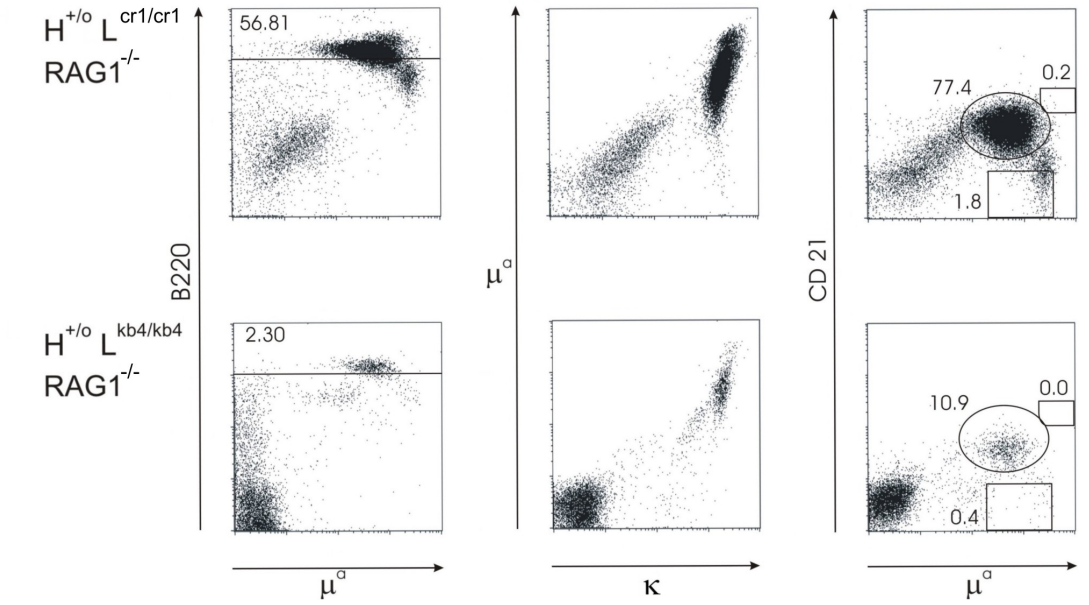

Figure 4.6. **The Ig alleles of the B cell nuclear transfer mouse.**

Flow cytometric analysis of peripheral blood lymphocytes (PBL) from RAG1-deficient ($RAG1^{-/-}$) mice with either cr1 or kb4 L-chain genes, combined with H^{+}. The mice were genotyped by PCR. PBL were prepared by hemolysis in a standard NH_4CL lysis buffer. Cells were stained 20 min with a PE-coupled anti-κ antibody or a biotin-coupled anti-μ^a antibody and with antibodies to B220 (B220-APC), CD21 (CD21-FITC) and μ^a (μ^a-biotin). PE-Cy5 conjugated streptavidin was used as a secondary marker. The mice were analyzed at 4-weeks of age. Numbers indicate percentages of mature (B220 high) B cells over total lymphocytes. The numbers in the boxed areas (transitional B cells) and encircled areas (mature B cells) are percentages of total lymphocytes. Lower box, T1, transitional state 1; upper box, T2, transitional state 2. The profiles were done four times. The mouse genotypes are given left of the profiles.

The FACS plots showed peripheral B cells from mice with either L allele expressed IgM^a (μ^a) on the surface, indicating either L chain, kb4 or cr1, was able to pair with the H chain respectively and could be presented on the cell surface, thus both are productive. Interestingly, the number of B cells in the kb4 mouse was strongly reduced. Analyzing Ig expression levels by means of receptor density on the other hand, demonstrated that B cells of mice with the genotype $H^{+/o}$ $L^{kb4/kb4}$ were as high as of mice of the $H^{+/o}$ $L^{cr1/cr1}$ genotype (Fig. 4.6, middle panels). Comparing the B220 expression levels, B cells with either kind of receptor show a mature phenotype, as both expression levels are above the threshold for mature B cells (Fig. 4.6, left panels). Comparing B-cell development by means of CD21 expression in kb4 and cr1 mice also showed the presence of mature B cells (encircled areas) and transitional B cells (boxed areas) (Fig. 4.6, right panels).

The most characteristic observation in these plots was the fact that there were much fewer B cells in the mouse with the H chain and the kb4 L chain than in the mouse with the H chain and the cr1 L chain. Despite the lower B cell count both mice still possess mature, IgM^a positive lymphocytes indicating a productive rearrangement on both L-chain alleles.

4.3.3 The Hxkb4 receptor is autoreactive

The finding that both L-chain rearrangements are productive combined with the fact that much fewer B cells are present in the RAG1-deficient mice with the HxL^{kb4} receptor than the HxL^{cr1} receptor, led to the hypothesis the HxL^{kb4} antibody might be autoreactive.

When testing this theory one has to keep in mind that the donor B cell was taken from an unchallenged mouse. One can therefore only speculate to what the putative antigen might be. If the first L-chain allele is autoreactive, the second L-chain recombination was most likely the result of receptor editing, that is secondary L-chain rearrangement. This is generally predicted in the spleen and in the bone marrow. Consequently, the approach was to look for the alleged autoreactivity in these organs. The serum of offspring of the nuclear transfer mouse was used to determine whether there was any reactivity with spleen or bone marrow cells. Serum was collected from a $H^{o/o}$ $L^{o/o}$ mice (wild-type Ig) and mice of the Ig genotypes $H^{+/o}$ $L^{kb4/o}$ and $H^{+/o}$ $L^{cr1/o}$, both on a regular and a $RAG1^{-/-}$ background, and from a $H^{+/o}$ $L^{cr1/kb4}$ mouse (all antibodies of Ig^a allotypetype). Once again $RAG1^{-/-}$ mice were used to exclude editing and have a monoclonal serum of Hxkb4 and Hxcr1 antibodies. To compare the binding of IgM antibodies from different mice to cells from selected organs, it was necessary to determine the titer of the IgM antibodies in the different sera by enzyme linked immunosorbent assay (ELISA). In a second step these antibodies were then normalized to equal concentration for use in flow cytometry. Initial assays revealed that the serum IgM concentration of $H^{+/o}$ $L^{kb4/o}$, $H^{+/o}$ $L^{cr1/o}$ and Ig wild-type mice were in the same range while the serum for $H^{+/o}$ $L^{kb4/o}$ and $H^{+/o}$ $L^{cr1/o}$ mice on a $RAG1^{-/-}$ background was at least 20 times lower (data not shown). The protocol was adapted by including a higher starting dilution for these samples to measure all samples in the same assay. In the ELISA shown here (Fig. 4.7) the initial dilution for the regular serum was 1:100,000. The sera of mice on a $RAG1^{-/-}$ background were pre-diluted 1:5,000. All samples were measured in duplicates. Displayed are the means of these

measurements. To calculate back the concentrations, a standard of known IgM concentration was also included.

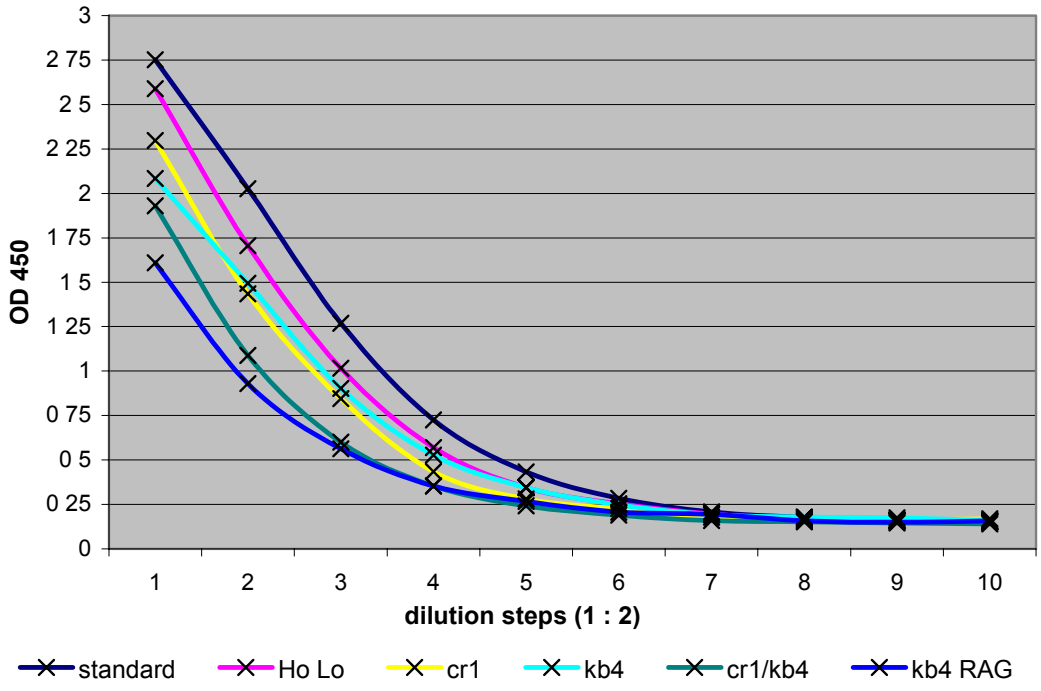

Figure 4.7. **Serum-IgM titer in various mouse strains.**

Indirect capture Enzyme-linked immunosorbent assay (ELISA) for serum-IgM concentration from mice of various genotypes: HoLo ($H^{o/o}$ $L^{o/o}$, Ig wild-type), cr1 ($H^{+/o}$ $L^{cr1/o}$), kb4 ($H^{+/o}$ $L^{kb4/o}$), cr1/kb4 ($H^{+/o}$ $L^{kb4/cr1}$), kb4 RAG ($H^{+/o}$ $L^{kb4/o}$ $RAG1^{-/-}$). ELISA Plates were coated with 4 ng/ml of goat anti-μ^a diluted in PBS. Blocking was done with 1% bovine serum albumin (BSA) in PBS. Washes were done with PBS containing 0.1% of Tween-20. The initial dilution for the standard was 1:500; for $H^{+/o}$ $L^{kb4/o}$; $RAG1^{-/-}$ it was 1:5,000; all other sera were diluted at 1:100,000. A horseradish peroxidase (HRP)-coupled goat anti-mouse Ig (H plus L chain) was used as a secondary antibody. TMB (3,3',5,5' tetramethylbenzidine) enzyme substrate was used for development. Antibodies from a mouse standard Ig panel were used as standards. Ordinate, optical density at 450 nm, abscissae, dilution steps of 1:2 respectively.

Analysis of the ELISA revealed similar concentrations for all mice on a RAG-sufficient background while serum IgM concentration was approximately 30 times lower in mice on a RAG-deficient background. Calculating back from the known standard, the wild-type ($H^{o/o}$ $L^{o/o}$) serum had a concentration of 14.7 mg/ml, the $H^{+/o}$ $L^{cr1/o}$ and the $H^{+/o}$ $L^{kb4/o}$ mouse had serum levels of 11.2 mg/ml and 11.1 mg/ml respectively. The $H^{+/o}$ $L^{kb4/cr1}$ mouse had IgM serum levels of 7.2 mg/ml. The lowest IgM levels were found in the $H^{+/o}$ $L^{kb4/o}$ mouse on a $RAG1^{-/-}$ background with 0.3 mg/ml. These experiments were repeated four times with the serum of different mice. The serum IgM-titer of a $H^{+/o}$ $L^{cr1/o}$

mouse on a RAG1-deficient background was measured in a separate ELISA (data not shown). It was found to be 0.3 mg/ml and repeated testing always indicated it to be in the same range as the RAG1-deficient $H^{+/o}$ $L^{kb4/o}$ mouse.

Because the cognate self-antigen was assumed to be in the spleen, single cell suspensions were prepared from the spleen of mice of Ig^b allotype and incubated with the various sera of Ig^a allotype. The different serum-IgM titers were determined before every experiment respectively, and normalized. To detect binding of the serum antibodies, a phycoerythrin (PE)-coupled antibody to μ^a was used as a secondary reagent; this way, positive staining of spleen cells must be mediated by the (autoimmune) serum. Furthermore, an allophycocyanin (APC)-coupled antibody to B220 allowed us to differentiate the spleen cells. To ensure that a possible positive result was not strain dependant, these tests were carried out on spleen cells of different strains of Ig^b allotype in separate experiments (C57BL/6, $AIRE^{-/-}$, $AIRE^{+/+}$ and $H^{o/o}$ $L^{o/o}$ $RAG1^{-/-}$ mouse). The results were the same in all analyzed strains, irrelevant of the genotype. Two of these flow cytometry experiments are shown in Fig. 4.8.

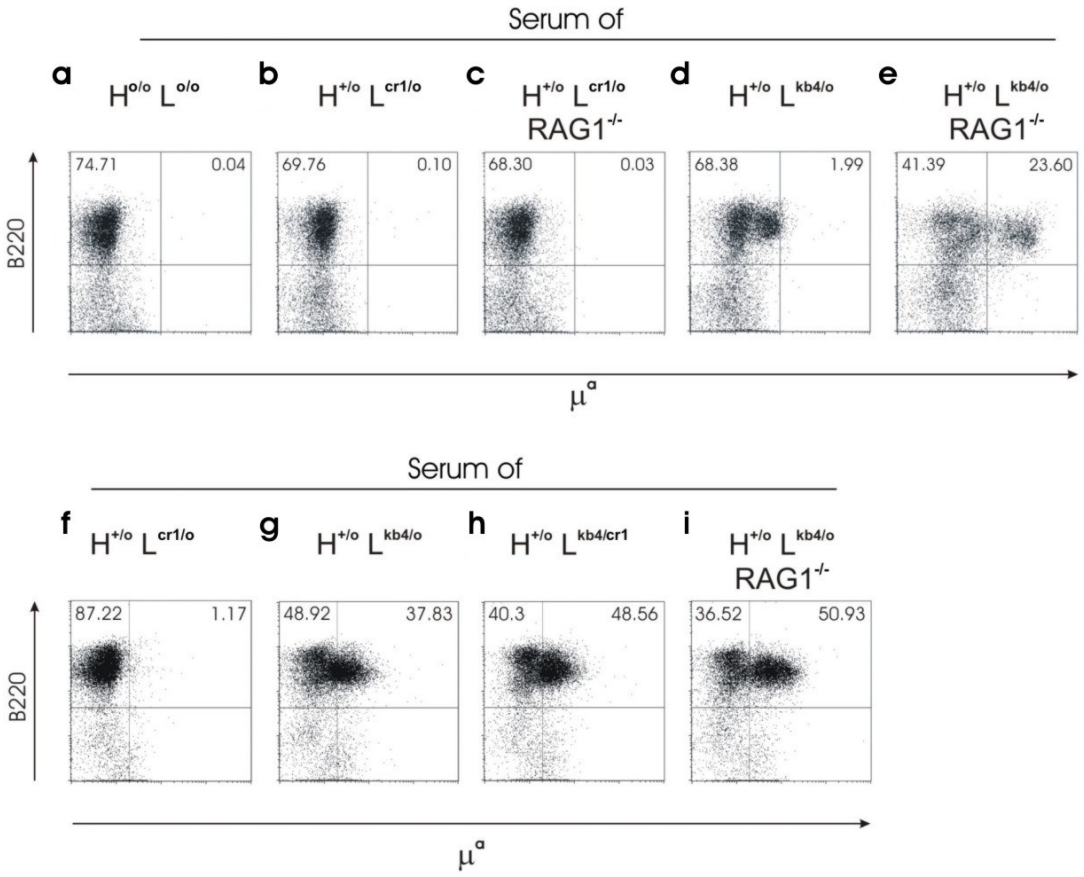

Figure 4.8. **Serum autoreactivity of various mouse strains.**
Sera from mice of various Ig genotypes (as indicated above), or $H^{o/o}$ $L^{o/o}$ (Ig wild-type) serum were incubated with single-cell suspensions of spleen cells of C57BL/6 (f-i) and $AIRE^{-/-}$ (a-e) mice, respectively, all of μ^b allotype. (Altogether, cells from four mouse strains were tested, two of which are shown. The fact that some of the mice were AIRE-deficient is not relevant here.) Samples were incubated with one of the normalized sera, of μ^a allotype. As a secondary antibody, PE-coupled anti-μ^a was added. APC-coupled anti-B220 was used to counterstain the cells. Ordinates, B220 antibody; abscissae, monoclonal anti-μ^a. Numbers in the quadrants indicate the percentages of the respective cell populations.

Analyzing the results of the experiment with spleen cells of C57BL/6 mice (Fig. 4.8f-i) and $AIRE^{-/-}$ mice (Fig. 4.8a-e), the wild-type ($H^{o/o}$ $L^{o/o}$) sera (Fig. 4.8, first upper profile) did not react with spleen cells, nor did the sera from $H^{+/o}$ $L^{cr1/o}$ mice, regardless whether it was on a RAG1-deficient background (Fig. 4.8c) or not (Fig. 4.8b, 4.8f). In contrast to this, serum from $H^{+/o}$ $L^{kb4/o}$ mice on a RAG1-deficient background did stain one third (Fig. 4.8e), or more than half of the cell, respectively (Fig. 4.8i). With receptor editing abolished in $RAG1^{-/-}$ mice, their serum is monoclonal. Therefore these experiments clearly demonstrate autoreactivity of the Hxkb4 receptor while no reactivity was seen with Hxcr1 or any wild-type antibodies. Interestingly, serum from a RAG wild-type $H^{+/o}$

$L^{kb4/o}$ mouse also exhibited autoreactivity (Fig. 4.8d, 4.8g), albeit with less intensity. Furthermore, serum from a mouse of $H^{+/o}$ $L^{kb4/cr1}$ genotype, reflecting the original donor lymphocyte, also showed autoreactivity (Fig. 4.8h), indicating that the kb4 allele contributed to at least some antibodies in that mouse. Similar experiments with bone marrow cells of the same mouse strains showed an identical pattern of autoreactivity (data not shown).

Although it was shown that the Hxkb4 antibody is autoreactive, the identity of the autoantigen has not yet been determined. Closer analysis of the stained cell population revealed that they trace back to the dead cell fraction in the forward and side scatter profiles (data not shown). In the same profiles the (live) lymphocyte gate, on the other hand, contained cells that stained with the Hxkb4 antibody, but they were few. Furthermore, although only the B220-positive cells stained, one cannot automatically assume that the self-antigen is present exclusively on dying B lymphocytes. B cells may be more sensitive to our experimental conditions. It must also be considered that dying or dead cells may give rise to artifacts in flow cytometry. However in all experiments, the target cells came from a single spleen cell preparation that was divided for incubation with the sera – yet reactivity of the B220-positive ($B220^{+}$) cells was associated only with sera containing the Hxkb4 antibody.

Concluding this analysis, it was possible to determine the presence of a yet undetermined self-antigen in the murine spleen and bone marrow to which only the autoreactive Hxkb4 receptor responds.

4.3.4 Deletion of B cells expressing Hxkb4

With the Hxkb4 receptor being autoreactive, the finding that RAG-deficient mice with the kb4 allele have fewer B cells than mice with cr1 allele is consistent (Fig. 4.4). Here, the objective was to determine the fate of B cells with a Hxkb4 receptor in a RAG-proficient mouse, as B cells with an autoreactive receptor do not maturate (Chen et al., 1995).

While analyzing various mice with the Hxkb4 receptor, it was noticed that RAG wild-type mice with two kb4 alleles ($H^{+/o}$ $L^{kb4/kb4}$) have a similar phenotype with low numbers of mature B cells in the periphery as RAG1-deficient $H^{+/o}$ $L^{kb4/o}$ mice (Fig. 4.9, second lower panel; Fig. 4.4, second lower panel). In these mice B-cell development is not restricted by RAG1 deficiency or lack of T-cell help. These mice are thus perfect to trace B-cell development. In the bone marrow, expression of the BCR on immature B cells marks the first stage in which B cells are subjected to selection events. After this first selection the IgM^+ B cells migrate as transitional B cells from the bone marrow via the bloodstream, exiting in the spleen. There are two major subsets of transitional B cells, the more immature transitional type 1 (T1) and more mature transitional type 2 cells (T2). Similar to the selection in the bone marrow during central development, B cells are once again subjected to BCR mediated negative selection during the peripheral stage of B-cell development in the spleen. The T2 cell stage is the last step before the lymphocytes are released into the blood as mature naïve B cells. Staining for IgM, CD21 and IgD, the three compartments bone marrow, blood and spleen were examined. B-cell development was studied by flow cytometry in mice of $H^{+/+}$ $L^{kb4/kb4}$ genotype and compared to $H^{o/o}$ $L^{o/o}$ (Ig wild-type) mice.

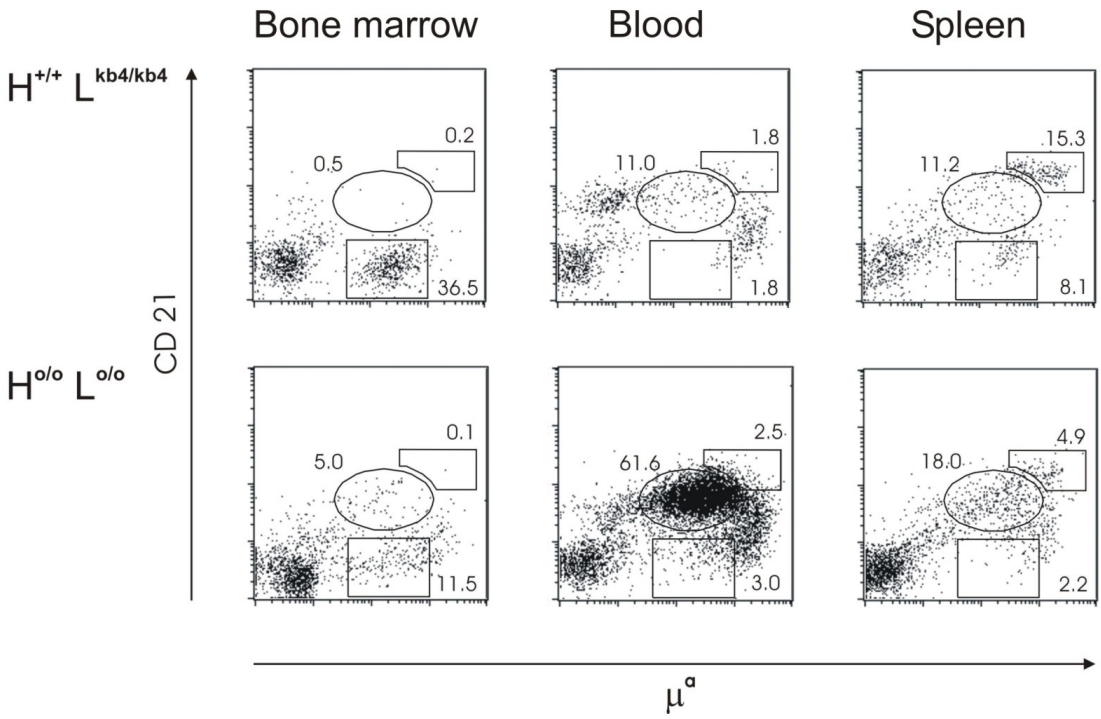

Figure 4.9. **Autoreactive B cells are blocked at the transitional stage 1.**

Flow cytometric analysis to follow B-cell development and selection in the bone marrow, spleen and peripheral blood of 4- to 6-week-old mice of genotype $H^{+/+}$ $L^{kb4/kb4}$ (upper panel) as compared to normal wild-type maturation, $H^{o/o}$ $L^{o/o}$ (lower panel). Lymphocytes from blood, bone marrow and spleen were prepared by hemolysis in a standard NH_4CL lysis buffer. The cells were stained with PE-coupled antibodies to μ^a and antibodies against CD21 (CD21-FITC). Ordinates, anti-CD21. Abscissae, anti-μ^a. The numbers in the boxed areas (transition stage B cells) and encircled areas (mature B cells) are percentages of total lymphocytes. Lower box, T1, transition state 1; upper box, T2, transition state 2.

Comparative analysis of the B-cell development in these two mice revealed that immature B cells in bone marrow and transitional B cells in the spleen of $H^{+/+}$ $L^{kb4/kb4}$ mice are blocked in development at the transitional stages 1 (T1), defined as $\mu^+/CD21^{neg}$ and 2 (T2) defined as $\mu^+/CD21^{hi}$, in the spleen (Fig. 4.9). There were more T1 cells in the bone marrow of a $H^{+/+}$ $L^{kb4/kb4}$ mouse (36.5%) than in a wild-type Ig (11.5%) mouse (Fig. 4.9). Similarly, there are more T2 cells in the spleen of the kb4 mouse than in the wild-type (15.3% versus 4.9%). However, in the peripheral blood, this situation was reversed. The $H^{+/+}$ $L^{kb4/kb4}$ mouse had few mature B cells (11%), defined as $\mu^+/CD21^{lo}$, as compared to 61.6% cells in the Ig wild-type (Fig. 4.9).

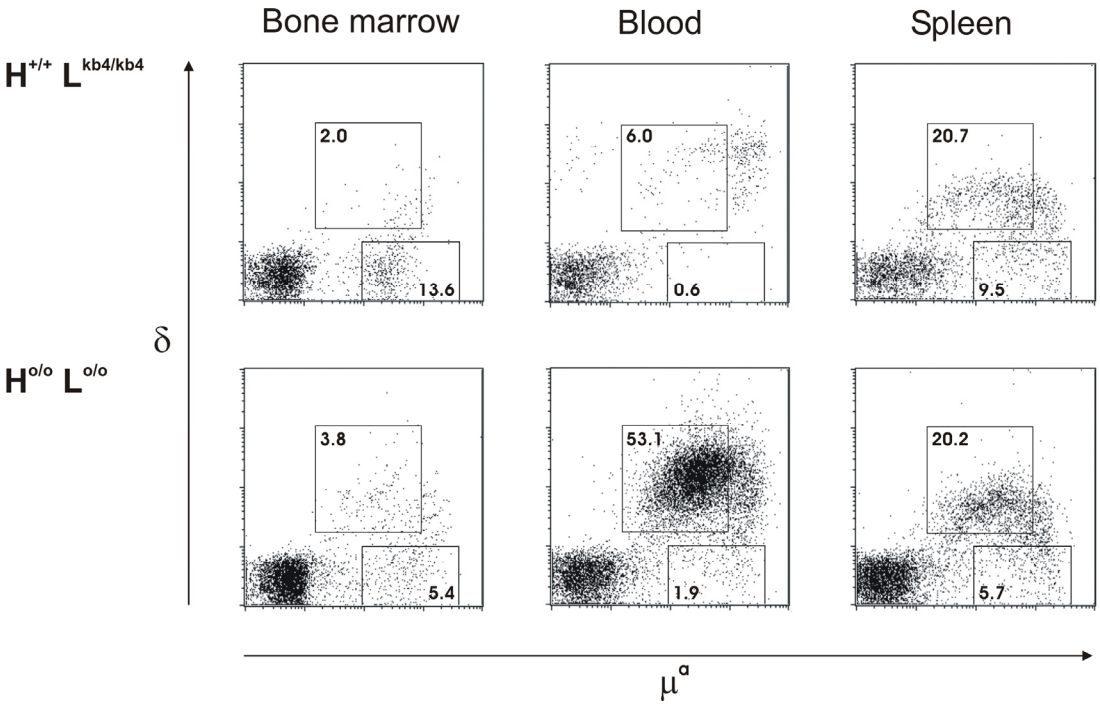

Figure 4.10. **Hxkb4 B cells are absent from the mature B cell pool.**
Flow cytometric analysis to follow B-cell development and selection in the bone marrow, spleen and peripheral blood of 4- to 6-week-old mice of genotype $H^{+/+}$ $L^{kb4/kb4}$ (upper panel) as compared to wild-type mice, $H^{o/o}$ $L^{o/o}$ (lower panel). Lymphocytes from blood, bone marrow and spleen were prepared by hemolysis in a standard NH_4CL lysis buffer. Lymphocytes were stained with PE-coupled antibodies to μ^a and biotinylated antibodies against δ (δ-biotin). Streptavidin coupled to PE-Cy5 was used as a secondary marker. Ordinates, anti-δ^α. Abscissae, anti-μ^a. Upper box, mature B cells; lower box, immature B cells.

In addition to CD21 and μ^a, IgD (δ) expression profiles were also assessed in the same mice as a second marker for mature B cells (Fig. 4.10). This allowed the verification of the previous results. Comparing early B-cell development, the $H^{+/+}$ $L^{kb4/kb4}$ mouse had 13.6% transitional B cells in the bone marrow, versus wild-type with 5.4% cells, again indicating a block in development. Similarly to previous plots, few of these cells were detected as mature B cells in the blood (6.0%), as compared to the wild-type (53.1%), which indicates negative selection.

This data speaks to a distinct block in B-cell development at the transitional B cell stage in $H^{+/+}$ $L^{kb4/kb4}$ mice, namely in the T2 cell stage in the spleen. This causes highly reduced numbers of mature naïve B cells in the peripheral blood.

4.3.5 Editing of receptors encoded by Hxkb4

Since the autoreactive Hxkb4 BCR causes a block in B-cell development in the spleen, the alleged location for B cell editing, different Ig combinations of the nuclear transfer mouse were analyzed for receptor editing. In normal mice, it is thought that during this process an autoreactive κ L-chain rearrangement is deleted by joining an upstream Vκ region to a downstream Jκ region gene. Alternatively, if productive recombination on the Igκ locus fails, the Igλ locus can be rearranged. In the nuclear transfer mouse, the V_{kb4} and V_{cr1} gene segments are joined to Jκ1 and Jκ2, respectively. Therefore, additional Vκ genes are present upstream as well as additional Jκ genes downstream of the respective alleles. Thus, like in Ig wild-type mice they can, in principle, be edited by either rearranging one of the κ alleles or the λ L-chain genes. Here, the peripheral blood of different mice was analyzed for signs of BCR editing.

Since RAG1 is required for receptor editing, it is not surprising that B cells with the Hxkb4 receptor were deleted in RAG1-deficient mice. Also, RAG1-sufficient mice with two kb4 alleles were more likely to delete their cells than not, even when editing their receptors (Fig. 4.9, 4.10, upper panels). Although in such cells three Jκ segments available for editing, compared to the four Jκ segments in germline configuration, the editing would have to be successful twice, unless an occasional new κ chain overrides the "autoimmune signal". However, cells with only one kb4 allele ought to edit their receptors and survive. To find out whether kb4, and/or cr1, are being edited, these genes were combined with an H^+ allele on a RAG1-sufficient background. Staining the peripheral B lymphocytes of the various mice including an Ig wild-type mouse for IgM and Igκ, receptor editing can be assessed by flow cytometry to determine whether these mice produce κ chains other than cr1 or kb4, respectively; or λ.

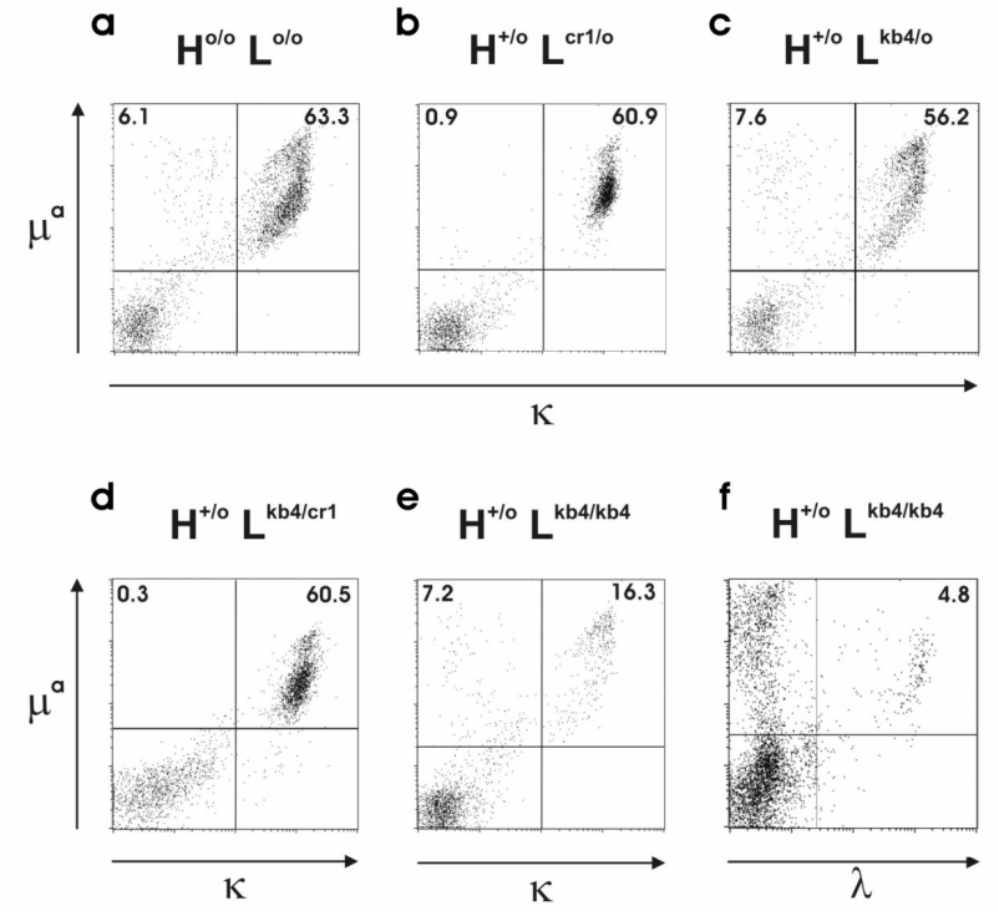

Figure 4.11. **Editing and allelic inclusion of the kb4 receptor.**

Flow cytometric analysis (one of five experients) of peripheral blood lymphocytes of 4- to 6-week-old wild-type and H^+ mice with various L-gene combinations. PBL were prepared by hemolysis in a standard NH_4CL lysis buffer. Samples were incubated with PE-coupled antibodies to κ and λ, respectively, and with antibodies to μ^a (μ^a –FITC). Ordinates, anti-μ^a; abscissae, anti-κ or anti-λ, respectively.

Analyzing the peripheral blood of $H^{+/o}$ $L^{cr1/o}$ mice, almost no κ-negative cells, which are consequently λ positive, were found (Fig. 4.11b). Furthermore, these B cells seemed to develop faster than regular B cells. All cells in the plot belong to either the double negative, non-B cell population or the mature B cell population, seemingly skipping the stages in between. They appeared as a distinct population in flow cytometry, which was homogenous and thus appeared monoclonal (Fig. 4.11b). This observation once again speaks in favor of our earlier conclusion that the cr1 allele most likely encodes the L chain that is part of a regular, non-self receptor, which is not edited. On the other hand, the kb4 allele did not prevent other κ (Fig. 4.11c) or λ genes (Fig. 4.11f) from being generated. The profile of the $H^{+/o}$ $L^{kb4/o}$ B cell population (Fig. 4.11c) looks similar to the B cell pool in an Ig wild-type mouse (Fig. 4.11a). It does not appear monoclonal and

displays the same diversity as the Ig wild-type. One can conclude this, because mice with one kb4 allele had a normal (polyclonal) B cell population, similar to an Ig wild-type mouse – presumably the result of L-chain editing. Studies in other mice showed that for an autoreactive Vκ joined to Jκ2, editing mediates tolerance with no apparent reduction in cell number (Halverson et al., 2004; Spanopoulou et al., 1994; Xu et al., 1998). As V_{kb4} is joined to Jκ1 the same should be true for these mice. In mice with two kb4 alleles again a low number of mature B cells was observed (Fig. 4.11e), which appeared in combination with a κ-negative population similar to the Ig wild-type (Fig. 4.11a). In studies on mice transgenic for an autoreactive Vκ-to-Jκ5 rearrangement – leaving λ as only editing option – editing is associated with diminished splenic B cell numbers (Halverson et al., 2004; Spanopoulou et al., 1994; Xu et al., 1998). Editing is aggravated in mice with two kb4 alleles, thus a similar situation might be true in this case. Here, B cells start of with an autoreactive receptor that can not easily be destroyed due to its presence on both alleles, resulting in reduced B cell numbers in the periphery. Consistent with the conclusion that the cr1 allele encodes a non-self receptor, which is not edited, the B cell compartment of mice with the genotype $H^{+/o}$ $L^{kb4/cr1}$ (Fig. 4.11d), were indistinguishable from the $H^{+/o}$ $L^{cr1/o}$ mouse (Fig. 4.11b).

In conclusion, these flow cytometry plots speak for accelerated B-cell development in mice with the Hxcr1 receptor with no signs of editing. In $H^{+/o}$ $L^{kb4/o}$ mice, editing is activated, causing a normal B cell pool. In $H^{+/o}$ $L^{kb4/kb4}$ mice on the other hand, B cell editing is aggravated, leading to B cell elimination. The strong resemblance of $H^{+/o}$ $L^{kb4/cr1}$ and $H^{+/o}$ $L^{cr1/o}$ profiles suggests a dominance of the non-self reactive allele over the autoreactive one.

4.3.6 Allelic inclusion

The autoreactive Hxkb4 receptor mediates B cell editing. As editing puts a strain on allelic exclusion, the question arose if allelic inclusion, the expression of two L-chain alleles, may have occurred in the donor B cell. This would contradict the general presumption that B cell editing always destroys the autoreactive rearrangement by recombining on the same allele and thereby maintaining allelic exclusion.

Although the kb4 allele did not prevent editing and further gene rearrangement, on a single cell level, isotypic (and perhaps allelic) exclusion may be maintained by the (now edited) kb4 allele, at least to some extent. In six mice of $H^{+/o}$ $L^{kb4/o}$ genotype, there were 6% to 7% κ-negative cells (7.6% shown in Fig. 4.11c), comparable to the 5% to 10% generally found in wild-type mice, of genotype $H^{o/o}$ $L^{o/o}$ (6.1% shown in Fig. 4.11a). These data raise an interesting paradox: in the donor B cell, which gave rise to the nuclear transplant mouse, the kb4 chain was edited in the sense that another L chain was added, but it did not cause allelic exclusion. This course of events is perplexing, because in general, the process of editing destroys self-reactive receptors. Unless there was some transcriptional or translational control, or other events, differentiating between the two allelic κ mRNAs, the kb4 L chain may have been expressed along with the cr1 chain. At any rate, in the mice with the genotype $H^{+/o}$ $L^{kb4/cr1}$, this seems to be the case.

To assess the extent of allelic exclusion, a germline κ allele (κ^o) was introduced in which the constant region (Cκ) had been replaced by the human Cκ sequence (Cκh)(Casellas et al., 2001). In expression profiles of heterozygous mice, this allele can be distinguished from the normal mouse κ allele. If a cell containing the kb4 transgene is edited and directs L-chain synthesis from the other allele, it should be detected by an antibody specific to human κ.

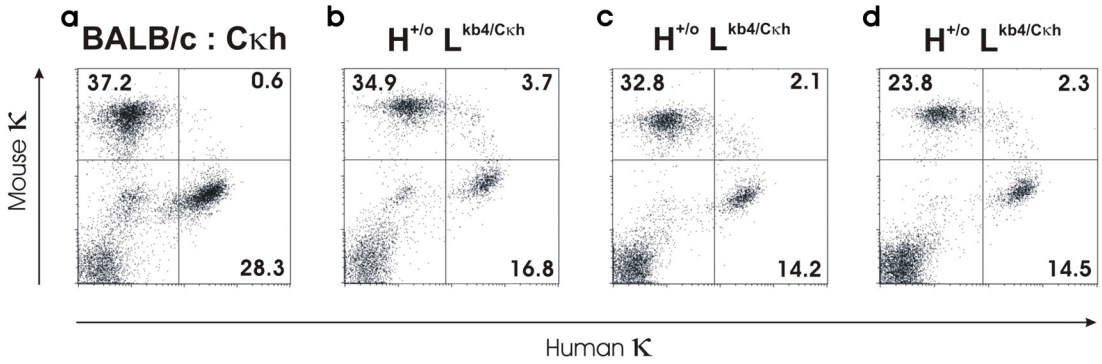

Figure 4.12. **Editing and allelic inclusion of the kb4 L chain.**

Flow cytometric analysis of mice with κ alleles that can be distinguished using different antibodies. PBL from 4- to 6-week-old mice were prepared by hemolysis in a standard NH_4CL lysis buffer. Samples were incubated with FITC-coupled antibodies to human κ and with antibodies to mouse κ (anti mouse κ–PE). Ordinates, goat anti-mouse κ; abscissae, goat anti-human κ. First panel from left, mixture of white blood cells from BALB/c mice and mice with two human Cκ alleles (Cκh); second, third, and fourth panels, mice with genotype $H^{+/o}$ $L^{kb4/Cκh}$. Numbers in the quadrants indicate the percentages of the respective cell populations over all lymphocytes.

In the three mice of the genotype $H^{+/o}$ $L^{kb4/Cκh}$, approximately 15% of the total population and one third of all B cells expressed human Cκ (Fig. 4.12b-d), clearly indicating editing. In these cells, editing might have replaced the autoreactive kb4 allele by an alternative, but unproductive rearrangement. Further attempts to recombine the remaining Jκ segments yielded only unproductive L chains, exceeding the editing potential on this allele. Thus, the second allele is recombined using the human Cκ allele. Alternatively, the kb4 allele might also have been silenced while the human Cκ allele was recombined and expressed. This possibility was not further investigated. More interestingly, another fact can be gleaned from this experiment: while a mixture of cells from a mouse with mouse κ genes and cells from a mouse with human $C_κ$ (denoted BALB/c : Cκh) gave very little background in the double-positive gate (0.6%, Fig. 4.12a), in the three mice of the genotype $H^{+/o}$ $L^{kb4/Cκh}$, there were from 2% to 3.7% cells that expressed both mouse and human κ (Fig. 4.12b-d). This percentage, however, does not reflect the extent of editing that replaces the kb4 allele by another mouse rearrangement. The 34.9% of mouse Cκ cells may or may not express the original kb4. But on the other hand, one can say that up to 18% of the cells with human κ still express mouse κ. This number calculated by dividing the 3.7% of double producers, by 20.5%, the sum of all human Cκ producers (3.7% + 16.8% = 20.5%), multiplied by 100. This data may give a clue as to the

expression status of the L alleles of the donor B cell from which the nuclear transfer mouse was generated. Clearly, the transcription unit of the kb4 allele was not destroyed, but it cannot be known whether or not the kb4 allele was silenced in other ways, for example, by methylation, or by a translational block. Because non-B cells do not rearrange Ig genes, for sure both alleles were generated in the donor mouse rather than in the nuclear transfer mouse.

The experiments described above allowed tracing the editing history of the donor B cell as follows: The pro-B cell started with the rearrangement of the H locus on the first allele. This rearrangement yielded a productive gene (H^+), and the cell continued to proliferate and to recombine the kb4 gene at the κ locus. The L chain was rearranged in-frame and the BCR presented on the B cell surface. Before or while traveling into the spleen, the immature B cell with the Hxkb4 receptor encountered self-antigen, which led to receptor editing. But instead of destroying the kb4 allele, rearrangement of the other κ allele generated the cr1 gene instead rescuing the B cell. However, the self-reactive allele may not have been silenced, but generated antibodies as fellow travelers, and thus contributed to the pool of autoantibodies in the serum.

4.4 transgenic Ig-gene expression

A lot of what is known today about the generation and function of B lymphocytes is based on experiments performed in mice transgenic for various H- and L-chain genes. This is especially true for autoreactivity and B-cell editing. Common to all Ig transgenic mice is the presence of pre-rearranged H- and/or L-chain genes in the germline. In normal, non-Ig transgenic mice these genes are unrearranged in the germline. During B-cell development RAG is activated to recombine the H- and L-chain genes. The Ig genes can only be expressed after RAG mediated recombination. Afterwards, receptor editing is only activated if the expressed BCR is autoreactive.

Unlike in normal mice, in the offspring of the nuclear transfer mouse the H- and L-chain loci are already pre-recombined in the germline, making recombination unnecessary. Such Ig genes could, in principle, be expressed before RAG is synthesized. This raises the critical question: When are transgenic H- and L-chain genes expressed during B-cell development? This question has broad implications for the study of B-cell development in

Ig transgenic mice, especially in regard to autoreactivity and editing. If RAG expression comes on before pre-rearranged transgenes are synthesized, the sequence of Ig genes might be altered before it is presented on the cell surface. Therefore, the editing process studied in such mice would be an experimental artifact, as it was not induced by autoreactivity. Only if the synthesis of a preformed BCR receptor precedes RAG expression in all cells, and an autoreactive receptor still causes editing, would this indicate a receptor-driven editing process. This important question has not previously been addressed in Ig transgenic mice. The two different BCRs of the nuclear transfer mouse were used to answer this question.

4.4.1 Hxcr1 receptor expression precedes RAG1 expression

To compare the expression of wild-type and transgenic H- and L-chain genes in relation to RAG1, Ig genes were crossed to mice in which the RAG1 gene had been replaced by a green fluorescent protein (GFP) cassette (Kuwata et al., 1999). The generated offspring were heterozygous for wild-type RAG1 on one allele and the GFP knock-in on the other allele, denoted $RAG1^{GFP/wt}$. In these mice, expression of the non-marked RAG1 allele, as well as the RAG2 locus, is concordant with the onset of GFP fluorescence (Hirose et al., 2002; Igarashi et al., 2002). Furthermore, as the non-marked RAG1 allele is still functional, these $RAG1^{GFP/wt}$ mice are still RAG proficient. Because RAG1 expression is easily detectable, these mice provide a valuable tool to study the influence of pre-rearranged H- and L-chain genes on RAG1 expression.

The results presented are preliminary. Here, the bone marrow and spleen of individual $H^{+/o}$ $L^{cr1/o}$, $H^{+/o}$ $L^{kb4/o}$, $H^{o/o}$ $L^{kb4/o}$ and $H^{o/o}$ $L^{o/o}$ mice were analyzed, all of them on a $RAG1^{GFP/wt}$ background (Fig. 4.13). A second mouse of the $H^{+/o}$ $L^{cr1/o}$ $RAG1^{GFP/wt}$ genotype was analyzed in a separate experiment (data not shown). In general, RAG expression is restricted to B and T cells during BCR and TCR recombination and editing. Thus, the unrearranged TCR in these mice represents an internal positive control for RAG expression. The respective thymuses of these mice were therefore also included in the experiments.

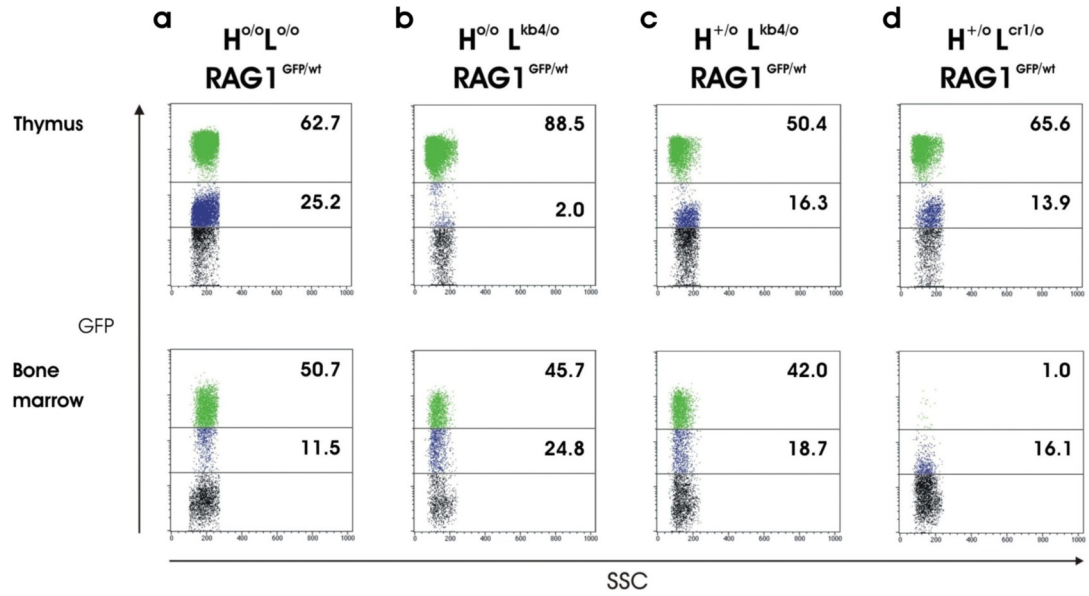

Figure 4.13. **RAG1/GFP expression in the bone marrow and thymus of Ig transgenic mice.**
Flow cytometric analysis of RAG1/GFP expression in the bone marrow and thymus of 4- to 8-week-old mice of genotypes $H^{+/o}$ $L^{cr1/o}$, $H^{+/o}$ $L^{kb4/o}$, $H^{o/o}$ $L^{kb4/o}$ and $H^{o/o}$ $L^{o/o}$ (Ig wild-type) mice, all on a RAG1/GFP background. Thymus and bone marrow lymphocytes were prepared by hemolysis in a standard NH_4Cl lysis buffer and washed. Ordinates, GFP expression; abscissae, side scatter. All mice were heterozygous for wild-type RAG1 and a knocked-in GFP under a RAG1 promoter ($RAG1^{GFP/wt}$). High-GFP-expressing cells (GFP^{hi}) are shown in green; low-GFP-expressing cells (GFP^{lo}) are shown in blue; and no-GFP-expressing cells (GFP^{neg}), shown in black. Numbers in gates indicate percentages of the respective cell population over all lymphocytes.

In the flow cytometry plots of $RAG1^{GFP/wt}$ mice, the GFP signal can be separated into three populations: high-GFP-expressing cells (GFP^{hi}), shown in green; low-GFP-expressing cells (GFP^{lo}), shown in blue; and no-GFP-expressing cells (GFP^{neg}), shown in black (Fig. 4.13). In RAG1-GFP mice, only the green GFP^{hi} population co-expresses RAG1 and GFP; in the other populations, RAG1 expression was undetectable by PCR (Igarashi et al., 2002). Analyzing the influence of different transgenic BCRs on RAG activation in the bone marrow (Fig. 4.13, lower panels), RAG activity was found in mice of the $H^{+/o}$ $L^{kb4/o}$ (42%, Fig. 4.13c, lower panel) and $H^{o/o}$ $L^{kb4/o}$ (45.7%, Fig. 4.13b, lower panel) genotypes, as well as in the Ig wild-type mouse (50.7%, Fig. 4.13a, lower panel). In contrast, two analyzed mice of the $H^{+/o}$ $L^{cr1/o}$ genotype demonstrated only 1.0% and 2.7% of GFP-positive cells, respectively (Fig. 4.13d, lower panels, and data not shown). As a positive control, the FACS plots showed that thymic T cells expressed GFP, and hence RAG1, in all analyzed mice. As they rearranged their TCRs, these cells were between 50.4% and 88.5% GFP positive (Fig 4.13a-d, upper panels). In normal B-cell

development, RAG is expressed during H- and L-chain rearrangement in the bone marrow, but is shut down once a productive BCR is presented on the cell surface (Nemazee et al., 2002).

Therefore, these experiments demonstrate that RAG1 is expressed before the pre-rearranged Hxcr1 BCR is synthesized, at least in some cells. Interestingly, the Hxkb4 receptor, using the same H chain as the Hxcr1 BCR, did not prevent RAG1 expression in the bone marrow. These results support our findings that the autoreactive Hxkb4 BCR causes receptor editing. In comparison, an Ig wild-type mouse (50.7%, Fig. 4.13a, lower panel) shows a phenotype that is similar to that of a mouse with the Hxkb4 receptor (42.0%, Fig 4.13c, lower panel). In the mouse analyzed here, the kb4 allele was present on only one allele ($H^{+/o}$ $L^{kb4/o}$). This allowed the B cell to easily alter the L chain by editing and thereby demonstrate normal B-cell development. These results are in agreement with the earlier finding that the peripheral B-cell pool of $H^{+/o}$ $L^{kb4/o}$ mice is very similar to that of $H^{o/o}$ $L^{o/o}$ (Ig wild-type) mice (Fig. 4.11a). RAG1/GFP was expressed in the bone marrow of the $H^{o/o}$ $L^{kb4/o}$; $RAG1^{GFP/wt}$ mouse (45.7%, Fig. 4.13c, lower panel), because the H chain still has to be rearranged during B-cell development.

4.4.2 RAG1/GFP is expressed in the blood and spleen of Hxkb4 mice

In addition to bone marrow and thymus, the spleen and the peripheral blood of the same mice were also analyzed. Individual $H^{+/o}$ $L^{cr1/o}$, $H^{+/o}$ $L^{kb4/o}$, $H^{o/o}$ $L^{kb4/o}$ and $H^{o/o}$ $L^{o/o}$ mice were studied all of them on a $RAG1^{GFP/wt}$ background (Fig. 4.13). A second mouse of the $H^{+/o}$ $L^{cr1/o}$ $RAG1^{GFP/wt}$ genotype was analyzed in a separate experiment (data not shown). Here, the B-cell marker B220 was used to distinguish GFP fluorescence in the B and T cells of these compartments (Fig. 4.14). Therefore, the different GFP populations in these studies are gated accordingly, with only the green cell population representing RAG-expressing cells.

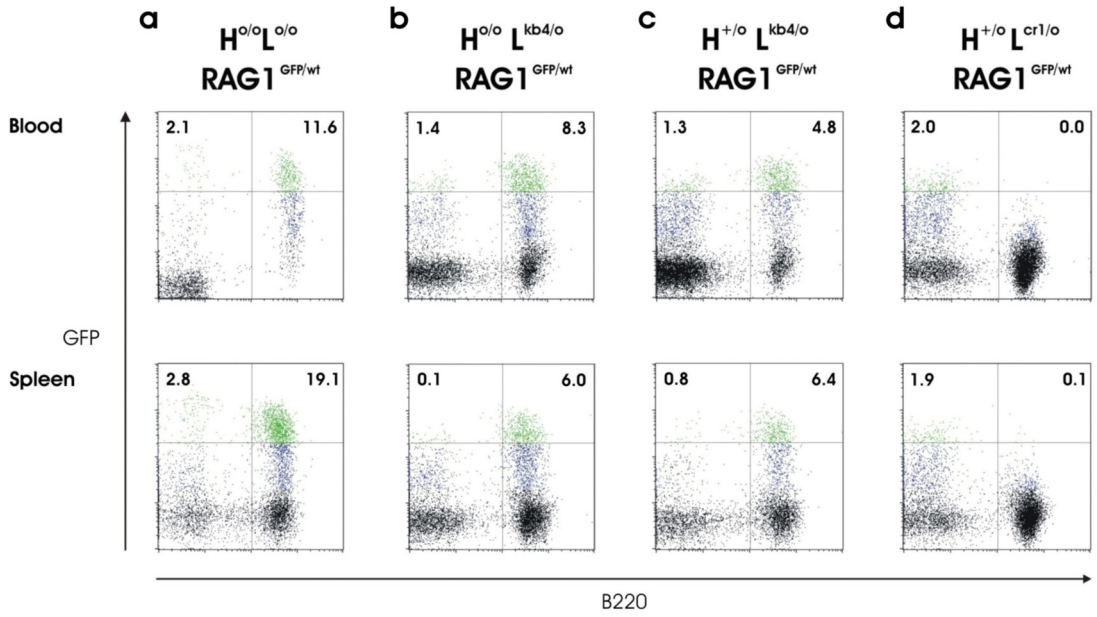

Figure 4.14. **RAG1/GFP expression in the blood and spleen of Ig transgenic mice.**
Flow cytometric analysis of RAG1/GFP expression in the peripheral blood and the spleen of 4- to 8-week-old mice of genotypes $H^{+/o}$ $L^{cr1/o}$, $H^{+/o}$ $L^{kb4/o}$, $H^{o/o}$ $L^{kb4/o}$ and $H^{o/o}$ $L^{o/o}$ (Ig wild-type) mice, all on a $RAG1^{GFP/wt}$ background. Blood and spleen lymphocytes were prepared by hemolysis in a standard NH_4Cl lysis buffer. Samples were incubated with APC-coupled antibodies to B220. Ordinates, GFP; abscissae, anti-B220 antibodies. High-GFP-expressing cells (GFP^{hi}) are shown in green; low-GFP-expressing cells (GFP^{lo}) are shown in blue; and no-GFP-expressing cells (GFP^{neg}), shown in black. Numbers in quadrants indicate percentages of the respective cell population over all lymphocytes.

Similar to the bone marrow, the $H^{+/o}$ $L^{cr1/o}$ mice showed an almost complete absence of GFP^{hi} B cells, with 0% or 0.9% GFP^{hi} B cells in the blood (Fig. 4.14d, upper panel and data not shown) and 0.1% or 0.2% GFP^{hi} B cells in the spleen (Fig. 4.14d, lower panel and data not shown). This is in contrast to the RAG1/GFP expression in the blood and spleen of the $H^{+/o}$ $L^{kb4/o}$ mouse (4.8% and 6.4%, respectively, Fig. 4.14c), $H^{o/o}$ $L^{kb4/o}$ mouse (8.3% and 6.0%, respectively, Fig. 4.14b) and the $H^{o/o}$ $L^{o/o}$ (Ig wild-type) mouse (11.6% and 19.1%, respectively, Fig. 4.14a). As a control, the B220-negative T cells in all mice displayed overall smaller but consistent GFP^{hi} populations at rates between 1.3% and 2.1% in the blood (Fig. 4.14a-d, upper panels) and 0.1% to 2.8% in the spleen (Fig. 4.14a-d, lower panels). While these results confirmed the findings in the bone marrow, the GFP^{hi} B- and T-cell populations in the blood seemed to challenge the published RAG expression pattern (Fig. 2.2) (Grawunder et al., 1995). However, these findings have been confirmed by other groups working with RAG1/GFP knock-in mice. As an explanation, it was found that in such mice GFP has a much longer half-life than the RAG protein. This

results in continued GFP fluorescence even after RAG1 expression has ceased (Hirose et al, 2002; Igarashi et al., 2002). Interestingly, the GFP^{hi} B-cell population in wild-type and $H^{+/o}$ $L^{kb4/o}$ mouse is larger in the spleen (19.1% and 6.4%, respectively, Fig. 4.14a, lower panel and 4.14c, lower panel) than in the blood (11.6% and 4.8%, respectively, Fig. 4.14a, upper panel and 4.14c, upper panel).

Taken together, these results show that, in the offspring of the nuclear transfer mouse, the H- and L-chain transgenes are expressed before RAG is synthesized. Furthermore, it appears that development was accelerated in B cells with the Hxcr1 receptors. In these B cells, RAG is not activated for V(D)J recombination or editing. In mice with the autoreactive Hxkb4 receptor, on the other hand, receptor editing and expression of RAG1 are activated.

5. Discussion

In the study of B-cell development, transgenic mice carrying pre-rearranged H- and L-chain genes have been very useful. Among these transgenic mice, the nuclear transfer mouse has a unique role in that it is the only one to be directly derived from a B lymphocyte. It was the goal of this work to use this mouse to trace the editing history of a single B lymphocyte.

5.1 The nuclear transfer mouse

In the history of Ig transgenic mice, the nuclear transfer mouse can be regarded as the "third generation" (Melchers, 2004; Verkoczy et al., 2004). The first Ig transgenic mice were made by Brinster et al. (κ chain) and Grosschedl et al. (H chain). In "first-generation" transgenic mice—the conventional Ig transgenics—rearranged Ig genes were randomly integrated into the mouse genome (Brinster et al., 1983; Grosschedl et al., 1984). The genes, regulated by their proper control elements, were thus expressed from nonphysiological chromosomal locations. To overcome this in "second-generation" transgenics, the Ig genes were knocked-in by gene-targeting, i.e., they express the Ig genes from proper chromosomal sites (Cascalho et al., 1996; Chen et al., 1995; Prak and Weigert, 1995; Sonoda et al., 1997). Therefore, the physical order of the Ig gene segments was maintained on the L chain. However, the knocked-in H-chain rearrangement in these mice replaced the respective germline J-region genes, with the result that the altered locus still contained all D regions upstream of the knocked-in VDJ exon, whereas no extra D regions are present in normal B cells (Verkoczy et al., 2004). Because, in these transgenics, all other V_H and D gene segments are still present in the pre-rearranged allele, they can participate in noncanonical D- to VDJ-type joints (Chen et al., 1995; Taki et al., 1995).

In the progeny of the nuclear transfer mouse (third generation), antibodies are expressed from physiologically rearranged Ig loci at their original location. Because it faithfully reproduces the configuration of all Ig loci as present in the donor lymphocyte, this mouse currently is the most physiological model for studying B-cell development. Nevertheless, it still does not perfectly replicate normal B-cell development: Obviously, no recombination is necessary before H and L chain can be synthesized. Therefore, the

temporal expression of the H- and L-chain genes may not reflect the physiological time course.

The most surprising feature of the original B cell from which the nuclear transfer mouse was cloned is the presence of two in-frame L-chain rearrangements (Hochedlinger and Jaenisch, 2002). In the normal B-cell pool, such cells were originally believed to be rare (Pernis et al., 1965; Weiler, 1965). However, in more recent studies it has been speculated that they may be more frequent and important than previously thought (Diaw et al., 2001). The donor lymphocyte might therefore represent this “rare” B-cell population. Looking at the nuclear transfer mouse from a technical point of view, there is no reason why the two L-chain rearrangements should have conferred on this B cell a selective advantage in the cloning process over a lymphocyte carrying only one L-chain allele. From that point of view, the cloning of this particular B cell was a matter of chance and a fortunate event, as it allows the study of the fate of double L-chain-producing B cells.

During normal B-cell development, random V(D)J recombination creates an enormous repertoire of antibody genes in the B-cell pool of each individual (Tonegawa, 1983). Consequently, unlike in other areas of research, where the function of a gene can be determined by a single knock-in or knock-out mouse, a single Ig transgenic mouse represents only one out of the vast repertoire of B cells present in an individual. With these qualifiers in mind, the nuclear transfer B-cell mouse provides a welcome addition for the study of B-cell autoreactivity and editing, and this is a first phenotypic analysis of this mouse.

5.2 B cell autoreactivity

The enormous antigen receptor diversity of the B-cell pool ensures an efficient immune response in a world of evolving pathogens. However, this process also has the potential to produce self-reactive antibodies that are harmful. Because the initial antibody repertoire is created early in B-cell development in a process blind to self-reactivity, autoreactive B cells are part of the initial B-cell repertoire. Such B cells are then selected against during development; indeed, a majority of nascent immature B cells are deleted in the spleen before they reach the mature B-cell stage (Diaw et al., 1997; Goodnow et al., 1988;

Ichiyoshi and Casali, 1994; Nemazee and Burki, 1989; Norvell et al., 1995; Teale and Klinman, 1980).

B-cell autoreactivity has been studied primarily by using transgenic mice carrying antibody genes that encode self-reactive antibodies derived from autoimmune MRL/lpr mice (anti-DNA, 3H9) or immunized mice (anti-MHC, 3-83), after selection for reactivity and specificity (Chai et al., 1994; Gay et al., 1993; Nemazee and Burki, 1989; Pelanda et al., 1997; Radic et al., 1993; Radic et al., 1991; Tiegs et al., 1993). Other mice were generated with antibodies specific to hen egg lysozyme (anti-HEL, Ig/HEL), and the cognate antigen was either injected or expressed as a transgene (Goodnow et al., 1988). In contrast to these models, the nuclear transfer mouse was created by cloning a random B cell (Hochedlinger and Jaenisch, 2002). Unfortunately, however, unlike in the first nuclear transplantation experiment (which generated tadpoles from frog lymphocytes with selected specificity to dinitrophenyl), the Ig receptor specificity of the original B cell was not selected for in the creation of the mouse (Hochedlinger and Jaenisch, 2002; Wabl et al., 1975). So, because the donor lymphocyte was destroyed in the nuclear transfer process, one can only reconstruct, in a historical sense, what the original lymphocyte expressed and to which antigen it reacted.

While recreating the editing history of the original B cell, it was a goal of this study to determine if one of the two L chains conferred autoreactivity. The search for the putative self-antigen was guided by the possibility that an autoreactive BCR would lead to receptor editing. Since receptor editing is thought to occur in bone marrow and spleen, cells from these organs were screened for self-antigen that would bind to one of the antibody combinations Hxcr1 or Hxkb4. To ensure that the putative antigen is not present in only a single mouse strain, four different strains were analyzed. Despite the different genetic backgrounds, the results were the same in all mice analyzed. No self-antigen was found for the Hxcr1 antibody; however, the Hxkb4 antibody bound to dead cells in bone marrow and spleen. While dead or dying cells may cause artifacts, in all experiments the only binding was in sera containing the putative autoimmune Hxkb4 antibody. In both bone marrow and spleen, B cells are the major cell type. It is therefore not surprising that the autoreactive population consisted of B220-positive cells. Both tissues are major sites of cell differentiation and selection, harboring considerable numbers of dead and dying cells of different types (Melchers et al., 1995; Osmond, 1991; Rolink et al., 2001). However, one cannot conclude from this that the antigen is exclusively present on dying

B cells. The putative antigen might be a common cellular component present in different cell types, including dying B cells. The identity of the putative self-antigen remains unknown.

Apart from the staining of bone marrow and spleen cells by Hxkb4 antibodies, there is further evidence for an autoreactive BCR in the nuclear transfer mouse: Mice with a pre-rearranged H and two autoreactive kb4 alleles ($H^{+/o}$ $L^{kb4/kb4}$) have a block in B-cell development at the transition from the immature to mature B-cell stage, where autoreactive B cells are usually eliminated. These results are similar to findings of Nemazee and Bürki: In their seminal experiments using conventional Ig transgenic mice with an autoreactive BCR expressed from ectopically integrated transgenes, editing was prevented, which led to the elimination of the autoreactive B cells (Nemazee and Burki, 1989). In the $H^{+/o}$ $L^{kb4/kb4}$ mouse, however, the presence of two autoreactive alleles in the germline impairs the editing: To destroy both autoreactive kb4 joints, Vκ replacement had to be successful on both alleles to rescue the cell.

5.3 Editing

Autoreactive B cells are inherent to the initial B-cell repertoire. To preserve tolerance, two basic mechanisms eliminate self-reactive antibodies (Melamed et al., 1998). The first is receptor editing, in which autoreactive B cells destroy the self-reactive BCR through secondary L-chain gene rearrangement (Gay et al., 1993; Radic et al., 1993; Tiegs et al., 1993). In the second mechanism, B cells that escape central censorship are clonally deleted by apoptosis or rendered unresponsive, i.e., anergic (Goodnow et al., 1988; Nemazee and Burki, 1989). In either case, B cells are thought to express a BCR from only one H- and one L-chain gene (Pernis et al., 1965; Weiler, 1965). But, as described here, the nuclear transfer mouse expresses two functional L chains. This finding led to an effort to recreate the developmental path of the original B cell, and to characterize B-cell development with the different H- and L-chain combinations in mice with an Hxcr1 receptor or an Hxkb4 receptor.

In $H^{+/o}$ $L^{cr1/o}$ mice, there was no sign of receptor editing; instead, B-cell development was accelerated, and the B-cell pool in these mice was similar to the one in other transgenic mice with a non-autoreactive BCR (e.g., Pelanda et al., 1997). Furthermore, no secondary

recombination was observed. Thus, the Hxcr1 antibodies can be regarded as nonself-reactive, i.e., innocuous. But in mice with the H chain and one kb4 allele ($H^{+/o}$ $L^{kb4/o}$), the BCR was autoreactive, and so editing would be expected to occur (Casellas et al., 2001; Nemazee and Weigert, 2000; Pelanda et al., 1997; Retter and Nemazee, 1998; Yamagami et al., 1999). Indeed, editing did happen, but in contrast to the general mechanism, in the progeny of the nuclear transfer mouse the autoreactive L-chain allele was not destroyed in all B lymphocytes; instead, the second κ allele was rearranged in some B cells. It seems, therefore, reasonable to believe that in the donor B cell that gave rise to the nuclear transfer mouse, the autoreactive $H^{+/o}$ $L^{kb4/o}$ genes were expressed first, and their autoreactivity then led to receptor editing and thus to the generation of the cr1 L chain on the other allele. This, in turn, created the $H^{+/o}$ $L^{kb4/cr1}$ configuration of the original lymphocyte.

B cells with two L-chain rearrangements after receptor editing have previously been described in only one other mouse, which has a gene-targeted H chain, designated 3H9H/56R, but no transgenic L chain. Unlike in the nuclear transfer mouse, which has two κ alleles, single B cells of the 3H9H/56R mouse express both κ and λ on the cell surface (Li et al., 2002). Even though the effect seems to be the same, there are distinct differences between the two mice. In the nuclear transfer mouse, autoreactivity is mediated by the Hxkb4 combination, and not by one of the Ig chains alone. Furthermore, a non-autoreactive BCR can consist of the H chain in association with various κ and λ L chains. In 3H9H/56R mice, on the other hand, the BCR specificity of the anti-DNA reactive antibody is determined by the H transgene alone (Li et al., 2002). The 3H9H/56R H chain can pair with very few L chains. When combined with any λ chain, 3H9H/56R is autoreactive and is self-tolerant only in association with a few selected Vκ genes (3 out of 93) (Li et al., 2002; Li et al., 2004). Consequently, the editing processes in the two mice are under different constraints.

In the 3H9H/56R mouse, two L-chain rearrangements presumably arise if a B cell productively rearranges the λ locus first. Since any λ chain is autoreactive in combination with 3H9H/56R, the B cell edits the L chain, only to produce one autoreactive λ rearrangement after the other. In the end, a productive λ rearrangement using the last available Vλ region cannot be destroyed and thus persists. In such a cell, because the BCR is still autoreactive, the κ locus will also open for recombination. The editing

process can only be stopped by one of the few nonself-reactive Vκ genes, which will create mixed molecules with the Hxλ combinations and thereby possibly mitigate the effects of self-reactivity. All B cells unable to create these combinations are eliminated (Li et al., 2002). Consequently, in 3H9H/56R mice, B cells with two L-chain rearrangements, λ and κ, are positively selected (Li et al., 2001). Guided by different recombination signal sequences, in normal mice the κ locus is usually more frequently recombined than the λ locus. In combination with the 3H9H/56R H chain, all but 3 Vκ regions are autoreactive, and therefore almost all κ expressing B cells are counter selected. In the nuclear transfer mouse, however, there is no apparent positive selection for double-producing cells. Since V_{kb4} is joined to Jκ1, replacement of this κ rearrangement with an upstream Vκ and a downstream Jκ region should not be restricted (Halverson et al., 2004; Spanopoulou et al., 1994; Xu et al., 1998). Thus, in the nuclear transfer mouse an autoreactive κ allele could be deleted in the same way as in a normal mouse.

The presence of two κ L-chain rearrangements in the nuclear transfer mouse raises the question of whether both L chains were expressed on the cell surface of the donor lymphocyte at the same time. Indeed, dual L-chain-expressing B cells were found in the B-cell pool of $H^{+/o}$ $L^{kb4/c\kappa h}$ mice, and autoreactivity was present in the serum of $H^{+/o}$ $L^{kb4/o}$ mice. The aspects of allelic inclusion are discussed below, but it seems clear that autoreactivity has persisted despite receptor editing in the nuclear transfer mouse. This is in contrast to the general view of B-cell tolerance, in which B cells that escape central censorship are believed to be clonally deleted by apoptosis or rendered anergic (Goodnow et al., 1988; Nemazee and Burki, 1989). However, it is known that, despite editing, apoptosis, and anergy, autoreactive antibodies exist in the serum of normal humans and mice (Chai et al., 1994; Coutinho et al., 1995; Dighiero et al., 1985; Imai et al., 1994; Lacroix-Desmazes et al., 1998). The results presented here may, therefore, provide a possible explanation for the origin of residual serum autoreactivity. In this view, during receptor editing the self-reactive allele is not always destroyed, but recombination on the second allele might rescue the B cell from deletion.

5.4 Allelic exclusion

In Burnet's clonal selection theory, each Ig-producing B cell expresses one antigen receptor with a unique specificity. However, each B cell has two alleles for the H-chain locus and two alleles for the multiple L-chain loci in its genome. It could, in principle, produce different antibodies from various combinations of these loci. Nevertheless, it is an experimental fact that over 99% of individual B cells express a surface Ig receptor of one H chain only, a phenomenon termed allelic exclusion (Pernis et al., 1965; Weiler, 1965). There are various hypotheses about the establishment of allelic exclusion, but the mechanism is still unresolved. However, it is clear that, during B-cell development, assembly of the H-chain gene precedes that of the L-chain genes. Therefore, to preserve allelic exclusion of the H chain, rearrangement of H-chain genes must not occur during L-chain rearrangement. Accordingly, the molecular mechanisms enforcing H-chain and L-chain allelic exclusion may differ (Bassing et al., 2002). At least for the L-chain locus, a widely accepted view is that RAG is turned off after a functional (non-autoreactive) BCR is presented on the cell surface (Alt et al., 1982; Coleclough et al., 1981; Wabl and Steinberg, 1982).

The results of this study indicate that an autoreactive allele might not always be destroyed during receptor editing, but the cell may be rescued by a rearrangement on the second allele. This results in the expression of two alleles, i.e., allelic inclusion. In T cells, frequent allelic inclusion of the TCRα genes poses an autoimmune "hazard" due to expression of autoreactive receptors (Sarukhan et al., 1998). The same might be true in B cells.

In the experiments to recreate the developmental steps leading to the rearranged alleles, a considerable number of B cells showed dual receptor expression. However, the expression status of the two L chains in the original B cell remains unknown. Because the original B cell was lost in the generation of this mouse, it is impossible to know whether the kb4 allele was silenced, for example, by methylation or by a transcriptional block; the nuclear transfer could have removed the impediments for expression of this allele. But one also has to consider that the preformed Ig genes in Ig transgenic mice may be expressed earlier than those in wild-type mice (Oberdoerffer et al., 2003; Pelanda et al., 1997). Thus, the preformed Ig genes in the analyzed progeny may rush B cells through differentiation. The rearrangement of two productive L-chain alleles might therefore be

less frequent than if both alleles had to be generated through consecutive recombination steps.

While in the past, the incidence of L-chain double producers was estimated to be very low, they have been found more and more frequently in recent years, sparking speculations about their frequency and importance (Casellas et al., 2001; Cebra et al., 1966; Diaw et al., 2000; Diaw et al., 2001; Hardy et al., 1986; Kwan et al., 1981; Pernis et al., 1965). In lymph nodes, there are not many autoreactive double-producing B cells (<5%) (Casellas et al., 2001). On the other hand, in mice with a preformed $V_\kappa 8$ allele and a 3H9 H-chain allele, 5% of the B cells retain expression of the $V_\kappa 8$ allele, while also presenting κ chain from the non-recombinant κ allele after recombination (Casellas et al., 2001). But among all transgenic mice, a significant number of B cells with two different BCRs on the surface have been found only in the 3H9H/56R mice. For that mouse, it was proposed that a second innocuous receptor could conceal autoreactivity by hindering cross-linking of the self-reactive receptors on the cell surface, thus stopping the editing process (Li et al., 2004). In other words, the innocuous receptor would dilute the autoimmune receptor, which exists along with it. It is hard to say how frequent this would be in the normal B-cell repertoire.

The experimental protocol in this work was not specifically designed to test the various hypotheses for allelic exclusion at the L-chain locus. However, a high frequency of functional double producers would tend to weaken the idea of "monoallelic accessibility" of κ genes for VJ recombination as a mechanism of allelic exclusion (Goldmit et al., 2002). Nevertheless, probabilistic enhancer activation and allelic competition are thought to contribute to allelic exclusion (Liang et al., 2004). In the work described here, the working hypothesis is the minimal one: Once a functional L chain has been produced, the recombinase is shut off. It seems obvious that a “functional L chain” needs to allow the H chain to reach the surface of the cell and not induce self-reactivity of the complete antibody.

5.5 RAG expression

During normal B-cell maturation, RAG is expressed in two phases. It is first increased in the pro-B-cell stage to rearrange the H-chain locus. Once a functional H-chain gene has

been assembled, it is expressed and presented on the cell surface as part of the pre-BCR. At this point the pre-B cell starts to proliferate, and RAG is synthesized at a much lower level. After proliferation, the second RAG expression phase begins with the recombination of the L-chain locus. Once a functional, non-autoreactive BCR is present on the cell surface, RAG synthesis is terminated. RAG synthesis is thus halted by the pre-BCR in pre-B cells or by the BCR in immature B cells.

Receptor editing is an event secondary to V(D)J recombination, induced by an autoreactive receptor. It can be distinguished from recombination only in that it is activated after an autoreactive BCR is presented on the cell surface. However, this distinction becomes important when receptor editing is studied in Ig transgenic mice. For this, it is important to know when exactly the preformed H- and L-chain genes are expressed. In this work, the question of whether the Ig transgenes of the nuclear transfer mouse are expressed before or after RAG synthesis is addressed. If RAG is expressed before the H-chain transgene is synthesized, the sequence of the pre-rearranged gene could be altered before it is expressed. The result would look like receptor editing, but since it is not caused by BCR autoreactivity, it would be an artifact. For the analysis described here, offspring of the nuclear transfer mouse were crossed with mice with a marker transcribing GFP whenever RAG1 is transcribed (RAG1-GFP mice) (Hirose et al., 2002; Igarashi et al., 2002).

For the interpretation of results from GFP indicator mice, one has to consider the correlation between RAG transcription, RAG protein expression, and RAG activity, and, similarly, GFP transcription, expression, and fluorescence. In Ig wild-type RAG1-GFP mice, even though GFP is transcribed only when RAG is synthesized, there is a discrepancy between RAG activity and GFP fluorescence. This was explained by a difference in the half-life of the RAG protein and the GFP protein (Hirose et al., 2002; Igarashi et al., 2002). In addition, it is currently not known how RAG activity is regulated. In mice with GFP inserted into either the RAG1 or RAG2 gene, recombinase activity seems to be regulated by transcription (Monroe et al., 1999; Yu et al., 1999). Other studies suggest that RAG activity levels are also regulated by phosphorylation (Li et al., 1996). Hence, RAG activity might be altered by phosphorylation, which would not necessarily be reflected by GFP fluorescence. The correlation between shutdown of GFP fluorescence and loss of RAG activity is not very strong. However, in contrast to its imprecision in marking the end of RAG activity, the RAG1-GFP detection system is very

precise in determining the onset of RAG expression: No GFP fluorescence has been detected in cells without RAG activity (Hirose et al., 2002; Igarashi et al., 2002).

In this study, mice with the autoreactive H- and L-chain combination express RAG in the bone marrow and in the spleen. In animals with the innocuous BCR (with the same H chain), RAG expression was prevented in most B cells, while a small population of B cells in the bone marrow showed high and medium GFP expression levels. Furthermore, no L-chain editing was found in mice with a nonself-reactive BCR.

To interpret these results, in addition to the correlation between GFP and RAG expression, one has to consider the temporal sequence of H- and L-gene expression. As mentioned above, unlike in wild-type mice, the preformed H- and L-chain genes in Ig transgenic mice are expressed prematurely since they do not have to be recombined first (Pelanda et al., 1997). As a result, in the nuclear transfer mouse, the L-chain gene is expressed ahead of time (Oberdoerffer et al., 2003). Considering this, the patterns of GFP expression observed in this study demonstrate that, in the presence of the nonself-reactive H- and L-chain combination, RAG is shut down. The autoreactive receptor, on the other hand, induces GFP and RAG expression and, therefore, secondary L-chain recombination. Both results are consistent with previous observations in other Ig transgenic mice with innocuous and self-reactive antibodies (Casellas et al., 2001).

However, this does not explain the population of B cells with medium and high levels of GFP expression in mice with the nonself-reactive BCR. In Ig wild-type mice, RAG is expressed at low levels in some early lymphoid progenitor cells, while developing B cells start to express RAG in the later pro-B-cell stage. Such early lymphoid progenitor cells also exhibit recombinase activity, as measured by H-chain D-to-J recombination (Igarashi et al., 2002). If this is also true of the nuclear transfer mouse, it might explain the presence of RAG despite premature H-chain transgene expression and pre-BCR presentation. Furthermore, since no evidence for L-chain editing was found in nonself-reactive BCR mice, and because recombination is restricted to the H-chain locus during initial recombination in normal B-cell development, RAG activity in these cells might only affect the H-chain locus, creating non-canonical H-chain joints. Subsequently, RAG would be turned off after pre-BCR presentation on the cell surface, or with a prematurely expressed κ chain, after the innocuous BCR is presented. Therefore, in some B cells the H-chain locus could be altered before it is expressed and thus imitate receptor editing.

Similar conclusions can be drawn from studies on other site-directed Ig transgenic mice. In mice with residual D regions upstream of the knocked-in VDJ rearrangement, edited H chains sometimes show N nucleotides in their H-chain sequence (Cascalho et al., 1996; Chen et al., 1995; Sonoda et al., 1997). This suggests that, despite the H-chain transgene, VDJ recombination can occur during early development, because N nucleotides are added by the terminal deoxynucleotidyl transferase only during initial H-chain recombination and not during receptor editing (Komori et al., 1993; Wasserman et al., 1997).

Since these results represent a study in progress, it is impossible to conclude whether the H-chain transgene or RAG is expressed first during B-cell development in the nuclear transfer mouse. For the majority of cells, the H chain is expressed before RAG is expressed. However, in some cells, RAG synthesis might precede H-chain presentation. Therefore, further experiments are necessary. As a future direction, the H-chain locus of B cells with the innocuous BCR locus will be sequenced after GFP expression. Furthermore, to determine whether RAG is turned off by the pre-BCR or by the BCR, mice with the nuclear transfer H chain will be generated in which RAG1 is nonfunctional on one allele due to GFP insertion and there is a RAG1 knock-out on the other allele. However, RAG activation will still be monitored by GFP expression. In such mice, RAG can be shut down only by the pre-BCR, consisting of the H chain combined with the surrogate L chain, instead of the BCR. These results may bring further insight to the correlation between RAG synthesis and pre-BCR expression. Furthermore, they might also provide clues to the mechanism that establishes allelic exclusion.

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Eidesstattliche Erklärung

Hiermit versichere ich, daß die vorliegende Arbeit von mir selbständig und unter ausschließlicher Verwendung der angegebenen Quellen und Hilfsmittel angefertigt wurde.

Meppen, den 10. Juli 2006

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Unterschrift